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GENOMIC AND FUNCTIONAL CHARACTERIZATION OF CASP

by

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

at

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ABSTRACT

The function of specialized cells of the immune system, including lymphocytes, is dependent in part on the expression of cell-specific genes. Subtractive hybridization was previously used in our laboratory to identify natural killer (NK)/T lymphocyte specific transcripts. One partial cDNA isolated by this method and recently named CASP (cytohesin associated scaffolding protein), codes for a protein with at least two protein interaction domains, an N-terminal PDZ and a central coiled coil domain. CASP also contains a novel C-terminal domain of unknown function. CASP's domain profile suggests it may serve as an adaptor protein involved in organizing the higher architecture of a lymphoid-specific signaling complex. CASP was characterized at the genomic, transcriptional, and functional protein levels. Full length CASP cDNA was isolated by 5' rapid amplification of cDNA ends (5'RACE), identifying a final 1077 base open reading frame that codes for a 40 kDa CASP protein. The entire CASP gene spanning 29 kilobases was cloned and partially sequenced, revealing an 8 exon/7 intron gene structure. Cloning and sequencing of an additional 4 kilobases of upstream sequence revealed that the CASP promoter region lacks a conventional TATA box but contains binding sites for a number of lymphocyte-specific transcription factors. In accordance with these findings, CASP can be transcriptionally activated in the T cell line Jurkat by T cell receptor (TcR)-mediated signals in an early fashion (3 hours). TcR-mediated CASP activation proceeds through classical immediate TcR signaling pathways, including conventional protein kinase C (PKC) and the mitogen-activated protein kinases (MAPK) ERK (extracellular signal-regulated kinase) and p38. Furthermore, CASP activation is inhibited by the protein synthesis inhibitor cyclohexamide, suggesting a requirement for the *de novo* synthesis of unidentified transcription factors.

Yeast two-hybrid screening of a B cell library identified a CASP interaction with cytohesin, a guanine nucleotide exchange factor (GEF) for the small GTPases of the ADP ribosylation factor (ARF) family. ARF and cytohesin have been implicated in the control of vesicle trafficking in the Golgi and the regulation of endocytosis and actin rearrangement at the plasma membrane. CASP binding to the N-terminal coiled coil of cytohesin was confirmed *in vitro* and in COS-1 cells. The specificity of CASP's coiled coil is not restricted to cytohesin, however, since it is also capable of interacting with other members of the cytohesin/ARNO family, ARNO and ARNO3. CASP localizes to perinuclear tubulo-vesicular structures that are in close proximity to the Golgi. In epidermal growth factor (EGF)-stimulated COS-1 cells over-expressing cytohesin and CASP, cytohesin recruits CASP to membrane ruffles, revealing a functional interaction between the two proteins. These observations collectively suggest that CASP is a scaffolding protein that facilitates the function of at least one member of the cytohesin/ARNO family in response to specific cellular stimuli, either at the cell periphery or at the level of the Golgi. The inducible and cell type-restricted expression of CASP, suggest it may regulate a lymphoid-specific aspect of vesicular trafficking.

LIST OF ABBREVIATIONS

a.a.	amino acid
AP1	Activator Protein 1
AP-1	Adaptor Protein-1
ARF	ADP Ribosylation Factor
ARNO	ARF Nucleotide-binding site Opener
BFA	Brefeldin A
CASP	Cytohesin associated scaffolding protein
CD	Cluster Determinant
CMV	Cytomegalovirus
COP	Coat Protein
DAG	Diacyl glycerol
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
ERK	Extracellular signal-Regulated Kinase
GEF	Guanine nucleotide Exchange Factor
GRP1	general receptor for phosphoinositides 1
IL-2	Interleukin 2
LZ	Leucine Zipper
MAPK	Mitogen Activated Protein Kinase
NF-AT	Nuclear Factor of Activated T cells
NK	Natural Killer
PDZ	Postsynaptic Density-95, Discs Large, protein, Zonula Occludens
PH	Pleckstrin Homology
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol Myristate Acetate
PtdIns	Phosphatidylinositol
RACE	Rapid Amplification of cDNA Ends
STAT	Signal Transducers and Activators of Transcription
TcR	T cell receptor

1. GENERAL INTRODUCTION

1.1. CASP GENE OVERVIEW

CASP cDNA, originally called B3-1, was cloned in our laboratory as part of a project aimed at identifying Natural Killer (NK)-specific genes. A method of subtractive hybridization was used to identify genes expressed in resting human NK cells purified from blood. Subtractive hybridization was commonly used to identify cell type-specific genes [1, 2] before the development of PCR-mediated differential display techniques. In this approach, two closely related, but functionally different, cell types must be used. In brief, common housekeeping genes are removed, leaving only cell-specific mRNA species that can then be cloned and characterized. This method is advantageous since it allows the detection of weakly expressed genes. Unlike differential display methods capable of detecting subtle differences in expression levels of a certain gene, subtractive hybridization can only identify genes expressed in target cells if they are not expressed in the reference cells. When generating the NK subtractive cDNA library, resting Jurkat cells were used as a reference. Jurkat cells, with T helper-like characterictics, are somewhat related to NK cells but clearly distinct on a functional level. All genes isolated by the subtractive hybridization procedure, including CASP, are inherently expressed in NK cells but not in resting Jurkat cells.

The lymphoid-specific expression of CASP was determined by northern analysis of tissue/organ-specific cell lines and a number of purified cell types [3]. More specifically, CASP was detected at low levels in purified NK/ T cells, but not in resting circulating B cells or monocytes. Since isolated NK cells had a typical purity of 70% and were always contaminated with T cells, we concluded that CASP expression was NK/T cell-specific. Recent EST database searches, however, suggest the expression of CASP in other cell types such as CD34+ stem/progenitor cells, germinal center B cells, and activated T cells, as well as a number of cancers including adenocarcinoma, embryonal carcinoma, myeloma, melanoma and lymphomas. Furthermore, CASP was recently isolated by yeast two-hybrid screening of a differential expression dendritic cell library

(unpublished). Although the cell distribution of CASP is broader than we originally thought since it is expressed in a number of cancer cell types and multiple cell types of hematopoietic origin.

The original CASP cDNA consisted of 1724 bases and had an open reading frame (ORF) of 972 bases coding for a 324 amino acid (a.a) protein with an estimated molecular weight of 36 kDa. The proposed CASP start codon did not conform to a Kozak consensus [4, 5], suggesting that longer CASP transcripts containing another upstream start codon may exist. This appeared to be the case, since ESTs, later cloned and sequenced as part of the human genome mapping efforts, showed the presence of another ATG 108 bases upstream of the original start codon. This was confirmed by our own 5'-RACE (rapid amplification of cDNA ends) studies, yielding a final open reading frame (ORF) of 1077 bases coding for a 359 a.a. protein with an estimated molecular weight of 40 kDa. Both start codons were capable of efficiently initiating translation when CASP cDNA was transfected in eukaryotic cells under the control of the cytomegalovirus (CMV) promotor. It remains unclear which start codon is preferentially used in normal cells.

Sequence analysis of CASP cDNA revealed the presence of three ATTTA sequences in the 3' untranslated region. These sequences infer mRNA instability and are responsible for the short half life of a number of proto-oncogene and cytokine/lymphokine transcripts [6, 7]. These sequences are important for tightly controlling the levels of regulatory proteins and may explain the low levels of CASP detected in normal NK/T cells.

Initial analysis of CASP's deduced protein sequence revealed the presence of a central coiled coil motif consisting of an alpha helix with similarity to leucine zipper (LZ) domains. LZ domains consist of a stretch of approximately 35 residues containing 4-6 leucines separated by 6 amino acids [8]. LZ domains, typically found in a variety of transcription factors, form an alpha helix with the leucines aligned on one side of the helix. The interdigitation of the leucine residues of two LZ domains forms the basis of LZ-mediated protein/protein interactions. Interestingly, CASP's coiled coil was long enough to encompass two overlapping leucine zipper domains [3]. CASP also harbored a

putative nuclear targeting sequence (RKSRK) near the C terminus. These observations led to the speculation that CASP may be a transcription factor targeted to the nucleus and capable of interacting with various classes of LZ-containing proteins.

Repeated protein database searches later identified a PDZ protein interaction domain, described for the first time in Psd-95 [9], near the N terminus of CASP. PDZ domains typically interact with the C termini of other proteins in membrane-associated complexes (discussed later). The presence of two protein interaction domains suggest that CASP functions as a scaffolding protein regulating the higher architecture of an unknown signaling complex. The location of such a complex appeared to be cytoplasmic rather than nuclear, since transfection studies in a eukaryotic cell line showed CASP association with a perinuclear, Golgi-like region. The mechanism of CASP's function still eludes us and may involve novel aspects that have never been described, particularly since the C-terminus of CASP contains a domain of unknown function with no homology to any other known protein. CASP must, however, play a role in a hematopoietic-specific pathway, as judged by the expression profile of CASP and our ability to induce its transcription in Jurkat T cells in response to classical T cell activation through the TcR.

1.2. T CELL ACTIVATION

T lymphocytes activation by antigen presenting cells (APC) involves direct interaction of a number of cell surface molecules present on both cell types. The TcR (T cell receptor) complex and its accessory molecules (CD4 on T helper cells and CD8 on cytotoxic T cells) interact with antigen presented by class II MHC on APCs while CD28 interacts with the B7 family, primarily B7-1 (or CD80) and B7-2 (or CD86). These molecules constitute the basis for the widely accepted model of the two-signal requirement for T cell activation. Antibodies directed against both the TcR complex and CD28 receptor are widely used to trigger these molecules and study the signaling cascades they transduce, as well as the resulting T cell activation events. While CD28 is expressed on 95% of CD4+ T cells and 50% of CD8+ T cells [10], the TcR, which defines T cells, is expressed on all T lymphocytes.

Following TcR engagement of antigen in the context of MHC, ITAMs (immunoreceptor tyrosine-based activation motif) at the carboxy terminus of the TcR-associated CD3 complex and zeta chains are initially tyrosine phosphorylated by Lck. Other non-receptor tyrosine kinases of the Src and Syk families (Fyn and ZAP-70, respectively) are recruited to the TcR through their Src homology 2 (SH2) domain, resulting in the phosphorylation of a wide array of targets, including phospholipase C-γ (PLC-γ), phosphatidylinositol-3-kinase (PI3K), a number of adaptor proteins, and adaptor associated proteins required to generate the signaling cascades that follow. The activation of PLC-γ in the proximal steps of TcR activation results in the hydrolysis of membrane phosphatidylinositol 4,5-biphosphate to generate the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). The primary role of DAG is the activation of serine/threonine specific protein kinase C (PKC) while IP_3 causes Ca^{2+} mobilization from intracellular stores. Calcium is required for the activation of conventional PKC isoforms (discussed below), as well as other Ca^{2+}-dependent enzymes, including the phosphatase calcineurin. Activated PI3K, on the other hand, phosphorylates membrane phosphatidylinositol (PtdIns) to produce PtdIns-3-Phosphate (PtdIns-3-P) and consequently PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3. Both latter phosphatidylinositides are second messengers implicated in mediating downstream signaling events and mitogenesis [11], partly by acting on a number of PKC isoforms. Hence, DAG, calcium and phosphatidyl-inositides produced by PLC-γ and PI3K play a significant role during the early events of TcR activation, partly by inducing PKC activity.

Downstream of PKC, the signaling cascade leads to the activation of a number of GTPases including p21Ras and Rho, and three main MAPKs (mitogen-activated protein kinase): ERK1/ERK2 (extracellular signal-regulated kinase), p38, and JNK (Jun N-terminal kinase). While signals originating at the TcR can induce both the ERK and p38 pathways, JNK activation requires a costimulatory signal provided by CD28 [12]. JNK is therefore considered the pivotal MAPK in T-cell activation. The cooperation of all three MAPK pathways activate several transcription factors, including AP1, NF-AT, and NF-κB, resulting in cytokine production and cell proliferation. Interestingly, the same MAPK

pathways are involved in TcR and cytokine mediated signaling as well as T cell down-regulation and apoptosis induced by Fas or extracellular stresses, suggesting that the interplay between these MAPK pathways and their downstream effectors control a variety of cellular processes.

1.2.1. Protein Kinase C

PKC was long suspected of contributing to T cell activation, but direct evidence for PKC activation by the PLC-γ products DAG and IP_3 and its translocation to the membrane as a result of TcR ligation was presented in 1987[13, 14]. Since then, the use of phorbol esters, non-physiological DAG homologs that cause potent and prolonged PKC activation, showed that PKCs are involved in many TcR generated signals and cellular events. Phorbol esters are now commonly used to mimic TcR signals. When combined with calcium ionophores, they can fully activate T cell cytokine production and proliferation. Eleven PKC isoforms have been characterized to date. They fall in three categories depending on their domain structures and activation requirements (reviewed in [15]). Calcium-dependent PKCs (cPKC-α, βI, βII, and γ) are activated by phorbol esters and calcium, novel PKCs (nPKC- δ, ε, η, θ, μ) are calcium-independent but can be activated by phorbol esters, and atypical PKCs (aPKC- λ, ξ) are both calcium- and phorbol ester-insensitive. The catalytic portion of all PKC isoforms share an ATP-binding domain (termed C3) and a substrate binding domain (C4) but they differ substantially in their regulatory portion (Figure 1.1). cPKCs require the binding of calcium to the C2 domain, causing a conformational change that promotes C2 binding to membrane lipids, primarily phosphatidylserine. The C1 domain binds to membrane DAG, and the cooperation of both domains is required for cPKC activation. nPKCs regulatory portion contains a C2-like domain that does not require calcium binding, and a C1 domain that differs from cPKC C1 domain in its lipid specificity. nPKCs are activated by lipids other than DAG, including phosphatidylinositol and cholesterol sulfate. Unlike cPKCs, nPKCs are also activated by the PI3K metabolites PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 [16]. aPKCs regulatory region contains a C1-like domain with specificity to

lipids in the case of PKC-ξ and proteins in the case of PKC-λ. aPKC-ξ can be activated by PtdIns-3,4,5-P_3 [17], a combinations of lipids such as phosphatidylserine/cis-unsaturated fatty acids [18], or ceramide, a second messenger produced by sphingomeylinase [19]. aPKC-λ's regulatory domain on the other hand interacts with and is activated by the protein LIP (lambda-interacting protein) [19].

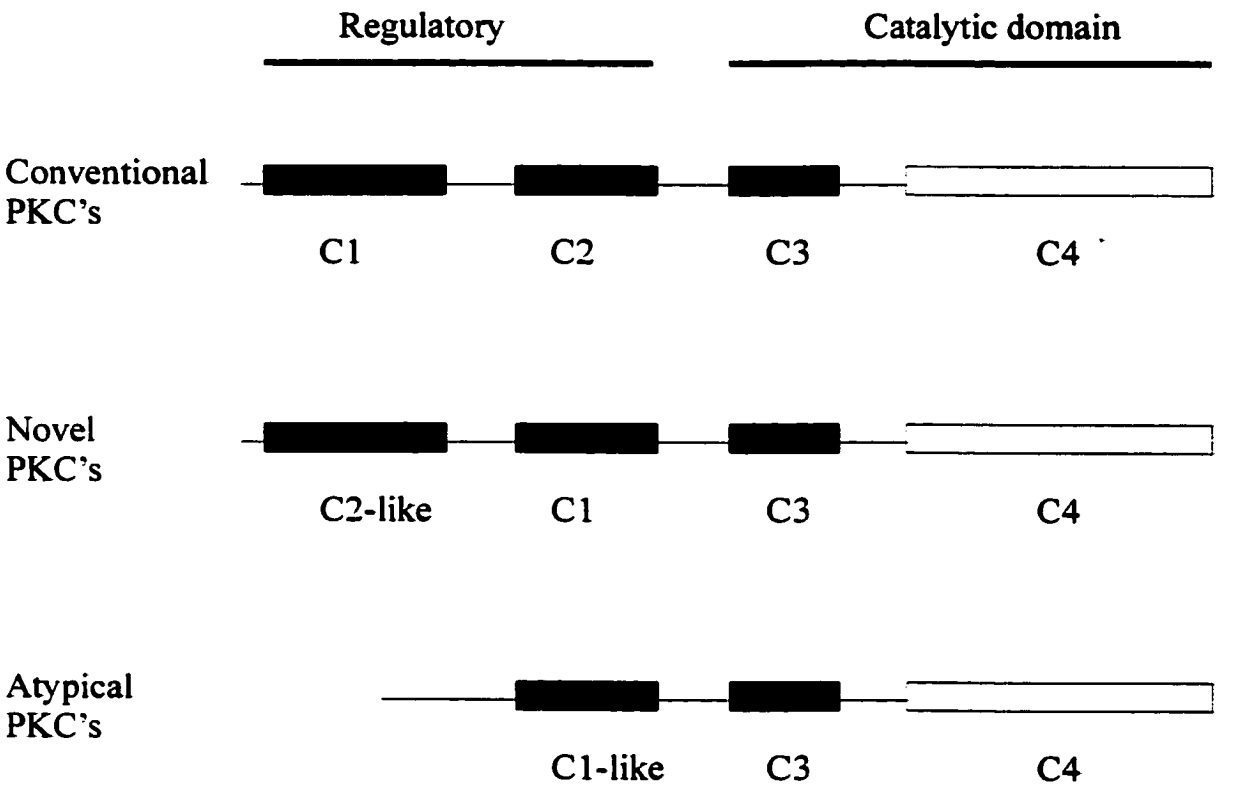


Figure 1.1. Structure of cPKCs, nPKCs, and aPKCs. adapted from [15]. All PKC isoforms have a catalytic region consisting of a C3 and C4 domain, and a regulatory region consisting of lipid binding domains (C1 and C2). cPKCs C2 domain binds to Ca^{2+}, and consequently to membrane phosphatidylserine. The C2 domain of nPKCs does not require Ca^{2+}. The C1 domain confers membrane lipid specificity and can bind to non-physiological phorbol esters while the C1-like domain of aPKCs, unlike cPKCs and nPKCs, is phorbol ester-insensitive.

Expression of cPKCs is ubiquitous with the exception of PKC-γ found primarily in the brain. While nPKC-δ is expressed in all tissues [20], most nPKCs have tissue specific expression patterns [21]. PKC-θ is predominantly found in hematopoietic cells and muscle cells [22, 23]. Cells of the representative T cell line Jurkat express PKC isoforms α, βI, βII, δ, ε, η, θ, μ, λ, ξ [20, 23-28]. The presence of several PKC isoforms in lymphocytes that can be activated through the TcR suggests that different PKC isoforms must act on multiple signaling pathways as a result of their substrate specificity, rather then serve a role of functional redundancy. The complexity of PKC signaling is evident when looking at the number of cellular events regulated by PKC pathways. PKC-α, for example, is required for interleukin 2 (IL-2) and IL-2 receptor (IL2-Rα/CD25) gene expression, as well as TNF-α cytokine production in Jurkat cells [24]. Additionally, multiple PKC isoforms can regulate individual activation events. PKC-α and θ are involved in IL2-Rα expression, while PKC-β, δ, and ε are involved in IL-2 production in normal blood lymphocytes [29]. Therefore a number of PKC pathways may be acting either in linear or parallel fashion to regulate cellular events like IL-2 production.

Interestingly, recent studies with PKC-θ in Jurkat cells show that this isoform (but not α, ε, or λ) specifically synergizes with calcineurin to activate a transfected IL-2 promotor through JNK [30, 31]. Furthermore, when an antigen-specific T cell clone is activated by APCs in a setting that mimics physiological activation of T cells, PKC-θ specifically translocated to the site of T cell/APC contact [32]. These observations collectively support a pivotal role for PKC-θ in T cell signaling. Other PKC isoforms clearly play a role in T cell activation events in general and IL-2 production in specific, possibly by targeting different elements of the more tightly controlled natural IL-2 promotor, or controlling the translation or secretion of IL-2. The interplay between the pathways transmitted by the different isoforms still needs to be elucidated.

Signaling pathways transmitted by PKC have been difficult to study due to the number of cellular processes they are involved in, their cell-specific activities, and the lack of specific PKC activators and inhibitors. PMA activates all cPKCs and nPKCs and

TcR activation results in collective PKC activation, rendering the study of individual PKC isoforms difficult. The use of PKC inhibitors is limiting as well. For example, the most specific PKC inhibitor to date, Gö6976, affects conventional PKCs, but cannot distinguish between the different cPKC isoforms. Some of these limitations were overcome in a number of studies by the use of antisense technology or the introduction of isoform specific antibodies, as well as transfections of constitutively active or dominant negative PKC isoforms. A study by Baier-Bitterlich G. and colleagues with phorbol myristate acetate (PMA)-activated Jurkat cells showed that nPKC-θ specifically induces AP1 activity and enhances NF-AT activity, an expected result since NF-AT activity is partially dependant on AP-1, but had no effect on a NF-κB promotor [33]. aPKC-α, like nPKC-θ, enhanced IL-2 promotor and NF-AT activity but, in contrast to PKC-θ, had no effect on AP1. It seemed that PKC-θ specifically controlled AP1 activity. Indeed, PKC-θ was recently reported to mediate the phosphorylation of Jun, a component of AP1, by specifically activating the Jun N-terminal kinase JNK[30, 31]. Furthermore, PKC-θ-mediated JNK activation could not be mimicked by PKC-α, ε, or λ indicating that these isoforms are not sufficient for AP1 activation. PKC-α can activate AP1, however, when combined with ionomycin, an ionophore that activates Ca^{2+}-dependent enzymes including calcineurin [31], or when transfected with an active form of Rho, a GTPase involved in T cell activation [34]. The role of cPKC-α in AP1 activation is not very well defined, but it may function in parallel with nPKC-θ to control other aspects of AP1 activation such as Fos (an AP1 component) expression or phosphorylation.

Novel PKC-ε has been implicated in AP1/NF-AT activation in a Ras-dependent manner as well as NF-κB activation in a Ras-independent manner [35]. A number of groups failed to detect an effect of this isoform on JNK activation, or any significant potentiation of IL-2 promotor activation, suggesting that PKC-ε plays a role in activation events other than IL-2 production. Atypical PKC-λ is implicated in NF-κB activation in Jurkat cells and fibroblasts [25]. However, NF-κB activation in fibroblast also involves cPKC-α or aPKC- ξ, depending on the mode of activation [19, 36], while LPS-activated macrophages require aPKC-α, βI, and δ [37]. This clearly demonstrates that the interplay

between PKC pathways is partly cell-specific, adding to the complexity of PKC signaling.

1.2.2. GTPases and T cell activation

One the pivotal steps in T cell activation downstream of PKC is the activation of p21Ras, the representative of the GTPase superfamily. Dominant negative Ras mutants abrogate IL-2 promotor activation by the TcR [38] and active Ras mutants can replace PKC activity for initiation of AP1 and NF-AT mediated transcription [39], indicating that p21Ras is both sufficient and necessary for T cell activation events. The small G protein superfamily includes members of the Ras, Rho, Rab, Arf and Ran families. Ras GTPases are involved in signaling, Rho family members are involved in signaling and cytoskeletal rearrangements, Rab and Arf families are involved in vesicle trafficking, and the Ran family is involved in nuclear transport. T cell activation requires signal transduction from membrane receptors, cellular polarization and cytoskeleton rearrangements, increased nuclear transport of transcription factors and cytokine secretion, all of which are mediated by GTPases of the different families. Current research on the role of GTPases in TcR mediated signaling has been focusing primarily on the Ras and Rho families of small G proteins.

Small GTPases exist in one of two conformations: a GDP-loaded inactive form and a GTP-loaded active form. The GDP/GTP exchange on GTPases is mediated by GTP-exchange factors (GEF). In essence, GEFs interact with inactive GDP/GTPases, causing a conformational change that promotes GDP to GTP replacement. The GTPases then interact with various effectors, recruiting them to the membrane or particulate fraction where they can be activated to transmit signals. Small GTPases are constitutively active in the GTP-loaded form until another class of proteins, GTPase activating proteins (GAP), interact with the small G proteins and activate the GTPase domain. GTP is then hydrolysed to GDP and the small G proteins return to the inactive conformation. GAPs therefore effectively inhibit the action of small G proteins. Another class of GTPase interacting proteins named GDI (GDP dissociation inhibitors) has been described for the

Rho and Rab families (reviewed in [40]), and recently for Ran GTPases [41]. A GDI binds to its corresponding GTPase and inhibits the GDP/GTP exchange, keeping the small G protein in the inactive conformation. GDIs clearly play an important regulatory role for some GTPases. To date, no GDIs have been described for the other GTPases of the Ras superfamily.

Ras function most certainly involves the activation of at least one GEF and possibly the inactivation of corresponding GAPs. Following TcR engagement, Ras activation by its primary GEF, SOS, involves phosphorylation of the adaptor protein Shc presumably by PKC [42], and the activity of phospho-tyrosine kinase Lck [43]. Shc recruits Grb2/SOS complexes to the membrane, facilitating the phosphorylation and activation of SOS by Lck. The physical interaction of SOS and Lck was recently confirmed as SOS was identified as a Lck-SH3 binding protein [44]. Another aspect of Ras activation involves the possible inactivation of p120GAP, an established Ras-GAP. P120GAP contains a Ca^{2+}-dependent lipid binding domain (CaLB), that targets the protein to the membrane following increases in intracellular Ca^{2+} levels [45], an event that occurs during T cell activation. Anti-CD3 treatment of T cells sequesters p120GAP in a complex that includes Lck/ Sam68/ PLC-γ/ PI3K at the intracellular tail of CD4 where it is inactivated by Lck-mediated phosphorylation [46, 47].

A number of Ras effectors have been isolated by the yeast two-hybrid method, and the binding is usually confirmed in vivo using fibroblasts. Some of the effectors include the serine/threonine kinase Raf [48, 49], the p110 subunit of PI3K [50], Ral-GEFs [51] and PKC-ξ [52]. PKC-ξ is activated by PtdIns-3,4,5-P_3 [17] so a model in which PKC-ξ is recruited by Ras to be activated by the PI3K metabolite is certainly appealing. It is not clear if this occurs in T cells however, especially that PKC-ξ activates NF-κB while active Ras cannot [35], suggesting that PKC-ξ signaling is independent from Ras at least in T cells. Ras activation of Ral through the Ral-GEFs has been established in fibroblasts [53, 54], but a link between Ras and Ral was never confirmed in lymphocytes. Similarly, PI3K activation by an active Ras mutant was reported in fibroblasts [55], but studies in Jurkat cells with the same Ras mutant failed to detect any

PI3K activity [56]. Raf kinase is the only Ras effector that appears to be universal to all cell systems [57]. PKC can directly phosphorylate and activate Raf-1 [58, 59], so a model in which Raf-1 is recruited to the membrane by Ras and consequently activated by PKC has been proposed. Raf-1 can then phosphorylate and activate MEK (MAPK/ ERK kinase) which in turn activates ERK [60]. The steps outlined above constitute the basis for PKC activation of the ERK pathway in a Ras-dependent manner.

Ras signaling leads to the activation of the Rho family of GTPases both in fibroblast and T cells. Rho-like GTPases control JNK, a pivotal MAPK in a variety of cellular events. Dominant negative mutants of the Rho-related GTPases RhoA, Rac, and Cdc42, inhibit Ras induced transformation of fibroblasts [61-63]. Furthermore, the Rho-related GTPases in fibroblasts are positioned along a linear pathway in which Cdc42, Rac and RhoA are sequentially activated [64]. This activation sequence has not been established in T cells. Dominant negative Rac inhibits Ras mediated transformation of Jurkat cells [56, 65], and Rac can substitute for p21Ras in one Jurkat system [65], or cooperate with Syk in another Jurkat system [66] to activate JNK. Cdc42 can activate JNK without the need for a costimulatory signal [67], suggesting that Cdc42 is located downstream of the site of signal convergence, and possibly downstream of Rac. Interestingly, Cdc42 is dispensable for IL-2 production during T cell activation by APC, but plays an important role in cell polarization and cytoskeleton rearrangements [68]. This suggests that even though Rac may be located upstream of Cdc42, Rac may be capable of activating JNK in a Cdc42-independent manner to drive IL-2 synthesis. RhoA GTPase specifically affects AP1 (but not NF-AT, Oct-1, or NF-κB) activation by PMA [34] and may be located on a linear pathway with Rac and/or Cdc42 in T cells, but this remains to be determined. Interestingly, RhoA cooperates with Cdc42 (but not Rac) to induce NF-κB by TNF-α [69], so Rho GTPases may be differentially regulated by different upstream signals to induce specific nuclear factors in response to these signals.

All three Rho-like GTPases have been implicated in cytoskeletal functions in fibroblasts. Several lines of evidence show that this also applies to T cells. The most convincing evidence come from studies with Vav, a GEF for the Rho family of GTPases; lymphocytes from Vav-deficient mice fail to form actin caps upon activation [34, 70].

Additionally, Cdc42 interacts with the effector WASP (Wiskott-Aldrich syndrome protein) [71], a protein required for T cell polarization during activation, and Rac (but not RhoA) colocalizes with Tailin, a cytoskeletal component, at the site of T cell/ APC contact [72]. Others have shown cytoskeleton localization of Cdc42 [34, 68], so Rac and Cdc42 may cooperate to transduce signals causing cell polarization for the directed release of cytokines towards the APC. RhoA is not involved in the same cytoskeletal processes as Cdc42 and Rac since it does not colocalize with the other Rho-like GTPases during T cell signaling, but its involvement in integrin-mediated adhesion has been reported in cytotoxic lymphocytes [73]. Recent studies with RhoA in EL-4 thymoma cells show this GTPase maintains cell structure since its inactivation results in major cytoskeletal reorganization [74]. It is believed that a balance of all three Rho-like GTPases is required for cytoskeleton maintenance in both T cells and fibroblasts.

The mechanism of Rac activation by Ras appears to be partly cell-specific since it is PI3K-independent in T cells [56] while it requires PI3K activity in fibroblast [55]. The complexity of Ras-mediated activation of Rac in T cells was further demonstrated in Vav-deficient lymphocytes [70], where JNK activation (downstream of Rac/Cdc42) was unaffected by the deficiency. This suggests that the mechanism of Rac activation by Ras in T cells may involve GEFs other than Vav, possibly TIAM-1 (T-lymphoma and metastasis), another Rho-GEF activated in fibroblasts in a PI3K-dependent manner [75].

Other small GTPases have been shown to play signaling roles in T cells including Rab5 of the Rab family and other members of the Ras family. Rab5 mediates TcR downregulation at the later stages of T cell activation by controlling TcR endocytosis [76]. Ral, a member of the Ras family, is activated by a number of GEFs including RalGDS (Ral GDP dissociation protein), RGL (Ral GEF-like), and Rlf (Ral GEF-like factor), all of which are Ras direct effectors. A downstream target of Ral is RLIP76, a GAP for Cdc42/Rac (but not RhoA) [77]. Ral may therefore mediate the negative regulation of some Rho-like GTPases by Ras after prolonged T cell activation. Other Ras family members affected by p21Ras activation include TC21, a small GTPase with potent oncogenic potential [78]. TC21 may contribute to Ras activity in T cells but the events downstream of TC21 have not been elucidated.

Research targeting the Rap GTPase, a member of the Ras family, have generated some interest in the role of this GTPase in T cells. Rap-1 maintains the anergic state of T cells and downregulates IL-2 expression [79]. Rap-1 (and Rap-2), like Ras, interacts with the Ral GEFs [80], and may regulate Rac/Cdc42 by activating Ral GTPases which then activate the Rac/Cdc42 inhibitor RLIP76. While this model remains speculative, another mode of Rap-mediated downregulation of Ras signaling by interfering with Raf has been established. Rap-1 competes with Ras for the serine/threonine kinase Raf-1 [81]; while Ras interaction with Raf results in its activation, Rap-1 sequestres Raf-1 from Ras and antagonize Ras activity.

