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Adipocyte Enhancer Binding Protein (AEBP1), a Multifunctional Protein Involved in Adipogenesis

by

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Submitted in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
August 8, 1997

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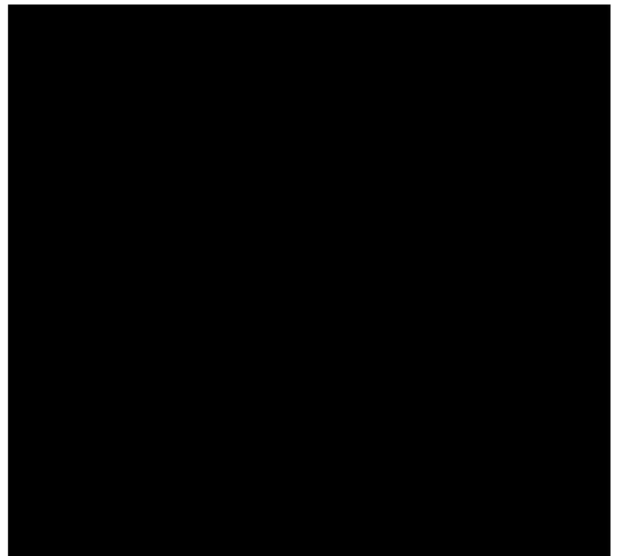

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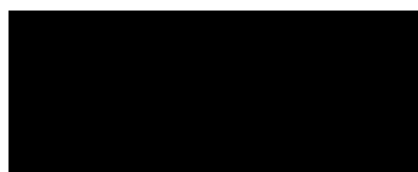

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Abbreviations

A	Absorbance
ADD1	adipocyte determination- and differentiation-dependent factor
AKAP79	A kinase anchoring protein
aP2	adipose P2 (422) gene
AE-1	adipocyte enhancer-1
AEBP1	adipocyte enhancer binding protein
ARE	adipocyte regulatory element
Arf	ADP ribosylation factor
ARF6	adipocyte regulator factor 6
βARK	β-adrenergic receptor kinase
BH	bleomycin hydrolase
cAMP	cyclic adenosine 3',5'-monophosphate
CAT	chloramphenicol acetyltransferase
C/EBP	CCAAT/enhancer binding protein
CF	coagulation factor (V) and (VIII)
CIAP	calf intestinal alkaline phosphatase
CP	carboxypeptidase
CPB-like	carboxypeptidase B-like
CMV	cytomegalovirus
dex-mix	dexamethasone-1-methyl-3-isobutylxanthine
DLD	discoidin-like domain
DR-1	direct repeat of the hormone response element
EGF	epidermal growth factor
EGFR	EGF receptor
ERK1/2	extracellular signal-regulated kinase 1/2
EST	expressed sequence tag
F	filamentous
FABP	fatty-acid-binding protein
FAK	focal adhesion kinase
FBS	fetal bovine serum
FFA	free fatty acids
FRK	Fos-regulating kinase
FSE	fat specific element

HMG-1/2	High mobility group protein 1/2
HSP27	Heat shock protein 27
GAP	GTPase activating protein
GLUT4	glucose transporter 4
GMSA	gel mobility shift assay
GPCR	G protein-coupled receptors
GRK	G protein-coupled receptor kinase
Grb2	growth-factor-bound protein 2
GPDH	glycerol-3-phosphate dehydrogenase
IGF-1	insulin-like growth factor-1
JAK	Janus kinase
JNK	Jun N-terminal kinase
JNKK	Jun N-terminal kinase kinase
LPA	lysophosphatidic acid
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
MBP	myelin basic protein
MEK	mitogen activated protein kinase/extracellular signal-regulated kinase kinase
MEL	murine erythroleukemia
MKP	mitogen activated protein kinase phosphatase
NGF	nerve growth factor
PAK	p21-activated kinase
PDGF	platelet derived growth factor
PH	pleckstrin homology
PI3K	phosphatidylinositol-3-kinase
PKA	cAMP dependent kinase
PKC	protein kinase C
PLCγ	phospholipase Cγ
PPAR	peroxisome-proliferator-activated receptor
Pref-1	preadipocyte factor 1
PRE	preadipocyte repressor element
PTG	protein targeting for glucose
PTPase	phosphotyrosine phosphatase
put	proline utilization operon
RACK	receptors for activated protein kinase C

Rb	retinoblastoma protein
RGS	regulators of G protein-signaling
RKK	RK kinase
RTK	receptor tyrosine kinase
RXR	retinoid X receptor
SAPK	stress-activated protein kinase
SCD2	steroyl-CoA desaturase-2
SH2/3	Src-homology 2/3
SEK	SAPK/ERK kinase
SOS	son of sevenless
SREBP1	sterol-response-element-binding protein
STAT	signal transducers and activator of transcription
TBP	TATA-binding protein
TCF	ternary complex factor
TCP	tubulin carboxypeptidase
TFIIB/D/E/H	transcription factor II B/D/E/H
TNFα	tumor necrosis factor α
UAS_G	yeast upstream activating sequence
3'AT	3-aminotriazole

ABSTRACT

Adipogenesis is a complex process involving a number of stages each requiring the coordinated expression of numerous genes and the activation or inactivation of numerous proteins. These studies have focused on AEBP1, a protein which is down-regulated during adipogenesis in 3T3 L1 preadipocytes, and have shown that AEBP1 is a preadipocyte-specific negative regulator of the adipogenic aP2 gene.

This study has identified AEBP1 as a DNA-binding protease. Sequence comparisons and kinetic studies using known carboxypeptidase substrates, activators, and inhibitors have characterized AEBP1 as a member of the regulatory-type B-like carboxypeptidase family. The C-terminal region of AEBP1 has been shown to bind to the AE-1 promoter region of the aP2 gene. Once AEBP1 is bound to DNA, an inherent carboxypeptidase activity is enhanced. This enhanced protease activity is required for AEBP1 to repress transcription of the aP2 gene by an active repression mechanism. This thesis has shown that the DNA-binding, the enzymatic activity, and the transcription repression function of AEBP1 are correlated, and strongly suggests that the DNA-bound AEBP1 uses its protease function to repress transcription.

Two-hybrid interaction studies have shown that AEBP1 interacts with heat shock protein 27 (HSP27), high mobility group 2 (HMG-2), and the Gγ5 subunit of a trimeric G protein. AEBP1 forms complexes with the Gβγ5 heterodimer in coimmunoprecipitation experiments. The *in vitro* interaction between AEBP1 and Gγ5 prevents AEBP1 from binding DNA, which inhibits the transcription repression function of AEBP1.

Furthermore, AEBP1 forms complexes in coimmunoprecipitation and gel filtration studies with both the active and inactive forms of the mitogen-activated protein kinase (MAPK). *In vitro* experiments have shown that MAPK phosphorylates AEBP1 in the C terminus. This *in vitro* interaction between AEBP1 and MAPK enhances the DNA-binding ability of AEBP1 and protects MAPK from inactivation by phosphatases.

In sum, AEBP1 appears to function as a negative transcriptional regulator of at least one adipogenic induced gene and may also be mediated by putative signaling pathways.

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Chapter 1:
Introduction

The struggle to find sustenance has driven man's development of efficient energy-storage mechanisms. Survival depended in part on the physiological ability to store energy as fat during bountiful times, and on the utilization of these energy stores in times of need. These metabolic adaptations to survive the feast-and-famine cycle developed over tens of thousands of years, and gave a competitive advantage to those most capable of storing energy when food was available. In today's fast-food culture there is a failure to maintain a healthy balance between nutritional intake and the general level of activity and exercise. One of the consequences of this dietary-energy imbalance is a society prone to obesity and its associated crippling diseases.

Obesity is a leading health risk in the Western world, and is strongly linked to adult-onset diabetes, hypertension, heart disease, and certain cancers. Individuals whose caloric intake chronically exceeds their energy expenditure may be deemed obese. These persons suffer from an expansion in size of pre-existing adipocytes (hypertrophy), as well as an increase in the number of adipocytes through differentiation of preadipocytes (hyperplasia).

The expansion in the number of adipocytes through differentiation of pre-existing preadipocytes occurs through a process known as adipogenesis. This is a tightly controlled process with the regulation of numerous proteins involved in maintaining the preadipocyte state, and initiating the differentiation processes. This thesis explores the regulation of adipogenesis at the level of transcription, and the signaling mechanisms involved in maintaining the preadipocyte phenotype and initiating adipogenesis.

1.I. Adipogenesis: Adipocytes and 3T3 L1 cells

Adipose tissue is composed of adipocytes, with intermingled blood cells, endothelial cells, pericytes, fibroblasts, and precursor adipocyte which are cells with varying degrees of differentiation (Geloen et al., 1989). Precursor adipocytes may be described as adipoblasts, which are unipotential cells that have been determined to only become adipocytes, but which are not expressing early adipocyte differentiation genes. Precursor adipocytes may also be described as preadipocytes which are defined by their ability to express early differentiation makers; but which are not capable of storing triacylglycerol or expressing late adipocyte genes (Ailhaud et al., 1992). These cells arise from multipotent stem cells of mesodermal origin that may also give rise to muscle or cartilage cells (Cornelius et al., 1994).

Cells isolated from adipose tissue can be induced to differentiate from preadipocytes to immature adipose cells and then to mature adipocytes (Ailhaud et al., 1992; 1994). Cell lines committed to the adipocyte lineage can be used to study adipocyte differentiation. In these clonal cell lines, changes in cell morphology and expression of adipocyte specific genes can be observed as cells differentiate from adipoblasts to preadipocytes to immature adipose cells and then to mature adipose cells.

The mouse 3T3 L1 cell line, selected from disaggregated mouse embryo cells, has been thoroughly studied as a model for adipocyte differentiation. These cells were chosen for their ability to accumulate triacylglycerides (Green et al., 1974), and are still used for study of the differentiation process.

A protocol has been established for the differentiation of these 3T3 L1 preadipocytes into greater than 90% adipocytes. This protocol includes the addition of dexamethasone (dex), a synthetic glucocorticoid agonist; 1-methyl-3-isobutylxanthine (MIX), a cAMP phosphodiesterase inhibitor; high levels of insulin, which act through

insulin-like growth factor-1 (IGF-1) receptors; and fetal bovine serum (FBS) (Cornelius et al., 1994).

The addition of dex-mix, insulin, and FBS initiates differentiation of 3T3 L1 cells, which leads to the morphological changes associated with adipogenesis, including the loss of fibroblast character and subsequent phenotypical rounding-up of the cell. Numerous studies have shown that 3T3 L1 cells behave like preadipocytes *in vivo*, and once differentiated function as mature adipocytes (Cornelius et al., 1994). One of the most compelling lines of evidence for the use of 3T3 L1 cells as a model adipogenic cell comes from electron micrograph studies which have shown that mature 3T3 L1 adipocytes possess the ultrastructural features of mature adipocytes *in situ* (Novikoff et al., 1980).

The initiation of differentiation of 3T3 L1 preadipocytes is dependent upon growth arrest in the G1/S stage of the cell cycle, usually signaled by cell-cell contact (Ailhaud et al., 1989). It is apparent that at least one round of DNA synthesis and cell doubling is required for terminal differentiation (Ailhaud et al., 1992). After the initiation of differentiation, 3T3 L1 cells begin to accumulate triacylglycerols and to express genes encoding the enzymes involved in the pathways for *de novo* fatty acid synthesis and triacylglycerol biosynthesis. These genes are expressed coordinately with the appearance of cytoplasmic triacylglycerol (Cornelius et al., 1994).

The expression of genes considered "markers" for various stages of adipogenesis is important for the monitoring of the differentiation process. Late markers for adipocyte differentiation (immature adipose cells) include the proteins glucose transporter-4 (GLUT-4), the fatty-acid-binding protein (FABP), and glycerol-3-phosphate dehydrogenase (G3PDH); the very late markers for differentiation (mature adipose cells) include the proteins adipsin and the acyl CoA binding protein (Ailhaud et al., 1992; 1994, and references therein).

Experiments designed to determine the nature of serum factors involved in the initiation of adipogenesis (reviewed in Cornelius et al., 1994) have shown that at least three independent signaling mechanisms are involved in adipogenesis. For example, receptor tyrosine kinase pathways (see section 1.II.A) are activated in preadipocytes by the binding of insulin to the growth factor-1 (IGF-1) receptor during the initiation of differentiation, and again through the binding of insulin to the insulin receptor during the later stages of adipogenesis. Likewise the activation of cAMP-dependent pathways through the stimulation of G protein coupled receptors (see section 1.II.E.) is necessary for the initiation of adipogenesis. Additionally, the glucocorticoid pathway has been shown to be necessary for this process (Cornelius et al., 1994).

1.II. Cell Signaling in Adipogenesis

1.II.A. Receptor Tyrosine Kinase Pathways

The initiation of differentiation of 3T3 L1 preadipocyte into mature adipocytes is induced by high concentrations of insulin, glucocorticoids, and cAMP (Ailhaud et al., 1992). Signal transduction pathways mediated by receptor tyrosine kinases (RTK) are critical for this initiation of adipogenesis.

Receptor tyrosine kinases (RTK; for a description of the receptor tyrosine kinase pathways, see Figure 1.1) are activated by the binding of either dimeric ligands, such as platelet derived growth factor (PDGF), or monomeric ligands, such as epidermal growth factor (EGF). The binding of a ligand to the receptor causes the homo- or hetero-dimerization of the receptor and the subsequent autophosphorylation of multiple tyrosine residues on the receptor by an intrinsic kinase activity. The now activated phosphorylated receptor acts as a signaling molecule through interactions with other proteins (Malarkey et al., 1995; Weiss et al., 1997).

Figure 1.1. Receptor Tyrosine Kinase Pathway in Mammalian Cells. Conventionally, the activation of a receptor tyrosine kinase through ligand binding causes the dimerization of the receptor and subsequent autophosphorylation by an intrinsic kinase activity. The phosphorylated tyrosine residues on the activated ligand-bound receptor allows adapter proteins, such as Grb2, to bind. Grb2 can then bind to and activate the SOS protein, a GTP-exchange protein. SOS activates Ras by allowing the exchange of GDP for GTP on Ras. Activated GTP-Ras can then bind and activate the protein kinase Raf-1. The activated Raf-1 phosphorylates MEK which in turn phosphorylates MAPK. Depending upon the cell type and type of RTK activated, the activation of MAPK may be transient or prolonged. Prolonged MAPK activation allows the kinase to be translocated to the nucleus where it phosphorylates transcription factors. A number of parallel pathways occur in cells in response to different stimuli and stress. At each level in the pathway (MAPK, MAPKK, and MAPKKK) there are different kinases which may be used or activated. These different components allow similar RTKs to use various combinations of kinases for different cellular outcomes (for review see Denhardt, 1996). (P) indicate proteins which are phosphoryled. Arrows indicate the activation of protein and major route of signal transduction. Shaded molecules indicate an alternative pathway leading to the activation of MAPK, which does not use Raf1 but instead MEKK.

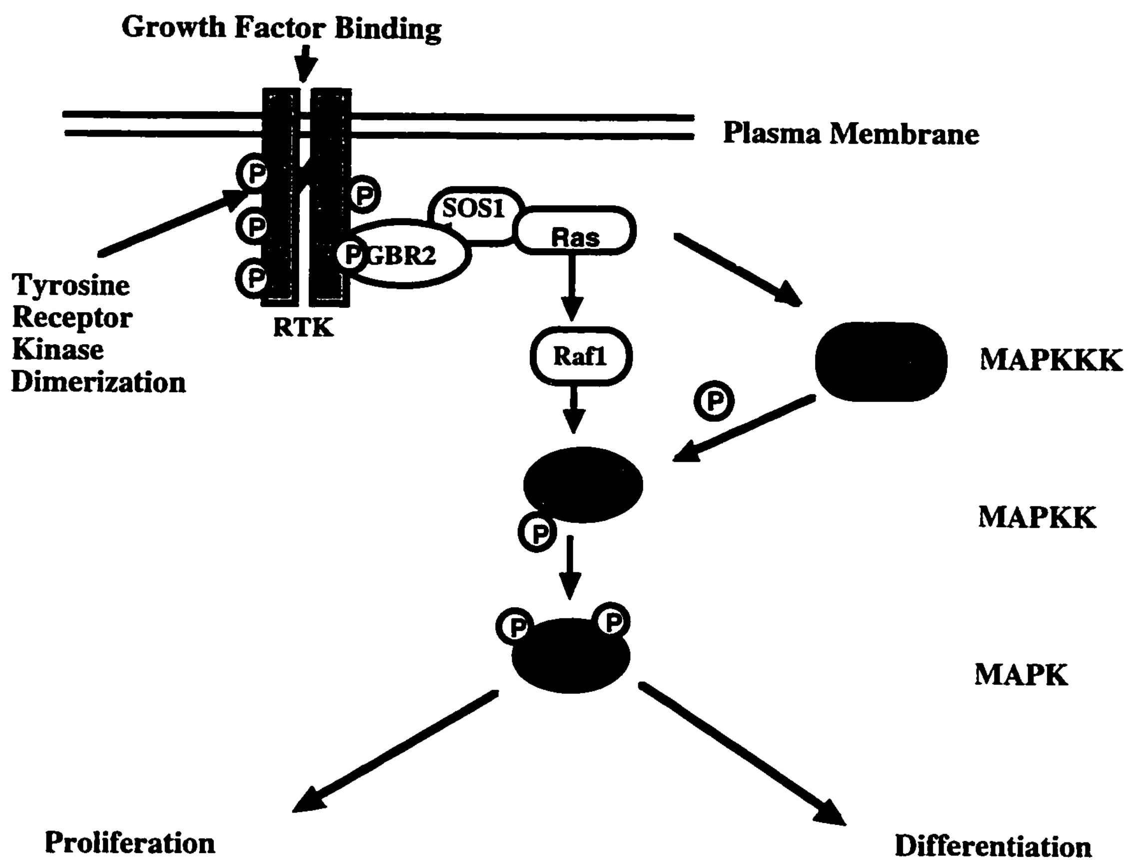


Figure 1.1 Typical Receptor Tyrosine Kinase Pathway in Mammalian Cells.

Phosphorylated tyrosine residues on the receptor are recognized by proteins containing Src-homology 2 (SH2) domains. Several proteins, including the growth-factor-bound-protein 2 (Grb2), GTPase activating protein (GAP), and phospholipase Cγ (PLCγ) (Malarkey, 1995), have SH2 domains and are capable of binding to RTKs. Many proteins containing receptor-binding SH2 domains also contain a different Src-homology 3 (SH3) domain which recognizes proline rich regions of other proteins (reviewed by Pawson, 1995). Proteins with both SH2 and SH3 domains allow the formation of signaling complexes at the receptor.

The binding of Grb2 to a RTK allows the localization of a guanine-nucleotide exchange factor termed son of sevenless protein (SOS), through a proline-rich SH3-binding region, to the membrane receptor. The binding of SOS in this fashion allows the activation of Ras by facilitating the exchange of GDP with GTP. The activated GTP-bound Ras is then able to bind to and activate other proteins, which are components of signal transduction pathways (Marshall, 1996). There are other mechanisms, not discussed here, by which Ras is activated by RTKs (Denhardt, 1996, see reference for review).

Studies have shown that nonphysiologically high concentrations of insulin influence differentiation of 3T3 L1 preadipocytes through a receptor tyrosine kinase, the IGF-1 receptor (Smith et al., 1988). Preadipocytes possess few insulin receptors but have large numbers of IGF-1 receptors, which can be activated by nonphysiologically high concentrations of insulin (Norgues et al., 1993). The activated IGF-1 receptor is capable of phosphorylating the insulin receptor substrate (IRS), the major substrate of the insulin receptor tyrosine kinase (Myers, and Morris, 1995). The determination that the activation of the IGF-1 receptor is a potent inducer of the differentiation process implies that a tyrosine-kinase-mediated signaling pathway is involved in adipocyte differentiation.

Other evidence implicating the involvement of receptor tyrosine kinase pathways in preadipocyte differentiation comes from phosphotyrosine phosphatase 1B experiments. Overexpression of this phosphatase blocks preadipocytes differentiation through the inactivation (dephosphorylation) of receptor tyrosine kinases (Liao and Lane, 1994). Furthermore, constitutive expression of Ras and Raf-1 (two components of RTK pathways; see Figure 1.1) can induce preadipocyte differentiation in the absence of insulin or IGF-1 (Porras et al., 1991; 1994). These studies further indicate that the initiation of adipogenesis is dependent on the activation of RTK mediated pathways.

1.II.B. Mitogen-Activated Protein Kinase (MAPK)

Mitogen-activated protein kinases (MAPKs) have been implicated as vital components of signaling pathways which determine whether a cell differentiates or proliferates. This section deals with the roles and regulation of MAPKs. For the scope of the studies described in this thesis MAPK refers to ERK1 (p44MAPK) and ERK2 (p42MAPK).

The MAPK family comprises a broad group of kinases which includes the extracellular-signal-regulated kinases 1 and 2 (ERK1 and ERK2), two ERK3 isoforms, ERK4, Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPKs), reactivating kinase/cytokine-suppressive anti-inflammatory drug-binding protein p38HOG1/RK/CSBP, p57 MAP kinases, and Fos-regulating kinase (FRK) (Cobb and Goldsmith, 1995; Su and Karin, 1996; Denhardt, 1996, and references therein). All of these MAPKs phosphorylate a minimum consensus sequence containing Ser or Thr adjacent to a proline, with an optimal consensus sequence of Pro-Xaa-Ser/Thr-Pro (Davis, 1993).

MAPK family members are activated by phosphorylation of a Tyr and Thr in a Thr-Xaa-Tyr sequence (where Xaa is a Glu, Pro, or Gly for ERKs, JNKs/SAPKs and p38/RK/CSBP, respectively) by a MAPK/ERK kinase (MEK or MAPKK, to be discussed below) (Denhardt, 1996). The phosphorylation of both the Thr and Tyr residues in the aforementioned consensus sequence is critical for activation of MAPK by MEK (Johnson et al., 1996). The phosphorylation of p42MAPK on Tyr-185 facilitates recognition and binding of substrates, whereas the phosphorylation of Thr-183 allows correct alignment of catalytic residues (Zhang et al., 1994). Both residues are essential for activation of MAPK.

MAPKs are phosphorylated by a family of proteins termed MAPK kinases (MAPKK) (see Figure 1.1) which includes MAPK/ERK Kinase (MEK), MAPK Kinase (MKK), JNK Kinase (JNKK), SAPK/ERK Kinase (SEK), and RK Kinase (RKK) (Denhardt et al., 1996). For example, the MEK subfamily MEK1a, MEK1b, and MEK2 phosphorylates the various ERKs, while the subfamily of SEK/JNKK/MKK4, phosphorylates JNK and SAPK (Denhardt et al., 1996, and references therein). These kinases are themselves activated by phosphorylation of Ser/Thr by a MAPKKK.

Within five minutes of growth factors binding to RTKs, MAPK and MEK (the kinase which phosphorylates MAPK; see Figure 1.1) are activated in the cytoplasm. If the activation of MAPK is prolonged for approximately 15-30 minutes, MAPK is translocated to the nucleus. However, MEK is quickly down-regulated upon activation of MAPK, and therefore does not accompany MAPK as it translocates to the nucleus (Alessi et al., 1995; Chen et al., 1992). The fact that MEK is down-regulated and not translocated with MAPK to the nucleus suggests that the regulation of activated MAPK occurs through both cytoplasmic and nuclear protein phosphatases, and not through continuous re-activation by MEK.

A number of protein Ser/Thr phosphatases have been identified, including PP1, PP4, PP5, PP2A, PP2B, PP2C (Yoder-Hill et al., 1995); as well, several protein tyrosine phosphatases have been identified including CDC45, SHP-1, and SHP-2 (Hunter, 1995). In addition, a dual Thr and Tyr protein phosphatase, CL100 (also known as MAP kinase phosphatase 1) is able to dephosphorylate Thr-183 and Tyr-185 of ERK2 *in vitro* (Alessi et al., 1995). Brondello et al., (1997) showed that MAP kinase phosphatase 1 (MKP1) and MKP2 are induced by the ERK1/ERK2 pathway. Following serum activation of fibroblasts, MKP1 and MKP2 are expressed within 30 to 60 minutes, respectively, and this expression is sustained for up to 14 hours. The activation of these phosphatases correlates with the inactivation of the MAPK suggesting that the activation of the MAPK phosphatases, by MAPK, causes an inhibitory feedback loop ultimately leading to the down-regulation of MAPK (Brondello et al., 1997).

In adipose cells evidence suggests that MKP1 does not inactivate ERK2, but rather influences other minor MAPK-homologues that play roles in distinct signal transduction pathways (Alessi et al., 1995). ERK1 and ERK2 are believed to be inactivated in the cytosol by dephosphorylation of Thr-183 by PP2A and dephosphorylation of Tyr-185 by an unidentified Tyr phosphatase (Alessi et al., 1995).

To understand fully the regulation of MAPKs, the regulation of MAPKKs must be understood. MAPKKs are activated by phosphorylation of a Ser/Thr by a MAPKKK (see Figure 1.1). At this level of regulation there are a number of modes of control whereby different signal pathways may be utilized. Raf-1 phosphorylates Ser-218 and Ser-222 in human MEK1 and other MEK family members. There appears to be a proline-rich region in the MEK family not found in other MAPKK members (JNKK/SEK1/MKK4), that facilitates this specific activation (Catling et al., 1995). Unlike Raf-1, MEKK (MEK kinase) also phosphorylates JNKK/SEK/MKK4. Although Raf-1 is the normal kinase responsible for phosphorylation of MEKs, MEKK

may also phosphorylate MEK *in vivo* (for review see Denhardt, 1996). These studies are important for the understanding of adipogenesis, as Ras has been shown to be a key component of adipogenesis (Benito et al., 1991), although activation of Raf-1 by Ras does not lead to the activation of MAPK in 3T3 L1 preadipocytes (Santos and Porras, 1996). Thus the activation of MAPK by signaling from Ras must be through another upstream kinase, possibly MEKK, which leads to the eventual differentiation of preadipocytes.

There is growing evidence that the duration of MAPK activation is pivotal in determining if a cell differentiates or proliferates. Studies of PC12 neuroblasts demonstrate the complexity in the regulation of different signal-transduction pathways within a specific cell type, and the different consequences of activating MAPK through different receptors. Treatment of these cells with fibroblast growth factor or nerve growth factor (NGF) leads to differentiation into neurites, whereas treatment with epidermal growth factor (EGF) leads to cell proliferation. These differences appear to be linked to temporal MAPK regulation. EGF treatment causes transient activation of MAPK, leading to cell proliferation, while NGF treatment causes sustained activation of MAPK, leading to differentiation (Marshall, 1995, and references therein). Sustained activation of MAPK would allow translocation of the kinase into the nucleus and the subsequent phosphorylation of nuclear transcription factors. Transient activation of MAPK would allow the phosphorylation only of transcription factors which are maintained in the cytoplasm and then translocated to the nucleus. The activation of different subsets of transcription factors may thus determine cell fate.

1.II.C. MAPK and Adipogenesis

Studies by Sale et al. (1995) showed that MAPK is a key element in adipogenesis, with MAPK activation being required for signaling initiated by insulin-

and serum-stimulation, for activation of DNA synthesis, and for the induction of differentiation in 3T3 L1 cells. Zhang et al. (1996) further showed that in 3T3 L1 cells, insulin stimulation activates MAPK. These studies also showed that insulin stimulates the phosphorylation of the peroxisome-proliferator-activated receptor (PPARγ2, a potent transcriptional activator of adipogenesis; see section 1.IV.A. below) in a MAPK-dependent manner, and showed that MAPK is an important mediator of cross-talk between insulin signal transduction pathways and PPARγ2 function.

MAPK phosphorylation of PPARγ2 directly reduces the ability of PPARγ to induce adipogenesis in NIH 3T3 cells and in cells ectopically expressing the insulin receptor (Hu et al., 1996). The activation of MAPK and its subsequent phosphorylation of PPARγ2 prevents PPARγ2 induction of preadipocyte differentiation.

Further studies by Adams et al. (1997) showed that in preadipocytes PPARγ2 is phosphorylated by MAPK in response to growth factors. This phosphorylation of PPARγ2 reduces its adipogenic activity in both a ligand-dependent and ligand-independent manner by preventing the receptor from interacting with other unidentified intermediary transcription proteins, thereby preventing transcriptional activation.

MAPK activation in preadipocytes appears to be required for differentiation, but MAPK is also capable of repressing adipogenic agents such as PPARγ2. The apparent conflicting roles of MAPK may be resolved by examining the differences in stimulation of MAPK, as MAPK will translocate to the nucleus upon prolonged activation, or by examining the activation of MAPK by different receptors types which utilize similar signaling pathways but cause drastic differences in cellular outcomes (Marshall, 1996). Adipogenic agents such as insulin may cause transient MAPK activation while mitogenic agents such as growth factors may cause prolonged MAPK activation. In support of this proposal, overexpression of the insulin receptor substrate 1 causes the activation of MAPK and induces cell proliferation in fibroblasts (Ito et al., 1996). Insulin normally causes these cells to differentiate, but prolonged activation of the IRS-

1 component of the insulin signaling pathway induces cell proliferation, again indicating an activation-dependent function of MAPK in adipogenesis.

1.II.D. Transcriptional Regulation by MAPK

A number of transcription factors with a variety of DNA-binding motifs, including ETS, MADS, zinc-finger, HMG and bZIP domains, are under the control and regulation of MAPK. The phosphorylation of transcriptional factors has several consequences. Phosphorylation may affect protein stabilization, may regulate the ability of the transcription factor to bind DNA, or may increase or decrease the ability of the transcription factor to act as a repressor or activator of transcription (for review see Treisman, 1996).

The interaction between a kinase and the protein it phosphorylates may allow signal-transduction regulation of specific transcription factors. The interaction of "signal transducers and activators of transcription" (STAT) proteins with the tyrosine kinase JAK and MAPKs allows specific activation the STAT family of transcription factors during receptor activation (Ihle, 1996). Also, different isoforms of JNK/SAPK interact with, and phosphorylate, Jun with different efficiencies, thereby altering the efficiency of Jun transcription regulation (Kallunki et al., 1994). Furthermore, both ERK2 and JNK/SAPK interact with the ternary complex factor (TCF), allowing different levels of control for this transcription factor (Gille et al., 1995). The activation of these transcription factors by signal-transduction-dependent kinases shows one example of the transcription regulation by specific kinases.

The aP2 gene promoter, an important gene involved in adipocyte differentiation (see Section 1.V. below), is positively regulated by the transcription factors c-Fos and c-Jun, which interact with an AP-1 DNA site in the proximal promoter region of the aP2 gene (Su and Karin, 1996). The phosphorylation of proteins involved in the

activation of *c-jun* and *c-fos*, including those involved in the formation of the Jun/Fos heterodimer, is tightly regulated by MAPK pathways (for a review see Karin and Hunter, 1995). The MAPKs ERK1 and ERK2 are able to phosphorylate and activate TCF, which in turn activates the expression of *c-fos*. *c-jun* expression is also increased because JNK/SAPK phosphorylates c-Jun and ATF2, which form a heterodimer that then binds an AP-1 site in the *c-jun* promoter (Angel and Karin, 1991). The c-Fos and c-Jun proteins may also be phosphorylated by growth-factor-activated kinase protein kinase (fos-regulating kinase; FRK) (Deng and Karin, 1994) and JNK/SAPK, respectively, causing these phosphoproteins to form heterodimers which allows transcriptional activation through the AP-1 promoter found in various genes (Karin and Hunter, 1995).

In summary, the regulation of the important DNA element AP-1, which is found in the promoters of a number of genes including the adipogenic gene, involves a complex series of phosphorylation events ultimately leading to transcriptional activation. The induction of both *c-fos* and *c-jun* is under the control of MAPK family members, and the formation of the c-Fos/c-Jun heterodimer is directly enhanced through MAPK phosphorylation.

1.I.E. G Protein Coupled Receptor Pathways

Another major receptor type involved in cell signaling is the G protein coupled receptor (GPCR). These receptors are implicated in the transduction of signals from the plasma membrane to the nucleus, and in the regulation of genes involved in adipogenesis. One of the major modes of signal transduction involved in the initiation of adipogenesis in preadipocytes is through the action of cyclic AMP (cAMP). Increased levels of intracellular cAMP are controlled through GPCRs and trimeric G proteins.

G protein coupled receptors (also known as seven-transmembrane-segmented or serpentine receptors) are activated by the binding of hormones or neurotransmitters, which cause the $\alpha\beta\gamma$ heterotrimeric G protein to dissociate into the α subunit and the $\beta\gamma$ heterodimer. The binding of a ligand to the GPCR initially causes the exchange of GDP for GTP on the α subunit of the trimeric G protein. This exchange allows the dissociation of the α subunit from the $\beta\gamma$ subunits (see Figure 1.2). Both the $\beta\gamma$ heterodimer and the α subunit are capable of acting as signaling molecules.

There are numerous GPCRs, all containing the characteristic seven transmembrane segments. These receptors are involved in transduction of signals from a diverse collection of ligands including hormones such as catecholamines, gonadotropins, parathyroid hormone, and glucagon, along with stimulants such as odorants and light (Dohlman et al., 1991).

There are over 21 types of G protein α subunits which can be grouped into four families. Members of the α_s family stimulate adenylyl cyclase, regulate calcium channels, and are modified by cholera toxin. Members of the α_i family inhibit adenylyl cyclase, regulate calcium and potassium channels, activate cGMP, and are modified by pertussis toxin. The α_q family activates phospholipase C (PLC), while the $\alpha 12$ family regulates sodium/potassium exchange (Neer, 1995).

A variety of α subunits is expressed in most tissues, with any particular cell expressing at least 4-5 different isoforms. The α subunit contains sites for the binding of receptors, effectors, and the G$\beta\gamma$ subunits, as well as a helical domain of unknown function. The α subunit also has a GTPase domain which is responsible for binding guanine nucleotides (Neer, 1995). Most α subunits are covalently modified by the addition of the fatty acid, palmitate, by a thioester bond to a cysteine residue located at the third amino acid in the N terminus (Muller and Lohse, 1994).

Figure 1.2. G Protein Coupled Receptor Pathways.

Upon activation of a GPCR through the binding of a hormone or neurotransmitter, a trimeric G protein is activated. The conformational change associated with the receptor-ligand interaction causes a conformation change in the Gα subunit. This conformational change releases bound GDP from the Gα subunit, which is replaced by GTP; the binding of GTP causes the dissociation of the βγ heterodimer. Both the βγ heterodimer and Gα subunit are able to act on effectors. An intrinsic GTPase activity associated with the Gα subunit causes the hydrolysis of GTP to GDP, which allows the re-association and inactivation of the Gα subunit and βγ heterodimer.

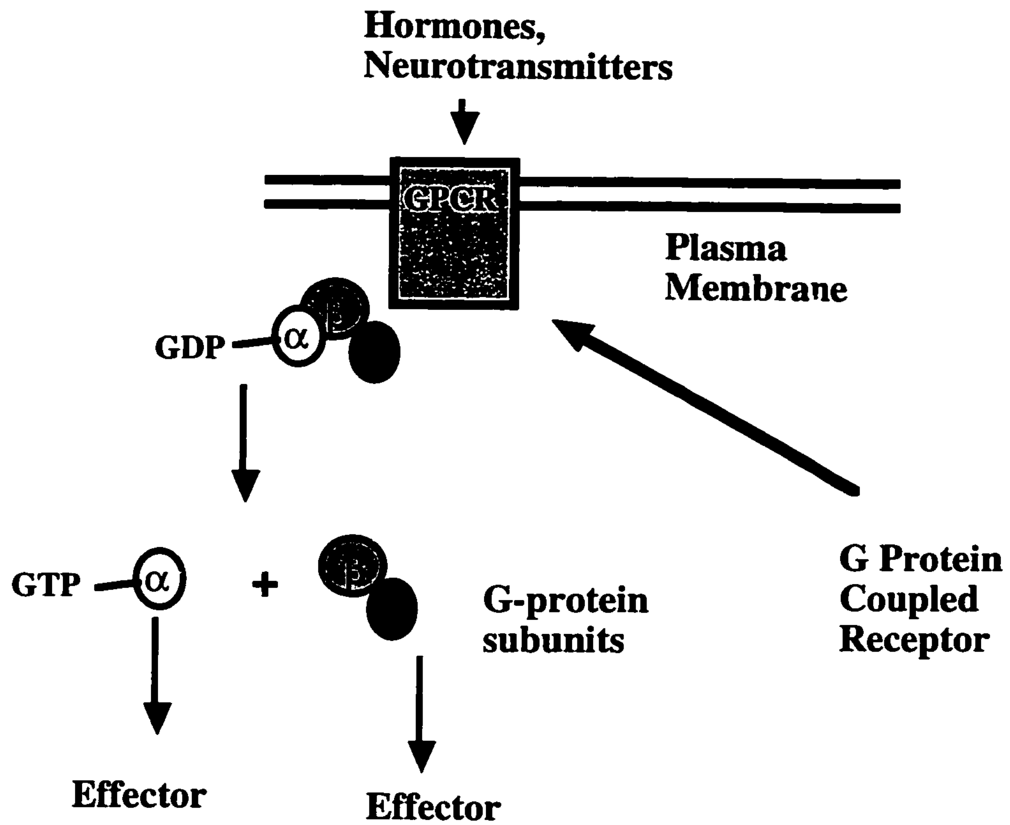


Figure 1.2. G Protein Coupled Receptor Pathways

A family of proteins termed regulators of G protein signaling (RGS) proteins has been identified. These proteins regulate G proteins by acting as GTPase-activating proteins (GAPs). It is suggested that this family of proteins negatively regulates the Gα subunit by accelerating the hydrolysis of GTP to GDP by the activated Gα subunit, thus inducing the formation of the inactive GDP-bound heterotrimeric G protein. Interestingly, RGSs may serve as multifunctional proteins. One specific RGS, RGS1, is stimulated by neural growth factor, and overexpression of RGS1 prevents nerve growth factorstimulation of MAPK (Dohlman and Thorner, 1997). Therefore, RGSs may function as regulators of cell signaling by inactivating G protein function.

There are at least five mammalian β subunits and twelve γ subunits (Sondek et al., 1996; Clapman, 1996). The variety of γ and β subunits allows numerous combinations of Gβγ heterodimers (although a number of combinations are not found), which contributes to the specificity of Gβγ effector function and strength of activation. Only certain combinations of βγ heterodimers will interact with specific effectors, thus allowing specific activation of effectors by the heterodimers.

The α subunit may interact with either the γ subunit, β subunit, or both, as the γ subunit has been shown to bind an α subunit affinity column *in vitro* (Rahmatullah and Robishaw, 1994), and the β subunit has recently been shown to interact with the α subunit by the elucidation of the trimeric G protein structure (Chapman, 1996). The binding of GDP by the α subunit of the trimeric G protein may cause a conformational change in the Gβγ heterodimer or may prevent effectors from binding to the Gβγ heterodimer (Neer, 1995).

The Gγ subunit is membrane associated through a lipid modification, by isoprenylation at a consensus sequence (a Cys-α-α-Xaa motif: Cys, cysteine; α, aliphatic amino acid; Xaa, any amino acid) found in the C terminus. If the Xaa amino acid is leucine or phenylalanine, the protein is geranylgeranylated (C20 isoprenoid); if Xaa is any other amino acid, the protein is farnesylated (C15) (Boyartchuk et al.,

1997). The $\gamma 1$ subunit is farnesylated, while all other known γ subunits are geranylgeranylated (Muller and Lohse, 1995). The γ subunit is isoprenylated by a transferase that modifies the cysteine residue in the C-terminal consensus sequence through a thioether bond. Once prenylation occurs the three remaining amino acids in the consensus sequence are proteolytically removed and the carboxyl group of the modified cysteine residue is methylated (Parish et al., 1996). In yeast, proteins termed Afc1 and Rce1 are responsible for this proteolytic cleavage. A loss of function of these proteolytic proteins prevents H-ras from localizing to the plasma membrane, instead localizing it to the endoplasmic reticulum (Boyartchuk et al., 1997). These finding suggest that the proper processing of the C terminus of the γ subunit may be necessary for correct intracellular localization. G$\beta\gamma$ heterodimers that are not isoprenylated fail to associate with membranes, activate effectors, or interact with the α subunit (Muller and Lohse, 1995).

The G$\beta\gamma$ subunit also interacts with a variety of proteins, including the G protein coupled receptor kinases (GRKs), other than the α subunit. GRKs, particularly the β-adrenergic receptor kinase (βARK) and rhodopsin kinase (Neer, 1995), appear to play a vital role in the regulation of cell signaling. Upon activation of a GPCR and subsequent dissociation of the associated trimeric G proteins, GRKs are sequestered to the plasma membrane by interaction with the G$\beta\gamma$ subunit through a pleckstrin homology (PH) domain. The PH domain is a 90- to 110- residue region first identified in pleckstrin and now found in a variety of proteins (for reviews see Shaw, 1996; Lemmon et al., 1996). The PH domain consists of seven anti-parallel β-sheets and a C terminus amphiphilic α-helix (Luttrell et al., 1995). The substrates for these GRKs are the ligand-bound activated forms of the G protein coupled receptors (Muller and Lohse, 1995). The phosphorylation of the GPCR by a GRK causes the desensitization of the receptor. This receptor phosphorylation may allow the binding of inhibitory proteins, such as arrestin, to the receptor, which may function to block cell signaling (Freedman

and Lefkowitz, 1996). The activation of GPCRs causes the release of distinct pools of Gβγ subunits which bind to different GRKs. GPCRs binding to specific Gβγ allows the specific translocation of different GRKs to the plasma membrane in response to ligand-bound stimulation of receptors (Daaka et al., 1997). Studies using several different γ and β subunits in various combinations showed varying degrees of GRK stimulation. These differences in receptor activation by GRKs may be due to a γ subunit determining specific interactions with the GRK, while the β subunit specifies GPCR recognition (Muller et al., 1993).

In vitro studies have shown that a variety of other proteins are capable of interacting with the Gβγ heterodimer through PH domains, including the GTPase-activating protein for Ras, spectrin, PLCγ, Rac (related to A and C kinase), and the oxysterol-binding protein, as well as IRS-1, SOS, and Ras-GEF protein (Touhara et al., 1994; Luttrell et al., 1995). These interactions have yet to confirmed *in vivo* and there are no known cellular functions for these *in vitro* protein-protein interactions. The Gβγ subunit is also found to bind to the serine/threonine kinase Raf-1 (Pumiglia et al., 1995). This interaction may function to sequester Raf-1 to the plasma membrane, or to negatively regulate Raf-1 by preventing it from interacting with GTP-Ras. The interaction of the Gβγ subunit with these important signaling molecules may represent another level of regulation in cell signaling with possible influences in the control of adipogenesis.

Recently, De Waard et al. (1997) showed that the Gβγ heterodimer of a trimeric G-protein binds directly to voltage-dependent calcium channels. This interaction results in the inhibition of these calcium channels. Studies by Zamponi et al. (1997) showed that PKC phosphorylated the channel in a region known to bind to Gβγ, thereby antagonizing Gβγ–induced inhibition of the calcium channel by preventing the heterodimer from binding and inhibiting the channel. The Gβγ heterodimer has also been shown to interact with the inwardly rectifying potassium channels (Wickman et

al., 1994). These studies again show that $G\beta\gamma$ plays more than a passive role in cell signaling.

With the recent elucidation of the protein structure of the trimeric G protein and the $G\beta\gamma$ heterodimer, the roles of the heterodimer are becoming more evident (Sondek et al., 1996; Lambright et al., 1996; Wall et al., 1995). This entire signaling complex is described as a 'nanomachine' consisting of a lever (receptor), switch ($G\alpha$), and propeller ($G\beta\gamma$) (Clapman, 1996). The $G\beta$ subunit exists as a 7-bladed "propeller" with a stretch of amino acids tapering from the seventh blade. This tapering region of the $G\beta$ subunit interacts with the $G\gamma$ subunit, which is partially draped over the face of the $G\beta$ subunit and interacts with the tapering of the $G\beta$ subunit in what is described as a 'intertwined thread' (Clapham, 1996). The lipid modification of the $G\alpha$ subunit inserts into the membrane at the same location as does the $G\gamma$ subunit lipid modification.

There are two recognition sites between the $G\beta$ subunit and the $G\alpha$ subunit. The first occurs at the lipid-modification site of the $G\alpha$ subunit, while the second is between the $G\alpha$ subunit switch face and an electronegative face of the $G\beta$ subunit. Upon activation of a G protein coupled receptor, the receptor flips the "switch domain" causing a conformational change in the $G\alpha$ subunit, which allows the exchange of GDP for GTP and the release of the $G\beta\gamma$ heterodimer (Clapham, 1996). This in turn allows the activation of the $G\alpha$ and $G\beta\gamma$ heterodimer. Wall et al. (1995) suggest that the position of the GPCR also allows broad access of both the $G\beta$ and $G\gamma$ subunits to the receptor.

1.II.F. Trimeric G Proteins and Adipogenesis

There have been studies detailing the role of G proteins in adipogenesis. Shinohara et al. (1991) examined the role in adipocyte differentiation of the family of G proteins which are sensitive to pertusis toxin. This sub-family of G proteins was found

to negatively regulate differentiation. Uehara et al. (1994) found that the levels of pertusis sensitive G protein decrease during adipogenesis, while Huppertz et al. (1993) showed that G protein levels increased after differentiation of the adipocyte cell. McFarlane-Anderson et al. (1993) showed that with the induction of differentiation there was a redistribution of $G\alpha_i$ and Gβ associated with loss of organization of stress fibers, and consistent with the cell rounding that is associated with adipogenesis. These studies indicate that G proteins are involved in the maintenance of the preadipocyte phenotype, are down-regulated during differentiation, and then are required for maintenance of the adipocyte phenotype after differentiation.

G proteins may also be involved in the insulin-mediated pathway during the later stages of adipogenesis. Robinson et al. (1992) proposed that the binding of insulin to its receptor causes signaling activation of the glucose transporter 4 (GLUT 4). This signaling may require ATP in initial stages of signaling, and require G proteins in later stages. More recently, Moxham and Malbon (1996) developed transgenic mice harboring inducible expression of antisense RNA to the mRNA for $G\alpha_{i1}$. They found that $G\alpha_{i1}$ was vital for insulin action in adipose tissue, and that $G\alpha_{i1}$ deficiency increases protein-tyrosine phosphatase activity and attenuates insulin-stimulated phosphorylation of IRS-1. Studies by Wang and Malbon (1996) showed that the levels of $G\alpha_s$ decrease in response to inducers of differentiation in 3T3 L1 cells, while the levels of $G\alpha_{i2}$ are enhanced. They found that either the suppression of $G\alpha_s$ or constitutive expression of $G\alpha_{i2}$ dramatically stimulated adipogenesis. These studies indicate that G proteins may play a major role in the regulation of adipogenesis.

Begin-Heick (1996), using cells from white epididymal adipose tissue from the obese *ob/ob*, mice found that these cells had very low lipolytic activity in comparison to normal adipose tissue, possibly due to a defect in G protein signaling. This decrease in enzymatic activity could be caused by low adenylyl cyclase activity which is activated by a GPCR, the β-adrenergic receptor. The failure of this GPCR to stimulate lipolysis

in the *ob/ob* mice may be caused by an impaired G protein function in response to stimulus.

The regulation of G proteins is apparently vital for the initiation of adipogenesis, and again in the later stages of differentiation. Further study into the mechanism of G protein regulation may lead to important insight into the regulation of adipogenesis.

1.II.G. Integration of the Gβγ Heterodimer and MAPK Pathways

The initiation of adipogenesis involves the coordination of several signals acting through diverse pathways. The convergence of these signals requires cross-talk between these distinct pathways. That is, different classes of membrane receptors can influence cellular processes such as proliferation and differentiation by using proteins which are involved in more than one signaling pathway. The Gβγ heterodimer has been implicated as one of these molecules.

There is growing evidence that the Gβγ heterodimer of trimeric G proteins is involved in regulating the mitogen-activated protein kinase pathway. MAPK activation mediated through GPCRs and RTKs is postulated to occur through a common signaling pathway (Daub et al., 1996). Studies showed that Shc activation, through tyrosine phosphorylation, allows the formation of a Shc-Grb2-Sos complex, which enhances the activity of Sos and leads to the activation of MAPK (See Figure 1.3). However, this activation is not through a direct interaction between the Gβγ subunit and Sos (van Blesen et al., 1995). The activated Shc acts as an adapter protein binding both RTK and Grb2.

Dikic et al. (1996) showed that upon stimulation of GPCR with bradykinin and lysophosphatidic acid, Pyk2 is tyrosine phosphorylated. The activated Pyk2 activates

Figure 1.3. Crosstalk between Tyrosine Kinase Receptor and G Protein Coupled Receptor Pathways.

The activation of GPCRs allows the exchange of GDP for GTP on the Gα subunit, causing the disassociation of the Gα from the Gβγ heterodimer. The Gβγ heterodimer then activates PI3K or Pyk2, leading to the activation of a Src-like kinass, which can tyrosine phosphorylate Shc to cause the formation of a Receptor-Shc-Grb2 complex. The activated Src-like kinase can also tyrosine phosphorylate a receptor tyrosine kinase (RTK), leading to the recruitment of the adapter protein Grb2 and then Sos. The recruitment of Grb2 and Sos, by either mechanism, leads to the activation of Ras and the eventual activation of MAPK. (P) indicates proteins which are phosphorylated. Arrows indicate major route of signal transduction and the activation of a protein by a prior protein. Question marks (?) above arrows indicate that the proteins involved at this stage of the pathway are not known.

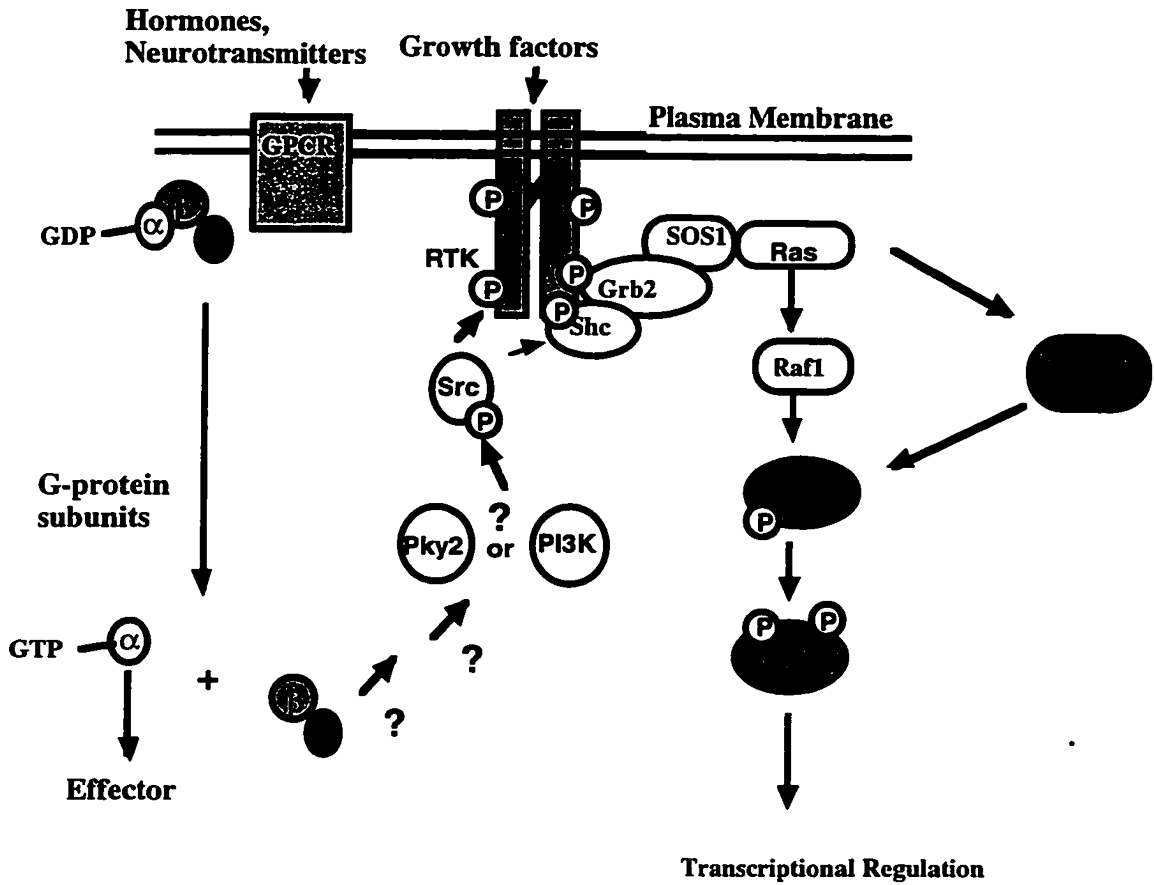


Figure 1.3. Crosstalk between Receptor Tyrosine Kinase and G Protein Coupled Receptor Pathways.

and binds Src through an SH2 domain. The Pyk2 activated Src-tyrosine kinase may then phosphorylate and activate Shc. The Src-like kinases Lyn and Syk are also found to be necessary for Gβγ activation of MAPK (Wan et al., 1996).

