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LOCALIZATION AND TARGETING OF B2-1, A GUANINE-NUCLEOTIDE EXCHANGE FACTOR FOR ADP-RIBOSYLATION FACTORS

by

Stella Yu-Chien Lee

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

at

Dalhousie University
Halifax, Nova Scotia
May, 2000

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Localization and Targeting of B2-1, a Guanine-Nucleotide Exchange Factor for ADP-Ribosylation Factors"

by Stella Yu-Chien Lee

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

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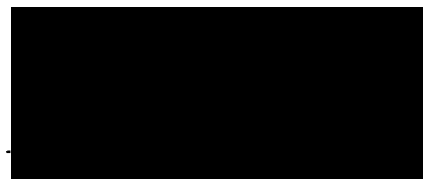

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Abstract

B2-1 (cytohesin-1) is a member of a group of proteins (including ARNO and ARNO3) that are all of similar size and domain composition. The three proteins contain an N-terminal coiled-coil domain, followed by Sec7 and pleckstrin homology (PH) domains. Recently, several research groups have shown that B2-1 has varied cellular functions and subcellular locations. One of these is an association of the B2-1 Sec7 domain with the plasma membrane, binding to the cytoplasmic portion of the integrin β2 chain (CD18) and a postulated involvement in inside-out signaling. Other groups have shown that B2-1 and the related proteins are guanine nucleotide-exchange factors that act upon ADP ribosylation factors (ARFs) and are localized to the Golgi or plasma membrane. Here we report the subcellular localization of B2-1 protein. Interestingly, B2-1 does not localize to the plasma membrane, but rather associates with a distinct Golgi complex compartment. B2-1's distribution was disrupted by brefeldin A, a drug that rapidly disrupts the Golgi apparatus by inhibiting ARF activity. Transient transfection of GFP-tagged B2-1 showed Golgi complex targeting. Excessive overexpression of transfected B2-1 also caused partial Golgi dispersion. While it is well established that the Sec7 domain has GEF activity and the PH domain anchors the proteins to membrane phosphoinositols, the function of the N-terminal coiled-coil region is unknown. Here it was shown that B2-1's N-terminus (residues 1-54) is necessary and sufficient to target the protein to the Golgi. The Sec7+PH domains of B2-1 (residues 55-398) were not sufficient for Golgi localization. Further deletion analysis and point mutagenesis indicated that the coiled-coil domain within the N terminus is responsible for Golgi targeting. Furthermore, ARNO and ARNO3 N termini also have the same capability for targeting to the Golgi. It was concluded that the N-terminal α-helical coiled-coil domain is used to target this family of proteins to the Golgi complex.

List of Abbreviations

ARF	ADP-ribosylation factor
BFA	brefeldin A
CC	coiled coil
DH	Dbl homology
EGF	epidermal growth factor
ER	endoplasmic reticulum
GAP	GTPase-activating protein
GDF	GDI displacement factor
GDI	GDP-dissociation inhibitor
GEF	guanine-nucleotide exchange factor
GFP	green fluorescent protein
GST	glutathione-S transferase
LSM	laser scanning microscope
PH	pleckstrin homology
PtdIns	phosphatidylinositol

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Chapter 1

General Introduction

B2-1 is a guanine-nucleotide exchange factor (GEF) for ADP-ribosylation factors (ARFs) during the process of vesicle formation. ARF is a subfamily of the small GTPase superfamily. Regulation of the small GTPases involves cycling between their active and inactive forms, and GEFs are one of the regulators of these small GTPases. In the first part of this introduction, some general features of the various GEFs for the small GTPases are reviewed. B2-1 and other more specific ARF-GEFs will be discussed in the second part of the introduction.

Part I: Guanine Nucleotide Exchange Factors for Small GTPases

Guanine-nucleotide binding proteins (G proteins) cycle between active, GTP-bound and inactive, GDP-bound forms. Several types of guanine-nucleotide binding proteins have been studied. For example, heterotrimeric G proteins, which consist of three subunits, Gα, Gβ, and Gγ, are involved in cell communication between the outside and inside of the cell. Another type of G protein, the elongation factor EF-Tu, transports transfer-RNA to the messenger RNA-ribosome complex during protein synthesis. A third type of G protein, the monomeric small GTPase, controls a variety of cellular events.

The first small GTPase Ras was discovered in 1980, which at the time was known as p21src, a transforming gene (v-H-*ras*) product of Harvey murine sarcoma virus (Chang et al., 1980; Shih et al., 1980). Later, the proto-oncogene (c-H-*ras*) was identified in vertebrates (Langbeheim et al., 1980). Since then, the number of known Ras-like small GTPases has been expanding. There are five major subfamilies in the Ras-like superfamily of GTPases, including Ras, Rab, Ran, Rho, and ARF (for reviews, see Mackay and Hall, 1998; Moore, 1998; Moss and Vaughan, 1998; Schimmoller et al., 1998; Vojtek and Der, 1998). Ras-like small GTPases function in many cellular processes, such as cytoskeleton organization (Rho/Rac), cell growth (Ras), nuclear transport (Ran) and vesicular trafficking between organelles (ARF and Rab).

Three classes of regulators for small GTPases are well characterized. They include guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and GDP-dissociation inhibitors (GDIs) (Figure 1.1). Another recently identified regulator, GDI displacement factor (GDF) (Dirac-Svejstrup et al., 1997), is not currently well understood. Guanine nucleotide exchange factors (GEFs) release GDP from GTPases, thus allowing GTP to bind GTPases. After the GDP/GTP exchange, GTPases switch on and interact with their downstream effectors. Some subfamilies of Ras-like GTPases have multiple effectors. For example, Ras GTPase activates a kinase cascade by binding to Raf, an upstream kinase of the Raf-Mek-Erk kinase cascade. Eventually, various transcription factors are activated in the nucleus. Besides Raf, Ras also binds to other effectors. Studies with yeast have shown that Ras interacts with Byr2, a MAPK kinase kinase, and Scd1, a GEF for the Rho GTPases family (Chang et al., 1994). Another class of regulator, GAP, works in a negative manner by stimulating hydrolysis of

GTP and leaving the GTPase in a GDP-bound, inactivated state. In contrast to GEFs and GAPs, which interact with every family of GTPase and are required for completion of GTPase GDP/GTP exchange cycles, GDIs are not found with all families of Ras-like small GTPases. So far only Rab and Rho families have corresponding GDIs (for review, see Geyer and Wittinghofer, 1997). GDI binds to the prenylated C-terminal tail of the GDP-bound small GTPase and forms a cytosolic complex. Although it is postulated that GDF acts in concert with GDI (Soldati et al., 1994), only one GDF has been identified (Dirac-Svejstrup et al., 1997).

As activators of G proteins, GEFs regulate a variety of cellular processes by activating GTPases. This chapter is focused on families of GEFs for small GTPases, their structures, interaction with GTPases, localization, and regulation mechanisms.

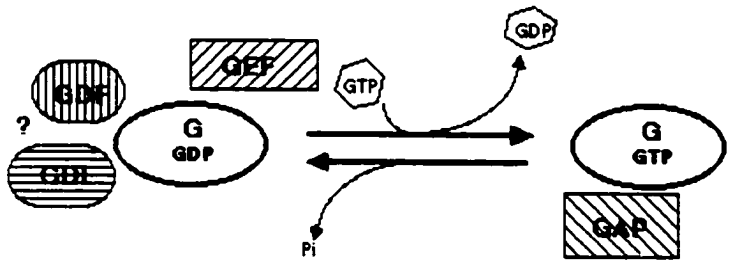


Figure 1.1 The small GTPase GDP/GTP exchange cycle. GTPases are regulated by two or four classes of regulators depending on the GTPase subfamilies. GEF stimulates the GDP/GTP exchange whereas GAP facilitates the GTPase to hydrolyse GTP. GDI binds to the prenylated tail of GDP-bound Rab or Rho and thus forms a cytosolic complex; GDF dissociates GDI from the GTPase. The question mark indicates that the identity of GDF is unclear.

General Features of GEFs

Not until recently were GEFs for the Ras-like small GTPases discovered. Although Ras-like GTPase subfamilies have similar features, their regulators are quite different. Figure 1.2 diagrams several examples of GEFs for some small GTPase families, each with different size and secondary structure.

The CDC25-like domain is the signature of Ras GEFs. This domain was first found in the yeast protein CDC25, which was the first identified GEF for a small GTPase (Jones et al., 1991). Later more Ras GEFs were found, including Sos (Simon et al., 1991) and RasGRP (Ebinu et al., 1998), all with the CDC25-like domain (Boguski and McCormick, 1993). This domain is approximately 250 residues long and is composed of three structurally conserved regions (Boguski and McCormick, 1993). Biochemical assays show that the CDC25-like domain carries out the GEF activity (Coccetti et al., 1995; Martegani et al., 1992). Mammalian CDC25 (mCDC25), also known as RasGRF, and Sos, not only have the CDC25-like domain but also the Rho GEF signature, DH and PH domains (see below).

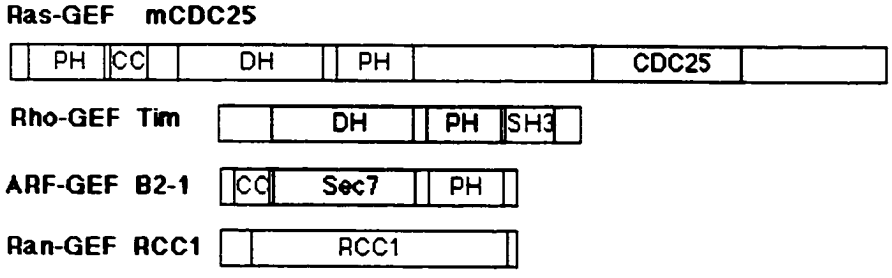


Figure 1.2 Schematic representation of the GEF families for the Ras, Rho, ARF, and Ran GTPases. Each GEF family has its specific homology domain(s) as shown in shaded boxes. The RCC1 domain is possibly specific for Ran-GEF. CC represents a coiled-coil domain. These domains are drawn roughly to scale.

All identified Rho GEFs contain a Dbl homology (DH) domain of about 180 residues followed by a pleckstrin homology (PH) domain of approximately 100 residues. The DH domain is involved in the GEF activity and the PH domain is thought to mediate membrane association. More than 20 Rho GEFs have been identified and the similarities between these proteins are restricted to the DH and PH domains. Besides the homologous region, Rho GEFs usually possess other distinct functional domains, such as the SH2 domain in Vav, the SH3 domain in Vav and Dbs, the diacylglycerol binding domain in Vav and Lfc, and the serine/threonine kinase domain in Bcr and Trio.

The Sec7 domain is found in ARF GEFs. B2-1, of which the gene product is an ARF GEF, was first documented in this lab (Liu and Pohajdak, 1992). Later, a homologue of B2-1, termed ARNO for ARF nucleotide-binding site opener, was found and shown to have GEF activity toward ARFs (Chardin et al., 1996). B2-1 family members (B2-1, ARNO and ARNO3) also contain a PH domain in tandem with the Sec7 domain. Besides this family, there are other proteins that contain the Sec7 domain. Yeast Gea1 and human p200 have GEF activity towards ARFs (Morinaga et al., 1997; Peyroche et al., 1996). The GEF activity of several other Sec7-domain-containing proteins, such as arabidopsis EMB30 and yeast Sec7p remains to be tested. Recently some proteins have been identified as ARF-like small GTPases (ARLs) which are structually similar to ARFs (Cavenagh et al., 1994; Clark et al., 1993). It is of great interest to know whether the GEFs for ARLs also contain the Sec7 and/or PH domains.

Two proteins were identified as Rab-GEFs, Mss4 (mammalian suppressor of Sec4) and Rab3-GEF, with different specificities towards Rabs (Wada et al., 1997). Rab3-GEF stimulates guanine nucleotide exchange specifically for the Rab3 GTPase,

whereas the substrates of Mss4 include Rab1, Rab3, Rab8, Rab10, Sec4, and Ypt1 (Burton et al., 1994). There is no sequence similarity between these two proteins. At least 30 Rabs exist in eukaryotic cells. Each of them localizes to a distinct compartment in the cell, and are thought to mediate vesicle fusion at a specific compartment (Chavrier et al., 1991; Gorvel et al., 1991; Goud et al., 1990). Considering the variety of Rabs it is likely that more Rab-GEFs exist.

The regulator of chromosome condensation, RCC1, was the first protein found with Ran-GEF activity. It has a unique motif of seven tandem repeats with each repeat being about 50 residues long. Four other proteins that have the "seven tandem repeats" motif, also known as the RCC1-like domain, were found later (Devilder et al., 1998). However, their GEF activity for Ran has yet to be proven. Intriguingly, one of the four proteins called p532, with two "seven tandem repeats", has GEF activity for ARF1 and Rab subfamily members (Rosa et al., 1996), but not for the Ran GTPase subfamily.

Tertiary structures of GEFs

Before GEF tertiary structure is discussed, the structure of GTPase must be understood. G proteins, such as the Gα subunit, the translation elongation factor EF-Tu, and the Ras-like small GTPase, share a conserved catalytic domain structure (Sprang, 1997). Taking Ras as an example, a comparison of the active (GTP-bound) and inactive (GDP-bound) forms of Ras shows a major conformational difference at residues 30 to 38, the switch I region, and residues 60 to 76, the switch II region. (Milburn et al., 1990) (Figure 1.3). The switch I region corresponds to loop 2 (L2) that includes part of the Mg^{2+}-binding site (Thr35). The switch II region consists of L4 and α2 and contains the

GTP γ-phosphate binding site. The β phosphate of the guanine nucleotide is located near the L1 (thus named the phosphate loop or P-loop).

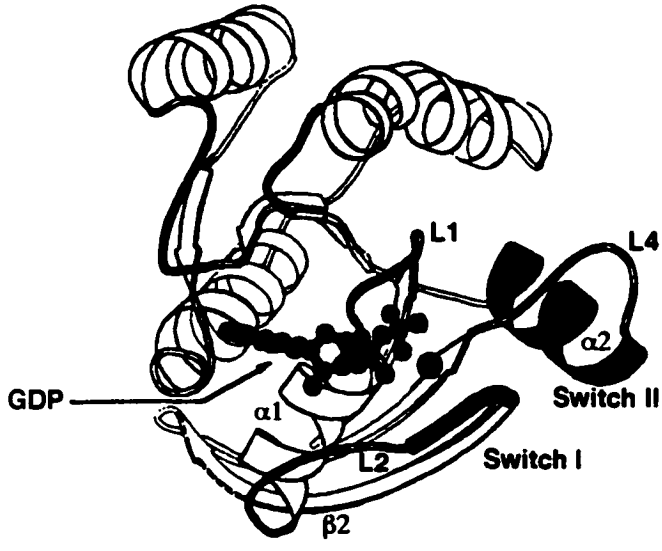


Figure 1.3 The tertiary structure of the GDP-bound Ras GTPase. Some secondary elements and the regions, switch I and switch II, which are involved in conformational change upon GDP/GTP binding, are labeled. The single solid sphere represents Mg^{2+}. This figure is adapted from Sprang (1997).

Unlike the Ras-like small GTPases, which all share similar tertiary structures, the different GEF family members have unique structures. Some tertiary structures of GEFs have been solved, either as the GEF alone or complexed with its corresponding small GTPase.

The first GEF tertiary structure to be established was that of human Mss4, a GEF of the Rab family (Yu and Schreiber, 1995). It contains a central seven-stranded β-sheet with one small three-stranded sheet on one side and a β hairpin on the other. It also binds Zn^{2+} through Cys23, Cys26, Cys94 and Cys97. The Rab binding site is at the Zn^{2+}-binding region and a neighbouring loop. Because the substrates of Mss4, such as Rab1, Rab3, and Rab8, share a highly conserved sequence at residues 63 to 77, corresponding to the switch II region, it is thought that Mss4 binds to Rab through this region.

The crystal structure of the catalytic domain of Sos, a Ras GEF, complexed with human H-Ras has been determined (Boriack-Sjodin et al., 1998) (Figure 1.4). Sos disrupts the interaction of Ras with its nucleotide by the insertion of an α-helix structure at the CDC25 homology domain. As the α-helix region is conserved in all Ras-GEFs (Boguski and McCormick, 1993), it is likely that they all share a similar structure. In this complex, Ras is surrounded by the oblong bowl-shaped Sos and the hydrophilic interface includes the P-loop, switch I, switch II regions and α3 helix-L7 region. In addition, the Ras-Sos complex does not hinder the binding site for either GTP or GDP. Therefore the structure allows nucleotide GDP release and rebinding in favor of GTP due to its intracellular abundance compared with that of GDP.