1.2.3. Mitogen activated protein kinases (MAPKs): ERK, p38 and JNK

Activating various GTPases during T cell signaling results in the phosphorylation of a number of MAPKs by a series of kinases with various classifications in the literature (see table 1.1). Extracellular signal regulated kinases (ERK) 1 and 2, also known as p44MAPK and p42MAPK respectively, are the classical MAPKs involved in T cell activation. ERK2 is more prelevant than ERK1 in T cells but both are activated in parallel by TcR ligation or phorbol ester treatment. ERK1/ERK2 (collectively referred to as ERK), are threonine/tyrosine phosphorylated by the MAPK kinase, MEK-1, which itself is phosphorylated and activated by the MEK kinase, Raf-1. The serine/threonine kinase Raf-1 is a direct Ras effector and is phosphorylated by PKC, therefore Raf-1 and MEK-1 provide the link between PKC/Ras and ERK. The signal requirements for ERK activation in T cells are well characterized. It is generally accepted that full ERK activation occurs upon anti-CD3 or phorbol ester treatment, without the requirement for CD28 or ionophore-mediated signals. This observation extends to both T cell lines and normal murine T cells [82]. Interestingly, normal human CD4+ T cells show significant ERK activity when treated with anti-CD3 antibodies but CD28 synergizes with CD3 to enhance ERK activation [83], suggesting that circulating human T cells may be more tightly regulated. Some of the ERK substrates include the ribosomal S6 kinases p90Rsk1/Rsk2/Rsk3 [84], and the transcription factor Elk-1.

The other MAPK pathways involved in T cell activation are the JNK (p46JNK1 and p54JNK2) and p38 MAPK pathways. JNK and p38 can also be activated in T cells by extracellular stresses including UV or γ-irradiation, as well as oxidative or osmotic stresses [85-88]. They are therefore classified as stress-activated protein kinases (SAPK). JNK primarily phosphorylates Jun whereas p38 substrates vary widely from transcription factors such as CREB (c-AMP response element binding protein), ATF2, CHOP (c-EBP homologous protein) and MEF2C (myocyte-enhancer factor 2C), to other kinases such as MAPKAPK-2 and MAPKAPK-3 (MAPK-activated protein kinase) and the HSP27 kinase PRAK (p38 regulated/activated kinase). The requirement of 2 signals for efficient JNK activation in T cells has been established in cell lines and primary T lymphocytes [12, 83], but the signaling requirements for p38 activation vary significantly depending on the T cells examined. P38 activity induced by the TcR in murine Th1 clones was not enhanced by CD28 costimulation [89], whereas CD28 treatment of normal human T cells significantly increased p38 activity without the need for TcR signals [90]. Normal murine T cells treated with CD28 or CD3 alone showed little p38 activity, but CD28 synergized with CD3 and greatly enhanced CD3-mediated p38 activation [82, 91]. Studies in Jurkat cells show that CD3 and CD28 (albeit to a lesser extent) can individually activate P38, but CD3/CD28 cooperate to induce higher p38 activity [82, 90]. PMA also significantly activated p38 in Jurkat cells, an effect that was further enhanced by ionophore costimulation. While CD28 synergizes with CD3 in normal T cells to activate p38, CD28 effects in Jurkat cells may be partly additive.

Kinase Level	**Other Classifications**	**Kinases leading to ERK, JNK, and p38 activation**		
MAPKKK	MKKK, MEKK	Raf-1	MEKK-1	?
MAPKK	MKK,MEK, SEK	MEK-1	SEK-1/MKK4, MKK7	MKK6
MAPK	MAPK, SAPK	ERK	JNK	P38

Table1.1. Kinase hierarchy downstream of Ras or Rho-like GTPases, leading to MAPK/ SAPK activation. ERKs are located downstream of Ras while JNK and p38 are located downstream of Rho-like GTPases.

A number of upstream kinases (MAPKK) that activate p38 have been identified. They include MKK3, MKK6, and the JNK kinase MKK4 (or SEK-1). MKK6 appears to be the main MAPKK involved in activation of T cells. MKK6 kinase activity parallels p38 activity and IL-2 production in activated Jurkat cells. Dominant negative MKK6 inhibits NF-AT and IL2 promotor-driven transcription, clearly establishing a role for this kinase in IL-2 gene activation in Jurkat cells [92] and normal murine T cells [82]. MKK6 leadfing to p38 activity is also necessary for both transcriptional and post-transcriptional aspects of TNF-α production in a T cell line (A3.01) activated by CD3/CD28 antibodies [93] and for IFN-γ production by murine Th1 cells [94].

The role of p38 in normal human T cells, however, does not seem to parallel its role in murine T cells. The specific p38 inhibitor SB203580 has little effect on IL-2 production and proliferation of peripheral human T cells, while it affects the secretion of a number of cytokines (IL-4, IL-5, IL13, TNF-α) in a post-transcriptional manner. IFN-γ and IL-10 seem to be the most significantly affected at the transcriptional levels by SB203580 treatment [95].

The reduced p38 activity levels found in murine anergic T helper cells support a role for p38 in activation of T cells [89]. A recent study by Zhang J. and colleagues using primary murine T lymphocytes suggests that p38 plays a more important role than JNK in CD3/CD28 signal integration for cell proliferation and cytokine secretion [82]. This conclusion stems from the observation that p38 rather than JNK is activated by costimulation of murine T cells, in contrast to Jurkat cells in which both p38 and JNK are activated after costimulation. A role for JNK in murine T cell activation cannot be ignored though, since anergic murine CD4+ clones have reduced JNK activity [96] and peripheral T cells from JNK2 knockout mice have defective CD3/CD28-induced cytokine production (IL-2, IL-4 and IFN-γ) and IL-2-dependent proliferation [97, 98]. Interestingly, T cells from JNK1-deficient mice hyper-proliferate, exhibit less activation-induced apoptosis and over-produce cytokines as a result of increased NF-AT nuclear translocation [99]. This suggests that JNK1 modulates T cells by controlling NF-AT activation and T cell apoptosis whereas JNK2 positively regulates cytokine production.

JNK-1 downregulatory effects may be mediated by the direct activation of the dual threonine/tyrosine MAPK phosphatases Pyst1 and Pyst2 [100].

The established activators of JNK1/JNK2 so far are the MAPKKs MKK4/SEK-1 and MKK7. Both SEK-1 and MKK7 are activated in Jurkat cells and murine thymocytes following CD3/CD28 or phorbol ester/ionophore costimulation, although MKK7 activity is more prominent in both cell types [92]. The role of SEK-1 and MKK7 through JNK has been established in lymphocyte proliferation and IL-2 production [67, 101]. While the links between Ras and ERK are well known, the direct effectors of Rho GTPases leading to JNK activation in T cells are not very well characterized. Rac/Cdc42 interact directly with and activate MEKK-1 (a SEK-1 kinase) in COS cells [102], but this observation was never confirmed in T cells. PAK (p21Rac/Cdc42-activated kinase), a serine/threonine kinase that is activated by autophosphorylation upon its interaction with Rac/Cdc42 [103, 104], can phosphorylate MEKK-1 in the EL4 murine thymoma line [105]. The PAK/MEKK-1/SEK-1/JNK pathway may not be universal, however, since some T cell lines with active PAK do not express MEKK-1 [67]. Other SEK-1 kinases may be involved.

Direct GTPase effectors linking the GTPases to downstream p38 activation in T lymphocytes have not yet been identified, primarily because p38 research in T cells is relatively new. SEK-1 may regulate p38 in T cells in a manner similar to JNK. Another likely candidate, MLK-3 (mixed lineage kinase-3), containing a CRIB (Cdc42/Rac interactive binding) domain, is capable of directly phosphorylating SEK-1 and MKK6, the p38-specific activator [106, 107]. MLK-3 over-expression in T cells induces JNK and p38 [108], suggesting that MLK-3 may regulate both SAPKs.

1.3. ARF GTPASES AND THEIR GEFS: GOLGI OR PLASMA MEMBRANE?

Vesicular transport occurs at various locations within the cell, particularly within the ER/Golgi compartments and at the site of endocytosis at the cell periphery. The regulation of vesicle formation and traffic has been associated with a number of related small GTPases known as ADP ribosylation factors (ARFs). ARF GTPases are divided

into three classes based on their gene structure: class I ARFs (ARFs 1-3) are Golgi-associated GTPases regulating vesicle formation [109-111]. Little is known about class II ARFs (ARFs 4 and 5) except that ARF5 may be involved in BFA-resistant Golgi/ER retrograde traffic [112] and Trans-Golgi Network (TGN) vesicle traffic [113]. ARF6, the only member of Class III ARFs, associates with cell membranes and is involved in endocytosis and actin rearrangements [114-116]. It is clear that different members of the ARF family regulate vesicles at specific locations within the cell.

The study of ARF function has been focused primarily on the ER/Golgi and associated structures where different anterograde and retrograde vesicle trafficking pathways occur. It is generally accepted that coat protein II (COPII) coated vesicles budding from the ER carry cargo proteins to the ER/Golgi intermediate compartment where they are replaced by COPI coated vesicles involved in retrograde traffic and intra-Golgi vesicle movement [117]. Sar1 is the major small GTPase implicated in the formation of COPII vesicles [118, 119] while the ARFs control COPI as well as clathrin coated vesicle formation and traffic in and around the Golgi [120-122]. Clathrin is recruited to vesicles through adaptor protein (AP) complexes AP-1 to AP-3 that are closely related on a structural basis. A newly identified adaptor protein complex, AP-4, shares the same structural hierarchy as other AP complexes, but is incapable of binding clathrin[123]. AP-4 is also recruited to the Golgi, particularly the TGN, in an ARF1-dependent manner [124].

ARF1 and ARF5 promote the recruitment of coat protein I (COPI), adaptor proteins (AP) -1 and -3 complexes to the Golgi [121, 125]. While ARF5 recruits AP-1 complexes more efficiently than ARF1, both ARF1 and ARF5 recruit COPI and AP-3 onto Golgi membranes equally well. Other evidence suggest that ARF1, but not ARF5, interacts with AP-3 on Golgi-associated immature secretory granules [126], so the association of ARF5 with AP-3 at physiological levels in various compartments of the Golgi still needs to be addressed. Interestingly, COPI vesicles recruitment by ARF1 requires lower ARF1 levels than AP-1 complexes, suggesting that ARF1 peferentially recruits COPI to the Golgi while AP-1 complex recruitment occurs at higher ARF1 levels [125, 127]. On the other hand, the association of ARF2 and ARF3 with specific adaptor

complexes is not clear, but the localization of ARF2 and to a lesser extent ARF3 to the Golgi [111, 128] suggest that these two GTPases interact with a COPI and/ or AP complex.

AP-2 is generally associated with receptor-mediated endocytosis and vesicle formation and traffic to early endosomes [129-132]. One main receptor endocytosis route occurs via clathrin coated pits and vesicles that are formed by AP-2 complexes. ARF6 localizes to cell membranes and regulates receptor endocytosis in an inducible fashion [114, 133, 134], so a mechanism by which ARF6 regulate AP-2 function is certainly appealing. ARF6, however, does not physically interact with AP-2 components [126], and evidence for an ARF6-regulated AP-2-independent endocytic pathway has been reported [135]. Furthermore, ARF6 function at the plasma membrane of a number of cell systems has been associated with actin rearrangements [115, 116, 136]. Interestingly, the adaptor protein beta-arrestin facilitates ARF6 function at sites of endocytosis and couples receptor endocytosis to clathrin and AP-2 complex [137]. ARF6 may then regulate vesicle formation of a non-clathrin dependent endocytic pathway as well as cooperate with classical clathrin-dependent endocytosis by facilitating actin remodeling at the plasma membrane.

The activity of ARFs requires the exchange of GDP with hydrolysable GTP by guanine exchange factors (GEF). All GEFs are characterized by a Sec7 domain, first described in yeast [138]. The Sec7 domain physically interacts with ARF and catalyses GDP/GTP exchange by inducing a conformational change in the nucleotide binding site of ARF [139]. A number of ARF GEFs have been described and can be classified according to their sensitivity towards the fungal metabolite Brefeldin A (BFA). BFA physically interacts with a Sec7/ARF intermediate [140, 141] and effectively inhibits GDP/GTP exchange on ARFs. As a result, anterograde traffic stops and net retrograde traffic redistributes some Golgi components into the ER [142] while other Golgi-resident proteins localize to tubulo-vesicular clusters [143]. BFA also causes tubulation of the TGN and endosomal system [144, 145], partly as a result of its inhibitory effect on ARF1 activity and recruitment of the AP-1 complex to the TGN [146].

BFA sensitive GEFs include a family of large proteins named BFA-inhibited guanine exchange factors BIG1 (originally known as p200 GEP1) and BIG2 [147]. BIG1 activates ARF1, ARF3, and ARF5 (to a lesser extent) but not ARF6 [148]. BIG2, on the other hand, shows specificity for ARF1, ARF5 and ARF6 [147]. A third member of the BIG family, BIG3, has been submitted to Genbank (accession AAL04174), but its specifity is still unknown. BIG1 and BIG2 copurify with Golgi markers [149], but recent work by Zhao *et.al.* shows that BIGs generally overlap with the TGN marker TGN38 [150].

Another large GEF implicated in ARF signaling is the 206 kDa GBF1 (Golgi-specific BFA resistance factor 1). GBF1 is resistant to BFA, associates with the cis-Golgi [112, 150], and aids in the formation of COPI vesicles [151]. Furthermore, GBF1 shows specificity to class II ARF5 [112]. Based on these findings GBF1 activity is suspected in COPI vesicles retrograde traffic. GBF1 and BIGs are suspected to regulate vesicle formation in different sub-compartments of the Golgi.

A family of small ARF GEFs with molecular weights around 50 kDa has emerged is the past few years as major regulators of ARF function in a BFA resistant manner. The first was originally cloned in our laboratory and was designated B2-1, a homolog of yeast SEC7 [152]. It was later renamed cytohesin-1 by others [153] and its GEF activity on ARF in a mammalian cell system was later confirmed [154]. There are currently four known members of the cytohesin family. ARNO (ARF nucleotide-binding site opener) [155], also known as cytohesin-2, and ARNO3 [156], the human homolog of mouse GRP1 (general receptor for phosphoinositides 1)[157]. Another member of the cytohesin/ARNO family, cytohesin-4, was recently identified in blood cells [158].The specificity of cytohesin/ARNO members to the various ARFs appears to be mediated primarily by the Sec7 domain. All cytohesin/ARNO members activate ARF-1 [156, 158], while cytohesin-1, ARNO and ARNO3 (but not cytohesin-4) activate ARF6 [158-160]. Cytohesin-1 can activate ARF3 [154, 161] while both cytohesin-1 and 4 can activate ARF-5.

All four members of the family are highly similar on a structural basis. They all have a similar domain distribution consisting of a sec7 homology domain, a pleckstrin

homology (PH) domain, and an N-terminal coiled coil. The carboxy-terminal PH domain allows cytohesin/ARNO interactions with membranes by binding to various polyphosphoinositides [162-165]. While the PH domains of cytohesin-1 and ARNO seem to bind non-selectively to various phosphoinositides, ARNO3 shows increased affinity to PtdIns-3,4,5-P_3, a product of PI3-Kinase activation [164, 166]. Generally, while the PH domain anchors the cytohesin/ARNO GEFs to membrane structures, the Sec7 domain facilitates the function of ARF in vesicle formation. The N-terminal coiled coil motif is involved in protein/protein interactions and most likely recruits scaffolding and other factors to the site of cytohesin/ARNO function. GRP1, the mouse homolog of ARNO3, recruits the scaffolding protein GRASP to the cell periphery where it co-localizes with an unidentified ARF [167]. GRASP can also interact with ARNO in an *in vitro* and *in vivo* binding assay. We recently reported the interaction of all members of the cytohesin/ARNO family with the coiled coil domain of CASP, a GRASP-related scaffolding protein [168] (discussed later).

One last group of GEFs, recently described as regulators of plasma membrane ARF activity, constitute the EFA6 family. EFA6 proteins partially resemble members of the cytohesin/ARNO family on a structural basis. EFA6 GEFs, like cytohesin/ARNO members, have a Sec7 domain, a PH domain and a coiled coil motif. They differ from their cytohesin/ARNO counterparts by size, location of the coiled coil (C-terminal in EFA6 versus N-terminal in cytohesin/ARNO), and the presense of proline rich regions that are absent in cytohesin/ARNO factors. EFA6 function may resemble that of cytohesin/ARNO by facilitating guanine exhange through Sec7 domain while being anchored to membranes through their PH domain and recruiting additional factors through the coiled coil and proline rich regions. No factors binding to the EFA6 coiled coil or proline regions have been identified, but the C terminus of EFA6 promotes actin rearrangement [169] possibly by regulating Rac1 GTPase [170]. At least two EFA6 proteins have been described to date (EFA6A and EFA6B) but database analysis revealed the presence of up to four members in the EFA family [169]. EFA6A and EFA6B localize to the cell membrane and act exclusively on ARF6 [169, 171]. BFA sensitivity of

EFA6 family members was never addressed primarily because of its association with plasma membrane rather than Golgi related structures.

The complexity of ARF signaling is becoming more apparent, not only because of the variety of ARF proteins, the variety of associated AP complexes, and the increasing number of GEFs and GAPs that regulate ARF functions, but also because of the number of processes affected by ARF GTPases and the multiple locations where they exert their effects. In addition to vesicle coat assembly in various locations of the anterograde and retrograde transport pathway between the Golgi and plasma membrane, ARF1 regulates actin assembly on the Golgi [172], ARF6 regulate actin rearrangement at the plasma membrane, and there is evidence of cross talk between ARF and various Rho GTPases [136, 173-176].

2. CASP GENOMIC ORGANIZATION AND TRANSCRIPTIONAL ACTIVATION IN JURKAT CELLS

2.1. INTRODUCTION

T cell activation is primarily initiated at the TcR, and involves a wide array of immediate, early, and late signaling pathways that involve various protein kinase C isoforms (PKC), as well as a number of mitogen-activated protein kinase (MAPK) pathways leading to gene expression. A number of PKC isoforms have been characterized to date. They fall in three categories depending on their activation requirements (reviewed in [15]). cPKCs (α, βI, βII, and γ) are activated by phorbol esters and calcium, novel PKCs (nPKC- δ, ε, η,θ, μ) are calcium-independent but can be activated by phorbol esters and atypical PKCs (aPKC- λ, ξ) are both calcium- and phorbol ester-insensitive. Expression of cPKCs with the exception of PKC-γ is ubiquitous, whereas nPKCs have tissue specific expression patterns [21]. PKC-θ is predominantly found in hematopoietic cells and muscle cells [23]. The presence of several PKC isoforms in lymphocytes that can be activated through the TcR indicates that different PKC isoforms must act on different signaling pathways as a result of their substrate specificity. Indeed, it seems that PKC-α and θ are involved in IL-2 receptor (IL-2R) activation, while PKC- β, δ, ε are involved in IL-2 activation [24, 29]. Recent studies with PKC-θ in Jurkat cells show that this isoform (but not α, ε, or λ) specifically synergizes with calcineurin to activate IL-2 transcription through JNK, and may therefore play a pivotal role in T cell activation events [30, 31]. Many PKC isoforms are clearly involved in TcR-mediated activation of T cells but the interplay between the various pathways transmitted by different isoforms is largely unknown.

Signals generated at the TcR are transduced through at least three MAPK pathways: ERK1/ERK2, p38, and the stress-activated protein kinase JNK (or SAPK). All three pathways are PKC-dependant [31, 89, 177]. Anti-CD3 or PMA treatment alone activate the ERKs as well as p38 [178], but JNK activation requires an additional co-

stimulatory signal provided by CD28 ligation on T cells. While JNK plays a pivotal role in T-cell activation and IL-2 production, the ERK1/ERK2 and p38 pathways are required for the production of various cytokines, and the inhibition of these pathways is detrimental to T-cell activation and proliferation [90, 92, 178].

We have previously cloned a novel cDNA from a human NK/T cell population by a method of subtractive hybridization which we designated B3-1 [3]. This cDNA codes for a 40 kDa protein with a domain profile consisting of an N-terminal PDZ domain, an internal coiled coil motif, and a C-terminal domain of unknown function. B3-1 was later renamed Cybr (Cytohesin binder and regulator) by others [179] and CASP (Cytohesin Associated Scaffolding Protein) by our group [168] based on its interaction with the guanine exchange factor cytohesin. Cytohesin was initially cloned in our laboratory (original designation B2-1) and identified as a Sec7 containing protein. It was later renamed cytohesin by others based on its effect on integrin adhesion [153]. CASP expression was originally established and later confirmed in lymphocytes [3, 179], and recent EST database searches suggest expression in other cell types of hematopoietic origin, such as CD34+ stem/progenitor cells, germinal center B cells, activated T cells, as well as a number of cancers including adenocarcinoma, embryonal carcinoma, myeloma, melanoma and lymphomas.

CASP's interaction with cytohesin is mediated by the internal coiled coil motif of CASP and the N-terminal coiled coil of cytohesin both *in vitro* and *in vivo* [168, 179]. Cytohesin/ARNO proteins constitute a group of Sec7-containing proteins that act as guanine nucleotide exchange factors (GEF) for ADP ribosylation factors (ARFs) implicated in vesicle formation and trafficking. The only other identified protein of the CASP family, known as GRASP or Tamalin, has a similar protein structure and acts as a scaffolding protein that facilitates GEF signaling at the periphery of PC19 cells [167, 180]. The lymphoid expression of CASP suggests a specialized role for CASP in lymphocyte-specific pathways involving the ARF family of small GTPases and the GEFs that activate them, particularly cytohesin.

In this study, we report the genomic organization of CASP and the transcriptional activation of CASP by TcR signaling pathways. CASP expression in Jurkat cells was

induced with anti-CD3 antibodies as well as the phorbol ester PMA. The early signaling pathways required for CASP activation, particularly PKC and MAPK, were targeted.

2.2. EXPERIMENTAL PROCEDURES

2.2.1. Cells and chemicals

Jurkat and YT (NK-like) cells were maintained in RPMI 1640 media supplemented with 10% heat-inactivated FCS (Gibco), 50 units/ml of penicillin G, and 50μg/ml of streptomycin. All agonists and inhibitors used were from Calbiochem. Bisindolylmaleimide I, Gő 6976, Gő 6983, and dibutyryl-cAMP were kindly provided by Dr. D.M. Byers (CRC, Halifax). Forskolin was a gift from Dr. M. Kelly (Dalhousie, Halifax). Cyclosporin was provided by Dr. D. Hoskin (Dalhousie, Halifax). OKT3 and anti-CD28 (m9.3) monoclonal antibodies were generous gifts from Dr. A. Issekutz (IWK hospital, Halifax) and Dr. C. June (Naval Medical Research Institute, Bethesda, MD), respectively.

2.2.2. Genomic cloning

A method of DNA walking by PCR was employed using a TaKaRa kit supplied by PanVera Co., Madison, WI. Manufacturer's instructions were followed with slight modifications. In brief, human genomic DNA was digested using various restriction enzymes (*Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Pst*I, *Xba*I), the appropriate cassette was ligated to the digested DNA and two PCR reactions were performed in sequence using nested primers matching the cassette's nucleotide sequence and CASP's coding sequence. The first PCR was performed with the following parameters: 95 °C/30 seconds, 55 °C/40 seconds, 72 °C/1 minute, for 35 cycles. The second PCR was performed with the same parameters but for only 24 cycles. PCR products were cloned into a PCRII plasmid (Invitrogen, San Diego,CA) and sequenced using the dideoxy method to identify exon/intron boundaries.

Long range PCR performed with a Long Expand PCR Kit (Bohringer Manheim) was also used to clone a number of introns into the PCRII plasmid. Long range PCR parameters were 95 °C/30 seconds, 62 °C/30 seconds, 68.5 °C/6 minutes, for 35 cycles. PCR products were partially sequenced as above to identify exon/intron boundaries.

2.2.3. 5' RACE

Jurkat cell were treated with 1 μM PMA overnight. RNA from YT and activated Jurkat cells was purified using Triazol (Life Technologies) according to manufacturer's instructions. A method of mRNA oligo capping (Generacer kit, Invitrogen) was used to amplify the 5' end of CASP mRNA from 1 μg of total RNA. Reverse transcription of oligo capped mRNAs was performed with random hexamers and M-MLV RT (Gibco BRL) at 37 °C for 1 hour. Nested CASP primers used for the RACE were Mar-AS10 (5'-CATTATCCTGCTTCTCCACAG-3') and Mar-AS11 (5'-CTTTGAGACCAGGAAAAGTCAC-3'). PCR products were cloned and sequenced as described earlier.

2.2.4. RT/PCR and expression analysis

5 x 10^5 Jurkat cells were placed in 1 ml of RPMI 1640 media (supplemented with FCS/antibiotics) for each treatment. Cells were induced with 1μg/ml of OKT3, with or without 1μg/ml of anti-CD28, or with 1μM PMA, or with 100 ng/ml anisomycin as indicated for the reported periods of time. All inhibitors, when used, where added to cells 20 minutes prior to activation. Cells appeared healthy after all treatments as judged by trypan blue exclusion. Cells were then lysed in 250 μl of Triazol (Gibco) and RNA was isolated following the manufacturer's instructions. The resulting RNA was resuspended in 20 μl of DEPC-treated water. 200 ng of total RNA (1μl) was reverse transcribed in a 10 μl reaction (50mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 1 mM dNTP's, 25 ng of random hexamer and 200 units of M-MLV RT purchased from Gibco) at 37 °C for 1 hour. Following a denaturing step (90°C/ 5minutes), PCR was performed on 1 μl of reverse transcription reaction using an Uno II thermocycler (Biometra) with the following parameters: denaturing at 94°C for 30 seconds, annealing at 56°C for 40 seconds, and elongation at 72°C for 1 minute. CASP was amplified for 35 cycles, IL-2 was amplified for 33 cycles, and beta-actin was amplified for only 21 cycles. Primers used were Mar1 (5'-TCAACCATGTGCTAGCTGGAG-3')/ Mar-AS11 for CASP, IL-2for (CCAAACTCACCAGGATGCTCAC)/ IL-2rev (AGTGTTGAGATGATGCTTTGAC) for Il-2, and Act-1 (5'-

CTGGAGAAGAGCTATGAGC-3')/ Act-2 (5'-TTCTGCATCCTGTCAGCAATG-3') for beta-actin. The specificity of the primers was confirmed by manual dideoxy sequencing of the three PCR products. PCR products were seperated by 7% polyacrylamide gel electrophoresis then viewed and photographed on a UV-transilluminator.

2.3. RESULTS

2.3.1. Genomic organization of CASP.

The CASP gene consists of eight exons and seven introns that span approximately 29 kilobases of genomic DNA (Figure 2.1). The introns vary in size with the smallest intron being 207 bases and the largest being 9101 bases. Introns 2 and 4, the smallest CASP introns (275 and 207 bases, respectively), were completely sequenced while the other introns were partially sequenced to identify exon/intron boundaries (Table 2.1). All of the exon-intron junctions conform to consensus splice sites. Intron sizes determined by gel electrophoresis analysis were in accordance with genomic data that span the CASP locus (accession AC019201), with the exception of intron 2. Intron 2 was 273 bases in our clones due to a CT deletion in a region of CT repeats, most likely the result of intronic polymorphism. Interestingly, the largest exon (exon 8, 1125 bases) contains 467 bases coding for the entirety of the C-terminal domain of unknown function.

5' RACE analysis of CASP in PMA-activated Jurkat cells revealed that CASP transcription starts 75 bases upstream of the first start codon (Figure 2.2). CASP transcription initiation in YT cells, on the other hand, occurs 38 bases upstream of the first ATG (Figure 2.2, double arrow). Interestingly, YT cells have unusually high levels of constitutive CASP expression (data not shown), possibly the result of the transformed phenotype of these cells.

2.3.2. CASP is an early gene activated by the TcR through PKC

We previously showed that CASP was expressed at low levels in resting T cells while it was virtually undetectable in resting Jurkat cells [3]. The lymphoid-specific expression of CASP prompted us to study CASP activation in stimulated T cells, particularly in Jurkat cells with no detectable endogenous CASP expression. Jurkat cells were initially stimulated with the phorbol ester PMA, an activator of PKC, and with PMA/ionomycin, a combination that mimics TcR/CD28 signaling and results in IL-2

transcription [181]. CASP expression was monitored by a method of semi-quantitative RT/PCR. In both cases, CASP transcription was increased, with no detectable synergy of ionomycin with PMA. This activation was completely inhibited with the addition of staurosporine, a PKC inhibitor (Figure 2.3A). In T cells, TcR engagement leads to PKC activation so we tested whether CASP transcription was initiated following TcR engagment. When Jurkat cells were incubated with the monoclonal anti-CD3 antibody OKT3, CASP mRNA was detected as early as 3 hours (Figure 2.3B). The addition of anti-CD28 had neither a temporal nor a quantitative effect on CASP activation, while it resulted in the drastic increase in IL-2 mRNA levels (lanes 2, 4, Figure 2.3B). We concluded that transcription of CASP was a direct result of TcR engagement and was unaffected by signals generated by CD28.

The detection of CASP mRNA following 3 hours of stimulation suggested to us that CASP belongs to a group of early genes that required the *de novo* synthesis of transcription factors. This was confirmed with the use of the known protein synthesis inhibitor, cyclohexamide (CHX), which completely inhibited CASP activation while it only resulted in a reduction of IL-2 mRNA levels at the end of the 12-hour activation period (Figure 2.4). While the prolonged activation of IL-2 requires renewal of transcription factors, its early activation can proceed in the absence of protein synthesis, and the half life of the mRNA is substantially prolonged [182]. CASP activation, on the other hand, was clearly dependent on the generation of new transcription factors following TcR-mediated Jurkat cell activation.

2.3.3. Involvement of conventional PKCs in TcR-mediated CASP activation

Since maximal CASP levels were reached by 10 hours of anti-CD3 treatment (data not shown), an activation protocol of 12 hours was used in the majority of experiments unless indicated otherwise. We established that CASP induction was inhibited by staurosporine, a general inhibitor of PKC activity (Figure 2.3). Preliminary studies with the calcium chelator EGTA suggested that conventional PKC(s) rather than the calcium-independent novel PKCs mediated CASP activation (Figure 2.5). Low

EGTA concentrations (1.5 mM supplemented with 2.5 mM magnesium chloride) that are well tolerated by Jurkat cells in a 12 hour activation assay inhibited OKT3-induced CASP transcription. This suggests that calcium-dependent enzymes, presumably cPKC(s), mediate CASP activation. The calcium-dependent phosphatase Calcineurin activated by the TcR was not involved in CASP activation since cyclosporine A, a know inhibitor of the phosphatase, had no effect on CASP activation while it drastically inhibited IL-2 transcription following CD3/CD28 co-stimulation (Figure 2.4A). In order to further characterize the involvement of conventional and novel PKCs in the early events leading to CASP transcription, we used more specific PKC inhibitors, namely bisindolylmaleimide I (also known as Gö 6850or GF 109203X), Gö 6983, and the cPKC-specific inhibitor Gö 6976. All three compounds inhibited CASP activation in OKT3-treated (Figure 2.6A) and PMA-treated (Figure 2.6B) Jurkat cells. When PMA-activated Jurkat cells were pretreated with Gö 6976, however, lower levels of CASP transcription were detected (lane 3, Figure 2.6A). This suggested that while one or more cPKCs are required for TcR-mediated activation of CASP, nPKCs, non-specifically activated by PMA, partially activated CASP transcription.