Further studies by Lopez-Ilasaca et al. (1997) showed that phosphoinositide 3-kinases (PI3Ks) are able to activate tyrosine phosphorylation of Shc, which leads to the activation of MAPK. The Gβγ heterodimer may localize PI3K to the plasma membrane, thereby giving this enzyme access to a large lipid substrate pool. This recruitment of PI3K to the plasma membrane activates Src-like kinases, which in turn activates Shc. To further add interest to these observations, studies by Luttrell et al. (1997) showed that activation of Src by Gβγ causes Src to tyrosine phosphorylate the epidermal growth factor (EGF) receptor. This activation allows direct recruitment of Shc and Grb to the EGF receptor, and leads to the eventual activation of MAPK. These studies illustrate the complex regulation mediated by Gβγ in the receptor tyrosine kinase signaling pathways.

The Gβγ heterodimer is also thought to activate MAPK through Raf-1 (Pumiglia et al., 1995) although the nature of the activation is not yet known (for review see Malarkey et al., 1995). It has been suggested that the Gβγ heterodimer targets Raf-1 to the membrane, acting analogously to Ras.

These studies demonstrate the complexity of integrating numerous signals. The numbers of receptors and signaling molecules involved in transducing a cellular response, presents a surmountable challenge for the cell. The cell must be able to distinguish among signals from many different receptors which utilize the same proteins in order to elicit cellular responses including differentiation and cell proliferation. The mechanism by which these molecules are ultimately regulated to elicit these cellular responses is still under investigation.

1.II.H. Protein Scaffolding

The regulation of complex cellular events that are mediated by numerous signals, such as adipogenesis, may be directed by scaffolding proteins. These scaffolding proteins physically bring together a number of proteins that participate in a signaling pathway to enhance the signal, or may bring together proteins from distinct signaling pathways to integrate multiple signals.

The Ste5p protein has been shown to be a scaffold protein vital for the mating process in the haploid budding yeast *S. cerevisiae* (Whiteway et al., 1996). This protein utilizes components of the G protein coupled receptor pathways (Hasson et al., 1994) and kinases associated with receptor kinase pathways (Choi et al., 1994) to transduce a signal. The binding of a pheromone to its GPCR causes a trimeric G protein to disassociate into the α and βγ subunits. The βγ heterodimer anchors the scaffold protein Ste5p, which appears to function as a homodimer (Yablonski et al., 1996), to the plasma membrane where it is able to mediate the formation of a kinase complex, or kinase module. The scaffold binds the PAK protein Ste20 (which phosphorylates the MEKK protein), Ste11 (which phosphorylates the MEK protein Ste7), Ste7 (which in turn phosphorylates the MAPK Fus3 and Kss1p), and the MAPK Fus3 and Kss1p. MAPK can then phosphorylate the transcription factor Ste12, which ultimately leads to the transcriptional activation of genes required for pheromone arrest. The scaffold protein Ste5 may also bind Bem1, a protein involved in regulating actin and the cytoskeleton (Faux and Scott, 1996; Leberer et al., 1997). Several other proteins including Ste50, Akr1, Syg1, Gpa1, and Cdc24 have been found to interact with Ste4 (Gβ), and are believed to further regulate the Ste5 mediated mating response (Leberer et al., 1997). Interestingly, many of these proteins, as well as Ste5, require the Gγ subunit to interact with the Gβ subunit (even in yeast two-hybrid experiments), indicating that the Gγ subunit is critical for interaction of the aforementioned proteins

with the $G\beta\gamma$ heterodimer (Leberer et al., 1997). It is proposed that several scaffold proteins exist to facilitate responses from different stimuli, and thereby allow different modules of kinases to be brought together to elicit a cellular response (Leberer et al., 1997).

The first protein shown to act as a scaffold was the yeast protein Ste5. Since the elucidation of Ste5 function in yeast, several mammalian proteins have also been shown to act as scaffolds. For example the A-kinase anchoring protein (AKAP79) has been implicated as a scaffolding protein (Klauck et al., 1996). This protein binds cAMP-dependent kinase (PKA), phosphatase 2B, and protein kinase C (PKC), and co-localizes these proteins at postsynaptic densities in neurons. The localization by a scaffold protein places two second-messenger-regulated kinases and a phosphatase in a signaling complex that is positioned to receive signals transduced across the synapase. Similarly, anchoring proteins termed "receptors for activated protein kinase C" (RACKs), may act in a similar fashion and be required for the binding and activation of PKC (Mochly-Rosen et al., 1995).

In 3T3 L1 adipocytes, a protein termed "protein targeting for glucose " (PTG) has been implicated as a scaffolding protein (Printen et al., 1997). In addition to binding glycogen and protein phosphatase PP1, PTG complexes with phosphorylase kinase, phosphorylase a, and glycogen synthase, the primary enzymes involved in hormonal regulation of glycogen metabolism. PTG is only expressed in insulin-responsive cells and may form a "metabolic molecule" with the aforementioned enzymes and the glycogen particle, which may play a role in the regulation of insulin action on glucose and lipid metabolism.

As both the G protein coupled receptors (GPCR) and tyrosine kinase receptors (RTK) have been shown to be involved in the differentiation pathway in preadipocytes (Shinohara et al., 1991; Uehara et al., 1994; Porras et al., 1994; Porras and Santos, 1996), it is possible that the formation of a scaffold would bring together signals from

both the GPCR and RTK to direct and enhance the differentiation process. It is interesting to speculate that the signaling required for the initiation of adipogenesis is regulated by a scaffolding protein, as the differentiation process has analogies to the yeast mating response including cell-cycle arrest, modulation of transcription, and changes in cellular morphology. A scaffolding protein may integrate the signals known to induce adipogenesis through the activation of key, or "master" genes that are involved in this process.

1.III. Transcriptional Regulation of Adipogenesis: Activators

The transduction of signals, through the cell-signaling pathways described above, ultimately leads to modulations in the transcription levels of genes required to maintain the preadipocyte phenotype and to initiate differentiation. The biochemical and morphological changes that accompany adipogenesis occur with coordinate alterations in the levels of expression of numerous proteins. These changes are due mostly to alterations in the transcriptional levels of corresponding genes (for a comprehensive summary see Table 1 in Cornelius et al., 1994). There are many studies characterizing the *cis*-regulatory elements and *trans*-acting factors which are involved in the coordinated regulation of genes required during preadipocyte differentiation, and growing evidence that a discrete number of proteins act as "master" regulators responsible for this coordinate expression. The most likely candidate regulators are the CCAAT/enhancer-binding protein α (C/EBP α), the peroxisome-proliferator-activated receptor γ (PPARγ), and the adipocyte determination- and differentiation-dependent factor, or sterol-response-element-binding protein-1 (ADD1/SREBP1). This section outlines some of these key transcription factors known to contribute to the differentiation of preadipocytes into adipocytes.

1.III.A. Peroxisome-Proliferator-Activated Receptor γ (PPAR γ)

PPARγ is implicated as a "master" regulator in adipocyte differentiation, able to activate fat-specific genes and initiate the differentiation process in preadipocytes (Spiegelman and Flier, 1996). PPARγ belongs to the peroxisome proliferator-activated receptor subfamily of nuclear hormone receptors, and exists as two alternative splicing isoforms (γ1 and γ2) (Zhu et al., 1995). PPARγ2 is found to form a heterodimer with retinoid-X receptor (RXRα); both RXRα and PPARγ2 are components of the adipocyte regulatory factor 6 (ARF6), which binds to DNA sites termed the adipose regulatory element (ARE6 and 7) found in the distal promoter region of the adipose P2 gene (aP2) (Tontonoz, 1994a; discussed in section 1.VI.). The DNA sequence PPARγ2 binds to is termed the direct repeat of the hormone response element (DR-1). A number of synthetic and natural ligands are known to activate PPARγ2. The synthetic ligands belong to a class of drugs known as thiazolidinediones, while natural eicosanoid derivatives such as prostaglandins D2 and J2 have also been shown to activate PPARγ2 (Brun et al., 1996).

Tontonoz et al. (1994b) showed that PPARγ2 is specifically expressed at high levels in adipocytes, and that ectopic expression of PPARγ2 in fibroblast cells led to their conversion into adipocytes, thus indicating that PPARγ2 was able to convert a cell line not predestined to become adipocytes into mature adipocytes. PPARγ2 must therefore be able to activate genes which are programmed to induce cells to differentiate into adipocytes.

PPARγ2 is phosphorylated by a mitogen-activated protein kinase (MAPK), a key kinase activated by RTKs including those for insulin and IGF-1 (see Figure 1.1 for regulation of MAPK by RTK, and MAPK activation of transcription factors), and this phosphorylation reduces its transcription activation ability (Hu et al., 1996a). Both ERK2 and JNK are found to phosphorylate PPARγ1 and 2 at Ser84 and Ser118

respectively, in the N-terminal A/B domain (Adams et al., 1997). The phosphorylation of PPARγ2 by MAPK inhibits both ligand-dependent and ligand-independent *trans*-activation functions of the receptor (Adams et al., 1997). MAPK phosphorylation of PPARγ2 directly reduces its ability to induce adipogenesis (Hu et al., 1996a). These studies indicate that phosphorylation of PPARγ2 by MAPK may be a mechanism by which signals to initiate differentiation are blocked, leading to the maintenance of the preadipocyte phenotype.

Interestingly, epidermal growth factor (EGF) and transforming growth factor α, acting through the EGF receptor, inhibit adipogenesis, as does tumor necrosis factor α (TNFα). TNFα and EGF stimulate MAPK, which in turn phosphorylates and inhibits PPARγ2, thus blocking adipogenesis (Adams et al., 1997). Furthermore, elevated levels of intracellular cAMP, a prerequisite for adipogenesis, are known to block MAPK activity in rat adipocytes. cAMP-dependent stimulation of protein kinase A also directly inhibits Raf-1, leading to the eventual inhibition of EGF-mediated MAPK activation (Graves and Lawrence, 1996; Adams et al., 1997). Thus elevated levels of intracellular cAMP also prevents MAPK from phosphorylating and inhibiting PPARγ2. The direct regulation of PPARγ2 by MAPK, through a number of receptor-activated pathways, illustrates the complex forms of regulation of a transcription factor (PPARγ) through a number of distinct signaling pathways.

1.III.B. The CCAAT/Enhancer Binding Protein (C/EBP) Family

Another important group of transcription factors involved in adipogenesis is the C/EBP family of proteins. There are a number of C/EBP isoforms which are expressed during different stages of adipocyte differentiation. These proteins play a vital role in the initiation of adipogenesis and throughout the differentiation process.

C/EBPβ and C/EBPδ are transiently expressed during the initiation of preadipocyte differentiation and play a key role in the initiation of adipogenesis. Ectopic expression of these proteins induces adipogenesis in fibroblasts through enhancement of expression of the transcriptional activator PPARγ2 (Yeh et al., 1995; Wu et al., 1995). Elevated levels of PPARγ2 mRNA are found in cells co-expressing C/EBPβ and C/EBPδ, suggesting that these factors may directly regulate PPARγ2 expression, as the promoter region of PPARγ2 has two C/EBP binding sites (Zhu et al., 1995; Wu et al., 1996).

Expression of a third member of the C/EBP family, C/EBPα, occurs after the differentiation process has began. Expression of C/EBPα at higher than physiological levels also induces adipogenesis in fibroblasts (Freytag et al., 1994), whereas expression of antisense C/EBPα RNA suppresses adipogenesis in 3T3 L1 cells (Lin et al., 1992). These results suggest that C/EBPα is a key regulator essential for adipogenesis. Furthermore, when C/EBPα is expressed at physiological levels it acts in synergy with PPARγ2 to induce adipogenesis in fibroblasts and myoblasts (Tontonoz et al., 1994b). Thus C/EBPα is capable of converting cells destined to become muscle cells (myoblasts) into adipocytes, again indicating that C/EBPα plays a vital role in mechanism which results in adipogenesis.

A protein believed to influence C/EBP and to play a role in adipogenesis is the retinoblastoma protein (Rb). Rb interacts with and activates members of the C/EBP family, but only during adipocyte differentiation (Chen et al., 1996). Specifically, Rb binds to C/EBPβ, which enhances C/EBPβ binding to its DNA sites thereby activating C/EBPβ. Interaction of Rb proteins with C/EBPβ may activate expression of PPARγ, as C/EBPβ is known to activate transcription of the PPARγ gene (Hu et al., 1996b), thus influencing the activation of adipogenesis.

1.III.C. The Adipocyte Determination- and Differentiation-Dependent Factor

The Adipocyte Determination- and Differentiation-Dependent Factor (ADD1), also known as the sterol regulatory element-binding protein (SREBP), is another transcription factor that influences adipogenesis. ADD1 is able to induce genes involved in fatty acid synthesis, cholesterol homeostasis, and glycerolipid synthesis, including glycerol-3-phosphatase acyltransferase, fatty acid synthetase, and lipoprotein lipase (Ericsson et al., 1997), but alone is not capable of inducing adipogenesis (Kim and Spiegelman, 1996). However, ectopic expression of ADD1 in preadipocytes increases the percentage of cells that undergo adipogenesis when fibroblasts are induced to differentiate (Kim and Spiegelman, 1996). These effects are thought to be mediated, in part, by the activation of PPARγ expression, as co-transfection of PPARγ with ADD1 causes a further 3-4 fold increases in PPARγ activity. Similarly, a dominant negative form of ADD1 prevents expression of the vital adipogenic genes PPARγ, adipsin, aP2, and C/EBPα, which ultimately leads to inhibition of adipogenesis (Kim and Spiegelman, 1996). These results indicate that ADD1 plays an important role in the initiation of adipogenesis and is crucial for the expression of other key regulators of the differentiation process.

The relationships between PPARγ, C/EBP, and ADD1 are critical for adipogenesis (for review see Brun et al., 1996; Spiegelman and Flier, 1996). In summary, initiation of the transient expression of C/EBPβ and C/EBPδ by 1-methyl-3-isobutylxanthine and dexamethasone causes the stimulation of expression of PPARγ2, possibly through interaction with C/EBP-binding sites in the promoter of the PPARγ2 gene. ADD1 also increases the expression of PPARγ2, possibly through the production of an endogenous ligand that binds to and activates PPARγ2. Insulin and IGF-1 have been shown to increase the transcriptional activation of PPARγ2 mediated by ADD1,

which may explain why PPARγ2 activity is enhanced in response to insulin (Brun et al., 1996). ADD1 also induces several genes involved in fatty acid metabolism and cholesterol homeostasis.

The formation of the activated PPARγ-RXRα heterodimer mediates the continuation of adipogenesis and induces of C/EBPα expression. The induction of C/EBPα in turn mediates the continued expression of PPARγ, possibly through binding to C/EBP sites in the PPARγ promoter region. Both C/EBPα and PPARγ act in synergy to maintain the fully differentiated adipocyte. This may occur through the direct binding of C/EBPα and PPARγ to C/EBP elements, which are found in the upstream promoter regions of both genes, through a direct interaction between PPARγ and C/EBPα.

1.IV. Negative Regulation of Adipogenesis

Although the majority of studies have focused on the initiation of adipogenesis, others have dealt with the negative regulation of adipogenesis and the maintenance of the preadipocyte state. It is becoming evident that the inactivation of negative regulators of transcription is an important component in the control of adipogenesis.

1.IV.A. Active and Passive Repression

Transcriptional repressor molecules have been grouped into two broad classes termed passive and active (Cowell, 1994). Passive repressors are targeted against activators, and use a number of strategies to down-regulate transcription. These repressors may compete for DNA-binding sites, thereby preventing activators from binding, or may 'squelch' or sequester activators by forming heterodimers that prevent the activator from binding to an enhancer region. Similarly, a repressor may mask the

activation domain of a transcriptional activator, thereby allowing the activator to bind to a promoter but preventing it from activating transcription.

Active repression, on the other hand, involves the down-regulation of the transcriptional process itself. It has been suggested that active repressors inhibit transcription by interfering with the pre-initiation transcription complex, through interactions with the general transcription machinery. Inhibitory factors have been shown to associate with the general transcriptional machinery, in particular TFIID and TBP (Inostroza, 1992). The repressors may encourage the formation of inactive initiation complexes. Active repressors may also interact with other transcription factors to form complexes that occlude the binding of activators (Cowell, 1992). In sum, active repressors directly influence the transcriptional machinery, whereas passive repressors influence transcription by interfering with other transcription factors, such as activators.

1.IV.B. C/EBP Family Members

C/EBP proteins may play a role in the negative regulation of adipogenesis. Two proteins, LIP and CHOP-10, are involved in passive transcriptional regulation by forming heterodimers with the aforementioned C/EBP proteins. LIP, which does not have a *trans*-activating domain, forms heterodimers with C/EBP proteins, thereby preventing the activation of transcription *in vitro* (Descombes and Schibler, 1991). CHOP-10, which is unable to bind DNA, also forms heterodimers with C/EBP proteins in this case preventing the C/EBP proteins from binding DNA and activating transcription (Ron and Habener, 1992). Although these proteins are expressed differentially during adipogenesis, their roles in this process remain to be elucidated (for review see Cornelius et al., 1994).

Another transcription factor found to negatively regulate the C/EBP family of transcription factors and to inhibit adipogenesis is c-Myc. In 3T3 L1 cells

overexpression of c-Myc blocks the normal induction of C/EBPα and prevents adipogenesis. c-Myc, by binding to the promoter of the C/EBPα gene thereby repressing C/EBPα gene expression, or by activating other factors which negatively regulate C/EBPα, may inhibit differentiation (Freytag and Geddes, 1992). By inhibiting the induction of the potent transcriptional activator of adipogenesis, C/EBPα, c-Myc is able to block differentiation and promote proliferation in these fibroblast-like cells.

1.IV.C. Tumor Necrosis Factor α (TNFα)

The cytokine tumor necrosis factor α (TNFα) also plays a role in the negative regulation of adipogenesis. The addition of TNFα to preadipocytes prevents the accumulation of triacylglycerol and inhibits the expression of adipocyte-specific mRNAs when these cells are induced to differentiate (Torti et al., 1985). *In vitro*, TNFα is found to affect lipid metabolism by suppressing the synthesis of key enzymes involved in triacylglycerol accumulation associated with adipogenesis, including lipoprotein lipase, glycerolphosphate dehydrogenase, and acetyl coenzyme A carboxylase (Zhang et al., 1996). Furthermore, TNFα is able to 'dedifferentiate' (cause the loss of the differentiated phenotype) mature fat cells by suppressing expression of adipocyte-specific mRNAs (reviewed in Zhang et al., 1996). The TNFα-mediated inhibition of adipogenesis occurs through transcriptional inhibition of C/EBPα, a potent transcription activator of adipogenic genes, or by the activation of c-Myc, a transcription repressor of C/EBPα (Ninomiya-Tsuji et al., 1993). TNFα also inhibits expression of PPARγ in 3T3 L1 cells prior to inhibition of expression of C/EBPα and the adipocyte fatty acid-binding protein; these inhibitory effects can be overcome by overexpression of PPARγ (Zhang et al., 1996). These studies indicate that

dedifferentiation process mediated by TNFα is due to inhibition of PPARγ, which occurs during the early events of this process.

Studies of obese rodent models linked TNFα to obesity-associated insulin resistance common to adult-onset diabetes (non-insulin dependent), as adipose tissue from these mice exhibited elevated levels of TNFα (Hotamisligil et al., 1993). TNFα-treated cells showed both decreased receptor tyrosine phosphorylation and decreased activation of the insulin receptor and the insulin receptor substrate (IRS-1) (Hotamisligil et al., 1994). Interestingly, cells treated with TNFα showed induced serine phosphorylation of the insulin receptor substrate. This serine phosphorylation converts IRS-1 into an inhibitor of the insulin receptor, possibly by blocking tyrosine phosphorylation or autophosphorylation of the receptor, or by localizing inhibitory proteins to the receptor (Hotamisligil et al., 1996). Thus by regulating molecules involved in the RTK pathway (see Figure 1.2), including the insulin receptor and IRS-1, TNFα is able to induce insulin resistance in adipocytes, which is associated with obesity.

Treatment of 3T3 L1 cells with TNFα also causes the down-regulation of another well characterized family of transcription factors, the "signal transducers and activators of transcription" (STAT). Upon binding of a cytokine (including TNFα) to a receptor, STAT proteins are phosphorylated on a tyrosine residue by the Janus kinase (JAK). The phosphorylated STAT proteins then dimerize and translocate with the kinase to the nucleus. Stephens et al. (1996) found that when preadipocytes were treated with TNFα to block differentiation, the expression of the adipogenic inducers STAT1 and STAT5 was suppressed. These results illustrates the broad range of inhibitory effects attributed to TNFα.

From these results, it is apparent that TNFα plays an important role in the regulation of adipogenesis and influences a number of the positive factors (PPARγ and C/EBPα) known to initiate and sustain adipocyte differentiation. The ability of TNFα to

dedifferentiate mature adipocytes shows that it influences factors vital for the maintenance of the mature adipocyte phenotype.

1.IV.D. The Preadipocyte Factor 1 (pref-1)

The preadipocyte factor 1 (pref-1) is a novel member of the epidermal growth factor (EGF) -like family of proteins. This protein is abundant in preadipocytes but is completely down-regulated during differentiation. Constitutive expression of pref-1 in preadipocytes blocks adipocyte differentiation (Smas and Sul, 1993). As EGF activation of the EGF receptor is known to inhibit adipogenesis, possibly through the prolonged activation of MAPK (as described in section 1.II.), it is speculated that pref-1 may function analogously to growth factors, binding to the EGF receptor and activating the cell to proliferate. The cessation of pref-1 negative influence on adipogenesis may occur during the growth arrest in the G1/S stage of the cell cycle, which is a prerequisite for the initiation of adipogenesis. It is also speculated that pref-1 may modulate cell-cell interactions by interacting with protein interaction domains termed EGF-repeats of unidentified proteins, thereby playing a role in the structural remodeling processes associated with adipogenesis (Smas and Sul, 1993).

1.IV.E. *cis*-Acting Repressor Elements

A number of genes involved in adipogenesis contain *cis*-acting elements which may be bound by repressors. These *cis*-acting elements may allow negative regulators to maintain the preadipocyte phenotype through repression of critical adipogenic genes including PPARγ, C/EBPα, and ADD1, or upon the initiation of adipogenesis inhibit the expression of genes involved in the maintenance of the preadipocyte phenotype (i.e. pref-1).

A preadipocyte repressor element (PRE) is found in the promoter of the stearoyl-CoA desaturase-2 (SCD2) gene. This *cis*-acting element is believed to be bound by proteins which repress this gene prior to differentiation. An unidentified 58-kDa protein found in preadipocytes but not present in adipocytes is found to bind to PRE and inhibit transcription (Swick and Lane, 1992). Binding of this unidentified protein to the PRE promoter region of the SCD2 gene and to the promoters of other genes containing the PRE elements may be necessary for maintenance of the preadipocyte in an undifferentiated state.

The 422/aP2 (Ro and Roncari, 1991) and adipsin (Wilkison et al., 1990) genes contain overlapping negative and positive regulator elements. These negative elements may bind factors which repress expression of these genes in preadipocytes. An understanding of the mechanisms by which unknown proteins repress transcription of these important genes which are activated during adipogenesis, and the mechanisms by which these repression functions are alleviated, would give valuable insight into the regulation of adipogenesis.

1.V. Adipocyte Fatty Acid Binding Protein - FABP

The alterations in the expression of several proteins during adipogenesis allows the newly differentiated adipocyte to perform one of its primary functions, the storage of triacylglycerols. The expression of one protein, the adipocyte fatty acid-binding protein (FABP), which is highly expressed in adipocytes, is considered a marker for adipocyte differentiation.

FABP is encoded by the adipose P2 (aP2/422) gene and belongs to a super-family of highly tissue-specific proteins. These proteins are capable of binding hydrophobic ligands, including fatty acids, retinoids, and prostaglandins, and may also function as substrates for tyrosine kinase receptors (Jaworski and Wistow, 1996).

FABP is phosphorylated by the insulin receptor (Buelt et al., 1991) and this phosphorylation occurs during, and may be critical for, the differentiation process (Blake and Clarke, 1990). Studies by Jarvis et al. (1989) also showed that FABP is phosphorylated on a Ser residue by an unidentified kinase, indicating a further level of regulation of this protein.

Hotamisligil et al. (1996) showed that aP2-deficient mice developed dietary obesity, but did not develop the insulin resistance associated with diabetes, whereas control mice did. These aP2-deficient mice also did not express TNFα, indicating that TNFα may be involved in insulin resistance associated with diabetes. As mentioned in section 1.IV.C., overexpression of TNFα causes adipocytes to dedifferentiate, and TNFα also inhibits the insulin receptor and IRS-1 in a manner commonly associated with insulin resistance. Hotamisligil et al. (1996) suggest that the aP2 gene product, FABP, may be a central player in the pathway that links obesity to insulin resistance through connecting fatty acid synthesis with TNFα expression. Free fatty acids (FFA) may induce insulin resistance by increasing the expression of TNFα or other genes which interfere with insulin action. FABP may enhance FFA induction of TNFα expression by shuttling FFAs to targeted cellular compartments (Hotamisligil et al., 1996). Therefore FABP would shuttle FFAs to cellular compartments, which would ultimately cause increased expression of TNFα by an unknown mechanism. In aP2-deficient mice, the lack of FABP would prevent this enhanced expression of TNFα by FFAs, which in turn would prevent TNFα mediated insulin resistance.

The regulation of transcription of the aP2 gene has been highly studied and has provided critical insight into the regulation of preadipocytes and the development of adipogenesis. The aP2 gene is regulated by an upstream promoter region known to function as either a positive or negative element in the regulation of aP2 gene expression, with two positive-acting transcription factors, C/EBP and AP-1, having consensus binding sites (Herrera et al., 1989; Ro and Roncari, 1991).

A Fat-Specific element (FSE2) located between -122 to -101 from the transcription start site of the aP2 promoter, and also found in the G3PDH promoter (another gene activated during differentiation), has been shown to bind the proto-oncogene product *c-fos* complex in mouse preadipocyte and adipocyte extracts (Distel et al. 1987). Furthermore, this complex contains the product of the proto-oncogene c-*jun* (Bohmann et al., 1987). c-Jun has been shown to interact with c-Fos, forming heterodimers that stimulate the ability of c-Jun to bind DNA (Cowell, 1994). The binding of this c-Jun–c-Fos complex, termed AP-1, is through the recognition of an AP-1 site located in FSE2 (Rauscher et al., 1989). This suggests that c-Fos and c-Jun-like proteins interact and influence the regulation of the aP2 gene.

The upstream proximal promoter region of the mouse aP2 gene (nucleotides -159 to -125 from the transcription start site, termed the AE-1 sequence), along with related sites in the promoters of various other genes, contains binding sites for the CCAAT/ enhancer-binding protein (C/EBP) (Herrera et al., 1989). Three forms of C/EBP, C/EBPβ, and δ, (expressed early in differentiation), and C/EBPα (expressed later in differentiation), all bind to this AE-1 site (Cao et al., 1991; explained in section 1.III.B). This indicates that the aP2 gene is activated throughout the adipogenesis process.

A 500-base pair fragment of the aP2 distal promoter region (-5.4 to -4.9 kb) was also found to be important in activating the aP2 gene during adipogenesis (Graves et al., 1990). Within this enhancer region are a number of protein binding sites termed adipose regulatory elements (ARE). NF-1, along with a number of other adipocyte regulatory factors (ARFs), has been shown to enhance transcription of the aP2 gene by binding to the adipose regulatory elements. Graves et al. (1992) showed that the sequences ARE2 and ARE4 were bound by the nuclear factor ARF2 which is present in many cell types. Another factor, ARF6, was found to bind to the adipose specific sites ARE6 and ARE7, with mutations in these sites greatly reducing the enhancer activity of

the ARF6 transcription activator complex (Graves et al., 1990). Tontonoz et al. (1994a, b) found that ARF6 was composed of PPARγ and RXRα (discussed in section 1.III.A.). These studies suggest a mechanism by which PPARγ activates transcription a number of important adipogenic genes, including aP2, in its role as a 'master' regulator of adipogenesis.

1.VI. Adipocyte Enhancer-1 Binding Protein - AEBP1.

In 3T3 preadipocytes, an AE-1 binding factor termed AEBP1 is found to bind to the AE-1 site and has been implicated as a silencer of aP2 gene expression (Distel et al., 1987; Herrera et al., 1989; Ro and Roncari, 1991). Thus AEBP1 may be involved in the regulation and maintenance of the preadipocyte phenotype.

The cDNA encoding the human homologue to AEBP1 has been cloned from osteoblasts. The protein is almost identical to the mouse AEBP1, except for an additional 105 amino acids located in the N terminus. This AEBP1 homologue was found to exist only in adipocyte tissue (a mixture of preadipocytes and adipocytes) and in osteoblasts, and was implicated as an osteoblast-specific gene which is down-regulated during differentiation and not found in mature bone. A similar transcription regulatory role for AEBP1 in osteoblast differentiation was suggested, although no target gene has been uncovered (Ohno et al., 1996).

The deduced protein sequence of the cDNA encoding mouse AEBP1 (Figure 1.4) shows that the protein is made up of three distinct domains (Figure 1.5). The N terminus contains a region that shows homology to the discoidin I protein of *Dictyostelium discoideum*. Similar discoidin domains, termed discoidin-like domains (Figure 1.6), have been found in several extracellular membrane proteins and are

Figure 1.4. Sequence of AEBP1.

The cDNA and deduced amino acid sequence of AEBP1 clone. The underlined stop codon at the 5' untranslated region (UTR) is in the same reading frame as the first methionine codon at nucleotide (nt) 160. A putative polyadenylation signal (aataaa) in the 3' UTR is underlined. Underlined restriction sites were used to generate mutants described in section 3.I.D.. The potentially interesting primary structure motifs highlighted in bold letters include a region (amino acids 622 to 656) that is rich in serine, threonine, and proline (STP-like sequences), which is flanked by a basic (amino acids 598 to 619, 46% arginine), and an acidic (amino acids 660 to 699, 58% glutamic acid) region. To isolate cDNAs encoding proteins that interact with the AE-1 site (nt -159 to -125 of the aP2 gene) in 3T3 preadipocytes, cDNAs from a 3T3-L1 preadipocyte cell library in the a Uni-Zap XR vector (Stratagene) were screened by the Affinity Screening procedure with random concatamers of the AE-1 sequence. Three independent phage plaques contained fusion proteins interacting specifically with the AE-1 sequence. Further analysis revealed that one cDNA clone encoded mRNA whose expression was down-regulated during adipocyte differentiation. This partial cDNA (~0.7 kb) clone was used as a probe to isolate a full-length cDNA from the same library. One clone contained a ~2.5 kb cDNA, and this cDNA was completely sequenced (He et al., 1995).

```
EcoR I Adaptor
tttcccttgctgtcggtctgaaccactagcccacgaoggtgtgtgcacatgcagagtgcccacctattgggatgggagt
cacccgcattgaggacaaccagatccgtgcctcctccatgctgcgccacggcctcggagcccagcgggccgggctcaac
                                                                          Nae I
160/1                                   190/11
ATG CAG GCT GGT GCC AAT GAA GAT GAC TAC TAT GAC GGG GCA TGG TGT GCT GAG GAC GAG
Met gln ala gly ala asn glu asp asp tyr tyr asp gly ala trp cys ala glu asp glu
220/21                                  250/31
TCG CAG ACC CAG TGG ATC GAG GTG GAC ACC CGA AGG ACA ACT CGG TTC ACG GGC GTC ATC
ser gln thr gln trp ile glu val asp thr arg arg thr thr arg phe thr gly val ile
280/41                                  310/51
ACT CAG GGC CGT GAC TCC AGC ATC CAT GAC GAC TTC GTG ACT ACC TTC TTT GTG GGC TTC
thr gln gly arg asp ser ser ile his asp asp phe val thr thr phe phe val gly phe
340/61                                  370/71
AGC AAT GAC AGC CAG ACC TGG GTG ATG TAC ACC AAT GGC TAC GAG GAA ATG ACC TTC TAT
ser asn asp ser gln thr trp val met tyr thr asn gly tyr glu glu met thr phe tyr
400/81                                  430/91 Sac I
GGA AAT GTG GAC AAG GAC ACA CCT GTG CTG AGC GAG CTC CCT GAG CCA GTT GTG GCC CGT
gly asn val asp lys asp thr pro val leu ser glu leu pro glu pro val val ala arg
460/101                                 490/111
TTC ATC CGC ATC TAT CCA CTC ACC TGG AAC GGT AGC CTG TGC ATG CGC CTG GAG GTC CTA
phe ile arg ile tyr pro leu thr trp asn gly ser leu cys met arg leu glu val leu
520/121                                 550/131
GGC TGC CCC GTG ACC CCT GTC TAC AGC TAC TAC GCA CAG AAT GAG GTG GTA ACT ACT GAC
gly cys pro val thr pro val tyr ser tyr tyr ala gln asn glu val val thr thr asp
580/141                                 610/151
AGC CTG GAC TTC CGG CAC CAC AGC TAC AAG GAC ATG CGC CAG CTG ATG AAG GCT GTC AAT
ser leu asp phe arg his his ser tyr lys asp met arg gln leu met lys ala val asn
640/161                                 670/171                          Bgl II
GAG GAG TGC CCC ACA ATC ACT CGC ACA TAC AGC CTG GGC AAG AGT TCA CGA GGG CTC AAG
glu glu cys pro thr ile thr arg thr tyr ser leu gly lys ser ser arg gly leu lys
700/181                                 730/191
ATC TAC GCA ATG GAA ATC TCA GAC AAC CCT GGG GAT CAT GAA CTG GGG GAG CCC GAG TTC
ile tyr ala met glu ile ser asp asn pro gly asp his glu leu gly glu pro glu phe
760/201             Bam HI              790/211
CGC TAC ACA GCC GGG ATC CAC GGC AAT GAG GTG CTA GGC CGA GAG CTC CTG CTC CTG CTC
arg tyr thr ala gly ile his gly asn glu val leu gly arg glu leu leu leu leu leu
820/221                                 850/231
ATG CAA TAC CTA TGC CAG GAG TAC CGC GAT GGG AAC CCG AGA GTG CGC AAC CTG GTG CAG
met gln tyr leu cys gln glu tyr arg asp gly asn pro arg val arg asn leu val gln
880/241                                 910/251
GAC ACA CGC ATC CAC CTG GTG CCC TCG CTG AAC CCT GAT GGC TAT GAG GTG GCA GCG CAG
asp thr arg ile his leu val pro ser leu asn pro asp gly tyr glu val ala ala gln
940/261                                 970/271
ATG GGC TCA GAG TTT GGG AAC TGG GCA CTG GGG CTG TGG ACT GAG GAG GGC TTT GAC ATC
met gly ser glu phe gly asn trp ala leu gly leu trp thr glu glu gly phe asp ile
1000/281                                1030/291
TTC GAG GAC TTC CCA GAT CTC AAC TCA GTG CTC TGG GCA GCT GAG GAG AAG AAA TGG GTC
phe glu [illegible] leu trp ala ala glu glu lys lys trp val
1060/3[illegible]                       1090/31[illegible]
CCC TAC [illegible] ATC CCT GAG CGT TAC CTG TCC CCA GAT GCC
pro tyr arg val pro [illegible] ile pro glu arg [illegible] pro asp ala
1120/321                                1150/331
ACG GTC TCC ACA GAA GTC [illegible] GTG CTG
thr val ser thr glu val [illegible] val leu
1180/341                                1210/351
GGT GCC AAT CTG AAC GGC [illegible] TGG ACA
gly ala asn leu asn gly gly [illegible] ala arg thr
1240/361                                1270/371
CCT AGC CAG GAG CAG CTG TTG GCC GAG GCA CTG GCA GCT GCC CGC GGA GAA GAT GAT GAC
pro ser gln glu gln leu leu ala glu ala leu ala ala ala arg gly glu asp asp asp
1300/381                                1330/391
GGG GTG TCT GAG GCC CAG GAG ACT CCA GAT CAC GCT ATT TTC CGG TGG CTG GCC ATC TCA
gly val ser glu ala gln glu thr pro asp his ala ile phe arg trp leu ala ile ser
1360/401                                1390/411
TTT GCC TCC GCC CAT CTC ACC ATG ACG GAG CCC TAC CGG GGA GGG TGC CAG GCC CAG GAC
phe ala ser ala his leu thr met thr glu pro tyr arg gly gly cys gln ala gln asp
1420/421                Hinc II         1450/431
TAC ACC AGC GGC ATG GGC ATT GTC AAC GGG GCC AAG TGG AAT CCT CGC TCT GGG ACT TTC
tyr thr ser gly met gly ile val asn gly ala lys trp asn pro arg ser gly thr phe
1480/441                                1510/451 Sac I
AAT GAC TTT AGC TAC CTG CAC ACA AAC TGT CTG GAG CTC TCC GTA TAC CTG GGC TGT GAC
asn asp phe ser tyr leu his thr asn cys leu glu leu ser val tyr leu gly cys asp
1540/461                                1570/471
AAG TTC CCC CAC GAG AGT GAG CTA CCC CGA GAA TGG GAG AAC AAC AAA GAA GCG CTG CTC
lys phe pro his glu ser glu leu pro arg glu trp glu asn asn lys glu ala leu leu
1600/481                                1630/491
ACC TTC ATG GAG CAG GTG CAC CGT GGC ATT AAG GGT GTG GTG ACA GAT GAG CAA GGC ATC
thr phe met glu gln val his arg gly ile lys gly val val thr asp glu gln gly ile
1660/501                                1690/511   Sty I
CCC ATT GCC AAT GCC ACC ATC TCT GTG AGT GGC ATC AAC CAT GGT GTG AAG ACA GCA AGT
pro ile ala asn ala thr ile ser val ser gly ile asn his gly val lys thr ala ser
1720/521                                1750/531
GGA GGT GAC TAC TGG CGC ATT CTG AAC CCG GGT GAG TAC CGT GTG ACA GCT CAC GCA GAG
gly gly asp tyr trp arg ile leu asn pro gly glu tyr arg val thr ala his ala glu
1780/541                                1810/551
GGC TAC ACC TCA AGT GCC AAG ATC TGC AAT GTG GAC TAC GAT ATT GGG GCC ACT CAG TGC
gly tyr thr ser ser ala lys ile cys asn val asp tyr asp ile gly ala thr gln cys
1840/561                                1870/571
AAC TTC ATC CTG GCT CGA TCC AAC TGG AAG CGC ATT CGG GAG ATC TTG GCT ATG AAC GGG
asn phe ile leu ala arg ser asn trp lys arg ile arg glu ile leu ala met asn gly
1900/581              Hinc II           1930/591
AAC CGT CCC ATT CTC CGA GTT GAC CCC TCA CGA CCC ATG ACC CCC CAG CAG CGG CGC ATG
asn arg pro ile leu arg val asp pro ser arg pro met thr pro gln gln arg arg met
1960/601                                1990/611
CAG CAG CGC CGT CTA CAG TAC CGG CTC CGC ATG AGG GAA CAG ATG CGA CTG CGT CGC CTC
gln gln arg arg leu gln tyr arg leu arg met arg glu gln met arg leu arg arg leu
2020/621                                2050/631
AAT TCT ACC GCA GGC CCT GCC ACA AGC CCC ACT CCT GCC CTT ATG CCT CCC CCT TCC CCT
asn ser thr ala gly pro ala thr ser pro thr pro ala leu met pro pro pro ser pro
2080/641                                2110/651                   Pst I
ACA CCA GCC ATT ACC TTG AGG CCC TGG GAA GTT CTA CCC ACT ACC ACT GCA GGC TGG GAG
thr pro ala ile thr leu arg pro trp glu val leu pro thr thr thr ala gly trp glu
2140/661                                2170/671
GAG TCA GAG ACT GAG ACC TAT ACA GAA GTA GTG ACA GAG TTT GAG ACA GAG TAT GGG ACT
glu ser glu thr glu thr tyr thr glu val val thr glu phe glu thr glu tyr gly thr
2200/681                                2230/691                          Stu I
GAC CTA GAG GTG GAA GAG ATA GAG GAG GAG GAG GAG GAG GAG GAG GAA GAG ATG GAC ACA
asn leu glu val glu glu ile glu glu glu glu glu glu glu glu glu glu met asp thr
2260/701                                2290/711
GGC CTT ACA TTT CCA CTC ACA ACA GTG GAG ACC TAC ACA GTG AAC TTT GGG GAC TTC TGA
gly leu thr phe pro leu thr thr val glu thr tyr thr val asn phe gly asp phe ---
2320                                    2360
gactgggatctcaaagccctgcccaattcaaactaaggcagcacctcccaagcctgtgccagcagacacatagcccatcag
2400                                    2440
atgtccctgggtggaccccactcccccagtgtgggacatcaaagctaccgggactctgcatagactctggtctacccgcc
2480                                    2520
ccagctctacctgccagcctttggggaggggcaggcaaggaagccaacgtcacatcaataaaacaagctcatgacaccaa
2560
aaaaaaaaaaaaaaaaactcgag
                Xho I
```

Figure 1.5. Domains of AEBP1 protein.

"DLD" is the discoidin-like domain. The "Carboxypeptidase" domain resembles a B-like carboxypeptidase. The "basic" and "acid" regions are possible DNA-binding motifs. "STP" is a region rich in serine, threonine, and proline. Numbers at the bottom of the cartoon depict amino acid number, 1 being the N terminus, 719 being the C terminus.

Figure 1.5. Domains of AEBP1 protein.

Figures 1.6. Discoidin-like Domains.

Amino acid sequence alignments of the N-terminal domain of AEBP1 with discoidin I (Disc), the A5 protein, the receptor tyrosine kinase (DDR), a milk fat globule protein (MFG-E8), and the coagulation factors V (CF-V) and VIII (CF-VIII) with ~35% sequence identity. The vertical lines and dots indicate identical and similar amino acids, respectively; ---, gaps introduced to optimize the alignments. Numbers indicate amino acid position (He et al., 1995, and references therein).

Figure 1.6 Discoidin-like Domains

Figure 1.7. Carboxypeptidase Domains.

Amino acid sequence alignments of AEBP1 with regulatory-type carboxypeptidases (CPH/E, CPN and CPM). Amino acids thought to be important in the active centers of bovine CPA and B are marked as follows: #, Zn-binding; o, catalytic; *, substrate-binding. The vertical lines and dots indicate identical and similar amino acids, respectively; ---, gaps introduced to optimize the alignments. Numbers indicate amino acid position (He et al., 1995, and references therein).

Figure 1.7 Carboxypeptidase Domains

believed to be involved in protein-protein interactions. The main body of the protein is highly similar (46-50% identity) to the regulatory carboxypeptidases E (CPE), CPM, CPH, and CPN (Figure 1.7). The C terminus contains a basic, STP (serine, threonine, and proline), and acidic domains. These peptide motifs have been implicated in DNA binding, as a polypeptide encoding this C-terminal region of AEBP1 was cloned by specifically binding to the AE-1 site of the aP2 gene (He et al., 1995).

1.VI.A. AEBP1 C-terminal Motifs

An interesting feature of AEBP1 is its possible DNA-binding region. In fact, the C-terminal of AEBP1 was cloned by screening cDNAs that encoded proteins that bound to the AE-1 site of the aP2 promoter region (He et al., 1995). Within this C terminus there is a region rich in arginine, and another region rich in glutamic acid (Figures 1.4 and 1.5). These regions may be responsible for the DNA-binding ability attributed of AEBP1, as other putative DNA-binding transcription factors utilize similar motifs to bind DNA. The ability of AEBP1 to bind DNA indicates that it may function as a transcriptional regulator of the aP2 gene by interacting with the AE-1 sequence found in the aP2 promoter.

In the C terminus of AEBP1 there is a serine, threonine, and proline rich region, which contains possible MAPK consensus phosphorylation sites (Pro-Xaa-Ser/Thr-Pro; Alvarez et al., 1991). Other transcription factors have been shown to be phosphorylated and regulated by MAPK isoforms. Therefore, AEBP1 may be phosphorylated in this C-terminal region by a proline-directed kinase like MAPK.

1.VI.B. AEBP1 Carboxypeptidase Domain

The most fascinating aspect of AEBP1 is a domain similar to the proteolytic carboxypeptidase enzymes. Carboxypeptidases are proteases that cleave C-terminal amino acids from polypeptides. There are two broad classes or families of carboxypeptidases: the digestive type, and the regulatory B-like type. The digestive carboxypeptidases play an important role in general protein and peptide degradation. Carboxypeptidase A cleaves C-terminal aromatic and aliphatic amino acids, whereas carboxypeptidase B cleaves basic amino acids and glycine. The lysosomal carboxypeptidase B removes all C-terminal amino acids except proline (Fricker, 1988).

The second family of carboxypeptidases is the B-like or regulatory carboxypeptidases. These enzymes remove C-terminal arginine and lysine, and play an important role in the processing of certain polypeptides and hormones. Member of this family of enzymes participate in the release of active peptides, alter membrane receptors specificity for polypeptides, and terminate polypeptide activity (Skidgel 1988; see Table 1 and reference therein). The physiological importance of these B-like carboxypeptidases is exemplified by carboxypeptidase E, which is involved in insulin processing. Mature insulin is produced by a series of proteolytic cleavages. Pro-insulin is cleaved by an endoprotease at dibasic Arg-Arg and Lys-Arg sequences. Carboxypeptidase E then removes the remaining Arg-Arg sequences found at the C terminus, resulting in the mature insulin molecule.

Carboxypeptidases have been shown to be involved in the onset of obesity. Naggert et al. (1995) showed that mice homozygous for the *fat* mutation are obese and hyperglycemic. The mutation responsible for this phenotype was found in the carboxypeptidase E gene. A single amino acid substitution, Ser-202 to Pro, in a highly conserved region of carboxypeptidase E destabilizes and abolishes enzyme activity.

Another interesting example of the diversity and importance of carboxypeptidases is seen in the function of tubulin carboxypeptidase. This enzyme cleaves C-terminal tyrosines from subunits of tubulin, a modification that occurs during neural and muscle differentiation. The modification of tubulin by this carboxypeptidase may be required to modulate tubulin during these differentiation processes (Webster et al., 1992).

Carboxypeptidase M is a membrane-bound enzyme that removes C-terminal arginine or lysine from various polypeptides (Skidgel, 1988). McGuire and Skidgel (1995) showed that carboxypeptidase M cleaves epidermal growth factor (EGF) at the C-terminal Arg to form des-Arg53-EGF. This modified form of EGF was found to bind to its receptor better, although the physiological function of this cleavage is unknown.

The above examples demonstrate the diverse functions of the regulatory B-like carboxypeptidases in a several cell functions including differentiation, and cell signaling. The main body of AEBP1 (amino acids 136-551) shows sequence similarity to several regulatory type carboxypeptidases (Figure 1.7).

1.VI.C. Other Proteolytic Enzymes Regulate Transcription

As AEBP1 may function as a DNA-binding protein and as a protease, it is interesting to review other proteins with similar functions. The targeting of a proteolytic enzyme to a DNA sequence in itself is not novel. There are several examples of enzymes that are involved in transcriptional repression. For example, the filamentous fungi *Aspergillis nidulans* and *Neurospora crasssa* use proteins with sequences similar to a putative pentafunctional enzyme as transcriptional regulators. A transcriptional repressor protein with sequence similarity to the C terminus of the enzyme, and a transcriptional activator with similarity to the N terminus of the same protein, are

believed to have originated from a pentafunctional enzyme which catalyzes five consecutive steps in the anabolic shikimate pathway leading to the synthesis of aromatic amino acids (Hawkins et al., 1993). Although the repressor and activator are not believed to have functional enzyme activity, the activator has developed the ability to bind DNA, and both have the ability to influence transcription of the genes encoding the enzymes involved in the shikimate pathway (the repressor binds the activator and inhibits transcription by an unknown mechanism). This shows an example of a protein evolving a new and interesting transcriptional regulatory function although losing its enzymatic capabilities.

T7 lysozyme is an amidase, which cleaves amide bonds in peptidoglycan layer of the bacterial cell wall, and is able to form a complex with T7 RNA polymerase and inhibit transcription (Moffatt and Studier, 1987). The formation of the lysozome–RNA polymerase complex provides a negative-feedback mechanism during a late transcription phase of T7 infection, and stimulates DNA replication. The alignment of the zinc ligands in T7 lysozyme with those of carboxypeptidase A and thermolysin shows structural similarities with the amidase. This sequence comparison suggests that this amidase may function by a similar catalytic mechanism to that of AEBP1. The two functions of the lysozyme are not separated into distinct domains but the protein can only perform one function at a time (Cheng et al., 1994). This example shows that a functional enzyme can act as a transcriptional regulator.

In the biotin operon of *E. coli*, BirA is a transcriptional regulatory protein and also an enzyme (biotin-protein ligase) that catalyzes the covalent attachment of biotin to certain proteins in carboxylation and decarboxylation reactions. BirA binds to the biotin operon and represses transcription of this operon (Cronan, 1989). This repression occurs when biotinoyl-AMP (a intermediate in the biotinoylation reaction) complexes with BirA and accumulates, thereby allowing BirA to bind to the *bio* operon. This is a

unique example where an activated enzyme associated with a reaction intermediate is able to bind DNA and repress transcription of its own operon.

In the bacterium *Salmonella typhimurium*, the proline utilization operon (*put*) consists of the *putP* gene, which encodes a proline transporter, and the *putA* gene which encodes an enzyme that functions as both a proline dehydrogenase and a 1-pyrroline-5-carboxylate dehydrogenase that catalyzes the oxidation of proline to glutamate. The PutA protein also functions as a transcriptional repressor of the *putP* and *putA* genes in response to proline. PutA is unique as it functions both as a membrane-associated enzyme and as a DNA-binding protein that represses transcription of its own operon. In the absence of proline, PutA remains in the cytoplasm where it binds to the *put* operon, preventing gene expression. In the presence of proline, PutA binds proline and associates with the electron-transport chain in the membrane where it functions as an active enzyme (Muro-Pastor and Maloy, 1995). The PutA protein shows an interesting example of an enzymes having two distinct functions in different cellular compartments.