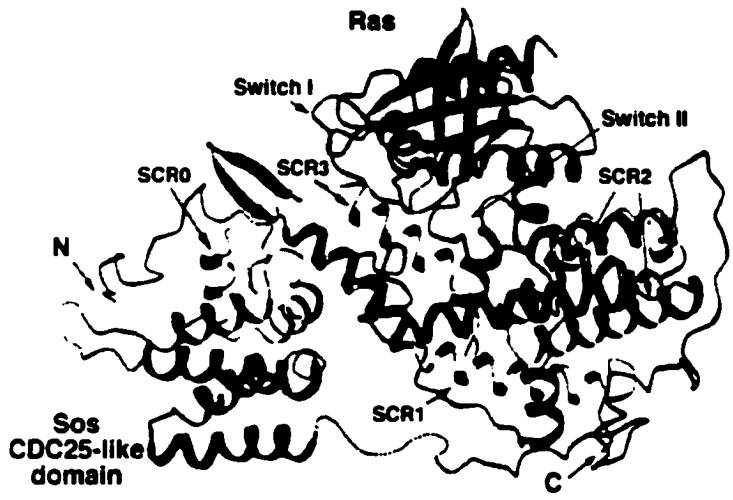


Figure 1.4 The tertiary structure of the complex of the CDC25-like domain of human Sos1 with human H-ras. The structurally conserved regions of the CDC25-like domain are labeled as SCR0-SCR3. H-ras switch I and switch II regions are indicated. N, amino-terminus; C, carboxyl-terminus. This figure is adapted from Boriack-Sjodin, et al. (1998).

Recently the structure of the Sec7 domain which contains the ARF-GEF activity of ARNO, B2-1, and yeast Sec7p has been solved (Betz et al., 1998; Cherfils et al., 1998; Goldberg, 1998; Mossessova et al., 1998) (Figure 1.5). Although from different proteins, the Sec7 domains have very similar structures. The Sec7 domain comprises exclusively α-helices and is a rod-shaped structure with no resemblance to other known GEF family structures. A hydrophobic groove of the Sec7 domain interacts with ARF switch I and switch II regions. Upon GEF binding, the ARF switch II conformation is changed, resulting in the displacement of switch I. This causes the nucleotide-binding site to open up.

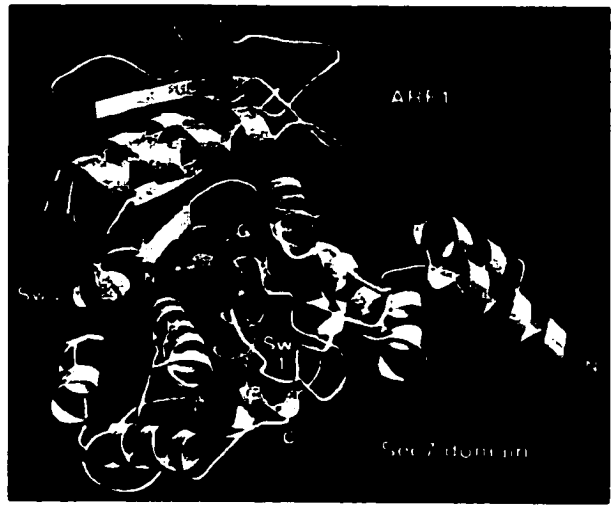


Figure 1.5 The tertiary structure of the Sec7 domain-ARF1 complex. The Sec7 domain is from a yeast protein Gea2 and ARF is a nucleotide-free human ARF1. The switch I (Sw 1) and switch II (Sw 2) of ARF1 are involved in the interaction with Sec7 domain. N, amino-terminus; C, carboxyl-terminus. This figure is adopted from Goldberg (1998).

The crystal structure of the DH domain of Rho-GEF has also been solved (Liu et al., 1998; Soisson et al., 1998) (Figure 1.6). This domain is comprised exclusively of α-helices. Mutagenesis studies indicated that the GTPase binding and exchange activity sites reside in two α-helices at the surface of the DH domain. As shown in Figure 1.6, the DH and PH domains of human Sos1 form an L-shaped structure with two distinct structural modules. The GTPase binding site lies near the DH and PH domain interface.

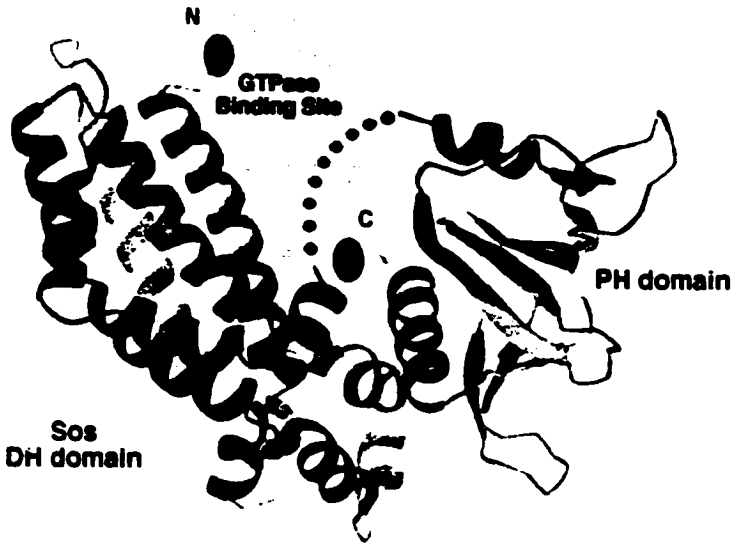


Figure 1.6 The tertiary structure of Sos DH and PH domains. The GTPase binding site may be near the interface between the DH and PH domains. N, amino-terminus; C, carboxyl-terminus. This figure is adapted from Soisson et al. (1998).

Structural analyses support the idea that GEFs induce conformational changes in GTPases and stabilize the nucleotide-free state, rather than the GTP-bound form, of the small GTPases. Through different structural elements and foldings, GEFs interact with common structures in the GTPases (switch I and switch II regions). Yeast genetic screen studies employed to identify the GEF binding residues in different small GTPase families also demonstrate that mutations at the switch I, switch II, and α3-L7 regions prevent GEF interaction (Day et al., 1998; Mosteller et al., 1994). It is thus suggested that GTPases of the Ras superfamily use the same structural regions and mechanism to interact with GEFs, even though all of the GEF families are not evolutionarily related.

Localization of GEFs: how does GEF know where to go?

Most G proteins work at a site next to the membrane. For example, heterotrimeric G proteins work at the inner side of the plasma membrane. Through a G-coupled receptor, which functions as a GEF for G protein, G protein exchanges GDP for GTP and then switches to an "active" form. The activated small GTPases Ras, Rab, ARF, and Rho, also locate to membranes. Because binding of GEF to the GTPase facilitates the GDP/GTP exchange and thus activates the GTPase, it is important that the GTPases are activated at the right place. So far there are no membrane-bound GEFs characterized for the Ras-like small GTPases.

How do GEFs locate to their respective membrane? Are there any targeting signals in their secondary structures? The PH domain could be a candidate since *in vitro* it binds to phosphoinositols. Also, many GEF proteins contain the PH domain. As mentioned earlier, PH domains are thought to be an anchor for proteins associating with membranes (Hemmings, 1997b). Our experimental data (Lee and Pohajdak, 2000), however, showed that the PH domain does not function in isolation as a targeting signal. The PH domain may orient the GEF at the correct angle and thus cause it to associate tightly with the membrane. In our experiments we showed that the N-terminal coiled-coil region of the small ARF-GEF family members (which include B2-1, ARNO, and ARNO3) is responsible for its localization. A Rac-GEF, Tiam1, targets to the plasma membrane through its N-terminal PH domain, a tandem coiled-coil region, and additional flanking region (Stam et al., 1997). The PH domain, or coiled-coil region alone, was not sufficient for its targeting. GEF-H1, a GEF for Rho/Rac GTPases, uses its unique carboxy-terminal coiled-coil domain, instead of its PH domain, to colocalize with

microtubules and subsequently bring the Rho/Rac GTPases to specific target sites (Ren et al., 1998).

Do other GEF proteins have a coiled-coil region in their sequences? Is there a common targeting mechanism among the GEF families? In seeking a possible region that serves as a targeting signal, some of the GEF sequences were compared by running the "COILS" program (Lupas et al., 1991) to predict the coiled-coil regions. Interestingly, many GEF proteins for Ras, Rho, and ARF families contain a putative coiled-coil region, while GEFs for Rab and Ran do not have this potential coiled-coil domain. Some coiled-coil regions are located in front of the DH and PH domains, such as in Dbl, Ost, Tiam-1, mCDC25, and RasGRF2. On the other hand, the coiled-coil regions of Lsc, p115, and GEF-H1 are located behind the DH and PH domains (Figure 1.7).

Some evidence indicates that the GEF coiled-coil regions are involved in protein binding and thereby targeting of the GEFs. The proto-oncogene of lbc has recently been cloned. In comparison to the sequence of the lbc oncogene, the proto-oncogene encodes an additional carboxy-terminal α-helix region followed by a proline-rich region (Sterpetti et al., 1999). The lbc product is a GEF for Rho. Comparing the lbc proto-oncogene and oncogene products (proto-Lbc v.s. onco-Lbc), the proto-Lbc localizes to the particulate (membrane) fraction whereas the onco-Lbc is cytosolic. When analysed the "COILS" program, it is predicted that the α-helix region, which is absent in the onco-Lbc, is a putative coiled-coil region. Taken together, the data suggested that the coiled-coil region plays a major role in the subcellular localization of proto-Lbc.

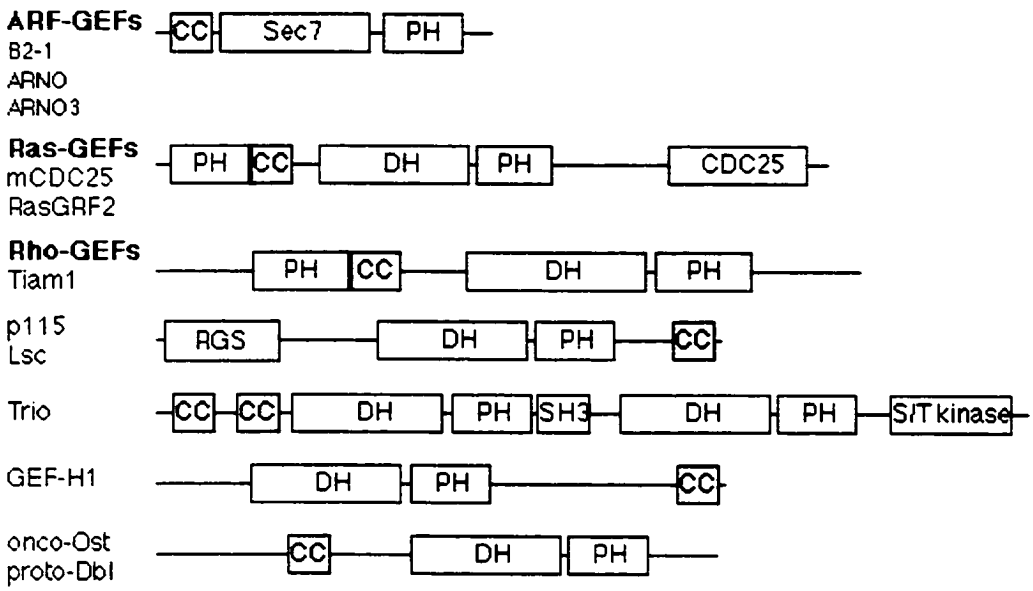


Figure 1.7 Schematic representation of the coiled-coil domains in GEF proteins. Other domains are indicated. CC, coiled-coil domain, as shown in shaded boxes; RGS, regulator of G protein signaling domain. These domains are not drawn to scale.

Many coiled-coil containing proteins have been identified and their structures solved (Lupas, 1996). These proteins are diverse, including structural proteins such as those used to assemble intermediate filaments, transcription factors, DNA polymerases, and G protein βγ subunits. Through their coiled-coil domains, these proteins form either hetero- or homo-dimers, trimers, or tetramers. Some of the GEF proteins may form heterodimers with their binding partners through the coiled-coil regions and thus localize to specific membranes.

Regulation of GEFs

All of the small GTPases require GEFs to exchange GDP for GTP. How do the GEFs know when and where to function? Localization as just discussed may provide part of the answer. But when and how are they regulated? For cell signaling it is very important to have the different enzymes well regulated. So far, it appears that there are several different ways that cells regulate GEFs. Some examples are given below.

Protein-protein binding regulation: Epidermal growth factor (EGF) regulates Ras GTPase by recruiting Sos to the plasma membrane (Buday and Downward, 1993). Binding of EGF to cell surface receptors results in homo-dimerization and tyrosine autophosphorylation of the receptors. This phosphorylation recruits the SH2-containing protein Grb2 (growth factor receptor-bound protein 2). Inside the cell, cytosolic Sos, a Ras GEF, binds to the SH3 domain of Grb2 through its proline-rich domain and is consequently recruited as a complex with Grb2 to the plasma membrane. After association with the plasma membrane, Sos stimulates the Ras signaling pathway (Figure 1.8).

Many GEF proteins contain not only the signature domains that are necessary for the guanine nucleotide exchange activity, but other functional domains involved in protein-protein interactions, such as SH3, SH2, and coiled-coil domains. The GEFs may use these domains to interact with other proteins and thereby be regulated.

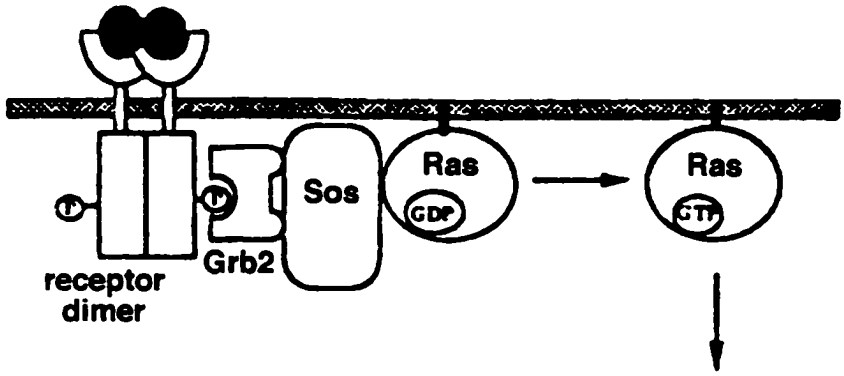


Figure 1.8 Recruitment of Sos to the plasma membrane activates the Ras signaling pathway. The Sos-Grb2 complex is recruited to the plasma membrane by the phosphorylated, homodimerized EGF receptors. Sos then stimulates the exchange of the GDP/GTP for the Ras small GTPase. This figure is adapted from Aronheim et al. (1994).

Phosphorylation: Vav is a postulated GEF for Rho/Rac due to its sequence homology, but initial studies to analyze its enzyme activity were in vain. Vav becomes extensively phosphorylated upon protein kinase activation. It was then shown that phosphorylation of Vav on tyrosine residues stimulates the Rac-1 nucleotide exchange (Crespo et al., 1997).

Second messenger activation: Some GEFs for the Ras GTPases are regulated by second messengers, such as cAMP, calcium, and diacylglycerol. Epac, a recently identified GEF for Rap1, a Ras-like GTPase, contains a cAMP-binding motif. Epac binds cAMP *in vitro* and its GEF activity is strongly induced by cAMP (de Rooij et al., 1998). By using a PKA mutant and inhibitor, it was shown that the activation of Epac by cAMP is not through the PKA related pathway, but rather directly through cAMP binding. Deletion of

the cAMP-binding motif causes activation of Epac, suggesting that this domain functions as a inhibitory module until the binding of cAMP.

Another Ras GEF, RasGRP, contains a diacylglycerol-binding domain and a calcium-binding motif, the "EF hands". Binding of a diacylglycerol analog PMA increases the GEF activity of RasGRP (Ebinu et al., 1998). The GEF activity of mCDC25 is enhanced by interaction with Ca^{2+}/calmodulin through its IQ (calmodulin binding) domain (Farnsworth et al., 1995).

Does GDI, an upstream regulator of GEF, function as a GDP-dissociation inhibitor?