2.3.4. CASP activation is inhibited by PKA

Some PKC inhibitors, including staurosporine and Gö 6850, can also inhibit PKA, albeit at much higher concentrations. Since TcR signaling can lead to PKA activation at later stages, we explored if PKA activation can lead to CASP transcription. We found instead that PKA activation by forskolin, an adenylate cyclase activator upstream of PKA, completely inhibited OKT3-induced activation of CASP (Figure 2.7). In the case of PMA activation on the other hand, low levels of CASP were detected when cells were pretreated with forskolin. The same results were observed when PKA was activated with the cAMP analog dibutyryl-cAMP (data not shown). While PKA clearly acts, at least partially, downstream of PKC, our data suggest that PKA also acts upstream of PKC, inhibiting CASP transcription in OKT3-activated Jurkat cells. The presence of

lower levels of CASP mRNA in PMA-induced Jurkat cells clearly suggests that CASP transcription can partially proceed through a PKC-dependant, PKA-insensitive pathway.

2.3.5. Involvement of the MAPK cascades (ERK and p38)

Based on our observations with the effect of PKA on CASP transcription, we suspected that CASP induction proceeds through at least two pathways downstream of PKC. Since anti-CD3 can activate both the ERK and p38 (but not JNK) pathways, we explored the role of these two pathways using MAPK-specific inhibitors. PD98059, a specific inhibitor of ERK kinase (MEK), inhibits the activation of ERK1/ERK2. This was confirmed in our experimental system with the use of phospho-ERK-specific antibodies and western blot analysis (data not shown). PD98059 treatment resulted in the partial activation of CASP by PMA (Figure 2.8). SB203580, a specific inhibitor of p38, had a similar effect on CASP transcription (Figure 2.8). The effects of both inhibitors were additive, suggesting that CASP is independently activated through the ERK and p38 pathways downstream of PKC.

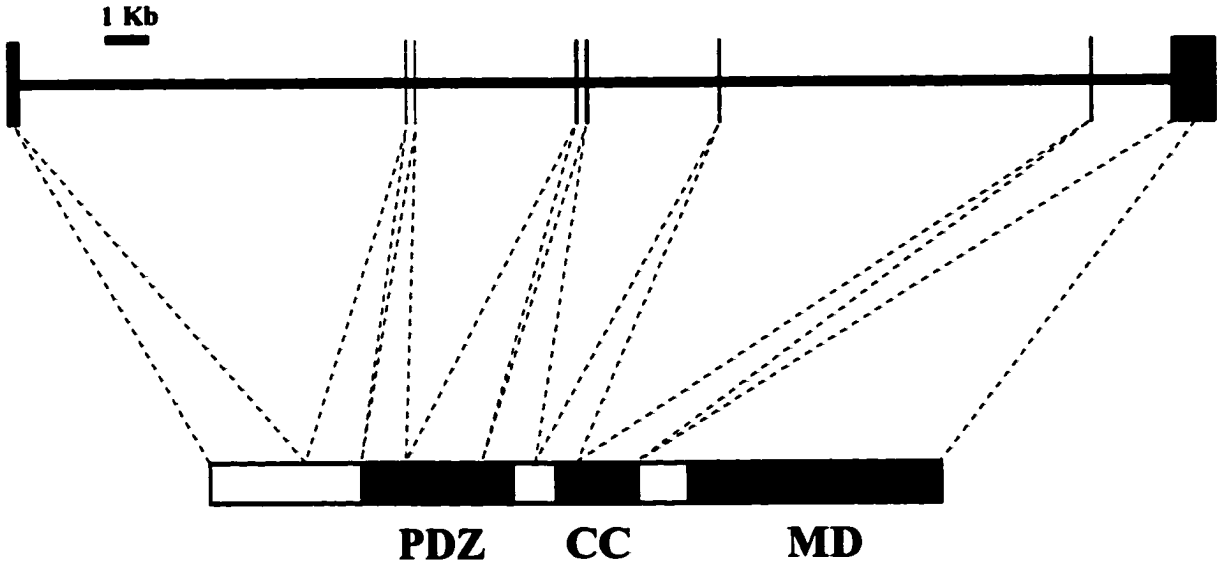


Figure 2.1. Organization of the CASP gene. The CASP gene with 8 exons and 7 introns is drawn approximately to scale. Exons 1 and 2 code for the N-terminus of CASP protein, exons 3,4, and 5 code for the PDZ domain. Exons 6 and 7 code for the coiled coil motif (CC). Exon 8 code for the C-terminal domain of unknown function (mystery domain, MD).

A)

CASP Exon	Size (bases)
1	249
2	50
3	55
4	103
5	94
6	70
7	67
8	1125

B)

Intron	5' boundary	3' boundary	Size (bases)
1	AAAGCAGgtatgattgctattttgtacccctttagagt	ggataactatatgtctttgtgtttttcagCTTGCTT	9101
2	CTCAAAGgtaaagagattgaattattttgtgtctctc	cagtttctttttttaaacatttttattacagAAAGCTT	275
3	AATTCAGgtgggcaattttcacattttctagacttcc	agtaactatatatcatcttaatttttacagTCTTACA	3407
4	CAAGCTGgtaacttaaatttttgctcatgttaggaat	ataacactcatttttcttggcatctcccagGTGATGT	207
5	TGCTAACgtaactatctgtctagttcccgtggggctt	caccttcttctttttttttccttaaaacagGATAGAG	3138
6	TTTAAAGgtaatttaatttaatgcagtgaggcacatt	aacttttttttttttttttttaatctcacagCAAACTT	8761
7	CTTCATGgtaaatgcaatttcctgttcagaaaccaca	atccatttcacagtagactttgtgctctagGTGATGC	2379

Table 2.1. Exon and intron description of the CASP gene. A) CASP exon sizes. B) Intron sizes and boundary sequences.

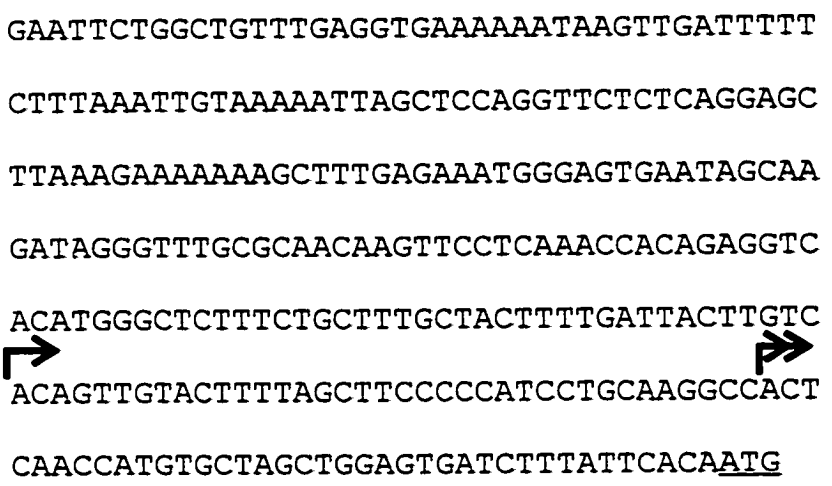


Figure 2.2. Transcription initiation sites of CASP. 5'RACE analysis of CASPin Jurkat and YT cells show two different initiation sites (single arrow and double arrows, respectively).

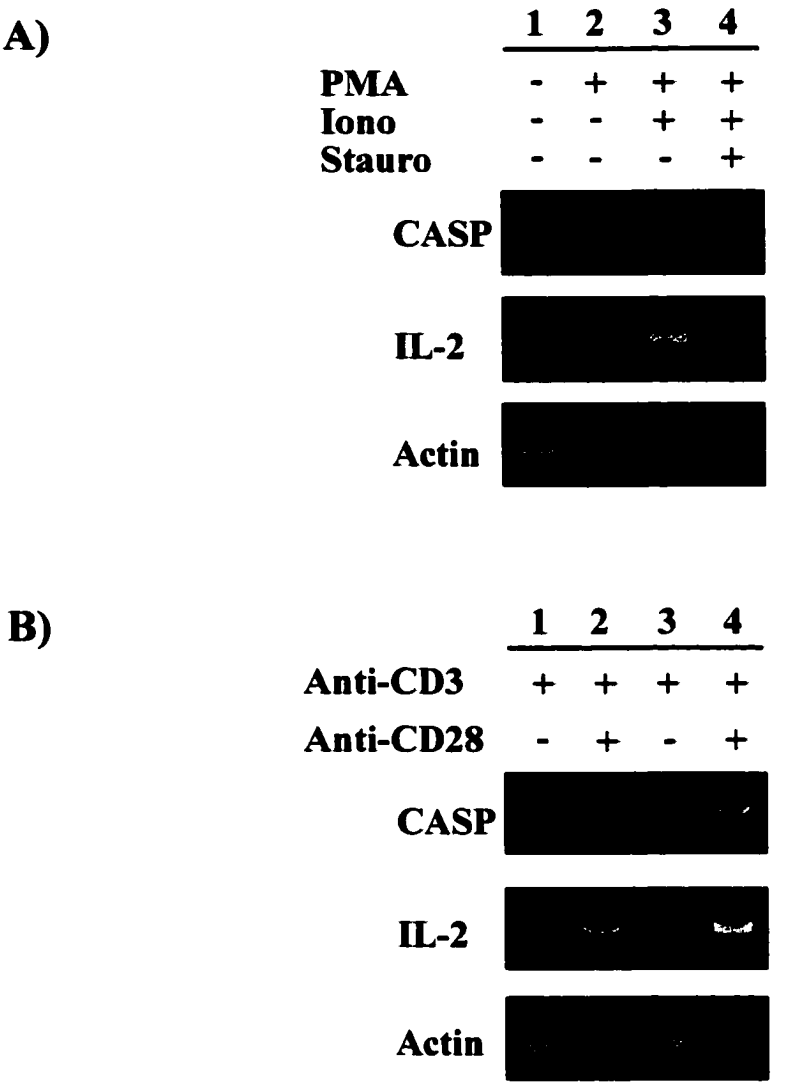


Figure 2.3. PMA and anti-CD3 antibodies induce CASP expression. A) Jurkat cells were activated with PMA alone (lane 1), or with PMA/ionomycin (iono)(lanes 3-5). Addition of the PKC inhibitor Staurosporine (stauro, 50 nM) (lane 4) inhibited the expression of CASP and IL-2. **B)** Jurkat cells were stimulated through CD3 and CD28 for 3 hours (lanes 1 and 2), and 5 hours (lanes 3 and 4). CD28 co-stimulation (lanes 2 and 4) had no effect on CD3-activated CASP expression while IL-2 expression was dramatically increased. Actin levels remained unchanged with all treatments.

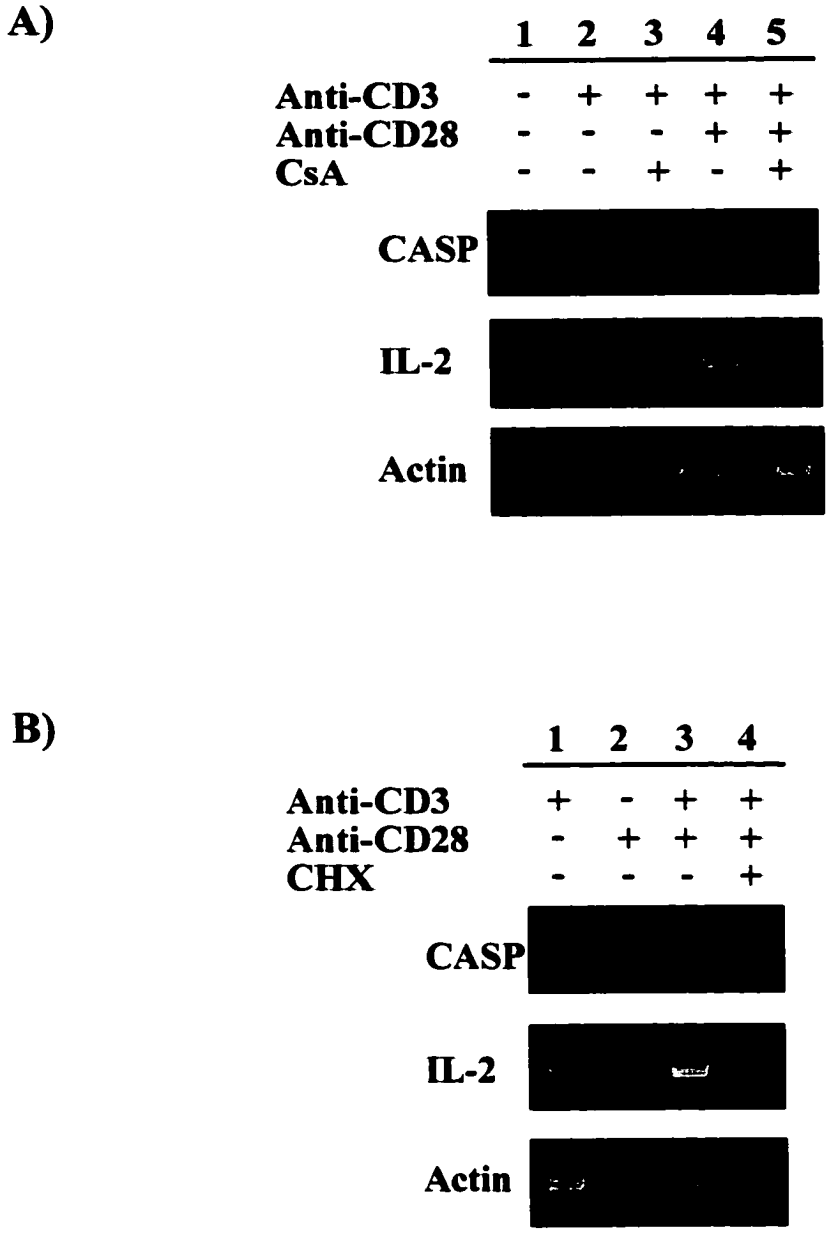
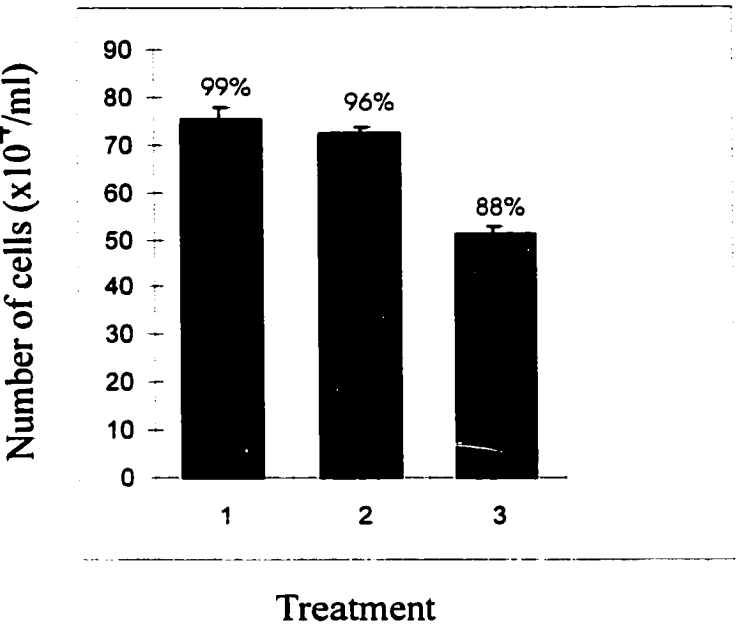


Figure 2.4. CASP expression is cyclosporin A-insensitive and requires protein synthesis. Jurkat cells were activated through CD3 and CD3/CD28 as indicated. A) Pre-treatment with cyclosporin A (8 microM) had no effect on CASP activation while it reduced IL-2 levels following co-stimulation. B) Pre-treatment with cyclohexamide (CHX, 1 microM) (lane 4) completely inhibited CASP activation while only reducing IL-2 levels. Actin levels remained relatively unchanged with all treatments.

A)



B)

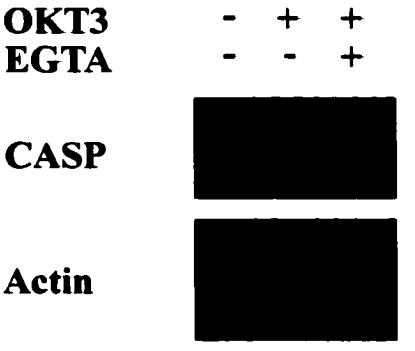


Figure 2.5. Effect of EGTA on CASP expression in OKT3-activated Jurkat cells. A) Effect of EGTA concentrations on Jurkat cell division and viability. 5 x 10^5 Jurkat cells /ml were plated in a 6 well plate, left untreated (treatment 1), incubated with 1.5 mM EGTA/ 2.5 mM magnesium Chloride (treatment 2), or 5 mM EGTA (treatment 3) for 12 hours at 37 degrees celcius/ 5% CO_2. Cells were counted using a heamocytometer and % viability was determined by Trypan Blue exclusion. 5 mM EGTA was detrimental to cell division and cell viability (88%). All treatments were performed in triplicates. **B)** Jurkat cells were incubated with or without 1.5 mM EGTA/ 2.5 mM MgCl2 and activated with OKT3 for 12 hours as indicated. EGTA clearly inhibits CASP activation by TcR signals.

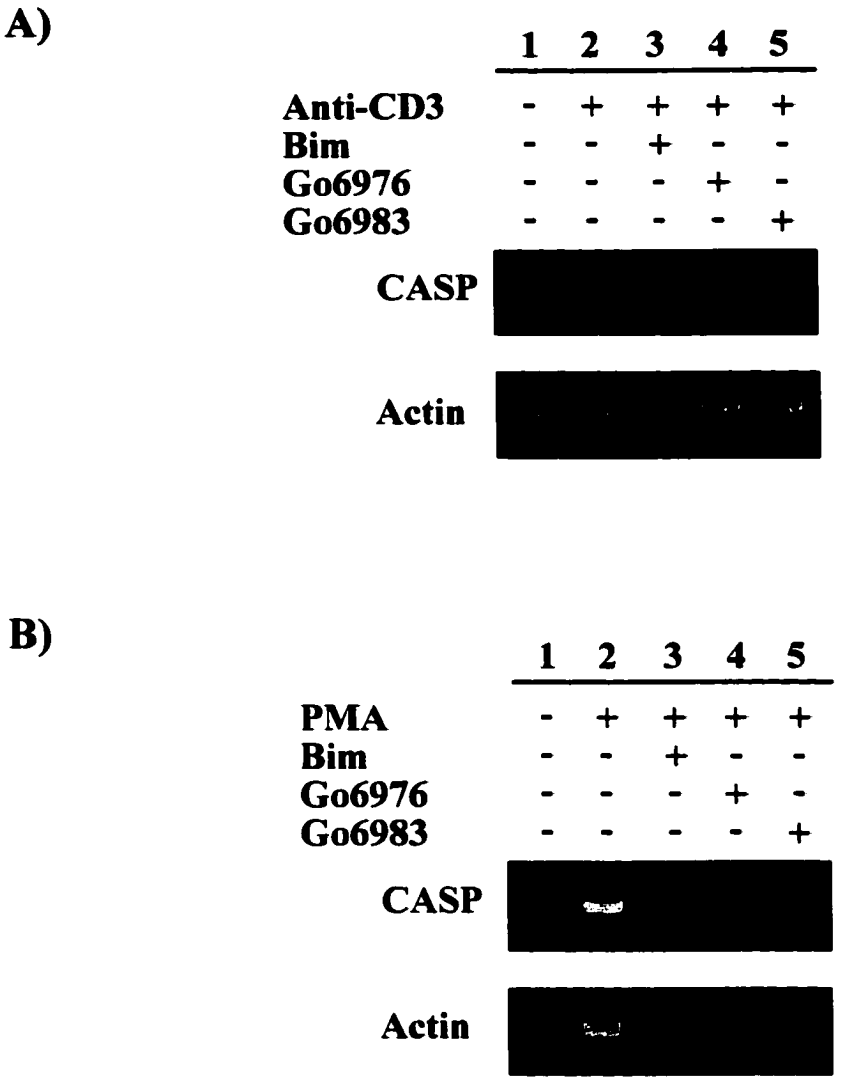


Figure 2.6. Effect of PKC-specific inhibitors on anti-CD3 and PMA induced CASP transcription. Anti CD3-activated **(A)** and PMA-activated **(B)** Jurkat cells were pre-treated with Bisindolylmaleimide I (Bim, 10 microM) (lanes 3), Gö 6983 (1 microM) (lanes 4), and Gö 6976 (1microM) (lanes 5). All three PKC inhibitors exhibited a marked reduction in CASP activation. Actin levels remained unchanged with all treatments.

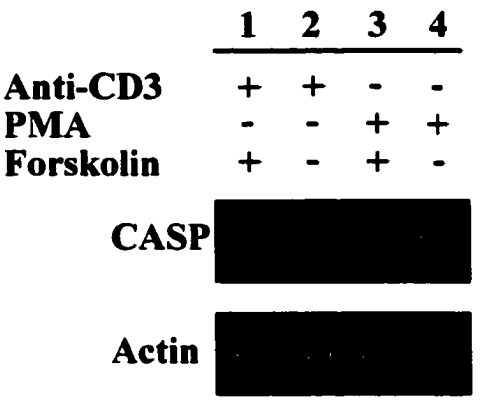


Figure 2.7. PKA inhibits CASP transcription. Anti-CD3-activated (lanes 1 and 2) and PMA-activated (lanes 3 and 4) Jurkat cells were pre-treated with forskolin (10 microM) (lanes 1 and 3). CASP transcription was markedly inhibited. Actin levels remained unchanged with all treatments.

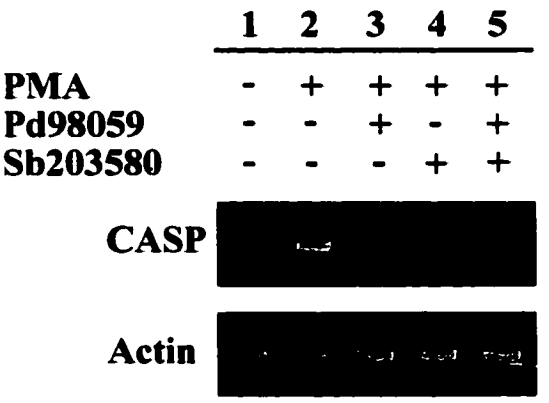


Figure 2.8. CASP transcription requires MAPKs ERK and p38. PMA-activated Jurkat cells (lanes 2-5) were pre-treated with Pd98059 (20 microM) (lane 3), SB203580 (10 microM) (lane 4), or both inhibitors (lane 5). PD98059 and SB203580 markedly reduced CASP transcription. Both inhibitors were required for complete inhibition of CASP transcription. Actin levels remained unchanged with all treatments.

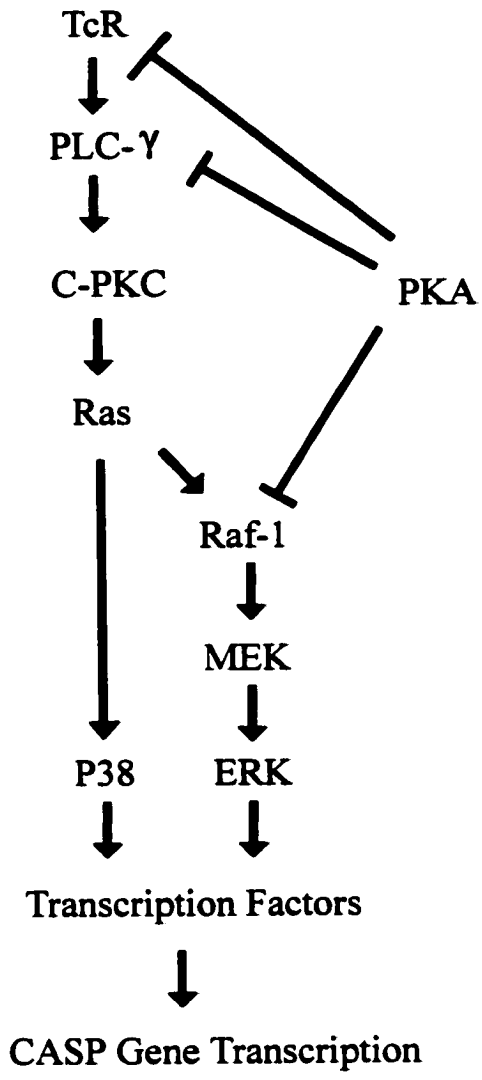


Figure 2.9. Proposed pathways leading to CASP transcription in Jurkat. Anti-CD3 activation of CASP proceeds though cPKC's and the MAPKs ERK and p38. The synthesis of transcription factors is required for transcriptional activation. PKA inhibits CASP activation both upstream of PKC, most likely at the level of PLC-gamma and the TcR, and downstream of PKC, most likely at the level of Raf. See Discussion for a description of PKA effects on TcR signaling.

2.4. DISCUSSION

CASP was originally cloned in our laboratory from NK-enriched human lymphocytes by a method of subtractive hybridization using Jurkat as a reference cell line. CASP is expressed in normal resting lymphocytes, HUT78 T cells, as well as the YT (NK-like) line. Recent reports showed that CASP is a scaffolding protein that interacts with cytohesin/ARNO guanine nucleotide exchange factors (GEFs), and potentially plays a role in signaling pathways generated by the ARF family of small GTPases. Here we report the genomic structure of the CASP gene, the site of transcriptional initiation, and we identify some of the immediate signaling pathways leading to CASP transcriptional activation in stimulated Jurkat T cells.

After identifying the transcriptional initiation site of CASP, we analyzed upstream genomic sequences for the presence of consensus sequences for transcription factors. Surprisingly, no significant regulatory elements, including TATA boxes or Sp1 sites could be identified. CASP therefore belongs to the growing group of TATA-less genes. The upstream region of CASP contains a number of consensus binding sites for transcription factors, some of which are lymphocyte-specific (discussed further in section 5). In order to determine some of the CASP transcriptional activation requirements in lymphocytes, we activated Jurkat cells with classical stimuli including anti-CD3 antibodies and the non-specific activator, PMA. The use of Jurkat cells in the subtractive hybridization procedure confirms the absence of CASP mRNA in resting Jurkat cells, and any detectable CASP transcripts would therefore be the result of *de novo* gene transcription. Both anti-CD3 and PMA treatments activated CASP expression in Jurkat cells. Costimulatory signals provided by anti-CD28 or mimicked by the use of ionomycin had no effect on CASP mRNA levels while they greatly enhanced IL-2 transcription. It is generally accepted that CD28 signals contribute to IL-2 activation on a transcriptional as well as a post-transcriptional level [183]. Clearly, CD28 does not contribute to CASP transcriptional activity and nor does it enhance CASP levels by potentially stabilizing CASP mRNA's. Although CASP 3' untranslated region (UTR) contains a number of

A+U-rich sequences (AUUUA) also found in the IL-2 3'UTR and believed to confer mRNA instability [184], the contrasting effects of CD28 signals on CASP and IL-2 mRNA levels may be due in part to the effects of CD28 on other destabilizing sequences found within the IL-2 3'UTR [185] as well as the IL-2 coding region [186]. No similar destabilizing elements could be found in the CASP cDNA. Additionally, costimulatory signals provided by CD28 and enhancing transcription of IL-2 clearly do not exert their effect on transcriptional regulatory sequences of the CASP gene.

CASP activation in Jurkat cells requires the synthesis of new transcription factors during the first few hours following TcR activation. In this study, we focused on the immediate factors rather than the newly synthesized transcription factors leading to CASP expression. PKCs are activated within minutes of TcR engagement [187]. Jurkat cells express PKC isoforms α, βI, βII, δ, ε, η, θ, μ, λ, ξ [20, 23-28]. Studies targeting individual PKC isoforms (α,β, δ, ε, θ) in T cells revealed that they transmit signals leading to various activation events including IL-2, IL2 receptor (IL2-Rα/CD25) expression, and TNF-α production [24, 29]. Recent reports support the notion that PKC-θ plays a pivotal role in T cell signaling and IL-2 activation [30, 31]. Moreover, PKC-θ specifically translocates to the site of T cell/APC contact when an antigen-specific T cell clone is activated by APCs in a setting that mimics physiological activation of T cells [32]. While PKC-θ may be crutial for T cell activation, other PKC isoforms clearly play important roles in various aspects of T cell activation. The use of the cPKC-specific inhibitor Gö 6976 in our study clearly shows that OKT3-dependent CASP activation proceeds through one or more cPKC isoform(s). nPKC-θ is unlikely to be involved in CASP activation, especially since CD28 costimulation normally leading to nPKC-θ activation [67] and optimal IL-2 activation as observed in our experiments, had no effect on CASP levels. Other nPKCs including PKC-θ may be partially involved in PMA-induced CASP activation, but that is most likely the result of cross-talk between PKC pathways in response to the artificially prolonged activation potential of the phorbol ester.

PKA is generally accepted as a negative regulator of lymphocyte activation [177, 188, 189]. CASP transcription is markedly inhibited by PKA activators, including cAMP and forskolin. The site of PKA activity resulting in CASP inhibition is most likely exerted both upstream of PKC, at the level of PLC-gamma [190, 191] and other TcR-proximal signaling pathways [192], and downstream of PKC, most likely at the level of Raf [193, 194] (Figure 2.9).

The reduction in CASP expression in response to ERK and p38 MAPK inhibitors clearly shows the involvement of transcription factors downstream of MAPKs. However, it is still unclear whether immediate MAPK downstream effectors act directly on CASP or whether multiple transcription factor activation events are required before CASP expression. CASP expression in YT cells is constitutive and unaffected by PKC or MAPK inhibitors (data not shown). The transformed phenotype of these cells most likely induces the expression of transcription factors that bypass all PKC and MAKP signaling pathways to activate CASP. In the case of YT cells, the different transcription initiation site may be the result of a transcription factor profile resulting in abnormally high expression of CASP. Further studies are needed to identify transcription factors that exert their effect directly on CASP in activated Jurkat cells, as well as YT cells.

In summary, CASP expression can be induced in T cells activated through the T cell receptor. The association of CASP with cytohesin and the effects of cytohesin on ARF suggest that CASP may be a regulator of ARF function in response to T cell activation stimuli. Our findings may represent one aspect of a potential mechanism for lymphocyte specific ARF function.

3. CASP INTERACTS WITH THE CYTOHESIN/ARNO FAMILY OF GUANINE EXCHANGE FACTORS

3.1. INTRODUCTION

The cytohesin/ARNO family of guanine nucleotide exchange factors (GEFs), characterized by an N-terminal coiled coil, a Sec7 homology domain, and a C-terminal pleckstrin homology (PH) domain, have emerged as regulators of the ARF family of small GTPases [154, 155, 195]. ARF GTPases are divided into three classes based on their gene structure. Class I ARFs (ARFs 1-3) are Golgi-associated GTPases regulating vesicle formation [109-111]. Little is known about class II ARFs (ARFs 4 and 5) except that ARF5 may be involved in BFA-resistant Golgi/ER retrograde traffic [112] and TGN vesicle traffic [113]. ARF6, the only member of Class III ARFs, associates with cell membranes and is involved in endocytosis and actin rearrangements [115, 116, 134]. The study of ARF function has been focused primarily on the ER and Golgi where different anterograde and retrograde vesicle trafficking pathways occur. It is generally accepted that COPII coated vesicles budding from the ER carry cargo proteins to the ER/Golgi intermediate compartment where they are replaced by COPI coated vesicles [117]. Sar1 is the major small GTPase implicated in the formation of these vesicles [118, 119] while the ARFs control COPI as well as clathrin coated vesicle formation and traffic in and around the Golgi [120-122].