One of the best known examples of an enzyme directly regulating transcription by proteolytic means is found in the SOS regulon of *E. coli*. In SOS regulation, the lexA protein inhibits transcription of 17 genes, known as the *din* genes, by binding to control regions of these genes. The RecA protein functions to catalyze DNA recombination, and also inactivates the LexA protein. In response to DNA damage a DNA fragment binds to the RecA protein, which allows the now-activated RecA to bind to LexA, which activates, by allosteric interaction, a serine protease activity in LexA resulting in the autocleavage. The proteolyized, fragmented lexA protein is unable to bind DNA, and unable to repress transcription of the *din* genes involved in DNA repair. This allows the *din* genes to be transcribed and the process of DNA repair to be carried out. Once DNA is repaired, RecA is down-regulated as it is no longer required in an active state, which allows newly synthesized LexA protein to again repress the *din*

genes (Shinagawa, 1996; Takahashi et al., 1996). Here is an example of a DNA-bound enzyme LexA and "coprotease" RecA using a protease activity to regulate transcription.

Finally, bleomycin hydrolase (BH), a cysteine protease, has been found to bind DNA and act as a repressor in the yeast *GAL* gene regulatory system (BH has been termed Gal6 in yeast). Bleomycin is a glycometallopeptide produced by *Streptomyces verticillus* and is used as an anti-cancer drug because of its ability to cleave double-stranded DNA. BH detoxifies bleomycin by hydrolyzing an amide bind, and cancer cells with increased amounts of BH are resistant to bleomycin. A cDNA encoding the yeast form of BH was unexpectedly cloned as a DNA-binding factor that interacts with the upstream activating sequence $(UAS)_G$ of the *GAL* system, and the Gal4 protein has been shown to regulate expression of the *GAL6* gene (BH) by binding to a site in the *GAL6* promoter. When *GAL6* is deleted, the levels of *GAL* gene RNA increase, indicating that Gal6 acts as an repressor of the *GAL* genes. It has been suggested that the enzymatic and regulatory function of BH are intertwined, and that an active protein may bind DNA (Joshua-Tor et al., 1995). The binding of BH to DNA may cause a conformational change in BH that facilitates enhanced access to the inner active site of the enzyme. The two apparently interrelated functions of BH show an exciting example whereby a bifunctional protein's enzyme activity may be enhanced though the binding of DNA.

From these examples, it may be expected that a proteolytic enzyme activity may be utilized in transcription repression. AEBP1 may provide such an example.

1.VI.D. AEBP1 Discoidin-like Domain

The N terminus of AEBP1 shares 35% similarity with a protein termed discoidin found in *Dictyostelium discoideum* (Poole et al., 1981) (Figures 1.5 and 1.6). Several other proteins contain discoidin-like domains (DLD), which are believed

to be involved in cell adhesion, and may interact with specific cell surface molecules (Alves et al., 1995). DLDs are found in the extracellular domain of A5 neural antigen of *Xenopus laevis* (Takagi et al., 1991), are implicated in anionic phospholipid binding in milk fat-globule membrane proteins (Johnson et al., 1993), and are found in coagulation factors V (CF-V) (Kane and Davie, 1986) and CF-VIII (Wood et al., 1984), and in certain receptor tyrosine kinases (Stubbs et al., 1990). Therefore, this DLD region in AEBP1 may interact with other proteins, which may act as substrates for, or regulators of, AEBP1.

1.VII. Purpose of Thesis

The study of adipogenesis has provided critical insight into the regulation of obesity, as well as providing a powerful tool (a differentiating 3T3 L1 cell line) for the study of complex biochemical mechanisms, including transcription and cell signaling. As the relationships between the major players (PPARγ, C/EBP, and ADD1) in adipogenesis unfold, there are still a number of unanswered questions concerning the maintenance of the preadipocyte phenotype, and the intracellular signals that stimulate the onset of adipogenesis. This thesis explores the possible role of AEBP1, a characterized preadipocyte protein, in the transcriptional regulation of the preadipocyte state and in the transduction of signals which induce adipogenesis of preadipocytes.

These studies were carried out by exploring the functions of distinct domains of AEBP1 as described above in section 1.VI. Since the cDNA encoding AEBP1 was cloned by its ability to bind a discrete DNA sequence found in the promoter of the fat-specific aP2 gene, the ability of AEBP1 to bind DNA is addressed. The most likely regions for DNA binding are the acidic and basic regions located in the C terminus. As AEBP1 binds the adipocyte aP2 gene and is down-regulated during differentiation,

AEBP1 may act as a transcriptional repressor. The role of AEBP1 as a transcriptional repressor is also explored.

In the C terminus of AEBP1 are a number of possible phosphorylation sites. MAPK is a well-characterized enzyme which plays an indispensable role in the signaling which determines whether a cell proliferates or differentiates. The active MAPK is known to phosphorylate several transcription factors found in the nucleus and cytoplasm. The possible phosphorylation of AEBP1 by MAPK is explored, as well as a possible role of AEBP1 in MAPK-regulated signaling pathways.

The N terminus of AEBP1 contains a Discoidin-like domain, which has been implicated in protein-protein interaction for a number of other proteins. This thesis also describes studies to determine if AEBP1 interacts with other proteins.

The main body of AEBP1 contains a domain with a high degree of similarity to the family of B-like carboxypeptidases. A study of the AEBP1 carboxypeptidase domain determines if the protein is indeed a functional carboxypeptidase, and characterizes the proposed enzyme activity. As AEBP1 may be able to bind DNA, repress transcription, and act as a protease, studies are carried out to determine if these functions are correlated.

Chapter 2: Materials and Methods

The Materials and Methods chapter is divided into four main sections. The first three sections concern the maintenance and manipulation of the different cell systems used: *Escherichia coli*, *Saccharomyces cerevisiae*, and mouse cultured cell lines. The fourth section deals with *in vitro* studies using the products and materials described in the first three sections. All materials used for the preparation of yeast and bacteria liquid and solid media were purchased from Difco Laboratories. All materials used in the maintenance of cultured cells were purchased from GIBCO. All other materials were purchased from Sigma Chemical Co. unless otherwise indicated.

2.I. Bacterial Procedures

2.I.A. Transformation of Competent Cells

Plasmid DNA was introduced into *Escherichia coli* cells through a calcium chloride transformation method (Sambrook et al., 1989). An appropriate amount of DNA (20-50 ng of a ligation product or 100 ng of plasmid DNA) was mixed with 50-100 µl of competent cells (see Table 2.1 for a list of the competent cells used) and incubated on ice for 30 minutes. The mixture was heat shocked at 37°C for 40 seconds, placed on ice for 2 minutes, and 950 µl of Luria-Bertani medium (LB: 0.5% [w/v] yeast extract, 1% [w/v] peptone, 1% [w/v] NaCl; for solid medium add 2 % [w/v] agar; prepared as described in Sambrook et al., 1989) was added. The cells were then incubated at 37°C for 1 hour with mild shaking (250 rpm). The cells were spread onto LB solid medium (prepared as described in Sambrook et al., 1989) supplemented with

Vector	Usage	Cell type
pET-16b	Protein expression, purification	DH5α, DE3
pACT	Two-hybrid, library expression	DH5α, Y153
pGBT9	2-hybrid, bait	DH5α, Y153
pBlueScriptSKII(+)	Sequencing, sub-cloning	DH5α
pGEM-3Zf(+)	*In vitro* transcription/ translation	DH5α
pGALTKCAT	Reporter for CAT assay	DH5α, NIH 3T3
pG4	Expression vector for CAT assay	DH5α, NIH 3T3
pSKV10	Expression vector for CAT assay	DH5α, NIH 3T3
pUC-CAT	Control reporter plasmid for CAT assay	DH5α, NIH 3T3
pTKCAT	Control reporter plasmid for CAT assay	DH5α, NIH 3T3
pRXV40CAT	Control reporter plasmid for CAT assay	DH5α, NIH 3T3
pAU3CAT	Control reporter plasmid for CAT assay	DH5α, NIH 3T3
pHermes-LacZ	Expresses LacZ - adjusting cell extracts by β-galactosidase assay	DH5α, NIH 3T3

Table 2.1. Vectors and Cell Types Used.

ampicillin (50 μg/ml) and incubated overnight at 37° C. Single colonies were then selected for plasmid DNA purification or for recombinant protein expression.

2.I.B. Preparation of Plasmid DNA

Plasmid DNA was isolated from transformed *E. coli* cells by a protocol described by Sambrook et al. (1989) with minor modifications. A single transformed colony of *E. coli* (strain dependent upon experiment; see Table 2.1) was picked and grown overnight at 37°C in 5 ml of LB medium containing ampicillin (50 μg/ml) with constant shaking (300 rpm).

The cells were collected by centrifugation at 5000 rpm for 5 minutes and the supernatant was discarded. The pellets were resuspended in 350 μl of STET buffer (8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0) by gentle vortexing, and lysed by incubation with 25 μl lysozyme (10 mg/ml) for 5 minutes at room temperature. Samples were boiled for 40 seconds and then chilled on ice for 10 minutes. The samples were centrifuged at 14 000 rpm for 10 minutes and the aggregated cell debris was removed with a toothpick. The plasmid DNA was precipitated by adding an equal volume of isopropanol, incubating the samples for 10 minutes at room temperature, and then centrifuging the samples for 10 minutes at 14 000 rpm. The DNA pellet was washed with 70% ethanol.

The samples were resuspended in 200 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) supplemented with RNase A (10 μg/ml) and incubated at 37°C for 15 minutes. The DNA was extracted once with phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1). The plasmid DNA was precipitated by adding 0.1 volume of 7.5 M sodium acetate (pH 8) and 2.5 volume of 100 % ethanol followed by incubation at -20° C for at least 2 hours. The plasmid DNA was precipitated by centrifugation at 14 000 rpm at 4° C for 15 minutes and then washed twice with 70%

ethanol. The dried pellet was resuspended in TE buffer and used for transformations or restriction digests.

2.I.C. Enzymatic Manipulations Using Purified Plasmid DNA

Restriction enzyme digests were carried out under standardized conditions (37°C for 1 hour) with the reactions containing 5 units of restriction enzyme (BioRad Laboratories, BRL, or New England Biolabs, NEB), 1x reaction buffer (BRL or NEB), plasmid DNA, and distilled water (to a final volume of 20 µl). For partial digests the amount of restriction enzyme and duration of incubations were varied.

Blunt-end DNA fragments were generated by Klenow or T4 DNA polymerase (BRL) after restriction digests were performed. After a restriction digest, 0.5 µl of 2 mM dNTP mixture (Boehringer Mannheim) was added along with Klenow enzyme to fill 5' overhangs; T4 polymerase was used for removing 3' overhangs. Both reactions were carried out at 30° C for 15 minutes.

Calf intestinal alkaline phosphatase (CIAP; Promega) was used to dephosphorylate DNA fragments to prevent religation. After a restriction enzyme digest, 1 unit of CIAP along with CIAP reaction buffer was added to the digest and incubated at 37°C for 1 hour.

To ligate an insert into a plasmid, T4 DNA ligase (BRL) was used. The reaction contained 1 unit of T4 ligase, 1x ligase buffer, and 1 mM ATP. All ligation mixtures were incubated at 4° C overnight.

2.I.D. DNA Fragment Isolation and Agarose Electrophoresis

Agarose gel electrophoresis was performed as described by Sambrook et al. (1989). Electrophoresis-grade agarose (BRL) was dissolved in 1x TAE buffer (10x

TAE: 40 mM Tris-acetate, 1 mM EDTA), and ethidium bromide (500 ng/ml) was added before casting the gel. DNA samples were prepared by the addition of 5x loading buffer (50% glycerol, 0.3% xylene cyanol, 0.3% bromophenol blue). The molecular size markers used were *Hind*III-digested lambda ladder (BRL). DNA fragments were visualized using a UV transilluminator and gel fragments were excised with a razor blade.

To isolate DNA fragments generated by restriction endonuclease enzyme reactions, Geneclean (BIO 101 Inc.) was used according to the manufacturers' instructions. For DNA isolated from TAE agarose gels, 4.5 ml NaI (6 M) was added per gram of excised gel. Gel fragments were incubated at 50°C for 5 minutes or until all gel fragments were melted. For DNA in solution, 3 volumes of NaI was added.

For DNA isolated from agarose gels or free in solution, 5 µl of Glassmilk (BIO 101 Inc.) was added, and the samples were incubated on ice for 10 minutes. The Glassmilk with its bound DNA was pelleted by centrifugation at 14 000 rpm for 10 seconds and then washed 3x with 500 µl New Wash Solution (BIO 101 Inc.). The pellet was suspended in 5 µl of water and incubated at 50°C for 5 minutes to release the DNA. The Glassmilk was separated by centrifugation and the supernatant containing the DNA was removed. The final incubation process was repeated to remove residual DNA from the Glassmilk.

2.I.E. Induction and Purification of Recombinant Protein

Induction

Inserts cloned into the vector pET-16b were transformed into DH5α cells, and transformants were screened by isolation of plasmid DNA followed by endonuclease restriction digests to identify positive clones. Once clones were identified, they were transformed into DE3 cells. Single colonies were selected and grown overnight in 2 ml

LB medium containing ampicillin (100 μg/ml) at 37°C. From these overnight cultures, 1 ml was transferred to 500 ml of LB medium containing ampicillin (100 μg/ml) in 1 litre flasks. The cells were grown at 37°C for approximately 4 hours until the absorbance at 600 nm (A_{600}) reached 0.5 to 0.6. To induce protein expression, isopropylthio-β-D-galactoside (IPTG, 2 mM final concentration) was added and the cells were grown at 37°C for a further 3.5 hours.

To determine the efficiency of induction, the cells in 1 ml samples from the induced and pre-induced cultures were collected by centrifugation at 5000 rpm for 5 minutes and resuspended in 1x loading buffer (NEB). The total protein from the samples was resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 30:1, acrylamide/bisacrylamide; prepared and run as described in Ausubel et al., 1994).

Cells were collected from a 500 ml culture by centrifugation at 5000 rpm for 5 min. The pellet was resuspended and lysed by incubation in lysis buffer (50 mM NaH_2PO_4, 10 mM Tris-HCl , 6 M guanidinium-HCl , 100 mM NaCl, adjusted pH to 8; 2.5 ml per 50 ml of culture) with gentle mixing for 1 hour at room temperature. The resuspended material was sonicated for 30 to 60 seconds and then centrifuged at 12 000 rpm for 10 minutes to remove cell debris. The soluble cell lysate produced in this way was stored at -20°C until further purification.

Purification

Purification of His-tagged recombinant protein was carried out using Talon Metal Affinity Resin (Clontech) according to the manufacturer specifications with a number of minor modifications. The resin (2 ml) was prepared by centrifuging at 700 rpm to remove the 20% ethanol in which the resin was suspended. The resin was then equilibrated by incubation with lysis buffer.

Lysate was added to the equilibrated resin and incubated for 1 hour at room temperature with constant agitation. After the incubation, the resin was collected by centrifugation at 700 rpm for 5 minutes and the eluent was discarded. The resin was washed with 10 ml lysis buffer, mixed by gentle shaking for 10 minutes, and collected by centrifugation for 5 minutes at 700 rpm, and the supernatant was discarded. This washing step was repeated twice. The washed resin was resuspended in 2 ml lysis buffer and transferred to a BioRad column. Once the resin had settled, the column was allowed to drain.

The resin was washed with 4-6 ml of lysis buffer followed by wash buffer (50 mM NaH_2PO_4, 8 M urea, 100 mM NaCl; adjust pH to 8 for the 1st wash and pH 7 for the 2nd wash) until the A_{280} was less than 0.01. The two pH wash steps were repeated at each pH until the desired absorbance reading was obtained.

The recombinant protein was eluted from the resin with 6 ml of elution buffer (50 mM NaH_2PO_4, 8 M urea, 20 mM PIPES, 100 mM NaCl; adjust pH to 6.3) and 1 ml fractions were collected, with protein concentrations monitored at A_{280}. A second elution was performed using elution buffer adjusted to pH 5, with the fractions similarly collected and monitored.

SDS-PAGE was performed with samples of each fraction to determine which fractions contained purified recombinant protein. The most concentrated recombinant protein fractions determined by SDS-PAGE were renatured through a step-wise dialysis procedure at 4°C using the following series of dialysis solutions:

1. Wash buffer + 4 M urea, pH 7.5, 2 hr
2. Wash buffer + 2 M urea, pH 7.5, 2 hr
3. Wash buffer + 1 M urea, pH 7.5, 2 hr
4. Wash buffer + 0.5 M urea, pH 7.5, 2 hr
5. Wash buffer + 0 M urea + 10% glycerol, pH 7.5, overnight

After this renaturation procedure, protein concentrations were determined by using a Coomassie blue dye-binding method (Bio Rad; Bradford, 1976), and a bovine gamma globulin as a standard. The renatured recombinant protein samples were aliquoted and stored at -20°C.

II. Yeast Procedures: Two-Hybrid System

The yeast two-hybrid system was used to identify proteins that interact with AEBP1 (Figure 2.1). Procedures as described by Chien et al. (1991) and Bartel et al. (1993) with a number of minor modifications. The Gal4 DNA-binding domain vector pGBT9, the pACT vector containing a human cDNA library from HeLa cells fused to the GAL4 activation domain, control plasmids pSE1111 (SNF4 cloned into the pACT vector containing the activation domain of Gal4) and pSE1112 (SNF1 cloned into the pAS1 vector containing the DNA-binding domain of Gal4) and the *Saccharomyces cerevisiae* strain Y153 (*MATα gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::gallacZ LYS2gal::HIS3*) were provided by Stephen Elledge (Department of Biochemistry, Baylor College of Medicine, Houston, Texas).

Figure 2.1. Yeast Two-Hybrid System

The yeast two-hybrid system utilizes a property of the Gal4 transcriptional activator to screen for novel protein-protein interactions. The Gal4 protein has separable DNA-binding and transcriptional activation domains. A human cDNA library is fused to a vector that expresses the Gal4 activation domain, and cDNAs for AEBP1 and AEBP1-ΔHinc are fused to a vector that expresses the Gal4 DNA-binding domain. When a protein containing the Gal4 DNA-binding interacts with another containing the Gal4 activation domain and then binds to the upstream activation sequence (UAS_G) of *lacZ* and *HIS3* the positive interaction results in gene activation, which can be monitored in certain yeast strains by growth on histidine-free medium and by monitoring β-galactosidase activity.

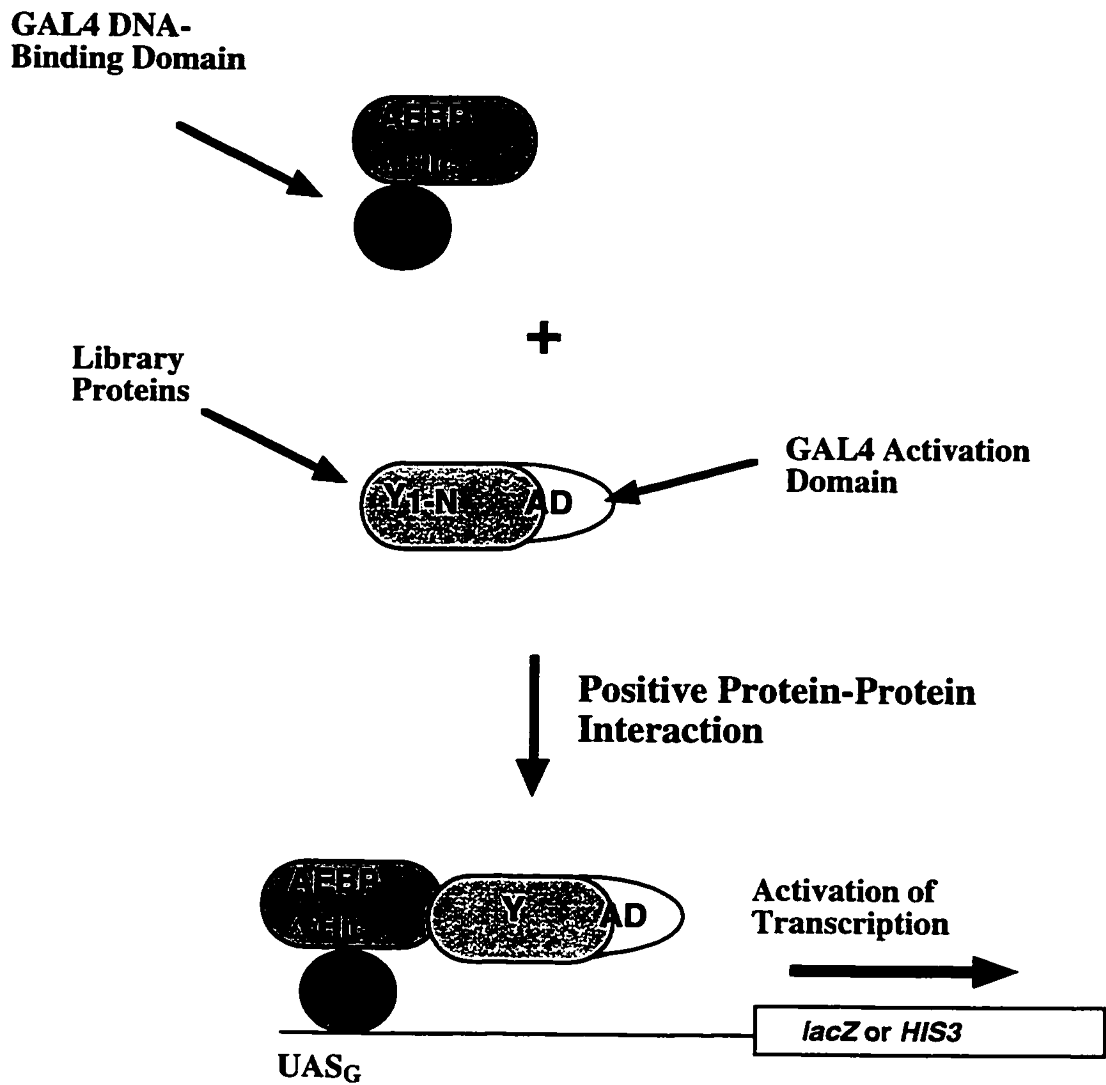


Figure 2.1. Yeast Two-Hybrid System

2.II.A. Yeast Lithium Acetate Transformation

Plasmids expressing AEBP1 (pGBT-AEBP1) or a mutant form of AEBP1 (pGBT-AEBP1ΔHic; described in section 3.I.D.) fused to the Gal4 DNA-binding domain of vector pGBT9 were transformed separately into yeast cells by the lithium acetate method (Ito et al., 1983). A single colony of Y153 cells was inoculated into 100 ml of YPD rich medium (containing per litre: 20 g Bacto-peptone, 10 g Bacto-yeast extract, 20 g glucose; for solid medium add 20 g agar; Ausubel et al., 1994) and grown overnight at 30°C on a shaking platform. When the cell density reached 1-2 x 10^7/ml, the cells were pelleted by centrifugation at 3000 rpm and washed with 20 ml of distilled water, resuspended in 5 ml of lithium acetate solution (0.1 M lithium acetate, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA) and pelleted as before. The cells were then suspended in 250 µl lithium acetate solution and shaken gently for 1 hour at 30°C.

The treated cells were divided into 100 µl portions, and 10 µg of plasmid DNA and 10 µg of carrier DNA (single-stranded salmon sperm DNA) was added. Control samples contained pSE1112 and carrier DNA (positive control) or only carrier DNA (negative control). These mixtures were incubated at 30°C for 30 minutes without shaking. To these samples, 1 ml of PEG solution (40% w/v polyethylene glycol 3350, 0.1 M lithium acetate, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA) was added, and the cells were incubated for 45 minutes. The cells were then heat shocked at 42°C for 5 minutes, collected by centrifugation at 14 000 rpm for a few seconds, resuspended in 0.4 ml TE solution (100 mM Tris, pH 7.5, 50 mM EDTA), resedimented, and resuspended in 0.5 ml TE solution. For each individual transformation mixture, 100 µl portions were spread onto the surface of SC-Trp drop-out plates ("SC" indicates synthetic complete medium: YNB supplemented with ammonium sulfate 1%, and 20 µg/ml adenine, uracil, and arginine, aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, and valine, each for solid medium Bacto-

agar (20 μg/l) was added; "SC-X" indicates that X is the amino acid omitted from the medium; Ausubel et al., 1994). To select for cells containing pGBT-AEBP1 plasmids, cells were grown on medium lacking tryptophan (SC-Trp) as the pGBT9 vector uses tryptophan as a selectable marker.

After 4 days incubation at 30°C, cells from individual colonies were spread onto fresh selective SC-Trp solid medium and the resulting single colonies were inoculated into 5 ml SC-Trp medium and grown overnight at 30°C. Cells were collected by centrifugation at 3000 rpm for 5 minutes and lysed by vortexing with acid-washed glass beads (Ausubel et al., 1994). To determine the expression levels of the Gal4 binding domain – AEBP1 chimeric proteins, Western blot analysis, as described in section 2.IV.D. below, was carried using anti-AEBP1 and anti-Gal4 antibodies.

2.II.B. Selection on 3-aminotriazole plates

One of the selection criteria used for protein-protein interactions in the yeast two-hybrid system is the activation of the *HIS3* gene, which allows growth on SC-His plates. The *HIS3* gene encodes the imidazole-glycerolphosphate (IGP) dehydratase enzyme, which is involved in histidine synthesis (Struhl and Davis, 1980), and is deleted from its normal genetic locus in Y153 cells. HIS3 function was provided by *HIS3* coding sequences expressed under the control of Gal4-binding sites and inserted at the *LYS2* locus. Thus the interaction between proteins fused to the activating and DNA-binding domains of Gal4 activates expression of this *HIS3* gene, which supplies HIS3 function. As the expression of this fusion gene is leaky (expressed without a positive protein-protein interaction), 3-aminotriazole (3'AT), a competitive inhibitor of IGP dehydratase (Struhl and Davis, 1980), was added to the SC-His plates to inhibit growth that was based on low-level *HIS3* expression. To determine the concentration of 3'AT required to prevent growth of pGBT-AEBP1 and pGBT-AEBP1ΔHinc

transformed cells, the cells were spread onto SC-His,Trp solid medium containing 0, 20, 40, 60, or 80 mM 3'AT and incubated at 30°C for 4 days.

2.II.C. Library Transformation

Library transformations were performed as described in section 2.II.A, with the following modifications. All volumes were increased five fold and cells were initially grown overnight in SC-Trp medium and then transferred to YPD medium for 4 hours to ensure optimal growth conditions immediately prior to the transformation.

The following additional controls were added to the library transformation protocol: Y153 cells alone plus library plasmids (negative control); pSE1112 alone or pACT alone (negative controls); pGBT9 vector with pSE1111 (negative control); pGBT9 with library (negative control); pACT and library (negative control); and pSE1111 and pSE1112 (positive control). To the Y153 cells transformed with pGBT-AEBP1 and pGBT-ΔHinc and to the Y153 cells containing library controls, 200 ug of plasmid library DNA was added.

After the heat shock-step, each sample was incubated in 200 ml of SC-Trp,Leu,His medium at 30°C for 4 hours. Cells were pelleted by centrifugation at 3000 rpm for 5 minutes, and the cells were resuspended in 6 ml of SC-Trp,Leu,His medium. The cells were plated at 300 μl/plate onto SC-Trp,Leu,His solid medium containing 60 mM 3'AT. A hundred-fold dilution of cells was spread onto YPD medium to determine transformation efficiency. The cells were allowed to grow for 10 days at 30° C, after which colonies were replica plated to freshly made SC-Trp,Leu,His solid medium containing 60 mM 3'AT and grown for an additional 6 days.

Cells from individual colonies were spread onto SC-Trp,Leu,His medium containing 60 mM 3'AT and grown for an additional 4 days, or until further testing for β-galactosidase activity.

2.II.D. β-Galactosidase Assay

β-galactosidase assays were performed to confirm protein-protein interactions. Colonies were spread onto filter papers which were placed on SC-Trp,Leu,His medium containing 60 mM 3'AT and grown overnight at 30°C. The filter papers were transferred to YPD solid medium and incubated for an additional two hours at 30°C. The filter papers were then submerged in liquid nitrogen for 1 minute and incubated with Z buffer (60 mM Na_2HPO_4, 400 mM NaH_2PO_4, 100 mM KCl, 100 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol, pH 7.0) and 5-bromo-4-chloro-3-indolyl-β-galactosidase (X-gal; 1 mg/ml) at 30°C. Colonies of cells expressing β-galactosidase enzyme turned blue after 2 to 16 hours of incubation.

2.II.E. Isolation of Yeast Plasmid DNA

Plasmid DNA was isolated as described by Ausubel et al. (1994). Single colonies from positive clones were incubated at 30°C overnight in SC-Trp,Leu,His medium containing 60 mM 3'AT to allow the loss of library plasmids not expressing proteins involved in protein-proteins interaction. Cells were pelleted by centrifugation at 3000 rpm for 5 minutes and suspended in SC-Trp,Leu,His medium containing 60 mM 3'AT and then spread onto SC-Trp, Leu, His solid medium containing 60 mM 3'AT and grown for 4 days. Single colonies were inoculated into SC-Leu medium and incubated at 30° C overnight without Trp selection to allow loss of the pGBT-AEBP1 or pGBT-AEBP1ΔHic plasmids. Cells were pelleted by centrifugation at 3000 rpm for 5 minutes, resuspended in SC-Leu medium and then transferred onto SC-Leu solid medium and grown for 4 days.

Single colonies were inoculated into 2 ml of SC-Leu medium and incubated at 30°C overnight. The cells were transferred to microfuge tubes and centrifuged for 5 seconds at 14 000 rpm, the supernatant was discarded, and the pellet was briefly vortexed by a bench top vortex. The cells were then resuspended in 200 µl of breaking buffer (25% v/v Triton X-100, 1% v/v SDS, 100 mM NaCl, 100 mM Tris-HCl, pH 8, and 1 mM EDTA) and acid-washed glass beads (0.3 g) were added along with 200 µl of phenol/chloroform/isoamyl alcohol (24:24:1). The samples were vortexed at high speed for over 10 minutes (Ausubel et al., 1989). The samples were then centrifuged for 5 minutes at 14 000 rpm and the aqueous layer containing the plasmid DNA was removed.

The isolated DNA was transformed into DH5α cells as described in Section 2.I.A., with ampicillin resistance used as the selection marker. DNA was isolated as described in Section 2.I B. and plasmid DNA was restricted with the endonucleases *Xho*II and *Eco*RI to obtain restriction maps of the library clones and to determine the number of unique clones.

2.II.F. Testing of Positive Clones

To determine if the protein-protein interactions were specific to AEBP1 or AEBP1ΔHinc and the expressed library clones, positive interacting library clones were tested along with a number of controls. The pACT-library clones were transformed into Y153 cells already harboring pGBT9 or pSE1112 (negative controls) to determine if the library clones interacted with the Gal4-binding domain alone, and pGBT-AEBP1 and pGBT- AEBP1ΔHinc to show the positive protein-protein interaction. pSE1111 and pSE1112 were transformed together as a positive control. All transformations were plated onto SC-Trp,Leu solid medium as described in Section 2.II.A..

From the transformed cells, single colonies were streaked onto filter paper and β-galactosidase activity was assessed as described in Section 2.II.F.. Library clones that had positive results for the X-gal assay when transformed into Y153 cells harboring pGBT-AEBP1 or pGBT-AEBP1ΔHinc and negative results when transformed into Y153 cell harboring pGBT9 or pSE1112 were deemed to generate specific protein interactions between proteins expressed from library clones and AEBP1 or AEBP1ΔHinc.

Positive protein-protein interaction library cDNAs were sub-cloned into pBlueScript vector at *Xho*II sites and transformed into DH5α cells for sequencing purposes. All sequencing was carried out by automated Licor sequencing at the Institute for Marine Biology, (NRC, Halifax, Nova Scotia).

2. III. Mammalian Cell Procedures

2.III.A. Cell Lines Used In Study

The following information was cited from the ATCC Catalogue of Cell Lines and Hybridomas (7th edition, 1992):

NIH 3T3: established from Swiss Mouse embryo culture.

3T3 L1: a continuous sub-strain from 3T3 (Swiss albino) developed through isolation.

2.III.B. Cell Line Maintenance

All cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% calf serum or fetal calf serum (GIBCO) at standard growing conditions, 37°C and 5% CO_2 atmosphere. The medium was changed every 2 days.

2.III.C. Plating and Subculturing of Cultured Cells

Vials containing frozen cells were thawed at 37°C in a water bath, and then immediately subcultured or plated to an appropriate number of plates. Medium was changed 4-5 hours after plating.

To subculture cells, the medium was removed and the cells were washed twice with PBS (0.137 M NaCl, 2.7 mM KCl, 4.2 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.3). Cells were treated with trypsin (GIBCO) at room temperature for 5 minutes (1 ml 1x Trypsin per 80 mm dish). The trypsin was removed and the cells were lifted from the plates by gentle tapping. The cells were resuspended in medium and then aliquoted into the appropriate number as determined by the amount to cells required for specific experiments.

Cells to be stored were lifted from the plates by gentle tapping and resuspended in freezing medium [regular culture medium supplemented with 10% DMSO (Sigma)] and frozen at -70°C. The following day the cells were transferred to liquid nitrogen storage.

2.III.D. Adipocyte Differentiation Protocols

To induce differentiation of 3T3 L1 preadipocytes, cells growing in DMEM (10% calf serum) were treated at confluence (day 0) with dexamethasone (0.25 μM) and 1-methyl-3-isobutylxanthine (0.5 mM) (dex/mix) and insulin (5 μg/ml) in DMEM (10% fetal bovine serum, FBS) and incubated for 48 hours. At day 2, the growth medium was changed to DMEM containing 10% FBS and 5 μg/ml insulin, and the cells were grown for 6 additional days.

2.III.E. Immunoprecipitation

3T3 L1 cells were washed twice with cold PBS and lysed and collected in cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 50 mM Na_2PO_7•7 H_2O, 0.25 % sodium deoxycholate, 0.1% Nonidet P-40, 1 mM Na_3VO_4, and 1 mM NaF, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg aprotinin/ml, 10 μg leupeptin/ml, 5 mM EDTA, and 5 mM EGTA by scraping with a rubber policeman. Cell debris was removed by centrifugation at 12 000 rpm for 10 minutes. The soluble cell lysates were incubated with protein A-agarose (Santa Cruz Biotechnology) for 1 hour at 4°C, then centrifuged at 12 000 rpm for 10 minutes. The beads were discarded and the supernatants were incubated, overnight at 4°C with specific antibodies (all from Santa Cruz Biotechnology except anti-AEBP1) and protein A-agarose. Samples were then centrifuged at 12 000 rpm for 10 minutes and the pellets were washed 4 times with RIPA buffer. The precipitated samples were suspended in 1x loading buffer (NEB), with the protein A-agarose beads remaining out of solution, and then resolved by SDS-PAGE (10, 12, or 15%) and analyzed by the Amersham ECL blotting system (see Section 2.IV.D.).

2.III.F. Transient Transfections

Transfection experiments in NIH 3T3 cells were carried out as described by Kawai and Nishizawa (1984) with minor modifications. Cells were grown to approximately 60-80% confluence, at the time of transfection the growth medium was removed and replaced with DMEM (no serum) containing polybrene (30 μg/ml). Plasmid DNA consisting of 2.5 μg of the reporter plasmid, 5 μg of the AEBP1 expression plasmid or control plasmid, and 1 μg of pHermes-lacZ plasmid was added to the cells, which were then incubated at 37° C for 6 hours with occasional swirling.

After this incubation, the medium was removed and replaced with 30% DMSO in PBS and the incubation was continued for 2-4 minutes at room temperature. The DMSO was removed and replaced with DMEM (10% calf serum) and the cells were grown for 48 hours, after which the cells were harvested and CAT activity was determined as described in section 2.III.G. below.

2.III.G. Chloramphenicol Acetyl Transferase (CAT) Assay

CAT assays were carried out similar to those described by Gorman et al. (1982). Briefly, cells were washed twice with cold PBS and then harvested in PBS by scraping the dishes with a rubber policeman, collected by centrifugation at 12 000 rpm for 10 minutes and resuspended in 100 µl CAT-TE (250 mM Tris pH 8.0, 5 mM EDTA). The cells were lysed by 4 freeze-thaw cycles (liquid nitrogen for 3 minutes, then 37°C).

Before CAT assays were done, cellular extracts were normalized by β-galactosidase assays. The β-galactosidase assays contained 3 µl of 100x Mg-buffer (0.1 M $MgCl_2$, 4.5 M β-mercaptoethanol), 66 µl *o*-nitrophenol-β-D-galactopryanoside (ONPG; 4 mg/ml in 100 mM $NaHPO_4/Na_2PO_4$, pH 7.5), 30 µl cell extract, 201 µl of 100 mM $NaHPO_4/Na_2PO_4$. The reactions were incubated at 37°C for periods ranging from 30 minutes to 16 hours. After the incubation period, reactions were stopped with the addition of 500 µl of 1 M Na_2CO_3. The enzyme activity was monitored at A_{554}.

Following the β-galactosidase assays, the remaining crude extract was heated at 60°C for 10 minutes to inactivate deacetylases which may be present in the extract, and cell debris was removed by centrifugation at 12 000 rpm for 10 minutes, with the supernatant being used in the CAT assay.

The CAT assay contained cell extract (volume determined by β-galactosidase assay) and 10 µl of assay buffer (containing 0.2 mg acetyl Co A [Pharmacia] and 0.05

μCi ^{14}C-chloramphenicol [ICN]), and was adjusted to 60 μl with CAT-TE. The assay mixtures were incubated at 37°C for 0.5 to 4 hours, and then extracted with 500 μl ethyl acetate with the top layer removed, dried in a Speedvac for at least 20 minutes, and resuspended in 10 μl of ethyl acetate. These samples were spotted onto silica gel thin-layer chromatography plates (Sigma) and developed in a solvent system of 95% chloroform and 5% methanol for 30 minutes. The plates were air dried, coated with Enhance Spray (DuPont NEN), and exposed to X-ray film for at least one day.

2.III.H. Nuclear Extract Preparation

Preadipocyte and adipocyte nuclear extracts were prepared by the following procedure. Cells were washed twice with cold PBS and harvested with a rubber policeman, collected by centrifugation at 2000 rpm for 2 minutes at 4°C, resuspended in 100 μl of Buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT), and incubated on ice for 10 minutes. The cells were homogenized, transferred to an Eppendorf tube, and centrifuged 2000 rpm for 2 minutes at 4° C. The resulting pellet was resuspended in Buffer A and centrifuged at 13500 rpm for 30 minutes at 4° C. The pellet was resuspended in 100 μl of Buffer C (20 mM Hepes pH 7.9, 20 % glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT; after the pellet was resuspended, 5 mM NaCl was added). The samples were incubated on ice for 30 minutes and then centrifuged at 13 500 rpm for 30 minutes at 4°C. The supernatant was collected and dialyzed against Buffer D (20 mM Hepes pH 7.9, 20 % glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). Protein concentrations were determined using the Bradford assay, and samples were stored at -80°C until use in gel mobility shift assays (section 2.IV.B.).

2.III.I. Gel Filtration Studies

3T3 L1 cells were serum starved 24 hours by incubation in medium containing only 0.1% calf serum prior to stimulation with 10% FBS and 5 μg/ml insulin with cell extracts prepared as described in section 2.III.E. The extracts were collected at the time of stimulation, and after 10- and 30-minutes, and 2- and 24-hours of stimulation. Blue dextran (50 ng) was added to 1 mg of extract, which was layered into a 1-m column containing Sephacryl S-300 High Resolution gel (Pharmacia). The column was calibrated using molecular weight standards (Bio Rad) containing Blue Dextran. Samples were run through the column and eluted in PBS containing 1 mM PMSF, 10 μg aprotinin/ml, 10 μg leupeptin/ml, 5 mM EDTA, and 5 mM EGTA and then collected in 0.5 ml fractions. Column fractions were concentrated by precipitating with 18% trichloro-acetic acid and then washed with acetone. Western blot analysis (section 2.IV.D.) was performed on individual fractions.

2.IV. Applications

2.IV.A. Carboxypeptidase Assays

Carboxypeptidase activity was measured by a method developed by Folk (1960) and modified by Fricker and Devi (1990). This method spectrophotometrically measures the conversion of hippuryl-L-arginine (Sigma) to hippuric acid by absorbance at 254 nm. Purified recombinant AEBP1 protein (section 2.I.E.) was mixed with 1 μg/ml of hippuryl-L-arginine (dissolved in 0.1 M sodium acetate, pH 5.5) and incubated at 37°C, and enzyme activity was measured at 4 minute intervals at A_{254} by spectrophotometer.

For all assays with activators or inhibitors, recombinant AEBP1 was incubated

with the activator or inhibitor for 1 hour on ice before enzyme assays were carried out.

2. IV. B. Gel Mobility Shift Assay (GMSA)

AE-1 oligonucleotide probes were labeled at the 5' ends by T4 DNA kinase (BRL) in 30 μl reactions containing 300-400 ng of oligonucleotide, 1x Kinase buffer (BRL), 100 μCi of [γ-^{32}P] ATP (3000 Ci/mmol, DuPont), and 10 units of T4 polynucleotide kinase. The reactions were incubated at 37°C for 30 minutes and then stopped by heating at 90°C for 5 minutes. The labeled probes were purified by diluting the labeling reactions to 100 μl and passage through NucTrap (Stratagene) columns.

Gel mobility shift assays were carried out by a procedure similar to the one described by Distel et al. (1987). Recombinant AEBP1 protein (500 ng) was incubated with the ^{32}P-labeled AE-1 oligonucleotide, binding buffer (100 mM Tris pH 7.5, 100 mM KCl, 50 mM $MgCl_2$, 10 mM DTT, and 25% glycerol) and water in a total volume of 20 μl for 20 minutes at room temperature, after which 2 μl of loading buffer (binding buffer plus bromophenol blue) was added. For competition gel mobility shift assays, the protein was incubated in presence of ~200-fold molar excess of unlabeled oligonucleotide competitors before addition of the labeled probe. For *in vitro* transcription/translation products (section 2.IV.C) and nuclear-extract gel mobility shift assays, 2 μg poly dI:dC was added to the reaction mixture at the time of incubation. For gel mobility shift assays utilizing antibodies, 500 ng of a specific antibody was incubated with protein prior to binding reactions for 10 minutes on ice. For reactions involving more than one recombinant protein, the proteins were pre-incubated together on ice for 30 minutes.

Non-denaturing (5%) polyacrylamide gels (30:1, acrylamide/bisacrylamide in 0.25x, 1x TBE buffer [containing 90 mM Tris base, 90 mM boric acid, 2 mM EDTA]; gels were run in 0.25x TBE buffer) were pre-run at 200 volts until the current dropped

to half the initial value. Samples were then loaded and run at 200 volts for approximately 3 hours until the dye front migrated two-thirds down the gel. The gels were removed, dried on filter paper, and exposed to X-ray film for 2 to 24 hours.

2.IV.C. *In vitro* Transcription-Translation

In vitro transcription and translation experiments were carried out according to the Technical Bulletin provided with the TnT Coupled Wheat Germ Extract System (Promega). The 50 µl reactions consisted of 25 µl Wheat Germ Extract, 2 µl TnT reaction buffer, 1 µl T7 polymerase, and1 µl amino acid mixture (1 mM), 1 µl RNasin ribonuclease inhibitor (40 u/µl), 1 µg DNA template, to a final volume of 50 µl in nuclease-free water (all reagents were from the TnT Coupled Wheat Germ Extract System kit). Reaction mixtures were incubated for 2 hours at 30°C. Translated products were used for gel mobility shift assays (section 2.IV.B. above).

2.IV.D. Western Blot Analysis

Western blot experiments were performed by using the Amersham ECL Western blotting system as described in ECL-Western blotting protocols (Amersham). Cell lysates (sections 2.II and 2.III) were resolved on either 10, 12, or 15% SDS-PAGE mini-gels along with pre-stained markers (NEB). Gels were then placed in a blotting apparatus (BRL) with nitrocellulose (Micron Separations) on top of the gel, and the chamber was filled with transfer buffer (15.1 g Tris base and 72 g glycine dissolved in 1 litre H_2O, then diluted 5x in 80% H_2O and 20% methanol). For transfer of large proteins (greater than 40 kDa), transfer reaction were carried out for 1-2 hours at 100 volts (less than 100 mA) on ice; for small proteins, transfers were carried out at 50 volts (less than 50 mA) for 20-30 minutes on ice, or at 70°C as described by

Robishaw and Balcueva (1993). Nitrocellulose membranes were then incubated in blocking buffer (5% milk powder in TBST, pH 7.5: 20 mM Tris base, 137 mM sodium chloride, 1 M acetic acid, 0.1% Tween-20) overnight at 4°C with constant agitation.

Membranes were washed 3x with TBST buffer for 15 minutes at room temperature. Membranes were then cut according to size as determined by protein markers, and individual sections of the membrane were incubated in primary antibody (100 ng/ml for all Santa Cruz Biotechnology Inc. monoclonal antibodies, and 200 ng/ml for polyclonal anti-AEBP1 antibody) in blocking buffer overnight at 4°C with constant shaking. After the incubation, membranes were washed 3x with TBST for 15 minutes at room temperature with constant shaking. Samples were then incubated with secondary antibody (1:2000 dilution of anti-rabbit IgG-HRP; Santa Cruz) at room temperature for 1 hour. Membranes were then washed twice with TBST, and once with TBS (TBST minus Tween), each for 15 minutes at room temperature.

Membranes were then incubated into a mixture of equal volumes of detection solutions 1 and 2 (ECL, Amersham) for precisely 1 minute, and wrapped in SaranWrap and exposed to X-ray film for 30 seconds to 2 hours.

2.IV.E. Phosphorylation and MAPK Assays

3T3 L1 cells were stimulated for 10 minutes with DMEM containing 10% FBS and extracts were prepared in cold RIPA buffer containing 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM EDTA, and 5 mM EGTA. Either a specific inhibitor of cell cycle-regulating kinases and ERK1 (100 mM olomoucine) or a general kinase inhibitor with lesser specificity for these kinases (100 mM iso-olomoucine) was added separately to 3T3 L1 cell extracts and incubated for 2 hour on ice. Kinase reactions (30 μl) were then carried out for 30 minutes at 37°C in a kinase buffer

containing 1 μg recombinant AEBP1, 30 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM sodium orthovandate, 1 mM dithiothreitol, and 30 μCi [γ-^{32}P] ATP (Amersham). Reactions were stopped by the addition of SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer (NEB). Samples were resolved by SDS-PAGE (15%) and transferred to nitrocellulose membranes. Radioactivity was detected by autoradiography. Immunoprecipitated ERK (2.III.E.) was used in the same fashion, replacing cell extract.

For the MBP kinase reaction, MAPK was immunoprecipitated (2.III.E.) from the cell lysates (500 μg) with agarose-conjugated anti-ERK1 antibodies (Santa Cruz) overnight. The precipitated pellets were collected and washed 3X with RIPA buffer, washed and equilibrated 3X in kinase buffer containing 30 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM sodium orthovandate, 1 mM dithiothreitol, and 15 μCi [γ-^{32}P] ATP. To the samples, 10 μg myelin basic protein (MBP; Santa Cruz) was added and the suspensions were incubated for 30 minutes. Samples were resolved by 15% SDS-PAGE and transferred to nitrocellulose membranes. Radioactivity was detected by autoradiography. Total ERKs were detected by the Amersham ECL western blotting system with anti-ERK1 serum (100 ng/ml).

2.IV.F. Serum Stimulation of MAPK Activation (Phosphorylation)

3T3 L1 cells were serum starved by incubation in DMEM containing only 0.1% calf serum for 24 hours, then treated with DMEM containing 15% FBS. Total cell lysate proteins (5 μg) were resolved by 8.5% SDS-PAGE and transferred to nitrocellulose membrane. Both forms of ERK1 and 2 were detected by immunoblotting with anti-ERK1 antibodies (50 ng/ml).

2.IV.G. Phosphatase Protection Assay

3T3 L1 cells were stimulated for 10 minutes with DMEM plus 10% FBS, and MEK1 was immunoprecipitated overnight with anti-MEK1 antibodies (1 μg/ml) as described in section 2.III.E. Recombinant MAPK (1 μg, Santa Cruz) was phosphorylated in a non-radioactive kinase reaction (2 hours) as described (section 2.IV.E.) with the addition of immunoprecipitated MEK and 100 μM ATP. The activated phosphorylated MAPK was recovered by incubating with an anti-phosphotyrosine antibody and protein A-agarose (Santa Cruz) overnight at 4°C. The immunoprecipitated MAPK was washed 5x with TE and then samples were incubation with either 1 μg of AEBP1 or control protein for 1 hour on ice. One sample was incubated with 2 units of alkaline phosphatase (NEB) for 1 hour at room temperature; another was incubated with 2 units heat inactivated alkaline phosphatase (15 minutes 70° C); and another was untreated. MAPK was precipitated and pellets were collected and washed 4x with RIPA buffer, equilibrated 3x in kinase buffer, and then incubated with 15 μCi [γ^{32}P] ATP and 10 μg MBP in a 20 μl reaction for 30 minutes at 30° C. Samples were resolved by 15% SDS-PAGE and transferred to nitrocellulose membranes. Radioactivity was detected by autoradiography.

Chapter 3:
Results and Discussion

The Results and Discussion chapter is divided into three main sections. The first section deals with the role of AEBP1 as a carboxypeptidase and a DNA-binding transcription factor. In this section the ability of AEBP1 to bind to DNA, to repress transcription, and the characterization of its protease activity are explored. Correlation between these three activities is shown and discussed.

The second section presents the results of the yeast two-hybrid experiment. The proteins demonstrated to interact with AEBP1 in the yeast system are identified and discussed in this section. The possible significance of these protein-protein interactions is also discussed.

The third and final section of the Results and Discussion chapter investigates the role of AEBP1 in the receptor tyrosine kinase signal-transduction pathways. The significance of the phosphorylation of AEBP1 and interaction with a mitogen-activated protein kinase (MAPK) is examined, along with the role of AEBP1 in receptor tyrosine kinase and G protein coupled receptor pathways.

I. AEBP1: A carboxypeptidase and DNA-Binding Transcriptional Repressor

3.I.A. AEBP1, a DNA-Binding Protein

A cDNA encoding AEBP1 was cloned as a protein with an estimated 79 kDa that binds to the AE-1 promoter region of the aP2 gene (He et al., 1995). This AEBP1 protein may represent the preadipocyte-specific negative regulator which is found to bind to the AE-1 site *in vivo* (Ro and Roncari, 1991).

To investigate the ability of AEBP1 to function as a DNA-binding protein, recombinant AEBP1 protein was produced. The cDNA encoding AEBP1 was cloned into the His-tag vector pET-16b and induced in the *E. coli* strain DE3. The recombinant protein was purified by binding to a metal affinity column (Clontech), eluted, and renatured. The purification process allowed the isolation of nearly homogeneous recombinant protein of an estimated molecular weight of 83 kDa (Figure 3.1).