GDP-dissociation inhibitor was named after the first discovery of its ability to inhibit the dissociation of GDP from RhoB (Ueda et al., 1990). The exact role of GDI remains to be defined. As the dissociation rate of GDP from small GTPases is very low, one wonders why a GDI is needed to "inhibit" dissociation. GDIs bind to the prenylated tail of members of the GDP-bound small GTPase Rab and Rho families. Indeed, the post-translational lipid modification is required for GDIs to bind the substrates (Hori et al., 1991). Either one or two geranylgeranyl (C_{20} or 2x C_{20}) tails are added at or near the C-terminus of Rab proteins by post-translational lipidation. In the Rho family, only a single geranylation (C_{20}) occurs. These lipid tails make the GTPase more hydrophobic and enhance membrane binding. Cytosolic Rabs and Rhos are found exclusively in GDI-bound forms. Also GDIs prefer GDP-bound to GTP-bound GTPases as substrates. All this information leads to the argument that the main function of GDI is to translocate the most hydrophobic subfamilies, Rab and Rho GTPases, by extracting Rabs and Rhos from

the membrane and making them cytosolic. Without GDI, it would be hard for the GDP-bound Rab and Rho to leave the membrane and start another cycle. Compared to Rho and Rab, the lipid tails of myristoylated ARF (C_{14}) and farnesylated Ras (C_{15}) are shorter and less hydrophobic than that of Rabs and Rhos (Black, 1992). The fact that *in vitro* the GDP-bound form of myristoylated-ARF is soluble (Antonny et al., 1997) supports the idea here that due to its lesser hydrophobicity, myristoylatd-ARF can be recruited from the membrane without the help of other regulators such as GDI and GDF.

Yeast Rab-GEF Dss4p does not facilitate the GDP/GTP exchange of GDI-bound Sec4p (yeast rab) (Moya et al., 1993). The GDI-bound forms of Rho family members were also poorly activated by Rho-GEFs (Kikuchi et al., 1992). It was then postulated that another factor may disrupt the GDI-GTPase complex before it is activated by GEF. One Rab GDI displacement factor (GDF) dissociates the GDI-GTPase complex (Dirac-Svejstrup et al., 1997). The ERM family is thought to serve as GDFs for the Rho family members (Takahashi et al., 1997). After the displacement of GDI, GEFs exchange GDP for GTP for the small GTPases.

Besides translocation, it is possible that GDI and GDF offer extra steps in regulating Rab and Rho GTPase subfamilies. GDI may bind to the inactivated GDP-bound GTPase until an incoming signal restarts the GDP/GTP cycles.

Cross-talk between Ras-like small GTPase superfamilies through regulators

As mentioned earlier, some Ras GEFs have domains that are the same as those in Rho GEFs, and some of them have GEF activity for both GTPase subfamilies. For

example, a Ras-GEF, mCDC25, also known as Ras-GRF, has DH and PH domains which are signatures of Rho-GEFs. Mutations within the DH domain that block the Rho-GEF activity prevent Ras-GEF activation (Freshney et al., 1997). Sos, another GEF for Ras, also with both homology domains, stimulates guanine nucleotide exchange on Rac through its DH domain (Nimnual et al., 1998). Abr and Bcr proteins are GEFs for the Rho family, but also have GAP domains and activities towards Rho (Chuang et al., 1995). These results indicate a potential mechanism for coupling cellular signaling pathways of Ras and Rho GTPase subfamilies. It has been suggested that Rho family proteins are downstream effectors of the Ras pathway (Zohn et al., 1998).

p115, a GEF for Rho family members, was recently identified as a GAP for the heterotrimeric G protein Gα12 and Gα13 subunits (Kozasa et al., 1998). In addition, Gα13 can stimulate the exchange activity of p115 RhoGEF (Hart et al., 1998). p115 contains a RGS domain (regulator of G protein signaling). RGS containing proteins act as GAPs for Gα subunits (Dohlman and Thorner, 1997). Another recently identified GEF for Rho GTPases, PDZ-Rho GEF, binds to Gα12 and Gα13 through its RGS domain, which was referred to as the LH domain (Fukuhara et al., 1999). Taken together, these RGS domain-containing Rho GEF proteins may serve as downstream regulators of G protein and upstream regulators of Rho, thereby connecting these two signaling pathways.

The protein p532, as mentioned earlier has two RCC1-like domains, has GEF activities for both ARF1 and Rab at its RCC1-like domains (Rosa et al., 1996). This protein contains many motifs in addition to its RCC1-like domains, including seven β-repeat domains characteristic of the Gβ, three SH3 binding sites, and a putative leucine-

zipper. It is possible, through these different domains, that p532 regulates multiple cellular processes, such as vesicle transport regulated by ARF and Rab.

Besides GEFs, cross-talk occurs between GAPs as well. In one study, a Ras-GAP called p120 RasGAP and p190 RhoGAP were shown to form a complex through their two SH2 domains and two tyrosine phosphorylation sites, respectively. By this binding the conformation of p120 RasGAP changed and thus it may interact with another unidentified protein through p120 RasGAP's SH3 domain (Hu and Settleman, 1997).

Proto-oncogenes

Many proteins involved in the small GTPase cycle, including the GTPase and its regulators, are oncogenic. Ras mutations in codon 12, 13, or 61 occur in more than 15% of human tumors with varied frequency in different tumor types (Bos, 1989). These point mutations result in activated Ras due to its insensitivity to Ras GAP and thereby, Ras acquires transforming ability.

Independent studies have identified a group of oncogenic proteins that contain the Dbl homology domain, many of which were later proven to be GEFs for the Rho/Rac GTPase family (Cerione and Zheng, 1996; Whitehead et al., 1997). Their mechanism for causing cell transformation is different from that of the Ras oncogene. Instead of point mutations, many Dbl family oncogenes are translocated and therefore encode a truncated and/or fusion protein. For instance, the oncogenic Dbl lacks the amino-terminal 497 residues (coiled-coil region) of proto-Dbl protein (Hart et al., 1994). Another member, Bcr, acquires transforming activity when fused with Abl (Bcr-Abl) (McWhirter and Wang, 1997). The phenotype of cells that have been transformed by the oncogenic Dbl

family proteins is similar to that of oncogenic Rho/Rac transforming cells (Khosravi-Far et al., 1994), suggesting that the Dbl family proteins contribute transforming activity by upregulating Rho/Rac GTPases through their GEF activities.

Conclusions

It has been almost two decades since the first small GTPase was discovered. Since then, more and more families of small GTPase have been characterized, followed by the discovery of their regulators, GEF, GAP, GDI, and the most recent GDF. GEFs play an important role in GTPase regulation, facilitating localization of GTPases, and then exchanging guanine nucleotide for the GTPases. Many ways of regulating GEFs have been identified, such as phosphorylation, protein-protein interaction, and second messenger binding. Considering the diversity of GEF proteins, more pathways, some of which may be tissue and cell type specific, that convey the signals to individual GEFs and thereby turn on the GTPase cycles, may exist. The complete regulatory mechanisms for the small GTPases may be far more sophisticated than we currently suspect.

Part II: Research History of Sec7-Related Proteins

Sec7-domain-containing proteins have drawn a lot of attention in recent years in the ARF-GEF field, because the Sec7 domain is a signature of the GEFs for the small GTPase ARFs. The Sec7 protein was initially characterized as a required component for protein transport within the yeast Golgi apparatus (Achstetter et al., 1988). In 1992 we cloned a cDNA, *B2-1* (Liu and Pohajdak, 1992), which was originally thought to be a natural killer cell specific gene. This gene encodes a protein that contains the amino terminal coiled-coil, central Sec7, and carboxyl pleckstrin homology domains. Due to similarity to the Sec7 protein, it was postulated that B2-1 functions in vesicle transport, similar to the Sec7 protein.

The first link between "Sec7" and ARF was established by Deitz et al. (1996). In an attempt to identify the mammalian homologues of the Sec7 protein, this group screened clones that rescue the *sec7* lethal mutants in yeast. Instead of a Sec7 homologue, the human ARF4 protein was identified as a rescuer of the yeast *sec7* mutant. Another approach was taken by Vaughan's group. They isolated a protein from bovine extracts that had the GEF activity for ARF. Sequenced fragments of this protein have similar sequences to the Sec7 protein in yeast (Morinaga et al., 1996). Later in the same year, a protein in yeast, Gea1, was identified as a suppressor of the growth defects caused by the *ARF* mutation (Peyroche et al., 1996). Further analysis showed that Gea1 has guanine nucleotide exchange activity. This protein contains a region with similarity to part of the Sec7 protein. At the same time, Chardin et al. used the "Sec7 domain" sequence to search the Genbank database to look for other genes that may serve as GEFs.

ARNO was thus identified as an ARF-GEF in humans (Chardin et al., 1996). Since then, numerous proteins that contain the Sec7 domain have been found, and many of them have GEF activity.

Besides the genes mentioned above, several other genes found earlier, such as *EMB30* in *Arabidopsis* (Shevell et al., 1994), and an open reading frame in *Caenorhabditis elegans* cosmid K06H7, also encode the Sec7 domain. A mutation at one of the conserved residues (E658K) in the Sec7 domain of EMB30 caused a different seedling phenotype, indicating this domain is important for function, even though there were no visible changes in Golgi morphology (Shevell et al., 1994).

Comparison of Sec7-domain-containing proteins

Based on size and domain composition, Sec7-domain-containing proteins are divided into three major groups, large, intermediate, and small proteins (Figure 1.9). The common region in which all the proteins aligned is the Sec7 domain, which is around 200 amino acid residues long. Besides the Sec7 domain, large size members have similarities throughout long stretches of the rest of the protein. In *Saccharomyces cerevisiae*, there are only large Sec7-domain-containing proteins present. These include Gea1p, Gea2p, and Sec7p. To this date, the large Sec7-domain-containing proteins identified in mammals are BIG1, BIG2, and GBF1. Based on the sequence similarity between these large Sec7-domain-containing proteins, the large size group has been divided into two subgroups: Gea1/2p, GBF1 and EMB30 in one subgroup; Sec7p, and BIG1/2 in another (Jackson and Casanova, 2000). In Figure 1.9 only Sec7p represents the large Sec7-domain-containing proteins. Small size members, B2-1 and two highly similar proteins

ARNO and ARNO3 found in humans, an ORF in *C.elegans*, and SecG in *Dictyostelium discoideum*, have another region beside the Sec7 domain that is well aligned, adjacent to the Sec7 domain. This region is the pleckstrin homology (PH) domain. The protein EFA6, the only intermediate member found so far, also has a weak homology to the PH region. In addition to the Sec7 and PH domains, the small size members have a coiled-coil region in front of the Sec7 domain.

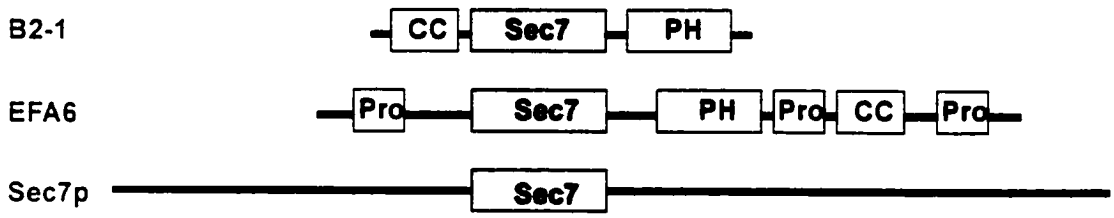


Figure 1.9 Domain composition of three groups of Sec7-domain-containing proteins. B2-1 represents the small size group; EFA6 represents the intermediate size group; and Sec7p represents the large size group. Abbreviations: CC, coiled-coil; Sec7, Sec7 domain; PH, pleckstrin homology domain; Pro, proline-rich region.

To take a detailed look at the Sec7domain, a multiple alignment was performed by using Clustal W (1.74), a computer sequence alignment program. Several different sized proteins originating from different organisms were chosen as described (Figure 1.10). Partial sequence of the SecG (including the complete PH domain and carboxyl end of the Sec7 domain) was first found in Genbank (accession number U78755). Further

Figure 1.10 Multiple alignment of Sec7 domain sequences. The Sec7 domain containing proteins compared here are as follows: B2-1, from *Homo sapiens*, accession number Q15438; ARNO, from *Homo sapiens*, accession number X99753; SecG, from *Dictyostelium discoideum*, unpublished sequence; K06H7, an open reading frame from the Cosmid K06H7 of *Caenorhabditis elegans*, accession number M89330; EFA6, from *Homo sapiens*, accession number NP002770; BIG1, from *Homo sapiens*, accession number NP006412; GBF1, from *Homo sapiens*, accession number NP004184; Sec7p, from *Saccharomyces cerevisiae*, accession number J03918; Gea1, from *Saccharomyces cerevisiae*, accession number P47102; EMB30, from *Arabidopsis thaliana*, accession number Q42510. B2-1, ARNO, SecG, and K06H7 are categorized in the small Sec7-domain-containing protein group. EFA6 is the only intermediate group thus far isolated. BIG1, GBF1, Sec7p, Gea1 and EMB30 belong to the large size group.

```
B2-1    FNMDPKKGIQFLIENDLLKN--TCEDIAQFLYKGE-GLNKTAIGDYLGERDEFNIQVLHA
ARNO    FNMDPKKGIQFLVENELLQN--TPEEIARFLYKGE-GLNKTAIGDYLGEREELNLAVLHA
SecG    FNSHPKKGIEFIVANGVISE-KNPKEVAHFLLTHS-ELSKQSIGEYIGEGDDFNLQVLHA
K06H7   FNQDPWKALDWLASRNVVAK--DPQALALWMKAGE-GLSKSAIGEILGDNRPFALETLDR
EFA6    STDRLALGSTDTLSNGQKADLEAAQRLAKRLYRLD-GFRKADVARHLGKNNDFSKLVAGE
BIG1    FNKKPKRGIQYLQEQGMLGT--TPEDIAQFLHQEE-RLDSTQVGEFLGDNDKFNKEVMYA
GBF1    FNQKPKKGIQFLQEKGLLTIPMDNTEVAQWLRENP-RLDKKMIGEFVS--DRKNIDLLES
Sec7    FNNKPKKAIPVLIKKGFLKD-DSPISIAKWLLETE-GLDMAAVGDYLGEGDDKNIAIMHA
Gea1    FNEKAKKGIQMLIEKGFIDS-DSNRDIASFLFLNNGRLNKKTIGLLLC--DPKKTSLLKE
EMB30   FNRDPKKGLEFLQGTHLLPDKLDPQSVACFFRYTA-GLDKNLVGDFLGNHDEFCVQVLNE
          .     .                    :*  :      :    :.  :

B2-1    FVELHEFTDLNLVQALRQFLWSFRLPGEAQKIDRMMEAFAQRYCQ---------------
ARNO    FVDLHEFTDLNLVQALRQFLWSFRLPGEAQKIDRMMEAFAQRYCL---------------
SecG    FVDELNFFGLDFDVALRKYLLTFRLPGEAQKIDRMMEKFASQFYQ---------------
K06H7   FTKEHKLHDVPIVPALRQYLFSFRLPGESQKINRILEKFAEVYAN---------------
EFA6    YLKFFVFTGMTLDQALRVFLKELALMGETQERERVLAHFSQRYFQ---------------
BIG1    YVDQHDFSGKDFVSALRMFLEGFRLPGEAQKIDRLMEKFAARYLE---------------
GBF1    FVSTFSFQGLRLDEALRLYLEAFRLPGEAPVIQRLLEAFTERWMN---------------
Sec7    FVDEFDFTGMSIVDALRSFLQSFRLPGEGQKIDRFMLKFAERFVD---------------
Gea1    FIDLFDFKGLRVDEAIRILLTKFRLPGESQQIERIVEAFSSKYSADQSNDKVELEDKKAG
EMB30   FAGTFDFQYMNLDTALRLFLETFRLPGESQKIQRVLEAFSERYYMQ---------------
        :     :    .  *:*  *  : * **    :*.:  *:  :

B2-1    CNNG----------VFQSTDTCYVLSIIMLNTLHNPNVKDK---TVERFIAMNRGI
ARNO    CNPG----------VFQSTDTCYVLSVIMLNTLHNPNVRDK---GLERFVAMNRGI
SecG    HNPENK--------VFVNQDAVYVLAVIMLNTAHNPQHKEE---TKAEFLRNNSGI
K06H7   QNPS----------YGNADQAHTVACIMVNTLHNPNVKDK---SLEKYIEMNEQL
EFA6    CNPEA----------LSSEDGAHTLTLMLLNTLHGHNIGKR---TCGDFIGNLEGL
BIG1    CNQGQT--------LFASADTAYVLAIIMLTTLHSPQVKNK---TKEQYIKMNRGI
GBF1    CNGSP----------FANSDACFSLAVIMLNTQHNHNVRKQNAPTLEEFRKNLKGV
Sec7    QNPG----------VFSKADTAYVLSLIMLNTLHSSQIKNK---SLQEFLENNEGI
Gea1    KNGSESMTEDDIIHVQPDADSVFVLSIIMLNTSHNPQVKDH---TFDDYSNNLRGC
EMB30   ---SP---E-----ILANKDAALVLSIIMLNTQHNVQVKKK---TEEDFIRNNRHI
                      . *    :: : :::.*  *. :  ..    :   :

B2-1    NDGGDLPEELLRNLYESIKNEPFKIPED
ARNO    NEGGDLPEELLRNLYDSIRNEPFKIPED
SecG    NSGDDLPPDFMENLYDKIVTNEIKMERD
K06H7   LEKGAITIEQLTEVYESVSVTQFKIPDE
EFA6    NDGGDFPRELLKALYSSIKNEKLQWAID
BIG1    NDSKDLPEEYLSAIYNEIAGKKISMKET
GBF1    NGGKDFEQDILEDMYHAIKNEEIVMPEE
Sec7    DNGRDLPRDFLEGLFNEIANNEIKLISE
Gea1    YNGKDFPRWYLHKIYTSIKVKEIVMPEE
EMB30   NGGNDLPREFLSELFHSICNNEIRTTPE
             :    :  ::  :    :
```

Figure 1.10

complete sequence of the Sec7 domain and coiled-coil region of this gene was done in collaboration with Dr. van Haastert (University of Groningen, The Netherlands) (Lee et al., unpublished results). The Sec7 domain used in this alignment is from residue 73 (F) to 249 (D) (FNMDP---KIPED) of B2-1.