The cytohesin/ ARNO GEFs regulate ARFs through the Sec7 homology domain by facilitating a GDP/GTP exchange, converting inactive GDP-bound ARFs to their active GTP-bound state. There are currently four known members of the cytohesin/ ARNO family. The first was originally cloned in our laboratory and was designated B2-1 [152]. It was later renamed by others to cytohesin-1 [153]. ARNO is also known as cytohesin-2 and ARNO3 is the human homolog of mouse GRP1 [156]. Another member of the cytohesin/ARNO family, cytohesin-4, was recently identified in blood cells [158]. To simplify nomenclature, we will follow the designations published in genbank:

cytohesin-1, ARNO, ARNO3 and cytohesin-4. The specificity of cytohesin/ARNO members to the various ARFs appears to be mediated primarily by the Sec7 domain. All cytohesin/ARNO members activate ARF-1 [156, 158], while cytohesin-1, ARNO and ARNO3 (but not cytohesin-4) activate ARF6 [158-160]. Cytohesin-1 can activate ARF3 [154, 161] while both cytohesin-1 and 4 can activate ARF-5.

All four members of the family are highly similar on a structural basis. In addition to the Sec7 homology domain, the carboxy-terminal PH domain allows cytohesin/ARNO interactions with membranes by binding to various polyphosphoinositides [162-165]. While the PH domains of cytohesin-1 and ARNO seem to bind non-selectively to various phosphoinositides, ARNO3 shows increased affinity to PtdIns-3,4,5-P_3, a product of PI3-Kinase activation [164, 166]. Generally, while the PH domain anchors the cytohesin/ARNO GEFs to membrane structures, the Sec7 domain facilitates the function of ARF in vesicle formation.

The N-terminal coiled coil motif, reminiscent of leucine zipper domains, is a signature domain of all the cytohesin/ARNO members and still the most elusive. Recently, we showed that this domain targets the cytohesin/ARNO proteins to the Golgi [196, 197]. The coiled coil motif most likely interacts with at least one adaptor protein that contains a similar domain and facilitates the higher architecture of signaling complexes that regulate vesicle formation. The only protein known to interact with the N terminus of a cytohesin/ARNO protein (mouse homolog of ARNO3, GRP1), is GRASP, a scaffolding protein of unknown function containing a coiled coil domain [167]. GRASP was also named Tamalin by others [180]. Here we report the interaction of cytohesin/ARNO proteins, particularly cytohesin, with a GRASP related scaffolding protein, CASP, originally cloned in our laboratory from NK enriched human lymphocytes [3]. CASP and GRASP share a similar domain profile, with an N-terminal PDZ domain, a central coiled coil motif, and C-terminal domain of unknown function.

3.2. EXPERIMENTAL PROCEDURES

3.2.1. Plasmids and cells

A Cytohesin fragment coding for residues 1- 68 (Cytohesin-N) [197] was subcloned in frame into the *Nco*I site of the plasmid vector pAS2-1 (Clontech) downstream of sequences encoding the Gal4 DNA binding domain (Gal4 BD). A Human B-cell cDNA library subcloned into the *Xho*I site of the activation domain plasmid pACT2 (Clontech) and the PJ69-4A yeast strain for the yeast-two-hybrid analysis were generous gifts from Dr. C. McMaster (Biochemistry, Dalhousie University, Halifax, NS, Canada).

A cytohesin-N *Bam*HI fragment coding for a.a. 1-54 was subcloned in pRSET A (Invitrogen) for generating recombinant 6His- cytohesin-N fusion protein. Plasmids for generating recombinant glutathione S transferase (GST)/CASP fusions (CASP a.a. 151-201 and a.a. 151- 241) were prepared by amplifying the CASP cDNA region coding for the coiled coil motif, TA cloning of the PCR fragments (Invitrogen) then subcloning into the appropriate pGEX vector (Amersham). The sense primer used for amplifying the coiled coil region was 5'-AAGCTTATCAGATCGTCCGGAAACCTGC-3'. Antisense primers were AS5 (5'-AGACGATGTTCCTGTAACTGC-3') and bish2 (5'-TGGATAATCGATTCCGGTCC-3'). Recombinant GST/CASP proteins lacking a significant portion of the coiled coil domain (a.a. 179-195) were generated by a similar manner using a CASP cDNA with an internal *Pst* I deletion.

CASP cDNA with the stop codon removed and CASP cDNA coding for the coiled coil domain (CASP (CC)) were subcloned into a modified (leader sequence removed) Sec Tag vector (Invitrogen), designated Sec CMV. In these constructs, the CASP full cDNA and the CASP coiled coil cDNA portion were cloned in frame with downstream sequences encoding myc and 6-His tags, and were under the control of the cytomegalovirus (CMV) promotor. *Pst I* deletion mutants of CASP and CASP (CC) lacking the majority of the coiled coil motif (CASP* and CASP (CC*), respectively)

were subcloned into Sec CMV in a similar manner. Primers used for amplifying the CASP (CC) and (CC*) cDNA were ZipATG (5'-GACCTGATGAGATCGTCCGGAAACCTGCTAAC-3') and ZipAS1 (5'-CAGACAATTCATCCAAGTCCATG-3'). Cytohesin full cDNA containing a stop codon was cloned into the Sec CMV vector downstream of an HA tag. Cytohesin-N, ARNO2-N and ARNO3-N fragments corresponding to the N termini of cytohesin, ARNO and ARNO3 respectively [197] were subcloned into a CMV/HA/Myc plasmid in frame with upstream sequences encoding an HA tag and downstream sequences encoding a Myc tag, under the control of the CMV promotor. COS-1 cells used for transfections were generously provided by Dr. K. Too (Biochemistry Department, Dalhousie University, Halifax, Nova Scotia), and were maintained in D-MEM media supplemented with 10% FBS and antibiotics.

3.2.2. Yeast Two Hybrid Analysis

The cytohesin-N/pAS2-1 bait vector was transformed into PJ69-4A cells using a standard LiAc transformation protocol. Yeast were plated on minimal medium deficient in Trp. Resistant yeast clones were grown overnight at 30 degrees in the same medium and Gal4 BD/B2-1 fusion protein production was confirmed by western blot analysis of yeast lysates using anti-Gal4 BD monoclonal antibodies (Santa Cruz Biotech). Yeast containing the bait construct were then transformed with 25 micrograms of the human B cell cDNA pACT2 library, plated on minimal medium lacking Trp/Leu/His +50 mM 3-aminotriazole (3-AT), and incubated at 30 degrees for 5 days until colonies appeared. Colonies were patched onto a new Trp^-/Leu^-/His^-/ 3-AT plate, grown overnight and transferred onto nitrocellulose filter paper for a secondary β-galactosidase screen. Positive yeast clones were grown overnight and lysed with glass beads to retrieve pACT2 plasmids. pACT2 inserts were amplified by PCR using primers gad5 (5'-GCGTTTGGAATCACTACAGGG-3') and gad3 (5'-GGTGCACGATGCACAGTT-GAA-3"), cloned into the PCR II vector (Invitrogen) and sequenced commercially using

an automated fluorescent Licor sequencer. Sequences were analyzed online using the BLAST search program at the National Center for Biotechnology Information website.

3.2.3. In vitro recombinant protein interaction assay

To purify GST fusion proteins, DH5 alpha cells harboring the CASP/pGEX constructs were induced with 1 mM IPTG for 3 hours at 37 °C. Cells were sonicated briefly in PBS 1% TX-100, and lysates were incubated with glutathione beads (Sigma) for 1 hour at room temperature. Beads were washed three times in PBS 1% TX-100 and resuspended as a 50% slurry in PBS 0.5% TX-100. In order to purify 6-His/ Cytohesin-N, ARNO-N or ARNO3-N fusion proteins, 100 ml BL21(DE3) pLysS cells (Invitrogen) harboring each of the Cytohesin, ARNO and ARNO3 N-terminal pRSET constructs were induced with 1 mM IPTG for 3 hours at 37 C. Cells were sonicated briefly in 6M Guanidine-HCl pH 8.0 prior to incubation with 200 µl of Ni-beads (Qiagen). Beads were washed twice with 8M urea pH 8.0 and proteins were refolded on the beads by sequentially washing in decreasing concentration of urea and increasing volumes of PBS 0.5% TX-100. Beads were finally washed twice in PBS 0.5 %TX-100 and bound proteins were eluted with 700 µl of 0.5 M immidizole in PBS. 10 mM β-mercaptoethanol (β-ME) was added to maintain solubility.

Binding assays were performed by incubating 10 µl of glutathione beads with bound GST fusion proteins with 50 µl of 6-His/ cytohesin-N, ARNO-N or ARNO3-N eluate in 500 µl of TBS-0.5% tween20/ 10 mM β-ME for 30 minutes at room temperature. Glutathione Beads were washed twice with TBS-tween and subjected to PAGE. Bound 6-His/ Cytohesin-N, ARNO-N or ARNO3-N fusion proteins were detected by monoclonal 6-His antibodies (Santa Cruz Biotech) and enhanced chemiluminescence (ECL, Amersham).

3.2.4. COS-1 transfection and protein binding assays

COS-1 cells, seeded in 6-well plates, were tranfected the following day with 0.5 µg of the appropriate CASP/Sec CMV construct (CASP (CC) or CASP (CC*)), 0.5 µg of

either cytohesin-N, ARNO-N or ARNO3-N/ CMV/HA/myc construct and 4 µl of superfect (Qiagen), in the presence of FCS and antibiotics. Cells were lysed 24 hours post-transfection in 1 ml of 1 x TBS-T supplemented with 0.5% NP-40, 1 uM PMSF and Leupeptin. First, lysates were cleared by centrifugation at 16,000xg for 5 minutes and then incubated with 1 ug of polyclonal anti-HA antibodies (Santa Cruz) and 10 ul of 50% agarose bead slurry (Santa Cruz) at room temperature with constant agitation for 30 minutes. Beads were washed once with 1 x TBS-T and subjected to PAGE. CASP and cytohesin/ARNO/ARNO3 proteins were detected by western blotting using monoclonal anti-Myc antibodies (Santa Cruz) and enhanced chemiluminescence (ECL, Amersham).

3.2.5. Immunofluorescence

COS-1 cells, grown on glass coverslips in 6-well plates, were transfected with 1µg of CASP/ CMV plasmid and 4 µl of superfect (Qiagen). 0.5 µg of CASP or CASP* were also doubly transfected with 0.5 µg of Cytohesin in the same manner. 22 hours post-transfection, cells were starved for 2 hours in a balanced salt solution (136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, 5 mM sodium phosphate, 2 mM $NaHCO_3$, and 25 mM Hepes pH 7.4), then stimulated with 100 ng/ml of murine EGF for 3 minutes at 37 degrees celcius. Unstimulated and EGF-stimulated cells were fixed in 4% paraformaldehyde in PBS (pH 7.2) for 20 minutes, permeabilized with 0.1% TX-100 in PBS, and blocked with goat serum at 1:100 dilution in PBS 0.1% TX-100. Primary and secondary antibodies (1:1000 in PBS 0.1% TX-100) incubations were 20 minutes each at room temperature. Receptor grade murine EGF was purchased from Sigma. Anti-myc antibodies were from Santa Cruz. Polyclonal anti-cytohesin antibodies were a generous gift from Dr. S. Bourgoin (Laval). CY3-conjugated anti-rabbit and anti-mouse antibodies were purchased from Sigma. Alexa488-conjugated anti-rabbit and anti-mouse antibodies were purchased from Molecular Probes.

3.3. RESULTS

3.3.1. The N terminus of cytohesin interacts with CASP in yeast

Yeast two-hybrid screening based on the Gal4 system and using cytohesin a.a. 1-54 as bait identified three potential clones. Interaction of all three clones with the N terminus of cytohesin was confirmed in yeast by patch plating and repeated secondary β-galactosidase screenings. All three clones were sequenced, two of which corresponded to CASP, a gene originally cloned in our laboratory from a human Natural Killer enriched population of lymphocytes [3]. The third clone corresponded to a tRNA gene and was unlikely to be a true binding partner of cytohesin. Both isolated CASP clones were identical and most likely represent multiple copies of the same clone in the library. The interacting CASP clones code for a truncated CASP protein that includes the carboxy terminus and the entirety of the coiled coil domain (Figure 3.1). The presence of coiled coil motifs in both the bait and the target proteins suggested to us that the cytohesin/CASP interaction was mediated by these motifs and prompted us to confirm this interaction *in vitro* and in a cellular system.

3.3.2. The N terminus of cytohesin interacts with the coiled coil domain of CASP *in vitro*

The N terminus of cytohesin harbors a coiled coil motif that most likely interacts with another coiled coil domain. The presence of such a domain in the truncated CASP protein expressed in yeast prompted us to confirm the interaction of cytohesin with the CASP coiled coil *in vitro*. We were unable to produce recombinant CASP protein efficiently in *E. coli* as it had a tendency to precipitate. We therefore made shorter GST fusion proteins that included specifically the coiled coil domain of CASP (Figure 3.2, A and B). These proteins were more soluble, particularly if they were used shortly after they were produced. We also circumvented the solubility problem by keeping the GST recombinant proteins coupled to the glutathione beads before performing the binding

assays. Additionally, we produced deletion mutants of the same CASP proteins lacking a significant portion of the coiled coil. In addition to the removal of key elements of the coiled coil, this deletion also affected the secondary structure of the remaining alpha helix. Recombinant N-terminal cytohesin (cytohesin-N) corresponding to a.a.1-54 and fused to a 6-His tag was produced in E.coli and tested for its interaction with the CASP/GST proteins. Recombinant cytohesin-N could only be captured *in vitro* by CASP bound to glutathione beads when the coiled coil of CASP remained intact. The deletion mutants of CASP on the other hand (LC* and SC*) failed to interact with cytohesin-N (Figure 3.2, C).

3.3.3. Cytohesin and CASP coiled coil domains interact in COS-1 cells

We attempted to confirm the validity of the cytohesin/ CASP interaction that we observed *in vitro* by co-transfecting COS-1 cells with cytohesin-N and CASP coiled coil domain (CASP (CC)) and testing for an interaction in COS-1 lysates. CASP expressed in eukaryotic cells cannot be detected with our anti-CASP antibodies generated against recombinant protein, possibly as a result of fundamental differences in CASP protein folding and/or post-translational modifications in eukaryotic cells. It was therefore necessary to fuse CASP (CC) with a myc tag for detection by western blotting. A 6-His tag was also fused to CASP (CC) in the hope of using nickel (Ni) beads on COS-1 lysates to co-purify cytohesin-N, but the Ni beads showed high non-specific affinity to COS-1 lysates. We therefore attempted to co-immunoprecipitate cytohesin-N and CASP (CC) using anti-HA and anti-cytohesin antibodies. Cytohesin-N was also fused to a myc tag for detection by western blotting using anti-myc antibodies. CASP (CC) readily co-precipitated with cytohesin-N from transfected COS-1 lysates using anti-HA antibodies (Figure 3.3). This interaction is specific to the coiled coil domain of CASP since the deletion mutant CASP (CC*) lacking the same portion of the coiled coil domain as the recombinant mutant CASP constructs used in the *in vitro* assay, showed no interaction with cytohesin-N (Figure 3.3, B). Furthermore, CASP (CC) was not precipitated with protein A agarose beads and antibodies without the presence of cytohesin-N. Expression

of the appropiate proteins in COS-1 cells was confirmed by immunoprecipitating myc-labelled proteins from lysates of the same transfections using anti-myc antibodies (Figure 3.3, C).

3.3.4. CASP interacts with other members of the cytohesin/ARNO family

All members of the cytohesin/ ARNO family are characterized by an N-terminal coiled coil motif. We therefore examined the binding specificity of CASP to the various members of this family of GEFs, including ARNO and ARNO3. Recombinant proteins corresponding to the N-termini of ARNO and ARNO3 (ARNO-N and ARNO3-N) harboring the coiled coil motif were produced in *E. coli* and tested for their ability to interact with GST/CASP recombinant proteins *in vitro*. Both ARNO-N and ARNO3-N were capable of interacting with an intact CASP coiled coil domain but not with the deletion variant of the same protein (Figure 3.4). These interactions were confirmed in COS-1 cells by co-transfecting HA-tagged ARNO-N or ARNO3-N with either CASP (CC) or CASP (CC*) and co-immunoprecipitation with anti-HA antibodies. CASP (CC) but not the deletion variant co-precipitated with both ARNO-N and ARNO3-N from COS-1 lysates (Figure 3.5, A). CASP showed no differential specificity to the various members of the cytohesin/ ARNO family in both our *in vitro* and COS-1 binding assays.

3.3.5. CASP intracellular localization is perinuclear in COS-1 cells

We have previously shown that the cytohesin, ARNO, and ARNO3 localize to the Golgi through their coiled coil motifs. We suspected that CASP may be a Golgi protein since it interacts with all three members of the cytohesin/ARNO family. Immunolocalization of CASP in transfected COS-1 cells clearly shows a perinuclear signal that is characteristic of the Golgi. To our surprise however, CASP did not co-localize with the Golgi marker Manosidase II (Figure 3.6) or Giantin (not shown) nor did it co-localize with the ER marker GRP78 (data not shown). It did, however, partially overlap with the ER/Golgi intermediate marker ERGIC-53. This partial overlap was more

evident when COS-1 cells were treated with Brefeldin A (BFA), causing the redistribution of both ERGIC-53 and CASP into similar tubular structures (Figure 3.7). BFA caused the relocation of Manosidase II into the ER as expected [198, 199]. The CASP stained tubulo-vesicular structures were in proximity to, but clearly distinct from, the Golgi and the ERGIC-53 associated structures.

3.3.6. Co-localization of cytohesin and CASP is coiled coil dependent

Cos-1 cells were co-transfected with the CASP coiled coil (myc-tagged) and the cytohesin N terminus fused to the green fluorescent protein (GFP). Over-expressed GFP normally localizes in the nucleus of COS-1 and as a result, the cytohesin N/GFP fusion exhibited nuclear staining (Figure 3.8, A). The coiled coil of CASP co-localizes with the green fluorescent fusion protein (Figure 3.8, B), demonstrating an interaction between the two proteins. The deletion mutant of CASP (CC) with a disrupted coiled coil was evenly distributed throughout the cytoplasm and did not co-localize with cytohesin (Figure 3.8, E).

Cytohesin localizes to the Golgi when expressed at low levels in COS-1 cells [196, 197], but exhibits cytoplasmic distribution when over-expressed in CHO and PC-12 cells [163]. Furthermore, over-expressed cytohesin can be targeted to CHO and PC-12 membranes by the appropriate extracellular stimuli. Similarly, redistribrution of GRP1 (ARNO-3) was observed by others in COS-1 cells stimulated with epidermal growth factor (EGF) [160]. We examined the localization of cytohesin in COS-1 cells upon EGF stimulation and found that cytohesin, like GRP1, translocated to the plasma membrane (Figure 3.9A and B). Full length CASP and CASP*, a deletion mutant of CASP lacking a portion of the coiled coil domain, exhibited perinuclear localization that was unaffected by EGF stimulation (Figure 3.9, C-F). When cytohesin was co-expressed with CASP however, EGF stimulation caused the translocation of both cytohesin and CASP to membrane ruffles (Figure 3.10, D-F). In non-stimulated cells, both CASP and cytohesin exhibited a diffuse cytoplasmic distribution with little membrane association. CASP perinuclear localization in these cells was disrupted presumably as a result of cytohesin

sequestering CASP through the coiled coil-mediated interaction. EGF-induced redistribution of CASP to membrane ruffles in the presence of cytohesin is clearly dependent on CASP's coiled coil motif, since the deletion mutant CASP* failed to relocate under the same conditions (Figure 3.10, J-L). Furthermore, CASP recruitment to membrane ruffles is mediated by cytohesin since CASP could not relocate to ruffles when expressed alone (Figure 3.9).

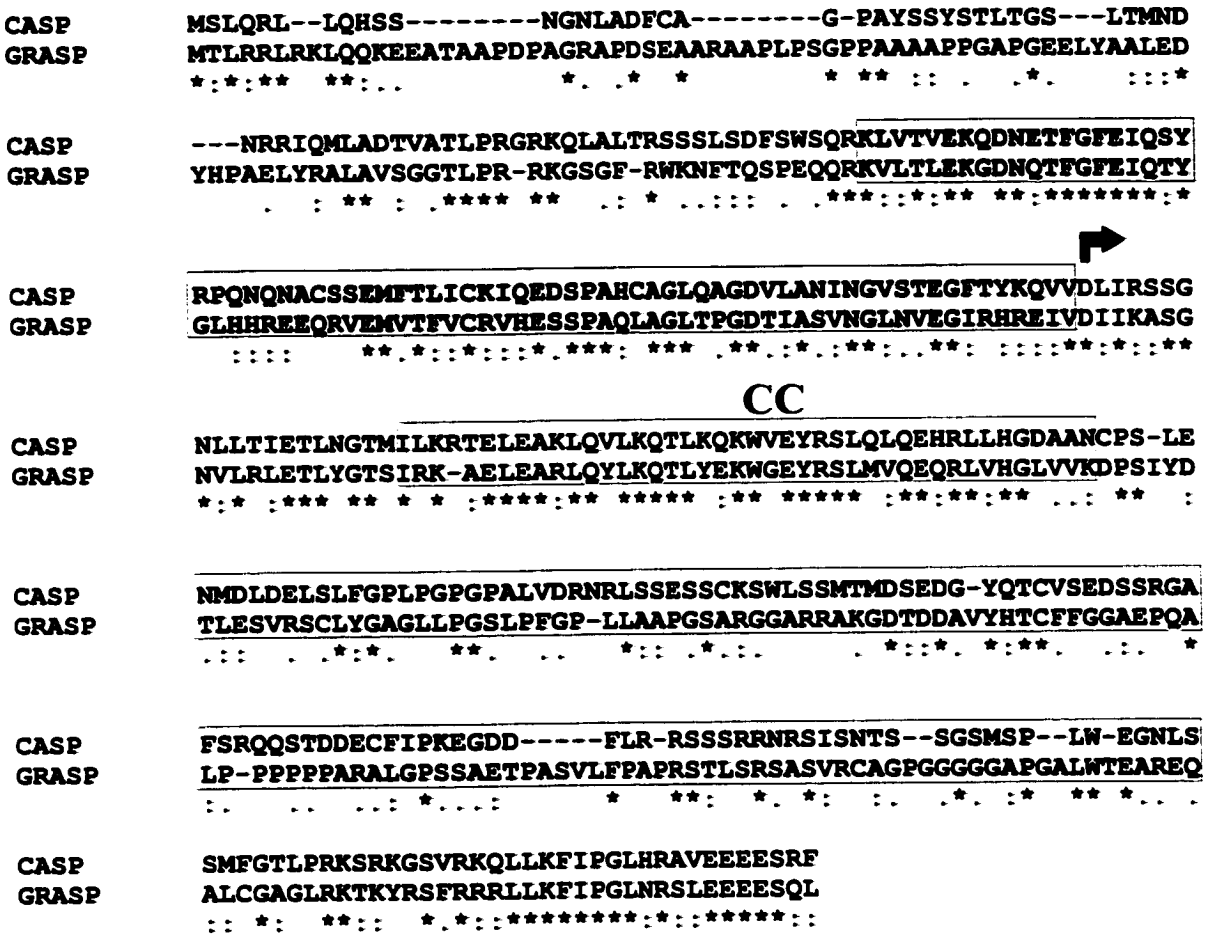


Figure 3.1. Comparison of CASP and GRASP proteins. The complete deduced amino acid sequence of CASP was aligned with GRASP, the only other known member of the CASP family, using Clustal W. (*) denotes matching amino acids, (:) denotes conserved substitutions and (.) represent semi conserved substitutions. The yellow box represents The N-terminal PDZ domain while the gray box represent the C terminal mystery domain of unknown function. The coiled coil region of CASP is shown in a clear box (CC). The greatest sequence divergence between CASP and GRASP is present at the N terminus and within the C-terminal mystery domain. The arrow shows the start of the partial CASP protein interacting with the cytohesin N-terminus bait used in the yeast two-hybrid screening.

Figure 3.2. CASP *in vitro* interaction with cytohesin is mediated by the coiled coil domain. A) Schematic representation of CASP coiled coil (CC) constructs fused to GST. A long construct (LC) contains parts of the PDZ domain and the mystery domain, MD. A deletion mutant of LC (LC*) lacks a significant portion of the coiled coil motif (dashed box). A short construct (SC) harbors the entirety of the coiled coil motif and part of the PDZ domain. A deletion mutant of SC (SC*) lacks the same region of the coiled coil as LC*. **B)** Coiled coil construct LC and SC and the corresponding deletion mutants (LC* and SC*, respectively), bound to glutathione agarose beads were visualised by western blotting using monoclonal anti-GST antibodies. A background band, possibly a GST truncation product that can be detected in all preparations (single arrowhead), and did not affect the outcome of the experiment. **C)** Recombinant 6-His-tagged N-terminal portion of cytohesin corresponding to a.a. 1-54 interacts *in vitro* with LC and SC constructs but not with the deletion mutant LC* and SC*. Recombinant cytohesin-N was detected with anti-His antibodies.

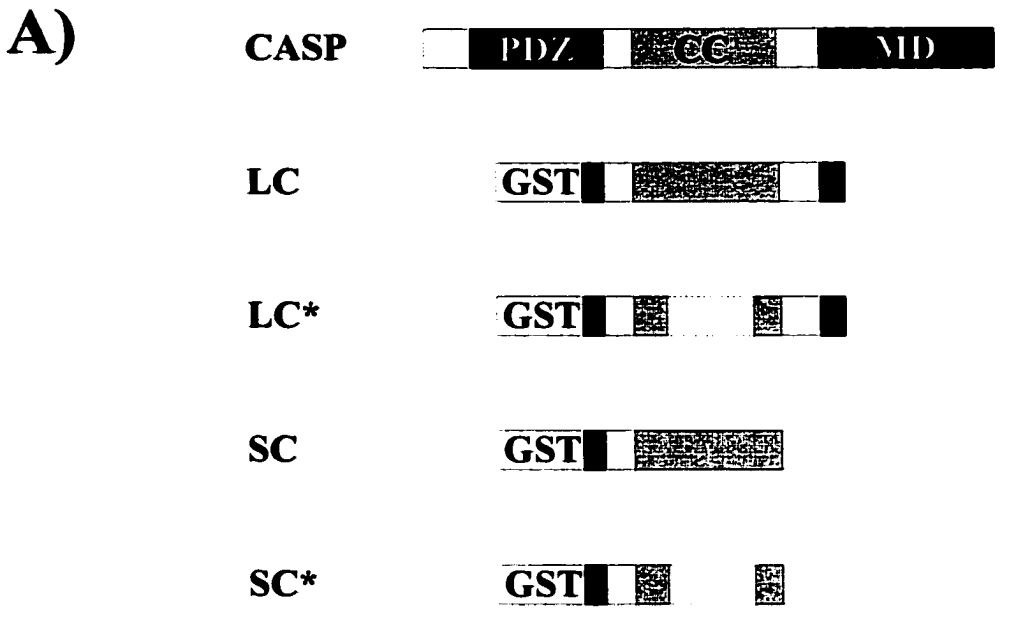

A)
CASP
PDZ
CC
MD
LC
GST
LC*
GST
SC
GST
SC*
GST
B)
Glutathione beads
LC LC* SC SC*
C)
Glutathione beads
LC LC* SC SC*

Figure 3.2

Figure 3.3. CASP *in vivo* interaction with cytohesin-N is mediated by the coiled coil domain. A) Schematic representation of cytohesin-N and CASP (CC)/CASP (CC*) plasmid constructs used for COS-1 transfection and *in vivo* protein interaction analysis. Cytohesin-N was cloned downstream of an HA tag sequence and upstream of a Myc tag sequence. CASP's coiled coil, CASP (CC), was cloned upstream of myc and 6-His tag sequences. CASP (CC*) is a deletion mutant of CASP (CC) lacking the same portion of the coiled coil domain as LC* and SC* described earlier. All constructs were under the control of the CMV promotor. **B)** COS-1 cells were transfected with cytohesin-N and/or CASP (CC) /CASP (CC*) plasmids as indicated. Following immunoprecipitation with polyclonal anti-HA antibodies and protein A agarose beads, cytohesin-N (double arrowhead) and CASP (CC)/ CASP (CC*) (single arrowhead) proteins were detected by western blotting using monoclonal anti-myc antibodies. **C)** Expression of cytohesin-N, CASP (CC) and CASP (CC*) in COS-1 cells was confirmed by immunoprecipitation of myc tagged proteins from the same lysates with polyclonal anti-myc antibodies followed by western blotting using monoclonal anti-myc antibodies. Arrows are same as above.

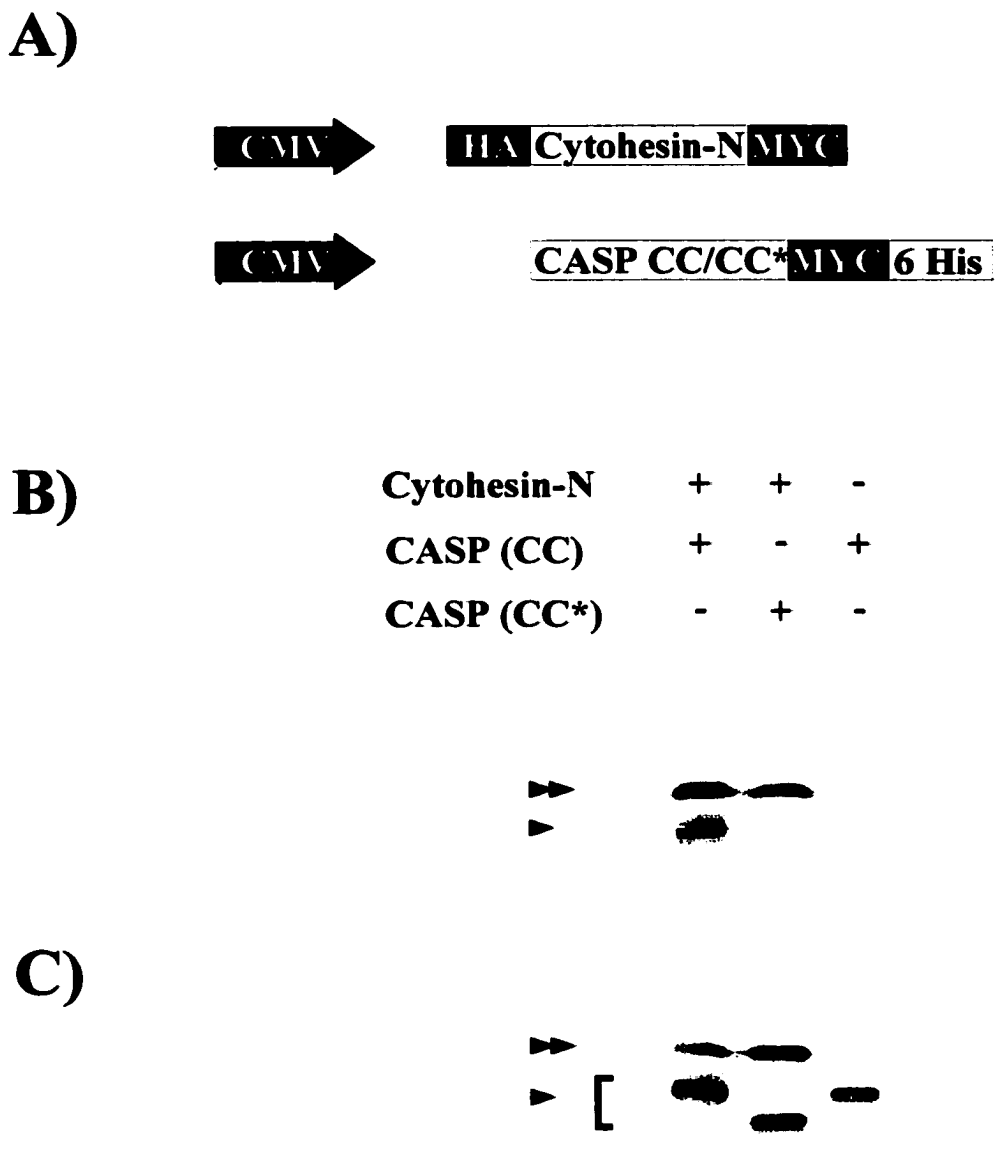

A)
CMV
HA Cytohesin-N MYC
CMV
CASP CC/CC* MYC 6 His
B)
Cytohesin-N + + -
CASP (CC) + - +
CASP (CC*) - + -
C)

Figure 3.3

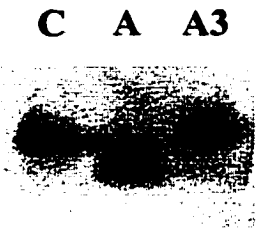
Figure 3.4. Interaction of CASP's coiled coil with cytohesin-N, ARNO-N, and ARNO3-N *in vitro*. **A)** Multiple alignment of the coiled coil motif found in the N termini of Cytohesin, ARNO and ARNO3. **B)** Recombinant 6-His tagged proteins corresponding to cytohesin a.a. 1-54 (C), ARNO a.a. 1-53 (A) and ARNO3 a.a. 1-58 (A3) were purified and then visualised by western blotting using anti 6-His antibodies. **C)** Recombinant C, A and A3 proteins interact *in vitro* with the GST tagged CASP construct LC bound to glutathione beads. No interaction could be detected with the deletion mutant LC*.