Recombinant protein was used in a gel mobility shift assay to determine if AEBP1 could bind to DNA. The following oligonucleotide termed AE-1 equivalent to the AE-1 site located in the 5' upstream promoter region of the aP2 gene as shown below, was radiolabeled and used for all GMSA studies:

```
 -159            -144 -139            -125
GATCCAGGGAGAACCAAAGTTAGAAATTTCTATTAAA
    GTCCCTCTTGGTTTCAACTTTTAAAGATAATTTCTAG
```

When AEBP1 was incubated with the AE-1 DNA (Figure 3.2 Lane 2) a mobility shift caused by the DNA-protein interaction was observed in comparison to Lane 1 containing labeled AE-1 DNA alone. To determine if the interaction between AEBP1 and AE-1 DNA is specific, competition studies were done. In this experiment, AEBP1 was pre-incubated with unlabeled AE-1 DNA prior to the addition of labeled AE-1 DNA. The presence of unlabeled AE-1 DNA should sequester the majority of the recombinant protein and result in a decrease in the amount of protein-DNA complex. Figure 3.2, Lane 3, shows the expected decrease in the amount of AEBP1–AE-1 complex after AEBP1 was pre-incubated with unlabeled AE-1 DNA. As controls, DNA sequences SP1 and AP3 (Figure 3.2, Lanes 4 and 5) were incubated with AEBP1 in a similar fashion. If AEBP1 specifically interacts with the AE-1 DNA, pre-incubation with other sequences should not influence the amount of AEBP1 protein available for protein-DNA complex formation. Lanes 4 and 5 of Figure 3.2 show that AEBP1

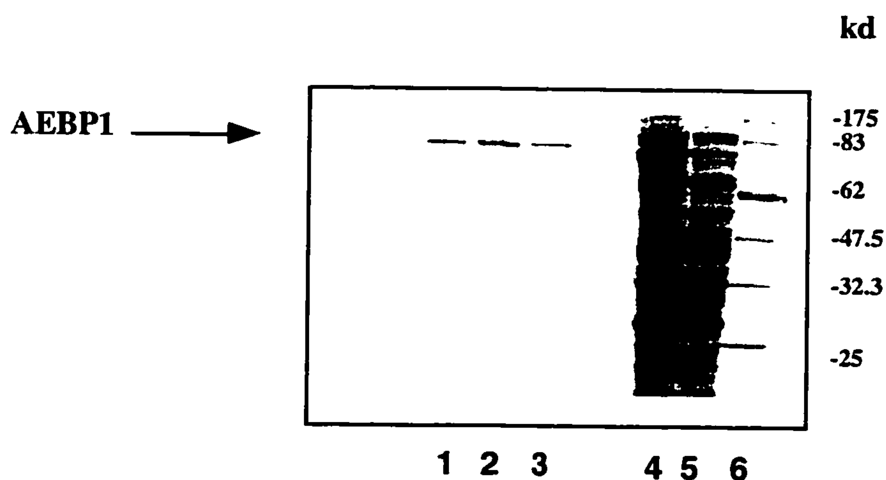


Figure 3.1 Purification of Recombinant His-tagged AEBP1

Coomassie blue stained SDS-PAGE gel containing purified recombinant His-tagged AEBP1 protein. Lanes 1 to 3 show fractions eluted from metal affinity column containing recombinant His-tagged AEBP1. Lane 4 shows total protein from bacteria induced to express AEBP1, and lane 5 shows total protein from pre-induced bacteria. Lane 6 shows molecular weight markers (NEB). Arrow indicates recombinant His-tagged AEBP1.

Figure 3.2. AEBP1 Binds to the AE-1 Site

Gel mobility shift assay shows that recombinant His-tagged AEBP1 binds to the AE-1 promoter region of the aP2 gene. Lane 1 shows radiolabeled AE-1 DNA alone. Lane 2 shows the mobility shift caused by the AEBP1–AE-1 DNA interaction. Lane 3 shows competition studies with a decrease in the amount of protein-DNA complex after AEBP1 is pre-incubated and sequestered by unlabeled AE-1 DNA. Lanes 4 and 5 show that the control DNA sequences SP1 and AP3 used for competition studies do not affect the AEBP1–AE-1 mobility shift. Protein-DNA samples were resolved by acrylamide non-denaturing gels, and the radiolabeled DNA was detected by autoradiography. Arrow indicates the AEBP1–AE-1 complex (He et al., 1995). This experiment was duplicated with similar results.

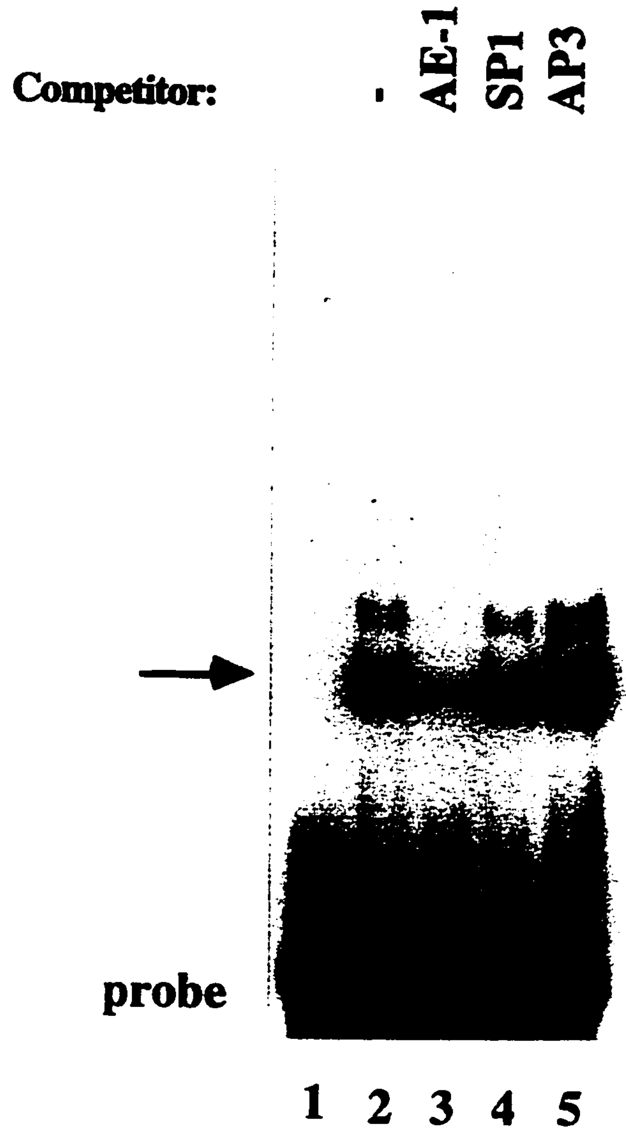


Figure 3.2 AEBP1 Bind to the AE-1 Site

binding was not affected by pre-incubation with other DNA sequences. The results from these gel mobility shift experiments show that AEBP1 binds to the AE-1 site from the promoter region of the aP2 gene. Competition studies show that this interaction is specific, as unrelated sequences did not affect the AEBP1–AE-1 complex formation, while unlabeled AE-1 DNA reduced the amount of AEBP1 available for binding to the labeled AE-1 DNA.

To further show that AEBP1 directly interacts with the AE-1 DNA, a "GMSA-antibody" experiment was performed. In this experiment antibodies directed towards AEBP1 (prepared by Dr. Ro; raised in New Zealand white rabbits using recombinant His-tagged AEBP1 protein; IgG purified) were pre-incubated with AEBP1. If the antibody interacts with the DNA-binding region of AEBP1, thus masking it, a reduction in the amount of AEBP1–AE-1 complex would be expected; conversely, if the antibody interacts with other domains of AEBP1, a larger "supershifted" complex corresponding to the antibody–AEBP1–AE-1 complex would be expected. Lane 3. Figure 3.3 showed that anti-AEBP1 antibody caused a significant reduction in the amount of AEBP1–AE-1 complex as compared to the reaction in Lane 2, which did not contain any anti-AEBP1 antibody. A non-specific antibody (S_{24}), prepared in a similar fashion as that directed towards AEBP1, was used as a control. As lane 4 shows, the anti-S_{24} antibody had no affect on the formation of the AEBP1–AE-1 complex. These results further show that AEBP1 interacts directly with AE-1 DNA.

To determine if AEBP1 is the unidentified *trans*-acting factor that was found to bind to the AE-1 site and act as a negative regulator of the aP2 gene (Ro and Roncari, 1991), GMSA-antibody experiments were done using nuclear extracts prepared from both 3T3 L1 preadipocytes and differentiated adipocytes.

Figure 3.3. GMSA-Antibody Experiments

Recombinant His-tagged AEBP1 was incubated with anti-AEBP1 antibody thus preventing interaction with the labeled AE-1 DNA. Lane 1 shows the radiolabeled AE-1 DNA alone. Lane 2 shows the mobility shift of the AEBP1–AE-1 complex. Lane 3 shows the reduction in complex formation in the presence of anti-AEBP1 antibody. Lane 4 shows that the unrelated anti-S_{24} antibody does not affect the AEBP1–AE-1 complex. Arrow indicates the AEBP1–AE-1 complex. Protein-DNA samples were resolved by acrylamide non-denaturing gels, and the radiolabeled DNA was detected by autoradiography. This experiment was duplicated with similar results.

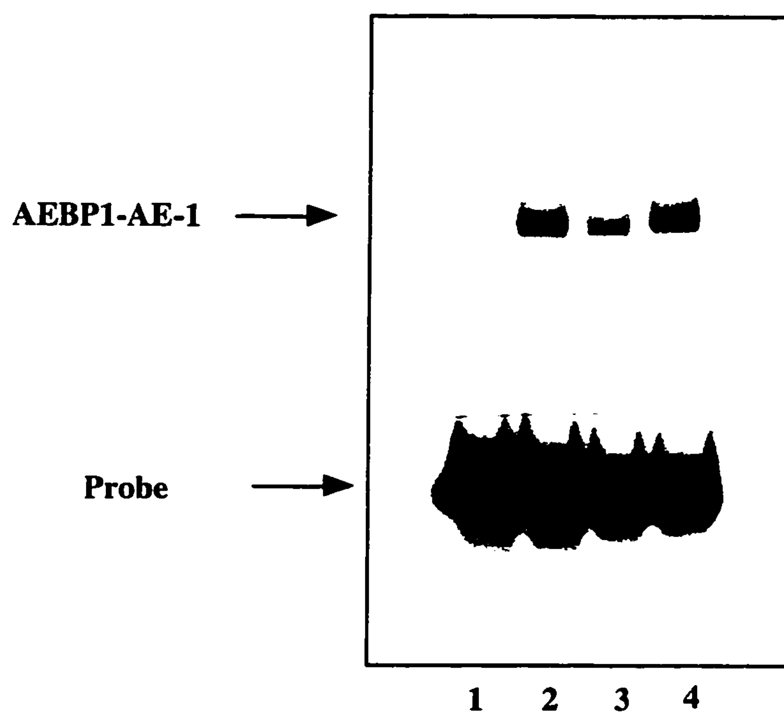


Figure 3.3 GMSA-Antibody Experiments

Nuclear extracts were pre-incubated with anti-AEBP1 antibody to determine if AEBP1 from preadipocyte extract interacts with the AE-1 site. Figure 3.4, Lane 2, shows the binding of a nuclear factor by the labeled AE-1 DNA. Lane 3 shows that incubation with the anti-AEBP1 antibody prevented this factor from binding to the AE-1 DNA, whereas a unrelated anti-S_{24} antibody (Lane 4) had no effect. These results indicate that the factor described by Ro and Roncari (1995) which binds to the AE-1 site in preadipocyte is in fact AEBP1. Lanes 5 shows that the *trans*-acting factor was either not present or incapable of binding the AE-1 DNA in nuclear extracts prepared from 3T3 L1 differentiated adipocytes. These results suggest that AEBP1 may act as the previously described *trans*-acting factor which binds to and negatively regulates the aP2 gene. This protein is either not present or incapable of binding the AE-1 site in adipocytes.

To determine if AEBP1 interacts with the AE-1 as a multimer (a common feature among DNA-binding transcriptional regulators), salt concentrations in the binding buffer were varied as described by Robidoux et al. (1993). Increasing the concentration of KCl in the binding buffer from 100 mM to 150 mM caused the apparent multimerization of AEBP1 protein capable of interacting with AE-1 DNA. Figure 3.5 shows this possible multimerization: Lane 1 contains AE-1 DNA alone, while Lane 2 shows the apparent AEBP1 multimer interacting with the AE-1 DNA. The presence of several protein-DNA complexes suggests that AEBP1 forms dimers, trimers, and possibly tetramers. A number of other transcriptional regulators have been shown to form multimers, including the subfamilies of C/EBP, Fos, Jun, ATF/CREB, and the yeast protein HSF, which forms a trimer (Sorger and Nelson, 1989; Calkhoven and Ab, 1996; Mitchell and Tjian, 1989). For many of these transcription factors, multimerization is a requirement for DNA binding, but for others multimerization may function to regulate the transcriptional factor.

Figure 3.4. GMSA-Antibody Experiments with 3T3 L1 preadipocyte and adipocyte cells

Lane 1 contains radiolabeled AE-1 DNA alone. Lane 2 contains nuclear preadipocyte extract plus the AE-1 DNA. Lane 3 contains nuclear extract preincubated with anti-AEBP1 antibody and shows a reduction in complex formation. Lane 4 shows that the unrelated anti-S_{24} antibody does not affect the AEBP1–AE-1 complex. Lanes 5, shows the *trans*-acting factor is either not present or incapable of binding the AE-1 DNA in nuclear extracts prepared from 3T3 L1 differentiated adipocytes. Arrow indicates the AEBP1–AE-1 complex. Protein-DNA samples were resolved by acrylamide non-denaturing gels, and the radiolabeled DNA was detected by autoradiography. This experiment was duplicated with similar results.

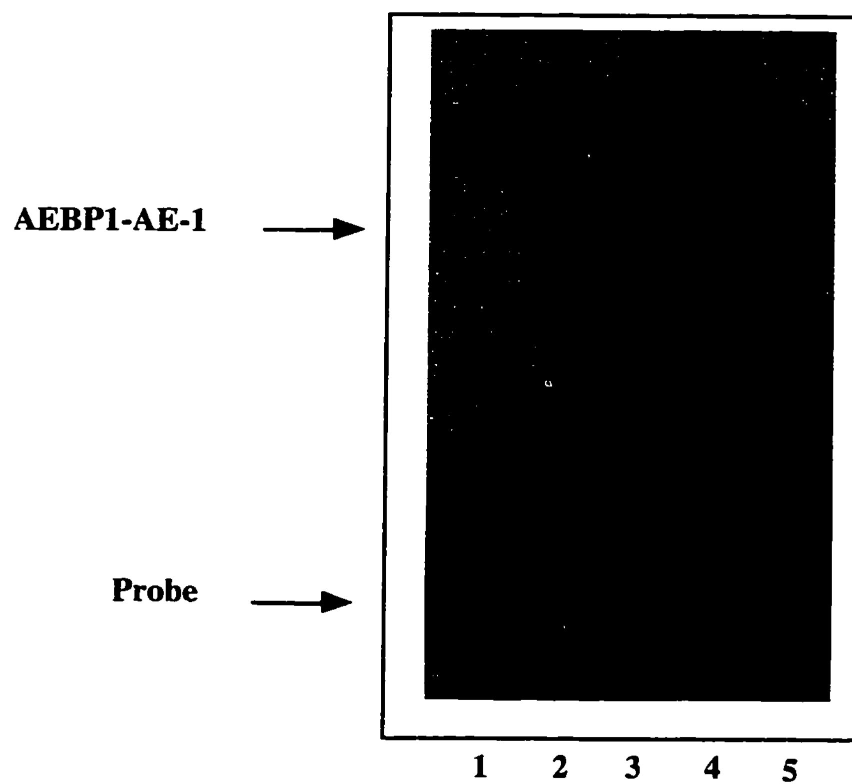


Figure 3.4 GMSA-Antibody Experiment with 3T3 L1 Preadipocyte and Adipocyte cells

Figure 3.5 Multimerization of AEBP1

Studies using different concentrations of KCl in the gel mobility shift binding buffer show that multimers of recombinant His-tagged AEBP1 are capable of binding the AE-1 DNA. Lane 1 contains radiolabeled AE-1 DNA. Lane 2 contains AEBP1 and the AE-1 DNA with 150 mM KCl binding buffer. Several shifted bands can be observed indicating the presence of multimers of AEBP1 binding to the AE-1 DNA. Lane 3. contains AEBP1 and the AE-1 DNA with 100 mM KCl binding buffer. Two shifted bands can be observed, indicating the less binding of similar multimeric forms. All other GMSA experiments were performed with 100 mM KCl in the binding buffer. Arrows indicate AEBP1–AE-1 complexes. Protein-DNA samples were resolved by acrylamide non-denaturing gels, and the radiolabeled DNA was detected by autoradiography. This experiment was duplicated with similar results.

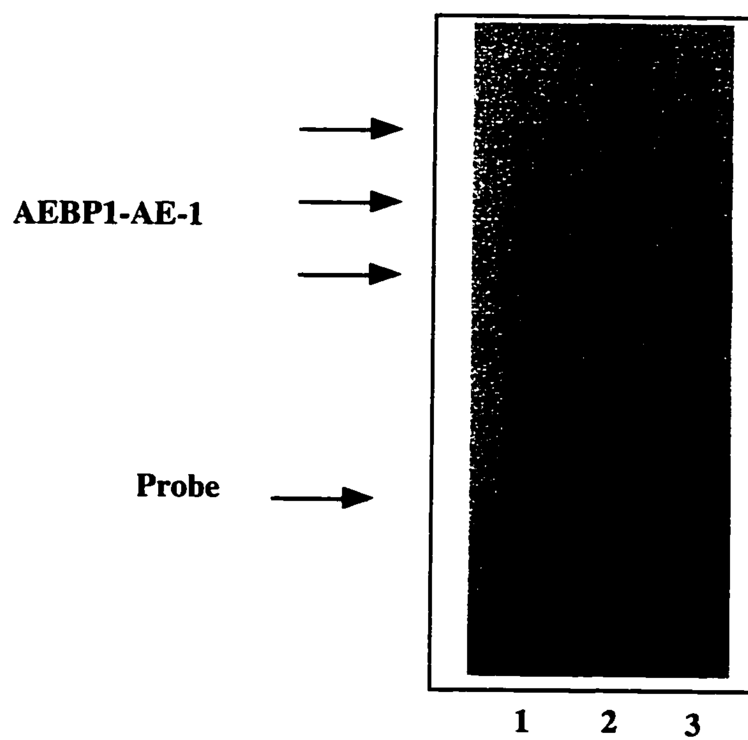


Figure 3.5 Multimerization of AEBP1

The significance of this *in vitro* multimerization is not obvious. Transcription factors may form multimers to enhance DNA-binding capabilities (Mitchell and Tjian, 1989), or to facilitate DNA-binding in an active conformational form (Joshua-Tor, 1995). Although AEBP1 appears to bind to DNA in both monomeric and multimeric forms, the function of this *in vitro* multimerization is not clear, and it is not known if AEBP1 multimers form or bind to DNA *in vivo*. It is possible that other truncated forms of AEBP1 may interact with AEBP1 to assemble inactive multimers in a fashion analogous to the interaction of LIP and CHOP with C/EBP proteins. CHOP, by masking the DNA-binding domain of C/EBPβ, and LIP, by masking the activation domain of C/EBPβ, inhibit the transcriptional activation function of C/EBPβ (Vasseur-Cognet and Lane, 1993). Further study into the multimerization of AEBP1 may demonstrate a possible mechanism by which this protein-DNA interaction is regulated.

Determining the region of AEBP1 responsible for its DNA-binding capability may give important insight into mechanisms by which AEBP1 is regulated. The C-terminal positon of AEBP1 has acidic and basic regions (Figure 1.5) which may act as DNA-binding regions. To determine if this region was responsible for DNA binding, a mutant form of AEBP1 termed AEBP1ΔSty (see Table 3.2 for a description of this mutation and details of its construction), containing a functional protease (section 3. I. C.) was used in the GMSA. This mutant protein is missing the C-terminal amino acids 514 to 719 encompassing the acidic and basic regions. As Figure 3.6 shows, AEBP1ΔSty was not capable of binding DNA in this assay. Recombinant His-tagged AEBP1 binds the AE-1 site (Lane 3), while recombinant His-tagged AEBP1ΔSty does not display this DNA-binding capability (Lane 2).

These results indicate that the C-terminal region of AEBP1 is responsible for the DNA binding, although the possibility that conformational changes caused by the deletion of the C-terminal sequences in the mutant AEBP1ΔSty may masks DNA

binding areas in other regions of AEBP1, thereby preventing the protein from binding DNA, cannot be ruled out.

The C-terminal region of AEBP1 also contains MAPK consensus phosphorylation sites, so it is interesting to speculate about possible phosphorylation regulation of AEBP1. Phosphorylation of transcription factors has been shown to affect protein stabilization, regulate DNA binding, and influence transcriptional regulatory activities (Triesman, 1996). As the dephosphorylated form of recombinant His-tagged AEBP1 binds DNA in GMSA studies, it is interesting to speculate that phosphorylation of AEBP1 may regulate DNA binding. Further study into the affects of AEBP1 phosphorylation may provide insight into the regulatory mechanisms mediating AEBP1–AE-1 complex formation.

In summary, GMSA results show that recombinant His-tagged AEBP1 directly interacts with the AE-1 promoter region of the aP2 gene *in vitro* and indicate that AEBP1 is capable of binding DNA as a multimer. Although it is not known if these multimers form *in vivo*, their formation may enhance DNA binding, or may represent a form of AEBP1 regulation. GMSA studies also indicate that the DNA-binding domain of AEBP1 is located at the C terminus. Within this region are acidic and basic regions that may be responsible for the DNA-binding function of AEBP1.

Within the AE-1 site is a consensus DNA-binding site for members of the C/EBP family of proteins. Nonetheless, methylation interference analysis shows that other factors also bind within this AE-1 site (Ro and Roncari, 1991). The gel mobility shift assays performed with nuclear extracts and antibodies directed towards AEBP1 show that AEBP1 may be a *trans*-acting factor described by Ro and Roncari (1991) which binds to the AE-1 site in preadipocytes. Furthermore, these GMSA results suggest that AEBP1 is capable of binding to the AE-1 site only in preadipocytes; therefore AEBP1 may be responsible for negative transcriptional regulation of the aP2 gene.

Figure 3.6. AEBP1 DNA-Binding Domain is Located in the C terminus A mutant form of AEBP1 lacking the entire C-terminal region, AEBP1ΔSty, was used to determine if the DNA-binding region was located in the C terminus. GMSA were carried out as described. Lane 1 contains labeled AE-1 DNA alone. Lane 3 contains AEBP1 plus AE-1 DNA, and a mobility shift was observed. Lane 2 contains the AEBP1 mutant AEBP1ΔSty plus AE-1 DNA, and no protein-DNA complex was observed. Arrows indicate AEBP1–AE-1 complex. Protein-DNA samples were resolved by acrylamide non-denaturing gels, and the radiolabeled DNA was detected by autoradiography. This experiment was duplicated with similar results.

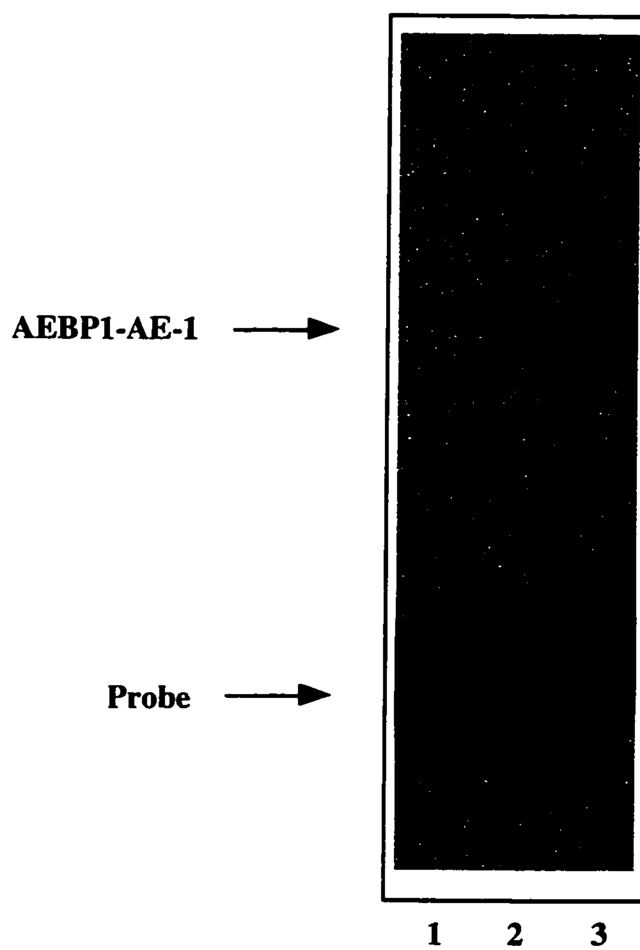


Figure 3.6 AEBP1 DNA-Binding Domain is Located in the C terminus

3.I.B. AEBP1, a Transcriptional Repressor

The ability of AEBP1 to bind to the AE-1 DNA sequence only in 3T3 L1 preadipocytes indicates that it may function as a negative transcription regulator of the aP2 gene. To study this possibility, CAT assays were carried out by Dr. G.-P. He to determine if AEBP1 could repress transcription.

The approach taken to study the role of AEBP1 as a transcriptional regulator was to link the promoter region of the aP2 gene, containing the AE-1 site, to the chloramphenicol acetyltransferase (CAT) gene. The reporter plasmid paP2(-168)CAT contains the promoter of the aP2 gene (sequences -168 to +21 in relation to the transcription start sequence), including the AE-1 site, inserted upstream of the CAT gene in the plasmid pUC-CAT. This construct allowed direct monitoring of CAT gene expression as regulated by AEBP1. The cDNA encoding AEBP1 was cloned downstream of the pSV40 promoter in the plasmid pKSV10 in both the positive (pSVAEBP1) and negative [pSVAEBP1(-)] directions. These constructs allowed the study of AEBP1 expression in transiently transfected cells. pSVAEBP1(-) was not produced as the opposite orientation contained a premature stop codon and provided a negative control for transcriptional regulation studies. Transient transfection into NIH 3T3 cells, and CAT assays were carried out as described in sections 2.III.F. and G..

Figure 3.7 shows that AEBP1 represses expression of the CAT gene under the control of aP2 promoter sequences that include the AE-1 site. Comparison of Lane 1 [pSVAEBP1(-)] with Lane 2 (pSVAEBP1) shows a five-fold decrease in CAT activity when AEBP1 was expressed. As controls, the following reporter genes containing heterologous promoters lacking the AE-1 site were transfected along with pSVAEBP1

Figure 3.7. AEBP1 is a Transcription Repressor

Transient transfection experiments show that AEBP1 repressed transcription of the CAT gene regulated by the AE-1 promoter region of the aP2 gene. Transient transfections were done using the indicated reporter construct along with either the control plasmid pSVAEBP1(-) (lanes 1, 3, 5) or an expression vector encoding AEBP1 (pSVAEBP1; lanes 2, 4, 6). The reporter plasmid paP2(-168)CAT (lanes 1 and 2) contains the AE-1 site inserted upstream of the bacterial CAT gene from pUC-CAT. For description of the control plasmids lacking the AE-1 site, see text. Transcription activity is presented in absolute levels of CAT activity (% conversion of chloramphenicol [C] to acetylated chloramphenicol [AC]) driven by each promoter. CAT activity was assayed after adjusting the cell extracts by β-galactosidase activity as described in section 2.III.G. Radioactivity was detected by autoradiography and counted with a scintillation counter. Similar results were obtained in three different transfection experiments. CAT assays were performed by Dr. G.-H. He (He et al., 1995).

Reporters	paP2(-168)CAT		pTKCAT		pRXV40CAT	
pSVAEBP1(-)	+		+		+	
pSVAEBP1		+		+		+
% Conversion	15.0	2.7	35.0	26.0	44.0	50.0

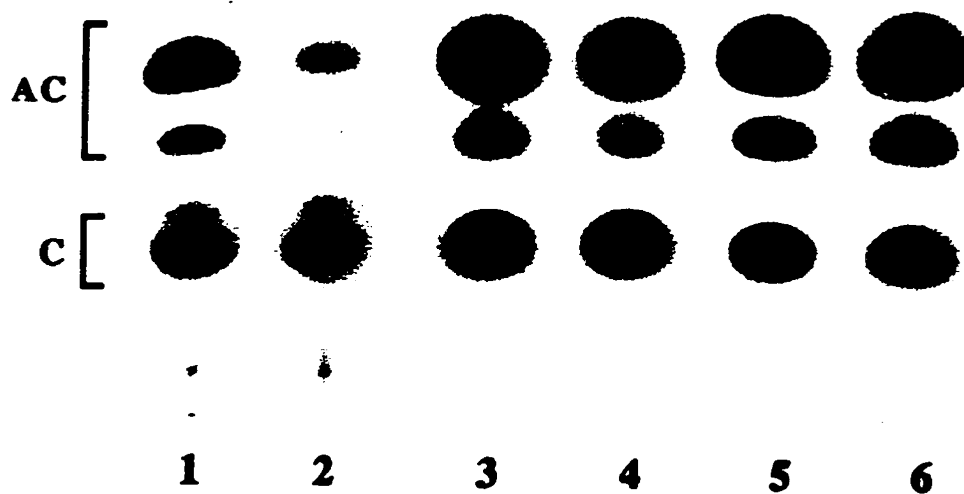


Figure 3.7 AEBP1 is a Transcription Repressor

and the control [pSVAEBP1(-)]: TK (pTKCAT; Lanes 3 and 4) and SV40 (pRXSV40CAT; Lanes 5 and 6). These reporters, lacking the AE-1 site, showed negligible CAT repression when AEBP1 was expressed. The results indicate that AEBP1 is capable of repressing the CAT gene *in vivo,* and that this repression is dependent on the AE-1 promoter region of the aP2 gene. Therefore AEBP1 may be an important regulator of the aP2 gene in the preadipocyte state.

The transcriptional repression caused by AEBP1 requires that the reporter plasmid to contain an AE-1 site. The observed repression may be due to a passive repression mechanism whereby AEBP1 prevents activators from binding DNA, and activating transcription. Alternatively, the observed repression may be due to a general non-specific shutdown of the RNA polymerase II machinery. However, a general non-specific shutdown of the RNA polymerase II machinery was unlikely as the control promoters, which do not contain the AE-1 site, were not influenced by AEBP1 expression. These results cannot determine if the transcriptional repression caused by AEBP1 is due to a passive process whereby AEBP1 blocks DNA sites which are normally bound by activators, or by an active mechanism whereby AEBP1 directly influences the general transcription machinery. To address this question, fusion proteins linking AEBP1 to the DNA-binding domain of Gal4 were constructed.

The cDNA encoding AEBP1 was cloned into the effector plasmid pG4 in both the positive (pG4-AEBP1) and negative [pG4-AEBP1 (-)] orientations, and transfected into NIH 3T3 cells along with the reporter plasmid pGALTKCAT containing five Gal4-binding sites upstream of the TK promoter that controls the CAT gene. Figure 3.8 shows that AEBP1 does repress transcription of the CAT gene when controlled by the Gal4-binding sites. Comparing lanes 2 (pG4-AEBP1) and 3 [pG4-AEBP1 (-)] shows that CAT activity was reduced six-fold when the Gal4-AEBP1 fusion protein

Figure 3.8 AEBP1-Gal4 Fusion Proteins Represses Transcription of the CAT Gene Controlled by Gal4-Binding Sites

These representative CAT assays show the repression function of G4-AEBP1 fusion proteins. Transient transfection analysis was done using the indicated reporter with either pG4, expressing the Gal4 DNA binding domain alone (lane 1 and 6), pG4-AEBP1 (lane 2 and 7), or the control pG4-AEBP1(-) (lane 3 and 8). Lanes 4 (pG4-AEBP1ΔSty) and 5 (pG4-AEBP1ΔHic) show CAT assays with mutant forms of AEBP1 and are discussed in section 3.I.D. Transcription activity is presented in absolute levels of CAT activity (% conversion of chloramphenicol [C] to acetylated chloramphenicol [AC]) expressed by each reporter. CAT activity was assayed after adjusting the cell extracts by β-galactosidase activity as described in section 2.III.G. Radioactivity was detected by autoradiography and counted with a scintillation counter. Similar results were obtained in three different transfection experiments. CAT assays were performed by Dr. G.-H. He (He et al., 1995).

pGALTKCAT pTKCAT Reporters

pG4 pG4-AEBP1 pG4-AEBP1(-) pG4-AEBP1ΔSty pG4-AEBP1ΔHic pG4 pG4-AEBP1 pG4-AEBP1(-) Effectors

15.4 2.8 16.8 3.8 16.4 26.9 28.5 24.5 % Conversion

AC

C

1 2 3 4 5 6 7 8

Figure 3.8 AEBP1-Gal4 Fusion Proteins Repress Transcription of the CAT Gene Controlled by Gal4-Binding Sites

was expressed. A control reporter lacking the Gal4-binding sites [Lanes 6, pG4; Lane 7, pG4-AEBP1; and Lane 8, pG4-AEBP1(-)] was not influenced by the expression of AEBP1.

These results indicate that AEBP1 is not repressing transcription through a passive mechanism, whereby AEBP1 blocks activator DNA-binding sites or masks activator domains responsible for DNA-binding. The transcriptional repression attributed to AEBP1 does not appear to occur through the prevention of transcriptional activators from binding to a specific AE-1 site, as the Gal4 binding promoter does not contain an AE-1 site, although it is possible that AEBP1 blocks activator binding sites in both the AE-1 site and Gal4 binding promoter region. However, the G4-AEBP1 fusion protein must be targeted the Gal4 DNA-binding site for transcriptional repression to occur.

These results suggest that AEBP1 may act by an active repression mechanism whereby the protein directly influences the general transcriptional machinery resulting in the inhibition of transcription. The ability of AEBP1 to repress transcription of the CAT gene by binding to the AE-1 site, and the ability of the Gal4-AEBP1 fusion protein to repress transcription of the CAT gene by binding to the Gal4-binding sites, indicates that directing AEBP1 to distinct DNA regions is vital for its repressor function.

3.I.C. AEBP1, a Functional Carboxypeptidase

Sequence comparisons of AEBP1 with regulatory B-like carboxypeptidases (CPB-like) indicate that AEBP1 may have carboxypeptidase activity (Figure 1.7). Comparisons between AEBP1 and the different CPB-like members (Figure 1.7) show that these proteins vary greatly in the C and N termini (Gebhard et al., 1989). As these differences are thought to determine the location of the carboxypeptidase in the cell and

AEBP1 also varies in the C and N termini of the protease region, having an extra 136 amino acids in the N terminus and 168 amino acids in the C terminus, these additional amino acids may contribute to a multifunctional role for AEBP1 within the cell.

Although the mechanism by which CPB-like proteases remove C-terminal basic amino acids is unknown, comparisons of the Zn^{2+}-binding residues, substrate-binding residues, and the catalytic residues with similar residues in carboxypeptidase A (CPA) (Christianson et al., 1987) may give insight into possible mechanisms of the CPB-like protease. AEBP1 has the conserved Zn^{2+}-binding residues His 207, Glu 210, and the similar residue Asn 345 (His 69, Glu 72, and His 196 are found in CPA; Gebhard et al., 1989; Roth et al., 1991), and the conserved catalytic residue Glu 452 (Glu 270 in CPA; Gebhard et al., 1989) found in all carboxypeptidases. AEBP1 also has conserved catalytic residues Gly 346 and Gly 347 found in carboxypeptidases N and E (residues 197 and 198, and 207 and 208 respectively; Gebhard et al., 1989). Other residues (Arg 127, Ser 197, and Try 198; Gebhard et al., 1989) found to be important in CPA activity are not conserved in any of the CPB-like proteins, and conserved residues Arg 145 and Tyr 248 of CPA are not conserved in AEBP1.

Although these sequence comparisons suggest that the CPB-like proteases function using a mechanism similar to that defined for CPA, the number of non-conserved catalytic residues between the CPB-like and CPA enzymes indicates that a slightly different mechanism may be involved in CPB-like protease activity. Sequence comparisons also suggest that AEBP1 may function similarly to the CPB-like proteases.

Residues His 207 and Glu 452 (His 69 and Glu 270 in CPA) have been determined to be involved in a mechanism important in a wide variety of metallo-peptidases, including bovine carboxyl-dipeptidase angiotensin-converting enzyme, rabbit neutral endopeptidase, enkephalinase, human fibroblast collegenase, bacterial thermoylsin (Gebhard et al., 1989), and AEBP1. Figure 3.9 shows the conserved

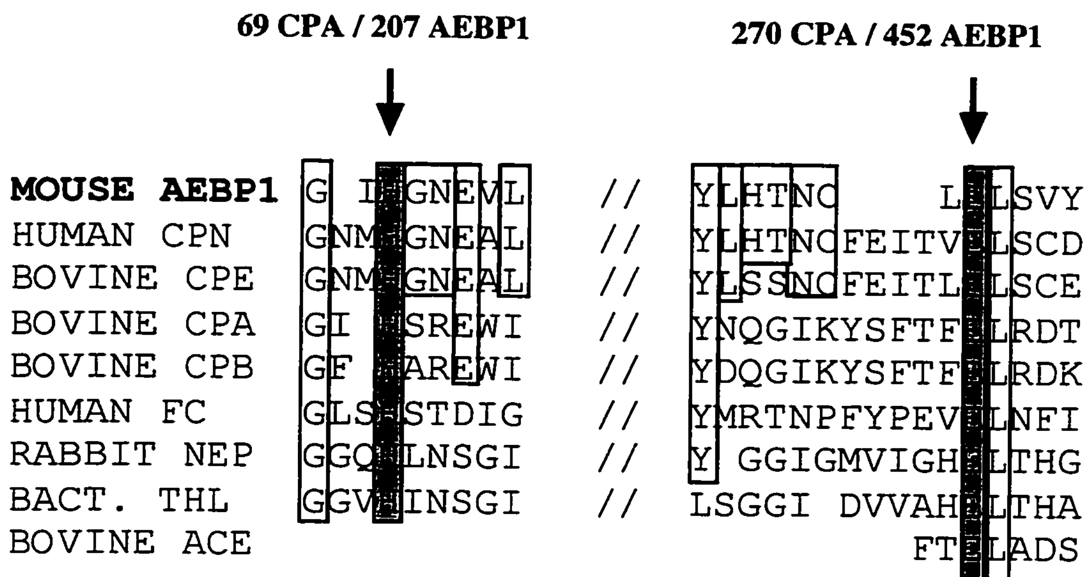


Figure 3.9. Common Mechanistic Residues Found in Metallo-Proteases This figure, modified from Gebhard et al. (1989, Figure 4), shows the similarity around residues His 69 and Glu 270 of CPA among AEBP1, carboxypeptidases (CP) A, B, E, and N, and the following metallo-endopeptidases: angiotensin-converting enzyme (ACE), fibroblast collagenase (FC), neutral endopeptidases (NEP), and bacterial thermolysin (BACT. THL). Identical residues are boxed.

regions. These comparisons suggest that a wide variety of metallo-peptidases employ common mechanisms in their protease functions, and possibly that AEBP1 employs a similar protease mechanism.

To determine if the AEBP1 carboxypeptidase domain is functional, enzyme assays were performed using the synthetic substrates hippuryl-Arg, hippuryl-Lys and hippuryl-Phe. Upon cleavage of the amino acid, the product hippuric acid can be spectrophotometrically measured by absorbance at 254 nm (A_{254}). Figure 3.10 shows that recombinant His-tagged AEBP1 was capable of cleaving the synthetic substrates hippuryl-Arg and hippuryl-Lys, indicating that the protein has functional carboxypeptidase activity. AEBP1 did not cleave the synthetic substrate hippuryl-Phe, a substrate for CPA. These results further indicate that AEBP1 functions similarly to CPB-like enzymes.

To ensure that the enzyme activity observed was due specifically to AEBP1, a number of controls were carried out. As the His-tagged AEBP1 assayed for enzymatic activity was a recombinant protein expressed and purified from bacteria, controls containing other His-tagged recombinant proteins, and controls containing bacterial extracts put through similar purification procedures, were tested in the carboxypeptidase assay. These purified His-tagged recombinant proteins and purified bacterial extracts showed no carboxypeptidase activity, indicating that the carboxypeptidase activity observed in the AEBP1 assays was due to AEBP1 and not to other proteins that may co-purify during the purification procedure.

To further explore the enzymatic activity of AEBP1, known carboxypeptidase activators and inhibitors were tested. The carboxypeptidase activators $NiCl_2$, $CoCl_2$, and $ZnCl_2$ (CPN, Skidgel et al., 1984; CPE and CPD, Song and Fricker, 1995; and CPM, McGuire and Skidgel, 1995) all were found to enhance AEBP1 carboxypeptidase activity. Figure 3.11 summarizes these activator studies and shows

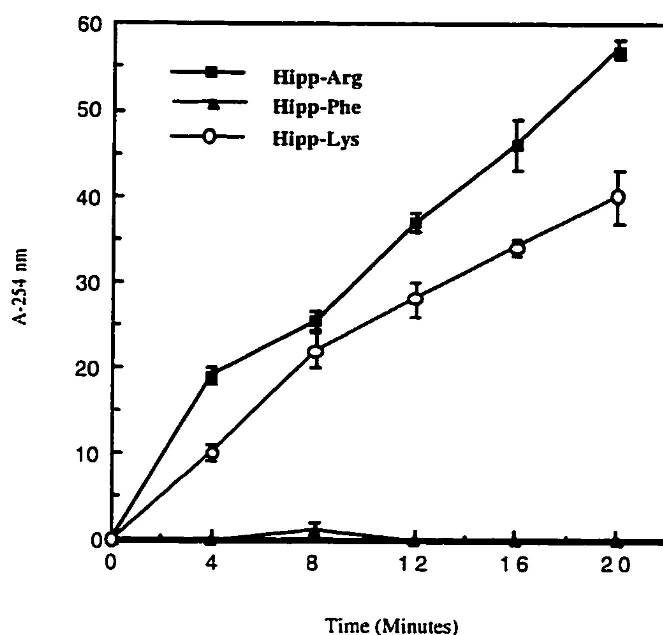


Figure 3.10. AEBP1 has Carboxypeptidase Activity

Carboxypeptidase assays were carried out using the recombinant His-tagged AEBP1 enzyme. The conversion of hippuryl-Arg (■), -Lys (○), and -Phe (▲) to hippuric acid was measured spectrophotometrically at 254 nm. The y ordinate gives absorbance in arbitrary units. All values were reported as means ± standard deviation (SD). All assays were repeated 4 times, n = 4. Where SD error bars are not visible they are contained within the dimensions of the symbol.

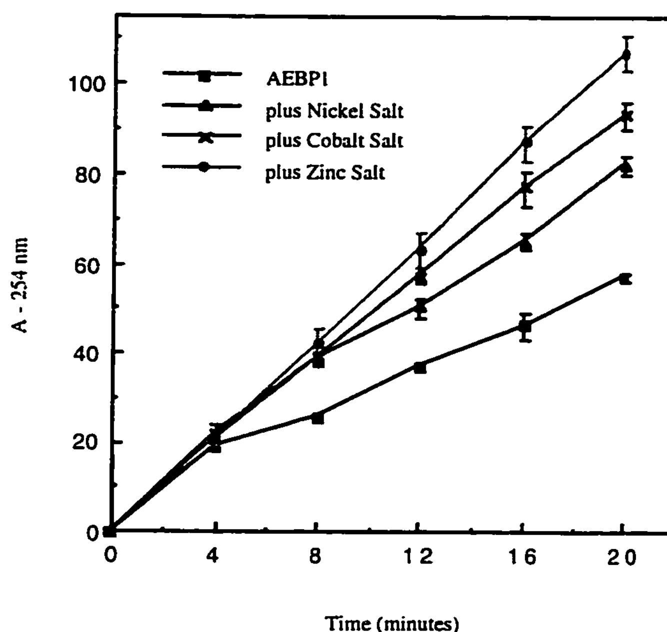


Figure 3.11 AEBP1 Carboxypeptidase Activity is Activated by Metal Salts

Carboxypeptidase assays were carried out for the recombinant His-tagged enzyme in the presence of metal activators. These salts were incubated with AEBP1 for 1 hour, after which conversion of hippuryl-Arg to hippuric acid was measured spectrophotometrically at 254 nm. The reaction contained AEBP1 alone (■), plus 1 mM $NiCl_2$ (▲), 1 mM $CoCl_2$ (x) and 1 mM $ZnCl_2$ (●). The y ordinate gives absorbance in arbitrary units. All values were reported as means ± standard deviation (SD). All assays were repeated 4 times, n = 4. Where SD error bars are not visible they are contained within the dimensions of the symbol.

that a 1 mM concentrations caused a 1.4-fold activation for $NiCl_2$, a 1.6-fold activation for $CoCl_2$, and a 1.9-fold activation for $ZnCl_2$ after a 20-minute assay. In Figure 3.12 the general carboxypeptidase inhibitor and chelator *o*-phenanthroline (CPM, Deddish et al., 1990; CPN, Barabe and Huberadeau, 1991; CPE and CPD, Song and Fricker, 1995; and CPM, McGuire and Skidgel, 1995), and the specific carboxypeptidase competitive inhibitor captopril (CPN, Barabe and Huberadeau, 1991), both inhibited AEBP1 carboxypeptidase activity. The specific inhibitor captopril (1 μM) reduced AEBP1 carboxypeptidase activity 54%, while 10 μM inhibitor completely abolished enzyme activity (data not shown). The general chelator *o*-phenanthroline (5 mM) reduced carboxypeptidase activity 40%, while 20 mM inhibitor completely abolished AEBP1 carboxypeptidase activity (data not shown). These studies further indicate that AEBP1 functions as a CPB-like enzyme, as it responds in a similar fashion as other CPB-like proteases to known activators and inhibitors.

The ability of AEBP1 to bind to a specific DNA sequence (section 3.I.A.) derived from the AE-1 promoter region of the aP2 gene raised the possibility that the AEBP1-DNA complex may affect AEBP1 enzymatic activity. To test this idea, AEBP1 was incubated with the AE-1 DNA sequence and then used in the carboxypeptidase enzymatic assay. As Figure 3.13 illustrates, AEBP1 carboxypeptidase activity was enhanced 1.9 fold in the presence of the specific DNA sequence. To ensure that the activation by the AE-1 oligonucleotide was not due to non-specific activation by DNA, a control assay containing the SP1 oligonucleotide (used as a control in the gel mobility shift assay; Figure 3.2) was performed. This control DNA sequence had no effect on AEBP1 carboxypeptidase activity (Figure 3.13). To further show that this was a specific activation of AEBP1 carboxypeptidase activity, due to AE-1 DNA, the assay was carried out using the C-terminal deletion-mutant form of AEBP1, AEBP1ΔSty,

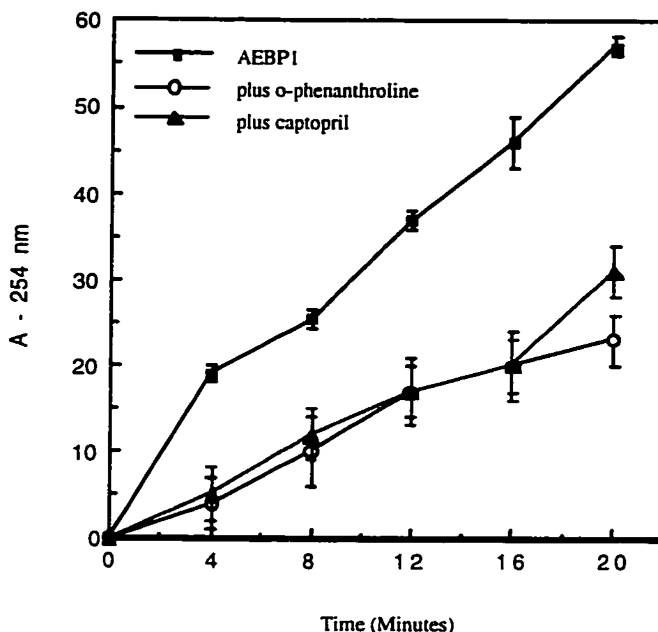


Figure 3.12. AEBP1 Carboxypeptidase Activity is Inhibited by Carboxypeptidase inhibitors

Carboxypeptidase assays were carried out using the recombinant His-tagged enzyme and putative carboxypeptidase inhibitors. These inhibitors were incubated with AEBP1 for 1 hour, after which the conversion of hippuryl-Arg to hippuric acid was measured spectrophotometrically at 254 nm. The reactions contained AEBP1 alone (■), plus 1 μM captopril (▲), or 5 mM *o*-phenanthroline (○).The y ordinate gives absorbance in arbitrary units. All values were reported as means ± standard deviation (SD). All assays were repeated 4 times, n = 4. Where SD error bars are not visible they are contained within the dimensions of the symbol.

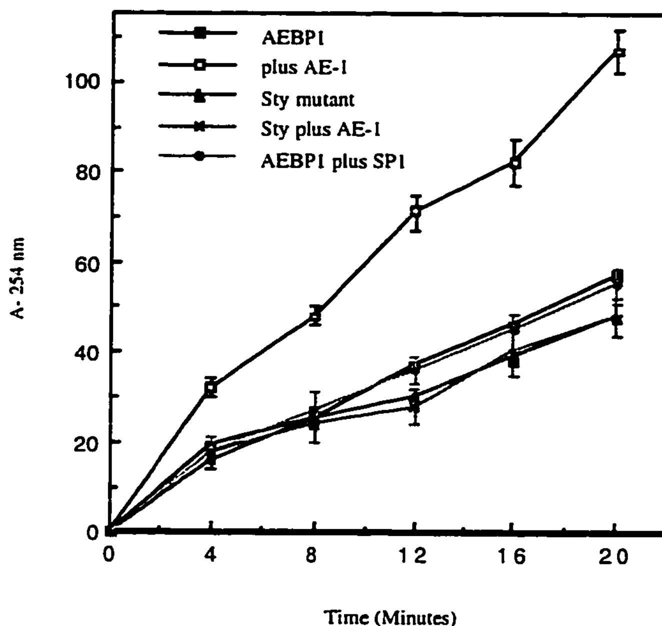


Figure 3.13. AEBP1 Carboxypeptidase Activity is Stimulated by the AE-1 DNA Sequence

The AE-1 DNA sequence or control DNA sequence was incubated with His-tagged AEBP1 (■) or AEBP1ΔSty (▲) for 1 hour, after which conversion of hippuryl-Arg to hippuric acid was measured spectrophotometrically at an absorbance (A) of 254 nm. The reactions contained AEBP1 (■) alone, or with 100 ng of AE-1 DNA (□) or SP1 DNA (●) added. Control reactions contained AEBP1ΔSty (▲) alone, or with 100 ng of AE-1 DNA (x) added. The y ordinate gives absorbance in arbitrary units. All values were reported as means ± standard deviation (SD). All assays were repeated 4 times, n = 4. Where SD error bars are not visible they are contained within the dimensions of the symbol.

which was not capable of binding DNA (Figure 3.6). AEBP1ΔSty displayed carboxypeptidase activity (Figure 3.13) but was not activated by the AE-1 oligonucleotide.

These results indicate that AEBP1 is activated by a novel mechanism whereby the direct binding of DNA enhances its protease activity. These studies show that by specifically binding to the AE-1 region of the aP2 promoter AEBP1 carboxypeptidase activity is enhanced. Other DNA molecules do not activate AEBP1 carboxypeptidase activity, and the enzymatic active mutant form of AEBP1 not capable of binding DNA was not influenced by the AE-1 DNA sequence. These results suggest that the ability of AEBP1 to bind DNA, its protease activity, and its transcriptional repression function are all correlated.