GEF activity of the Sec7 domain

The crystal and solution structures of the Sec7 domains from several proteins are solved (Figure 1.5)(Betz et al., 1998; Cherfils et al., 1998; Goldberg, 1998; Mossessova et al., 1998). As aforementioned the Sec7 domain consists of 10 α-helices (labeled αA to αJ). The important regions for interaction with ARF locate at the loop between αF and αG and at αH (Figure 1.5), as shown in Figure 1.10, in blue letters. The glutamate residue (yellow highlighted "E"), which is conserved in all Sec7 domains, even in the most divergent EFA6, is essential for its interaction with ARF (Beraud-Dufour et al., 1998; Goldberg, 1998). It lays within a hydrophobic groove of the Sec7 domain and interacts directly with the switch I region of ARF (mentioned in chapter 1). Interestingly, the EMB30 mutation mentioned earlier is also at this glutamate residue. Mutation of this glutamate to lysine, which is the same replacement in the EMB30 mutation, dramatically decreases the exchange activity of ARNO and B2-1 (Beraud-Dufour et al., 1998; Betz et al., 1998).

Sensitivity of ARF-GEFs to brefeldin A

ARF-GEFs have been postulated to be the targets for the fungal metabolite brefeldin A (BFA). BFA blocks the anterograde Golgi transport pathway. The intracellular transport of secretory proteins is blocked when cells are treated with BFA (Misumi et al., 1986). BFA causes the dissociation of β-COP, one subunit of the coated proteins of COPI vesicles, and therefore blocks assembly of coatomers and consequently vesicle budding. Pretreatment of the cells with GTPγS or AlF_4^- prevents the effect of BFA on the dissociation of β-COP from Golgi membranes (Donaldson et al., 1991). Both AlF_4^- and GTPγS block G proteins in the active, GTP bound state. Further analysis revealed that an enzyme, which catalyzes the guanine nucleotide exchange for ARF, in a Golgi-enriched fraction is inhibited by BFA (Donaldson et al., 1992b; Helms and Rothman, 1992). At that time no guanine nucleotide exchange factors for ARF had been found.

Not until several Sec7-domain-containing proteins were identified as ARF-GEFs has it become possible to compare the sequences and to study how the ARF-GEFs interact with BFA. Evidently, not all Sec7-domain-containing proteins are BFA sensitive. Initially, the large size Sec7-domain-containing proteins were BFA-sensitive while the small sized ones were BFA-insensitive until GBF1 (large in size) was found, which was identified as a Golgi BFA resistant factor (Claude et al., 1999). The Sec7 domains of the small ARF-GEF members are less sensitive to BFA than that of the large ARF-GEFs (except GBF1). Due to this fact, residues within the Sec7 domain that are different between these two groups were given much attention. In 1999, it was shown that certain residues within the Sec7 domain might be the targets of BFA, thus blocking

GEF activity for ARF (Peyroche et al., 1999; Sata et al., 1999). Two reports found the BFA-susceptible region of the Sec7 domain by different approaches. By random mutagenesis, Peyroche et al. (1999) showed that the 35 amino-acid residue region around the second conserved motif (second blue-letter region in Fig. 1.10) is related to BFA sensitivity. Two residues (green highlighted positions in Fig. 1.10) were further identified in this region that are responsible for BFA sensitivity. In the characterized BFA-insensitive small members (B2-1 and ARNO in figure 1.10), Phe (F) and Ala (A) occupy these positions, whereas in the BFA-sensitive large members these positions are Tyr (Y) and Ser (S). Interestingly, GBF1 bears a hybrid sequence YA. Another group identified a similar region by making chimeras of the large and small Sec7-domain-containing proteins (Sata et al., 1999). By swapping the Sec7 domains between the Sec7 protein and cytohesin-1/B2-1, an 11-amino-acid region within the Sec7 domain was located. As shown in Fig 1.10 the two pink-highlighted residues are the susceptible residues. Although there is no difference in these two residues among large members, GBF1 has three extra amino acids in this region. More information on BFA sensitivity could be obtained by checking the sensitivity of the other non-characterized Sec7-domain-containing proteins (e.g., SecG, K06H7, and EFA6).

Studies of the pleckstrin homology domain in the small Sec7 domain family proteins

One interesting feature of the small Sec7 domain family proteins is that these proteins contain a PH domain at the carboxyl end of the Sec7 domain. The PH domain is a well-defined phospholipid binding domain. First characterized in 1993, there are now

more than 100 proteins found to contain the PH domain, many of which are involved in signal transduction and cytoskeleton organization. Several GEFs and GAPs for small G proteins also have PH domains.

GRP1 (mouse homologue of ARNO3) was first cloned as a general receptor for phosphoinositides (Klarlund et al., 1997), specific for phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) through its PH domain. The GEF activity of ARNO is stimulated through its PH domainARNO's PH domain has been shown to be stimulated by PtdIns(4,5)P_2 *in vitro*, although PtdIns(3,4,5)P_3 was not tested in that study (Chardin et al., 1996).

Detailed studies concerning the specificity of various PH domains have been carried out (Kavran et al., 1998; Rameh et al., 1997). Based on the these specificities, PH domains are grouped into three major categories: firstly, those that bind PtdIns(3,4,5)P_3 with high affinity; secondly, those that bind PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 equally well; and finally, those that bind specifically to PtdIns(3,4)P_2. Some other PH domains do not show any preference among different PIs. The PH domains of the small ARF-GEFs belong to the group that binds specifically to PtdIns(3,4,5)P_3 (Kavran et al., 1998; Klarlund et al., 1998)

GEF specificity towards ARFs

In mammals, there are six ARFs and at least three groups of different GEFs for ARFs that have been identified. Is there any specificity among them, or are they functionally redundant? In *S. cerevisae*, ARF1 and ARF2 are 96% identical and functionally redundant. Therefore, their ARF-GEFs, Gea1, Gea2, and Sec7p probably

interact with both equally well *in vitro*. A double deletion of Gea1 and Gea2 is lethal, as is a Sec7p deletion, suggesting that there are no overlapping functions between Gea1/Gea2 and Sec7. This indicates that even though they can interact with both ARFs, there must be other factors that affect their specific functions *in vivo*, presuming in distinct compartments. The small ARF-GEFs contain an amino-terminal coiled-coil region besides the Sec7 and PH domains. Their PH domains are believed to bind to the PtdIns(3,4,5)P_3 and there is no evidence for interaction with ARF. The Sec7 domain interacts with an ARF binding site, but there are no clues on the specificity towards different ARFs. Biochemical assays showed promiscuous results of exchanging activity on different types of ARFs. Large Sec7-domain-containing proteins can use their other uncharacterized domain as the specificity binding region. EFA6, on the other hand, is colocalized with ARF6 to the plasma membrane and endocytotic vesicles.

Mammalian ARFs are grouped into class I (ARF1, ARF2, and ARF3), class II (ARF4 and ARF5), and class III (ARF6). The small ARF-GEF family members, B2-1/cytohesin-1, ARNO, and ARNO3 can activate class I ARFs (Franco et al., 1998; Meacci et al., 1997). Conflicting results have been observed for ARNO and GRP1 (the mouse homologue of ARNO3) exchanging activity on ARF6 (Frank et al., 1998; Langille et al., 1999). GBF1 is specific for ARF5 (Claude et al., 1999). BIG1/BIG2 activate ARFs 1,5, and 6 (Togawa et al., 1999). EFA6 is a GEF for ARF6 and colocalized with ARF6 to the plasma membrane (Franco et al., 1999).

In this thesis the subcellular localization of the small ARF-GEF and its targeting mechanism were studied. The results showed that the coiled-coil region is responsible for Golgi targeting. The Sec7 domains among the three small ARF-GEFs are more than

84% identical and 96% conserved. By binding to an adaptor protein, the small ARF-GEFs obtain substrate specificity.

Chapter 2

B2-1, a Sec7- and Pleckstrin Homology Domain-Containing Protein, Localizes to the Golgi Complex

(This chapter is published in Experimental Cell Research (2000) 256, 515-521)

Introduction

Using the technique of subtractive hybridization (NK cells minus T helper cell line Jurkat), we isolated several unique cDNA clones. One of the clones, B2-1, is highly expressed in NK cells and peripheral T cells (Liu and Pohajdak, 1992). Various other cell lines have lower levels of B2-1 expression and it was shown that B2-1 was not NK cell specific. The B2-1 cDNA encodes for a 398-amino-acid long protein. The gene was mapped to chromosome 17qter (Dixon et al., 1993). The internal domain of B2-1 was first reported to have a high similarity to a portion of the yeast *SEC7* gene product (Sec7p) (Achstetter et al., 1988) and this region is now generally termed the "Sec7 domain". Several groups independently showed that the Sec7-domain-containing proteins are guanine exchange factors (GEFs) for various ADP ribosylation factors (ARFs) (Chardin et al., 1996; Meacci et al., 1997; Morinaga et al., 1997; Pacheco-Rodriguez et al., 1998; Peyroche et al., 1996). ARF is a small GTP-binding protein and is necessary for coatomer-coated vesicle formation (Donaldson et al., 1992a). There are two major groups of Sec7-domain-containing proteins, one that is large in size (Sec7p, Gea1p, Gea2p, p200 and GBF1) and another group that is smaller in size (ARNO, B2-1 /cytohesin, and ARNO3). Recently, structure studies of the Sec7 domain also show the

possible interaction between this domain and ARF (Betz et al., 1998; Cherfils et al., 1998; Mossessova et al., 1998). B2-1 was shown to be specific for ARF1 and ARF3 but not ARF5 (Meacci et al., 1997). ARNO was shown to be effective on both ARF1 (Chardin et al., 1996; Frank et al., 1998) and ARF6 (Frank et al., 1998). However, others have not been able to reproduce an effect of ARNO on ARF6 (Franco et al., 1998). The third member, GRP1 (homologue of ARNO3), was active on ARF1 and ARF5 (Klarlund et al., 1998). Recently another Sec7-domain-containing protein EFA6, which is intermediate in size, was shown to be specific for ARF6 (Franco et al., 1999). All of the small Sec7-domain-containing proteins also have a flanking pleckstrin homology domain which binds to phosphoinositols including phosphatidylinositol 4,5-bisphosphate (Harlan et al., 1994) and phosphatidylinositol 3,4,5-trisphosphate (Klarlund et al., 1997).

The fungal metabolite brefeldin A (BFA) inhibits GDP exchange activity towards various ARFs by binding to the GEF-GDP-ARF complex (Peyroche et al., 1999). Consequently the vesicular trafficking is blocked and the Golgi complex disrupted. Interestingly, only the large Sec7-domain-containing members are found to be BFA-sensitive. Mutation of the large yeast Sec7p results in the accumulation of glycoproteins within the Golgi apparatus and the exaggeration of Golgi cisternae (Franzusoff and Schekman, 1989). Sec7p is required for endoplasmic reticulum (ER)-derived vesicle fusion with the Golgi complex (Lupashin et al., 1996). Overexpression of ARFs can rescue the *SEC7* mutation in yeast (Deitz et al., 1996). However, a non-transport function was also given to B2-1 (renamed cytohesin-1). Cytohesin-1 was shown to bind to the intracellular carboxyl terminus of the β2-integrin molecule and its Sec7 domain is responsible for inducing LFA-1 binding to ICAM (inside-out signaling) (Kolanus et al.,

1996). However, the Sec7 domain of B2-1 does not interact with β2-integrin in solution (Betz et al., 1998).

While there is a growing amount of evidence suggesting a role of these Sec7-domain-containing proteins in vesicular transport in mammalian cells, there is conflicting information on the *in vivo* subcellular localization of the small Sec7 domain family members. Overexpression of ARNO shows a cytosolic distribution with somewhat poor plasma membrane localization (Frank et al., 1998). This subcellular distribution is expected since one of the ARFs targets for ARNO is ARF6, which has previously been found associated with the plasma membrane (Cavenagh et al., 1996). Recently, it was also shown that ARNO does not have any affects on ARF6 (Franco et al., 1998). Others have found that ARNO is cytoplasmic and only localizes to the plasma membrane following stimulation with insulin (Venkateswarlu et al., 1998b). In contrast, it was recently shown that ARNO overexpression causes disassembly of the Golgi and is postulated to play a role in Golgi to ER transport (Monier et al., 1998). ARNO3 overexpression also shows a similar effect on the Golgi (Franco et al., 1998). The last member of this family, cytohesin-1, was shown to associate with the plasma membrane (Nagel et al., 1998). Here we report the immunolocalization of endogenous and transfected B2-1 protein. The B2-1 protein is found in a perinuclear compartment that colocalizes with Golgi complex markers. B2-1's distribution is affected by BFA. In addition, by transfection of green fluorescent protein (GFP)-tagged B2-1, we showed a similar Golgi complex association as with endogenous B2-1. Furthermore, cells that overexpressed transfected B2-1 resulted in a major alteration of the Golgi complex.

Materials and Methods

Cell Culture

Cos-1 cell line was obtained from Dr. C. Too (Dalhousie University, Halifax, Nova Scotia, Canada) and was propagated in DMEM containing 10% fetal bovine serum and antibiotics at 37°C in 5% CO_2.

Antibodies

Polyclonal antiserum was raised in New Zealand White rabbits against full-length recombinant B2-1 expressed in *Escherichia coli*. Rabbits were immunized with 150 μg of protein in Freund's Complete Adjuvant and bled 8 weeks postimmunization. Anti-GRP78 monoclonal antibody was purchased from StressGen. Antibodies against ERGIC-53, giantin and TGN 46 were kindly provided by Dr. H. -P. Hauri (Biozentrum, University of Basel, Basel, Switzerland) (Schweizer et al., 1988), Dr. A.D. Linstedt (Carnegie Mellon University, Pittsburgh, PA) (Linstedt and Hauri, 1993) and Dr. M. Fukuda (Burnham Institute, La Jolla, CA) (Kain et al., 1998). FITC-conjugated, and Alexa (488)-conjugated goat anti-rabbit and goat anti-mouse antibodies were purchased from Sigma and Molecular Probes, respectively. Cy3-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Sigma.

Protein gel and Western Blotting

Protein samples were loaded on SDS-PAGE and either stained with Coomassie brilliant blue or analyzed by Western blotting. For Western blotting, signals were detected by the enhanced chemiluminescence method (Amersham) using HRP-conjugated goat anti-rabbit antibodies (Amersham).

Expression of Recombinant Proteins

6xHistidine-tagged B2-1. Partial digestion of B2-1 cDNA with *Nco*I generated a fragment coding for amino acid 1-398 (full-length B2-1). This fragment was ligated to a 6xhistidine tag plasmid pRSET B (Invitrogen, Carlsbad, CA) *Nco*I site. Recombinant protein was expressed in *E. coli* BL21(DE3)pLysS. Induction of protein expression and purification on Ni-NTA resin were followed using the Qiagen protocols.