A)

```
Cytohesin-N   1 MEEDDSY----VPSDLTAEERQELENIRRRKQELLADIQRLKDEIAEVANE
Arno-N        1 ME-DGVYE----PPDLTPEERMELENIRRRKQELLVEIQRLREELSEAMSE
Arno3-N       1 MDEDGGGEGGGVPEDLSLEEREELLDIRRRKKELIDDIERLKYEIAEVMTE

Cytohesin-N     IENLGST 54
Arno-N          VEGLEAN 53
Arno3-N         IDNLTSV 58
```

B)



C)

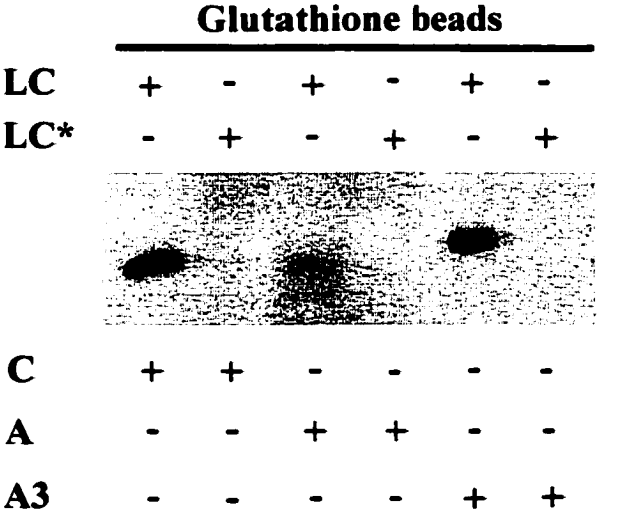


Figure 3.4

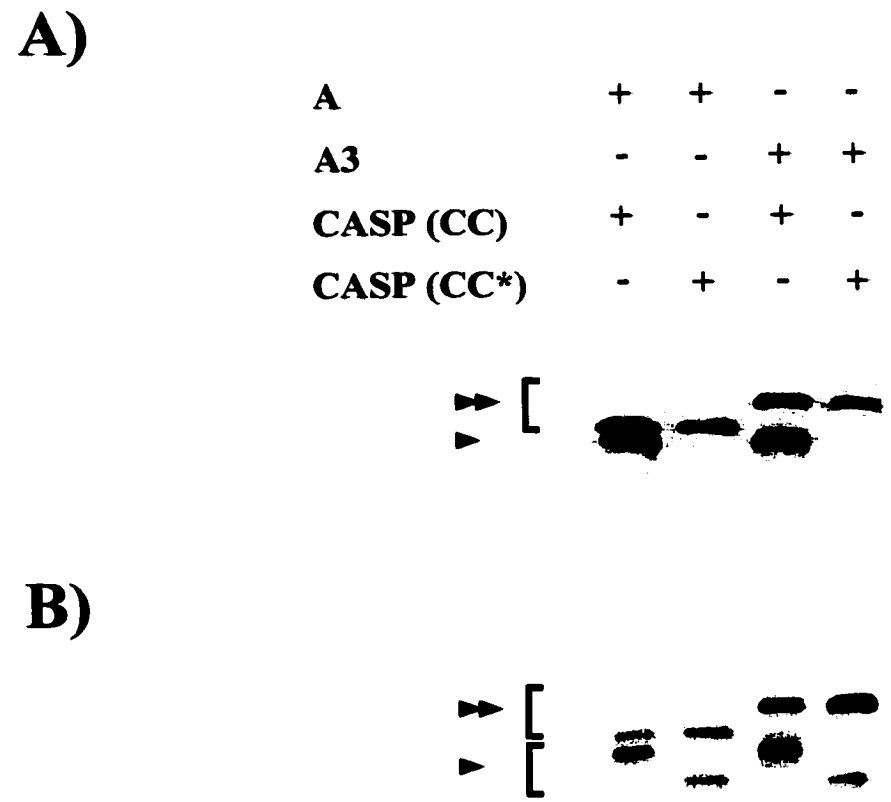


Figure 3.5. CASP interaction with ARNO-N and ARNO3-N *in vivo* is mediated by the coiled coil domain. A) cDNA sequences coding for ARNO a.a. 1-53 (A) and ARNO3 a.a. 1-58 (A3) were cloned downstream of an HA tag sequence and upstream of a myc tag sequence under the control of the CMV promotor. COS-1 cells were transfected with A, A3 and CASP (CC)/CASP (CC*) plasmids as indicated. A, A3, CASP (CC) and CASP (CC*) proteins were detected by western blotting using monoclonal anti-myc antibodies. **B)** Expression of A, A3, CASP (CC) and CASP (CC*) in COS-1 cells was confirmed by immunoprecipitation of myc-tagged proteins with polyclonal anti-myc antibodies followed by western blotting using monoclonal anti-myc antibodies. A and A3 are indicated with double arrowheads. CASP (CC) and CASP (CC*) are indicated with single arrowheads.

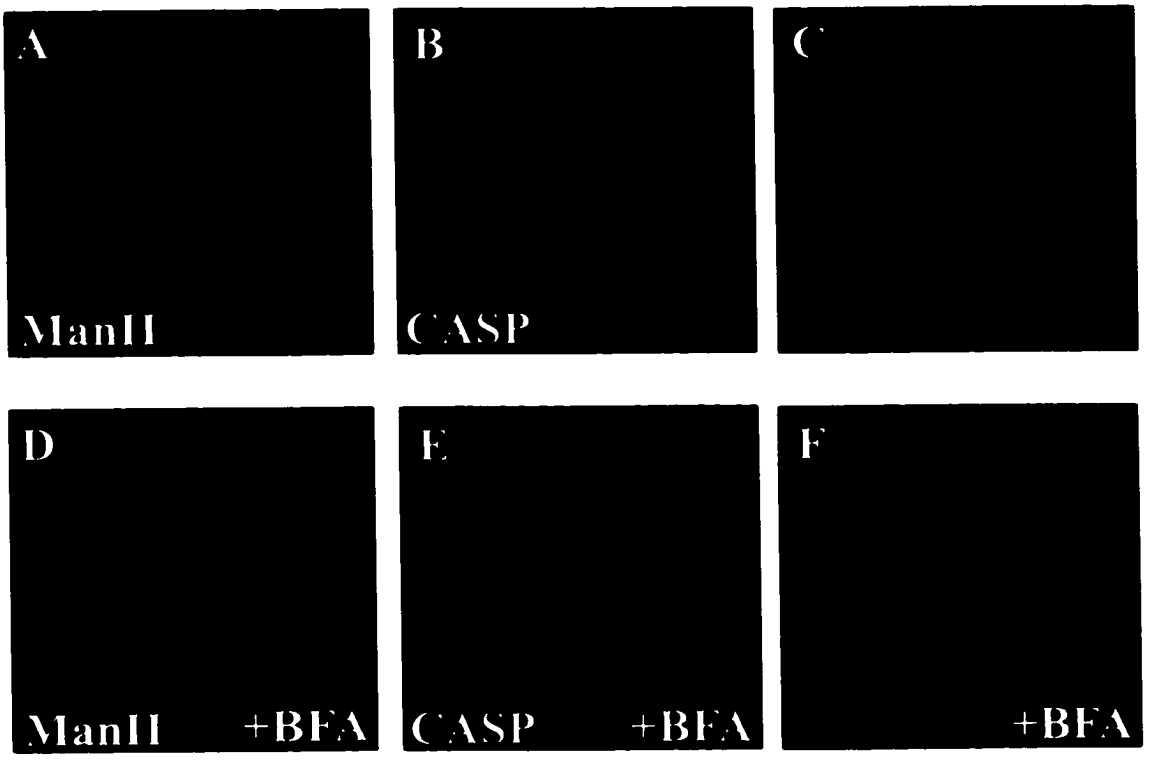


Figure 3.6. CASP localizes to a perinuclear region in COS-1 cells. COS-1 cells were transfected with the full length CASP cDNA. The cis-Golgi was stained with polyclonal anti-Mannosidase II and Alexa488-conjugated secondary antibodies **(A, D)**. CASP was detected in transfected COS-1 cells using monoclonal anti-myc and Cy3-conjugated secondary antibodies **(B, E)**. BFA treatment caused the diffusion of Mannosidase II staining and the redistribution of CASP into vesicular structures. Doubly stained images without BFA **(C)** or with BFA **(F)** were generated by superimposing images **A** and **B** or **D** and **E**, respectively.

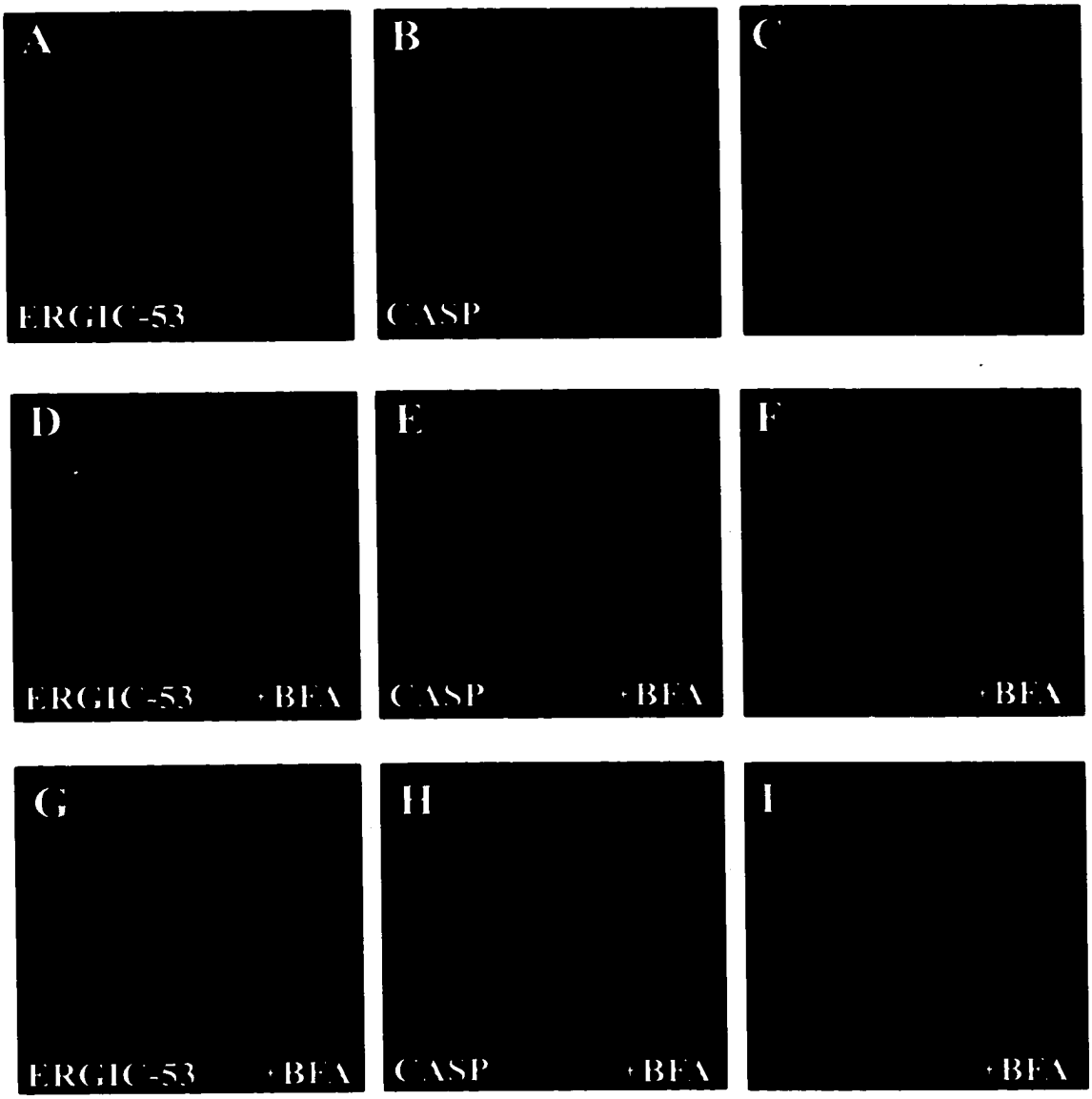


Figure 3.7. Partial overlap of CASP and ERGIC-53 in COS-1 cells. CASP-transfected COS-1 cells were doubly immunostained with monoclonal anti-ERGIC-53/Alexa488-conjugated secondary antibodies **(A, D)** and polyclonal anti-myc/Cy3-conjugated secondary antibodies **(B, E)**. BFA treatment caused the redistribution of both ERGIC-53 and CASP into vesicular structures. ERGIC-53 and CASP partial overlap (superimposed images **C** and **F**) was observed with and without BFA treatment. The same results **(I)** were observed when transfected COS-1 cells treated with BFA were stained with anti-ERGIC-53/Cy3-conjugated secondary antibodies **(G)** and polyclonal anti-myc/ Alexa488-conjugated secondary antibodies **(H)**.

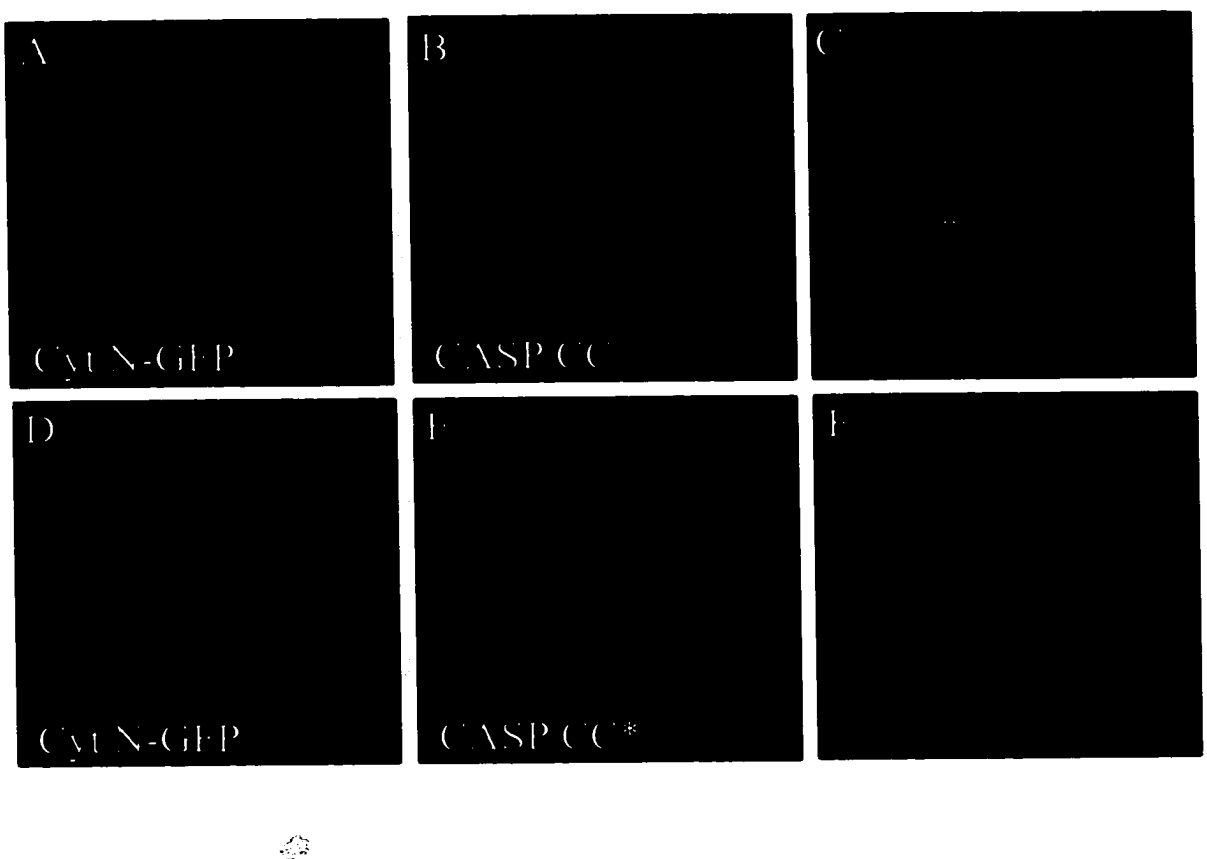


Figure 3.8. Interaction and colocalization of CASP and cytohesin coiled coils in COS-1 cells. CASP (myc-tagged) coiled coil (CASP CC) and the coiled coil deletion variant CASP CC*were co-transfected with cytohesin N-terminal coiled coil fused to GFP (Cyt N-GFP). CASP (CC) and CASP (CC)* proteins were visualized using monoclonal anti-myc antibodies and Cy3-labeled secondary antibodies. **C** and **F** represent superimposed images of **A/B** and **D/E**, respectively.

Figure 3.9. Effect of EGF on cytohesin, CASP and CASP* localization. COS-1 cells were transfected with full-length cytohesin (HA tagged), full length CASP (myc-tagged), or CASP* (lacking a portion of the coiled coil) then induced with EGF for 3 minutes **(B, D, F)**. Cytohesin was detected with polyclonal anti-HA antibodies and Alexa-488 secondary antibodies **(A, B)**. CASP and CASP* proteins were detected with monoclonal anti-myc antibodies and CY3-conjugated secondary antibodies (**C/D** and **E/F**, respectively).

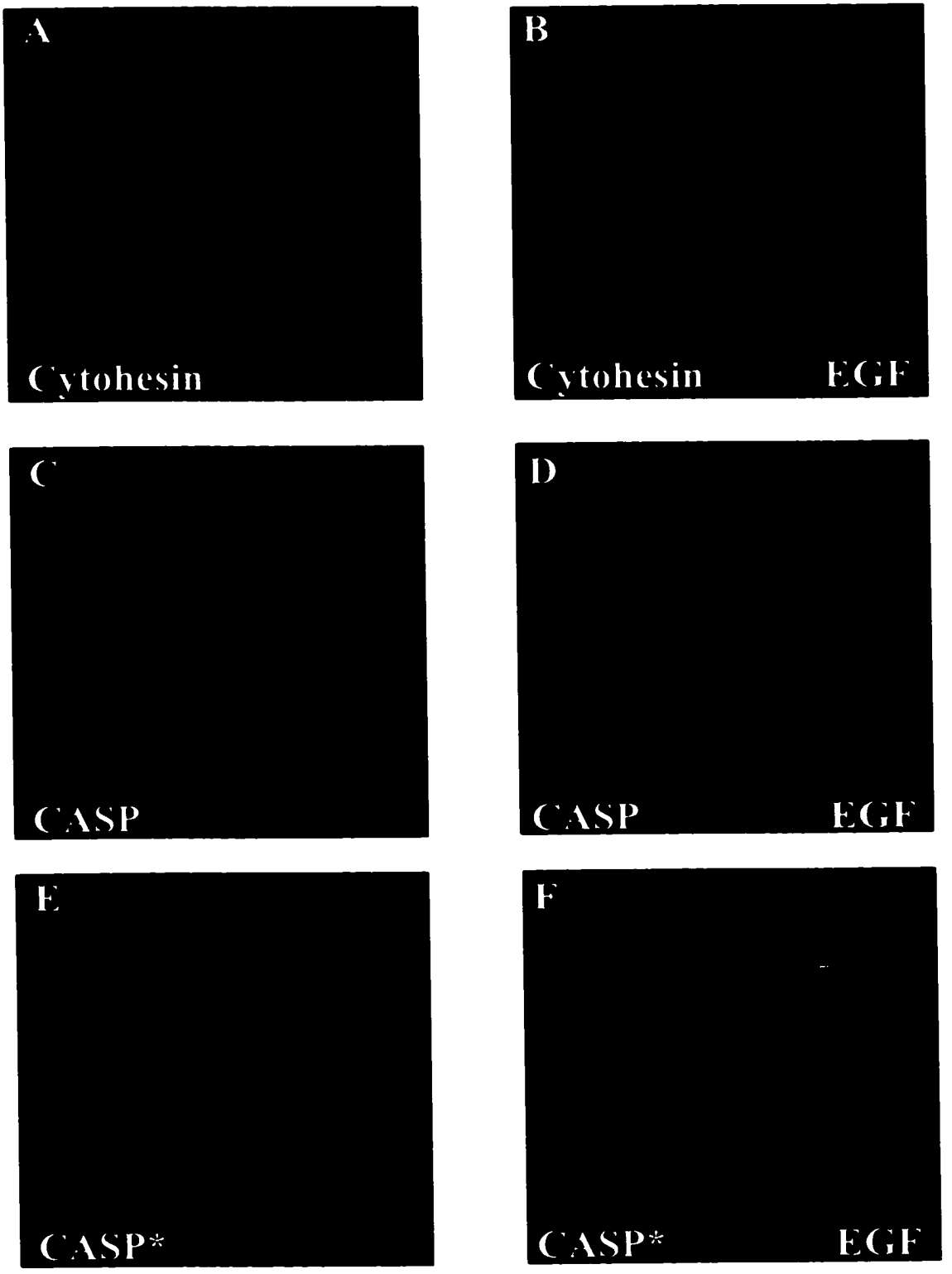


Figure 3.9

Figure 3.10. Colocalization of CASP and cytohesin in EGF-stimulated COS-1. COS-1 cells were transfected with cytohesin (HA-tagged)/CASP (myc-tagged) **(A-F)** or cytohesin/CASP* **(G-L)** then induced with EGF for 3 minutes **(D-F, J-L)**. Cytohesin was detected with polyclonal anti-HA antibodies and Alexa-488 secondary antibodies **(A, D, G, J)**. CASP and CASP* proteins were detected with monoclonal anti-myc antibodies and CY3-conjugated secondary antibodies **(B, E, H, K)**. Cytohesin/ CASP and cytohesin/ CASP* images were superimposed **(C, F, I, L)**. Identical results were obtained when cytohesin was detected with a polyclonal anti-cytohesin antibody (data not shown).

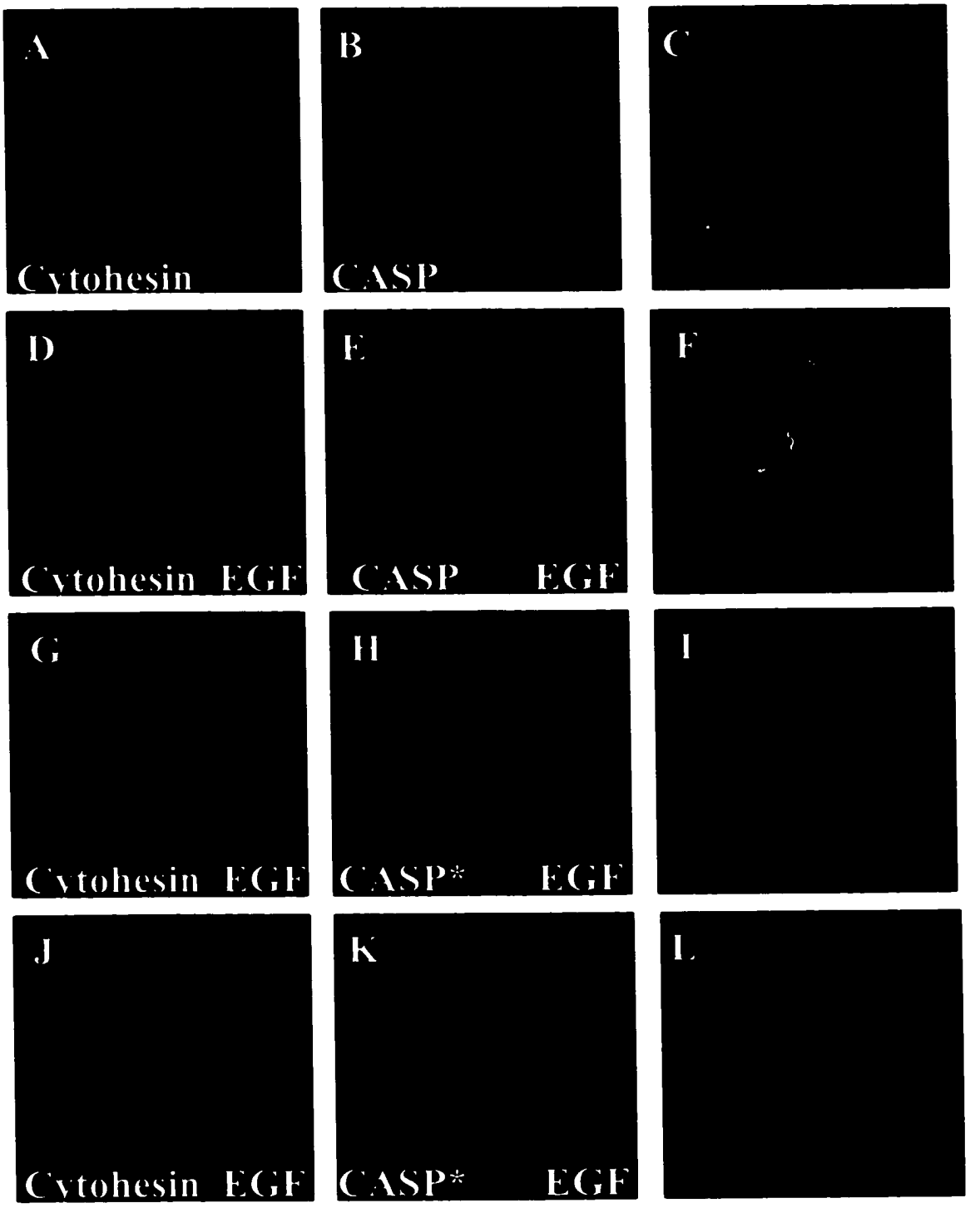

A
Cytohesin
B
CASP
C
D
Cytohesin EGF
E
CASP EGF
F
G
Cytohesin EGF
H
CASP* EGF
I
J
Cytohesin EGF
K
CASP* EGF
L

Figure 3.10

3.4. DISCUSSION

We took a yeast two-hybrid approach to identify proteins that specifically interact with the coiled coil domain found in the N terminus of cytohesin. Using cytohesin amino acids 1-54 as bait, we identified CASP as a potential binding partner. CASP was originally cloned in our laboratory from an NK/T cell population. EST database searches suggests the expression of CASP in other cell types such as CD34+ hematopoietic stem/progenitor cells, germinal center B cells, and activated T cells, as well as a number of cancers including adenocarcinoma, embryonal carcinoma, myeloma, melanoma and lymphomas. CASP contains at least two known protein interaction domains: an N-terminal PDZ domain and a coiled coil motif. The presence of a coiled coil in CASP suggested to us that the cytohesin/CASP interaction is mediated by this motif. *In vitro* binding assays with partial CASP recombinant proteins containing primarily the coiled coil motif and deletion mutants of the same protein in which the coiled coil motif is impaired, clearly demonstrates that this region of CASP specifically interacts with the coiled coil of cytohesin. Additionally, interaction assays in COS-1 cells expressing coiled coil constructs of cytohesin, CASP, and coiled coil deletion mutants of CASP show that the cytohesin/ CASP interaction is specifically mediated by the coiled coil motifs. CASP was identified by others as a cytohesin interacting protein by yeast two-hybrid screening of a differential expression dendritic cell library, and was submitted to genbank as a cytohesin-binding-protein (accession AF068836). In that case, however, the entirety of cytohesin was used as bait and the protein segments responsible for the interaction were never published. We are the first to confirm such an interaction both *in vitro* and in a cellular system, as well as identify the domains responsible for this interaction.

The specificity of the CASP coiled coil domain was tested by examining the interaction of CASP with other members of cytohesin/ARNO family, particularly ARNO and ARNO3. All three members are associated with the Golgi of COS-1 cells [197] and most likely play specific roles in ARF-mediated vesicle formation. CASP is capable of interacting with all three members of the family, at least in our experimental system.

There may be differential specificity with the various ARNOs at a lower expression level than that induced by the CMV promotor, but that remains to be tested. We were unable to test such an interaction by co-immunoprecipitating proteins from normal cell lysates due to the lack of functional CASP antibodies. Nonetheless, our data suggests that CASP may regulate or facilitate a specialized aspect of vesicle transport that involves at least one member of the cytohesin family in hematopoietic cells.

The interaction of CASP with cytohesin/ARNO/ARNO3 in COS-1 cells suggests an association of CASP with the Golgi complex. Immunofluorescence experiments clearly showed the association of CASP with Golgi proximal structures. Co-localization studies with Golgi markers, on the other hand, showed that CASP was not directly associated with the Golgi. The only marker tested that exhibited partial overlap was ERGIC-53, a well recognized component of the ER-Golgi intermediate region [200]. This partial overlap persisted even after BFA treatment, which caused the redistribution of both CASP and ERGIC-53 into similar but not identical tubulo-vesicular structures. Others have shown that BFA treatment causes the dissociation of the Golgi stack and the recycling of some Golgi components such as manNOSidase-II into the ER, while other components such as ERGIC-53 and the Golgin GM130 cluster in distinct tubular structures [199]. It appears that CASP is associated with a dynamic compartment that normally interacts with the Golgi and fuses with vesicular Golgi remnants after BFA treatment. This compartment may be part of the ER/Golgi intermediate region, but is clearly distinct from the ERGIC-53 associated structures. The physical interaction of CASP with cytohesin and the apparent association of CASP and cytohesin proteins with different but overlapping compartments of the perinuclear region, most likely reflect the dynamic or inducible nature of CASP's function. The physical interaction of endogenous CASP with cytohesin and/or ARNO/ARNO3 at the Golgi may require stimuli that remain unidentified to date.

Intracellular localization studies in EGF-stimulated COS-1 cells over-expressing CASP and cytohesin clearly show the functional interaction of the two proteins. Furthermore, the coiled coil interaction is responsible for the colocalization observed.

The translocation of CASP in the presence of cytohesin upon EGF stimulation is likely mediated by cytohesin's PH domain, a property of cytohesin reported by others in PC-12 cells [163]. Cytohesin translocation to membranes is similar to GRP1 and ARNO translocation reported by other groups [162, 201], and is consistent with the ability of all three proteins to activate ARF6 in vitro and more importantly membrane bound ARF6 *in vivo* [159, 202]. CASP over-expressed alone failed to localize to the membrane, most likely as a result of the overwhelming CASP levels compared to endogenous cytohesin (and potentially ARNO/ARNO3) levels. CASP's inability to disrupt cytohesin translocation to the membrane in response to EGF is expected since cytohesin's interaction with CASP and membranes is mediated by two different domains: the coiled coil and the PH domain, respectively. The association of CASP and cytohesin at membranes following EGF stimulation suggests that cytohesin is capable of recruiting CASP to the appropriate site of activity in response to specific stimuli.