Kinetic constants were determined using recombinant purified His-tagged AEBP1, with the results illustrated in Figure 3.14 and summarized in Table 3.1. The results showed that AEBP1 has a higher affinity for the substrate hippuryl-Arg than it did for the substrate hippuryl-Lys (Figure 3.14, A and B). The greater than 4-fold difference in K_m values for the substrates suggests that arginine may be the preferred C-terminal amino acid of an unknown substrate which AEBP1 cleaves.

AEBP1 has a higher affinity for the activator $ZnCl_2$ than the other metals $CoCl_2$ and $NiCl_2$, and is more sensitive to the specific carboxypeptidase competitive inhibitor captopril than to the general chelator *o*-phenanthroline. These results indicate that both the substrate-binding and Zn^{2+}-binding residues are important for AEBP1 carboxypeptidase activity, as a known inhibitor which chelates zinc inhibits carboxypeptidase activity, and a known carboxypeptidase competitive inhibitor also inhibits enzymatic activity. These experiments, illustrated in Figure 3.14 and summarized in Table 3.1, ultimately show that AEBP1 behaves like other members of the CPB-like carboxypeptidases in terms of its response to substrates, activators and inhibitors.

Hippuryl-Lys	K_m	5.0 ± 0.4 mM
Hippuryl-Arg	K_m	1.2 ± 0.3 mM
AE-1 DNA	K_a	110 ± 23 ng/ml
$NiCl_2$	K_a	0.71 ± 0.11 mM
$CoCl_2$	K_a	0.55 ± 0.09 mM
$ZnCl_2$	K_a	0.29 ± 0.09 mM
captopril	I_{50}	4.5 ± 0.8 μM
o-phenanthroline	I_{50}	7.2 ± 0.4 mM

Table 3.1. Properties of AEBP1 Carboxypeptidase

Kinetic studies of carboxypeptidase activity of the recombinant His-tagged enzyme. For K_m determination, substrate concentrations were varied and the conversion of hippuryl-Arg or -Lys to hippuric acid was measured. For K_a studies 1 mM hippuryl-Arg was used, and for I_{50} studies 1.5 mM hippuryl-Arg was used. Concentrations of activators and inhibitors were varied to determine I_{50} and K_a values. All assays were measured spectrophotometrically at an absorbance of 254 nm. All values are reported as means ± standard deviation (SD). All assays are repeated 4 times, n = 4.

Figure 3.14. Kinetic Properties of AEBP1 Carboxypeptidase

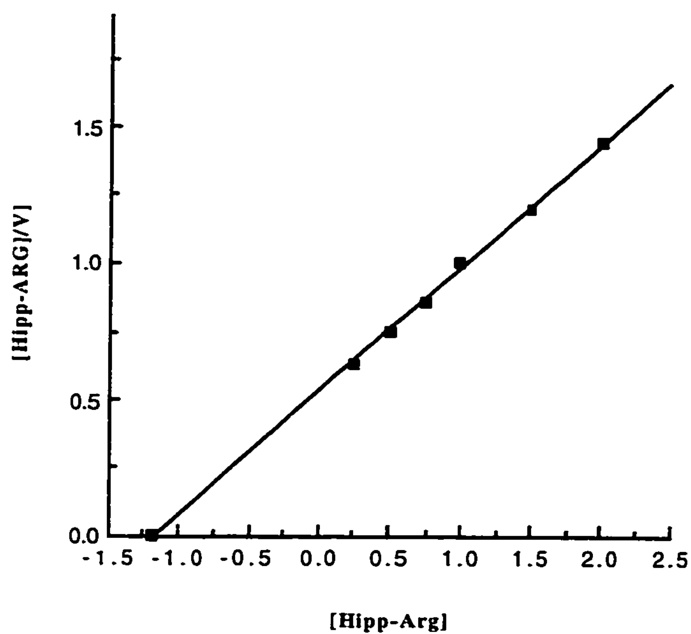
Kinetic studies were performed with the recombinant His-tagged AEBP1 carboxypeptidase. These are representative plots which illustrate the effects of varying substrate, activator, and inhibitor concentrations on the carboxypeptidase activity of AEBP1.

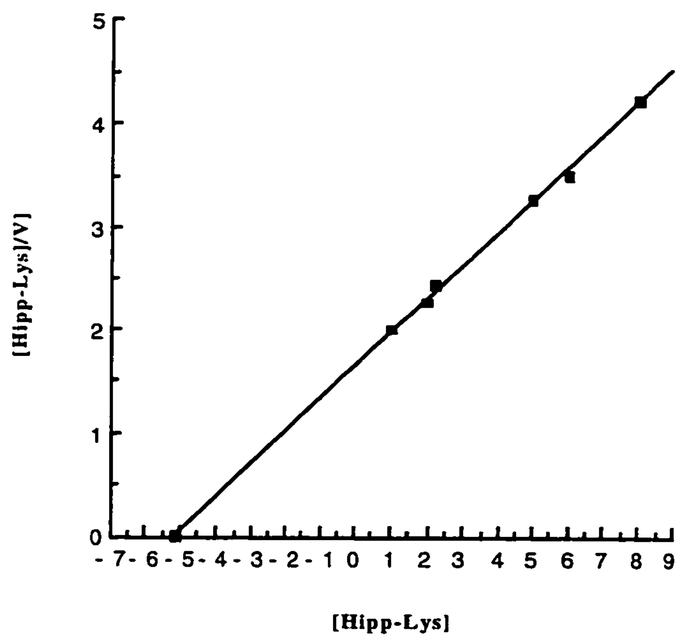
A. and B. The K_m values for the substrates Hippuryl-Arg (**A.**, upper panel) and Hippuryl-His (**B.**, lower panel) were determined by plotting [substrate]/Velocity versus [substrate] in a Hanes plot. The intercept with the x-axis determined the K_m value.

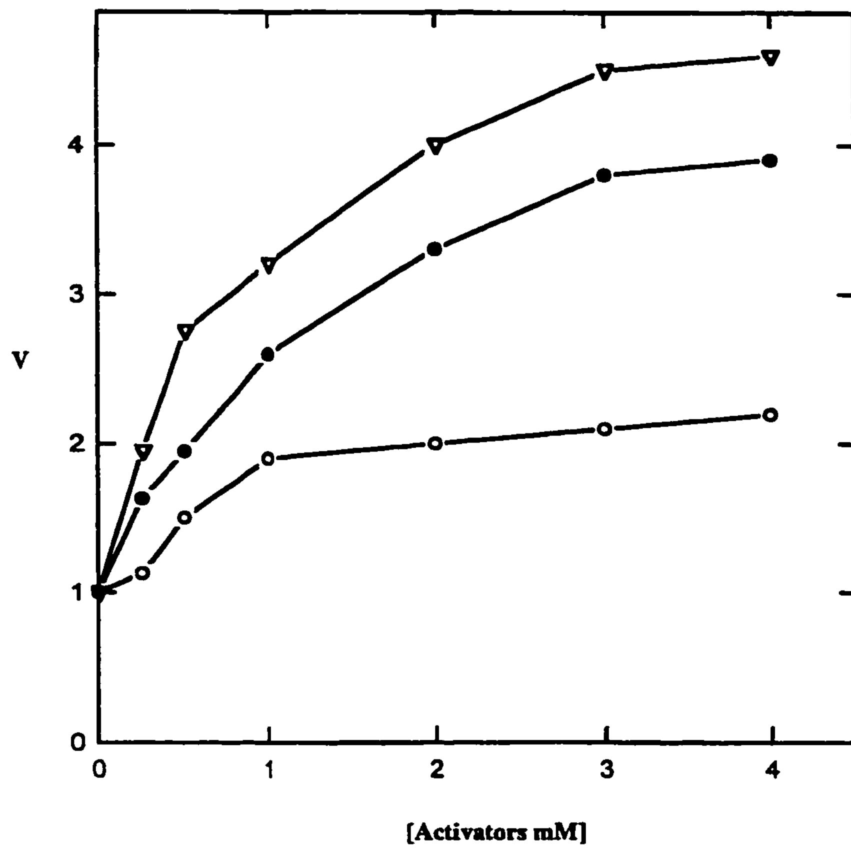
C. The K_a values for the activators for the $NiCl_2$ (○), $CoCl_2$ (●), and $ZnCl_2$ (▽) were determined through plotting V_{max} versus [activator].

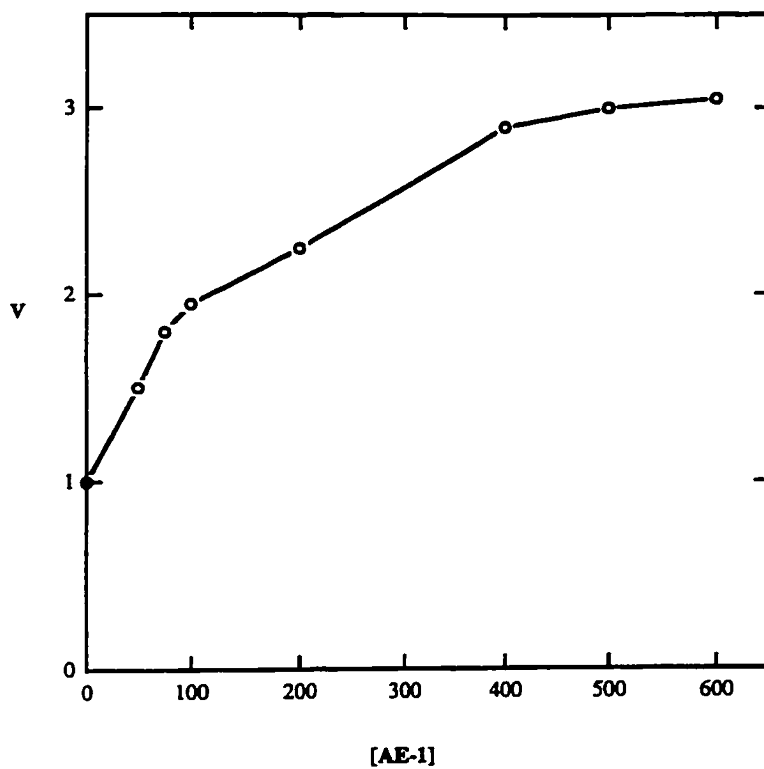
D. The K_a values for the activators AE-1 DNA was determined through a plotting V_{max} versus [activator].

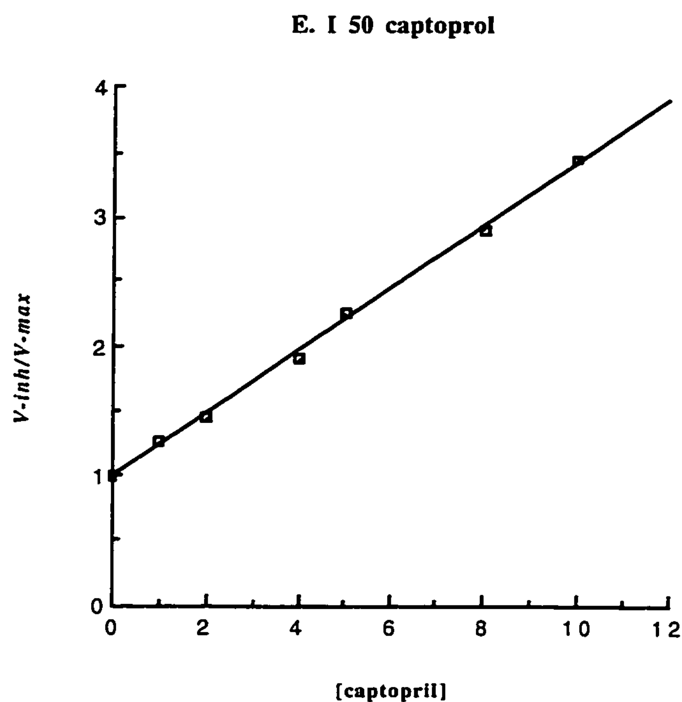
E. and F. The I_{50} values for the inhibitors captopril (**E.**, upper panel) and *o*-phenanthroline (**F.**, lower panel) were determined by plotting $V_{inhibitor}/V_{max}$ versus [inhibitor]. Concentration of captopril was in μM, and for *o*-phenanthroline was mM.


A. Km Hipp-Arg
[Hipp-ARG]/V]
1.5
1.0
0.5
0.0
-1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0 2.5
[Hipp-Arg]


B. Km Hipp-Lys
[Hipp-Lys]/V]
5
4
3
2
1
0
-7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9
[Hipp-Lys]


C. Activators
V
0
1
2
3
4
0
1
2
3
4
[Activators mM]


D. AE-1 Activation
V
0
1
2
3
0
100
200
300
400
500
600
[AE-1]



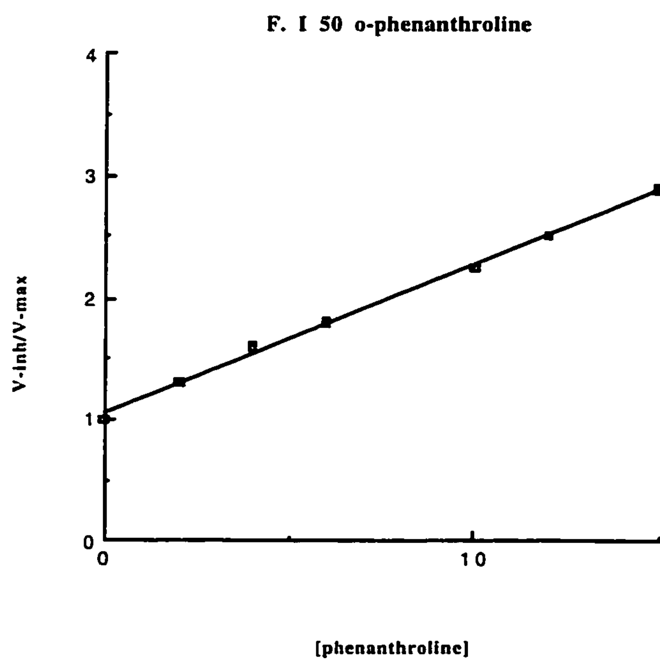


Figure 3.14. Kinetic Properties of AEBP1 Carboxypeptidase

3.I. D. AEBP1 is a a DNA-binding Protein, a Transcriptional Repressor, and Carboxypeptidase

AEBP1 possesses a protease activity capable of cleaving basic amino acids from synthetic substrates. AEBP1 also directly binds to the AE-1 upstream promoter region of the aP2 gene. The interaction between AEBP1 and the specific AE-1 DNA sequence stimulates AEBP1 carboxypeptidase activity. Furthermore, AEBP1 represses transcription when directed to the AE-1 promoter region, and Gal4-AEBP1 fusion proteins repress transcription when directed to upstream Gal4-binding sites. These transcriptional studies indicate that AEBP1 represses transcription through an active mechanism, by directly influencing the general transcription machinery. These results indicate that the binding of AEBP1 to the AE-1 promoter region of the aP2 gene leads to activation of AEBP1 carboxypeptidase activity, and that this enhanced enzymatic activity may then be used to repress transcription of the aP2 gene in preadipocytes. To explore this intriguing hypothesis, mutational analysis of AEBP1 was carried out, assaying the mutants for both carboxypeptidase and transcriptional repression activities.

Mutations were constructed in both the C and N termini of the carboxypeptidase domain of AEBP1, and larger deletions encompassing the entire C-terminal region (residues 551-719) of the protein were also used (see Table 3.2 for detailed information concerning the nature and construction of mutations). The mutant AEBP1 cDNAs were made in pBlueScript SKII(+) vector and then subcloned into the pET-16b vector for protein expression and enzymatic assays. All mutations were also subcloned into the pG4 fusion-protein vector and tested in the Gal4-binding CAT assay system to allow mutants missing C-terminal DNA-binding regions to be assessed as Gal4 fusion proteins that would be directed towards the Gal4-binding sites. All CAT assays were carried out by Dr. He (He et al., 1995).

Table 3.2. Construction and Description of AEBP1 Mutants

AEBP1 mutants were constructed and tested for carboxypeptidase activity and for the ability to repress transcription. Mutants were named according to restriction site in AEBP1 cDNA that was used to create the mutation, or according to mutations caused by Bal 31 nuclease digestion. Restriction sites are indicated in Figure 1.4. Reading frame "shift" indicates that a frame shift was caused in the construction of the mutation in the AEBP1 cDNA, while "continuous" indicates that the reading frame was maintained in construction of the mutation in the AEBP1 cDNA.

Name	Construct of mutation	Amino Acids Deleted	Reading Frame
ΔSty	*Sty*I site (nt 1700) Blunt and Religate	205 aa of C-terminal	Shift
ΔSac	*Sac*II site (nt 1283) Blunt and Religate	344 aa of C-terminal	Shift
ΔBam	*Bam*HI site (nt 770) Blunt and Religate	513 aa of C-terminal	Shift
ΔBal-1	Bal 31 Nuclease digestion at *Sac*I (nt 1515)	aa 504-520	Continuous
ΔBal-2	See ΔBal-1	aa 504-545	Continuous
ΔBal-3	See ΔBal-1	aa 502-545	Continuous
ΔBal-4	See ΔBal-1	218 aa of C-terminal	Shift
ΔBal-5	See ΔBal-1	aa 417-525	Continuous
ΔBal-6	See ΔBal-1	250 aa of C-terminal	Shift
ΔBal-7	See ΔBal-1	256 aa of C-terminal	Shift
ΔBal-8	See ΔBal-1	aa 461-545	Continuous
ΔHic	*Hic*II (nt 1441) to *Hic*II (nt 1932) Fragment removed	aa 429-587	Continuous
ΔBal-9	See ΔBal-1	aa 448-472	Continuous
ΔBal-10	See ΔBal-1	aa 432-483	Continuous
ΔBal-11	Bal-31 Nuclease Digestion at *Bam*HI Site (nt 770)	aa 206-210	Continuous

ΔBal-12	See ΔBal-11	aa 203-208	Continuous
ΔBg/Ba	*Bgl*II (nt 703) to *Bam*HI (nt 770) removed	aa 182-206	Continuous
ΔBal-13	See ΔBal-11	aa 197-206	Continuous

Table 3.2. Construction and Description of AEBP1 Mutants

The AEBP1 mutants constructed are depicted in the following figures: Figure 3.15 , C-terminal deletions; Figure 3.16, deletions in the carboxypeptidase domain C terminus; and Figure 3.17, deletions in the carboxypeptidase domain N terminus. The results are summarized in Table 3.3.

The first set of mutants all have C-terminal truncations. The ΔSty mutant has the entire C terminus of AEBP1 deleted along with 37 amino acids of the carboxypeptidase C-terminal domain. This mutant still possessed carboxypeptidase and transcriptional repression activities. The two larger truncations ΔSac and ΔBam both have the majority of the C terminus of the CP region along with the entire C terminus of AEBP1 removed. Not surprisingly, these two mutants did not display carboxypeptidase or transcriptional repression activities. The ΔSac and ΔBam mutants have large truncations which encompass most of the protein; therefore the loss of both carboxypeptidase and transcriptional repression activities of these mutants cannot be correlated, as the folding of the remaining polypeptide is undoubtedly compromised.

The second set of mutants all contain deletions in the C terminus of the carboxypeptidase domain, and some of these have the entire C terminus of AEBP1 deleted. The mutants ΔBal-1, ΔBal-2, ΔBal-3, ΔBal-5, and ΔBal-8 all have various deletions at the C terminus of the carboxypeptidase domains, and all retain functional carboxypeptidase and transcriptional repression activities. These mutations are all localized to the carboxypeptidase region downstream from residues responsible for catalytic activity in other carboxypeptidases. Therefore, if the deletion did not affect protein folding it is not surprising that these mutants retain both enzymatic and transcriptional regulatory functions.

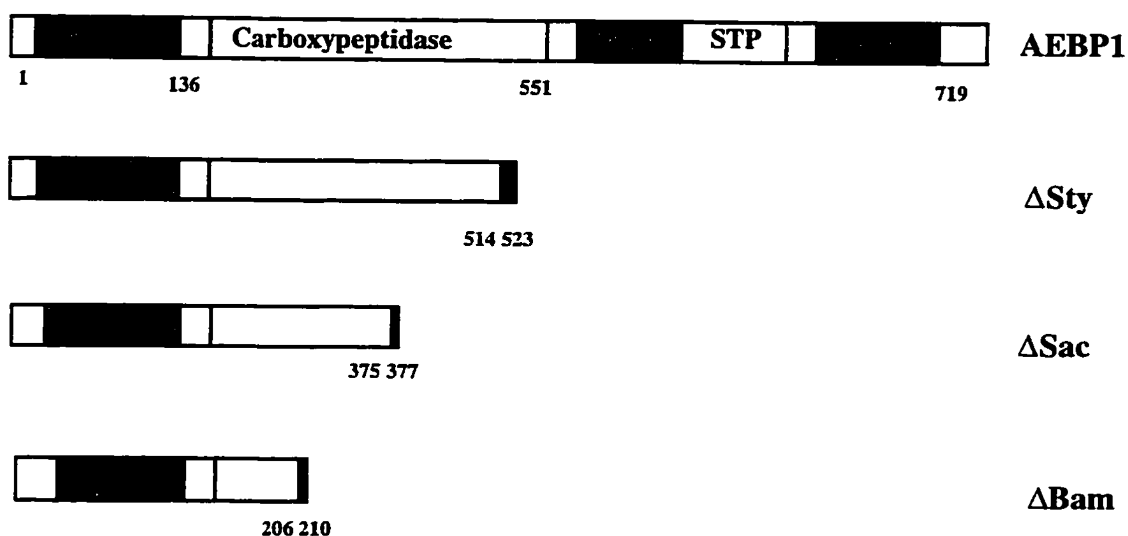


Figure 3.15. AEBP1 C-terminal Deletions

The cartoon, not drawn to scale, depicts AEBP1 and C-terminal mutants. These mutants are missing the entire C terminus and portions of the carboxypeptidase domain. DLD indicates Discoidin-like domain; STP indicates serine, threonine, and proline rich region. Numbers indicate amino acids, and dark a box at C-terminal end of a mutant indicates amino acid changes due to a frame shift caused during construction of the mutation.

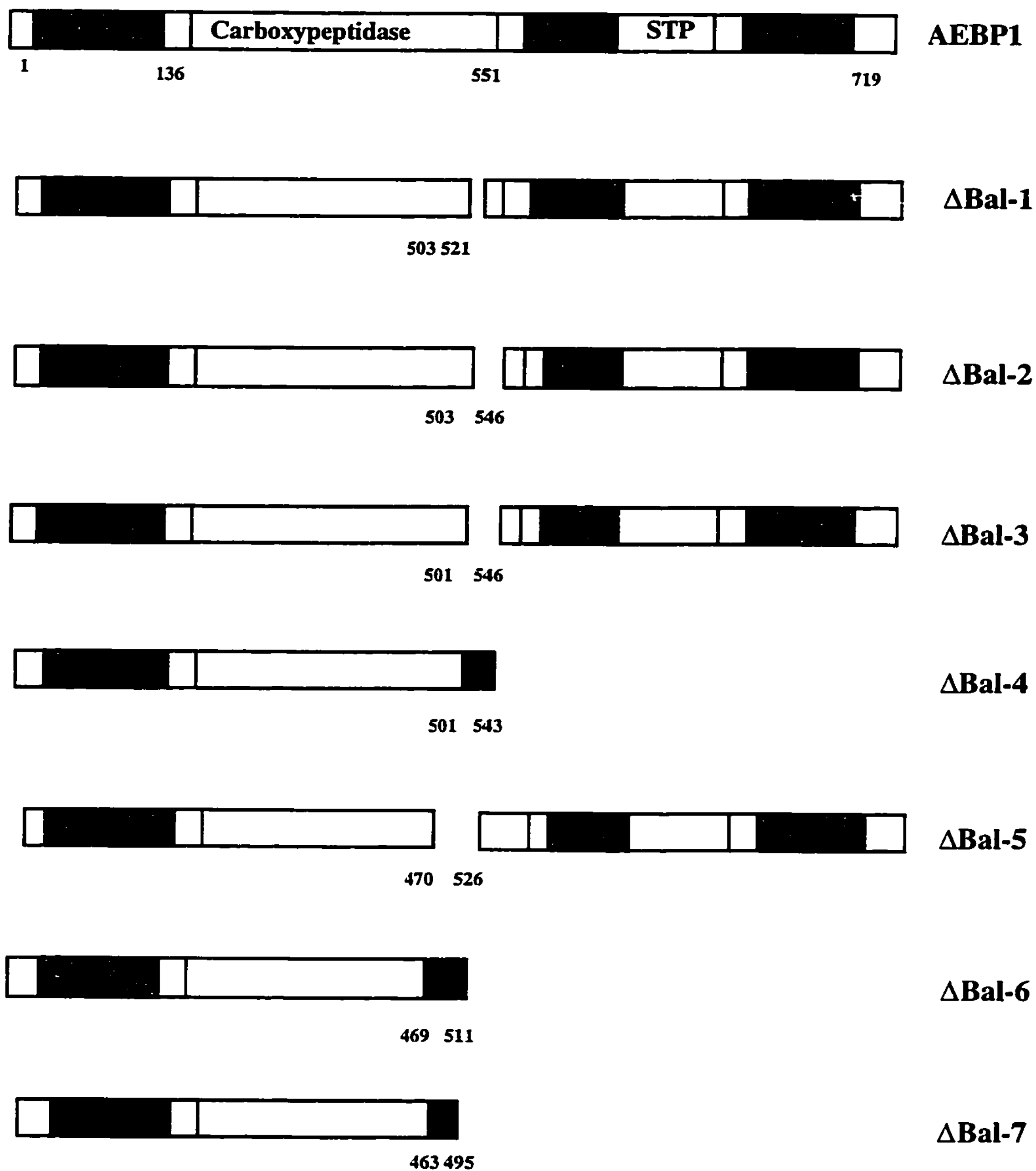
The mutants ΔBal-4, ΔBal-6, and ΔBal-7 all have C-terminal truncations encompassing the entire C terminus of AEBP1 and portions of the carboxypeptidase domain. These deletions do not remove any of the proposed functional residues of the carboxypeptidase region of AEBP1, and this group of mutants displays both carboxypeptidase and transcriptional repression activity.

The mutant ΔHic has 123 amino acids removed from the carboxypeptidase region and a further 36 amino acids deleted from the C terminus of AEBP1. The mutated protein, missing a number of residues believed to be involved in enzymatic activity, is unable to repress transcription or display carboxypeptidase activity.

The mutant ΔBal-9 has 25 amino acids removed from the carboxypeptidase domain of AEBP1. As this region was proposed to be important not only for the catalytic function of the family of carboxypeptidases but also for the peptidase activity of the endopeptidases discussed above (Figure 3.9), it would be expected that this mutant would have reduced enzymatic activity. As Table 3.3 shows, ΔBal-9 does indeed display reduced carboxypeptidase and transcriptional activity. The position of Glu 477 in the ΔBal-9 mutant in the same position relative to the N-terminal sequences of the missing Glu 452, may explain why the enzymatic activity of this particular mutant is not completely abolished. In this position Glu 477 may function in the catalytic mechanism but at a reduced efficiency. However, it is difficult to determine which residues are involved in the protease function of the mutant, as the removal of 25 amino acids may affect structural properties of the protein, and as the catalytic mechanism of AEBP1 and other B-like carboxypeptidases has not been elucidated, it is not certain that these residue are actually involved in the catalytic mechanism of AEBP1. Further study into this region of the protein may give insight into the mechanism by which this carboxypeptidase functions.

Figure 3.16. AEBP1 Carboxypeptidase C-terminal Mutants

The cartoon, not drawn to scale, depicts intact AEBP1 and carboxypeptidase C-terminal mutants. All mutants are missing the C-terminal portions of the AEBP1 carboxypeptidase domain. DLD indicates Discoidin-like domain; STP indicates serine, threonine, and proline rich region. Empty spaces in AEBP1 mutants depict deleted residues. All deletions have continuous reading frames unless indicated by a black box. Dark boxes at C-terminal end of mutants ΔBal-4, -6, and -7 indicate amino acid changes due to frame shifts caused during construction of the mutation, and numbers indicate amino acids.



Carboxypeptidase
STP
AEBP1
1
136
551
719
ΔBal-1
503 521
ΔBal-2
503 546
ΔBal-3
501 546
ΔBal-4
501 543
ΔBal-5
470 526
ΔBal-6
469 511
ΔBal-7
463 495

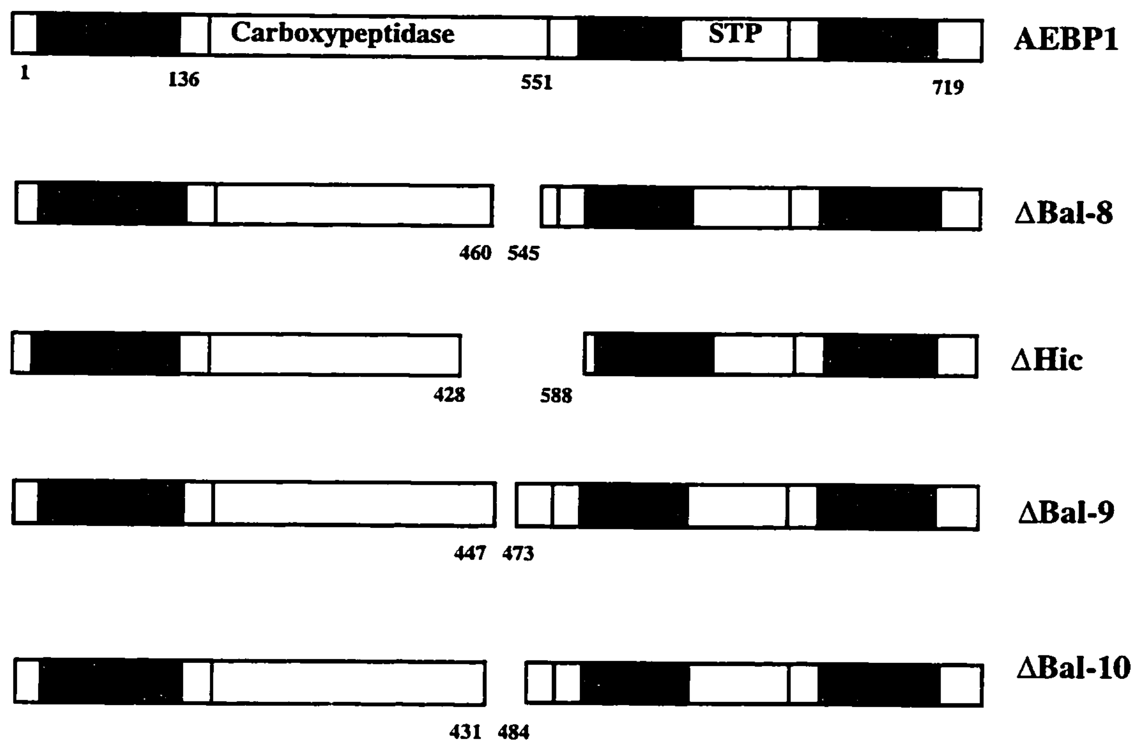


Figure 3.16. AEBP1 Carboxypeptidase C-terminal Mutants

The mutant ΔBal-10 has 52 amino acids deleted from the carboxypeptidase domain of AEBP1, including the proposed catalytic residue Glu 452. The deletion of this region of AEBP1 results in the loss of both the carboxypeptidase and repression activity of AEBP1.

The final set of mutants (Figure 3.17) has deletions in the AEBP1 carboxypeptidase N terminus. The mutant ΔBal-11 has 5 amino acids removed from the N terminus of the carboxypeptidase domain, including two of the three putative Zn^{2+}-binding residues. The removal of these residues is expected to reduce enzymatic activity. As Table 3.3 shows, both enzymatic and transcriptional repression activity are reduced in this mutation. The mutant ΔBal-12 has 6 amino acids removed from the N terminus of the carboxypeptidase domain, including one of the three putative Zn^{2+}-binding residues. This mutant would also be expected to have reduced enzymatic activity, except that the His 207 residue proposed to bind Zn^{2+} is replaced by Arg 201 which is similarily position relative to the downstream sequence due to the deletion, Arg 201 may therefore be capable of binding Zn^{2+} in this position. Table 3.3 shows that AEBP1 and ΔBal-12 have virtually the same enzymatic and transcriptional repression activities. However, it is difficult to determine which residues are involved in the Zn^{2+}-binding function of these mutants as the removal of 6 amino acids from ΔBal-12 and 5 amino acids ΔBal-11 may affect structural properties of the mutant proteins. It is also not certain that these resides are even involved in the Zn^{2+}-binding of AEBP1, as the catalytic mechanism of AEBP1 and other B-like carboxypeptidases has not been elucidated. Further study into this region of the protein may give insight into the mechanism by which this carboxypeptidase functions.

The mutant ΔBg/Ba has 25 amino acids removed from AEBP1 carboxypeptidase N terminus. This larger deletion abolishes both carboxypeptidase and transcriptional repression functions, indicating that this region is important to the function or structure of the protein.

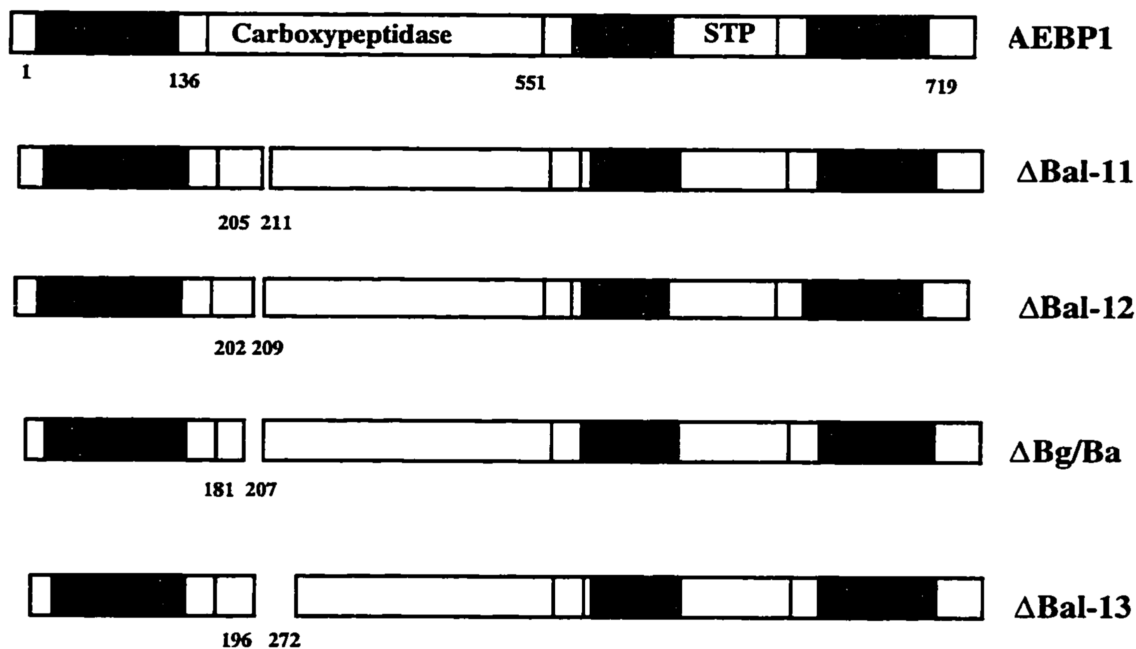


Figure 3.17. AEBP1 Carboxypeptidase N-Terminal Mutants

The cartoon, not drawn to scale, depicts intact AEBP1 and carboxypeptidase N-terminal mutants. All mutants are missing N-terminal portions of AEBP1 carboxypeptidase domain. DLD Discoidin-like domain; STP serine, threonine, and proline rich region. All deletions have continuous reading frames, and numbers indicate amino acids.

Mutant	CP Activity	Repressor Activity
AEBP1	++++	++++
ΔSty	+++	+++
ΔSac	–	–
ΔBam	–	–
ΔBal-1	+++	+++
ΔBal-2	+++	+++
ΔBal-3	+++	+++
ΔBal-4	+++	+++
ΔBal-5	+++	+++
ΔBal-6	+++	+++
ΔBal-7	+++	+++
ΔBal-8	+++	+++
ΔHic	–	–
ΔBal-9	++	++
ΔBal-10	–	–
ΔBal-11	+++	+++
ΔBal-12	++++	++++
ΔBg/Ba	–	–
ΔBal-13	–	–
ΔA/CPM	+++	+++

Table 3.3. Summary of Mutant AEBP1 Carboxypeptidase and Transcriptional Repression Activities

AEBP1 is considered to have full activity for both functions, as indicated by ++++. Mutants have either partial or no carboxypeptidase (CP) and transcriptional repression (repressor) activities, as compared to wild-type AEBP1. Partial activity is indicated by a number of (+)s, as determined in comparison to wild-type activity with ++++, and no activity is indicated by (–).

The mutant ΔBal-13 has 75 amino acids removed from the N terminus of the carboxypeptidase domain of AEBP1 including two of the three putative Zn^{2+}-binding residues. Again this mutant has both carboxypeptidase and transcriptional repression functions abolished, indicating that this region is important to the function or structure of the protein.

Western blot analysis of cell extracts using anti-GAL4 antibody was carried out to ensure that the proteins assayed in the CAT assay experiments summarized in Table 3.3 were expressed at equivalent amounts. He et al. (1995) showed that all of the proteins used for CAT assay experiments were expressed in transient transfected cells. Therefore the observed CAT assay results are not due varying protein expression levels of individual mutants. To ensure that the proteins expressed were capable of binding to the Gal4-binding site, GMSA experiments were carried out using *in vitro* transcribed and translated protein and a DNA fragment containing five copies of the Gal4-binding site. The results in Figure 3.18 show that the fusion proteins G4-AEBP1 (Lane 3), and G4-AEBP1ΔHic (Lane 4), are both able to bind to the Gal4-binding sites. This indicates that the AEBP1ΔHic mutant was capable of binding the Gal4-binding sites, and therefore the failure of this mutant to repress transcription in the CAT assay experiments summarized in Table 3.3 was not due to an inability of the mutant to bind DNA.

The results summarized in Table 3.3 show that the carboxypeptidase and transcriptional repression activities of AEBP1 are related. An obvious observation is that whenever AEBP1 carboxypeptidase activity is eliminated by mutation, the mutant protein is also no longer capable of repressing transcription. Likewise, whenever a version of AEBP1 displays enzyme activity it is also capable of repressing transcription. These results may not convincingly demonstrate that AEBP1 carboxypeptidase activity is required for repression of transcription, as any mutation that eliminates both functions may also cause conformational or folding defects that

Figure 3.18. G4-AEBP1 Fusion Proteins Bind to the Gal4-binding Sites

In vitro transcribed and translated G4-AEBP1 proteins were found to bind to labeled DNA containing five copies of the Gal4-binding site. Lane 1 contains labeled DNA alone; Lane 2 contains the parental expression vector pGEM-3Zf(-) and the Gal4-binding DNA; Lane 3 contains the fusion protein G4-AEBP1 and the Gal4-binding DNA; Lane 4 contains the fusion mutant G4-AEBP1ΔHic and the Gal4-binding DNA. The arrow indicates the protein-DNA complex.

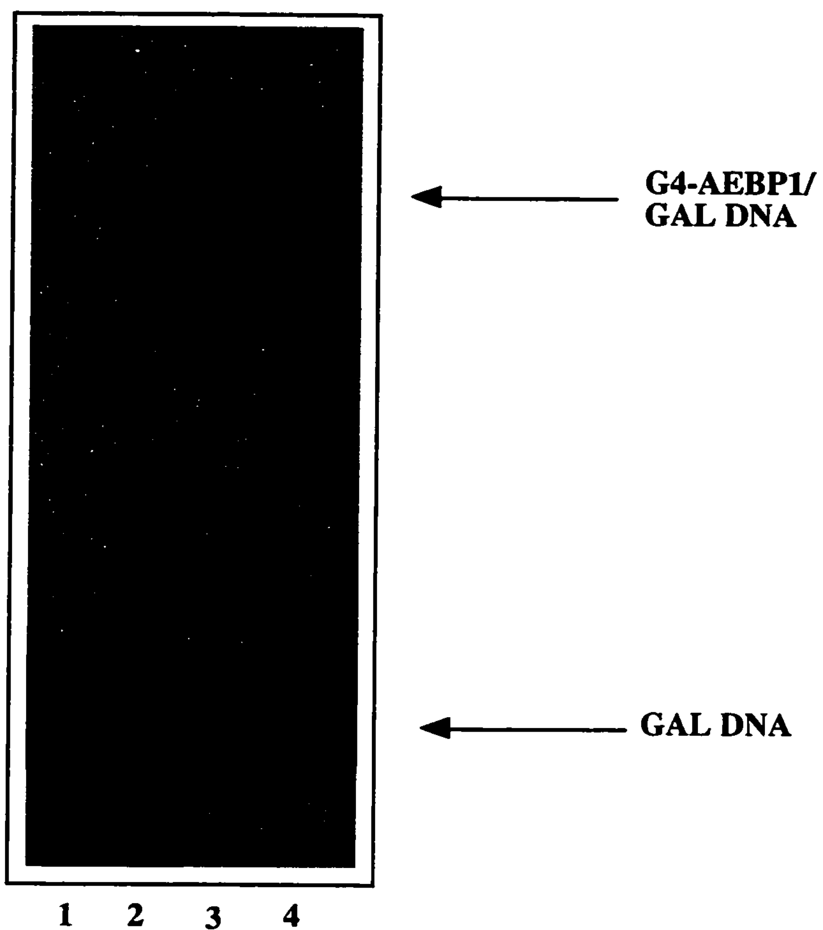


Figure 3.18 G4-AEBP1 Fusion Proteins Bind to the GAL4 DNA

prevent the mutant protein from performing these two functions. However, as Table 3.3 illustrates, the two functions appear to be interrelated. For all 18 mutants constructed, whenever a mutation reduces AEBP1 carboxypeptidase activity, there is a reduction in the transcriptional repression activity, suggesting that AEBP1 carboxypeptidase activity is vital for its ability to repress transcription.

The results indicate that AEBP1 may use an active mechanism of transcriptional repression whereby the protease is directed to DNA. The mutational analysis of AEBP1 shows that this transcriptional repression is not simply due to protein-protein interactions with the general transcriptional machinery. Truncations encompassing the entire C terminus are functionally inactive. This may be due to conformational disruptions associated with the removal of residues, which reduce or abolish activities. However, small deletions in both the N and C termini cause the loss of both carboxypeptidase and transcriptional repression functions. Again this may be due to conformational disruptions associated with the removal of residues, but similar deletions in these regions do remain active. Comparisons of the carboxypeptidase and transcriptional repression activities of the 18 mutants suggest that the protease activity of AEBP1 is required for its ability to repress transcription.

To further demonstrate that AEBP1 uses its carboxypeptidase activity to repress transcription, a protease-domain replacement was attempted. In this experiment the carboxypeptidase domain of AEBP1 was replaced with the entire carboxypeptidase domain of carboxypeptidase M (CPM; provided by R. A. Skidgel; McGuire and Skidgel, 1995; Deedish et al., 1990). As AEBP and CPM are similar throughout their carboxypeptidase regions (He et al., 1995), a chimeric protein with the carboxypeptidase domain of AEBP1 replaced with carboxypeptidase domain of CPM may be able to function as a transcriptional repressor when targeted to DNA. This chimeric protein (ΔA/CPM; Figure 3.19) was tested for carboxypeptidase and for

Figure 3.19. AEBP1 Carboxypeptidase Replacement Experiment

The cartoon, not drawn to scale, depicts the chimeric protein termed ΔA/CPM, which was constructed from AEBP1 and the membrane carboxypeptidase (CPM). The carboxypeptidase domain and C-terminal region of AEBP1 were removed and replaced with the carboxypeptidase region of CPM, minus its signal sequence and glycan attachment site. The chimeric protein, ΔA/CPM, had carboxypeptidase activity and was able to repress transcription when tested in the GAL4 CAT assay system. DLD Discoidin-like domain; STP serine, threonine, and proline rich region. Dark boxes at C-terminal end of mutants indicate amino acid changes due to frame shifts caused during construction of the mutation, and numbers indicate amino acids.

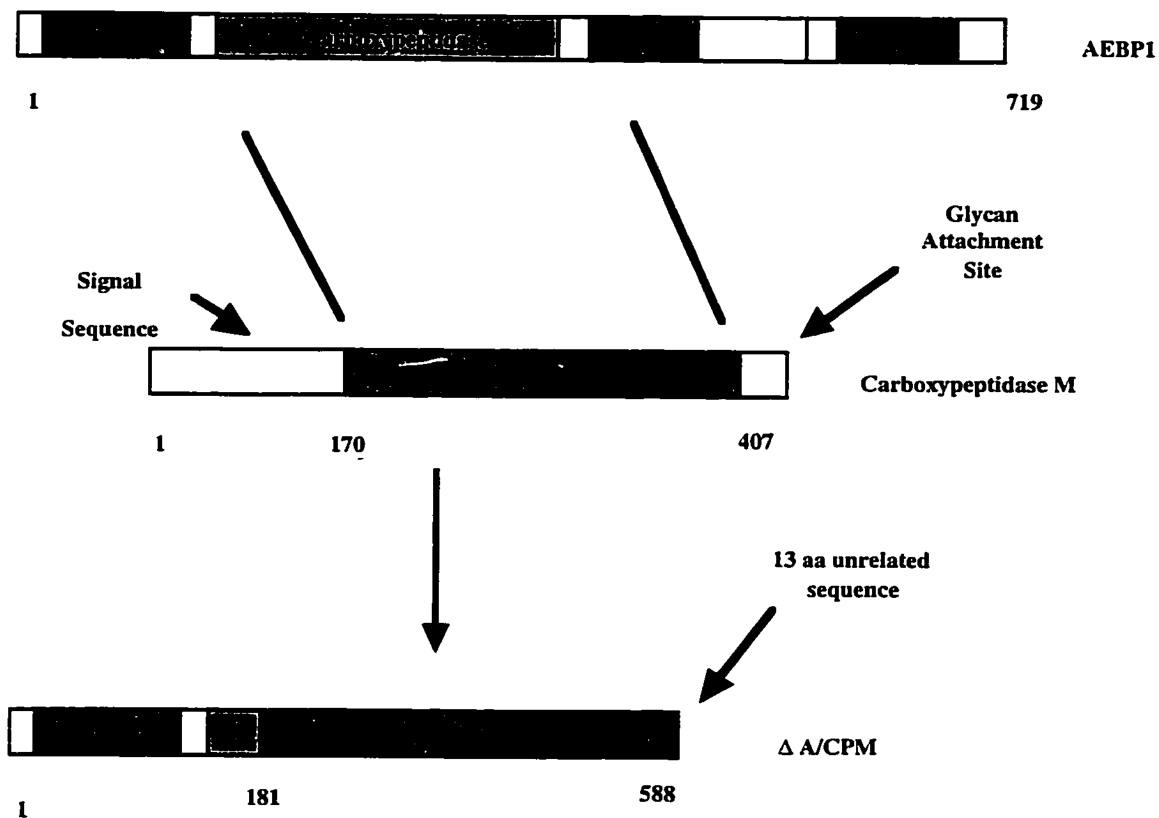


Figure 3.19. AEBP1 Carboxypeptidase Replacement Experiment

transcriptional repression activities in the Gal4 fusion protein CAT assay system. The purified recombinant His-tagged ΔA/CPM protein showed carboxypeptidase activity when assayed with the synthetic substrate hippuryl-Arg (Table 3.3). Moreover, the G4-ΔA/CPM fusion protein repressed transcription of the CAT reporter gene when co-transfected with the reporter pGALTKCAT gene (Table 3.3; He et al., 1995). These results suggest that the targeting of a functional carboxypeptidase to DNA allows the protease to repress transcription. The chimeric fusion protein ΔA/CPM is targeted to the Gal4-binding site. The bound ΔA/CPM may then use its carboxypeptidase activity to repression transcription of the CAT reporter gene. Although it is not clear how this enzyme activity is involved in this transcriptional repression, these results do further indicate that a carboxypeptidase activity is involved in this form of transcriptional repression.

In summary the targeting of a carboxypeptidase, either AEBP1 or the heterologous carboxypeptidase CPM fused to the N-terminal discoidin-like domain of AEBP1, allows the protease to function as a transcriptional repressor. The mutational analysis shows that AEBP1 enzymatic activity of the protein is correlated to its transcription repression activity. The carboxypeptidase replacement experiment and the mutational analysis, taken together, suggest that AEBP1 uses its protease activity to directly repress transcription. AEBP1 represses transcription when targeted to the AE-1 site, and when Gal4-AEBP1 fusion proteins are targeted to the Gal4-binding site. Although the mechanism by which AEBP1 represses transcription is not known, these studies indicate that when AEBP1 is targeted to DNA, it influences the general transcriptional machinery.

Transcriptional repressors have been grouped into two broad categories termed active and passive (Cowell, 1994; Johnson, 1995). Passive repressors block the binding of activators or general factors, or form inactive heterodimers with activators. Active repressors bind to independent sites (not bound by activators) and directly

interact with the general transcription machinery. This interaction prevents the formation of active polymerase complexes, or prevents active polymerase complexws from transcription elongation. The studies described here indicate that AEBP1 represses transcription by an active mechanism. The actual mechanism employed by AEBP1 is unknown. It is possible that AEBP1 prevents the formation of an active polymerase complex, that it inhibits elongation of the active polymerase complex, or that AEBP1 acts through an unknown active mechanism of repression. The targeting of an enzyme to a promoter to repress transcription through a protease activity would in itself represent a novel mechanism of active transcription regulation.

3.II. Two-Hybrid Results: Proteins which Interact with AEBP1

3.II.A. Testing for Protein-Protein Interactions

The yeast two-hybrid system described in section 2.II. was used to screen for proteins which may interact with AEBP1. Proteins fused to the Gal4 DNA-binding domain were designed to select for proteins which interact with AEBP1, and also to select for potential substrates of AEBP1 carboxypeptidase activity. To screen for possible substrates, the inactive carboxypeptidase mutant form of AEBP1, AEBP1ΔHic (see Figure 3.16 and Table 3.2), was used. Western blot analysis confirmed that both proteins were expressed in the Y153 yeast strain (data not shown).

Y153 colonies harboring proteins which may interact in the two-hybrid system, as determined through the initial screening process of growth on SC-Trp,Leu,His solid medium containing 60 mM 3'AT, were isolated. These colonies were replica plated onto selection medium to ensure that cells expressing proteins which interacted with, and activated, the *HIS3* gene were selected. Colonies (Table 3.4) were then tested for β-galactosidase activity in an X-gal assay. Colonies displaying β-galactosidase activity, which indicated a protein-protein interaction between the DNA binding G4–AEBP1 fusion protein and an unknown protein containing the Gal4 activation domain resulting in the transcription activation of the β-galactosidase gene, were isolated. From the initial colonies, no positive protein interaction wers found with the G4–AEBP1ΔHic mutant fusion protein when β-galactosidase activity was assessed. However, four individual colonies gave positive results with G4–AEBP1.