GST-tagged B2-1. B2-1 cDNA was ligated to a glutathione *S*-transferase (GST) tag plasmid pGEX. Recombinant protein was expressed in *E. coli* DH5α. Induction of protein expression and purification were followed using standard protocols (Amersham Pharmacia Biotech AB, Sweden). In brief, cells were induced with 1mM IPTG at 37°C for 3 h. Cell lysates were resuspended in sonication buffer (1xPBS containing 1.5% sarcosyl). After sonication, $CaCl_2$ (1 mM final) and Triton X-100 (2% final) were added before centrifugation. Recombinant protein was purified on glutathione-Sepharose 4B beads (Amershan Pharmacia Biotech AB, Sweden) under native conditions.

B2-1 GST protein competition assay

GST proteins (GST-tag alone and GST-B2-1) were purified on glutathione-Sepharose 4B beads (without elution). The protein concentrations were estimated by running an aliquot of beads on a SDS-PAGE protein gel. B2-1 antiserum, 1 μl, was diluted in 500 μl 1xPBS and preincubated with 1 μg GST-tag alone, 1 μg GST-B2-1, or equal amount of control beads alone, respectively. After a 1-h preincubation, the supernatants were used for immunofluorescence experiments.

Transient transfection and construct

A full-length B2-1 fragment was amplified by PCR with introduced primer *Bam*HI sites and was ligated into the *Bam*HI site at the N-terminus of GFP (pCS2+GFP

vector). The construct was sequenced and the protein expression was confirmed by Western blotting. Transient transfection was performed using Qiagen SuperFect reagent and following the manufacturer's protocol. The cells were fixed and used for immunofluorescence experiments after 24 h of transfection.

Immunofluorescence Microscopy

Cos-1 cells were seeded on glass coverslips the day before experiments. The cells were fixed in 4% paraformaldehyde in PBS buffer, pH 7.5, for 20 min, followed by permeabilization in 0.1% Triton X-100. Primary antibodies diluted in PBS containing 1% goat serum were added and incubated for 30 min at room temperature. The cells were then washed with PBS containing 0.1% Triton X-100 three times. The cells were incubated with secondary antibodies for 20 min at room temperature. Washing procedure was then repeated. As for primary antibodies, B2-1 antiserum and its preimmune serum were used in 1:500 dilution; ERGIC-53, giantin, and GRP78 mouse monoclonal antibodies were used in 1:1000 dilution; TGN46 rabbit polyclonal antibodies were used in 1:400 dilution. As for secondary antibodies, we used FITC-, Alexa (488)-, or Cy3-conjugated goat anti-rabbit antibodies and Alexa (488)- or Cy3-labeled goat anti-mouse antibodies (1:1000 dilution). For BFA treatment experiments, Cos-1 cells were incubated with 5 μg/ml BFA (Sigma, 5 mg/ml stock solution in ethanol) for various periods prior to fixation. Cells were observed using a Zeiss confocal microscope LSM.

Results

Localization of B2-1

Full-length recombinant B2-1 was made in *E.coli* and purified for raising polyclonal antibodies (Fig. 2.1A). B2-1 was purified under denatured condition and was renatured before elution. In all cell line lysates tested, a band about 75 kDa was recognized by the preimmune serum (Fig. 2.1B, lane 1). B2-1 antiserum, however, recognized an extra band at size 46 kDa, which is the expected size of B2-1 (Fig. 2.1B, lane 2). Due to the preimmune band recognized in Western blotting assay, we first checked the immunofluorescence staining pattern of preimmune serum on cells. As shown in Fig. 2.2A, on Cos-1 cells, only few random scattered dots were visible using preimmune serum. B2-1 antiserum stained the perinucleus with a concentrated vesicular pattern (Fig. 2.2B). Similar B2-1 perinuclear localization was found in the lymphoid cells (Appendix I, Fig. A.1). Affinity-purified antibodies also gave a similar staining pattern, although with less intensity (not shown). The stronger immunofluorescent staining of B2-1 antiserum (compared to Western analysis) may be due to the renatured purification condition of the B2-1 recombinant protein and that the raised antibodies can recognize native antigens better than denatured ones (Western blotting). Although B2-1 was initially cloned from an NK-subtracted cDNA library, it was further found by Northern analysis to be expressed in various non-NK cells. Several cell lines of lymphoid origin were shown to have high levels of B2-1 mRNA expression while other non-lymphoid lines have various levels. The higher expression of B2-1 in lymphoid cells was further substantiated by Kolanus et al. (1996).

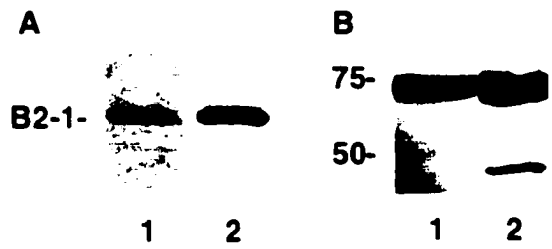


Figure 2.1 Production of recombinant B2-1 and detection with B2-1 antiserum. (A) Lane 1, Coomassie brilliant blue staining of purified full-length *E. coli* recombinant B2-1 protein containing the 6xhistidine-tag; lane 2, Western blotting, B2-1 antiserum against purified full-length recombinant B2-1 protein. (B) Western blotting of Cos-1 cell lysate using preimmune serum (lane 1) or B2-1 antiserum (lane 2).

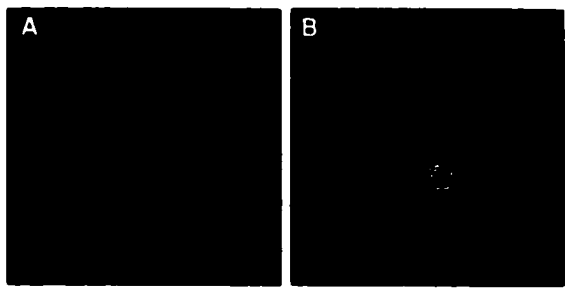


Figure 2.2 B2-1 subcellular localization. Immunofluorescent detection of B2-1 using B2-1 antiserum (B) or preimmune serum (A) in Cos-1 cells. Cy3-conjugated goat anti-rabbit secondary antibody was used.

To further verify B2-1 antiserum's specificity, GST-tagged B2-1 recombinant protein was added to compete away the antibodies for immunofluorescent staining. As shown in Figure 2.3, B2-1 antiserum that was preincubated with GST-beads alone (2.3B), as well as with beads only (2.3A), did not block the B2-1 signal. In contrast, preincubation of B2-1 antiserum with GST-B2-1-beads dramatically reduced the concentrated perinuclear vesicular staining signal (2.3C). This experiment was repeated several times with similar results. Because the GST-B2-1 recombinant protein bound the antibodies raised against the 6xhistidine tagged-B2-1 (see Materials and Methods) and consequently blocked the perinuclear immunofluorescent staining pattern, it also indicates that the B2-1 antibodies were specific to B2-1 proteins, rather than to the 6xhistidine tag.

Since B2-1's subcellular localization showed a perinuclear distribution similar to that of proteins associated with the ER and Golgi, we then tested, by dual labeling, antibodies directed against proteins found in these two compartments in Cos-1 cells. As shown in Figure 2.4, B2-1 partially overlapped with two Golgi-associated proteins, ERGIC-53 and giantin. The majority of the ERGIC-53 distribution is in the ER-Golgi intermediate compartment while giantin is a membrane protein localized in the Golgi complex (Linstedt and Hauri, 1993). In addition to B2-1's partial overlap with these two Golgi complex proteins (giantin and ERGIC-53), it also localized to an area adjacent to the Golgi complex with a punctate staining pattern. As evident in Fig. 2.4 (bottom panels), B2-1 did not colocalize with the ER retention protein GRP78. GRP78 contains the KDEL motif and is found primarily in the ER. It is clear that B2-1 localized to a perinuclear area from which GRP78 was excluded. In Jurkat cells B2-1 partially

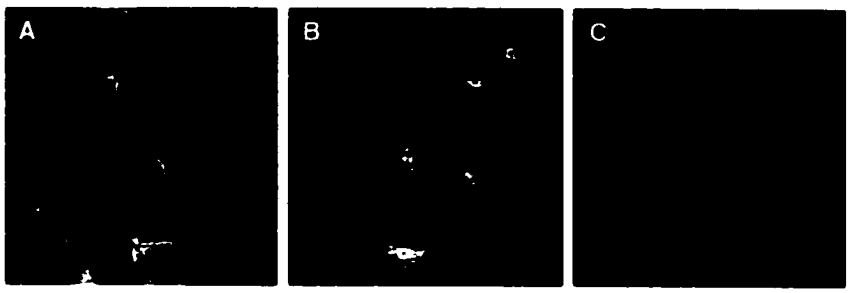


Figure 2.3 GST-B2-1 recombinant protein can block the B2-1 antiserum immunofluorescent staining. B2-1 antiserum was preincubated with beads only (A), GST-tag beads (B), or GST-B2-1-beads (C) before immunofluorescence detection. Cy3-conjugated goat anti-rabbit secondary antibody was used.

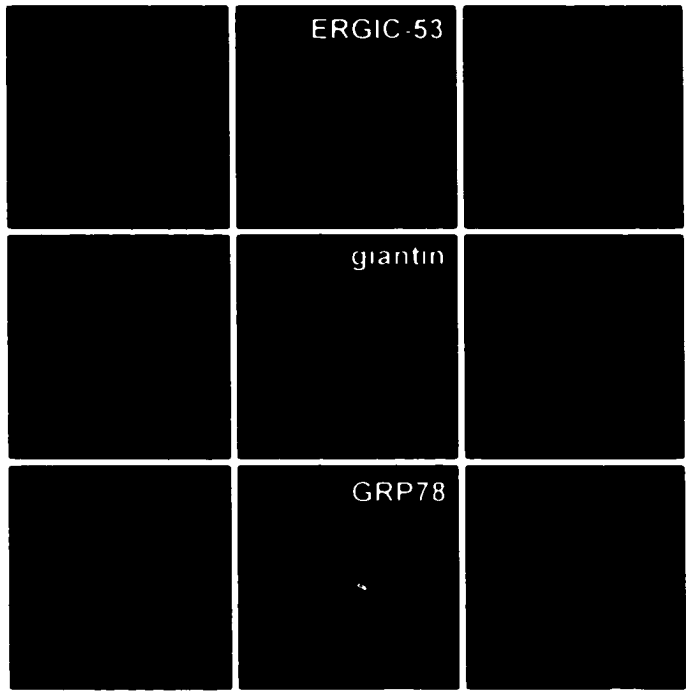


Figure 2.4 Double labeling of B2-1 and marker proteins. Cos-1 cells were double labeled with B2-1 antiserum and ERGIC-53, giantin or GRP78 antibodies as indicated. B2-1 was visualized by Cy3-conjugated goat anti-rabbit antibodies. ERGIC-53, giantin and GRP78 were visualized by Alexa-conjugated goat anti-mouse antibodies. Left panels are B2-1 staining; middle panels are marker proteins as indicated; right panels are superimposed images.

overlapped with β-COP (results not shown). B2-1 did not associate with LAMP or clathrin, two markers that are found associated with lysosomes and endosomes (Appendix I, Fig. A.2).

The antibiotic BFA has been shown to have rapid disruptive effects on the Golgi apparatus *in vivo* (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989) while leaving other organelles such as the ER intact but expanded. BFA complexes with the Sec7 domain and the GDP bound ARF protein and therefore inhibits ARF GDP/GTP exchange. Interestingly, only the large Sec7 domain containing proteins (p200, Gea1, Gea2) are sensitive while the smaller members are less sensitive or resistant to BFA. As indicated in Figure 2.5, treatment of Cos-1 cells with BFA for 10 min caused a disruption of B2-1 distribution and of the Golgi markers ERGIC-53 and giantin (compared to untreated cells in Fig. 2.4). In BFA-treated cells B2-1 showed a more punctate cytoplasmic staining. By 30 min of drug treatment, B2-1 and the Golgi markers showed a major disruption with a different redistribution pattern. This redistribution pattern of giantin now resembles that found with ER markers (e.g. GRP78 in Fig. 2.4).

Transfection of GFP-tagged B2-1

Since there is high homology between family members of ARF-GEF, we performed a transient transfection to rule out the possibility that the observed localization maybe due to antibody crossreaction with the other members. The transfected B2-1 was fused to the GFP. The Cos-1 cells transfected with GFP-tagged B2-1 showed an intensive perinuclear region (Fig. 2.6A) identical to that observed with endogenous B2-1 detection. By double labeling, this B2-1-concentrated compartment was also shown to be associated with the Golgi markers ERGIC53, giantin and TGN46 similar to the overlap found with

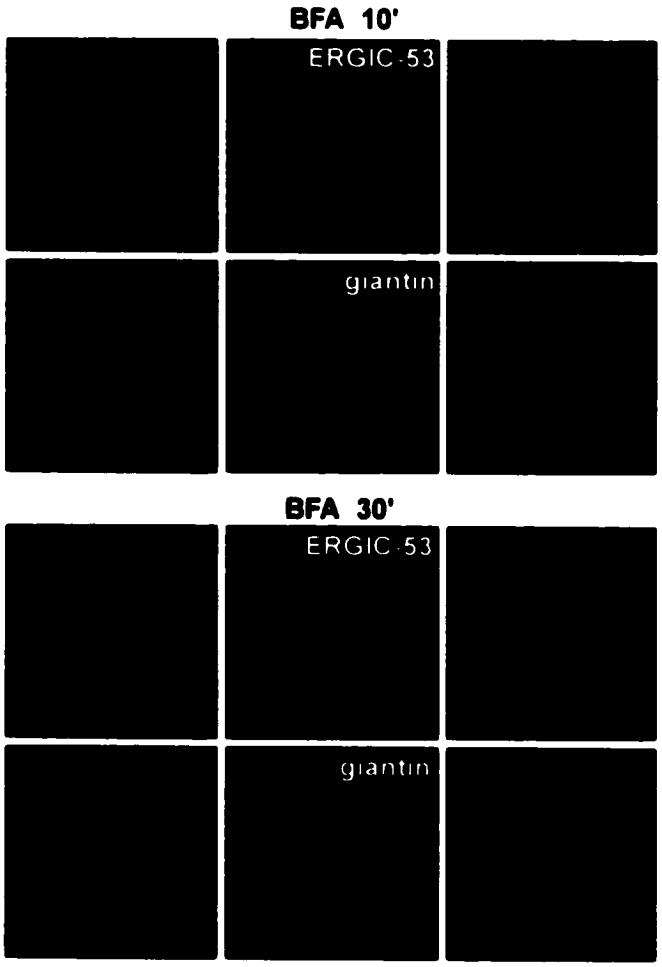


Figure 2.5 Effects of brefeldin A treatment on B2-1 subcellular localization. Cos-1 cells were treated with 5 μg/ml brefeldin A for 10 min or 30 min as indicated. Cells were probed with B2-1 antiserum and ERGIC-53, or giantin antibodies, respectively.