Interestingly, CASP is not the only protein capable of interacting with cytohesin/ARNO proteins through their N-terminal coiled coil domain. GRASP (GRP1 associated scaffolding protein), the only other known member of the CASP family, was recently cloned from a mouse library and shown to interact with both ARNO and GRP1 (ARNO3) [167]. GRASP expression is induced by trans-retinoic acid (tRA) in embryonal carcinoma PC19 cells and its interaction with GRP1 occurs at the cell periphery. The ability of GRASP to interact with cytohesin was never established since it is not expressed in PC19 cells. The structural similarity between CASP and GRASP and their capability of interacting with multiple members of the cytohesin/ARNO family suggest that the CASP/GRASP family of scaffolding proteins play a role in ARF-mediated vesicle formation at a number of cellular locations. CASP and GRASP are likely to be recruited by cytohesin/ARNO members and may act as scaffolding proteins to bring in other proteins to the site of activity. Other domains of CASP and GRASP, particularly the PDZ domain and the uncharacterized C-terminal domain, may also target those proteins to the site of their function, or may recruit additional proteins into a larger signaling complex. In any case, the roles of CASP and GRASP is clearly not ubiquitous, since

GRASP is only expressed in response to trans-retinoic acid stimulation, and both CASP and GRASP show tissue specific distributions.

In summary, we identified the first protein to interact with the N-terminal coiled coil domain of cytohesin. The hematopoietic expression of CASP suggests a role for CASP in a cell type specific inducible aspect of vesicle formation, either at the level of the Golgi or the plasma membrane associated signaling event, where it interacts with the appropriate cytohesin/ARNO member(s) and their target ARF(s). We are currently trying to further pinpoint the perinuclear compartment targeted by CASP as well as map the region responsible for this perinuclear localization.

4. CHARACTERIZATION OF CASP'S PDZ DOMAIN

4.1. INTRODUCTION

The PDZ domain consists of a region of 80-100 amino acids originally described in the neuron-specific Postsynaptic Density-95 protein (Psd-95), and subsequently in the drosophila Discs Large protein(Dlg) and the mammalian tight junction protein, ZO-1 (Zonula Occludens) [9, 203]. This domain was previously referred to as GLGF for the amino acid motif found within, or DHR (Disc-large Homology repeat), but it is now known as PDZ for the three proteins in which it was originally described (Psd-95/Dlg/ZO-1). All three proteins are members of the membrane-associated family of guanylate kinases (MAGuK), consisting of multiple PDZ domains, one SH3 domain and a protein binding domain similar to guanylate kinase. MAGuKs are associated with the plasma membrane and localize to synapses which are large organizational centers comprising a wide array of cytoskeletal, cytoplasmic and trans-membrane proteins such as receptors and ion channels (reviewed in [204]). More than 150 PDZ-containing genes have been cloned from human alone, the majority of which associate with membranous structures. Some PDZ containing proteins are not exclusive to membranes and can shuttle between membrane complexes and the nucleus where they exhibit transcriptional activity. The membrane associated MAGuK CASK/Lin2 for example, can enter the nucleus under some circumstances by interacting with the nuclear factor Tbr-1 [205]. L-periaxin, a membrane associated protein of myelin forming Schwann cells can translocate to the nucleus using nuclear localization signals found within the PDZ domain [206].

Cytoskeletal elements inherently interact with membrane associated complexes such as synapses. Some PDZ containing adaptor proteins have been shown to link membrane signaling complexes to the cytoskeleton [207, 208]. Other PDZ containing proteins have no apparent membrane related function and interact primarily with the cytoskeleton. These include LIM domain containing adaptor proteins that aid in the formation of cytoskeleton-associated signaling complexes [209-212]. LIM domains are 50-60 amino acid protein binding domains named after the product of three genes in

which they were originally described (Lin-11, Isl1 and Mec-3) [209]. Two LIM-PDZ containing serine/threonine kinases, LIMK1 and LIMK2, have also been shown to interact with the cytoskeleton and regulate actin reorganization by phosphorylating cofilin, an actin-binding protein that depolymerizes actin filaments [213-215]. Interestingly, LIMK1 can shuffle between the cytoskeleton and the nucleus due to presence of nuclear import and export sequences but the nuclear function of LIMK1 remains unknown [216].

Recently, a number of Golgi- and vesicle- associated PDZ-containing proteins have been described. GRASP65, a Golgi Reassembly Stacking Protein, interacts with GM130, a medial/trans Golgi associated matrix protein that serves as a vesicle docking receptor [217, 218]. GRASP65 remains Golgi cisternae- bound via an N-terminal myristic acid anchor, where it interacts with GM130 via its PDZ domain, and possibly regulates vesicle traffic and Golgi stacking. GIPC is another PDZ containing protein that interacts with RGS-GAIP, a GTPase activating protein (GAP) for G-alpha i subunits associated with clathrin-coated vesicles [219]. GIPC therefore is potentially involved in vesicle sorting and trafficking. With the movement of vesicles along a cytoskeletal framework and the association of PDZ domains with Golgi/vesicle as well as the cytoskeleton elements, it is not surprising that some PDZ containing proteins provide the physical link between vesicles and the cytoskeleton. Recently, Neurabin-I has been shown to interact with TGN38, a trans-Golgi integral membrane protein, and F-actin of the cytoskeleton [220], clearly establishing an interaction of the Golgi with the cytoskeleton. It appears PDZ domains play important roles in membrane related signaling pathways, either at the level of plasma membranes or Golgi stacks and vesicles. However, PDZ domains are not always associated with membrane/cytoskeleton complexes. For example, the prodomain of IL-16 harboring two PDZ domains is targeted to the nucleus [221]. Junction proteins of the ZO family (ZO-1, ZO-2, ZO-3) also exhibit nuclear localization in cell cultures grown at low density [222]. It is still not clear whether PDZ containing proteins are functionally active in the nucleus or whether they are simply sequestered away from their site of action in the cytoplasm. Nonetheless, the nuclear localization of some PDZ proteins reflects the complexity and variety of signaling pathways PDZ domains may be involved in.

4.2. BINDING SPECIFICITY OF PDZ DOMAINS

The nature of PDZ interaction with partner proteins have been extensively studied, particularly in the first member of the family, PSD-95. PDZ domains generally interact with the carboxy termini of other proteins containing the consensus sequence S/T-x-V [223]. PSD-95 interacts with NMDA receptor subunits harboring this carboxy motif. Crystal structure analysis of the third PSD-95 PDZ complexed with a S/T-X-V peptide revealed that it consists of six Beta strands (BetaA-F) and two alpha helices [224] (Figure 4.1). The S/T-X-V peptide lies in a binding pocket consisting primarily of the BetaB strand and the AlphaB helix. The hydrophobic C-terminal valine (position 0) of the peptide interacts with a carboxylate binding loop within the betaB strand. Peptide binding is stabilized by hydrogen bonding of the hydrophobic residue (position 0) and the S/T residue (position -2) with a conserved leucine or isoleucine (alphaB8) and a positively charged histidine (H) residue (alphaB1) of the alphaB helix, respectively. Crystal structure analysis of a Dlg PDZ domain with the peptide T-X-V revealed essentially the same features [225].

The simplicity of PDZ binding requirements suggest a limited specificity of PDZ domains, which is highly improbable with the number of complex signaling pathways involving PDZs and the required interactions with the appropriate partners. It was suggested that PDZ specificity could be increased by targeting PDZ containing proteins to the appropriate microenvironment and limiting the number of potential partners [204]. A higher degree of complexity in the mode of PDZ binding became apparent from peptide library screening with a number of PDZ domains [226, 227]. Residues at positions -1, -3, -4 and -5 also seemed to play a role in peptide binding. Furthermore, the complexity of the S/T-X-V motif was expanded to include other hydrophobic residues at position 0, such as leucine (L), isoleucine (I), and alanine (A). Interestingly, a new class of PDZ domains (class II) emerged from the same work, with specificity to hydrophobic residue phenylalanine (F) at position –2. Other hydrophobic residues at the same position (such as tryptophan (W), valine (V) and tyrosine (Y)) were later shown to confer specificity for class II PDZs [227]. The lack of hydroxyl groups at this position did not comply with the accepted model of PDZ binding where the alphaB1 histidine provided stabilizing hydrogen

bonds. Crystal structure analysis of a class II PDZ domain, human CASK, revealed that a second hydrophobic binding pocket interacts with the hydrophobic residue at the –2 position [228]. The alphaB1 histidine normally found in class I PDZs is replaced with the hydrophobic residue valine (V) in class II PDZs to accommodate this new mode of binding [229] (see figure 4.1).

A third class of PDZ domains with high affinity to peptides harboring negatively charged residues (aspartic acid, D or glutamic acid, E) at the –2 position was originally described in neuronal nitric oxide synthase (nNOS) [230]. Class III PDZs have the same 6 beta strands/ 2 alpha helices structure but differ primarily from class I and class II PDZ by the presence of a tyrosine residue rather than a histidine or a valine in the alphaB helix [229]. This tyrosine residue provides hydrogen bonds that interact with the aspartic acid side chain of the binding peptide. Mutation of this tyrosine to a histidine (as found in class I PDZ) changes the specificity of nNOS binding from D-X-V to S-X-V [230]. An arginine residue (alphaB9) stabilizes the negatively charged aspartic acid residue (position-2) [229]. Interestingly, Tiam-1 PDZ domain is specific for the peptide Y-X-F where an aspartic acid (D) residue in the alphaB helix interacts with the tyrosine (Y) at the –2 position of the peptide [229]. The D/Y interaction in Tiam-1 and its corresponding peptide is essentially the reverse of the nNOS interaction with its peptide. The presence of a D/Y interaction between both nNOS and Tiam-1 and their corresponding peptides has lead to the classification of Tiam-1 PDZ as a class III PDZ domain. Some consider Tiam-1 as a class II PDZ protein however, because of its specificity towards peptides with hydrophobic residues at position -2 ($^{-2}$Tyr and $^{-2}$Phe) [226].

Recently, a new consensus C-terminal peptide for the third PDZ of INAD was described and defined another class of binding specificity (class IV) [227]. Class IV PDZ binding peptides are characterized by an acidic residue at position 0 and drastically deviate from all other known PDZ binding peptides that contain hydrophobic residues at that position. In fact, PDZ3 of INAD acts as both a class II and a class IV as it can bind equally well to the consensus sequence $^{-2}\psi D\Phi^{0}$ and $^{-1}\psi D/E^{0}$ (ψ is for aromatic residues and Φ is for hydrophobic residues). The only other reported exception to the requirement of a

hydrophobic residue at position 0 is the case of Mint1-1 PDZ domain that binds to the consensus $^{-3}$D/ExWC/S^{0} [231]. The authors classify Mint1-1 as a class III PDZ domain, but it is clearly different from nNOS and Tiam-1 in its binding specificity and should be classified in a separate category.

A number of reports revealed a new mode of PDZ binding to internal motifs. At least four PDZ containing proteins, PSD-95 [232, 233], syntrophin [229], EBP50 [208] and INAD [234] have the ability to bind to internal sequences as well as C-terminal peptides. In all cases except INAD, the PDZ domains in question are involved in PDZ-PDZ interactions. INAD, in contrast, binds through its fifth PDZ domain (PDZ5) to a G box homology region within its binding partner, NORPA. PSD-95 PDZ2 and syntrophin can both interact with the extra long PDZ domain of nNOS. Structural analysis revealed that the binding pocket of both PSD-95 PDZ2 and syntrophin PDZ interact with a beta-finger formed by two beta sheets present at the carboxy end of the nNOS PDZ domain. The beta finger resembles carboxy peptide in that it forms a protrusion that can get inserted into the binding pocket. EBP50 homodimerization through PDZ-PDZ interactions was not as extensively examined but there seems to be no beta finger present based on secondary structure analysis of the EBP50 protein. Furthermore, INAD's PDZ5 binds to an alpha helical region of NORPA. Obviously, the mode of PDZ binding to internal motifs is quite variable and most likely reflects the presence of multiple PDZ classes capable of binding to internal peptides.

4.3. BINDING SPECIFICITY OF CASP'S PDZ

We took a number of approaches to determine the binding specificity of CASP's PDZ domain. In one approach, a panning assay using recombinant CASP PDZ was to be performed with a combinatorial peptide display library in collaboration with Dr M.Lin, John Hopkins University. In this assay, a random nanopeptide fused to the C terminus of the lac repressor [235] was to be tested for binding to immobilized recombinant CASP PDZ protein. No results were obtained possibly as a result of the quality of recombinant proteins provided. Other approaches we took were based on the yeast two-hybrid. First, we

planned to use the CASP PDZ domain to screening the same B cell expression library that revealed the CASP/cytohesin interaction. Second, we planned to use the same CASP PDZ bait to screen a carboxy-terminal peptide library fused to the GAL4 activation domain. Both yeast two-hybrid approaches could not be carried out since yeast expressing CASP's PDZ fused to the Gal4-binding domain (Gal4-BD) were not viable. We have recently had good success in generating viable yeast clones expressing Gal4-BD/CASP PDZ variants, and we hope to carry out the yeast two-hybrid experiments outlined above in the near future.

Based on the simplified requirements for binding specificity of PDZ domains outlined earlier, the residue alphaB1 of the CASP PDZ being a tyrosine suggests that it is a class III PDZ that binds to peptides with negatively charged residues at position -2 (D or E). It is very difficult to predict the peptide consensus beyond this point; although PDZ domains have similar three dimensional structures, a universal pattern governing which residues dictate peptide specificity has not be elucidated. Furthermore, there are numerous irregularities found in many PDZ domains. For example, the hydrophobic residue at position 0 is stabilized by the hydrophobic residues of the GLGF pocket and the conserved hydrophobic AlphaB8 residue [229], but PDZ domains of all classes, including the class IV PDZ3 of Inad that bind to negatively charged C-terminal residues, contain the same conserved residues at these locations. Conversely, residue alphaB9, a conserved lysine/arginine, stabilizes the negatively charged $^{-2}$D or $^{-2}$E of class III binding peptides, but all PDZ domains have a positively charged amino acid at alphaB9 (Figure 4.1). In another example, Valine (alphaB5) contributes to the stability of a hydrophobic residue at position 0 of the binding peptide [236], but a number of class II and class III PDZ domains lack the hybrophobic residue at alphaB5 while still maintaining specificity to position 0 hydrophobic residues. The presence of an alphaB5 valine in the CASP PDZ suggests specificity toward hydrophobic residues at position 0 of the binding peptide as it may further stabilize pocket interactions with C-terminal hydrophobic residues. Clearly, the binding specificity of PDZ domains needs to be confirmed on a case by case basis, and the only apparently universal criteria appears to be the identity of the residue at alphaB1,

conferring specificity for -2 residues of binding peptides. Based on these observations, the best guess at a consensus for the peptide binding to the CASP PDZ would be $^{-2}$D/E-X-Φ^0, with X representing any amino acid and Φ representing a hydrophobic residue. More experimental work is needed to confirm this consensus and further pinpoint the identity of residues at other positions of the peptide, particularly positions -1 and -3.

Figure 4.1. Multiple alignment of Class I, II and III PDZ domains. PDZ domains consist of 6 beta sheets and 2 alpha helices. The GLGF motif, the key alphaB1 residue, and the conserved alphaB9 residue are boxed. The PDZ domain of human CASP (hCASP) is classified as a class III since the residue at position alphaB1 is a tyrosine.

Class I

```
PDZ3*       REPRRIVIHRGST--GLGFNIVGGED------GEG--IFISFILAGGPADLSGELRKGDQILS
PDZ1*       MEYEEITLERGNS--GLGFSIAGGTDNPHIGDDPS--IFITKIIPGGAAAQDGRLRVNDSILF
PDZ2*       EKVMEIKLIKGPK--GLGFSIAGGVGNQHIPGDNS--IYVTKIIEGGAAHKDGRLQIGDKILA
Syn*        QRRRVTVRKADAG--GLGISIKGGR--EN--KMP---ILISKIFKGLAADQTEALFVGDAILS
```

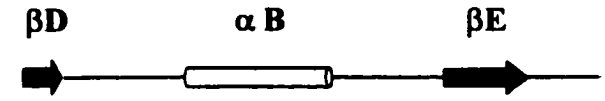
Class II

```
hCASK*      --RLVQFQKNTDE--PMGITLKM------NELN-H--CIVARIMHGGMIHRQGTLHVGDEIRE
hp55*       --RLIQFEKVTEE--PMGITLKL------NEKQ-S--CTVARILHGGMIHRQGSLHVGDEILE
```

Class III

```
Tiam-1*     VTHSIHIEKSDTAADTYGFSLSSVEED------GIRRLYVNSVKETGLASKKG-LKAGDEILE
hnNos*      NVISVRLFKRKVG--GLGFLVK-------ERVSKPP-VIISDLIRGGAAEQSGLIQAGDIILA
```

```
hB3-1*      QRKLVTVEKQDNE--TFGFEIQSYRPQNQNACSSEMFTLICKIQEDSPAHCAG-LQAGDVLAN
```



Class I

```
PDZ3*       VNGVDLRNASHEQAAIALKN--AGQTVTIIAQYK
PDZ1*       VNEVDVREVTHSAAVEALKE--AGSIVRLYVMRR
PDZ2*       VNSVGLEDVMHEDAVAALKN--TYDVVYLKVAKP
Syn*        VNGEDLSSATHDEAVQVLKKTGKEVVLEVKYMKD
```

Class II

```
hCASK*      INGISVANQTVEQLQKMLREMRGSITFKIVPSYR
hp55*       INGTNVTNHSVDQLQKAMKETKGMISLKVIPNQQ
```

Class III

```
Tiam-1*     INNRA----ADALNSSMLKDFLSQPSLGLLVRTY
hnNos*      VNGRPLVDLSYDSALEVLRGIASETHVVLILRGP
```

```
hCASP*      INGVSTEGFTYKQVVDLIR-SSGNLLTIETLNGT
```

Figure 4.1

5. GENERAL DISCUSSION AND CONCLUSIONS

In this study, we characterized a novel cDNA isolated in our laboratory, both genetically and functionally. Subtractive hybridization screening of a human NK/T cell library identified CASP (original code name B3-1) as a potential transcription factor with unusual features. This assumption was made based on the presence of a potential nuclear localization signal (^{329}PRKSRK^{334}) and more importantly, a long helix reminiscent of leucine zippers (LZ domains) found in a variety of helix-loop-helix (HLH) and basic-HLH transcription factors. With the discovery of PDZ domains, one of which is present at the N terminus of CASP, CASP emerged as an unusual protein that contains elements found in transcription factors and in membrane bound proteins. Furthermore, the carboxy terminus contained a potential domain that bears no significant homology to any proteins submitted to the Swiss-Prot database so far. Complementary DNA coding for this carboxy domain also has no homology to any sequences submitted to Genbank. The novel characteristics of the 359 a.a. deduced protein sequence and the lymphocyte specific expression profile warranted further characterization of CASP, as it may represent a novel regulator of lymphocyte functions. Starting with an incomplete cDNA that contained a putative start codon resulting in a shorter version of CASP (324 a.a.), we proceeded to isolate the full cDNA, determine the genomic structure of the gene, and isolate the promoter region for a better understanding of the control requirements of CASP. Most importantly, we functionally characterized CASP protein and associated it with a cellular process in lymphocytes.

5.1. CASP GENE CHARACTERISTICS

We took a number of approaches to isolate genomic and cDNA CASP sequences based on traditional library screening and PCR methods. Genomic library screening using a CASP cDNA probe resulted in one clone (clone F2) corresponding to the 3'end of the CASP gene containing the complete sequences of exon 8 and partial sequences of intron 7. Intron 7 is the last intron of the CASP gene, and no other clones containing additional intron/exon information could be isolated. In retrospect, this result is not

surprising since the majority of CASP exons, with the exception of exon 8, are relatively short (between 50 and 103 bases) and would not easily hybridize to the cDNA probe. We were also unsuccessful in isolating genomic clones encompassing the second largest exon (exon 1: 249 bases) and upstream genomic sequences although the cDNA probe contained sequences matching 160 bases of that exon.

In order to isolate additional genomic sequences, particularly sequences corresponding to the 5'untranslated region and containing regulatory elements, we resorted to a PCR based method of "genomic walking". Linkers harboring two primer sites were ligated onto the ends of total genomic DNA digested with the appropriate restriction enzymes. Nested polymerase chain reactions using CASP specific primers were then performed to amplify genomic sequences that contain exon/intron boundaries or genomic sequences corresponding to the promoter region. The majority of exon/intron distribution was defined using this method. The size of the introns however, particularly introns 1 and 6 (9101 and 8761 bases, respectively), could not be determined as they exceeded the limitation of the PCR. The use of commercial long range PCR products allowed us to amplify the missing sequences and determine the approximate sizes of all large introns. Limited sequence data for the 5'untranslated region was obtained using the genomic "walking method" since the low complexity of upstream genomic sequences offered no useful restriction sites within the distance limitations of the PCR. We then modified the "genomic walking" method by incorpororating the use of long range Taq and Pfu polymerase mixtures to allow the amplification of long upstream sequences. We successfully amplified a 4.4 kilobase DNA segment that contained 4 kilobases of upstream sequence. All intron sizes/locations and upstream genomic sequences later correlated well with genomic data obtained from the human genome project database.

Analysis of upstream sequences surprisingly revealed no signature regulatory sequences, particularly conventional TATA boxes, CAAT boxes or Sp1 binding sites. CASP clearly belongs to the growing group of TATA-less genes controlled by a specialized group of transcription factors. The hematopoietic expression of CASP suggested the presence of cell specific transcription factors. Analysis of upstream sequences (4073 bp) using MatInspector software [237] based on TRANSFAC databases

[238, 239] revealed potential binding sites for a number of helix-loop-helix (HLH) and zinc finger transcription factors (see appendix I). More importantly, the database search of proximal upstream sequences (500bp) identified binding sites for lymphocyte-specific factors or ubiquitous factors known to influence gene expression in response to lymphocyte signaling pathways. Some of these factors include activation protein complexes (AP1, AP2, and AP4), NF-AT (Nuclear factor of activated T cells), STATs (signal transducers and activators of transcription), and a lymphocyte-restricted transcription factor of the Ikaros family. The first 250 bases directly upstream of the transcriptional start site harbor 2 potential NF-AT sites (-165 and -119), one Oct-1/Oct-2 site (-233), one Ikaros binding site (-98), one STAT recognition sequence (-59), and one AP1 site (-47). Genomic sequences further upstream (-250 to -500) contained additional binding motifs for NF-AT (-261, -276, -306, -446), Ikaros (-285, -307, -447), AP complexes (-348, -404), Oct-1/Oct-2 (-339, -381), and STAT (-447). A number of additional consensus binding motifs with high core sequence similarity and matrix similarity scores (greater than 90%, see appendix I) are present in the upstream sequences we isolated, but the significance of these sites is still unknown. The extent of genomic sequence necessary and sufficient for controlling the CASP gene must first be identified experimentally. Many transcription factors binding to sites beyond 200 bases upstream of the transcriptional initiation site may still play a role as cis-acting enhancers.

NF-AT was originally identified as a T cell-specific transcription factor that is constitutively present in the cytosol in a phosphorylated form designated NF-ATp (pre-existing) or NF-ATc (cytosolic)[240]. The NF-AT family has at least ten members that represent the products of different genes as well as protein variants generated by alternative splicing. NF-AT is not restricted to T cells but is also expressed in other lymphoid cells (B cells, leukocytes, NK cells) and non-lymphoid tissues (muscle, testis, kidney). Dephosphorylation of NF-AT by the calcium/calmodulin-dependent phosphatase calcineurin promotes its nuclear translocation and therefore its transcriptional activity. Immunosuppressive drugs such as cyclosporin A (CsA) and FK506 targeting calcineurin are believed to downregulate T cells partly by inhibiting NF-AT translocation to the nucleus (128). TcR-mediated CASP transcription through NF-AT as a result of

calcineurin activation was ruled out since cyclosporine A pre-treatment of CD3-activated Jurkat cells had no effect on CASP transcription.

The AP1 family of transcription factors are characterized as Jun/Fos heterodimers or Jun homodimers that interact with a palindromic consensus AP1 site, also known as TRE (TPA-responsive element) (reviewed in [241]). Jun and Fos are constitutively expressed in cells and AP1-driven transcription is dependent on phosphorylation of AP1 complex components [242-244]. JNK phosphorylates Jun directly [242] while the ERK activated pp90(Rsk2) phosphorylates Fos [84, 245]. Additionally, ERK-activated Elk-1 upregulates transcription of the Fos gene, hence sustaining the activity of Fos-containing AP complexes. CD3 and CD28 signal convergence is required for JNK (and therefore Jun) activation in T cells. CD3-mediated CASP activation was unaffected by CD28 costimulation, suggesting that the initial steps of CASP gene activation do not require AP1 activity. Experiments using the protein synthesis inhibitor cyclohexamide clearly show that CASP activation by TcR signals requires *de novo* protein synthesis. CASP transcription cannot be initiated by AP1 or NF-AT factors that are rapidly phosphorylated to induce transcription without the need for protein synthesis.

STAT factors are not directly activated by the TcR [246], but they certainly play a role in T cell activation events as a result of autocrine signaling through several cytokine receptors. STATs are rapidly tyrosine phosphorylated by receptor-bound Janus kinases (JAKs), allowing the homo-or hetero-dimerization of STAT factors through a reciprocal SH2/phospho-tyrosine interaction. STAT dimers then translocate to the nucleus to induce transcription. STAT factors are characterized by a rapid response following receptor binding of its ligand as they relay signals directly from receptors to the DNA. STATs may be involved in later stages of TcR activation events, when cytokines are produced and secreted to induce autocrine signaling events. In this scenario, STATs may be involved in CASP transcription since cytokine production and secretion require *de novo* protein synthesis. However, the rapid response of STAT factors is accompanied by an efficient downregulation of STATs within 15 minutes to four hours of activation. Therefore, the sustained levels of CASP mRNA that we observed beyond 24 hours cannot be contributed to STAT induced transcription alone.

While most of the transcription factors described above are primarily involved in immediate signaling events (within 15 minutes) and may play a role at later stages, the delayed-early activation of CASP (3 hours) suggest that other transcription factors such as Oct-2 may be more relevant. Oct-2, a member of the POU (Pit-Oct-nematode Unc) family of transcription factors originally described as B-cell specific, is induced at later stages (3 hours) of T cells activation and persists beyond 24 hours [247, 248]. Oct-2 binds to an octamer site found in the promotors of all the Ig heavy and light chains [249] and several cytokines including IL-2, IL-3 and IL-4, where they normally cooperate with other transcription factors such as NF-AT to enhance transcription [250-253]. Oct-2 may therefore play a role in the prolonged activation of numerous cytokines. The similarities in activation profiles of Oct-2 and CASP suggest that Oct-2 may play a significant role in CASP transcriptional activation. Interestingly, all B cell tumor lines and the EL4 T cell line constitutively express Oct-2 [252], which may contribute to their transformed phenotype. Coincidently, CASP is constitutively expressed in the Burkitt's lymphoma cell line RAJI and EL4 T cells.

5.2. CASP PROTEIN CHARACTERISTICS

The identification of protein binding partners is the most direct approach for the functional classification of a given protein. Protein function can be elucidated by identifying an interacting protein that is already associated with a known cellular process. We attempted to isolate a CASP binding partner by co-immunoprecipiation of metabolically ^{35}S-labeled RAJI lysates using CASP-specific antibodies. RAJI was a good candidate cell line for this work since CASP is constitutively expressed and most likely functional in these cells. We could not identify any potential binding partners with this approach, as a result of the poor binding of our antibodies to CASP expressed in a eukaryotic system. Our CASP antibodies, raised against recombinant CASP, recognize the recombinant protein very well but do not react with CASP from any eukaryotic lysates on western blots. The same result was observed in lysates of transfected eukaryotic cell lines constitutively expressing CASP.

Screening cDNA expression libraries is another viable approach for identifying protein binding partners [254, 255]. MAX (Myc associated X) was identified with this approach as a leucine zipper binding partner of the proto-oncogene product Myc [256]. We had limited success with λgt11 cDNA library screening using recombinant CASP protein. Although our antibodies used for detection recognized recombinant CASP very well, the mis-folding of CASP produced in *E. coli* most likely affected the interaction of CASP with potential binding partners. We are currently using improved *E. coli* strains for efficiently producing properly folded recombinant proteins. Furthermore, we have generated smaller CASP variants based on the domain distribution of CASP that will be used to screen cDNA expression libraries and identify domain specific interacting proteins.

Yeast two-hybrid screening of a B cell library proved the most successful in identifying CASP interactions with other proteins. The coiled coil interaction between CASP and cytohesin was identified using the cytohesin N terminus as bait, then confirmed by *in vitro* and *in vivo* binding assays. Yeast cells expressing the complete CASP or the N terminus of CASP harboring the PDZ domain were not viable for unexplained reasons. Fusion of the C terminus of CASP, containing the mystery domain, to the Gal binding domain resulted in false expression of the reporter gene. Consequently, yeast two-hybrid analysis using the complete CASP, the N-terminal PDZ and the C-terminal mystery domain as bait could not be carried out successfully. We have recently developed PDZ domain and coiled coil constructs that were successfully fused to the Gal4 binding domain and expressed in yeast. Work in progress is aiming at identifying PDZ interacting proteins and additional coiled coil interacting factors (other than cytohesin).

Sequence comparison of human CASP with its murine counterpart (accession NP_631939, unpublished) revealed a high degree of homology throughout the entire protein (Figure 5.1). One significant difference however, is the presence of an alphaB1 tyrosine residue in the human CASP PDZ domain and a histidine residue in murine CASP at the same location. This suggests that human CASP and murine CASP contain PDZ domains of different classes (class III and class I, respectively). The amino acid

difference at the key alphaB1 position of the PDZ domain is intriguing and suggests that the carboxy terminus of the human and murine CASP PDZ-interacting proteins differ, particularly at the -2 position, to accommodate for the variation in PDZ classes. The murine PDZ-interacting protein most likely harbors a C terminus with the consensus S/T-X-V while the carboxy terminus of the human CASP PDZ-interacting protein most likely conforms to the consensus $^{-2}$(D/E)XΦ^0 as discussed earlier.

CASP remained unique in its domain distribution until a related protein termed GRASP (GRP-1 associated scaffold protein) was recently characterized [167]. The rat homolog of murine GRASP, named Tamalin, was described shortly thereafter [180]. GRASP/Tamalin resembles CASP on a structural basis and shares a similar domain distribution, suggesting that they belong to the same family. There are a number of key differences between CASP and GRASP however, notably in the N terminus preceding the PDZ domain and in the carboxy-terminal mystery domain (see figure 3.1). GRASP's N terminus contains an alanine/ proline-rich region with a putative SH3 binding site. It is still not known whether CASP's N terminus contains functional motifs that may contribute to its function. The lowest sequence homology between CASP and GRASP occurs in the mystery domain, which may reflect a difference in the target specificity of both proteins. The CASP and GRASP PDZ domains may also play a significant role in target specificity. Examination of the amino acid sequence of CASP's PDZ suggests that it belongs to class III PDZ domains. Tamalin on the other hand harbors a class I PDZ domain that interacts with the S-(S/T)-L carboxy terminus of G-protein-coupled neurotransmitter receptors of the mGluR family (metabotropic glutamate receptors) [180]. Ionotropic (Ion channels) and metabotropic (G-protein coupled) glutamate receptors have been studied primarily in the brain where they regulate a number of neuronal events (reviewed in [257]), but their expression has also been established in lymphoid cells [258-260]. CASP might therefore interact with lymphocyte metabotropic glutamate receptors, and possibly other receptors with carboxy termini following the consensus $^{-2}$(D/E)XΦ^0.