To confirm the specificity of these protein-protein interactions, each library plasmid expressing a protein fused to the Gal4 activator domain was isolated and then transformed into Y153 cells along with the G4–AEBP1, or with several controls. The negative controls used with the library plasmids were as follows: pGBT9, and

pSE1112 (expressing SNF1 fused to DNA-binding domain of Gal4 in pAS1). As a positive control, pSE1111 (expressing SNF4 fused to the activation domain of Gal4 in pACT) and its two-hybrid partner pSE1112 were transformed together. These controls were used to determine if a protein expressed by the library plasmid was interacting directly with AEBP1, or with the DNA-binding domain of the fusion protein to give what is termed a "false positive".

The four clones identified as expressing protein that interacted with AEBP1 in both the 3'AT resistance and the β-galactosidase assays all showed no interactions with the two negative controls. This finding implied that the gene activation leading to both 3'AT resistance and to β-galactosidase enzyme activity was due to an interaction between the AEBP1 part of the fusion protein and the unidentified library protein and not to interaction between the library protein and the Gal4-DNA-binding region of the AEBP1 protein. These 4 library clones, termed clones 1, 3, 5, and 7, were sequenced and the derived DNA sequence was compared to Genbank data banks for DNA and amino acid similarity. The results of these comparisons are summarized in Table 3.5.

Figure 3.20 shows the compete DNA sequence of cDNA insert of clone 5. This sequence showed no homology to nucleotide or amino acid sequences in Genbank, but showed complete nucleotide sequence homology to an expressed sequence tag (EST) from *Homo sapiens* Accession number 203372 3. This clone was not used in further studies.

DNA-binding Plasmid	Number of Colonies		
	Total	Growth on replica 3'AT plates	X-gal assay
AEBP1	2 x 10^6	128	4
ΔHic	5-6 x 10^6	103	0

Table 3.4. Summary of Two-hybrid Screen Results

Clone	Identity	Cloned Sequence	Interaction Studies
Clone 3	Gγ5 subunit of trimeric G protein	Entire protein	Two-hybrid, IP, GMSA
Clone 7	Heat Shock 27	Partial sequence	Two-hybrid
Clone 1	High Mobility Group 2 protein	Partial sequence	Two-hybrid
Clone 5	EST - *Homo sapiens* Accession # 203372 3	Partial sequence	Two-hybrid

Table 3.5. Proteins Interacting with AEBP1

IP indicates immunoprecipitation studies; GMSA indicates gel mobility shift studies.

```
  1: GTACCTCTCTGCTTCCCTGGCAGCCTGGGGAAGGGTGCAGGGCTCAGTGC
 51: GCTAAACCATGGTAAACATCTTCAATAGAACTACCCTAGAATTTAGTGAG
101: TGTGAGACTGAGATATTGCTCAGAATAAATTTATTCCATAGCCATTTAGG
151: ATTGCATGTTCTGGACCAACCTTGTCCAGTATGTTTTCTGTTTGAGCTTT
201: TTCATTCTTTTGTTAAGCCAACAAGTTGAGAATTTGGCCCTGCTGGGATC
251: CATGTAGTGGGCACTAGCTGCTCTTTGGCCAAGGCTTCATAAATGATTCA
301: GTCTCTCATTATCTGTCCTCTAGCCCCACACCCTGATTTAGACCGTGGCA
351: AAGGAAGAACTTGAGGTCAAGACCAACCAAATCTGTGAATTAAAGCTGTT
401: ATTTTTTTCTCTGCAAGGGCGCTTTGCTTCAGGTCTGGGCTATGTGCAGA
451: ACCTAAGCAGGCTGTGAGAGTTAGAAGAGGCAGTATTACATGTTAGGCCC
501: AGAACACCATGGGAAAAGGTTTATGTAGTGTATCTTAGTGGCCTGCCTAG
551: CTGCCTCTGGCCAGGCTGACTTCTGATGTCCACATTAGCTCGTACCTGAA
601: CCCTGTTGCTGAATGCCAGCCCTGTTCTCCTGTACTATTATATACGCCAT
651: GGCTGGGGGCATTGAAGGAAGTAAGCTCTCAGAGATCCTAACACTGGCTG
701: GGAACCTCTGACTCAGAGCATGTCTTAAAGAGTCCACATCTGGCCAGGCG
751: CGGTGGCACATGCTGTAATCCCAG    774
```

Figure 3.20. Complete Sequence of the cDNA insert ofClone 5 Identified by Two-hybrid Interaction with AEBP1

The cDNA was sequenced from the 5' upstream *Xho*I linker sequence at nucleotide 1, which was connected to the sequence encoding the Gal4 activation domain, to the 3' *Xho*I linker sequence at nucleotide 774. The numbers indicate nucleotide sequence.

3.II.B. High Mobility Group-2 (HMG 2) Protein Interacts with AEBP1 in the Yeast Two-hybrid System

Clone 1 was determined to encode a 35 residue region of the high mobility group 2 (HMG 2) protein (Figure 3.21). The clone contains a 105 nucleotide region which is in frame with the Gal4 activation domain and the *Xho*I linker sequence, and shows a 100% identity with the cDNA sequence for the human HMG 2 gene. Immediately downstream from the open reading frame is a translational stop sequence followed by approximately 300 nucleotides containing other stop sequences.The region of the HMG 2 protein corresponding to the polypeptide encoded by clone 1 was a 35 residue region from amino acid 132 to 167 of the 210 residue HMG 2 protein.

HMG proteins contain two non-identical HMG box domains termed A and B and an acidic C-terminal tail, and were discovered as non-histone components of chromatin. There is a now a family of proteins comprising over 121 members containing HMG boxes (Baxevanis and Landsman, 1995). The region of HMG 2 interacting with AEBP1 in the two-hybrid analysis is a partial HMG box located in the B domain of HMG 2. A possible reason that a full-length HMG 2 clone was not isolated in the two-hybrid screen is that the acidic domain of the HMG protein has been found to be toxic in bacteria and would therefore be selected against when the library cDNA plasmids were amplified (Baxevanis and Landsman, 1995).

Although HMG proteins have been studied extensively, the function of these proteins still remains unclear. HMG proteins are known to be involved in chromatin assembly, DNA replication, and transcription (Zwilling et al., 1995). HMG proteins have been implicated as architectural elements responsible for facilitating the formation

```
Clone 1    1     MSWEQSAKDKQPYEQKAAKLKEYQKDIAAYRAK   35
HMG 2      132   MSWEQSAKDKQPYEQKAAKLKEYQKDIAAYRAK   167
```

Figure 3.21. Clone 1 encodes a region of HMG 2

Expressed sequence from the two-hybrid library plasmid termed clone 1. The numbers indicate amino acid sequence. The cDNA sequenced is in the correct reading frame from the 5' upstream *Xho*I linker sequence and the Gal4-activation domain.

of higher-order nucleoprotein complexes (Grosschedl et al., 1994). HMG proteins have also been purified from a complex fraction termed USA which is required for efficient *in vitro* transcription of activator-dependent templates (Zwilling et al., 1995). HMG 2, in particular, has been shown *in vitro* to inhibit the general transcription machinery through a direct interaction with the TATA-binding protein (TBP), which prevents TBP from interacting with TFIIB and blocks the formation of the pre-initiation complex (Ge and Roeder, 1994).

HMG 2 also directly interacts with the octamer transcription factors Oct1 and Oct2 through an HMG box domain and a POU homeodomain of Oct1/2. The interaction enhances the Oct1/2 DNA-binding ability, and enhances its role as a transcription activator (Zwilling et al., 1995). It is suggested that HMG 2 assembles a functional transcription complex over the octamer motif in promoters. The POU homeodomain of Oct proteins also interacts with the viral transcription activator VP16 and the general transcription apparatus. Therefore the HMG 2 protein may be involved in directing this complex to appropriate DNA sequences (Zwilling et al., 1995). HMG proteins have also been shown to stimulate the binding of NF-κB to its binding site, and the binding of ATF2/c-Jun to one of its binding elements, through the assembly of nucleoprotein complexes (Zwilling et al., 1995).

HMG 2 inhibits transcription by RNA polymerase II. HMG 2 was purified as a transcription repressor which bound DNA and interacted with the polymerase once transcription was initiated (Stelzer et al., 1994). TFIIH, a member of the general transcription machinery with kinase and helicase functions, was able to relieve the repression caused by HMG 2 (Stelzer et al., 1994). Therefore the interaction of this DNA-binding enzyme with HMG 2 is able to alleviate the transcription repression caused by the interaction of HMG 2 with the general transcription machinery.

Other DNA-binding proteins containing HMG box domains are involved in transcription regulation, including the yeast ARS-binding protein, UBF, the

mitochondrial transcription factor mtTF-1, the lymphoid enhancer-binding protein, the sex-determining factor SRY, the Sox proteins, fungal regulatory proteins Mat-Mc, Mat-a1, Ste11 and Rox1 (Grosschedl et al., 1994; and references within), and transcription factor TFIIIB (Wang and Roeder, 1995).

As AEBP1 has been shown to interact with DNA and repress transcription (section 3.I.), it is interesting to speculate as to the relevance of the protein-protein interaction between AEBP1 and HMG 2. It is possible that the interacton between AEBP1–HMG 2 is functionally similar to the Oct1/2–HMG 2 interaction. The interaction between AEBP1 and HMG 2 may localize AEBP1 to the AE-1 promoter region of the aP2 gene, allowing it to bind efficiently, or may localize a complex containing AEBP1 to the AE-1 site. In both scenarios, the interaction of AEBP1 with HMG 2 would assist AEBP1 in localizing to the AE-1 site. Once AEBP1 is bound to the AE-1 DNA sequence it would be free to repress transcription. Another possible scenario would allow the HMG 2 protein to act as an adapter molecule bringing together the protease AEBP1 with potential substrates. The binding of AEBP1 to HMG 2 in a larger nucleoprotein complex may give AEBP1 access to a substrate which is also present in this complex.

Interestingly, HMG 1 has been found to be rapidly translocated to and accumulated on the cell surface in a membrane-associated fashion upon induction of differentiation by murine erythroleukemia (MEL) cells (Passalacqua et al., 1997). Furthermore, the release of HMG 1 into the extracellular medium by an unknown mechanism accelerates differentiation, while the addition of anti-HMG 1 antibody to the cells blocks differentiation of MEL cells when the inducer for differentiation is added (Passalacqua et al., 1997). HMG 1 is also reported to bind the membrane and is also believed to bind its own unidentified receptor (Passalacqua et al., 1997). These results are intriguing, as AEBP1 is involved in the regulation of preadipocyte differentiation

and is believed to be involved in receptor-linked signal-transduction pathways (section 3.III.).

Further study of the AEBP1–HMG 2 interaction may prove to be interesting. This study may assist in determining a substrate for the enzymatic transcription repression functions of AEBP1, as HMG proteins are involved in regulating the general transcription machinery and are known to influence other transcription regulators. It is interesting to speculate that HMG 2 may bring AEBP1 into proximity with DNA-bound general transcription factors, thereby facilitating its repression function. Study of this protein-protein interaction may also help in the understanding the transcription regulation of important genes involved in adipogenesis.

3.II.C. Heat Shock Protein (HSP) 27 Interacts with AEBP1 in the Yeast Two-hybrid System

DNA sequence analysis showed that clone 7 encodes the first 146 amino acids of the 205 residue heat shock protein (HSP) 27 (Figure 3.22). Both nucleotide and derived amino acid sequences show 100% homology with those of the human HSP27 cDNA and protein.

HSP27 was initially described as a molecular chaperone protein which is localized to the nucleus in cells under stress, but has since been found to localize to other cellular regions (Kim et al., 1984). The physiological role of HSP27 is unknown, but this multifunctional protein is involved in several cellular functions, and is localized to various cellular regions. The AEBP1–HSP27 interaction is discussed further at the end of section 3.II..

```
Clone 7      1   MTERRVPFSLLRGPSWDPRFDWYPHSRLFDQAFGL 35
HSP27        1   MTERRVPFSLLRGPSWDPRFDWYPHSRLFDQAFGL
Clone 7     36   PRLPEEWSQWLGGSSWPGYVRPLPAAIESPAVAAP 70
HSP27       36   PRLPEEWSQWLGGSSWPGYVRPLPAAIESPAVAAP
Clone 7     71   AYSRALSRQLSSGVSEIRHTADRWRVSLDVNHFAP 105
HSP27       71   AYSRALSRQLSSGVSEIRHTADRWRVSLDVNHFAP
Clone 7    106   DELVKTGVVEITGKHEERQDEHGYISRCFTRKYTL 140
HSP27      106   DELVKTGVVEITGKHEERQDEHGYISRCFTRKYTL
Clone 7    141   PPGVDP    146
HSP27      141   PPGVDP
```

Figure 3.22. Clone 7 encodes a region of HSP27

Expressed sequence from the two-hybrid library plasmid termed clone 7. The numbers indicate amino acid sequence. The cDNA sequenced is in the correct reading frame from the 5' upstream *Xho*I linker sequence and the Gal4 activation domain.

3.II.D. The Gγ5 Subunit of a Trimeric G protein Interacts with AEBP1 in the Yeast Two-hybrid System

Clone 3 was determined to encode the entire 68 residue Gγ5 subunit of a trimeric G protein. Both nucleotide and derived amino acid sequences show 100% homology with those of the human Gγ5 cDNA and protein (Figure 3.23).

Trimeric G proteins are important signaling molecules involved in the transduction of extracellular signals through G protein coupled receptors (GPCR) to a number of effectors (summarized in Figure 1.2). The binding of hormones or neurotransmitters to GPCRs cause conformational changes in trimeric G proteins which facilitates the exchange of GDP for GTP on the α subunit. The GTP-bound, activated α subunit dissociates from the βγ dimer, leaving both molecules free to act on effector molecules. GPCRs and trimeric G proteins are reviewed in detail in section 1.II.E.

Gγ5 was first purified from human placenta and liver tissues (Fisher and Aronson, 1992), and later from bovine spleen (Morishita et al., 1993), and is now known to have ubiquitous expression (Yan et al., 1996). Studies by Hansen et al. (1994) have shown that Gγ5 is localized to areas of focal adhesion, regions known to be important for signal transduction.

The γ subunits are by far the most divergent components of trimeric G proteins, with subunits having as little as 25 % similarity. There is a growing family of γ subunits with members grouped into distinct sub-classes based on amino acid sequence, post-translational modifications, and similar abilities to interact with different α and β subunits. One sub-class contains γ1 and γ11, a second contains γ2, γ3, γ4, and γ7, a third contains γ5, and a forth contains γ10 (Ray et al., 1995).

```
Clone 3    1   MSGSSSVAAMKKVVQQLRLEAGLNRVKVSQAAADL 35
Gγ5        1   MSGSSSVAAMKKVVQQLRLEAGLNRVKVSQAAADL
Clone 3   36   KQFCLQNAQHDPLLTGVSSSTNPFRPQKVCSFL   68
Gγ5       36   KQFCLQNAQHDPLLTGVSSSTNPFRPQKVCSFL
                                             *+++
```

Figure 3.23. Clone 3 Encodes Gγ5

Expressed sequence from the two-hybrid library plasmid termed clone 3. The numbers indicate amino acid sequence. The cDNA sequenced was in the correct reading frame from the 5' upstream *Xho*I linker sequence and the Gal4-activation domain. The (*) indicates the cysteine which is prenylated and carboxyl methylated. The (+) indicates amino acids which are removed by proteolysis after the * cysteine has been modified.

Another recently isolated γ subunit (γ12) is unique as it is phosphorylated by protein kinase C in response to GPCR activation. This phosphorylation causes the γ12 subunit to bind more tightly with the α subunit *in vitro,* indicating a regulatory role for this phosphorylation in response to stimulus (Morishita et al., 1995). This results is important, as it shows a new level of regulation of γ subunits through phosphorylation. Gγ5 has a serine residue in the same location (fourth N-terminal amino acid) as Gγ12, and therefore may be phosphorylated in a similar fashion.

Interestingly, the C-terminal region of Gγ subunits is isoprenylated. All Gγ subunits, except Gγ5 and Gγ12, have the consensus Cys-α-α-Xaa motif, where Cys is the cysteine that become prenylated, α are aliphatic amino acids, while Xaa is any amino acid. In contrast, Gγ5 has a Cys-Xaa-Xaa-Xaa motif (Cys-Ser-Phe-Leu) at the C terminus (see Figure 3.23). The C-terminal prenylation specified by this motif may be involved in plasma membrane localization. This slightly different Gγ5 prenylation signal, although Gγ5 is still geranylgeranylated, has been proposed to target Gγ5 to other membranes or to the region of focal adhesion where Gγ5 is found (Hansen et al., 1994). This proposed localization may not occur as the three amino acids following the prenylated Cys of Gγ5 are removed by proteolysis before such an event may occur.

Only γ5 and γ12 are found in a wide variety of tissues, and both contain the Cys-Xaa-Xaa-Xaa prenylation signal. It is speculated that these two proteins may be the major G proteins responsible for mediating signals in a variety of cells (Morishita et al., 1995).

The γ subunit complexes with the β subunit in the cytoplasm before post-translation modifications occur (Rehm and Ploegh, 1997). The failure of this complex to form causes improper intracellular localization and rapid degradation of the γ subunit (Pronin and Gautam, 1993). These modifications are also critical for the active γ subunit localization. Removal of the cysteine prenylation signal by mutagenesis prevents the $\beta\gamma$ heterodimer from attaching to the membrane (Simonds et al., 1991;

Kalman et al., 1995), and carboxyl methylation following protease cleavage of the three C-terminal amino acids is also required for membrane localization (Parish and Rando, 1994; Philips et al., 1995). This cysteine methylation is reversible and believed to represent a mechanism of regulation by which Gβγ heterodimer attachment to the membrane determines the cellular function of the Gβγ complex (Philips et al., 1995). Therefore, a non-plasma membrane pool of Gβγ may exist, although only transiently as the heterodimer may be continuously removed and reattached to the membrane (Philip et al., 1995; Rehm and Ploegh, 1997).

The γ5 subunit is found to localize in an area of focal adhesion (Hansen et al., 1994). Adhesion interactions are known to play a critical role in directing cell migration, proliferation, and differentiation (Clark and Brugge, 1995). This area brings together integrins (a major family of cell surface receptors) and links them to cytoskeletal complexes and bundles of actin (Clark and Brugge, 1995). Tyrosine kinases, including the focal adhesion kinase (FAK), phosphatases, and their substrates play an important role in the regulation of focal adhesion.

Extracellular signals transduced through plasma membrane receptors, RTK and GPCR, activate FAK, which forms signaling complexes that mediate the activation of several other signaling molecules, including MAPK (Clarke and Brugge, 1995). In particular, the activation of GPCR through the binding of various neuropeptides causes the activation of G proteins. The activated G proteins mediate the filamentous (F-) actin-dependent activation of FAK (Zhang et al., 1996). This form of actin is protected by HSP27 capping (discussed in 3.II.J.). These results point to the possibility that AEBP1 may be involved in the regulation of adipogenesis, as the proteins that AEBP1 interacts with (MAPK, HSP27, Gγ5) are all localized in areas of focal adhesions, areas known to be involved in the regulation of cell signaling pathways (discussed in 3.III.).

Immunocytochemistry studies have shown the γ5 subunit to be localized with vinculin, a structural protein involved in cytoskeletal-membrane attachment in focal

adhesions. Furthermore, the γ5 subunit was found to immuno-stain along actin fibers beyond the membrane associated focal adhesion vinculin staining, indicating that the protein may interact with another cytoskeletal protein, zyxin. Disruption of the cytoskeleton caused the redistribution of vinculin, actin, and Gγ5 staining. Surprisingly, however, Gγ5 and vinculin were redistributed together, indicating that the γ5 subunit specifically associates with one or more of the components that comprise a focal adhesion. These results indicate that the γ5 subunit plays a central role in G protein-coupled-signaling pathways present in focal adhesions. This association appears to be widespread, as γ5 colocalizes with vinculin in cardiac fibroblasts, rat aortic smooth muscle, and Schwann cells (Hansen et al., 1994).

Furthermore, a Gβγ subunit also localizes to a network of fibres, termed cytokeratin filaments, in starfish oocytes. The Gβγ subunit was found to coexist with the cytokeratin, and upon oocyte maturation the Gβγ subunit is dissembled (Chiba et al., 1995). These studies indicate that Gβγ subunits may exist in a localized membrane pool and be an integral part of the signal-transduction pathway thought to regulate focal adhesion.

In support of alternative roles and locations for these heterodimers, the Gβγ heterodimer was found to interact with the small GTP-binding protein termed ADP ribosylation factor (Arf) (Colombo et al., 1994; Franazzi et al., 1994). Trimeric G proteins are thought to play a role in vesicular transport through both endocytic and exocytic pathways. This regulation may occur by bringing cytosolic proteins, such as Arf, to the Golgi or other vesicle membranes, through direct interaction or through adapter proteins with PH domains which directly bind to the βγ heterodimers (Colombo et al., 1995; Franco et al., 1995). These studies show that the βγ heterodimers may have regulatory roles in vesicle transport, and may be associated with the membranes of these vesicles. However, it is important to note that no *in vivo* interactions between βγ heterodimers and PH domain proteins (excluding βARK) have yet been shown.

3.II.E. AEBP1 and Gγ5 Co-immunoprecipitate

To confirm that AEBP1 and Gγ5 interact *in vivo*, co-immunoprecipitation and Western blot experiments were performed (Figure 3.24). When an antibody directed towards AEBP1 was incubated with 3T3 L1 cell extracts and precipitated with protein A-agarose beads, Gγ5 was found to co-immunoprecipitate (Figure 3.24, Lane 2, lower panel). Likewise, when an antibody directed towards Gγ5 was incubated with 3T3 L1 cell extracts and precipitated with protein A-agarose beads, AEBP1 was found to co-immunoprecipitate (Figure 3.24, Lane 3, upper panel). To confirm that this co-immunoprecipitation was not due to non-specific binding to the protein A-agarose beads, a control incubation with protein A agarose beads and 3T3 L1 extracts alone was performed. Figure 3.24 Lane 1 (upper and lower panels) shows that neither AEBP1 nor Gγ5 interact with the protein A-agarose beads. To confirm that AEBP1 and Gγ5 did not interact with antibodies non-specifically, antibodies purified from preimmune serum were used. Figure 3.24 Lane 4 shows that neither AEBP1 nor Gγ5 interact non-specifically with antibodies from preimmune serum.

The results summarized in Figure 3.24 suggest that AEBP1 and Gγ5 interact in 3T3 L1 preadipocytes. Although this co-immunoprecipitation study does not show that the two proteins interact directly, this experiment coupled with the results of the two-hybrid assay suggests that AEBP1 and Gγ5 directly interact in preadipocytes.

Figure 3.24. AEBP1 and Gγ5 Co-immunoprecipitate

Equal amounts of 3T3 L1 cell extract were used in immunoprecipitation studies with antibodies directed towards either affinity purified AEBP1 (Lane 2) or Gγ5 (Lane 3). The immunoprecipitates were resolved electrophoretically and probed with either anti-AEBP1 (upper panel) or anti-Gγ5 (lower panel) antibodies. Lane 1 contains material precipitated with protein A agarose beads alone. Lane 4 contains material precipitated with antibodies purified from preimmune serum. Protein samples were resolved in SDS-PAGE gels and then transferred to nitrocellulose for immunoblotting. Pre-stained protein markers (NEB) were used to determine molecular weights. Bands were observed after ECL chemiluminescence reactions followed by autoradiography.

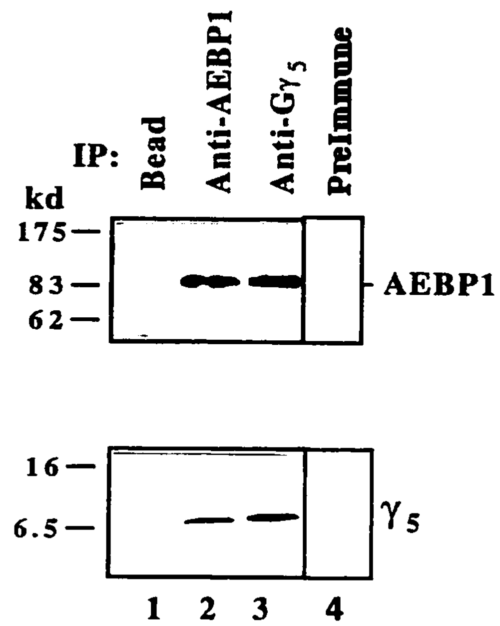


Figure 3.24 AEBP1 and Gγ5 Coimmunoprecipitate

3.II.F. AEBP1 Complexes with Gγ5 and Gβ

As all γ subunits have been found to be associated with a β subunit *in vivo*, it was necessary to determine if β subunit was present in a complex with Gγ5 and AEBP1. The Gγ5 subunit has been found to associate with Gβ1 and Gβ2 (Ray et al., 1995); therefore an antibody directed towards Gβ1-4 was used in co-immunoprecipitation experiments. As Figure 3.25 demonstrates, antibodies directed towards AEBP1 (Lane 2), Gβ1-4 (Lane 3), or Gγ5 (Lane 4) were able to precipitate AEBP1 (upper panel). Likewise, all three antibodies were able to precipitate Gβ (lower panel). Controls using protein-A agarose beads (Lane 1) or antibodies purified from preimmune serum (Lane 7) showed that these immunoprecipitations were not due to non-specific binding to the protein-A agarose beads or to non-specific antibody binding.

These results indicate that AEBP1 interacts with the Gβγ heterodimer. This finding is significant, as the Gβγ subunits are known to exist only in the heterodimer or trimer formation (Rehem and Ploegh, 1997), although it has been suggested that γ monomers may exist in certain systems (Herlitze et al., 1996). The formation of a Gγ5-AEBP1 complex without the β subunit would be highly unlikely, as the γ subunit has not been reported to exist as monomer.

Figure 3.25. AEBP1, Gγ5, and Gβ1-4 Co-immunoprecipitate

Equal amounts of 3T3 L1 cell extract were used in immunoprecipitation studies with antibodies directed towards either AEBP1 (Lane 2 and 5), Gβ1-4 (Lane 3 and 6) or Gγ5 (Lane 4). The immunoprecipitates were resolved electrophoretically and probed with either affinity purified anti-AEBP1 (upper panel) or anti-Gβ1-4 (lower panel) antibodies. Lane 1 contains material immunoprecipitated with protein A-agarose beads alone. Lane 7 contains material immunoprecipitated with antibody purified from preimmune serum. Protein samples were resolved in SDS-PAGE gels and then transferred to nitrocellulose for immunoblotting. Pre-stained protein markers (NEB) were used to determine molecular weights. Bands were observed after ECL chemilumenescence reactions followed by autoradiography.

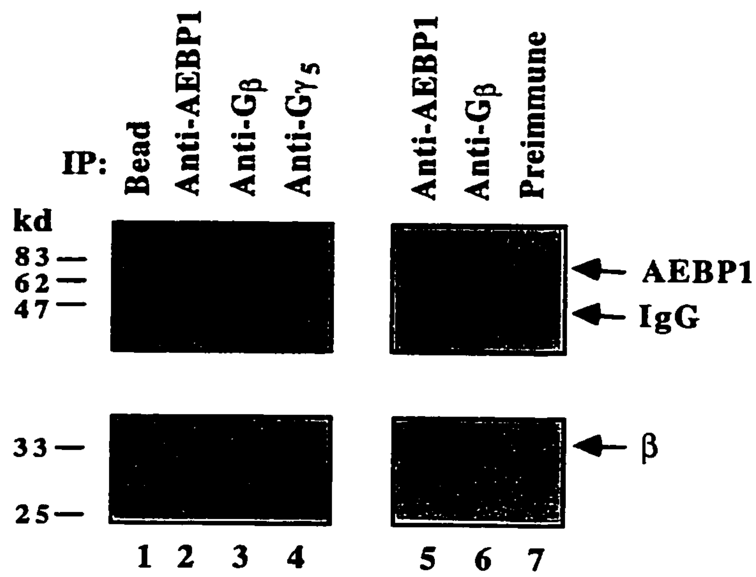


Figure 3.25 AEBP1, Gγ5, and Gβ Co-immunoprecipitate

3.II.G. Gγ5 Prevents AEBP1 Binding to DNA

To study this interaction further, cDNA expressing Gγ5 was subcloned into the pET-16b vector. Expression of this vector produced His-tagged Gγ5 protein with an estimated molecular weight of 16 kDa, which was purified by binding to a metal affinity column, eluted, and renatured. The purification process allowed the isolation of nearly homogeneous recombinant protein (Figure 3.26).

Recombinant His-tagged Gγ5 was used in a gel mobility shift assay to determine if the AEBP-Gγ5 interaction affects the ability of AEBP1 to bind DNA. AEBP1 and Gγ5 were incubated prior to the addition of labeled AE-1 DNA (the region of the aP2 gene to which AEBP1 binds, as described in section 3.I.A.). As Figure 3.27 illustrates, the Gγ5 subunit prevented AEBP1 from binding to the AE-1 site. Lane 7 shows that Gγ5 was not itself capable of binding DNA, and Lanes 10-12 show that increasing the amounts of Gγ5 in the gel mobility shift assay prevented AEBP1 from binding to the AE-1 site. In Lane 12, equivalent concentrations of AEBP1 and Gγ5 were present, and the ability of AEBP1 to bind to the AE-1 site was completely abolished. To ensure that the inability of AEBP1 to bind to the labeled AE-1 DNA observed in the gel mobility shift assay observed was not due to a non-specific mechanism which prevented AEBP1 from binding to the AE-1 site, a protein purified in a similar fashion (expressed cDNA of yeast Rab11, small GTP-binding protein, cloned into pET-16b vector, provided by Dr. Pak Poon) and BSA were used as controls. Figure 3.27 shows that BSA (Lane 1) and the control protein (Lanes 2-4) had no effect on the ability of AEBP1 to bind the AE-1 DNA.

The results illustrated in Figure 3.27 show that the interaction between AEBP1 and Gγ5 prevents AEBP1 from binding to the AE-1 site. Gγ5 may mask the DNA-binding domain of AEBP1, preventing it from binding to the AE-1 site, or Gγ5 may

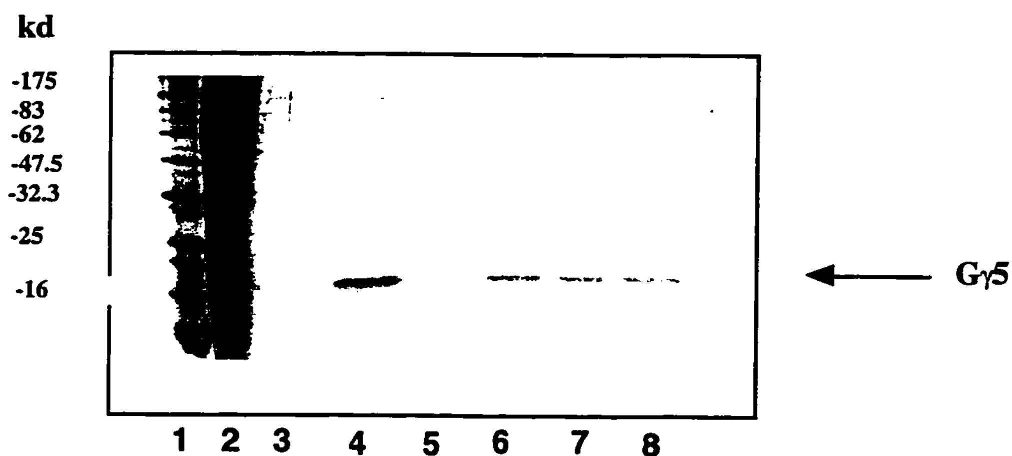


Figure 3.26 Purification of Recombinant His-tagged Gγ5

SDS-PAGE gel showing the purified recombinant His-tagged Gγ5. Lane 1 contains protein molecular weight markers. Lane 2 contains total protein from bacteria induced to express Gγ5. Lanes 3 to 8 contain fractions eluted from the metal affinity column which contains recombinant His-tagged Gγ5. Proteins were stained with Coomassie blue. Arrow indicates Gγ5.

Figure 3.27. Gγ5 Prevents AEBP1 from Binding to the AE-1 Site

Recombinant AEBP1 protein was incubated with radiolabeled AE-1 DNA to produce the mobility shift observed (see arrow). Lane 1, 2 μg AEBP1 plus 2 μg BSA; Lane 2-4, 2 μg AEBP1 plus 0.5, 1.0, and 2.0 μg of control protein (Rab-11), respectively. Lane 5, 2 μg control protein. Lane 6, AE-1 DNA alone; Lane 7, 2 μg Gγ5 alone; Lane 8, 2 μg AEBP1 plus unlabeled AE-1 DNA (competitively inhibited binding to the AE-1 site). Lane 9, 2 μg AEBP1 alone; Lane 10-12, 2 μg AEBP1 plus 0.5, 1.0, and 2.0 μg Gγ5, respectively. Protein-DNA samples were resolved by acrylamide non-denaturing gels and the radiolabeled DNA was detected by autoradiography.

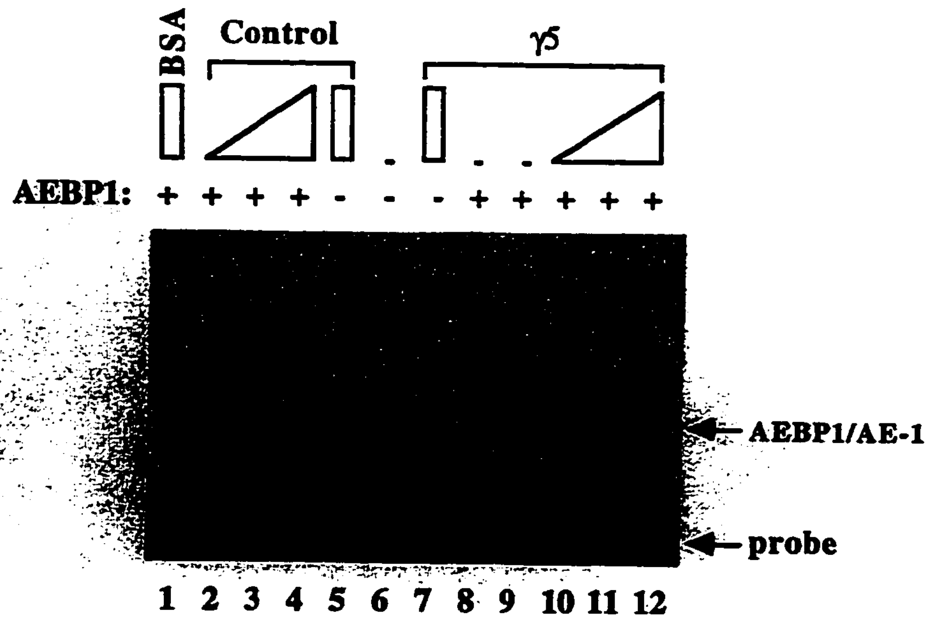


Figure 3.27 Gγ5 Prevents AEBP1 from Binding to the AE-1 Site

bind to another region of AEBP1, causing a conformational change which may prevent AEBP1 from binding DNA. In either case, this protein-protein interaction plays a regulatory role for AEBP1 *in vitro.*

3.II.H. Gγ5 Prevents AEBP1 from Repressing Transcription

The ability of Gγ5 to prevent AEBP1 from interacting with the AE-1 site indicates that this protein-protein interaction could inhibit the ability of AEBP1 to repress transcription. Dr. G.-P. He performed CAT and *in vitro* transcription assays to test this possibility. As Figure 3.28 shows, transient cotransfection of Gγ5 (pRc/CMVg5) along with AEBP1 (pSVAEBP1) and the reporter plasmid paP2(-168)CAT into NIH 3T3 cells caused on inhibition of the ability of AEBP1 to repress transcription of the reporter CAT gene, paP2(-168)CAT, which contains the AEBP1 binding site, AE-1. Increasing the amount of transfected Gγ5 caused an increase in CAT activity, indicating Gγ5 reversed the repression ("derepression") of transcription of the CAT gene attributed to AEBP1 binding to the AE-1 site. As a control, the Gγ5 was cloned into the pRc/CMV vector in the opposite orientation [pRc/CMVg5(-)], and when this plasmid was cotransfected with AEBP1 no derepression was seen. Therefore, this restoration of CAT activity in the presence of AEBP1 may be due to γ5 expression.

To show further that a Gγ5-AEBP1 interaction prevents AEBP1 from repressing transcription, an *in vitro* transcription system was developed. The transcription template pCMV/AE-1 contained the human cytomegalovirus (CMV) gene fragment (-829 to +363) with three copies of the AE-1 sequence installed upstream of the CMV promoter. Transcription activity was measured using an *in vitro*

Figure 3.28. Gγ5 Regulates the Transcription Function of AEBP1

Left Panel. Transient transfection assays were carried out with the reporter plasmid paP2(-168)CAT along with either pSVAEBP1(-) or an expression plasmid encoding AEBP1 (pSVAEBP1). The repression activity of pSVAEBP1 for CAT gene expression decreased when cells were cotransfected with the Gγ5 expression plasmid pRc/CMVg5, but not when cotransfected with the control plasmid pRc/CMVg5(-).

Right Panel. Transient transfection analysis was carried out with the reporter plasmid paP2(-168)CAT along with either pRc/CMV, pRc/CMVg5, and pRc/CMVg5(-), respectively, to show that Gγ5 does not enhance expression of the reporter plasmid paP2(-168)CAT.

All columns illustrate CAT assays from cells transfected with the reporter plasmid paP2(-168)CAT along with the effector plasmids indicated at the bottom of each column. Transcription activity is presented in relative levels of CAT activity (% conversion of chloramphenicol (C) to acetylated chloramphenicol (AC)) driven by each reporter. CAT activity was assayed after adjusting the cell extracts by β-galactosidase activity as described in section 2.III.G.. Radioactivity was detected by autoradiography and counted with a scintillation counter. The values represent averages from duplicate transfection experiments (He and Ro, unpublished data).

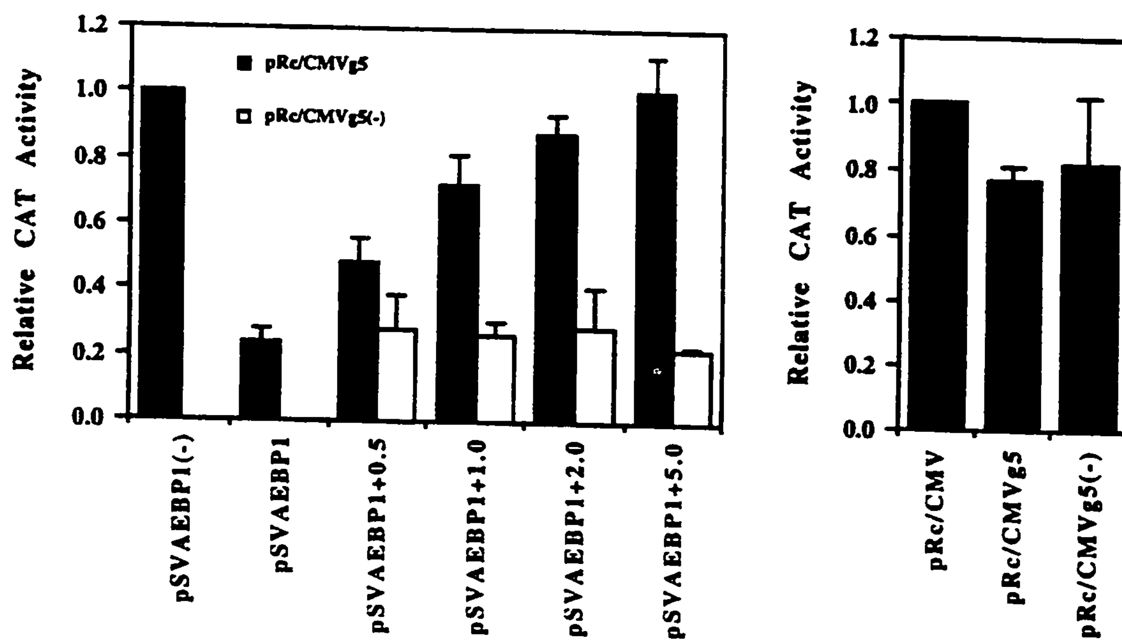


Figure 3.28 Gγ5 Regulates the Transcription Function of AEBP1

nuclear run-off transcription assay with HeLa nuclear extracts (NEB). When recombinant AEBP1 was added to the nuclear run-off transcription reaction along with the pCMV/AE-1 template, transcription activity was significantly decreased (Figure 3.29, Lanes 1 and 2). However, when recombinant AEBP1 was tested with the template pCMV, which does not contain the AEBP1 AE-1 binding site, no effects on transcription were observed (Lanes 7 and 8). These results further confirm that directing AEBP1 to the AE-1 site is a prerequisite for the ability of AEBP1 to repress transcription.

Next, Gγ5 was tested in the transcription assay. When recombinant Gγ5 was added to the aforementioned nuclear run-off assay, the repression caused by AEBP1 was virtually abolished. Figure 3.29, Lanes 4-6, show that increasing amounts of Gγ5 mediated increased transcription of the pCMV/AE-1 template. Lanes 8-10 show that increasing amounts of Gγ5 along with AEBP1 does not affect transcription of a template not under the control of AE-1 sites (pCMV), and Lane 3 show that Gγ5 alone was not able to affect transcription of the pCMV/AE-1 template.

The two experiments illustrated in Figures 3.28 and 3.29 show that the interaction between AEBP1 and Gγ5 prevents AEBP1 from repressing transcription. This inhibition of AEBP1 activity is most likely caused by Gγ5 preventing AEBP1 from binding to the AE-1 region of the aP2 gene, as studies suggest that AEBP1 must bind DNA to repress transcription (section 3.III.D.).

3.II.I. The Relevance of AEBP1 and Gγ5 Interactions

The Gγ5 subunit is a functional component of a trimeric G protein. These proteins are known to interact with G protein coupled receptors (GPCR) at the plasma

Figure 3.29. Gγ5 Directly Inhibits AEBP1 Transcription Repression Function

In vitro nuclear run-off reactions were done using HeLa nuclear extracts. Transcription of the pCMV/AE-1 template produced a 363 bp labeled transcript. The effects of recombinant His-tagged AEBP1 and Gγ5 upon transcription of this template were observed. The nuclear run-off reactions used either pCMV/AE-1 (Lanes 1 to 6) or pCMV (Lanes 7 to 10) templates: templates alone (Lane 1 and 7), plus 200 ng of AEBP1 (Lanes 2 and 8), or Gγ5 (Lanes 3 and 9), or plus 200 ng of AEBP1 and 50 (Lane 4), 100 (Lane 5), and 200 ng (Lanes 6 and 10) of Gγ5. Radioactivity was detected by autoradiography (He, Muise, and Ro, unpublished data).

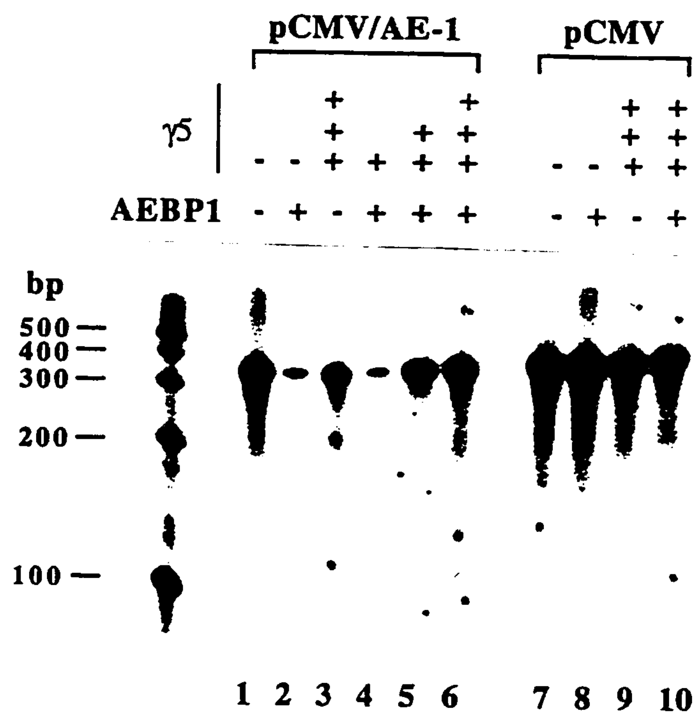


Figure 3.29 Gγ5 Directly Inhibits AEBP1 Transcription Repression Function

membrane. Therefore it is interesting to speculate about the cellular location of this protein-protein interaction. Within the Gγ subunit family there is little sequence similarity, and most Gγ subunits posses only 50% identity with each other. For example, Gγ5 shows 28 % identity with Gγ1 and less than 55 % identity with Gγ2, 3, 4, and 7 (Yan et al., 1996). As mentioned, Gγ5 is a unusual member of the Gγ subunit family with a high degree of diversity in the C-terminal region. At the C terminus of Gγ5 there is an unusual prenylation signal, a Cys-Xaa-Xaa-Xaa motif that does not correspond to the Cys-α-α-Xaa (α indicates aliphatic residues, and Xaa is any amino acid) motif found in most other Gγ subunits. The Cys-Xaa-Xaa-Xaa prenylation signal has been shown to be vital for membrane targeting, as mutation of this signal sequence prevented Gβγ heterodimers from attaching to the membrane (Simonds et al., 1991). As the C-terminal cysteine of Gγ5 is geranylgeranylated and the three residues following the prenylated cysteine are removed, the significance of this Gγ5 prenylation signal is unclear. However, it is postulated that it may be involved in targeting Gγ5 to focal adhesions or other unidentified membranes through an unknown mechanism (Hansen et al., 1994).

The Gα subunit of a trimeric G protein has been shown to translocate to the nucleus in response to growth factors, even though this subunit is known to be membrane-localized through myristoylation. In response to insulin and EGF treatment of Balb/c3T3 cells, the $G\alpha_i$ subunit (a member of the family of Gα proteins sensitive to pertussis toxin) has been shown to rapidly (within 1-4 hours) translocate to the perinuclear region within these fibroblasts, and within 2 days to localize in the nucleus, where it binds to the separating chromatin of dividing nuclei (Crouch, 1991; Crouch and Hendry, 1993).

Similar results have been seen in agonist-activated platelets (Crouch et al., 1989) and in haemopoietic cells (Townsend et al., 1993). When progenitor haemopoietic cells were induced to differentiate by TNF-α treatment, the Gα subunit

was found to translocate to the nucleus (Townsend et al., 1993). It is conceivable that the Gβγ heterodimer may also translocate to the nucleus, as the β subunit coimmunoprecipitates with the Gα subunit 20 hours after activation by EGF- and insulin-stimulation, but not after a 3-minute to 1-hour stimulation of fibroblast cells (Crouch and Hendry, 1993). McFarlane-Anderson et al. (1993) showed that in cell lines derived from adipose tissue $G_s\alpha$ was localized in the vesicular cytoplasm and the nucleus, and $G_i\alpha2$ and Gβ were localized with actin stress fibers and in the nucleus. Differentiation of these cells caused the reorganization of actin with $G_i\alpha2$ and Gβ. These results strongly suggest that G proteins may be translocated to the nucleus in preadipocytes. It is therefore also possible that the Gβγ heterodimer may also be localized to the nucleus.

More recently, Crouch and Simson (1997) showed that in Swiss 3T3 fibroblasts the α subunit and β subunit of the G_{i2} trimeric G protein translocate to the nucleus in response to serum, thrombin, and epidermal growth factor, where they bind to chromatin during mitosis. It is believed that protein kinase C is responsible for the activation of this translocation event. Although the γ subunit was not studied, it is suggested that the βγ heterodimer is translocated to the nucleus as a unit, possibly along with the α subunit. It is suggested that trimeric G proteins in the nucleus may regulate mitosis and other nuclear events. This study is interesting, not only for the description of the novel translocation of the G protein subunits to the nucleus, but also for evidence that both GPCR and receptor tyrosine kinase receptors influence this translocation event. This study raises the possibility that AEBP1 and the Gβγ heterodimer may interact in the nucleus, and that this interaction may prevent AEBP1 from performing its repression function.

In support of this hypothesis, preliminary immunofluorescence and cell-fractionation studies by Park and Ro (unpublished data) indicate that Gγ5 and Gβ are localized to the nucleus in 3T3 L1 preadipocytes. If these results hold true, it would be

possible for AEBP1 and the Gβγ heterodimer to interact in the nucleus of preadipocytes.

On the other hand, AEBP1 has also been cloned as a membrane localized protein which interacts with the epidermal growth factor receptor (EGFR) protein substrate (eps8) at the plasma membrane (De Foire, personal communication to Dr. Ro). The eps8 protein has a nuclear localization sequence (Fazioli et al., 1993) and is known to be translocated to the nucleus upon receptor stimulation (Castagnino et al., 1994). The eps8 protein binds to the EGFR by a novel SH2/pTyr-independent mechanism (Castagnino et al., 1994). The eps8 protein contains a SH3 domain which may recognize proline-rich regions, and a putative PH domain which may recognize Gβγ heterodimers (Castagnino et al., 1994).

In this case, it is interesting to hypothesize that AEBP1 may be maintained in a cytoplasmic pool. AEBP1 may be translocated along with eps8 to the nucleus in response to growth factor stimulation of 3T3 L1 cells where it would prevent transcription of differentiation-specific genes, thereby allowing the cell to proliferate. Conversely, the eps8 protein may bring together AEBP1 through its SH3 domain (AEBP1 has a proline rich region which may potentially interact with SH3 domains) and a Gβγ heterodimer through its PH domains (PH domains have been shown *in vitro* to interact with the Gβγ heterodimer) in response to differentiation stimulus. In this configuration, the AEBP1–Gβγ interaction may prevent AEBP1 from translocating to the nucleus, thereby inhibiting the AEBP1 repression of adipogenic specific genes.

3.II.J. HSP27 and AEBP1 Revisited

HSP27, the polypeptide encoded by clone 7 (section 3.II.C.), has been shown to exist in a 200 to 800-kDa multimeric complex located in the perinuclear region of the cytoplasm. When cells are subjected to heat stress, HSP27 proteins aggregate into a

larger 800 to 2000-kDa complex which is translocated into the nucleus (Satoh and Kim, 1995). HSP27 is also known to exist in three isoforms, including non-phosphorylated (mainly in unstressed cells), mono-phosphorylated, and diphosphorylated forms (Satoh and Kim, 1995).

A second cytoplasmic pool of HSP27 has been identified. HSP27 from this pool was found to be phosphorylated in response to treatment of cells with tumour necrosis factor, platelet growth factor, fibroblast growth factor, tumour-promoting phorbol esters, or heat shock (Stokoe et al., 1992, and references within). This phosphorylation of HSP27 was determined to occur through MAP kinase-activated protein kinase-2 (MAPKAP kinase 2) on a serine residue (Stokoe et al., 1992) and was down-regulated by protein phosphatases 2A and 2B (Satoh and Kim, 1995). MAPKAP kinase 2 has been shown to be activated by members of the MAPK family of kinases, including ERK1 and 2, and p38 MAPK, but not by the stress-activated MAPK kinase JNK/SAPK (Guay et al., 1997). This phosphorylation of HSP27 is interesting, as AEBP1 has been shown to be phosphorylated by MAPK (section 3.III.).

The reasons for HSP27 phosphorylation is unclear. The protein is known to be phosphorylated in response to mitogens and to heat shock, which may cause HSP27 to protect actin. But the function attributed to this phosphorylation event regarding actin protection is contradictory and confusing. What follows is a summary of the observed results of HSP27 phosphorylation.