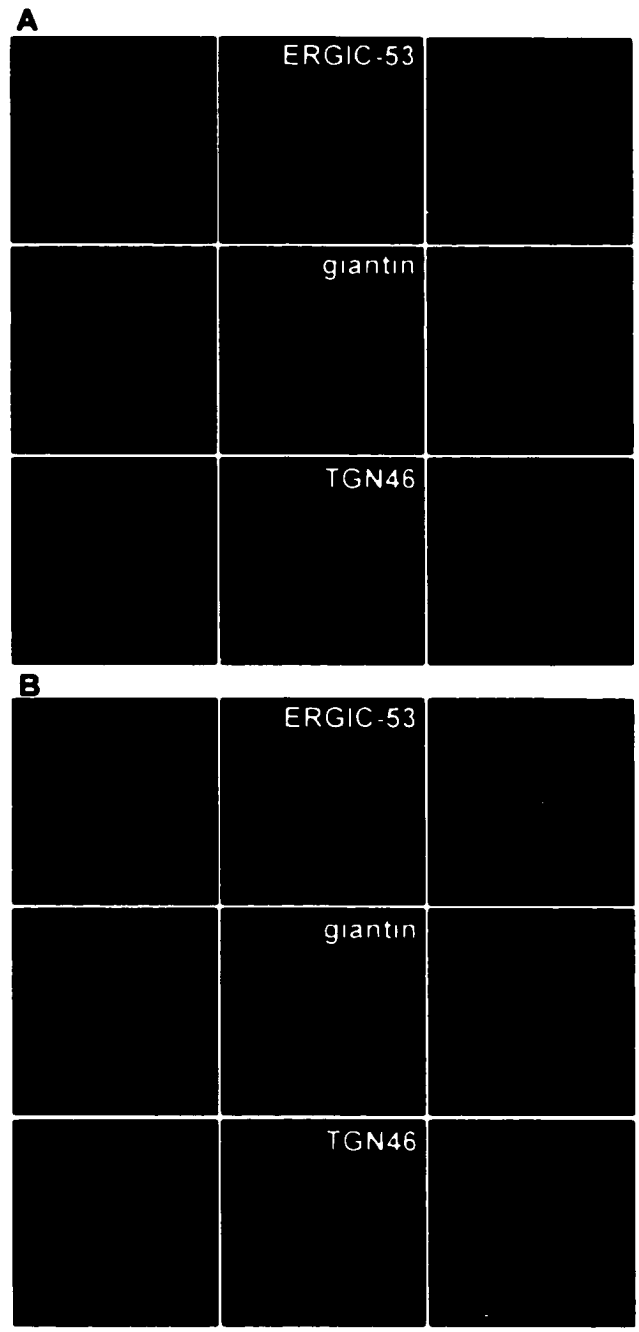


Figure 2.6 Localization of GFP-tagged B2-1 in transfected cells. Cos-1 cells were transfected with GFP-tagged full-length B2-1 and probed with ERGIC-53, giantin, or TGN46 (A). The bottom panels (B) were cells visually selected which showed excessive overexpressed B2-1 protein.

endogenous B2-1. Note that a dividing cell in Fig. 2.6A (giantin panels) lacked Golgi staining with both giantin and GFP-B2-1. Transfected GFP alone gave a nuclear localization (not shown). These experiments were also repeated using the Jurkat cell line with similar results to those presented here with Cos-1 cell line. The Golgi-associated marker mannosidase II also partially overlapped with transfected B2-1 in Jurkat (not shown).

A certain population of transfected cells observed had overexpressed levels of B2-1 protein (Fig. 2.6B). Interestingly, when these cells were examined for endogenous Golgi associated markers (ERGIC-53, giantin and TGN46) they also showed major Golgi alterations (increased size and dispersion) compared to nontransfected cells.

Discussion

In this report, we present the subcellular localization of B2-1. We report that B2-1 has a perinuclear staining that localizes to the Golgi complex. It is clear that B2-1 partially colocalized with the Golgi-associated markers ERGIC-53, giantin and TGN46. It is also evident that B2-1 was excluded from the region staining by GRP78, an ER marker. Additionally, B2-1 was not associated with the plasma membrane nor lysosomes/endosomes.

BFA, which causes rapid Golgi disintegration, had an effect on B2-1 localization. After BFA treatment, the Golgi components (both lipid and proteins) move into ER and leave no visible Golgi structure behind (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989). Recently, the inhibitory effect of BFA has been studied intensively. It has been shown that BFA stabilizes the ARF-GDP-Sec7 domain complex. The crucial residues in

the Sec7 domain that involve this "sensitivity of BFA" were mapped (Mansour et al., 1999; Peyroche et al., 1999; Sata et al., 1999). However the precise residues that are sensitive to BFA are still controversial. *In vitro* biochemical assays indicate that the Sec7 domains of the small Sec7-domain-containing proteins (B2-1/cytohesin, ARNO, and ARNO3) are BFA resistant (Chardin et al., 1996; Meacci et al., 1997). Since our immunolocalization data shows a major redistribution of B2-1 after BFA treatment, it could indicate two possibilities. One is that B2-1 Sec7 domain is not completely resistant to BFA's action. In support of this it was shown that BFA had a minor inhibition of ARNO-GEF activity (Peyroche et al., 1999). Second, and more likely, is that the Golgi contains other Sec7-domain-containing GEFs (i.e., the large size members) that are affected by BFA's inhibition. B2-1, while being directly resistant to BFA, may be dispersed due to secondary affects on the Golgi membranes.

The transfected B2-1 distribution was similar to the endogenous B2-1 pattern. Transfected B2-1 overlapped with giantin, ERGIC-53 and TGN 46, three well-established Golgi markers. Cells that were observed to be excessive overexpressing B2-1 had marked changes in B2-1 distribution and on the other Golgi markers. These B2-1-overexpressing cells had a more dispersed Golgi complex. This would indicate that GFP-B2-1 likely affected normal Golgi function. These results are similar to those showing that overexpression of ARNO or ARNO3 caused a major Golgi disruption (Franco et al., 1998; Monier et al., 1998).

In contrast to our results presented here and various papers reporting that the Sec7 domain proteins are GEFs, Kolanus et al. reported that both B2-1 (renamed cytohesin-1 in their study) and cytohesin-2 can bind to the cytoplasmic tail of CD-18 (Kolanus et al.,

1996). The CD-18-binding domain was specifically mapped to the Sec7 domain. We have not been able to coimmunoprecipitate CD-18 with our B2-1 antiserum (or vice versa with anti-CD-18) (results not shown). We have also not been able to detect a B2-1 immunofluorescent signal that localizes to the plasma membrane even though we are able to detect plasma membrane CD-18 by immunofluorescence in the cells (Jurkat) we use (results not shown). Furthermore, it has been shown by others that B2-1 (cytohesin-1) does not interact with CD18 (β2-integrin) in solution (Betz et al., 1998). Recently, in addition, the group that speculated that GRP1 is involved in cell surface signaling found that this protein is also a GEF and is involved in vesicular trafficking (Klarlund et al., 1998). The function and subcellular distribution of the other members (ARNO and ARNO3) has also been controversial in that some have located these proteins with the plasma membrane (Venkateswarlu et al., 1998b) while others have shown a Golgi complex association (Franco et al., 1998; Monier et al., 1998). We speculate that our Golgi compartment localization data support previous work that some of these Sec7-domain-containing proteins are involved in vesicle formation at the Golgi.

Chapter 3

N-terminal Targeting of Guanine Nucleotide Exchange Factors (GEF) for ADP Ribosylation Factors (ARF) to the Golgi

(This chapter is published in Journal of Cell Science (2000) 113, 1883-1889)

Introduction

B2-1 belongs to a family of proteins (including ARNO and ARNO3), which are all approximately the same size (400 aa) and have the same three-domain organization (an N-terminal coiled-coil, the Sec7 domain and a pleckstrin homology (PH) domain) (Chardin et al., 1996; Klarlund et al., 1997; Liu and Pohajdak, 1992). The Sec7 domain was originally found to have homology to a portion of the yeast Sec7 protein. Previously the Sec7 protein in yeast was shown to be involved in transporting vesicles from the ER to the Golgi (Achstetter et al., 1988; Franzusoff et al., 1992). Overexpression of human ADP-ribosylation factor 4 (ARF4) in yeast can rescue the Sec7 defect (Deitz et al., 1996). ARFs are small GTPases involved in vesicular trafficking between organelles. Several independent groups have discovered that the B2-1 family of proteins are guanine nucleotide exchange factors (GEFs) for ARFs and this activity has been specifically mapped to the Sec7 domain (Chardin et al., 1996; Pacheco-Rodriguez et al., 1998). So far there are three types of Sec7-domain-containing proteins identified (based on size and organization) including small (B2-1, ARNO and ARNO3) (Chardin et al., 1996; Klarlund et al., 1997; Liu and Pohajdak, 1992), intermediate (EFA6) (Franco et al., 1999), and the

large molecular mass family (Sec7p, Gea1p, Gea2p, p200, and GBF1) (Achstetter et al., 1988; Mansour et al., 1998; Morinaga et al., 1997; Peyroche et al., 1996). These proteins have all been shown to have GEF activity for various ARFs.

All members of the small and intermediate-sized ARF-GEF families contain a PH domain. Although it has been suggested that the PH domain may be involved in regulating or stimulating ARF activity, this domain is thought to recruit these proteins to membrane phosphoinositols (Hemmings, 1997a). The various PH domains have been shown to bind the polar phosphoinositol head of a variety of phosphoinositol lipids (Harlan et al., 1994; Rameh et al., 1997). For instance, the PH domains of the small ARF-GEFs bind specifically to phosphatidylinositol-3,4,5-trisphosphate (Klarlund et al., 1997). In addition, activation of phosphatidylinositol-3 kinase (PI-3 kinase) has been implicated in increasing membrane binding of these PH domain-containing proteins (Nagel et al., 1998; Venkateswarlu et al., 1998b).

While there is a growing amount of evidence suggesting a role of these Sec7-domain-containing proteins in vesicular transport in mammalian cells, there is conflicting information on the *in vivo* subcellular localization of these proteins. Overexpression of ARNO results in a cytoplasmic distribution with a somewhat poor plasma membrane colocalization (Frank et al., 1998). While some groups have shown a plasma membrane association of these proteins (Ashery et al., 1999; Frank et al., 1998), others have found that these proteins are cytoplasmic and only localize to the plasma membrane following stimulation (Venkateswarlu et al., 1998a; Venkateswarlu et al., 1999a; Venkateswarlu et al., 1998b). Recently, studies have indicated that overexpression of ARNO (Monier et al., 1998) and ARNO3 (Franco et al., 1998) caused fragmentation of the Golgi,

suggesting that their function is in the Golgi. This is supported by the evidence that all three of the small Sec7 members act on ARF1, which has been localized to the Golgi compartment (Peters et al., 1995). Previously, by using antiserum raised against recombinant B2-1 protein, we detected B2-1 protein in a discrete perinuclear region in various cell lines (Lee et al., 2000). This region colocalized with several established Golgi markers (giantin, ERGIC-53 and TGN46) but not with the ER marker (GRP78) or the endosome/lysosome markers (LAMP1, clathrin). Furthermore, transfection of tagged full-length B2-1 also showed a similar Golgi localization. In this study, we made various B2-1 constructs fused to the green fluorescent protein (GFP). We show that through its coiled-coil N terminus, B2-1 is localized to the Golgi apparatus in normal, non-stimulated Cos-1 cells. The N terminus is necessary and sufficient for the Golgi targeting. Furthermore, the coiled-coil N-termini of ARNO and ARNO3 also have the Golgi targeting capabilities.

Materials and methods

Cell culture

The Cos-1 cell line was obtained from Dr. C. Too (Dalhousie University, Halifax, Canada) and cells were propagated in DMEM containing 10% fetal bovine serum and antibiotics at 37°C in 5% CO_2.

Construction of GFP fusions

All fragments were inserted to the *Bam*HI restriction site and fused to the N terminus of GFP in pCS2+GFP vector.

For various domains of B2-1, PCRs were performed to introduce *Bam*HI or *Bgl*II restriction sites and to amplify the corresponding fragments. Constructs encoded the following: NSP, residues 1-398; N, residues 1-54; SP, residues 55-398; N1-22, residues 1-22; N1-33, residues 1-33; N23-SP, residues 23-398; N34-SP, residues 34-398.

For site-directed mutagenesis of B2-1 N terminus, the following mutagenic primers were used: I23A: 5'-CCGTCGGGCGTTCTCCAG; I23P: 5'-CCGTCGGGGGTTCTCCAG; I30A: 5'-GTCAGCCAGCGCCTCCTG; I30P: 5'-GTCAGCCAGCGGCTCCTG; I34A: 5'-CAGCCTCTGGGCGTCAGCCAG; I34P: 5'-CAGCCTCTGAGGGTCAGCCAG. Underlined sequences indicate the mutated nucleotides in the primers.

All constructs were sequenced and the fusion protein expressions were confirmed by western blotting using anti-GFP antibodies (Clontech).

Cloning of the N termini of ARNO and ARNO3

N termini of ARNO and ARNO3 were constructed with amino-terminal myc tag (pJ3M) vector. Reverse transcriptase-PCR (RT-PCR) was performed to clone the ARNO and ARNO3 N termini using the following primers:

ARNO, 5'-GGGGATCCATGGAGGACGGCGTTTATG and

5'-GGGGATCCCAGCCCCTCCACCTCGCT;

ARNO3, 5'-GGGGATCCATGGATGAAGACGGCGGC and

5'-GGGGATCCTAGATTGTCGATCTCTGT. The fragments were cloned into a myc tag vector pJ3M. The fragments encode residues 1-50 for ARNO and residues 1-55 for ARNO3. The constructs were sequenced prior to use.

Transfection

Cos-1 cells were seeded on glass coverslips the day prior to the transient transfection experiment. Transient transfections were performed using Qiagen SuperFect reagent and following the manufacturer's protocol. Cells were continuously cultured in the presence of serum. All fusion proteins obtained from the transfections were also confirmed by western Blotting.

Immunofluorescence microscopy

Following 24 hours of transfection, cells were fixed in 4% paraformaldehyde in PBS buffer, pH 7.5, for 20 minutes, followed by permeabilization in 0.1% Triton X-100. For the cells transfected with myc-tagged constructs, primary mouse monoclonal anti-myc antibodies diluted in PBS containing 1% goat serum were added and incubated for 30 minutes at room temperature. The cells were then washed with PBS containing 0.1% Triton X-100 three times. The cells were incubated with secondary Cy3-conjugated goat anti-mouse (Sigma) and Alexa(488)-conjugated goat anti-rabbit antibodies (Molecular Probes) for 20 minutes at room temperature. Washing procedure was then repeated. Cells were observed using a Zeiss confocal microscope LSM. TGN46 rabbit polyclonal antiserum was kindly provided by Dr. M. Fukuda (Burnham Institute, La Jolla, USA) (Kain et al., 1998).

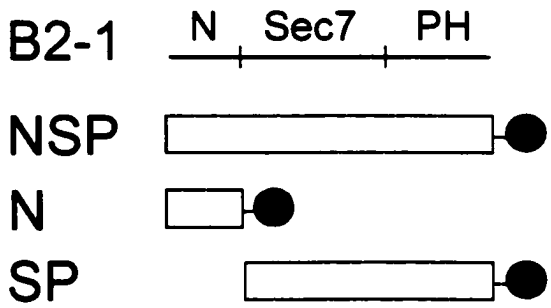
Results

The N termini of B2-1, ARNO, and ARNO3 contain Golgi complex targeting information

In a previous study, we showed the Golgi localization of endogenous and transient transfected B2-1 (Lee et al., 2000). To further examine the domain that is involved in targeting to the Golgi complex, we systematically constructed B2-1 mutants that contained various domains linked to the green fluorescent protein (GFP). We prepared several subdomain B2-1 constructs including N (N-terminus (aa 1-54), SP (Sec7 + PH, aa 55-398) and NSP (full length, N-terminus+ Sec7+ PH), all fused to the N-terminus of the GFP. Figure 3.1A includes a graphic representation of the constructs used for transfection. All transfections were performed in Cos-1 cells. Transfected GFP alone showed a nuclear distribution (Fig. 3.1B, GFP). Both transfected N-GFP and NSP-GFP showed cytoplasmic fluorescence with intensive perinuclear Golgi signals (Fig. 3.1B, N and NSP) while most of SP-GFP proteins remained nuclear (Fig. 3.1B, SP). To rule out any possible interaction of the GFP tag with our fusion proteins, transfection of myc-tagged B2-1 using both Jurkat and Cos-1 cell lines gave identical results (Appendix I, Fig. A.5). These results strongly suggest that B2-1 N terminus contains a protein domain or module that is responsible for this Golgi targeting.