One common characteristic of CASP and GRASP is their interaction with members of the cytohesin/ARNO family. We showed that CASP interacts with multiple

members of the cytohesin/ARNO family [168], while others have shown that GRASP can interact with at least ARNO and ARNO3 (the human homolog of GRP-1) [167, 180]. These interactions were demonstrated *in vitro* and by binding assays of over-expressed protein *in vivo*. The coiled coil domain of both proteins may be more selective at physiological levels of cytohesin/ARNO proteins, but that remains to be elucidated. In any case, cytohesin/ARNO proteins link CASP and GRASP to cellular processes that involve ARF GTPases. Interestingly, cytohesin also interacts with ARD1 (ARF domain 1), a protein that contains an N-terminal GAP domain and a C-terminal ARF domain [261]. ARD1 localizes to the Golgi where it may control vesicle trafficking [262] and CASP may interact with cytohesin to participate in ARD1 as well as ARF processes

EGF-induced translocation of CASP to the membrane of COS-1 cells is mediated by cytohesin in our experimental system. Receptor triggers can induce cytohesin, ARNO and ARNO3 translocation to the plasma membrane [202, 263], suggesting that any of these proteins can potentially recruit CASP to the periphery. ARF1 and ARF6 association with the plasma membrane has been reported in a variety of cellular systems [264-269], but the effects of EGF stimulation require the activity of ARF6 exclusively [202, 270]. Based on these observations, CASP is likely to be recruited by cytohesin in our COS-1 studies to the site of ARF6 activity. The perinuclear localization of CASP however, implies that CASP may play a role in vesicle trafficking at the level of the Golgi in association with a cytohesin/ARNO protein and its target ARF.

```
mCASP*      MSLQRFLQRQGSNGNL-EYCADSAYGSYSVLTGQLTMEDNRRIQVLADTVATLPRGRKQL
hCASP*      MSLQRLLQHS-SNGNLADFCAGPAYSSYSTLTGSLTMNDNRRIQMLADTVATLPRGRKQL
            *****:**:..***** ::**..**.***.***.***:******:**************

mCASP*      ALARSSSLGDFSWSQRKVVTVEKQDNGTFGFEIQTYRLQNQNICSSEVCTMICKVQEDSP
hCASP*      ALTRSSSLSDFSWSQRKLVTVEKQDNETFGFEIQSYRPQNQNACSSEMFTLICKIQEDSP
            **:*****.********:******** *******:** **** ****: *:***:*****

mCASP*      AHCAGLQVGDIFANVNGVSTEGFTHKQVVDLIRSSGNLLTIETLNGTMIHRRAELEAKLQ
hCASP*      AHCAGLQAGDVLANINGVSTEGFTYKQVVDLIRSSGNLLTIETLNGTMILKRTELEAKLQ
            *******.**::**:*********:************************ :*:*******

mCASP*      TLKQTLKKKWVELRSLHLQEQRLLHGDTANSPNLENMDLDESSLFGNLLGPSPALLDRHR
hCASP*      VLKQTLKQKWVEYRSLQLQEHRLLHGDAANCPSLENMDLDELSLFGPLPGPGPALVDRNR
            .******:**** ***:***:******:**.*.******** **** * **.***:**:*

mCASP*      LSSESSCKSWLSSLTVDSEDGYRSSMSEDSIRGAFSRQTSTDDECFHSKDGDEILRNASS
hCASP*      LSSESSCKSWLSSMTMDSEDGYQTCVSEDSSRGAFSRQQSTDDECFIPKEGDDFLRRSSS
            **************:*:*******::.:**** ******* ******* .*:**::**.:**

mCASP*      RRNRSISVTSSGSFSPLWESNYSSVFGTLPRKSRRGSVRKQILKFIPGLHRAVEEEESRF
hCASP*      RRNRSISNTSSGSMSPLWEGNLSSMFGTLPRKSRKGSVRKQLLKFIPGLHRAVEEEESRF
            ******* *****:*****.* **:*********:******:******************
```

Figure 5.1. Multiple alignment of mouse and human CASP (mCASP and hCASP, respectively) using clustalW software. The residue defining the PDZ class of CASP is shown in bold type. mCASP contains a histidine residue classifying it as a class I PDZ. hCASP on the other hand contains a tyrosine residue (class III PDZ) at the same location.

5.3. FINAL CONCLUSIONS

The analysis of CASP genomic sequence showed that CASP is a TATA-less gene potentially regulated by lymphoid-specific transcription factors. This is supported by the cell type-restricted expression of CASP and our ability to induce its transcription through a T cell specific signaling molecule, the TcR. While the immediate signaling requirement for this transcriptional activation has been elucidated, additional work is needed to identify the inducible transcription factors that act directly on the CASP promoter. Other receptors that activate CASP transcription also need to be identified, particularly in cells of hematopoietic origin other than T lymphocytes.

The ability to target CASP to the plasma membrane through an interaction with cytohesin and recent work by other groups on the related protein GRASP, suggest a role for CASP in facilitating an ARF process at the plasma membrane. The ability of CASP and GRASP to bind to various members of the cytohesin/ARNO family and the similarities in their domain distribution suggest that CASP regulates a lymphoid-specific process that parallels GRASP's function in neuronal cells. The non-selective interaction of CASP's coiled coil with cytohesin/ARNO proteins suggest that the specificity of CASP towards a particular ARF process is partially controlled by the specificity of a given cytohesin/ARNO to its target ARF. Our findings do not exclude a potential role for CASP in a cytohesin/ARNO controlled process in the vicinity of the Golgi. Other proteins that interact with CASP may contribute to the function of CASP at the proper location. In addition to the SH3 binding site found only in GRASP, The PDZ and mystery domains most likely contribute to the higher architecture of signaling complexes that organize the appropriate factors at the sites of CASP and GRASP function. Identifying PDZ interacting proteins will help pinpoint the true location of CASP's function. Finally, CASP studies should target cells of hematopoietic origin that endogenously express CASP and other factors required for its function.

APPENDICES

APPENDIX I. Genomic analysis of CASP upstream sequence.

4 kilobases of upstream genomic sequence were analysed using using MatInspector software available at http://transfac.gbf.de/. Core similarity and matrix similarity were set at 0.85. Data from 2 kilobases directly upstream of the transcriptional initiation site of CASP are presented.

Transcription factor	base	Core	Matrix	Sequence
V$CEBPB_01	2004 (-)	0.986	0.933	accttagGAAAatt
V$NFAT_Q6	2004 (-)	1.000	0.984	cttagGAAAatt
V$IK2_01	2005 (-)	0.826	0.856	ccttAGGAaaat
V$GFI1_01	2011 (+)	1.000	0.871	aaggtaAATCctaaaag
V$DELTAEF1_01	2011 (-)	1.000	0.896	atttACCTtag
V$TST1_01	2011 (-)	0.895	0.860	taggATTTaccttag
V$IK2_01	2017 (-)	0.826	0.854	ttttAGGAttta
V$NFAT_Q6	2024 (+)	1.000	0.858	taaaaGAAAaga
V$GATA3_03	2033 (+)	1.000	0.862	agAGATgttc
V$XFD2_01	2039 (+)	1.000	0.864	gttcTAAAcagtag
V$HFH1_01	2040 (-)	1.000	0.871	tactGTTTagaa
V$PADS_C	2050 (-)	0.865	0.872	gGTGTTcta
V$DELTAEF1_01	2052 (+)	1.000	0.941	gaacACCTttg
V$SRY_02	2056 (-)	1.000	0.853	acttACAAaggt
V$GFI1_01	2060 (-)	1.000	0.912	acaacaAATCcttgact
V$AP1_Q2	2061 (-)	1.000	0.903	ctTGACttaca
V$AP1FJ_Q2	2061 (-)	1.000	0.917	ctTGACttaca
V$AP1_Q4	2061 (-)	1.000	0.907	ctTGACttaca
V$AP1_C	2062 (+)	0.868	0.880	gTAAGTCAa
V$TCF11_01	2066 (+)	1.000	0.870	GTCAaggatttgt
V$SRY_02	2071 (-)	1.000	0.874	aacaACAAatcc
V$SRY_02	2074 (-)	1.000	0.903	ccaaACAAcaaa
V$AP1_Q4	2083 (-)	1.000	0.897	tgTGACttcca
V$AP1_Q2	2083 (-)	1.000	0.906	tgTGACttcca
V$AP1FJ_Q2	2083 (-)	1.000	0.942	tgTGACttcca
V$TCF11_01	2088 (+)	1.000	0.888	GTCAcattcccct
V$IK1_01	2090 (-)	1.000	0.893	taagGGGAatgtg
V$IK2_01	2091 (-)	1.000	0.901	taagGGGAatgt
V$GFI1_01	2094 (-)	1.000	0.858	gaaacagtAATCtttta
V$MZF1_01	2095 (-)	1.000	0.954	taaGGGGa
V$TATA_C	2097 (+)	0.853	0.909	ccCTTAAAAg
V$GATA3_02	2103 (+)	0.825	0.861	aaaGATTact
V$HFH8_01	2109 (+)	1.000	0.856	tacTGTTtctcca
V$VMYB_01	2109 (-)	0.820	0.852	agaAACAgta
V$CEBPB_01	2111 (-)	0.986	0.916	gggtggaGAAAcag
V$GATA_C	2121 (-)	1.000	0.928	tGATAAagggt
V$GATA1_03	2122 (-)	1.000	0.955	cttgtGATAaaggg
V$GATA1_04	2122 (-)	1.000	0.956	ttgtGATAaaggg
V$GATA1_02	2122 (-)	1.000	0.939	cttgtGATAaaggg
V$GATA3_02	2124 (-)	1.000	0.899	tgtGATAaag
V$GATA2_02	2124 (-)	1.000	0.923	tgtGATAaag
V$LMO2COM_02	2124 (-)	1.000	0.940	gtGATAaag
V$GATA1_06	2124 (-)	1.000	0.884	tgtGATAaag
V$GATA1_05	2124 (-)	1.000	0.906	tgtGATAaag
V$MYCMAX_02	2127 (+)	0.895	0.851	tatCACAaggga
V$IK2_01	2131 (+)	1.000	0.883	acaaGGGAcact
V$NKX25_01	2139 (-)	1.000	0.884	aaAAGTg
V$CDPCR3HD_01	2142 (+)	1.000	0.858	ttttGATCcg
V$GATA3_03	2143 (-)	0.812	0.886	acGGATcaaa
V$GATA2_03	2143 (-)	0.853	0.854	acgGATCaaa
V$GATA3_02	2143 (-)	0.831	0.850	acgGATCaaa
V$TCF11_01	2149 (-)	1.000	0.871	GTCActgctacgg

V$AP1_Q4	2156 (+)	1.000	0.910	agTGACtggca
V$AP1_Q2	2156 (+)	1.000	0.929	agTGACtggca
V$AP1FJ_Q2	2156 (+)	1.000	0.933	agTGACtggca
V$TCF11_01	2158 (-)	1.000	0.971	GTCAtgccagtca
V$CAAT_01	2158 (-)	0.847	0.878	tcatgCCAGtca
V$NF1_Q6	2159 (+)	1.000	0.855	gacTGGCatgacacagg
V$AP1_Q2	2165 (+)	1.000	0.887	caTGACacagg
V$AP1FJ_Q2	2165 (+)	1.000	0.912	caTGACacagg
V$AP1_Q4	2165 (+)	1.000	0.879	caTGACacagg
V$AP1_C	2166 (-)	0.860	0.853	cTGTGTCAt
V$AP1_Q4	2177 (+)	1.000	0.881	acTGACagaac
V$AP1FJ_Q2	2177 (+)	1.000	0.924	acTGACagaac
V$AP1_Q2	2177 (+)	1.000	0.900	acTGACagaac
V$IK2_01	2185 (+)	1.000	0.919	aacaGGGAaatg
V$NFAT_Q6	2186 (+)	1.000	0.905	acaggGAAAtga
V$TCF11_01	2186 (-)	1.000	0.964	GTCAtttccctgt
V$CREL_01	2190 (-)	1.000	0.853	ggtcatTTCC
V$BRN2_01	2191 (+)	0.854	0.858	gaaatgacCAATaaaa
V$NFY_01	2193 (+)	1.000	0.926	aatgaCCAAtaaaaat
V$CAAT_01	2193 (+)	1.000	0.930	aatgaCCAAtaa
V$AP1_Q4	2193 (+)	1.000	0.897	aaTGACcaata
V$AP1_Q2	2193 (+)	1.000	0.917	aaTGACcaata
V$AP1FJ_Q2	2193 (+)	1.000	0.923	aaTGACcaata
V$MEF2_02	2194 (+)	1.000	0.884	accaataaaAATAgact
V$RORA1_01	2194 (-)	1.000	0.870	ttttattGGTCat
V$NFY_Q6	2195 (+)	1.000	0.949	tgaCCAAtaaa
V$FREAC7_01	2196 (+)	1.000	0.884	gaccaaTAAAaataga
V$TST1_01	2196 (+)	0.895	0.873	gaccAATAaaaatag
V$XFD2_01	2198 (+)	1.000	0.870	ccaaTAAAaataga
V$RSRFC4_01	2198 (-)	1.000	0.870	agtCTATttttattgg
V$TATA_C	2198 (+)	0.890	0.928	ccAATAAAAa
V$TATA_01	2199 (+)	1.000	0.877	caaTAAAaatagact
V$HFH3_01	2200 (-)	0.955	0.893	gtcTATTtttatt
V$HNF3B_01	2200 (-)	1.000	0.854	tagtcTATTtttatt
V$GKLF_01	2203 (+)	0.810	0.857	aaaaatagacTAGG
V$RORA1_01	2208 (+)	1.000	0.937	tagactaGGTCaa
V$RORA2_01	2208 (+)	1.000	0.871	tagactaGGTCaa
V$AP1_Q4	2211 (-)	1.000	0.857	ttTGACctagt
V$DELTAEF1_01	2211 (-)	1.000	0.855	tttgACCTagt
V$AP1_Q2	2211 (-)	1.000	0.871	ttTGACctagt
V$AP1FJ_Q2	2211 (-)	1.000	0.903	ttTGACctagt
V$ER_Q6	2212 (-)	1.000	0.869	tacaaattttTGACcta
V$TCF11_01	2216 (+)	1.000	0.868	GTCAaaaattttgt
V$BRN2_01	2219 (-)	1.000	0.869	ttaattacAAATtttt
V$BRN2_01	2220 (+)	1.000	0.913	aaaatttgTAATtaac
V$S8_01	2222 (+)	1.000	0.974	aatttgtaATTAacag
V$S8_01	2224 (-)	1.000	0.966	acctgttaATTAcaaa
V$NKX25_02	2228 (-)	1.000	0.899	gtTAATta
V$E47_02	2230 (+)	1.000	0.952	attaaCAGGtgtgacc
V$VMYB_01	2230 (+)	0.820	0.874	attAACAggt
V$E47_01	2231 (+)	0.833	0.874	ttaACAGgtgtgacc
V$MYOD_01	2232 (+)	1.000	0.898	taaCAGGtgtga
V$TCF11_01	2232 (-)	1.000	0.875	GTCAcacctgtta
V$LMO2COM_01	2232 (+)	1.000	0.939	taaCAGGtgtga

V$DELTAEF1_01	2233	(-)	1.000	0.955	tcacACCTgtt
V$MYOD_Q6	2233	(-)	1.000	0.955	caCACCtgtt
V$USF_C	2234	(-)	0.856	0.922	aCACCTgt
V$T3R_01	2236	(-)	1.000	0.875	caatgtGGTCacacct
V$AP1FJ_Q2	2239	(+)	1.000	0.925	tgTGACcacat
V$AP1_Q2	2239	(+)	1.000	0.870	tgTGACcacat
V$AP1_Q4	2239	(+)	1.000	0.852	tgTGACcacat
V$PADS_C	2240	(-)	1.000	0.878	tGTGGTcac
V$OCT1_06	2245	(+)	0.833	0.887	cacattgatATGGg
V$GATA1_03	2246	(+)	1.000	0.911	acattGATAtgggc
V$GATA1_02	2246	(+)	1.000	0.959	acattGATAtgggc
V$GATA1_04	2247	(+)	1.000	0.917	cattGATAtgggc
V$CDPCR3HD_01	2247	(+)	0.842	0.936	cattGATAtg
V$LMO2COM_02	2249	(+)	1.000	0.961	ttGATAtgg
V$GATA_C	2250	(+)	0.868	0.937	tGATATgggct
V$NF1_Q6	2257	(+)	1.000	0.882	ggcTGGCtggatgtcag
V$CETS1P54_01	2262	(+)	0.852	0.921	gcTGGAtgtc
V$AP1FJ_Q2	2264	(-)	1.000	0.904	tcTGACatcca
V$AP1_Q2	2264	(-)	1.000	0.864	tcTGACatcca
V$ER_Q6	2272	(+)	1.000	0.919	gaggtactttTGACcct
V$RORA1_01	2282	(-)	1.000	0.870	aaacaagGGTCaa
V$SRY_02	2285	(-)	1.000	0.896	caaaACAAgggt
V$GKLF_01	2286	(-)	1.000	0.913	atacaaaacaAGGG
V$SRY_02	2290	(-)	1.000	0.886	ggatACAAaaca
V$GATA_C	2291	(-)	0.875	0.902	gGATACaaaac
V$GATA1_02	2292	(-)	1.000	0.888	acctgGATAcaaaa
V$GATA1_04	2292	(-)	1.000	0.863	cctgGATAcaaaa
V$GATA1_03	2292	(-)	1.000	0.885	acctgGATAcaaaa
V$LMO2COM_02	2294	(-)	1.000	0.878	tgGATAcaa
V$CETS1P54_01	2295	(-)	0.852	0.868	ccTGGAtaca
V$GATA_C	2299	(-)	0.875	0.862	gGATACctgga
V$DELTAEF1_01	2299	(-)	1.000	0.853	ggatACCTgga
V$GATA1_04	2300	(-)	1.000	0.878	atggGATAcctgg
V$GATA1_03	2300	(-)	1.000	0.914	aatggGATAcctgg
V$GATA1_02	2300	(-)	1.000	0.893	aatggGATAcctgg
V$LMO2COM_02	2302	(-)	1.000	0.896	ggGATAcct
V$OCT1_06	2303	(-)	0.944	0.903	aataatgggATACc
V$SS8_01	2303	(+)	1.000	0.857	ggtatcccATTAtttg
V$IK2_01	2303	(-)	1.000	0.955	taatGGGAtacc
V$NFAT_Q6	2313	(+)	1.000	0.869	tatttGAAAaat
V$CEBPB_01	2323	(-)	0.930	0.938	gtgttaaGTAAaat
V$E4BP4_01	2324	(-)	1.000	0.918	tgttaaGTAAaa
V$HLF_01	2325	(-)	1.000	0.869	GTTAagtaaa
V$VBP_01	2325	(+)	1.000	0.871	tTTACttaac
V$NKX25_01	2327	(-)	1.000	0.885	ttAAGTa
V$CEBPB_01	2335	(-)	0.986	0.912	aactcatGAAAtgt
V$AP1_C	2342	(-)	0.848	0.871	tTAACTCAt
V$AP1_C	2342	(+)	0.856	0.862	aTGAGTTAa
V$CEBP_C	2343	(+)	0.965	0.863	tgagttaaGTAATaagt
V$CEBPB_01	2344	(+)	0.930	0.948	gagttaaGTAAtaa
V$E4BP4_01	2345	(+)	1.000	0.936	agttaaGTAAta
V$HLF_01	2346	(+)	1.000	0.911	GTTAagtaat
V$VBP_01	2346	(-)	1.000	0.885	aTTACttaac
V$HLF_01	2346	(-)	0.902	0.854	ATTActtaac

V$NKX25_01	2347	(+)	1.000	0.885	ttAAGTa
V$S8_01	2348	(-)	1.000	0.855	aaatacttATTActta
V$NFAT_Q6	2359	(-)	1.000	0.981	aactgGAAAata
V$CETS1P54_01	2360	(-)	0.852	0.861	acTGGAaaat
V$VMYB_01	2364	(-)	0.876	0.874	agaAACTgga
V$CEBPB_01	2366	(-)	0.986	0.919	caattaaGAAActg
V$S8_01	2370	(-)	1.000	0.982	atcattcaATTAagaa
V$NKX25_02	2372	(+)	1.000	1.000	ctTAATtg
V$OCT1_06	2372	(+)	0.833	0.886	cttaattgaATGAt
V$TCF11_01	2373	(-)	0.807	0.878	ATCAttcaattaa
V$TCF11_01	2376	(-)	0.807	0.879	ATCAtcattcaat
V$GFI1_01	2381	(-)	1.000	0.953	atgtaAATCatagatca
V$CDP_02	2381	(-)	0.806	0.892	atcATAGatcatcat
V$CLOX_01	2381	(-)	0.807	0.863	atcATAGatcatcat
V$TCF11_01	2383	(-)	0.807	0.868	ATCAtagatcatc
V$GATA3_03	2383	(-)	1.000	0.887	atAGATcatc
V$CDPCR3HD_01	2384	(-)	1.000	0.941	cataGATCat
V$OCT1_Q6	2391	(-)	1.000	0.905	gctgatgtAAATcat
V$AP1_C	2391	(-)	0.802	0.852	gTAAATCAt
V$OCT1_05	2391	(+)	0.808	0.859	atgatttACATcag
V$OCT_C	2391	(+)	0.814	0.853	atgATTTAcatca
V$CEBPB_01	2393	(-)	0.930	0.893	agctgatGTAAatc
V$E4BP4_01	2394	(-)	1.000	0.855	gctgatGTAAat
V$VBP_01	2395	(+)	1.000	0.921	tTTACatcag
V$AP4_Q5	2400	(+)	1.000	0.883	atCAGCttaa
V$RORA1_01	2403	(+)	1.000	0.938	agcttaaGGTCag
V$T3R_01	2404	(+)	1.000	0.901	gcttaaGGTCagtatt
V$AP1FJ_Q2	2406	(-)	1.000	0.899	acTGACcttaa
V$DELTAEF1_01	2406	(-)	1.000	0.887	actgACCTtaa
V$AP1_Q2	2406	(-)	1.000	0.883	acTGACcttaa
V$ER_Q6	2407	(-)	1.000	0.877	cattgtaatacTGACct
V$TCF11_01	2411	(+)	1.000	0.870	GTCAgtattacaa
V$OCT1_06	2414	(-)	0.944	0.865	ttcattgtaATACt
V$SRY_02	2416	(+)	1.000	0.893	tattACAAtgaa
V$SOX5_01	2417	(+)	1.000	0.863	attaCAATga
V$NFY_01	2421	(-)	1.000	0.855	ttgtaCCAAttcattg
V$NFY_Q6	2424	(-)	1.000	0.867	gtaCCAAttca
V$SOX5_01	2430	(+)	1.000	0.855	ggtaCAATtt
V$AP1_Q2	2440	(-)	1.000	0.939	aaTGACtacta
V$AP1FJ_Q2	2440	(-)	1.000	0.932	aaTGACtacta
V$AP1_Q4	2440	(-)	1.000	0.913	aaTGACtacta
V$TATA_01	2441	(-)	1.000	0.931	ttaTAAAtgactact
V$TCF11_01	2445	(+)	1.000	0.983	GTCAtttataaaa
V$FREAC7_01	2447	(+)	1.000	0.884	catttaTAAAaatggg
V$XFD2_01	2449	(+)	1.000	0.897	tttaTAAAaatggg
V$TATA_C	2449	(+)	1.000	0.926	ttTATAAAAa
V$TATA_01	2450	(+)	1.000	0.964	ttaTAAAaatgggtg
V$CMYB_01	2460	(+)	1.000	0.920	gggtgagtcaGTTGgcc
V$AP1_Q4	2461	(-)	1.000	0.962	acTGACtcacc
V$AP1_Q2	2461	(-)	1.000	0.949	acTGACtcacc
V$AP1FJ_Q2	2461	(-)	1.000	0.952	acTGACtcacc
V$AP1_C	2462	(+)	1.000	0.997	gTGAGTCAg
V$AP1_C	2462	(-)	0.981	0.976	cTGACTCAc
V$NFE2_01	2462	(-)	1.000	0.901	aaCTGActcac

V$LMO2COM_01	2465	(+)	0.804	0.884	agtCAGTtggcc
V$TCF11_01	2466	(+)	1.000	0.860	GTCAgttggcctg
V$VMYB_01	2466	(-)	0.876	0.866	gccAACTgac
V$MYOD_Q6	2466	(-)	0.872	0.964	gcCAACtgac
V$VMYB_02	2467	(-)	0.820	0.891	gccAACTga
V$NF1_Q6	2469	(+)	1.000	0.911	agtTGGCctgagtgggt
V$NFY_Q6	2476	(-)	0.805	0.854	cacCCACtcag
V$CAAT_01	2477	(-)	0.827	0.890	gccacCCACtca
V$NKX25_02	2492	(-)	1.000	0.884	ctTAATat
V$CEBPB_01	2492	(+)	1.000	0.951	atattaaGCAAatg
V$BRN2_01	2500	(+)	1.000	0.866	caaatgtaTAATttcc
V$S8_01	2504	(-)	1.000	0.941	aaatggaaATTAtaca
V$OCT_C	2507	(+)	0.814	0.864	ataATTTCcattt
V$OCT1_Q6	2507	(-)	1.000	0.899	ccaaatggAAATtat
V$OCT1_05	2507	(+)	0.846	0.905	ataatttCCATttg
V$OCT1_06	2507	(+)	0.889	0.867	ataatttccATTTg
V$NFAT_Q6	2508	(-)	1.000	0.956	aaatgGAAAtta
V$OCT1_07	2509	(-)	1.000	0.866	caaatggaAATT
V$BRN2_01	2512	(-)	1.000	0.892	aaaatgccAAATggaa
V$OCT1_06	2514	(+)	0.889	0.861	ccatttggcATTTt
V$NF1_Q6	2516	(+)	1.000	0.911	attTGGCattttaaaat
V$OCT1_06	2522	(+)	0.944	0.854	cattttaaaATATt
V$TATA_C	2523	(+)	0.928	0.871	atTTTAAAAt
V$TATA_C	2523	(-)	0.928	0.871	atTTTAAAAt
V$BRN2_01	2529	(-)	1.000	0.891	tttattctTAATattt
V$BRN2_01	2530	(+)	0.854	0.862	aatattaaGAATaaaa
V$NKX25_02	2531	(-)	1.000	0.884	ctTAATat
V$EREAC7_01	2535	(+)	1.000	0.904	taagaaTAAAaattaa
V$TST1_01	2535	(+)	0.895	0.887	taagAATAaaaatta
V$XFD2_01	2537	(+)	1.000	0.883	agaaTAAAaattaa
V$TATA_C	2537	(+)	0.890	0.874	agAATAAAAa
V$HNF3B_01	2537	(-)	0.855	0.881	tttaaTTTTtattct
V$TATA_01	2538	(+)	1.000	0.864	gaaTAAAaattaaaa
V$HFH2_01	2538	(-)	0.823	0.879	taaTTTTtattc
V$S8_01	2538	(+)	1.000	0.932	gaataaaaATTAaaaa
V$BRN2_01	2542	(-)	1.000	0.866	aatattttTAATtttt
V$BRN2_01	2543	(+)	1.000	0.881	aaaattaaAAATattt
V$NKX25_02	2544	(-)	1.000	0.860	ttTAATtt
V$HFH2_01	2544	(-)	0.823	0.868	tatTTTTaattt
V$HNF3B_01	2544	(-)	0.855	0.851	aaataTTTTtaattt
V$HNF3B_01	2546	(-)	1.000	0.850	gtaaaTATTtttaat
V$HNF3B_01	2549	(+)	1.000	0.914	aaaaaTATTtaccta
V$XFD3_01	2550	(-)	0.826	0.853	taggtaAATAtttt
V$XFD1_01	2550	(-)	1.000	0.931	taggTAAAtatttt
V$HFH3_01	2551	(+)	0.955	0.872	aaaTATTtaccta
V$CEBPB_01	2554	(-)	0.930	0.924	aagttagGTAAata
V$E4BP4_01	2555	(-)	1.000	0.916	agttagGTAAat
V$DELTAEF1_01	2555	(+)	1.000	0.890	atttACCTaac
V$VBP_01	2556	(+)	1.000	0.871	tTTACctaac
V$HLF_01	2556	(-)	1.000	0.875	GTTAggtaaa
V$VBP_01	2556	(-)	0.800	0.891	gTTAGgtaaa
V$GFI1_01	2561	(-)	1.000	0.867	ccctaggAATCtgataa
V$GATA_C	2562	(-)	1.000	0.934	tGATAAagtta
V$GATA1_04	2563	(-)	1.000	0.966	atctGATAaagtt

V$GATA1_03	2563 (-)	1.000	0.925	aatctGATAaagtt
V$GATA1_02	2563 (-)	1.000	0.923	aatctGATAaagtt
V$GATA3_02	2565 (-)	1.000	0.870	tctGATAaag
V$GATA2_02	2565 (-)	1.000	0.920	tctGATAaag
V$GATA1_06	2565 (-)	1.000	0.886	tctGATAaag
V$GATA1_05	2565 (-)	1.000	0.934	tctGATAaag
V$LMO2COM_02	2565 (-)	1.000	0.948	ctGATAaag
V$AP4_Q5	2591 (-)	1.000	0.911	gaCAGCagtc
V$AP1_Q2	2593 (-)	1.000	0.876	gaTGACagcag
V$AP1FJ_Q2	2593 (-)	1.000	0.902	gaTGACagcag
V$S8_01	2597 (+)	1.000	0.858	tgtcatccATTAatag
V$CAAT_01	2598 (+)	0.856	0.877	gtcatCCATtaa
V$CDPCR3HD_01	2598 (-)	0.930	0.935	aatgGATGac
V$TCF11_01	2598 (+)	1.000	0.986	GTCAtccattaat
V$S8_01	2603 (-)	1.000	0.854	tcatcactATTAatgg
V$GFI1_01	2605 (-)	1.000	0.924	aaattaAATCatcacta
V$TCF11_01	2607 (-)	0.807	0.852	ATCAtcactatta
V$POLY_C	2608 (-)	0.927	0.865	aATTAAAtcatcactat
V$GATA3_03	2614 (+)	0.875	0.868	gaTGATttaa
V$S8_01	2617 (-)	1.000	0.930	gcacataaATTAaatc
V$NKX25_02	2619 (+)	1.000	0.860	ttTAATtt
V$HFH8_01	2645 (+)	1.000	0.873	aaaTGTTtttaaa
V$HFH3_01	2645 (+)	1.000	0.853	aaaTGTTtttaaa
V$GFI1_01	2649 (+)	1.000	0.916	gtttttaaAATCaatct
V$TATA_C	2650 (-)	0.928	0.877	atTTTAAAAa
V$TATA_C	2650 (+)	0.928	0.873	ttTTTAAAAt
V$GFI1_01	2653 (+)	1.000	0.887	taaaatcAATCtctctc
V$GATA3_03	2654 (-)	0.875	0.912	atTGATttta
V$CDPCR3HD_01	2655 (-)	0.886	0.933	gattGATTtt
V$IK2_01	2674 (-)	0.826	0.875	tattAGGAagac
V$DELTAEF1_01	2724 (+)	1.000	0.866	atatACCTgta
V$TATA_01	2732 (+)	0.802	0.857	gtaTACAtatctgta
V$GATA_C	2733 (-)	0.868	0.938	aGATATgtata
V$GATA1_04	2734 (-)	1.000	0.949	tacaGATAtgtat
V$GATA1_03	2734 (-)	1.000	0.879	gtacaGATAtgtat
V$GATA1_02	2734 (-)	1.000	0.932	gtacaGATAtgtat
V$LMO2COM_02	2736 (-)	1.000	0.941	caGATAtgt
V$GATA3_03	2736 (-)	1.000	0.864	acAGATatgt
V$EVI1_04	2739 (-)	1.000	0.867	aGATAtgtacagata
V$GATA_C	2743 (-)	0.868	0.948	aGATATgtaca
V$GATA1_04	2744 (-)	1.000	0.924	tataGATAtgtac
V$GATA1_02	2744 (-)	1.000	0.929	ttataGATAtgtac
V$GATA1_03	2744 (-)	1.000	0.876	ttataGATAtgtac
V$MEF2_02	2745 (+)	1.000	0.872	atctataAATAtagatt
V$LMO2COM_02	2746 (-)	1.000	0.932	taGATAtgt
V$GATA3_03	2746 (-)	1.000	0.864	atAGATatgt
V$CDPCR3HD_01	2747 (-)	0.842	0.906	tataGATAtg
V$FREAC7_01	2749 (+)	1.000	0.945	tatctaTAAAtataga
V$HFH3_01	2751 (-)	0.955	0.884	ctaTATTtataga
V$GFI1_01	2751 (-)	1.000	0.889	acgtaaAATCtatattt
V$HFH8_01	2751 (-)	0.816	0.876	ctaTATTtataga
V$XFD1_01	2751 (+)	1.000	0.878	tctaTAAAtataga
V$XFD2_01	2751 (+)	1.000	0.922	tctaTAAAtataga
V$TATA_01	2752 (+)	1.000	0.941	ctaTAAAtatagatt