HSP27 plays a role in cytoskeleton assembly (Zhu et al., 1994). In nonhematopoietic cells, HSP27, in response to mitogens or to heat stress, is phosphorylated and translocated from membrane pools to the cytoplasm, where it becomes associated with actin and is involved in cytoskeleton assembly (Zhu et al., 1994). It is suggested that HSP27 is a component of a signal-transduction pathway which regulates microfilament dynamics by protection against reorganization of the cytoskeleton (Madsen et al., 1995). HSP27 has also been described as a filamentous-

actin (F-actin) cap protein, preventing F-actin polymerization when fibroblast cells are deprived of serum or in response to cellular shock. Overexpression of HSP27 in rodent cell lines enhances growth factor-induced F-actin accumulation following mitogenic stimulation of quiescent cells, while overexpression of a non-phosphorylatable form of HSP27 exerts a dominant-negative effects and inhibits this mitogen response (Lavoie et al., 1993; Huot et al., 1996). The phosphorylation of HSP27 is proposed to reduce the binding affinity of HSP27 for actin, which modulates actin monomer addition upon mitogenic stimulation or heat shock (Lavoie et al., 1995)

Heat shock also causes the oligomerization of HSP27, which is a requirements for its protection function (Lavoie et al., 1995). Furthermore, upon the addition of serum and cytokines, such as tumor necrosis factor-α, to cells HSP27 is phosphorylated (Mehlen et al., 1995). These results indicate further that HSP27 is regulated by signal-transduction pathways and is involved in the regulation of actin polymerization.

Furthermore, the activation of a G protein coupled receptor (GPCR), the bombesin receptor, has been shown to mediate the activation of MAPK through a trimeric G protein. The activated G proteins causes the subsequent colocalization of MAPK and HSP27 from a membrane fraction to a cytosolic fraction in smooth muscle (Yamada et al., 1993), indicating that GPCRs may be responsible for the translocation and colocalization of both HSP27 and MAPK. This is interesting as AEBP1 has been found to interact with Gγ5 and HSP27, and is phosphorylated and interacts with MAPK (see section 3.III. below).

The physiological significance of the interaction between AEBP1 and HSP27 is not obvious, but it is interesting to speculate on the possible functions for this interaction. HSP27 is found to localize in the nucleus and the cytoplasm. HSP27 may interact with AEBP1 in the nucleus, where it may be involved in its transcription regulation role. HSP27 is also found to be phosphorylated by MAPKAP kinase, an

enzyme activated by MAPK. AEBP1 is phosphorylated by MAPK (see section 3.III. below) and may be involved in its signal-transduction pathway. It is possible that AEBP1, MAPK, and HSP27 may interact in a signal complex, as MAPK and HSP27 have been shown to colocalize upon the activation of G protein coupled receptors.

Finally, AEBP1 was also found to interact with the Gγ5 subunit of the trimeric G protein (section 3.II.D.). This G protein subunit is known to localize to a focal adhesion regions. As this region is involved in actin regulation, and MAPK is known to be involved in the regulation of this region during differentiation, it is possible that AEBP1, Gγ5, HSP27 and MAPK all may interact in this region during preadipocyte differentiation. HSP27 has also been described as a transient marker for differentiation induced growth arrest (Madsen et al., 1995), a process which is critical for the induction of adipogenesis in 3T3 L1 preadipocytes. It is intriguing to speculate that these proteins interact at the focal adhesion area during differentiation dependent growth arrest in preadipocytes, and are involved in the signaling which results in the subsequent morphological changes which accompany adipogenesis. Further study into the AEBP1–HSP27 interaction may provide insight into the regulation of the cytoskeleton during adipogenesis.

3.III. AEBP1 is Phosphorylated by and Interacts with the Mitogen-Activated Protein Kinase (MAPK)

3.III.A. AEBP1 is Phosphorylated by MAPK

In the C-terminal region of AEBP1 there are several possible phosphorylation sites (Figure 1.5). To determine if AEBP1 was phosphorylated *in vitro*, phosphorylation experiments were performed using recombinant His-tagged AEBP1 and extracts from 3T3 L1 cells. Figure 3.30, Lane 3, upper panel, shows that AEBP1 was phosphorylated by a protein kinase in the extract. Comparisons of Lanes 1 and 3 show that the phosphorylated protein was not present in the extract. Therefore the observed phosphorylated protein may be attributed to the recombinant His- tagged AEBP1 to the kinase reaction mixture. To verify that the phosphorylation of AEBP1 occurred in the C terminus of the protein, a mutant form of AEBP1 lacking the entire C terminus, AEBP1ΔSty (Figure 3.14)was used in the kinase reaction. Lane 2 shows that this truncated protein was not phosphorylated by a kinase in the 3T3 L1 cell extract, suggesting that AEBP1 was phosphorylated in the C-terminus. However, it is possible that conformational changes caused by the truncation in this mutant may mask potential phosphorylation sites in other regions of AEBP1.

Knowing that AEBP1 can be phosphorylated, several strategies were undertaken to identity the protein kinase responsible for this phosphorylation. To determine if the phosphorylation occurred on a tyrosine residue, an antibody directed towards phosphorylated tyrosine was incubated with extracts of 3T3 L1 cells that had been stimulated with 15% FBS and immunoprecipitated with protein A-agarose beads. Immuno-blots using anti-AEBP1 antibody were performed on the immunoprecipitate to determine if a tyrosine phosphorylated AEBP1 was immunoprecipitated. No tyrosine-

Figure 3.30. Phosphorylation of AEBP1 in the C terminus by MAPK
Recombinant His-tagged AEBP1 was phosphorylated by either a kinase from 3T3 L1 cell extracts (upper panel) or with immunoprecipitated MAPK (lower panel). Kinase reactions contained no AEBP1 (Lane 1), 2 μg AEBP1ΔSty (Lane 2), 2 μg AEBP1 (Lane 3), 2 μg AEBP1 plus 100 μM olomoucine (Lane 4), or 2 μg AEBP1 plus 100 μM iso-olomoucine (Lane 5). Protein samples were resolved by SDS-PAGE and the radiolabeled proteins were detected by autoradiography. Protein sizes were determined by comparison to pre-stained molecular weight markers (NEB). The arrow indicates radiolabeled AEBP1. IP indicates immunoprecipitated ERK1/2.

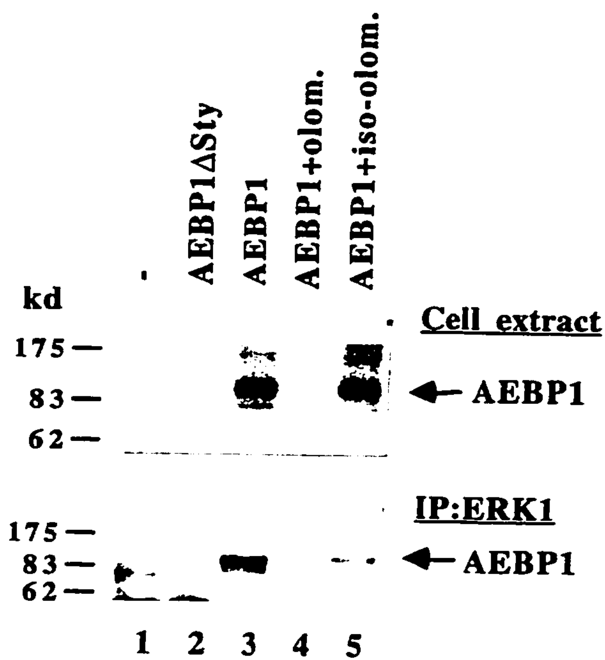


Figure 3.30 Phosphorylation of AEBP1 in the C terminus by MAPK

phosphorylated AEBP1 protein was detected in this experiment (data not shown). The converse experiment was performed using antibody directed towards AEBP1, with the immunoprecipitate blotted with anti-phosphotyrosine antibody. Again no tyrosine-phosphorylated AEBP1 was observed, indicating that AEBP1 was not phosphorylated by a tyrosine kinase (data not shown). In addition, recombinant purified protein kinase C (provided by D. Douglas) was also not capable of phosphorylating AEBP1 (data not shown).

Within the C-terminal STP region (serine, threonine, and proline; see Figure 1.5) of AEBP1 are a number of MAPK phosphorylation consensus sequences (PXS/TP; discussed in 1.II.B.). MAPK (ERK 1 and 2) immunoprecipitated from 3T3 L1 cells was tested for the ability to phosphorylate recombinant His-tagged AEBP1 (Figure 3.30, lower panel). Comparison of Lanes 1 and 3 shows that recombinant AEBP1 was phosphorylated by immunoprecipitated MAPK. Lane 2 shows that the C-terminally truncated mutant, AEBP1ΔSty, was not phosphorylated by immunoprecipitated MAPK, suggesting that AEBP1 is phosphorylated in the C terminus by MAPK.

To show further that MAPK was the kinase responsible for phosphorylation of AEBP1 in 3T3 L1 cell extracts, the MAPK competitive inhibitor olomoucine (Wieprecht et al., 1996) was tested. Figure 3.30, Lane 4, upper panel, shows that when olomoucine was added to the kinase reaction, phosphorylation of the recombinant His-tagged AEBP1 was abolished. Less inhibition was observed when the control inhibitor iso-olomoucine (a non-specific inhibitor with low affinity for MAPK; Wieprecht et al., 1996) was used in AEBP1 phosphorylation experiments (Lane 5), as this inhibitor has a much reduced affinity for MAPK. Similar experiments were done with immunoprecipitated MAPK (Figure 3.30, Lane 4 and 5, lower panel) and showed that MAPK was responsible for phosphorylation of the recombinant AEBP1.

Finally, to show that AEBP1 was in fact phosphorylated by MAPK, inactive unphosphorylated recombinant MAPK was used in a phosphorylation experiment. MEK, the kinase which phosphorylates MAPK, was immunoprecipitated with anti-MEK antibody and used in a kinase reaction (Figure 3.31) alone (lane 1), or with MAPK (lanes 2 to 5), AEBP1 (lanes 3 and 5), and the MAPK substrate myelin basic protein MBP (lanes 4 and 5). The results showed that recombinant MAPK was able to phosphorylate AEBP1 (lane 5) while at the same time phosphorylating MPB. Comparing lanes 4 and 5 shows that the level of phosphorylation of MBP was decreased when AEBP1 was present, indicating that AEBP1 was used as a substrate, decreasing the amount of MBP used as a substrate. This phosphorylation of AEBP1 suggests that MAPK was able to efficiently phosphorylate AEBP1 even when MBP was present in the same reactions.

Several transcription factors, including repressors are known to be phosphorylated by MAPK family members (see section 1.II D; reviewed by Treisman, 1996; Su and Karin, 1996). Although the function of the *in vitro* phosphorylation of AEBP1 is not apparent, phosphorylation of other transcription factors may provide clues. Phosphorylation of transcription factors is known to stabilize proteins, regulate DNA binding, affect the ability of proteins to regulate transcription, or determine nuclear localization. All of these are possible outcomes of AEBP1 phosphorylation. Further study into the function of this phosphorylation event may lead to important insight into mechanism by which AEBP1 is regulated.

Figure 3.31. AEBP1 is Phosphorylated by recombinant MAPK Immunoprecipitated MEK phosphorylates MAPK, which in turn phosphorylates AEBP1 and MBP. Immunoprecipitated MEK from serum stimulated 3T3 L1 cells was used in a kinase reaction with recombinant MAPK (Lanes 2-5), AEBP1 (Lanes 3 and 5), and MBP (Lanes 4 and 5). Protein samples were resolved by SDS-PAGE, and radiolabeled proteins were detected by autoradiography. Protein sizes were determined by comparison to pre-stained molecular weight markers (NEB). The arrow indicates radiolabeled AEBP1, MAPK, and MBP.

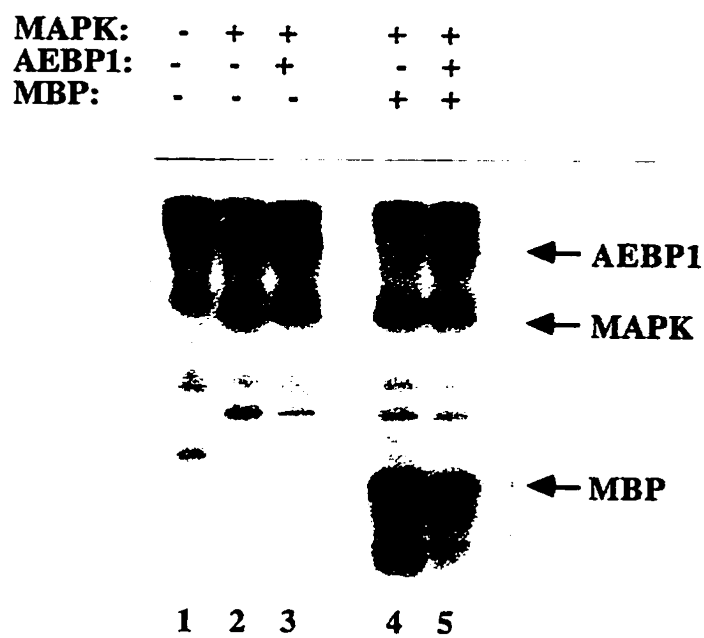


Figure 3.31 AEBP1 is Phosphorylated by Recombinant MAPK

3.III.B. AEBP1 Complexes with MAPK

Studies to determine the protein kinase responsible for the phosphorylation of AEBP1 using anti-phosphotyrosine antibody showed that two tyrosine phosphoproteins with approximate molecular weights those of the MAPK enzymes ERK1 and ERK2 co-immunoprecipitated with AEBP1 (data not shown). Furthermore, AEBP1 phosphorylation studies using immunoprecipitated MAPK showed that a protein which was phosphorylated by MAPK co-immunoprecipitated with MAPK and corresponded to the estimated size of the native AEBP1 (Figure 3.30, Lower panel, Lanes 1 and 2). These results indicated that AEBP1 may co-immunoprecipitate with MAPK.

To explore this possible interaction, other co-immunoprecipitation studies were undertaken. Figure 3.32, left panel, lanes 2 and 4, shows that antibodies directed towards AEBP1 and MAPK both immunoprecipitate AEBP1. Likewise, Figure 3.32 right panel, lanes 1 and 3, shows that antibodies directed towards AEBP1 and MAPK also both immunoprecipitate MAPK. Immunoprecipitation studies using controls showed that these proteins where not interacting non-specifically with the agarose beads (lane 1, 3, and 5 of Figure 3.32, left panel, and lane 5 of Figure 3.32, right panel) or non-specifically with other antibodies (lane 6 of Figure 3.32 left panel, and lane 4 of Figure 3.32 right panel). These results suggest that AEBP1 and MAPK interact in 3T3 L1 cells either directly or in a larger complex through other proteins.

Figure 3.32 AEBP1 Interacts with MAPK

Left panel

AEBP1 co-precipitates with MAPK. Equal amounts of 3T3 L1 cell extract were used in immunoprecipitation studies with protein A-agarose beads (Lane 1, 3, and 5), anti-AEBP1 antibody (Lane 2), anti-ERK1 antibody (Lane 4), or anti-S24 (small subunit ribosomal protein) antibody (Lane 6). The immunoprecipitated samples were immunoblotted with anti-AEBP1 antibodies.

Right panel

MAPK co-precipitates with AEBP1. Equal amounts of 3T3 L1 cell extract were used in immunoprecipitation studies with anti-AEBP1 antibody (Lane 1), anti-ERK1 antibody (Lane 3), anti-S24 (small subunit ribosomal protein) antibody (Lane 4), or protein A-agarose beads (Lane 5). Lane 2 contains molecular weight markers (NEB). The immunoprecipitated samples were immunoblotted with anti-ERK1 antibodies. These antibodies also precipitate ERK2.

Protein samples were resolved in SDS-PAGE gels and then transferred to nitrocellulose for immunoblotting. Pre-stained protein markers (NEB) were used to determine molecular weights. Bands were observed after ECL chemilumenescence reactions followed by autoradiography. IP indicates antibody used for immunoprecipitations. IB indicates antibody used for immunoblotting.

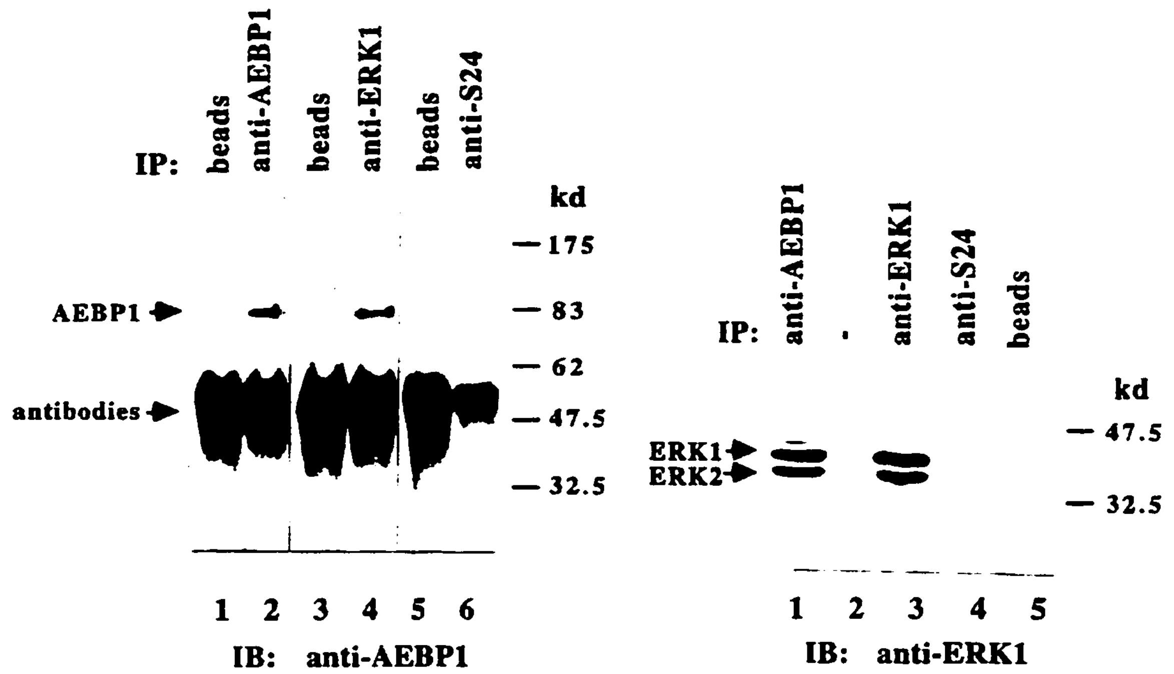


Figure 3.32 AEBP1 Complexes with MAPK

The interaction between protein kinases and the transcription factors which they phosphorylate is not unprecedented. Several transcription factors bind to and are phosphorylated by protein kinases. One of the better studied examples is the STAT proteins. Upon cytokine-ligand binding, the Janus kinase (JAK) tyrosine kinases interact with and phosphorylate cytokine receptors, ultimately leading to the activation of the receptor. The STAT proteins directly interact with Janus kinases on the receptor, and are phosphorylated upon ligand binding to the cytokine receptor. The phosphorylated STAT protein dimerizes and then translocates to the nucleus, were it acts as a transcription factor (Hill and Treisman, 1995). STAT proteins have also been shown to be phosphorylated by and interact with MAPK, which is thought to antagonize the JAK-mediated phosphorylation and activation of the STAT proteins (Ihle, 1996).

The stress-activated protein kinases (SAPK/JNK) interact with the transcription factor c-Jun. Inactive SAPK/JNK tightly binds c-Jun. Upon stress activation of SAPK/JNK, the protein phosphorylates c-Jun, which causes the disassociation of the transcription factor from the kinase. c-Jun is then translocated to the nucleus where it acts as a transcription regulator. It is interesting that both phosphorylated and unphosphorylated forms of c-Jun interact with SAPK/JNK, indicating a regulatory function for this interaction. The binding of inactive SAPK/JNK is believed to sequester the transcription factor and prevent it from performing its *trans*-activation function (Dai et al., 1995). Furthermore, MAPK has been shown to interact with c-Jun, possibly with the same regulatory function (Dai et al., 1995). In light of these studies, it would be interesting to determine if AEBP1 interacts with the phosphorylated, dephosphorylated, or both forms of MAPK to determine if the AEBP1–MAPK interaction was further involved in regulation of AEBP1.

3.III.C. AEBP1 Interacts with the Active and Inactive Forms of MAPK

To determine if AEBP1 interacts with the phosphorylated active form of MAPK (MAPK is phosphorylated on two residues, a Ser/Thr and a Tyr, and both residues must be phosphorylated for kinase activity), the inactive, dephosphorylated form (removal of both phosphates), or both, immunoprecipitation experiments were done with quiescent or serum stimulated 3T3 L1 cells. As Figure 3.33 illustrates, both the dephosphorylated and phosphorylated forms of the MAPK enzymes ERK1 and ERK2 can be immunoprecipitated by anti-MAPK antibody (lanes 1 and 2) and by anti-AEBP1 antibody (lanes 3 and 4).

These results indicate that AEBP1 complexes with both the active and inactive forms of MAPK. To explore the relevance of this interaction, gel-filtration studies were performed. These experiments were designed to determine if AEBP1 interacts with MAPK during various stages of induction of 3T3 L1 preadipocyte differentiation. Quiescent 3T3 L1 cells were stimulated with serum and insulin, and then collected at various times thereafter. After gel-filtration procedures, the extracts were immunoblotted for MAPK and AEBP1.

AEBP1 from quiescent and 10-minute serum- and insulin-stimulated cells eluted at an estimated molecular weight between 450 and 900 kDa (Figure 3.34, panels A, B, and C), and continued at an estimated molecular weight of 100 to 450 kDa (Figure 3.34, panels A, B, and C). AEBP1 was only located in lower molecular weight eluent from 2-hour insulin- and serum-stimulated cells (Figure 3.34, panel D).

In the quiescent and 10-minute serum-stimulated cells, MAPK was found to co-

Figure 3.33 AEBP1 Interacts with the Phosphorylated and Dephosphorylated Forms of MAPK

This figures shows both the inactive, dephosphorylated and "gel shifted" active, phosphorylated forms of MAPK which immunoprecipitate with anti-ERK1 and anti-AEBP1 antibodies. Equal amounts of 3T3 L1 cell extract from quiescent cells (Lanes 1 and 3) or serum-stimulated cells (Lanes 2 and 4) were used in immunoprecipitation studies with either anti-ERK1 (Lanes 1 and 2) or anti-AEBP1 (Lanes 3 and 4) antibodies and analyzed by immunoblotting with anti-ERK1 antibody. Protein samples were resolved in 12% SDS-PAGE gels and then transferred to nitrocellulose for immunoblotting. Pre-stained protein markers (NEB) were used to determine molecular weights. Bands were observed after ECL chemilumenescence reactions followed by autoradiography. IP indicates antibody used to immunoprecipitate, either α-AEBP1 (anti-AEBP1) or α-ERK1 (anti-ERK1). $pp44^{ERK1}$ and $pp42^{ERK2}$ correspond to the phosphorylated forms of MAPK. $p44^{ERK1}$ and $p42^{ERK2}$ correspond to the dephosphorylated form of MAPK. Number above the lanes indicate the duration of serum stimulation times.

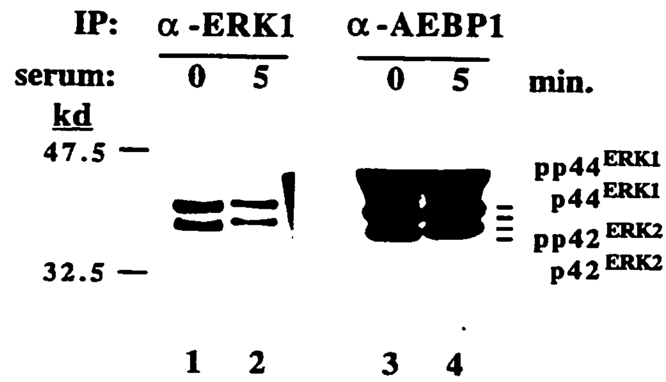
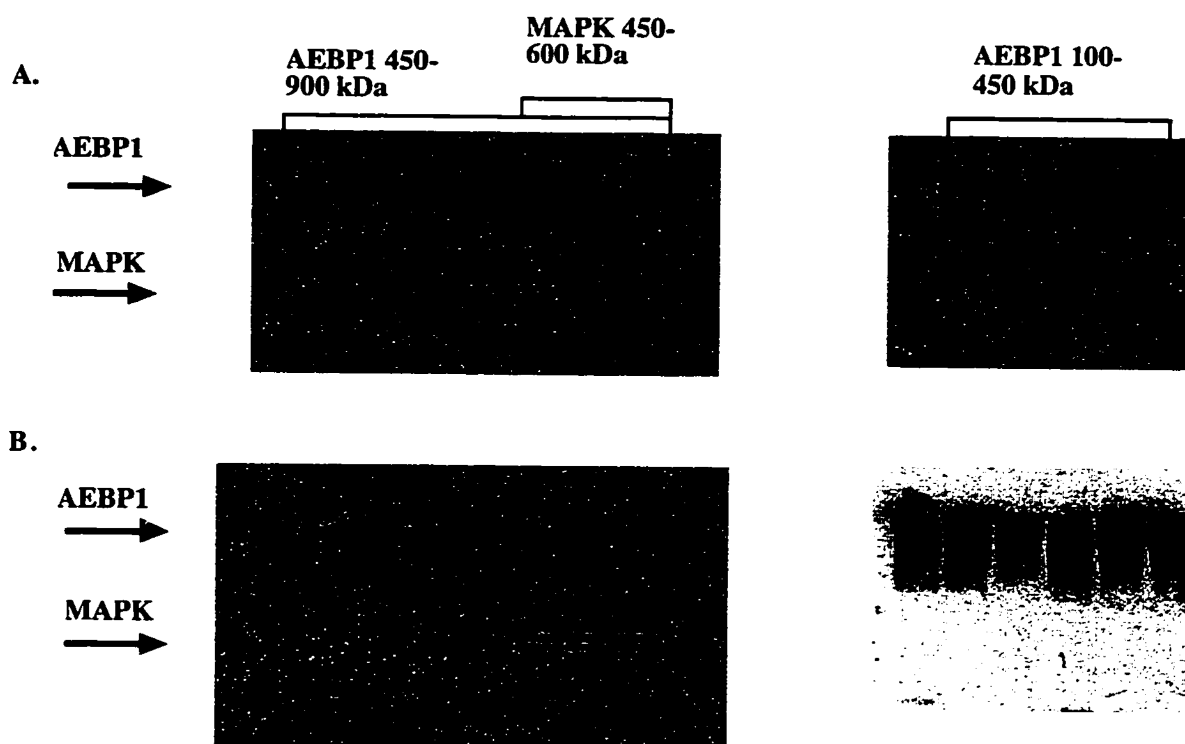


Figure 3.33 AEBP1 Complexes with the Phosphorylated and Dephosphorylated Forms of MAPK

Figure 3.34 AEBP1 and MAPK Co-Purify in Gel-filtration Studies

Quiescent (**panel A**), 10-minute (**panel B**), 30-minute (**panel C**), and 2-hour (**panel D**) serum- and insulin-stimulated cells were collected for gel-filtration studies. Panels A, B, and C show that AEBP1 elutes at an estimated molecular weight of 450 to 900 kDa, while panels A, B, C, and D show that AEBP1 also elutes in a smaller complex of an estimated molecular weight of 100 to 450 kDa. MAPK was found to co-elute with AEBP1 in a large complex (panels A and B) of an estimated molecular weight of 450 to 600 kDa, while panel C show MAPK in a smaller complex of an estimated molecular weight of 150 to 200 kDa. Samples were immuno-blotted with anti-AEBP1 and anti-MAPK (ERK1) antibodies. All fractions containing AEBP1 are are shown in panels A, B, C, and D.

Molecular weights were estimated by comparing the elution volume to that of known molecular weight standards, plotted (volume of eluent) versus 1/kDa. Protein samples were resolved in SDS-PAGE gels and then transferred to nitrocellulose for immunoblotting. Pre-stained protein markers (NEB) were used to determine molecular weights. Bands were observed after ECL chemilumenescence reactions followed by autoradiography.


A.
AEBP1 450-
900 kDa
MAPK 450-
600 kDa
AEBP1 100-
450 kDa
AEBP1
MAPK
B.
AEBP1
MAPK

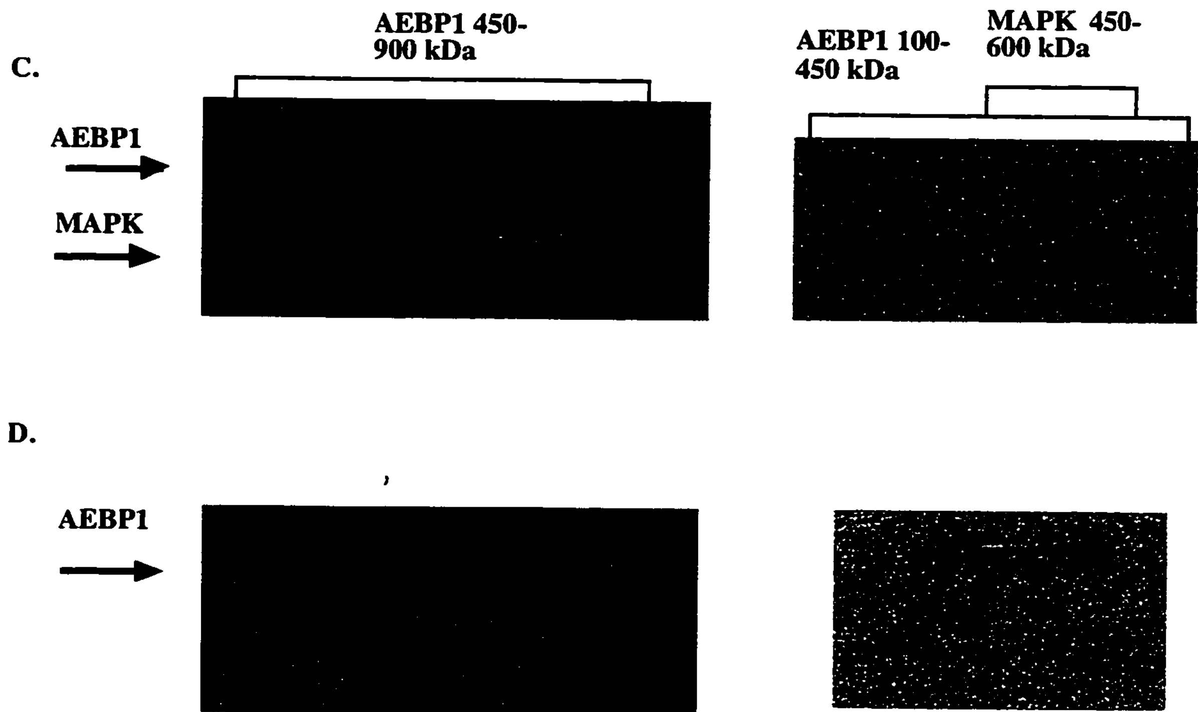


Figure 3.34 AEBP1 and MAPK Co-Purify in Gel-filtration Studies

purify with AEBP1 at a molecular weight of approximately 450 to 600 kDa (panel A and B). When 3T3 L1 cells were stimulated for 30 minutes, MAPK was found to co-elute with AEBP1 at a molecular weight of approximately 150 to 200 kDa (panel C). After two hours of stimulation MAPK was not found to co-elute with AEBP1 (panel D). These results do not demonstrate that the two proteins directly interact, but suggest that the proteins may be present together in large complexes, perhaps associated with other proteins.

Although the gel-filtration studies do not give insight into the function of the AEBP1–MAPK complexes, it is interesting to speculate on the functional role of this interaction. It is known that upon the binding of serum factors (Gonzalez et al., 1993) including growth factors (Lenormand et al., 1993) and insulin (Kim and Kahn, 1997), to a receptor tyrosine kinase, MAPK is activated at the plasma membrane and translocated to the nucleus. This MAPK activation must be sustained for at least 15 minutes if the kinase is to be translocated to the nucleus (Chen et al., 1992; Alessi et al., 1995; Grammer and Blenis, 1997). The gel-filtration results suggest that within 30 minutes of serum and insulin stimulation, MAPK may be released from a large complex in preadipocyte cells. The smaller complex may contain AEBP1 and MAPK and other unidentified components, and may be translocated to the nucleus. Mitogenic signals acting through receptor tyrosine kinases, such as EGFR, may signal AEBP1 along with MAPK to be translocated. Once in the nucleus AEBP1 may repress transcription of adipogenic induced genes, such as aP2, thus allowing cell proliferation. In the nucleus AEBP1 and MAPK may dissociate when AEBP1 binds to the AE-1 DNA (see section 3.III.E.).

In the larger complex present in quiescent cells, AEBP1 may interact with the inactive form of MAPK along with other proteins identified in the two-hybrid screen. In this case, the $G\gamma 5$ subunit may anchor the complex to the plasma membrane. This complex would undoubtedly contain the $G\beta$ subunit, along with AEBP1 and MAPK.

Although these studies do not attempt to clarify the function of the AEBP1–MAPK interaction, it is intriguing to imagine that AEBP1 interacts with MAPK in both a cytoplasmic and a nuclear complex, as AEBP1 co-elutes with MAPK in two complexes in gel-filtration studies. If this is the case, it is possible that AEBP1 is translocated to the nucleus along with MAPK upon sustained stimulation of preadipocytes. Likewise, AEBP1 may be present in a larger complex where to interacts with the inactive form of MAPK, and upon stimulation AEBP1 interacts with the activated form of the kinase. This would explain why AEBP1 co-immunoprecipitates both the active and inactive form of MAPK. The phosphorylation of AEBP1 by MAPK may signal the translocation of this complex or regulation of the interaction between AEBP1 and MAPK.

Studies by Hu et al. (1996) and Adams et al. (1997) have shown that mitogen activation of MAPK prevents preadipocyte differentiation by causing the phosphorylation of the adipogenic transcription factor PPARγ. However, activation of MAPK has been shown to be essential for insulin-dependent differentiation of 3T3 L1 (Porras and Santos, 1996; Sale et al., 1995). These results indicate that MAPK is activated by adipogenic factors and that this activation is required for the initiation of differentiation. Once the differentiation process has begun, prolonged MAPK activation may lead to the phosphorylation of PPARγ and the subsequent inhibition of adipogenesis. This view also indicates that prolonged MAPK activation in 3T3 L1 cells causes the opposite outcome than it does in neuroblast cells. In neuroblasts, EGF transiently activates MAPK leading to cell proliferation, while nerve growth factorcauses prolonged activation of MAPK, leading to differentiation (Marshall, 1995). In preadipocytes the opposite may occur, with mitogens causing prolonged activation of MAPK and proliferation of these cells, while adipogenic agents cause transient activation of MAPK and differentiation. In growth arrested fibroblasts,

prolonged MAPK activation is known to mediate cell proliferation (Brondello et al., 1995, 1997; Lenormand et al., 1996).

Therefore, the activation of MAPK may ensure that both proteins are translocated to the nucleus, and also ensure that both proteins inhibit adipogenesis through transcription repression. The prolonged activation of MAPK may enhance AEBP1 transcription repression function and inactivate PPARγ in 3T3 L1 preadipocytes, thereby blocking differentiation.

3.III.D. AEBP1 Protects MAPK Activity

MAPK is known to be activated by MEK phosphorylation in the cytoplasm, and if this activation is sustained, MAPK translocates to the nucleus. However, if this activation is transient, MAPK is prevented from translocating (Alesi et al., 1995). As MEK is known to be transiently activated by phosphorylation, the regulation of prolonged or transient MAPK activation may be through phosphatases (Alesi et al., 1995; Grammer and Blenis, 1997; discussed in section 1.I.D). To determine if the interaction between AEBP1 and MAPK influences the duration of MAPK activation, an *in vitro* phosphatase assay was designed using recombinant AEBP1 and MAPK.

In this assay (Figure 3.35), recombinant MAPK was activated by active immunoprecipitated MEK, isolated by immunoprecipitation with anti-phosphotyrosine antibody, and then incubated with AEBP1 (lanes 1 to 3) or with a control protein (Rab-11) which is not known to interact with MAPK (lanes 4 to 6). MAPK was then treated with calf intestinal alkaline phosphatase (CIAP) and purified by immunoprecipitation with anti-ERK1 antibody. To test for activity, MAPK treated in this way was incubated in a kinase reaction with the substrate MBP. Comparing lane 5 (no AEBP1) with lane 2 (plus AEBP1) of Figure 3.35 shows that AEBP1 protects MAPK from the phosphatase

in this reaction. As a control, heat-inactivated CIAP (lanes 3 and 6) is not able to inactivate MAPK.

These results indicate that AEBP1 was able to protect MAPK from inactivation by at least one phosphatase *in vitro*. The mechanism by which this protection occurs is not obvious. AEBP1 may interact with MAPK to mask its phosphorylated amino acid residues or cause a conformation change which prevents phosphatases from gaining access to these residues. It is possible that when AEBP1 enters the MAPK active site it causes a conformational change which protects the kinase from inactivation. In any case, the protection of MAPK activity from phosphatases by AEBP1 may explain the enhanced and sustained activation of MAPK in cell lines overexpressing AEBP1 which is discussed below.

These results may help rationalize the effects of AEBP1 and MAPK in a 3T3 L1 cell line overexpressing AEBP1 (Kim and Ro, unpublished results). In this cell line an expression plasmid pAEBP1/Neo, which constitutively expresses AEBP1, was stably transfected into 3T3 L1 cells. Stable cell lines expressing high levels of AEBP1 (as judged by Western and Northern blot analysis) were isolated. Kim and Ro (unpublished data) showed that in these cells lines MAPK activity was higher than in control cell lines which were stably transfected with the parental plasmid pWZLNeo alone. Furthermore, they have shown that MAPK activity was sustained over 8 hours in 3T3 L1 cells overexpressing AEBP1, in comparison to only 4 hours in control cells expressing normal levels of AEBP1, when these cells are stimulated by serum. Interestingly, these overexpressing cell lines are not able to differentiate as assessed by Northern blot analysis and the lack of ability to accumulate lipids (Kim and Ro,

Figure 3.35 AEBP1 Protects MAPK From Inactivation by Phosphatase Activity

AEBP1 protects MAPK from calf intestinal alkaline phosphatase (CIAP) treatment. MAPK was phosphorylated and activated by immunoprecipitated MEK isolated from equal amounts of serum-stimulated 3T3 L1 cells. The activated MAPK was then isolated by immunoprecipitation with anti-phosphotyrosine antibody, and incubated with either AEBP1 (Lanes 1 to 3) or Rab-11 control protein (Lanes 4 to 6). Materials resolved in Lanes 2 and 5 was then treated with CIAP, and materials in lanes 3 and 6 were treated with heat-inactivated CIAP (ΔCIAP). Samples resolved in Lane 1 and 4 was not treated. MAPK in each sample was then isolated by immunoprecipitation with anti-ERK1 antibody and used in a kinase reaction with the MAPK substrate MBP. Protein samples were resolved by SDS-PAGE, and the radiolabeled proteins were detected by autoradiography. Protein sizes were determined by comparison to pre-stained molecular weight markers (NEB). The arrow indicates radiolabeled MBP. This experiment was repeated three times with similar results.

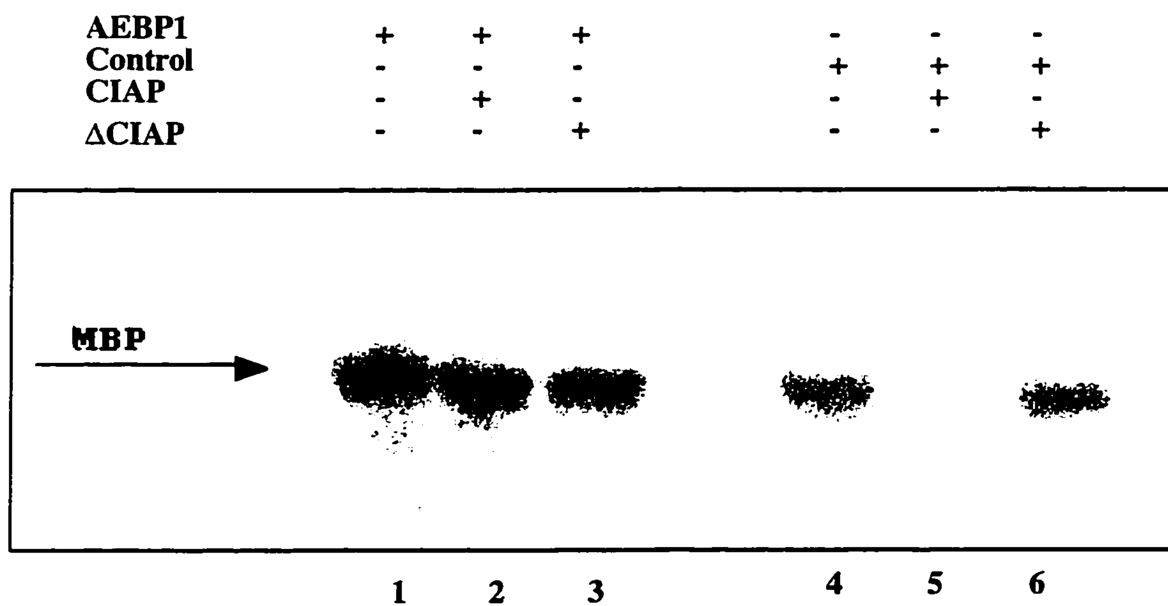


Figure 3.35 AEBP1 Protects MAPK from Inactivation by Phosphatase Activity

unpublished data). Although the *in vivo* reasons for the inability of 3T3 L1 cell lines overexpressing AEBP1 to differentiate and the prolonged activation of MAPK in these cells are undoubtedly complex, the *in vitro* protection of MAPK activation by AEBP1 may provide insight. In these cell lines, it is possible that AEBP1 interacts with MAPK and protects it from phosphatases that would otherwise inactivate the kinase, or that prolonged activation of MAPK may prevent activation of phosphatases that would otherwise down-regulate MAPK. In any case, the failure of phosphatases to dephosphorylate MAPK would allow prolonged activation of MAPK, which may inhibit adipogenesis, through the phosphorylation and inactivation of PPARγ, and the possibly through the activation of AEBP1 (see section 4.II.).

3.III.E. MAPK Enhances the Ability of AEBP1 to Bind DNA

It is conceivable that AEBP1 is phosphorylated by MAPK in the nucleus, where the kinase is known to translocate. This phosphorylation event may enhance or inhibit the ability of AEBP1 to repress transcription. The interaction between AEBP1 and MAPK may thus affect both proteins. As shown above, this interaction may protect MAPK from phosphatases which would otherwise inactive the kinase, and the sustained activation of MAPK may allow maintenance of the preadipocyte phenotype. The interaction of the two proteins may also facilitate the binding of AEBP1 to DNA. Proteins such as HMG 2 enhance the DNA binding of transcription factors like Oct1/2 but do not cause a "supershift " (the formation of a band of slower mobility due to a protein-protein-DNA interaction) in gel mobility shift assay, indicating that once the transcription factor interacts with DNA the HMG 2 protein disassociates (Zwilling et al., 1995). Similarly, the retinoblastoma protein (Rb) enhances the binding of the CCAAT/enhancer-binding proteins (C/EBP) to cognate binding sites, but does not cause a "supershift" in gel mobility shift assay. This finding indicates that the proteins

interact, and that when C/EBP binds to DNA, the Rb protein is released. Furthermore, this interaction only occurs in differentiating 3T3 L1 cells, with the interactions of the two proteins enhancing the transcriptional activation of genes required for the differentiation process (Chen et al., 1996). The interaction of AEBP1 and MAPK may function in a similar manner. MAPK may enhance the ability of AEBP1 to bind DNA, but disassociate once the AEBP1-DNA interaction takes place.

In support of this hypothesis, MAPK was found to enhance the ability of AEBP1 to bind to DNA in gel mobility shift assays (Figure 3.36). Lanes 11 to 13 show that increasing the amount of MAPK in the binding reaction enhanced the binding of AEBP1 to the AE-1 DNA. Lane 8 shows that MAPK alone does not bind this DNA, while lanes 1 to 4 show that BSA and the control protein yeast Rab-11 did not affect the DNA binding ability of AEBP1. The band of higher mobility seen when AEBP1 and MAPK are added together in the gel mobility shift assays may be due to conformational changes in the AEBP1–AE-1 complex brought about by the MAPK–AEBP1 interaction. Bands of lower mobility may be due to AEBP1 dimer and trimer interaction with the AE-1 DNA induced by MAPK. However, the possibility that AEBP1 and MAPK bind together to the AE-1 DNA to produce the higher or lower mobility bands cannot be ruled out.

These results suggest an explanation for the results observed in the gel-filtration studies. After stimulation of cells with serum and insulin, MAPK translocates to the nucleus (Kim and Kahn, 1997; Gonzalez et al., 1993; Lenormand et al., 1993). Therefore, AEBP1 and MAPK may be translocated together to the nucleus during the first 15 to 30 minutes of stimulation, which would account for the presence of the smaller complex seen in the gel-filtration studies. After 2 hours of stimulation, this complex is not seen, as AEBP1 DNA-binding may cause the disassociation of MAPK.

Figure 3.36. MAPK enhances the DNA-binding Ability of AEBP1
Recombinant AEBP1 protein was incubated with radiolabeled AE-1 DNA to produce the mobility shift observed (see arrow). Lane 1 contains 2 μg of AEBP1 plus 2 μg of BSA. Lanes 2-4 contain 2 μg of AEBP1 plus 0.5, 1.0, and 2.0 μg of control Rab-11 yeast protein, respectively. Lane 5 contains 2 μg of AEBP1 and Lane 6 contains 2 μg of Rab-11 alone. Lane 7 contains the labeled AE-1 DNA alone. Lane 8 contains 2 μg of MAPK alone. Lane 9 contains 2 μg of AEBP1 plus unlabeled AE-1 DNA (competitively inhibits binding to the AE-1 site). Lane 10 contains 2 μg of AEBP1 alone. Lane 11-13 contain 2 μg of AEBP1 plus 0.5, 1.0, and 2.0 μg MAPK, respectively. Protein-DNA samples were resolved by SDS non-denaturing gels and the radiolabeled DNA was detected by autoradiography.

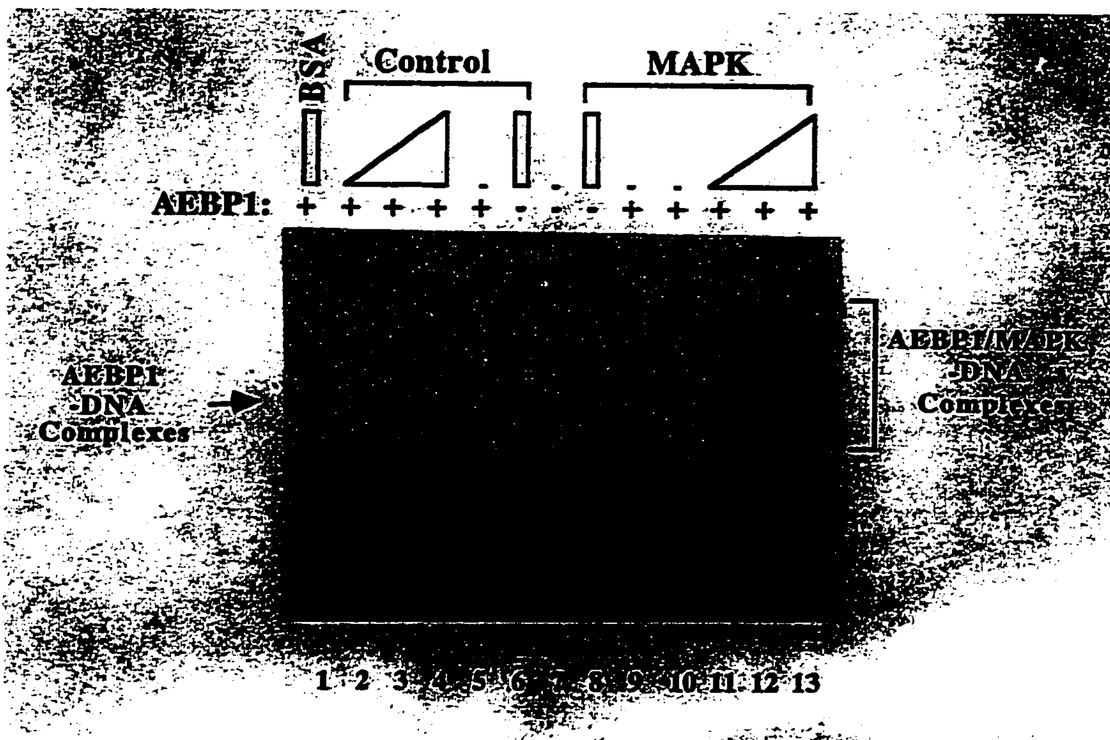


Figure 3.36 MAPK Enhances AEBP1 DNA Binding Ability

The DNA binding of AEBP1 would cause repression of the differentiation specific gene aP2. The sustained activation of MAPK may also prevent phosphatases from regulating AEBP1, and therefore would allow continued cell growth.

3.III.F. The Relevance of AEBP1 and MAPK Interactions

These studies demonstrate that *in vitro* AEBP1 is most likely phosphorylated in the C terminus by MAPK. Furthermore, AEBP1 coimmunoprecipitates with both the active, phosphorylated, and the inactive, dephosphorylated, forms of MAPK. Gel-filtration studies show that AEBP1 and MAPK co-elute in a large complex from quiescent and 10-minute serum- and insulin-stimulated cells, and in a smaller complex after 30 minutes of stimulation, but that they do not elute together after 2 hours of stimulation. *In vitro* studies show that recombinant AEBP1 was able to protect phosphorylated recombinant MAPK from dephosphorylation by calf intestinal alkaline phosphatase, and that this *in vitro* interaction enhances the DNA-binding ability of AEBP1.

Although these studies do not indicate a definite function for the phosphorylation of AEBP1 or for the interaction between AEBP1 and MAPK, taken together with the observations regarding cell lines overexpressing AEBP1, inferences can be made concerning the association of these two proteins.

AEBP1 and MAPK may exist in a cytoplasmic complex, which would explain why AEBP1 is co-immunoprecipitated with the inactive, dephosphorylated form of MAPK. Upon serum and insulin stimulation, MAPK becomes activated by phosphorylation. This active kinase may then phosphorylate AEBP1, which may signal the proteins to dissociate from the larger complex. The two proteins may then be translocated to the nucleus. Protein-protein interaction between AEBP1 and MAPK may protect MAPK from phosphatases which would otherwise inactive the kinase and

prevent translocalization to the nucleus. Once in the nucleus, MAPK enhances the DNA-binding ability of AEBP1, which causes the kinase to disassociate, allowing AEBP1 to repress transcription of the aP2 gene, and may allow MAPK to activate other transcription factors required to maintain the preadipocyte phenotype and to allow cell growth.

Recently Pouyssegur (communicated at FEBS Meeting on Cell Signaling, Amsterdam, 1997; Brondello et al., 1995, 1997; Lenormand et al., 1996) proposed that MAPK is regulated by MEK and the MAPK phosphatases MKP-1, -2 and -3. It was proposed that in growth arrested fibroblasts MAPK is anchored by physical interaction to MEK, which would allow MAPK to be quickly activated. Upon RTK or GPCR stimulation, MAPK is activated and, depending on the temporal MAPK activation, would remain in the cytoplasm or be translocated to the nucleus. The activation of MAPK by MEK would cause MAPK to phosphorylate MEK, which in turn would allow the dissociation of MAPK from MEK.