The other small ARF-GEF family members, ARNO and ARNO3, also contain this N-terminal region in front of the Sec7 domain. This region also has the largest amino acid variability between the three members. Interestingly, the N terminus of all three members contains a coiled-coil structure, as predicted by the "COIL" program (Lupas et al., 1991). Because of these observations, we tested whether ARNO and ARNO3 N

A



B

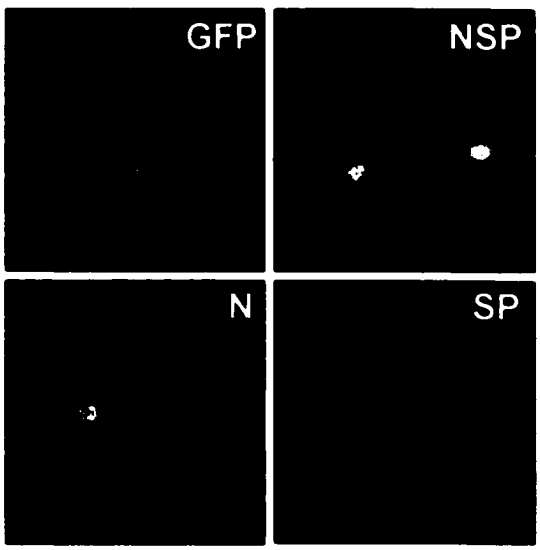


Figure 3.1 (A) A schematic representation of the constructs used for transfection. The full-length B2-1 domain composition is as indicated. Filled circle, the green fluorescent protein (GFP); N, N terminus (aa 1-54); SP, Sec7+PH domain (aa 55-398); NSP, the full-length B2-1 (residues 1-398). (B) Subcellular localization of the transient transfected B2-1 constructs in Cos-1 cells.

termini also have similar targeting capabilities. We constructed B2-1, ARNO and ARNO3 N termini linked to a myc tag. To confirm that the N terminus targets to the same region as the endogenous and full-length transfected B2-1 (Lee et al., 2000), double labeling was also employed. As shown in Fig. 3.2A, the N terminus of B2-1 colocalized with the Golgi marker TGN46. Double staining with the Golgi marker, giantin, gave a similar colocalization (not shown). Transfection of ARNO-N and ARNO3-N showed similar Golgi targeting (Fig. 3.2B) and colocalization with both Golgi markers (not shown). Identical results were also obtained with GFP-tagged ARNO-N or ARNO3-N (data not shown). These experiments were also repeated in the Jurkat cell line with identical results (Appendix I, Fig. A.7). This indicates that all three members use their varied N-terminal coiled-coil domains to target to the Golgi complex. These results are unlike those recently published, which suggested that transfected ARNO and ARNO3 show a primarily cytosolic distribution (Franco et al., 1998; Venkateswarlu et al., 1998b). All of our transfected cells also have a cytoplasmic distribution; however the signal is strongly enhanced at the Golgi complex. Furthermore, in support of the results presented here, overexpression of ARNO or ARNO3 does cause Golgi fragmentation (Franco et al., 1998; Monier et al., 1998; Venkateswarlu et al., 1998b). We have also observed that excessive overexpression of B2-1 in Cos-1 cells causes Golgi dispersion as detected by several established Golgi marker proteins (Lee et al., 2000).

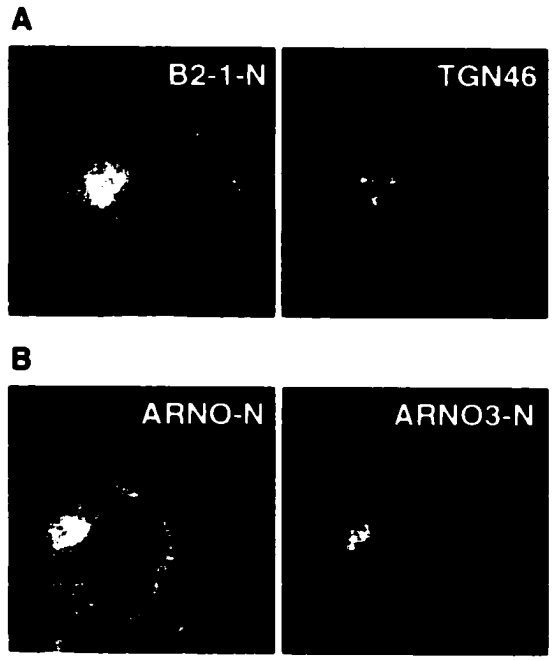


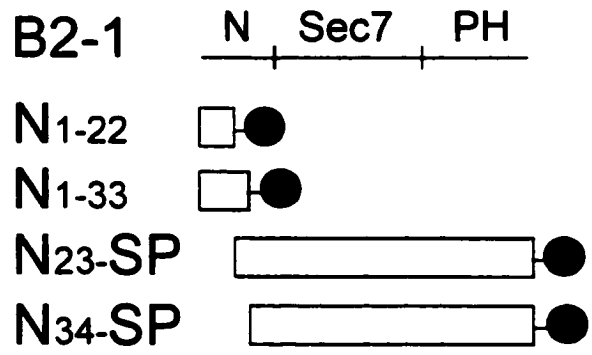
Figure 3.2 Subcellular localization of transfected myc-tagged N termini of small ARF-GEFs. B2-1, ARNO and ARNO3 in Cos-1 cells. (A) Double labeling of myc-tagged B2-1 N terminus and the Golgi marker TGN46. Cells were double labeled with primary myc mouse monoclonal antibody and TGN46 rabbit polyclonal antiserum. Cy3-conjugated goat anti-mouse and Alexa (488)-conjugated goat anti-rabbit secondary antibodies were used, respectively. (B) Myc-tagged N terminus of ARNO (ARNO-N) and of ARNO3 (ARNO3-N). Experiments were also performed with GFP-tagged ARNO-N or ARNO3-N (in the Jurkat cell line) with identical targeting (not shown). All constructs were sequenced to confirm their identity.

Detail-mapping the N-terminus of B2-1

To further map the region within the N terminus of B2-1, we prepared various constructs (GFP fusion) as indicated in Fig. 3.3A. Among the three N terminus (without Sec7 and PH domains) constructs, only N1-54 could localize to the Golgi (Fig. 3.1B, N). Neither N1-22 nor N1-33 was sufficient to target to the Golgi (Fig. 3.3B), suggesting that either the targeting region lies within the second half of this N terminus (residues 34-54) or the first half of the N-terminus is necessary but not sufficient for targeting. To test these two possibilities, we prepared the constructs N23-SP (residues 23-398, which includes most of this coiled-coil region) and N34-SP (residues 34-398, which includes the second half of the coiled-coil region). As shown in Fig. 3.3B, N23-SP could localize to the Golgi complex even though the first 22 amino acids are missing. N34-SP, unlike N23-SP, was unable to locate to the Golgi and was scattered in the cytoplasm. These experiments were also performed in Jurkat cells, with identical results (not shown). These results indicate that the first 22 amino acids are not involved in Golgi targeting and the coiled-coil region in the N terminus is required for proper B2-1 Golgi targeting.

To further test whether the coiled-coil structure is involved and to map which portion of the coiled-coil region is crucial for Golgi targeting we constructed several point mutations in this region. In the coiled-coil heptad repeat, residue positions (Fig. 3.4A) a and d are usually hydrophobic and crucial for helix structure. We thus chose to individually mutate three amino acids (I23, L30 and I34) at either position a or position d within the helix. These three positions are identical in B2-1, ARNO and ARNO3 (Fig. 3.4A). Minor hydrophobic substitutions, including I23A, L30A and I34A, had no effect on Golgi targeting (Fig. 3.4B). We then tested if substitution of any of these amino acids

A



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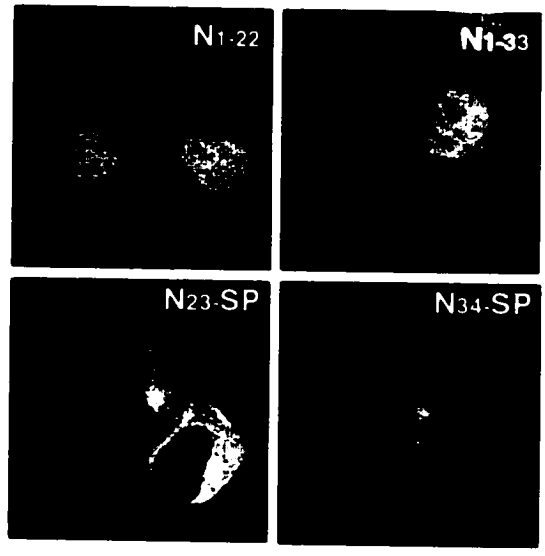
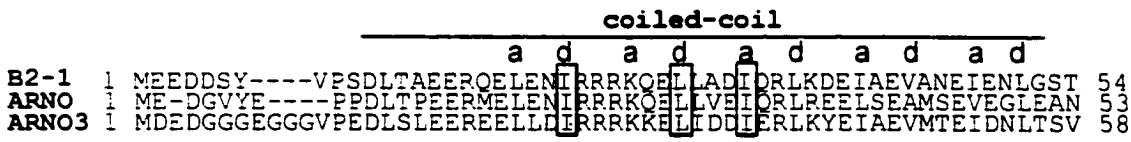


Figure 3.3 Mapping the location of the targeting signal in the N terminus of B2-1. (A) A schematic representation of the constructs used to map the N-terminal targeting region. Filled circle, green fluorescent protein (GFP); N1-22, residues 1-22; N 1-33, residues 1-33; N23-SP, residues 23-398; N34-SP, residues 34-398. (B) Subcellular localization of the transient transfected B2-1 constructs in Cos-1 cells.

A



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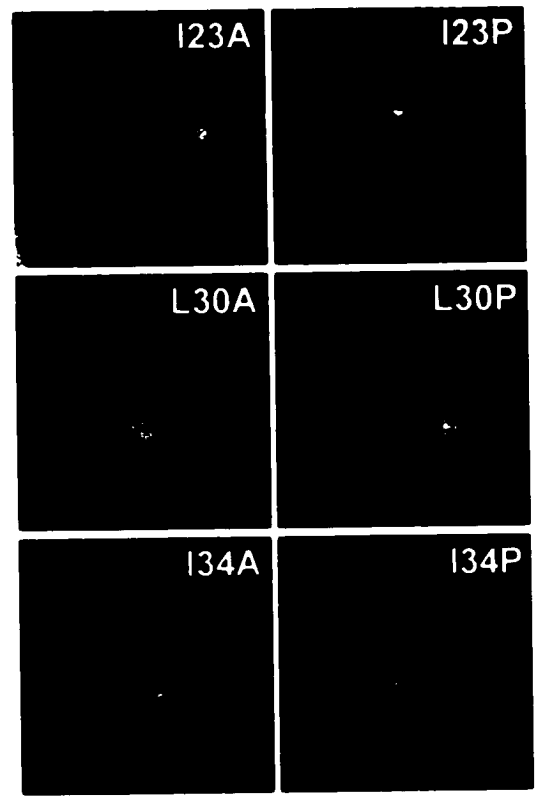

Figure 3.4 Site-directed mutagenesis of B2-1's N-terminus. (A) An alignment of B2-1, ARNO and ARNO3 coiled-coil N-termini. The heptad repeat positions a and d are indicated. The sites of which each point mutation was performed are boxed (residues I23, L30, and I34). (B) Transient expression of the N-terminal B2-1-GFP mutants. Two mutations of each position were constructed (I23A, I23P, L30A, L30P, I34A, and I34P).

with proline, a known helix disruptor, would alter Golgi targeting. Interestingly, the I34P mutant lost its targeting capability and remained in the nucleus, similar to control GFP alone. As shown in Fig. 3.4A, position I34 is in the middle of the coiled-coil structure, and mutating this isoleucine to a proline likely causes a dramatic conformational change of the structure, thereby disrupting the coiled-coil interaction. The other two proline substitutions, I23P and L30P, surprisingly had no effect on Golgi targeting. These results suggest that the most likely targeting region involves amino acids 31 to 54 (the second half of the coiled-coil structure) and that the complete N-terminal alpha helix region is not required for the targeting and adaptor-recognition event.

A model of GEF-ARF interaction on the Golgi membrane

These results suggest that B2-1, ARNO and ARNO3 all use their N-terminal amphipathic coiled-coil domains to target themselves to the Golgi complex. As shown in Fig. 3.5 we summarize the data presented here (Fig. 3.5A) and propose that the Golgi targeting sequence resides in the N-terminal, coiled-coil region. The most crucial area of the alpha helix centers around I34. We propose that the coiled-coil region binds to a Golgi membrane adaptor. The identity of the Golgi protein(s) that act as adaptors and facilitate this type of targeting are presently unknown. In this model (Fig. 3.5B), the PH domain functions as a membrane anchor to allow an interaction of B2-1 Sec7 domain with its appropriate ARF(s). Deletion constructs of B2-1 that contain only the Sec7 and PH domain do not target to the Golgi complex, indicating that the PH domain alone is not responsible for targeting. This targeting and arrangement of the B2-1 protein may

A

B2-1	Residues	Golgi
NSP	1-398	+
N_{23}-SP	23-398	+
N_{34}-SP	34-398	-
SP	55-398	-
N	1-54	+
N_{1-22}	1-22	-
N_{1-33}	1-33	-
N I23A	1-54	+
N I23P	1-54	+
N L30A	1-54	+
N L30P	1-54	+
N I34A	1-54	+
N I34P	1-54	-
ARNO-N		+
ARNO3-N		+

B

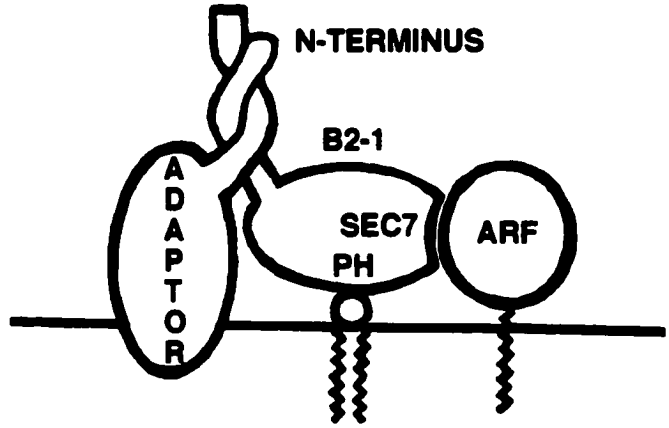

Figure 3.5 (A) Summary of the targeting capabilities of the various constructs used in this study. +, has targeting ability; -, no targeting ability. (B) A model of GEF-ARF interaction on the Golgi membrane. B2-1 uses its coiled-coil N terminus to bind to an adaptor, which may also contain a coiled-coil region. B2-1 is also anchored to the membrane by its PH domain, which attaches to a phosphoinositol. The proper orientation of B2-1 allows its Sec7 domain to activate GDP-GTP exchange on membrane associated ARF allowing proper insertion of its N-terminal myristate into the appropriate Golgi membrane. This ARF activation then induces vesicle formation.

facilitate the proper orientation and recruitment of ARF with the Golgi membrane following its activation by B2-1's Sec 7 domain. Membrane phospholipids are required to bind to ARF N terminus to facilitate GEF interaction (Franco et al., 1995; Franco et al., 1996; Paris et al., 1997). Following the interaction, a GDP-GTP exchange occurs in ARF, causing a conformational change in which the ARF's N-terminus myristate is anchored into the Golgi membrane (Goldberg, 1998).

Discussion

In this study, we have demonstrated that the N termini of the small ARF-GEF members, including B2-1, ARNO and ARNO3, are necessary and sufficient for the Golgi complex targeting. We have also identified that the coiled-coil region within the N terminus is crucial for this targeting. The point mutation I34P, which disrupted the coiled-coil structure, abolished the Golgi targeting. The coiled-coil regions of several proteins are necessary for their proper localization (Stacey and von Arnim, 1999; Stam et al., 1997), presumably through coiled-coil protein interactions. Many proteins use their α-helix sequences to target to the Golgi complex or to certain vesicles (Grote et al., 1995; Nelson et al., 1998). Several proteins use N-myristoylation or palmitoylation of specific sequences to target themselves to the Golgi (Liu et al., 1997). As B2-1's N terminus lacks any signals for these post-translational lipid attachments, this explanation for its Golgi targeting is unlikely. Recently, two of the larger mammalian Sec7-domain-containing members, p200 and GBF1, were also shown to localize to the Golgi complex (Claude et al., 1999; Mansour et al., 1999). The Golgi targeting region of p200 was mapped to the N-terminal third of the protein, which is the non-Sec7 region. Since the

large Sec7-domain-containing GEFs have no putative coiled-coil region, their mechanism to localize to the Golgi complex is different to that of the small GEFs. A Golgi-targeting domain has recently been located at the carboxyl termini of several extensive coiled-coil peripheral Golgi membrane proteins, such as golgin-97 and golgin-245/p230 (Barr, 1999; Kjer-Nielsen et al., 1999; Munro and Nichols, 1999). This Golgi targeting domain, which contains 50 amino acids and a very conserved tyrosine residue, interacts with Rab6 (Barr, 1999), indicating that the mechanism of targeting to the Golgi of these proteins is through the interaction with other proteins. As many protein domains involved in Golgi localization are starting to be identified, the coiled-coil domain interaction we demonstrate in this study can be added to the list of Golgi targeting mechanisms.