V$CDPCR3HD_01	2759 (+)	0.886	0.902	tataGATTtt
V$GATA3_03	2760 (+)	1.000	0.960	atAGATttta
V$XBP1_01	2763 (+)	1.000	0.855	gattttACGTgaatatc
V$ARNT_01	2763 (+)	1.000	0.870	gattttaCGTGaatat
V$VBP_01	2766 (+)	1.000	0.914	tTTACgtgaa
V$CREBP1_01	2767 (-)	1.000	0.912	tcACGTaa
V$CREBP1_01	2767 (+)	1.000	0.917	ttACGTga
V$OCT1_02	2768 (-)	0.980	0.904	aaagatATTCacgta
V$GATA_C	2770 (-)	0.868	0.888	aGATATtcacg
V$GATA1_03	2771 (-)	1.000	0.895	ataaaGATAttcac
V$GATA1_04	2771 (-)	1.000	0.912	taaaGATAttcac
V$GATA1_02	2771 (-)	1.000	0.911	ataaaGATAttcac
V$TATA_01	2772 (-)	1.000	0.904	ttaTAAAgatattca
V$GATA3_03	2773 (-)	1.000	0.896	aaAGATattc
V$LMO2COM_02	2773 (-)	1.000	0.927	aaGATAttc
V$FREAC7_01	2774 (-)	1.000	0.883	caattaTAAAgatatt
V$XFD2_01	2774 (-)	1.000	0.897	attaTAAAgatatt
V$S8_01	2780 (-)	1.000	0.971	atacatcaATTAtaaa
V$NKX25_02	2782 (+)	1.000	0.883	taTAATtg
V$CDPCR3HD_01	2785 (+)	0.930	0.938	aattGATGta
V$HNF3B_01	2791 (+)	0.855	0.895	tgtatTCTTtagttg
V$XFD1_01	2792 (-)	1.000	0.871	caacTAAAgaatac
V$HFH2_01	2793 (+)	0.814	0.866	tatTCTTtagtt
V$NF1_Q6	2794 (-)	1.000	0.936	cctTGGCaactaaagaa
V$RFX1_01	2800 (-)	1.000	0.907	aaataccttgGCAActa
V$RFX1_02	2800 (-)	1.000	0.900	aaataccttgGCAActa
V$DELTAEF1_01	2806 (-)	1.000	0.858	aaatACCTtgg
V$IK2_01	2818 (-)	1.000	0.887	aaaaGGGAgtgg
V$BARBIE_01	2818 (-)	1.000	0.862	ggcaAAAGggagtgg
V$GKLF_01	2823 (-)	1.000	0.871	gtagggcaaaAGGG
V$CEBPB_01	2825 (-)	1.000	0.895	gtgtaggGCAAaag
V$TATA_01	2832 (-)	1.000	0.860	acaTAAAtgtgtagg
V$VBP_01	2845 (+)	1.000	0.857	gTTACttacc
V$DELTAEF1_01	2848 (+)	1.000	0.882	acttACCTggc
V$CEBPB_01	2856 (+)	0.986	0.922	ggcttctGAAAacg
V$HFH8_01	2871 (+)	1.000	0.856	atgTGTTtttaat
V$PADS_C	2872 (+)	0.865	0.866	tGTGTTttt
V$TST1_01	2874 (-)	1.000	0.881	caggAATTaaaaaca
V$GFI1_01	2876 (-)	1.000	0.912	cggtctaAATCaggaat
V$S8_01	2876 (-)	1.000	0.935	aatcaggaATTAaaaa
V$BRN2_01	2878 (-)	0.854	0.854	taaatcagGAATtaaa
V$NKX25_02	2878 (+)	1.000	0.874	ttTAATtc
V$CETS1P54_01	2880 (-)	0.926	0.897	tcAGGAatta
V$TST1_01	2885 (+)	0.895	0.856	cctgATTTagaccgt
V$AP1_C	2886 (-)	0.802	0.859	cTAAATCAg
V$CMYB_01	2888 (+)	0.841	0.863	gatttagaccGTTAtat
V$VMYB_01	2894 (-)	1.000	0.944	tatAACGgtc
V$VMYB_02	2895 (-)	1.000	0.982	tatAACGgt
V$HFH8_01	2901 (+)	1.000	0.866	ataTGTTtgtggt
V$HFH3_01	2901 (+)	1.000	0.887	ataTGTTtgtggt
V$GATA1_02	2909 (+)	1.000	0.897	gtggtGATAtcact
V$GATA1_03	2909 (+)	1.000	0.879	gtggtGATAtcact
V$GATA1_04	2910 (+)	1.000	0.905	tggtGATAtcact
V$GATA_C	2910 (-)	0.868	0.896	tGATATcacca

V$GATA1_02	2911	(-)	1.000	0.891	aaagtGATAtcacc
V$GATA1_03	2911	(-)	1.000	0.879	aaagtGATAtcacc
V$GATA1_04	2911	(-)	1.000	0.906	aagtGATAtcacc
V$PADS_C	2911	(+)	0.904	0.882	gGTGATatc
V$GATA2_03	2911	(+)	1.000	0.895	ggtGATAtca
V$LMO2COM_02	2912	(+)	1.000	0.910	gtGATAtca
V$GATA_C	2913	(+)	0.868	0.888	tGATATcactt
V$LMO2COM_02	2913	(-)	1.000	0.910	gtGATAtca
V$GATA3_03	2913	(-)	0.875	0.887	agTGATatca
V$GATA3_02	2913	(-)	1.000	0.861	agtGATAtca
V$GATA2_03	2913	(-)	1.000	0.912	agtGATAtca
V$PADS_C	2914	(-)	0.904	0.897	aGTGATatc
V$NKX25_01	2919	(-)	1.000	0.871	caAAGTg
V$AP1FJ_Q2	2926	(-)	1.000	0.868	ttTGACatgga
V$TCF11_01	2931	(+)	1.000	0.874	GTCAaaattcaat
V$OCT1_06	2933	(+)	0.889	0.911	caaaattcaATTCc
V$TST1_01	2934	(-)	1.000	0.875	ctggAATTgaatttt
V$CETS1P54_01	2940	(-)	0.852	0.881	acTGGAattg
V$CEBPB_01	2940	(-)	0.873	0.857	ttatactGGAAttg
V$S8_01	2947	(-)	1.000	0.855	catgactcATTAtact
V$NFE2_01	2951	(+)	0.800	0.862	taATGAgtcat
V$AP1FJ_Q2	2952	(-)	1.000	0.941	caTGACtcatt
V$AP1_Q2	2952	(-)	1.000	0.933	caTGACtcatt
V$AP1_Q4	2952	(-)	1.000	0.943	caTGACtcatt
V$AP1_C	2953	(-)	0.981	0.982	aTGACTCAt
V$AP1_C	2953	(+)	1.000	0.992	aTGAGTCAt
V$TCF11_01	2957	(+)	1.000	0.993	GTCAtgctgtgat
V$GATA1_03	2962	(+)	1.000	0.876	gctgtGATAgagtt
V$GATA1_02	2962	(+)	1.000	0.934	gctgtGATAgagtt
V$GATA1_04	2963	(+)	1.000	0.923	ctgtGATAgagtt
V$LMO2COM_02	2965	(+)	1.000	0.942	gtGATAgag
V$GATA_C	2966	(+)	0.891	0.900	tGATAGagttt
V$GFI1_01	2973	(+)	1.000	0.873	tttttaaAATCtataat
V$TATA_C	2974	(+)	0.928	0.873	ttTTTAAAAt
V$TATA_C	2974	(-)	0.928	0.877	atTTTAAAAa
V$GATA3_03	2978	(-)	1.000	0.960	atAGATttta
V$CDPCR3HD_01	2979	(-)	0.886	0.902	tataGATTtt
V$BRN2_01	2979	(+)	1.000	0.890	aaaatctaTAATtggc
V$S8_01	2983	(-)	1.000	0.978	tgaagccaATTAtaga
V$NFY_01	2983	(-)	1.000	0.929	tgaagCCAAttataga
V$NKX25_02	2985	(+)	1.000	0.883	taTAATtg
V$NFY_Q6	2986	(-)	1.000	0.932	aagCCAAttat
V$CAAT_01	2987	(-)	1.000	0.949	tgaagCCAAtta
V$NF1_Q6	2988	(+)	1.000	0.922	aatTGGCttcaactttt
V$BARBIE_01	2993	(-)	1.000	0.870	ttcaAAAGttgaagc
V$GFI1_01	3010	(-)	1.000	0.899	atagtaaAATCtgaatt
V$S8_01	3011	(-)	1.000	0.943	aaatctgaATTAatag
V$NKX25_02	3013	(+)	1.000	0.874	atTAATtc
V$GATA3_03	3019	(+)	1.000	0.919	tcAGATttta
V$TATA_01	3024	(-)	1.000	0.932	ataTAAAtagtaaaa
V$MEF2_02	3024	(-)	1.000	0.884	ccaaatataAATAgtaa
V$HNF3B_01	3025	(+)	1.000	0.918	tttacTATTtatatt
V$XFD2_01	3026	(-)	1.000	0.914	aataTAAAtagtaa
V$RSRFC4_01	3026	(+)	1.000	0.873	ttaCTATttatatttg

V$XFD1_01	3026 (-)	1.000	0.895	aataTAAAtagtaa
V$FREAC7_01	3026 (-)	1.000	0.934	caaataTAAAtagtaa
V$HFH8_01	3027 (+)	0.816	0.884	tacTATTtatatt
V$HFH3_01	3027 (+)	0.955	0.928	tacTATTtatatt
V$CEBP_C	3034 (+)	1.000	0.867	tatatttgGCAATttct
V$CEBPB_01	3035 (+)	1.000	0.978	atatttgGCAAttt
V$NF1_Q6	3037 (+)	1.000	0.915	attTGGCaatttctact
V$NKX25_01	3050 (-)	1.000	0.900	tcAAGTa
V$TST1_01	3054 (-)	1.000	0.902	gatgAATTcaactca
V$TST1_01	3057 (+)	1.000	0.856	gttgAATTcatcttc
V$CEBPB_01	3071 (-)	0.873	0.853	gtgtactGGAAgtg
V$CETS1P54_01	3071 (-)	0.852	0.948	acTGGAagtg
V$NRF2_01	3071 (-)	1.000	0.900	actGGAAgtg
V$NKX25_01	3071 (-)	1.000	0.884	ggAAGTg
V$OCT1_06	3086 (-)	1.000	0.912	taaaatgaaATGTg
V$IRF1_01	3088 (-)	1.000	0.867	ctaaaatGAAAtg
V$GFI1_01	3095 (-)	1.000	0.916	gcaAATCtttgaactaa
V$GATA3_03	3104 (+)	1.000	0.860	aaAGATttgc
V$ER_Q6	3125 (+)	1.000	0.873	tgctagtctTGACcttc
V$TCF11_01	3127 (-)	1.000	0.850	GTCAagactagca
V$AP1FJ_Q2	3134 (+)	1.000	0.859	ctTGACcttca
V$DELTAEF1_01	3134 (+)	1.000	0.858	cttgACCTtca
V$GFI1_01	3135 (-)	1.000	0.925	tttaaAATCtctgaagg
V$RORA1_01	3135 (-)	1.000	0.943	ctctgaaGGTCaa
V$GATA3_03	3144 (+)	1.000	0.960	agAGATttta
V$TATA_C	3148 (+)	0.928	0.877	atTTTAAAAa
V$TATA_C	3148 (-)	0.928	0.873	ttTTTAAAAt
V$PADS_C	3154 (-)	0.865	0.866	tGTGCTttt
V$TCF11_01	3156 (-)	1.000	0.858	GTCAaatgtgctt
V$AP1FJ_Q2	3163 (+)	1.000	0.881	ttTGACttttt
V$AP1_Q2	3163 (+)	1.000	0.865	ttTGACttttt
V$TCF11_01	3164 (-)	1.000	0.870	GTCAaaaagtcaa
V$AP1FJ_Q2	3171 (+)	1.000	0.862	ttTGACttttg
V$GFI1_01	3174 (-)	1.000	0.868	ggaagAATCgaaacaaa
V$SRY_02	3175 (-)	1.000	0.941	cgaaACAAaagt
V$IK2_01	3202 (-)	1.000	0.875	gtagGGGAggca
V$MZF1_01	3206 (-)	1.000	0.957	gtaGGGGa
V$VMYB_01	3226 (-)	0.876	0.854	gagAACTggt
V$DELTAEF1_01	3240 (+)	1.000	0.858	aactACCTgcc
V$CMYB_01	3249 (-)	1.000	0.861	tttggctgagGTTGggg
V$NF1_Q6	3250 (-)	1.000	0.917	cttTGGCtgaggttggg
V$DELTAEF1_01	3251 (+)	1.000	0.853	cccaACCTcag
V$NFAT_Q6	3263 (+)	1.000	0.992	caaagGAAAaaa
V$HNF3B_01	3264 (-)	0.855	0.855	ttttttTTTTtccttt
V$HFH3_01	3268 (-)	0.838	0.869	gctTTTTttttc
V$HFH3_01	3269 (-)	0.838	0.871	tgcTTTTttttt
V$HFH2_01	3269 (-)	0.823	0.862	gctTTTTttttt
V$BARBIE_01	3272 (+)	1.000	0.851	aaaaAAAGcatctgt
V$TAL1ALPHAE47_01	3275 (-)	1.000	0.911	gatcaCAGAtgctttt
V$TAL1BETAE47_01	3275 (-)	1.000	0.908	gatcaCAGAtgctttt
V$TAL1BETAITF2_01	3275 (-)	1.000	0.893	gatcaCAGAtgctttt
V$LMO2COM_01	3277 (-)	0.822	0.905	tcaCAGAtgctt
V$MYOD_Q6	3278 (+)	0.915	0.914	agCATCtgtg
V$PADS_C	3284 (+)	0.904	0.929	tGTGATctt

V$GATA3_02	3284 (-)	0.831	0.890	caaGATCaca
V$GATA3_03	3284 (-)	1.000	0.900	caAGATcaca
V$GATA2_03	3284 (-)	0.853	0.890	caaGATCaca
V$ER_Q6	3304 (+)	1.000	0.897	tggtgccctTGACccat
V$AP1FJ_Q2	3313 (+)	1.000	0.902	ctTGACccata
V$AP1_Q2	3313 (+)	1.000	0.876	ctTGACccata
V$AP1_Q4	3313 (+)	1.000	0.857	ctTGACccata
V$RORA1_01	3314 (-)	1.000	0.895	gcatatgGGTCaa
V$RORA2_01	3314 (-)	1.000	0.900	gcatatgGGTCaa
V$OCT1_01	3317 (+)	1.000	0.860	ccaTATGcacatgcagg
V$OCT1_02	3317 (+)	1.000	0.912	acccatATGCacatg
V$LMO2COM_01	3323 (-)	0.822	0.914	ctgCATGtgcat
V$USF_C	3325 (+)	0.876	0.932	gCACATgc
V$USF_C	3325 (-)	0.817	0.855	gCATGTgc
V$IK2_01	3330 (+)	1.000	0.892	tgcaGGGAccaa
V$CMYB_01	3335 (-)	1.000	0.908	acattgcctGTTGgtcc
V$DELTAEF1_01	3358 (+)	1.000	0.951	tgccACCTtgg
V$CP2_01	3364 (-)	0.909	0.853	ggcaaacCAAG
V$VMYB_01	3372 (-)	0.820	0.851	ggtAACAggc
V$RFX1_01	3374 (-)	0.945	0.864	gggcaggaagGTAAcag
V$RFX1_02	3374 (-)	0.945	0.863	gggcaggaagGTAAcag
V$DELTAEF1_01	3375 (+)	1.000	0.858	tgttACCTtcc
V$CETS1P54_01	3379 (-)	0.926	0.909	gcAGGAaggt
V$BARBIE_01	3388 (-)	1.000	0.861	gaggAAAGgagtggg
V$GKLF_01	3388 (-)	0.873	0.869	aggaaaggagTGGG
V$NFAT_Q6	3393 (-)	1.000	0.925	gggagGAAAgga
V$GKLF_01	3394 (-)	0.937	0.865	caggggaggaAAGG
V$IK2_01	3397 (-)	1.000	0.868	ccagGGGAggaa
V$AP2_Q6	3400 (-)	0.905	0.850	gtCCCAggggag
V$MZF1_01	3401 (-)	1.000	0.948	ccaGGGGa
V$IK2_01	3403 (+)	1.000	0.943	ccctGGGAccac
V$PADS_C	3407 (-)	1.000	0.882	aGTGGTccc
V$MYCMAX_02	3409 (+)	0.810	0.864	gacCACTtgctg
V$USF_Q6	3410 (+)	0.864	0.860	acCACTtgct
V$USF_C	3411 (+)	0.836	0.919	cCACTTgc
V$NKX25_01	3412 (-)	1.000	0.946	gcAAGTg
V$AP4_Q5	3413 (-)	1.000	0.851	atCAGCaagt
V$CP2_01	3414 (-)	0.909	0.861	gcatcagCAAG
V$CEBPB_01	3422 (+)	1.000	0.876	tgctgccGCAAagt
V$SOX5_01	3454 (-)	1.000	0.852	ggcaCAATgt
V$NFAT_Q6	3475 (-)	1.000	0.917	gaaggGAAAgcc
V$IK1_01	3475 (-)	1.000	0.892	ggaaGGGAaagcc
V$IK2_01	3476 (-)	1.000	0.937	ggaaGGGAaagc
V$IRF2_01	3476 (-)	1.000	0.856	gggaaggGAAAgc
V$IK1_01	3480 (-)	1.000	0.892	caatGGGAaggga
V$IK2_01	3481 (-)	1.000	0.949	caatGGGAaggg
V$GKLF_01	3481 (-)	1.000	0.876	tacaatgggaAGGG
V$SRY_02	3486 (-)	1.000	0.875	aagtACAAtggg
V$SOX5_01	3487 (-)	1.000	0.860	agtaCAATgg
V$SRY_02	3493 (-)	1.000	0.859	ctttACAAagta
V$XFD1_01	3496 (+)	1.000	0.852	tttgTAAAgatcat
V$BARBIE_01	3497 (+)	1.000	0.860	ttgtAAAGatcatgg
V$GATA3_02	3501 (+)	0.831	0.867	aaaGATCatg
V$GATA3_03	3501 (+)	1.000	0.891	aaAGATcatg

V$TCF11_01	3505	(+)	0.807	0.850	ATCAtgggtaatt
V$BRN2_01	3505	(+)	1.000	0.936	atcatgggTAATtttt
V$S8_01	3509	(-)	1.000	0.952	ttacaaaaATTAccca
V$CEBPB_01	3514	(+)	0.930	0.925	aatttttGTAAgga
V$SRY_02	3515	(-)	1.000	0.865	ccttACAAaaat
V$VBP_01	3516	(-)	1.000	0.852	cTTACaaaaa
V$GKLF_01	3529	(+)	0.937	0.865	aaagtagtaaAAGG
V$TH1E47_01	3543	(-)	1.000	0.861	gcattcttCTGGttgt
V$NFY_C	3557	(+)	0.800	0.868	gctgAATGGttaca
V$CAAT_01	3559	(-)	0.856	0.934	tgtaaCCATtca
V$CAAT_01	3574	(-)	0.827	0.865	atctgCCACtga
V$GATA1_03	3578	(+)	1.000	0.872	tggcaGATAtaatg
V$GATA1_02	3578	(+)	1.000	0.895	tggcaGATAtaatg
V$GATA1_04	3579	(+)	1.000	0.931	ggcaGATAtaatg
V$GATA3_02	3580	(+)	1.000	0.853	gcaGATAtaa
V$GATA2_03	3580	(+)	1.000	0.921	gcaGATAtaa
V$GATA3_03	3580	(+)	1.000	0.920	gcAGATataa
V$LMO2COM_02	3581	(+)	1.000	0.916	caGATAtaa
V$GATA_C	3582	(+)	0.868	0.884	aGATATaatgt
V$BRN2_01	3602	(-)	1.000	0.886	tagatcatTAATgttc
V$TCF11_01	3602	(-)	0.807	0.878	ATCAttaatgttc
V$S8_01	3604	(-)	1.000	0.859	cctagatcATTAatgt
V$IK2_01	3626	(+)	1.000	0.917	ttcaGGGAaagt
V$IK1_01	3626	(+)	1.000	0.873	ttcaGGGAaagta
V$STAT_01	3626	(-)	1.000	0.961	TTCCctgaa
V$NFAT_Q6	3627	(+)	1.000	0.913	tcaggGAAAgta
V$ER_Q6	3660	(+)	1.000	0.921	agaacactaTGACcggc
V$PADS_C	3661	(-)	0.865	0.943	aGTGTTctc
V$GRE_C	3661	(-)	1.000	0.862	cggtcatagTGTTctc
V$TCF11_01	3662	(-)	1.000	0.985	GTCAtagtgttct
V$AP1FJ_Q2	3669	(+)	1.000	0.865	taTGACcggca
V$E47_02	3673	(-)	1.000	0.894	aattaCAGGtgccggt
V$DELTAEF1_01	3675	(+)	1.000	0.945	cggcACCTgta
V$MYOD_01	3675	(-)	1.000	0.927	ttaCAGGtgccg
V$LMO2COM_01	3675	(-)	1.000	0.967	ttaCAGGtgccg
V$MYOD_Q6	3676	(+)	1.000	0.924	ggCACCtgta
V$USF_C	3677	(+)	0.856	0.920	gCACCTgt
V$TST1_01	3678	(-)	1.000	0.887	caagAATTacaggtg
V$S8_01	3680	(-)	1.000	0.923	gggcaagaATTAcagg
V$OCT1_02	3692	(+)	0.980	0.904	gcccatATTCacaag
V$VMYB_01	3704	(+)	0.876	0.907	aagAACTgaa
V$GRE_C	3704	(-)	0.819	0.865	aggacattcAGTTctt
V$NMYC_01	3715	(+)	1.000	0.898	gtcctCGTGtgc
V$USF_Q6	3716	(-)	1.000	0.866	caCACGagga
V$USF_C	3717	(+)	0.813	0.871	cCTCGTgt
V$TCF11_01	3718	(-)	1.000	0.853	GTCAgcacacgag
V$AP4_Q5	3721	(-)	1.000	0.863	gtCAGCacac
V$AP1_Q4	3725	(+)	1.000	0.896	gcTGACttctg
V$AP1_Q2	3725	(+)	1.000	0.926	gcTGACttctg
V$AP1FJ_Q2	3725	(+)	1.000	0.933	gcTGACttctg
V$CHOP_01	3733	(-)	1.000	0.880	caaTGCAatgcag
V$OCT1_06	3734	(-)	1.000	0.867	agcaatgcaATGCa
V$BRN2_01	3738	(-)	0.854	0.914	aacattagCAATgcaa
V$BRN2_01	3739	(+)	1.000	0.931	tgcattgcTAATgtta

V$CEBPB_01	3749 (+)	0.930	0.944	atgttaaGTAAgag
V$E4BP4_01	3750 (+)	1.000	0.926	tgttaaGTAAga
V$VBP_01	3751 (-)	1.000	0.876	cTTACttaac
V$HLF_01	3751 (+)	1.000	0.865	GTTAagtaag
V$NKX25_01	3752 (+)	1.000	0.885	ttAAGTa
V$GKLF_01	3754 (+)	0.817	0.874	aagtaagagaATGG
V$GKLF_01	3759 (+)	0.873	0.913	agagaatggaTGGG
V$CDPCR3HD_01	3763 (+)	0.930	0.949	aatgGATGgg
V$IK2_01	3766 (+)	1.000	0.956	ggatGGGAaaca
V$IK1_01	3766 (+)	1.000	0.873	ggatGGGAaacaa
V$NFAT_Q6	3767 (+)	1.000	0.931	gatggGAAAcaa
V$SRY_02	3771 (+)	1.000	0.930	ggaaACAAatgg
V$MYCMAX_02	3783 (+)	0.810	0.872	tagCACTtgggg
V$USF_C	3785 (+)	0.836	0.908	gCACTTgg
V$NKX25_01	3786 (-)	1.000	0.932	ccAAGTg
V$IK2_01	3788 (+)	1.000	0.893	cttgGGGAcatt
V$MZF1_01	3788 (+)	1.000	0.965	cttGGGGa
V$HNF3B_01	3793 (+)	0.855	0.874	ggacaTTTTtctttc
V$HFH3_01	3795 (+)	0.838	0.860	acaTTTTtctttc
V$NFAT_Q6	3797 (-)	1.000	0.858	agaaaGAAAaat
V$EVI1_02	3803 (-)	1.000	0.851	agagAAGAaag
V$NFAT_Q6	3812 (+)	1.000	0.860	ctaaaGAAAaaa
V$HNF3B_01	3813 (-)	0.855	0.853	ccattTTTTtcttta
V$HFH3_01	3813 (-)	0.838	0.879	atttTTTTtcttta
V$GKLF_01	3814 (+)	0.817	0.928	aaagaaaaaaATGG
V$HNF3B_01	3814 (-)	0.855	0.884	gccatTTTTttcttt
V$HFH2_01	3814 (-)	0.823	0.862	atttTTTTtcttt
V$HFH2_01	3815 (-)	0.823	0.866	catTTTTttctt
V$BRN2_01	3821 (+)	1.000	0.869	aaaatggcAAATggtg
V$PADS_C	3833 (+)	1.000	0.867	gGTGGTatg
V$OCT1_06	3840 (-)	0.889	0.873	gcaaattgcATTCa
V$NF1_Q6	3841 (-)	1.000	0.854	ctgTGGCaaattgcatt
V$CHOP_01	3841 (+)	1.000	0.911	gaaTGCAatttgc
V$CEBPB_01	3847 (-)	1.000	0.893	ttctgtgGCAAatt
V$TH1E47_01	3871 (+)	1.000	0.890	taagaattCTGGctgt
V$DELTAEF1_01	3887 (-)	1.000	0.981	tttcACCTcaa
V$HFH3_01	3893 (-)	0.955	0.855	actTATTttttca
V$HNF3B_01	3893 (-)	1.000	0.877	caactTATTttttca
V$GFI1_01	3895 (-)	1.000	0.913	agaaaAATCaacttatt
V$HNF3B_01	3904 (+)	0.855	0.868	gttgaTTTTtcttta
V$HFH3_01	3906 (+)	0.838	0.892	tgaTTTTtcttta
V$CEBPB_01	3908 (-)	0.986	0.878	atttaaaGAAAaat
V$NFAT_Q6	3908 (-)	1.000	0.858	ttaaaGAAAaat
V$SRY_02	3917 (-)	1.000	0.890	ttttACAAttta
V$SOX5_01	3918 (-)	1.000	0.864	tttaCAATtt
V$XFD1_01	3920 (+)	1.000	0.861	attgTAAAaattag
V$S8_01	3921 (+)	1.000	0.947	ttgtaaaaATTAgctc
V$CETS1P54_01	3946 (+)	0.926	0.855	tcAGGAgctt
V$CEBPB_01	3952 (+)	0.986	0.859	gcttaaaGAAAaaa
V$NFAT_Q6	3954 (+)	1.000	0.854	ttaaaGAAAaaa
V$HFH3_01	3955 (-)	0.838	0.872	cttTTTTtcttta
V$HNF3B_01	3955 (-)	0.855	0.864	agcttTTTTtcttta
V$HFH2_01	3956 (-)	0.823	0.854	cttTTTTtcttt
V$HNF3B_01	3956 (-)	0.855	0.851	aagctTTTTttcttt

V$BARBIE_01	3960	(+)	1.000	0.881	aaaaAAAGctttgag
V$CEBPB_01	3967	(+)	0.986	0.952	gctttgaGAAAtgg
V$IK2_01	3975	(+)	1.000	0.927	aaatGGGAgtga
V$GKLF_01	3987	(+)	1.000	0.880	atagcaagatAGGG
V$GATA1_03	3989	(+)	1.000	0.909	agcaaGATAgggtt
V$GATA1_02	3989	(+)	1.000	0.970	agcaaGATAgggtt
V$GATA1_04	3990	(+)	1.000	0.950	gcaaGATAgggtt
V$LMO2COM_02	3992	(+)	1.000	0.991	aaGATAggg
V$GATA_C	3993	(+)	0.891	0.937	aGATAGggttt
V$CEBPB_01	3999	(+)	1.000	0.917	ggtttgcGCAAcaa
V$CEBPB_01	3999	(-)	1.000	0.928	ttgttgcGCAAacc
V$HLF_01	4001	(-)	0.820	0.873	GTTGcgcaaa
V$CEBPB_01	4011	(-)	0.873	0.901	ggtttgaGGAActt
V$RFX1_02	4011	(-)	0.882	0.855	gtggtttgaGGAActt
V$STAT_01	4014	(+)	1.000	0.882	TTCCtcaaa
V$PADS_C	4019	(-)	1.000	0.902	tGTGGTttg
V$RORA1_01	4023	(+)	1.000	0.916	ccacagaGGTCac
V$AP1FJ_Q2	4026	(-)	1.000	0.904	tgTGACctctg
V$ER_Q6	4027	(-)	1.000	0.883	agagcccatgTGACctc
V$LMO2COM_01	4030	(-)	0.822	0.882	gccCATGtgacc
V$SREBP1_01	4030	(+)	1.000	0.851	ggTCACatggg
V$TCF11_01	4031	(+)	1.000	0.879	GTCAcatgggctc
V$USF_Q6	4031	(+)	0.864	0.916	gtCACAtggg
V$USF_C	4032	(-)	0.817	0.859	cCATGTga
V$USF_C	4032	(+)	0.876	0.930	tCACATgg
V$BARBIE_01	4043	(-)	1.000	0.861	tagcAAAGcagaaag
V$BARBIE_01	4051	(-)	1.000	0.858	atcaAAAGtagcaaa

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