If the receptor-mediated stimulus causes transient MAPK activation, the kinase will be inactivated by MKP-3 within 20 minutes. If the receptor-mediated stimulus causes prolonged MAPK activation, the kinase will be translocated to the nucleus. The translocation is dependent on the protection of MAPK from MKP-3 by unknown proteins, possibly cytosol localized transcription factors, which are phosphorylated by MAPK and also responsible for this MAPK nuclear translocation. The activated MAPK may phosphorylate and regulate transcription factors in the cytosol or factors present in the nucleus.

After approximately 10 to 12 hours of prolonged MAPK activation, the expression of MKP-1 and -2 is induced. These phosphatases are localized only in the nucleus, and their specific down-regulation of MAPK is required for continued cell growth. MKP-1 and -2 were found to physically interact with MAPK, and MAPK was found to phosphorylate and down-regulate these phosphatases.

It is intriguing to speculate that AEBP1 may be the transcription factor in preadipocytes which is responsible for protecting MAPK from the cytosol-localized phosphatase MKP-3, and which allows MAPK to be translocated to the nucleus, which ultimately inhibits adipogenesis.

Chapter 4:
Conclusions

This thesis has explored the roles of the multifunctional protein AEBP1. The first section characterized AEBP1 as an enzymatic transcription regulator. In that section, the DNA-binding, the enzymatic, and transcription repression abilities of AEBP1 were explored. These three functions were correlated, strongly suggesting that the DNA-bound AEBP1 uses its protease function to repress transcription.

The second section identified and studied proteins which interact with AEBP1. Two-hybrid analysis showed that AEBP1 interacts with HSP27, HMG 2 and the $\gamma 5$ subunit of a trimeric G protein. Further analysis was done with the $\gamma 5$ subunit; these studies suggest that AEBP1 actually complexes with the $\beta\gamma$ heterodimer of a trimeric G protein in 3T3 L1 cells. This interaction *in vitro* prevented AEBP1 from binding DNA and repressing transcription.

The third section documented and discussed the phosphorylation of AEBP1 by MAPK, and the interaction of AEBP1 with this protein kinase. The phosphorylation of AEBP1 by MAPK may serve to regulate AEBP1. AEBP1 was found to complex with both the phosphorylated and dephosphorylated forms of MAPK. This interaction *in vitro* enhanced the DNA-binding ability of AEBP1, and protected MAPK from dephosphorylation by at least one protein phosphatase enzyme.

This chapter discusses the aforementioned aspects of AEBP1. The first section examines the role of AEBP1 as an enzymatic transcription regulator, and the second deals with the role of AEBP1 in the signaling pathways linked to G protein coupled receptors (GPCR) and receptor tyrosine kinases. Finally, the third section explores the role of AEBP1 in adipogenesis.

4.I. AEBP1 a Transcription Regulator

The aP2 gene product, the adipocyte fatty-acid-binding protein (FABP), is involved in the binding and transport of lipids within adipocytes. The expression of this protein is considered to be a marker for adipocyte differentiation. Therefore, an understanding of the negative regulation of this gene may provide critical insight into the maintenance of the preadipocyte phenotype and the initiation of adipogenesis. This gene is highly regulated, with binding sites for two proteins known to be major contributors to the differentiation process, C/EBPα (Herrera et al., 1989) and PPARγ (Tontonoz et al., 1994a/b). AEBP1 was first identified as a DNA-binding protein which specifically interacts with the aP2 gene proximal promoter region termed AE-1 (He et al., 1995). AEBP1 was found to be down-regulated during differentiation, as indicated by Northern and Western blot analysis (He et al., 1995).

This study has identified AEBP1 as the preadipocyte-specific negative regulator of the aP2 gene as described by Ro and Roncari (1991). In this study, recombinant AEBP1 was found to bind to an oligonucleotide AE-1 sequence. The DNA-binding function of AEBP1 was attributed to the C-terminal 168 amino acids. Within this region there are acidic, basic, and STP motifs which are similar to the DNA-binding motifs of other characterized transcription factors (Tjian and Maniatis, 1994).

This study also showed that AEBP1 is a DNA-binding protease. Sequence comparisons and kinetic studies using known substrates, activators, and inhibitors have characterized AEBP1 as a member of the regulatory, B-like carboxypeptidase family. This family of proteases plays vital regulatory roles in the cell. These carboxypeptidases are involved in the release and processing of active peptides, alteration of receptor specificity for substrates, and the termination of polypeptide activity (Skidgel, 1988). The binding of DNA is a novel function for these regulatory-type carboxypeptidases.

The binding of an enzyme to DNA is not unprecedented. Two members of the general transcription machinery, other than the RNA polymerase II itself, have enzymatic activity. TFIIH is capable of binding DNA and contains helicase and ATPase activities, and a protein kinase activity responsible for the phosphorylation of the CTD region of RNA polymerase II (Roeder, 1996; Ohkuma and Roeder, 1994). These enzymatic activities are believed to be involved in promoter clearance and transcription elongation, which allows proper RNA polymerase function (Svejstrup et al., 1996). Likewise, TFIID has a serine kinase activity and may phosphorylate TFIIF (Orphanides et al., 1996). It is interesting that TFIID also contains a HMG box similar to the region of HMG 2 which interacts with AEBP1. Also, the transcription elongation factor ELL contains a methyltransferase-like domain which may be functional (Reines et al., 1996). DNA-binding proteins involved in DNA repair and nucleotide excision also contain a number of enzymatic functions including helicase, endonuclease, and ATPase activities (Drapkin et al., 1994). Other enzymes which bind DNA include the P-element transposase, which prevents RNA polymerase II complex assembly at the P-element promoter (Kaufman and Rio, 1991), and ADP-ribosyltransferase, which binds to its own promoter and acts as a negative modulator of its own gene (Oei et al., 1994).

Several proteases also bind to DNA, as described in section 1.VI.C.. For example, in the bacterium *Salmonella typhimrium*, the proline utilization operon is regulated by one of its products, PutA. PutA functions both as a membrane-associated protein with dual enzymatic activities, which are involved in the conversion of proline to glutamate, and also as a DNA-binding transcription repressor, which regulates its own *put* operon. When bound to the membrane, PutA functions as an enzyme, but in the absence of proline the protein accumulates in the cytoplasm and repress the *put* operon. The distribution of PutA is ultimately determined by the availability of proline, which is both an inducer of the *put* operon and a substrate for PutA. When PutA binds to proline (its substrate), the enzyme is incapable of binding DNA. However, in the

absence of proline, PutA binds to and represses the *put* operon (Muro-Pastor and May, 1995). It has been suggested that this may be a common mechanism by which membrane-associated enzymes regulate their own operons.

Another DNA-binding enzyme is bleomycin hydrolase (BH), a serine protease. Bleomycin hydrolase is an endogenous enzyme that cleaves bleomycin, a small glycometallopeptide produced by *Streptomyces verticillus* and is used as a anticancer drug because of its ability to cleave double-stranded DNA (Joshua-Tor et al., 1995). BH has been found to be a DNA-binding repressor in the yeast *GAL* regulatory system, and is designated there as Gal6. The BH/Gal6 protein binds DNA in what is described as an active protease conformation, indicating that the binding of DNA may be required for functional BH enzymatic activity. It is suggested that the protease activity may be involved in the ability of BH to repress transcription, although the potential substrates for the enzymatic activity of BH and the mechanism by which this repression occurs are not known (Joshua-Tor et al., 1995). The fact that the function of BH proteins is widely conserved from bacteria to humans (Joshua-Tor et al., 1995) evokes speculation that targeting proteases to DNA to repress transcription may be a regulatory mechanism conserved though evolution.

In this study, it is shown that, like bleomycin, the carboxypeptidase AEBP1 binds DNA in an active form. Unlike BH, DNA binding enhances the protease activity of AEBP1 and has been shown to be critical for its repression function, indicating that this protease directly inhibits transcription.

Like BH, AEBP1 is capable of binding DNA in a multimeric form. BH has been shown to bind DNA in what is described as a "trimer of dimers". This BH DNA-binding conformation, which is described as an essential conformation for enzymatic activity, allows potential substrates access to catalytic residues (Joshua-Tor et al., 1995). Gel mobility shift assay studies show that AEBP1 also binds DNA in multimeric forms. Although a monomeric form of AEBP1 also binds DNA, it is not

known if these multimers or monomers exist *in vivo*; nonetheless it is interesting to speculate on the functional significance of the multimerization of AEBP1. The formation of dimers, trimers, or tetramers may be a requirement for full carboxypeptidase activity of AEBP1 *in vivo*, a situation that would be similar to that of other carboxypeptidases, including CPN and CPH (Gebhard et al., 1989; Skidgel, 1988). Alternatively, multimerization may enhance DNA binding, a situation which would be similar to that of BH (Joshua-Tor et al., 1995) and other transcription regulators, including STAT proteins and hormone receptors (Sorgen and Nelson, 1989; Calkhoven and Ab, 1989; Mitchell and Tjian, 1989). Perhaps *in vivo* the formation of multimers regulates the DNA binding activity of AEBP1 and modulates its transcription repression function through unknown mechanisms.

Transcription repressors have been classified in two loosely defined categories, active or passive, dependent upon the ability of the regulator to directly influence the general transcription machinery (Jackson, 1991; Cowell, 1994; Johnson, 1995). Passive repression entails the prevention of activators from influencing transcription. This form of repression can occur through competition for the DNA binding sites of specific transcription factors, although competitive inhibition is one of the less common mechanisms, probably due to the complexity and the large number of regulatory elements found in the upstream promoter regions of eukaryote genes.

The second type of passive transcription regulation is termed 'masking' (Johnson, 1991). This type of repression involves the physical interaction of a repressor with an activator, ultimately leading to repression. One of the most compelling examples of this form of repression occurs in preadipocytes. Members of the C/EBP transcription regulator family, CHOP10 and LIP, are able to repress transcription by interfering with other C/EBP members (Vasseur-Cognet and Lane, 1993). CHOP can form heterodimers with C/EBPα and C/EBPβ, which masks the DNA binding domain of these C/EBP proteins. CHOP therefore acts as a dominant-

negative inhibitor of C/EBPα and C/EBPβ by preventing the heterodimers from binding DNA (Ron and Habener, 1992). LIP, on the other hand, can form heterodimers with a form of C/EBPβ, and in doing so masks the activation domain of C/EBPβ. This heterodimer is capable of binding DNA, but not capable of activating transcription (Descombes and Schibler, 1991).

Studies described here indicate that AEBP1 does not act through a passive transcription regulation mechanism. The CAT assays done by Dr. G.-P. He have shown that AEBP1 must be targeted to the AE-1 site to repress transcription (He et al., 1995). This observation alone does not indicate that AEBP1 represses transcription of the aP2 gene through an active mechanism, but other studies using heterologous promoters and *in vitro* transcription experiments do support an active repression mechanism. The fusion protein containing the Gal4 DNA-binding domain linked to AEBP1 was able to repress transcription when directed towards a promoter controlled by the Gal4 binding sites. This fact indicates that AEBP1 does not simply block activator binding to the AE-1 site or preventing specific activators from influencing transcription. These results also indicate that AEBP1 must be directed toward a specific DNA element, either AE-1 or Gal4-binding sites, to inhibit the general transcription machinery. Added to this evidence, the *in vitro* transcription assay using recombinant His-tagged AEBP1 further shows that when AEBP1 is directed to the AE-1 site it represses transcription. This repression was not seen when a DNA template without AEBP1-binding sites was used. These results indicate that the transcription repression was not due to masking of activators or to non-specific protein disruption of the RNA polymerase II general transcription machinery. The results taken together indicate that AEBP1 represses transcription by an 'active' mechanism.

Active transcription repression is defined as the direct down-regulation of transcription, rather than the inhibition of specific transcription activators (Cowell, 1994). Several transcription repressors utilize active repression mechanisms, including

the *Drosophilia* developmental proteins Kruppel, even-skipped, and engrailed, the human Kruppel-related factor, the Wilms tumour gene product WT1, the human bZIP protein, and the virally induced thyroid-hormone receptor v-erbA (Cowell, 1994, and references therein). This class of repressors would also include HMG 2 proteins, which inhibit RNA polymerase II by a direct interaction (Stelzer et al., 1994). The active repression mechanisms previously described have been through either anti-enhancement, whereby a repressor causes the occlusion of general transcription factors from the promoter region, or by locking the general transcription machinery in the initiation stages, thereby preventing elongation (Cowell, 1994; Johnson, 1995). Johnson (1995) described active repressors as proteins which provide a cell with the mechanism to turn off expression of a gene regardless of the number and nature of activators present. AEBP1 appears to be one of these molecules, one which functions through an exciting atypical active repression mechanism.

The evidence presented here suggests that AEBP1 represses transcription through an active mechanism whereby proteolytic activity is not only used but required. A number of observations suggest that the AEBP1 protease, DNA-binding, and repression functions are inseparable. First, when AEBP1 is bound to the AE-1 DNA its enzymatic activity is stimulated. Kinetic studies show that AEBP1 behaves like other B-like carboxypeptidases in terms of responses towards substrates, activators, and inhibitors. Unlike these B-like carboxypeptidases, AEBP1 is more active when bound to DNA. Studies on bleomycin hydrolase suggest that the BH enzyme is active when bound to DNA (Joshua-Tor et al., 1996) but no enzymatic characteristics of that or any other DNA-binding protein have been described.

Mutational analysis of AEBP1 shows that its carboxypeptidase activity is essential for its transcription regulation function. Eighteen mutations were made in the carboxypeptidase domain of AEBP1. In every case, if carboxypeptidase activity was knocked out, so was the ability to repress transcription. Likewise, if the mutant had

carboxypeptidase activity it was able to repress transcription. Possibly more revealing were the mutants which possessed reduced activities: whenever carboxypeptidase activity was reduced there was a reduction in repression. These results were not due an inability to bind to DNA, as all mutant proteins tested in the Gal4 CAT-assay system were fusion proteins containing the Gal4 DNA-binding domain. There is a possibility that the absence of both activities can be attributed to changes in the conformation or folding of the protein, although Western blot analysis showed that in each case the protein was being produced, and gel mobility shift assays showed that the fusion proteins bound to the Gal4-binding sites in labeled DNA.

The carboxypeptidase domain replacement experiment shows that enzymatic activity is indispensable for transcription repression. A fusion protein containing a carboxypeptidase domain similar to that of AEBP1 (CPM), the N-terminal discoidin-like domain of AEBP1, and the Gal4 DNA-binding domain was able to repress transcription when targeted to a promoter containing the Gal4-binding sites, indicating that when a fusion protein with carboxypeptidase activity is directed to DNA it is capable of repressing transcription. It may appear that the transcription repression observed was due to the discoidin-like domain, as all mutants possessed this domain. However, some mutants with only small deletions had both functions abolished even though this domain was present, and mutants with reduced functions also had an intact discoidin-like domain. These observation indicate that the discoidin-like domain alone was not responsible for the observed repression. However, it is possible that AEBP1 requires both carboxypeptidase activity and a discoidin-like domain to repress transcription.

Together these results demonstrate that the carboxypeptidase activity of AEBP1 is involved in its transcription regulation function. The active repression demonstrated by AEBP1 may occur through cleavage of an unidentified general transcription factor. Studies to determine potential substrates for this carboxypeptidase did not provide any

candidates, but did give insight into a possible mechanism by which this repression takes place.

The two-hybrid analysis showed that AEBP1 interacts with the high mobility group (HMG) 2 protein. HMG 2 is a common DNA-binding protein intimately involved in a number of DNA-related processes including chromatin assembly, replication, and transcription (Zwilling et al., 1995). Although the AEBP1–HMG 2 interaction has not been confirmed *in vivo* and there is no obvious indication of a function for this protein-protein interaction, it is interesting to speculate about the possible outcomes of this interaction. HMG 2 has been shown to enhance the DNA-binding capabilities of other transcription regulators including Oct1/2 (Zwilling et al., 1995), so its interaction with AEBP1 may localize AEBP1 to the AE-1 site and enhance DNA binding. Alternatively, HMG 2 may function as an adapter protein, bringing together AEBP1 and a member of the general transcription machinery such as TFIID (Orphanides et al., 1996), TFIIIB (Wang and Roeder, 1995), or any one of several transcription activators (Grosschedl et al., 1994, and references therein) which have HMG-box domains similar to that interacting with AEBP1. It is possible that these proteins, through their HMG-box domains, interact with and are substrates for AEBP1. Further study of the *in vivo* nature of this interaction, and the testing of HMG box domain proteins as substrates, may give insight into the mechanism of AEBP1 active repression.

Interestingly, the $G\gamma 5$ subunit of a trimeric G protein also interacts with AEBP1. In terms of *in vitro* regulation of transcription, this interaction appears to down-regulate AEBP1. Although it is difficult to imagine that this interaction takes place in the nucleus, recent evidence suggests that these two proteins may indeed interact in the nucleus. McFarlane-Anderson et al. (1993) showed that G proteins are translocated to the nucleus in adipocyte precursor cells, while studies by Crouch and Simson (1997) show that the α subunit and β subunit, and by implication the γ subunit,

of trimeric G proteins translocate to the nucleus in Swiss 3T3 L1 cells in response to stimulation by serum, and growth factors including EGF, and thrombin. This work allows the possibility that the γ subunit may be present in the nucleus, where it can influence AEBP1-mediated regulation of transcription.

This Gγ5–AEBP1 interaction has been shown to prevent AEBP1 from binding DNA, thus inhibiting AEBP1 from repressing transcription. Preventing a transcription regulator from binding DNA is a common inhibitory mechanism, termed 'masking' (Johnson, 1991). The masking of AEBP1's ability to bind DNA leads to direct inhibition of the enzyme's repression ability as judged by CAT and nuclear run-off assays. Masking may have important functional consequences, as it may allow a protein to remain in an inactive state until a transduced activation signal arrives, or may prevent a regulatory molecule from interacting with DNA under certain circumstances (discussed below in section 4.II. and III.). It either case, further elucidation of cellular localization of the Gγ5–AEBP1 may give insight into this novel form of AEBP1 regulation.

MAPK, another signaling molecule, may also regulate the transcription repression function of AEBP1. MAPK was shown to enhance the DNA-binding ability of AEBP1 to the AE-1 sequence. Gel mobility shift assays indicate that MAPK dissociates from AEBP1 once AEBP1 is bound to DNA, in a manner similar to HMG 2 enhancement of Oct1/2 DNA-binding ability (Zwilling et al., 1995) and Rb enhancement of C/EBP DNA-binding ability (Chen et al., 1996). However, it is possible that the MAPK–AEBP1 complex binds to the AE-1 sequence. Again, further elucidation of cellular localization should clarify the significance of the effect of MAPK enhancement of AEBP1 DNA-binding (discussed below in section 4.II. and III.).

The phosphorylation of AEBP1 by MAPK may also play an important role in the regulation of AEBP1. Several transcription factors are phosphorylated, resulting in a variety of regulatory effects. MAPK phosphorylation of PPARγ prevents PPARγ

from inducing adipogenesis through inactivation of its transcription activation function (Hu et al., 1996; Adams et al., 1997).

Another mechanism by which phosphorylation regulates transcription factors is demonstrated by the STAT proteins. STAT proteins are maintained in the cytoplasm in an inactive state; upon the activation of a receptor the STAT proteins are phosphorylated, which causes their translocation to the nucleus where they act as transcription activators. In particular, the phosphorylation of STAT1 and 5 is required for their subsequent translocation to the nucleus, and is required for the activation of certain adipogenic genes influenced by these STAT proteins (Stephens et al., 1996). Similarly, C/EBPβ is phosphorylated by protein kinase C within the leucine-zipper domain, which causes a conformational change that enhances the protein's selective DNA-binding and transcription activation function (Mahoney et al., 1992; Wegner et al., 1992). CHOP and C/EBPβ, members of the C/EBP family described above, are phosphorylated by the MAPK p38. CHOP is able to act as a dominant-negative regulator of C/EBPβ binding to one class of DNA targets, and as an activator by directing CHOP- C/EBPβ heterodimers to another class of DNA targets. The phosphorylation of CHOP enhances its activation abilities and is necessary for CHOP inhibitory effects on adipogenesis (Wang et al., 1996). These examples show a wide range of regulation of transcription factors attributed to protein phosphorylation.

Studies described here show that AEBP1 is phosphorylated by MAPK and suggest that the site of phosphorylation is the C terminus, the putative DNA-binding region. Phosphorylation in this region may enhance or inhibit the ability of AEBP1 to bind the AE-1 sequence, although non-phosphorylated protein is able to bind to DNA. In the C-terminal there is also the RRLQYRLRMREQMRLRR sequence, which may correspond to a nuclear localization signal (as indicated by the localization program found at http://psort.nibb.ac.jp/cgi-bin/okumura; personal communication by Park and Ro). Also, stretches of glutamic acid residues, similar to the stretch of glutamic acid

residues located in the C terminus of AEBP1, have been shown to act as a nuclear localization signal in other proteins (Boulikass, 1996). If AEBP1 is phosphorylated at MAPK consensus phosphorylation sites which are located in between the putative nuclear localization signal (RRLQYRLRMREQMRLRR) and the glutamic acid rich region, it may be possible that phosphorylation at this site may influence nuclear localization of AEBP1. It is possible that these nuclear localization signals may be masked by regions of AEBP1, ("intramolecular masking") or by other proteins ("intermolecular masking"), which may prevent AEBP1 from entering the nucleus. These types of nuclear localization masking mechanisms have been shown to prevent localization of other proteins (reviewed in Jans and Hubner, 1996). The phosphorylation by MAPK of AEBP1 in this particular region may overcome the proposed masking. AEBP1 may be signaled to translocate to the nucleus by a mechanism analogous to the MAPK phosphorylation of c-Jun (Jans and Hubner, 1996, see reference for review of nuclear localization regulation by phosphorylation).

Alternatively, the phosphorylation of AEBP1 may regulate its interaction with other proteins, including Gγ5. This is an attractive possibility as unphosphorylated recombinant His-tagged AEBP1 interacts with Gγ5, which prevents AEBP1 from binding to the AE-1 sequence. The phosphorylation by MAPK of AEBP1 may cause the disassociation of AEBP1 from Gγ5, thereby allowing AEBP1 to repress transcription.

Overall, the phosphorylation of proteins is a powerful signaling mechanism which may result in a variety of effects. AEBP1 may be phosphorylated by MAPK in the C terminus, the region involved in DNA-binding. Study of this phosphorylation event *in vivo* and the significance of the aforementioned protein interactions with AEBP1 will undoubtedly provide insight into the regulation of the transcription factor AEBP1.

4.II. The Role of AEBP1 in Cell Signaling

The studies presented in this thesis indicate that AEBP1 is regulated by, and interacts with, putative signaling molecules (Table 4.1). The nature of these protein-protein interactions is not clear, but these studies provide insight into possible *in vivo* functions. It is interesting to note that most of the proteins listed in Table 4.1 have been shown to be involved in the differentiation processes of a variety of cell types, and most have been shown to be localized to both the cytoplasm and nucleus, indicating a role for AEBP1 in signaling pathways.

MAPK, the first protein listed in Table 4.1, is known to play a pivotal role in the signaling pathways which determine whether a cell differentiates or proliferates (Marshall, 1995). MAPK regulates a growing number of transcription factors which control genes involved in these processes. MAPK translocates to the nucleus in response to stimulation by serum (Gonzalez et al., 1993), growth factors (Lenormand et al., 1996), or insulin (Kim and Kahn, 1997) of fibroblasts and 3T3 L1 preadipocytes. Depending upon the receptor used to signal the activation of MAPK, the kinase experiences either transient or prolonged activation (Marshall, 1995). The consequences of prolonged or transient MAPK activation also vary in different cell types. Alessi et al. (1995) and Grammer and Blenis (1997) have shown that the MAPK activator MEK is quickly down-regulated upon MAPK activation, and in this situation MEK is not translocated to the nucleus. This finding indicates that once MAPK is activated it is regulated by phosphatases which inactive the kinase. The fact that AEBP1 interacts with and is phosphorylated by MAPK indicates that AEBP1 may be involved in and regulated by a signal-transduction pathway involving MAPK.

Protein	Interaction Studies in this Thesis	Cellular Localization	Involvement in Differentiation (not necessarily adipogenesis)
MAPK	co-IP, gel-filtration, and GMSA	Cytoplasm, focal adhesion and nucleus	yes
Gγ5	Two-hybrid, co-IP, and GMSA	Plasma membrane, focal adhesion, possibly nucleus?	yes
HMG 2	Two-hybrid	Nucleus and cytoplasm	yes
HSP27	Two-hybrid	Nucleus, cytoplasm, and actin-associated	yes

Table 4.1. Proteins which interact with AEBP1

co-IP indicates co-immunoprecipitation studies, and GMSA indicates gel mobility shift assay.

Studies by Kim and Ro (unpublished data) indicate that prolonged activation of MAPK results in the inhibition of adipogenesis by 3T3 L1 preadipocytes. The *in vivo* reasons for this activation of MAPK are no doubt complex, but *in vitro* studies show that AEBP1 may be directly responsible for this prolonged MAPK activity. *In vitro* protection assays shows that AEBP1 protects MAPK from phosphatase activity. Thus in 3T3 L1 cells overexpressing AEBP1 the prolonged activation of MAPK and inability of these cells to differentiate may be due to AEBP1 preventing MAPK inactivation by phosphatases.

Further evidence for the MAPK–AEBP1 complex being more than a casual kinase-substrate transient interaction comes from coimmunoprecipitation experiments. These experiments show that AEBP1 interacts with both the active (phosphorylated) and inactive (dephosphorylated) forms of MAPK. If AEBP1 only interacted with MAPK when the kinase phosphorylated AEBP1, the inactive, dephosphorylated MAPK would not be expected to coimmunoprecipitate with AEBP1 from quiescent 3T3 L1 preadipocytes.

Added to this evidence are gel-filtration experiments which show that MAPK co-elutes with AEBP1 in a large complex from quiescent and 10-minute serum- and insulin-stimulated cells, and in a smaller complex after 30 minutes of stimulation. Neither complex was observed after 2 hours of stimulation. This evidence does not show that the proteins are present in the same complex and does not give insight into a possible functional role for these complexes. However, it is interesting to speculate about the significance of these proposed complexes containing AEBP1 and MAPK.

The larger complex may be cytosolic and contain both AEBP1 and MAPK, along with other proteins. Upon receptor tyrosine kinase activation, AEBP1 and MAPK may be disassociated from the larger complex and then translocated to the nucleus in what appears as the smaller complex. In the nucleus AEBP1 inhibits transcription of differentiation induced genes such as aP2 and MAPK inactivates

adipogenic proteins such as PPARγ, and activates other proteins required to maintain the preadipocyte phenotype. Once the MAPK–AEBP1 complex enters the nucleus the interaction of AEBP1 with the AE-1 sequence may cause the disassociation of the complex, as the two proteins do not elute together after 2 hours insulin- and serum-stimulation. Continued stimulation with serum, insulin, dexamethasone, and 1-methyl-3-isobutylxanthine may cause the inactivation of both AEBP1 and MAPK through the activation of phosphatases, therefore allowing adipogenesis to be initiated. Alternatively, stimulation with these agents may cause only transient activation of MAPK, and in this case the MAPK–AEBP1 complex will not be translocated into the nucleus and the aforementioned effects would not be observed. The possible translocation of AEBP1 with MAPK to the nucleus may enhance the DNA-binding activity of AEBP1, leading to the activation of its role in the repression of adipogenesis. Determination of cellular localization of AEBP1 will be vital in determining its role in the initiation of adipogenesis.

As AEBP1 was also found in gel-filtration fractions not containing MAPK, the protein probably exists in complexes with other proteins. Gγ5, the second protein listed in Table 4.1 as interacting with AEBP1, is also an important and well-characterized signaling molecule. This protein is a subunit of the trimeric G proteins, which are activated by ligand binding to G protein coupled receptors (GPCR). The binding of hormones or neuropeptides to GPCRs causes conformational changes in trimeric G proteins which facilitate the exchange of GDP with GTP on the α subunit. The active GTP-bound α subunit then dissociates from the βγ heterodimer, leaving both molecules free to act on effector molecules. This particular γ subunit, Gγ5, is interesting for a number of reasons. It is one of a few γ subunits which is found to have ubiquitous tissue distribution, and the only one to be localized in the area of focal adhesion (Hansen et al., 1994).

These studies show that AEBP1 interacts with the $\gamma 5$ subunit and suggest that AEBP1 complexes with the $\beta\gamma 5$ heterodimer in 3T3 L1 preadipocytes, but the nature of this interaction is unclear. As discussed in section 4.I., this interaction *in vitro* prevents AEBP1 from binding to DNA, thus inhibiting the transcription repression function of AEBP1. The cellular location of this proposed protein-protein interaction is unclear, but preliminary studies by Park and Ro (unpublished results) indicate that $G\gamma 5$ is localized in the nucleus in 3T3 L1 preadipocytes. It is reasonable to suggest that $G\gamma 5$ is localized in the nucleus, as other G proteins have been found to be localized to the nucleus in cells capable of differentiating into adipocytes (McFarlane-Anderson et al., 1993). This possibility is further strengthened by the fact that the G protein G_i is translocated to the nucleus in Swiss 3T3 fibroblasts in response to serum, growth factors, and GPCR agonists. It is believed that all subunits of this trimeric G protein, including the γ subunit, are translocated to the nucleus, where they may play regulatory roles (Crouch and Simson, 1997). These results suggest a novel and exciting role for the γ subunit within the cell. In the nucleus it is possible that $G\gamma 5$ acts as a negative regulator of AEBP1 inhibiting AEBP1 in its regulation of transcription. If indeed these results hold true, it would be interesting to determine if $G\gamma 5$ is translocated to the nucleus in response to adipogenic inducers, where it may inhibit AEBP1 in its repression role. Park and Ro (unpublished data) have shown an increase in the abundance of $G\gamma 5$ but not AEBP1 in the nucleus in response to adipogenic inducers. Similarly, characterization of the functional interaction domains of both $G\gamma 5$ and AEBP1 may give insight into the mechanism by which this interaction takes place.

Although AEBP1 may be localized only to the nucleus, other cellular localization for this protein cannot be ruled out. Therefore it is important to discuss a possible cytosolic interaction of the $G\gamma 5$–AEBP1 complex. Formation of a complex consisting of AEBP1 with both the $\beta\gamma 5$ heterodimer and MAPK is reminiscent of the interaction involved in the yeast pheromone response scaffold. In the yeast system, the

binding of a pheromone to its GPCR causes the dissociation of a trimeric G protein. The βγ heterodimer localizes the homodimerized scaffold protein Ste5, which interacts with the protein kinases PAK, MEKK, MEK, and two MAPKs (Whiteway et al., 1996: Yablonski et al., 1996; Leberer et al., 1997). As both GPCRs (Shinohara et al., 1991; Uehara et al., 1994) and receptor tyrosine kinases activate MAPK (Porras et al., 1994; Porras and Santos, 1996) are involved in the initiation of adipogenesis, it is interesting to speculate that in adipogenesis, as in yeast pheromone signaling, a scaffold protein is required for the transduction of signals which ultimately lead to the initiation of differentiation. The Gβγ5 heterodimer may be involved in localizing AEBP1 and MAPK to the plasma membrane in response to adipogenic agents, or may localize these proteins to the plasma membrane where they remain in an inactive state, only to be translocated in response to various stimuli.

Several mammalian scaffold proteins have been recently discovered, including the A-kinase anchoring protein AKAP79 (Klauck et al., 1996), and the "protein targeting for glucose" PTG protein, which is found in 3T3 L1 adipocytes (Printen et al., 1997). Therefore it is not unrealistic that a scaffolding protein may function in adipogenesis. The employment of a scaffold protein in the initiation of adipogenesis would seem to be advantageous. The initiation of adipogenesis involves signals for three seemingly independent signaling pathways. Insulin acting through receptor tyrosine kinase, along with glucocorticoid-dependent and cAMP-dependent pathways, have all been shown to be necessary for adipogenesis (Cornelius et al., 1994). A scaffolding protein could bring together molecules from different signaling pathways and focus these signals to quickly and decisively initiate adipogenesis. Once this proposed signaling complex is formed, the transduced signal may overcome the inhibition of adipogenesis by proteins which maintain the preadipocyte phenotype.

Further study of the cellular localization of AEBP1 and Gγ5 through immunocytochemistry and sub-cellular fractionation at different stages of adipogenesis

may provide important insight into the role of AEBP1. Showing that MAPK and AEBP1 directly interact, and the determination of the domains involved in the AEBP1–Gγ5 and AEBP1–MAPK complexes, may help indicate an *in vivo* role for these protein-protein interactions.

Bem1p, a yeast protein which interacts with the scaffold protein Ste5, appears to also function as a scaffold involved in the pheromone response. Haploid yeast cells have developed the ability to direct mating-specific protrusions towards the pheromone source. This polarized cell growth is mediated by cytoskeletal elements. The Bem1 protein interacts with Ste5p, Ste20p, Cdc24p, Far1p, and actin, and may be involved in the morphogenesis involved in this response. It has been suggested that the formation of this complex is crucial for the activation of downstream signaling pathways and the transduction of signals from GPCRs to the cytoskeleton (Leeuw et al., 1995; Leber et al., 1997, and references therein).

The differentiation process in 3T3 L1 preadipocytes also involves morphological changes, including the loss of stress fibers (Novikoff et al., 1980) and the transcriptional down-regulation of actin and tubulin genes (Cornelius et al., 1994). As HSP27, the fourth protein listed in Table 4.1, is involved in actin polymerization and cytoskeletal assembly (Zhu et al., 1994; Lavoie et al., 1995) it is interesting to speculate on the possible function of the AEBP1–HSP27 interaction. AEBP1 may be involved in the signaling of cytoskeletal changes which occurs during adipogenesis. AEBP1 and HSP27 may be involved in a complex that signals the morphological changes involved in differentiation, as the actin protection attributed to HSP27 would have to alleviated. Given that Gγ5 also has been shown to be localized in the area of focal adhesion, a cellular region involved in cytoskeletal regulation and cell signaling (Hansen et al., 1994), and that HSP27 and MAPK have been shown to colocalize from a membrane fraction to a cytosolic fraction in response to activated GPCRs (Yamada et

al., 1995), it is interesting to speculate on a possible significance of these observation in terms of a possible role for AEBP1.

AEBP1 may be a member of a large protein complex which includes MAPK, Gγ5, HSP27, and other proteins. The proteins in this complex may be localized in the region of focal adhesion upon activation of GPCRs, depending on the nature of the activating signals. Upon the sustained stimulation by an adipogenic stimulus, proteins in the complex may dissociate, ultimately leading to the morphological changes associated with adipogenesis. Further study into the existence of this proposed complex may answer a number of questions concerning the transduction of signals involved in cytoskeletal rearrangements which occur during adipogenesis.

Finally, HMG protein, the third protein listed in Table 4.1, have also been shown to play a role in cellular differentiation. HMG proteins have been shown to be rapidly translocated to and accumulated at the cell surface in a membrane associated fashion upon induction of differentiation in murine erythroleukemia (MEL) cells (Passalacqua., 1997). The release of this protein from the nucleus to the cell surface accelerates differentiation in MEL cells, while disruption of this translocation inhibits differentiation, indicating that the release of HMG proteins plays an important role in differentiation of MEL cells. Although there is no obvious connection between HMG 2 and adipogenesis, further study into this phenomenon may prove interesting and may provide insight into the regulation of adipogenesis. It would be interesting to determine if HMG proteins are also translocated from the cytoplasm to the nucleus in preadipocytes during differentiation and, if so, the purpose for HMG protein interaction with membrane receptors. If the interaction of HMG 2 with AEBP1 occurs *in vivo*, it would be interesting to determine what regulatory purpose this interaction serves, both for the individual proteins and for adipogenesis. Cellular localization studies for both these proteins in 3T3 L1 preadipocytes may give insight into the possible function of this interaction.

4.III. The Role of AEBP1 in Adipogenesis

Adipogenesis is a complex process involving a number of stages, each requiring the coordinated expression of numerous genes and the activation or inactivation of numerous proteins. For the adipoblast 3T3 L1 cell line to differentiate a number of requirements must be met. Once these preadipocytes have ceased dividing at confluence they can be induced to differentiate by hormonal and mitogenic agents. The cells then undergo a number of rounds of mitotic clonal expansion, and DNA replication may alter the accessibility of promoter elements to *trans*-acting factors that activate or derepress regulatory genes required for the initiation of differentiation (Cornelius et al., 1994). One of these proteins activated during differentiation is the aP2 gene, which encodes the adipocyte fatty acid-binding protein.

These studies have focused on AEBP1, a protein which is down-regulated during adipogenesis in 3T3 L1 preadipocytes (He et al., 1995), and showed that AEBP1 is the preadipocyte-specific negative regulator of the aP2 gene described by Ro and Roncari (1991).

The aP2 gene contains consensus binding sequences for the following transcription activators: the c-Jun and c-Fos heterodimer (Distel et al., 1987), C/EBPα (Herrera et al., 1989), and PPARγ (Tontonoz et al., 1994a/b). These well-characterized transcription regulators activate the aP2 gene, as well as other genes regulated during adipogenesis, but the question of how the aP2 gene is maintained in a repressed state in preadipocytes has yet to be answered. Ro and Roncari (1991) have shown that a negative transcription regulator is involved in the maintenance of this aP2 gene in the preadipocyte state. This study shows that AEBP1 is indeed this negative regulator. Furthermore, AEBP1 uses a novel mechanism by which it imposes this repression. These studies have shown that AEBP1 uses a protease activity in its repression function

and a DNA-binding region to interact with the AE-1 site located at the proximal nucleotides -159 to -125 in the promoter region of the aP2 gene. Although the mechanism by which AEBP1 represses the aP2 gene is novel, the outcome of this repression is also important in regulating adipogenesis, for the activation of the aP2 gene has been shown to be a key component of adipogenesis.

Repression of the aP2 gene in the preadipocyte state may prevent activation by factors such as cAMP (Yang et al., 1989) and c-Jun and c-Fos (Herrera et al., 1989), which are known to activate this gene during differentiation. The active form of repression of aP2 exerted by AEBP1 ensures that this gene is not activated by activators, which might otherwise overcome repression caused by passive repressors which only compete for the DNA-bindings sites or mask activators. During circumstances which cause the activation of adipogenic signaling pathways but are not sufficient to initiate differentiation, AEBP1 may prevent the inappropriate stimulation of adipogenic genes. AEBP1, by binding to the AE-1 site and using a protease function to actively repress the general transcription machinery, may provide tight negative regulation of this important adipogenesis induced gene. The negative regulation attributed to AEBP1 may be overcome by C/EBPα, which also binds to the AE-1 site, or by PPARγ, which binds in the distal promoter region of the aP2 gene, ultimately leading to differentiation.

The fact that AEBP1 and C/EBP proteins both bind to regions in the AE-1 site points to an intriguing possibility that AEBP1 may be able to bind to the C/EBP consensus binding site and act as global negative regulator of a number of proteins involved in adipogenesis, similar to the proposed function of c-Myc regulation of C/EBP-dependent genes in several cell types (Mink et al., 1996). C/EBP-binding sites have been found in the promoters of adipogenic genes including PPARγ (Zhu et al., 1995) and C/EBPα (Christy et al., 1991), as well as the adipocyte-specific genes stearoyl-CoA desaturase (Christy et al., 1989) and the mouse obese (ob) gene (Hwang

et al., 1996; Hollenberg et al., 1997). AEBP1 may bind to the sequence TTTCT in the AE-1 sequence (Ro, personal communication). The promoters of C/EBP and PPARγ both have several copies of this sequence in their proximal promoter region (Zhu et al., 1995; Fakuoka et al., 1997). If AEBP1 is able to bind to the proximal promoters of these genes, AEBP1 may act as a 'master' negative transcription regulator in the maintenance of adipogenic genes in a preadipocyte state. Further study into the possible regulation by AEBP1 of other genes containing AEBP1-binding sites is being carried out, and may give invaluable insight into regulation of the genes known to be responsible for adipogenesis.

The phosphorylation of AEBP1 by MAPK, the interaction of AEBP1 with MAPK, the two-hybrid interaction of AEBP1 and Gγ5, and the 3T3 L1 cell lines over expressing AEBP1as developed by Kim and Ro all indicate that AEBP1 is involved in signal-transduction pathways of preadipocytes. When 3T3 L1 cells constitutively overexpressing AEBP1 are induced to differentiate by the standard protocol, differentiation is blocked. These effects may be attributed to the binding of AEBP1 to adipogenic genes which contain AEBP1-binding sites as previously discussed, but this hypothesis does not explain the prolonged activation of MAPK observed in these cells.

In 3T3 L1 cells, sustained activation of MAPK is known to inhibit adipogenesis, partially through the phosphorylation and inactivation of PPARγ (Hu et al., 1996; Adams et al., 1997). Furthermore, in fibroblasts and preadipocytes, MAPK translocates to the nucleus in response to stimulation by serum (Gonzalez et al., 1993), growth factors (Lenormand et al., 1996), or insulin (Kim and Kahn, 1997). Therefore, in 3T3 L1 cells prolonged MAPK activity appears to induce cell proliferation and inhibits adipogenesis, but activation of MAPK is a requisite for adipogenesis (Porras et al., 1995; Sale et al., 1995; Porras and Santos, 1996). Along these same lines, studies by Alessi et al. (1995) have shown that stimulation of a particular receptor, such as the EGF receptor, in different cell types has profoundly different effects on the duration of

MAPK activation. Stimulation of similar receptor tyrosine kinases within the same cell also may cause drastic differences in the duration of MAPK activation (Peng et al., 1995; Marshall, 1995). These differences appear not to be the result of MEK activation but rather stem from a MEK-independent pathway through which the MKP phosphatases which inactivate MAPK both in the cytoplasm and nucleus are regulated (Alessi et al., 1995; Brondello et al., 1995 and 1997; Grammer and Blenis, 1997).

These results indicate that several complicated factors determine the temporal activation of MAPK and the subsequent outcomes of MAPK activation. Studies of 3T3 L1 preadipocytes overexpressing AEBP1 indicated that prolonged activation of MAPK inhibits adipogenesis in this cell line (Kim and Ro, unpublished data). Studies described here suggest an *in vitro* reason for this inhibition. AEBP1 protects activated MAPK from phosphatase-mediated inhibition, allowing MAPK to phosphorylate MBP in an *in vitro* assay. If similar protection of MAPK occurs *in vivo*, then this AEBP1 interaction with MAPK may sustain MAPK activation, thus further inhibiting adipogenesis through the phosphorylation of endogenous PPARγ.

Support for this *in vitro* interaction comes from gel-filtration studies, which show that AEBP1 and MAPK co-elute in two distinct complexes. In quiescent 3T3 L1 and 10-minute serum-and insulin-stimulated cells, AEBP1 and MAPK are found to co-elute in a large complex. After 30 minutes of stimulation, AEBP1 and MAPK co-elute in a smaller complex, and neither complex is found after a 2-hour stimulation. These results may be explained by a translocation event whereby AEBP1 and MAPK interact in a large cytosolic complex, which upon stimulation dissociates, allowing the AEBP1–MAPK complex to be translocated to the nucleus.

There are undoubtedly numerous factors *in vivo* which ultimately decide if the cell differentiates, but the AEBP1–MAPK activation documented here may allow protection of MAPK from phosphatases, particularly MKP-3, which is known to down-regulate cytosolic MAPK thus preventing the kinase from translocating to the

nucleus (Brondello et al., 1995, 1997). If this protection occurs there would be a two-fold inhibition of adipogenesis and maintenance of the preadipocyte attributed to AEBP1. AEBP1 may enhance MAPK inhibition of differentiation as well as repress transcription of adipogenic induced genes. It is also interesting that gel mobility shift assays show that MAPK enhances AEBP1 DNA-binding ability, again indicating that these two proteins may act in synergy to inhibit differentiation.

Further study into the interaction of AEBP1 and MAPK may provide important insight into the regulation of adipogenesis. Most importantly, cytoimmunochemistry experiments and sub-cellular fractionation will determine where AEBP1 is localized, and show if AEBP1 and MAPK colocalize in preadipocytes, and in these cells after stimulation provided by growth factors, serum, and insulin. These studies may determine if the *in vitro* protection of MAPK from phosphatase activity attributed to AEBP1 occurs *in vivo*, and if MAPK enhances the DNA-binding ability of AEBP1 *in vivo*. The establishment of a 3T3 L1 cell line overexpressing AEBP1 provides a powerful tool for the study of AEBP1. The aforementioned cytoimmunochemistry and sub-cellular fractionation experiment with these particular cell lines may give further understanding into the cellular role of AEBP1.

Studies by Wang et al. (1993), Shinohara et al. (1991), McFarlane-Anderson et al. (1993) Uehara et al. (1994), and Wang and Malbon (1996) have shown that trimeric G proteins are involved in adipogenesis. The function of the *in vivo* interaction of AEBP1 and the $\gamma 5$ subunit of a trimeric G protein is unknown. *In vitro* studies show that $G\gamma 5$ was able to prevent AEBP1 from binding DNA, and inhibits the AEBP1 transcription repression function. Studies by McFarlane-Anderson et al. (1993) have shown that G proteins are present in the nucleus of cells which are capable of differentiating to adipocytes, and Crouch and Simson (1997) have shown that the α subunit, β subunit, and most likely also the γ subunit, are translocated to the nucleus in fibroblast cells upon sustained activation of receptor tyrosine kinase and G protein

coupled receptors. Preliminary studies by Park and Ro also indicate that the γ5 subunit is localized to the nucleus in 3T3 L1 preadipocytes.

Therefore it is possible that the AEBP1–Gγ5 interaction takes place in the nucleus. If this is the case, a novel mechanism of transcription regulation would take place. In the preadipocyte state, AEBP1 may repress the transcription of adipogenic genes through its protease activity. Upon GPCR activation by adipogenic agents, Gγ5 may be translocated to the nucleus, where it interacts with AEBP1 and prevents inhibition of adipogenesis-specific genes. This form of transcription regulation would be novel and interesting. Although Crouch and Simson (1997), McFarlane-Anderson (1993), and others have observed G protein translocation to the nucleus, a physiological function for this translocation has not been identified.

Alternatively, as discussed in section 4.II., a cytoplasmic AEBP1–Gγ5 interaction cannot be ruled out. This interaction may be involved in bringing together proteins involved in cell signaling, including MAPK and HSP27. A number of mammalian scaffold proteins have been identified (Klauck et al., 1996; Printen et al., 1997), indicating that the formation of scaffolds around architectural proteins may be an important mechanism in controlling complex cellular functions. The fact that AEBP1 interacts with MAPK, Gγ5, and HSP27 suggests that the formation of a large complex may occur in preadipocytes, and that this complex may play a role in adipogenesis. Further study into the interaction of these proteins with AEBP1, including cyto-immunochemistry and sub-cellular fractionation, may provide insight into the possible formation of complexes with AEBP1. Tracking sub-cellular localization of these proteins during different stages of adipogenesis may clarify the function of these protein-protein interactions.

In summary, AEBP1 has been identified as a preadipocyte-specific negative regulator of the aP2 gene. The C-terminal region of AEBP1 is capable of binding to the AE-1 promoter region of the aP2 gene. Once AEBP1 is bound to DNA, an inherent

carboxypeptidase activity is stimulated. This increased protease activity is required for active transcription repression of the aP2 gene mediated by AEBP1.

Interaction studies have shown that AEBP1 interacts with MAPK, Gγ5, HSP27, and HMG 2. The interaction of Gγ5 with AEBP1 *in vitro* prevents AEBP1 from binding to the AE-1 sequence, which inhibits the transcription repression function of AEBP1. The *in vitro* interaction of AEBP1 and MAPK enhances the DNA-binding ability of AEBP1, and protects MAPK from phosphatases. Furthermore, it appears that MAPK phosphorylates AEBP1 in the C terminus *in vitro*. The interactions of AEBP1 with MAPK and Gγ5 have been confirmed by coimmunoprecipitation studies.

Further study into the mechanism by which AEBP1 actively represses transcription, and the determination of other genes regulated by AEBP1, will provide insight into the regulation of adipogenesis, and into the general mechanism by which proteins are capable of repressing transcription. Similarly, study of the proteins found to interact with AEBP1 may provide insight into the mechanism by which AEBP1 is regulated. Determination of the consequences of AEBP1 phosphorylation by MAPK may reveal important insight into this regulation.

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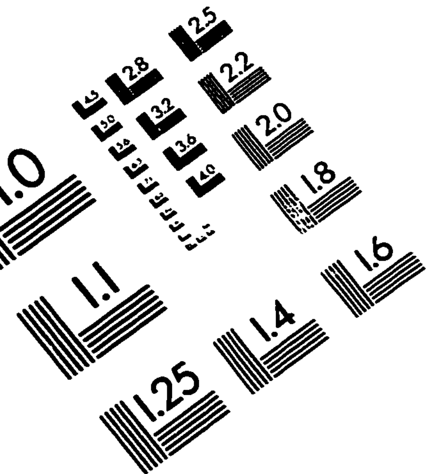
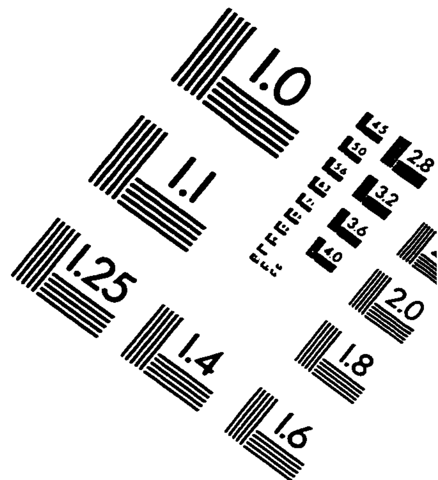
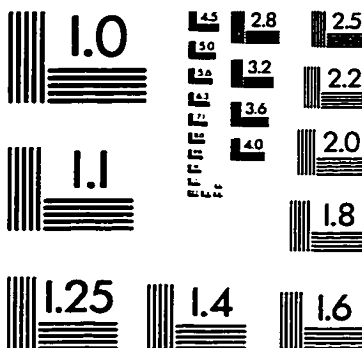
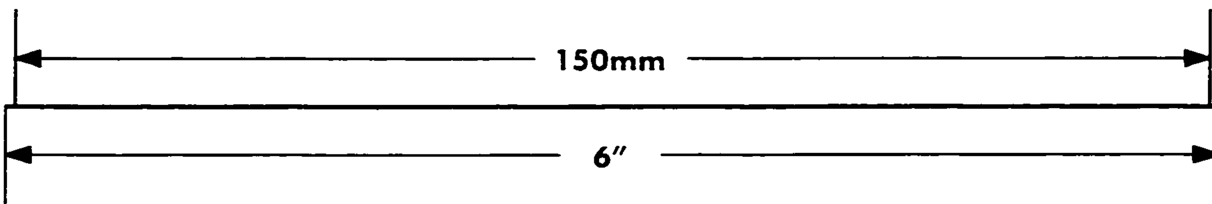
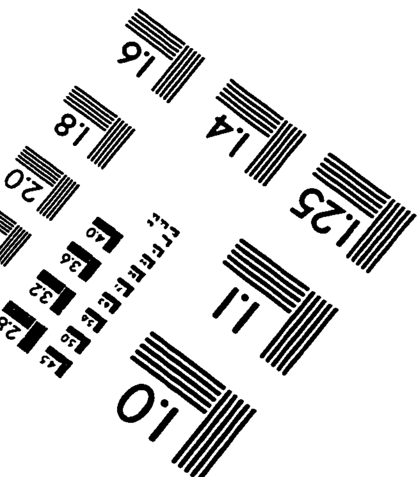
IMAGE EVALUATION TEST TARGET (QA-3)



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