B2-1's PH domain is not involved in targeting to the Golgi. Similar results were obtained with ARNO's PH domain (Levine and Munro, 1998). Venkateswarlu et al. (1998a; 1999a; 1998b) showed that cytohesin-1/B2-1, ARNO and GRP1/ARNO3 were translocated to the plasma membrane from cytosol through their PH domains upon either epidermal-growth factor, nerve growth factor or insulin stimulation. In their report, it was clear that without the N-terminal, coiled-coil domains the GFP-PH fusion proteins localized to the nucleus, as did the GFP alone. This was interpreted as due to the small size of the GFP fusion proteins, which allowed free access through the nuclear pore. As the N-terminal GFP fusion proteins constructed in our study (Fig. 3.1, N) were actually much smaller than the Sec7+PH domains fused with GFP (Fig. 3.1, SP) or the GFP-PH (used in their studies), it is evident that the N-terminus carries the Golgi targeting signal. We have also observed plasma membrane translocation of overexpressed GFP-Sec7+PH upon 20% serum stimulation (unpublished data). The physiological function of the PH

domain-dependent translocation to the plasma membrane is not clear. As ARF6 is characteristically localized at the plasma membrane, it was speculated that translocation of these GEFs may play a role in regulating ARF6 functions (Venkateswarlu et al., 1999a; Venkateswarlu et al., 1998b). However, *in vitro* biochemical assays have shown that these three GEF proteins have no effect on ARF6 (Franco et al., 1998). Recently, a GEF specific for ARF6, EFA6, has been identified and this protein localizes to the plasma/endosome membrane (Franco et al., 1999). It is possible that the B2-1 family members of ARF-GEFs are usually localized at the Golgi complex (through the coiled-coil domain) and serve as regulators of the Golgi vesicle transport, and that under certain physiological conditions it may be necessary to translocate these ARF-GEFs to the plasma membrane (via PH domain) for further unknown functions.

In the model proposed here, we suggest that the N-terminal, coiled-coil domain is used to recognize the adaptor protein(s) at the Golgi complex and thus is able to localize B2-1 to the Golgi. The proteins that may serve as adaptors for B2-1 and other family members are currently unknown. However, the Golgi complex contains several resident proteins with extensive coiled-coiled domains (Barr, 1999; Bascom et al., 1999; Erlich et al., 1996; Kjer-Nielsen et al., 1999; Linstedt and Hauri, 1993; Munro and Nichols, 1999), which may serve as adaptors for these proteins. Nevertheless, it may also be possible that another class of coiled-coil proteins is involved in this Golgi-targeting mechanism. These small ARF-GEF members mainly differ in their coiled-coil domains, suggesting that they may interact, through similar structure and protein interaction, with different adaptors in potentially different compartments within the Golgi or Golgi-associated compartments. Recently, Munc13-1, a brain specific phorbol ester receptor localized to

presynaptic transmitter release zones, was identified as a binding partner of msec7-1 (rat homologue of B2-1/cytohesin-1) by yeast two-hybrid screening (Neeb et al., 1999). The interaction is through the amino-terminal coiled-coil region of msec7-1 and the carboxy-terminal one-third of Munc13-1. Although the molecular mechanism by which Munc13-1 affects neurotransmitter release is not clear, one function of Munc13-1 may be to serve as an adaptor to recruit the msec7-1 to the presynaptic transmitter-releasing zones in brain tissues.

Chapter 4

General Discussion and Conclusions

Summary of results

In this thesis, the localization and targeting of B2-1 were studied. Indirect immunofluorescence staining with polyclonal antiserum raised against the full-length B2-1 recombinant protein was used to localize B2-1 in several cell lines. B2-1 was found in the perinuclear region with a punctate pattern. By double labeling with different organelle marker proteins, it was further determined that B2-1 localizes to the Golgi complex. Overexpressed B2-1, tagged with either GFP or myc, gave a similar Golgi staining and further confirms the endogenous B2-1 localization. Golgi localization was disrupted by incubating with brefeldin A before immunostaining, demonstrating that B2-1 localization is brefeldin A sensitive, either by direct binding of brefeldin A or an indirect effect on the Golgi membrane. According to others, small Sec7-domain-containing proteins (including B2-1, ARNO, and ARNO3) are brefeldin A insensitive in *in vitro* assays. Therefore, the effect of brefeldin A on B2-1 distribution is likely due to a secondary effect on other resident Golgi proteins.

The location of a cell protein is related to and important for its function. Therefore, the localization mechanism was further studied. By overexpressing domain-deleted mutants, the first 54 residues of the amino-terminus of B2-1 were shown to be necessary and sufficient for localization to the Golgi. The rest of B2-1, the Sec7 and PH domains, are not sufficient for targeting of B2-1 to the Golgi because the mutant without

the N-terminal sequence lost its Golgi targeting capability. Analysis of the N-terminal sequences revealed an α-helix coiled-coil region. Site-direct mutagenesis studies further proved that isoleucine at position 34 is important for targeting, presumably by maintaining the coiled-coil structure.

Discussion and conclusions

The discussion sections in chapters 2 and 3 were based on the research presented in each publication. In this chapter, a more general and speculative discussion regarding B2-1 is presented.

Localization

Human ARF1, 3, 4, and 5 are predominantly cytosolic and are recruited to Golgi membranes when GTPγS is added, indicating that the active, GTP-bound form of ARF is membrane bound (Cavenagh et al., 1996). Since the small ARF-GEF members use class I ARFs (human ARF1 and ARF 3) as substrates, they may localize to the same area as the class I ARFs. My data verify the small ARF-GEF Golgi complex localization.

In contrast to my findings, some groups found that transfected ARNO and ARNO3 are distributed in the cytoplasm and presumed that these proteins were cytosolic (Franco et al., 1998; Monier et al., 1998). Fractionation of HeLa cells showed that endogenous ARNO was predominantly in the cytosol (Monier et al., 1998). Some contradictory results to the cytoplasmic localization have been observed with ARNO and GRP1. While most of the immunostaining of ARNO seems cytoplasmic, there is some colocalization with co-transfected ARF6 in the plasma membrane (Frank et al., 1998). In addition, density gradient analysis demonstrated that endogenous ARNO does not

cofractionate with Golgi membranes. However, our immunofluorescent data showed that B2-1 was both cytoplasmic and Golgi membrane-associated through the N-terminal coiled-coil region. While others showed the cytoplasmic distribution of the overexpressed small ARF-GEFs, we present that endogenous, as well as transfected, B2-1 is localized to a specific area in the cell. The different results observed from various transfection experiments could be due to varied levels of protein expression. Stronger signals could disguise the localization patterns.

PH domain translocation

PH domains of B2-1, ARNO, and ARNO3 translocate to the plasma membrane upon insulin or epidermal growth factor stimulation (Venkateswarlu et al., 1998a; Venkateswarlu et al., 1999a; Venkateswarlu et al., 1998b). The translocation is thought to be PtdIns(3,4,5)P_3-induced because the PH domains of B2-1, ARNO, and ARNO3 bind PtdIns(3,4,5)P_3/IP_4, and translocation is blocked when a PI3-kinase inhibitor is added. However, the activity of ARF-GEF after translocation has not been addressed, raising the question whether the translocation is physiologically relevant. Venkateswarlu et al. are in favor of the idea that B2-1/cytohesin1, ARNO, and ARNO3 are translocated to the plasma membrane and consequently activate ARF6. Since the catalytic activity of the small ARF-GEFs on ARF6 has not been adequately established, it seems premature to assume that these ARF-GEFs function on ARF6 based on the translocation studies. Furthermore, the guanine nucleotide exchange activity of EFA6, a newly identified ARF-GEF, is ARF6 specific and both EFA6 and ARF6 localize to the plasma membrane. Thus EFA6 is a more likely GEF for ARF6 (Franco et al., 1999).

Several other PH domain translocations have been documented. For example, $GAP1^m$, a Ras GAP, and centaurin-α, a putative GAP for ARF, translocate to the plasma membrane via their PH domain when stimulated with EGF (Lockyer et al., 1999; Venkateswarlu et al., 1999b). Nevertheless the recruitment of $GAP1^m$ seems to have no effect on its activity (Lockyer et al., 1999). All of the PH domains that translocate to the plasma membrane upon agonist stimulation have a high binding affinity for PtdIns(3,4,5)P_3/Ins(1,3,4,5)P_4.

Among the PtdIns(3,4,5)P_3 binding proteins, two are constitutively associated with the plasma membrane through PH domains. One is PDK-1, which is a 3-phosphoinoside-dependent protein kinase that phosphorylates and activates membrane-associated PKB (Currie et al., 1999). However it has been reported that PDK-1 localizes to the cytosol and is translocated to the plasma membrane upon stimulation (Anderson et al., 1998). Another protein, GAP^{IP4BP}, a Ras GAP, constitutively localizes to the plasma membrane (Lockyer et al., 1997). Interestingly, the reported PtdIns(3,4,5)P_3 binding affinities of the PH domain of PDK-1 (Kd 1.6 nM for PtdIns(3,4,5)P_3 and 5.2 nM for PtdIns(3,4)P_2) and GAP^{IP4BP} (Kd ~10 nM for Ins(1,3,4,5)P_4) are extremely high, compared to other PtdIns(3,4,5)P_3 specific PH domains (Currie et al., 1999; Lockyer et al., 1997). Taken together, agonist-stimulated or constitutive binding to PtdInsP_3-containing membranes depends on the binding affinity of the PH domains, the amount of PtdInsP_3 that is available, and perhaps the balance between membrane PtdInsP_3 and cytosolic InsP_4.

Agonist-induced translocation of transfected PH domains usually occurs all around the plasma membrane. Does it happen under a physiological environment?

Recent studies suggest that there are lipid rafts (microdomains) present at the membranes (for review, see Jacobson and Dietrich, 1999). It is interesting to know how the PH doamain-mediated translocation is regulated spatially, if at all. Another thought is that some of the PH domain translocations may occur at the intracellular membranes in response to the local PtdIns(3,4,5)P_3 production during certain signal transduction pathways, although evidence to support the idea is lacking. However, possibly due to low concentrations, there is no sensitive method to detect intracellular PtdIns(3,4,5)P_3

PH domain in localization

The role of the PH domain in inositol phospholipid binding is now generally accepted. But how is the PH domain functionally involved in distinct proteins? And how is it regulated? As mentioned in chapter 2, the PH domains are grouped into three categories based on their binding specificity toward PtdIns. Some PtdIns(4,5)P_2 specific PH domain containing proteins are localized in the plasma membrane via the PH domain. Upon stimulation, phospholipase C hydrolyzes PtdIns(4,5)P_2 to IP_3 and diacylglycerol. Consequently, the amount of PtdIns(4,5)P_2 is reduced and some of the PH domain containing proteins dissociate from the plasma membrane into the cytosol. Whether this translocation is due to reduction of PtdIns(4,5)P_2 in the plasma membrane or the increase of IP_3 in the cytosol is controversial (Hirose et al., 1999; Raucher et al., 2000; Stauffer et al., 1998). By contrast, the PtdIns(3,4,5)P_3 specific PH domain containing proteins are found mostly in the cytoplasm and translocate to the plasma membrane when PtdIns(3,4,5)P_3 production is triggered by PI-3 kinase activation.

A number of PH domain-containing proteins with various functions are localized in the Golgi apparatus. For instance, βIII spectrin (Stankewich et al., 1998), protein kinase C μ (Prestle et al., 1996), evectins (Krappa et al., 1999), and an oxysterol-binding protein (OSBP) (Levine and Munro, 1998) contain a PH domain and localize to the Golgi. Their targeting mechanisms, however, are not clear. Some studies have been done for spectrin and OSBP. The constitutive Golgi binding signal of spectrin is not in the PH domain, but rather through the actin-binding domain and membrane-association domain 1 (MAD1) (Devarajan et al., 1997). The PH domain of OSBP targets to the Golgi (Levine and Munro, 1998). So far this is the only PH domain localized to the Golgi. OSBP translocates to the Golgi from the cytosol when it binds to oxysterols (Ridgway et al., 1992). However, the PH domain of OSBP is specifically bound to PtdIns(4,5)P_2, which is not restricted to the Golgi membrane, suggesting that another mechanism is involved in Golgi targeting.

The binding specificity of the PH domain needs to be taken into account when considering its involvement in localization, since the basal level of polyphosphate PtdIns varies. PtdIns(4)P and PtdIns(4,5)P_2 are constitutively produced, whereas PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are non-detectable in quiescent cells. Therefore, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 binding proteins would need another membrane targeting motif rather than the PH domain if these proteins are associated with membranes in unstimulated conditions. Since the binding specificity of some PH domains hasn't been characterized, sequence comparison was used to predict possible PtdIns targets based on Isakoff et al.'s study (1998). In their analysis, a consensus sequence in the β1-loop-β2 region of the PH domain was observed in those with high selectivity of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3.

As shown in Figure 4.1, I analysed some PH domains mentioned in the discussion, which either localize in the Golgi or have known binding specificity. B2-1, ARNO, GAP1^{m}, centaurin-α1, PDK-1, GAP1^{IP4BP}, and PKB have all been documented with high affinity to PtdIns(3,4,5)P_3 and /or PtdIns(3,4)P_2, which is consistent with the consensus alignment. On the other hand, spectrin, OSBP, PKCμ, and evectin-1, which all are Golgi associated proteins, do not match the consensus sequence for 3-phosphoinoside binding.

Taken together, B2-1's Golgi cellular localization appears to be distinct from other PtdIns(3,4,5)P_3 binding proteins, which localize predominantly in the cytoplasm. Compared to several Golgi proteins bearing a PH domain, B2-1 is unique in that its PH domain is PtdIns(3,4,5)P_3 specific, whereas others appears not to be 3-phosphoinositide specific.

Coiled-coil domains in protein-protein interaction

In the present study, we demonstrate that the coiled-coil region of the small ARF-GEF proteins is responsible for its Golgi localization. Chardin et al. (1996) observed that purified ARNO, wild-type and the PH domain deletion mutant, migrated as a dimer on gel filtration, whereas the Sec7 domain alone migrated as a monomer. This indicates that ARNO forms a homodimer via its coiled-coil region. Another group has recently identified Munc13-1 as a potential binding partner of msec7-1 (rat homologue of B2-1/cytohesin1) (Neeb et al., 1999). The interaction was further mapped to the coiled-coil region of msec7-1. Together, it is evident that the small ARF-GEFs form a homodimer, and possibly a heterodimer under physiological environment, via the amino-terminal coiled-coil region.

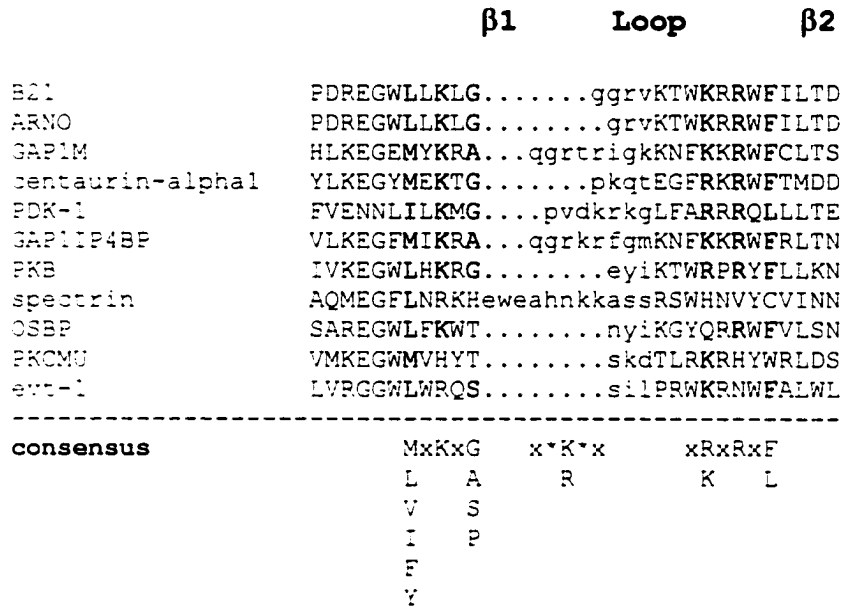

Figure 4.1 Alignment of the PH domain β1-loop-β2 region of several PH domain-containing protiens. The consensus sequence for 3-phosphoinoside specific binding (PtdIns(3,4,5)P_3/PtdIns(3,4)P_2) is highlighted in the alignment. The residues in the loop are lowercased. x, any single amino acid; *, varied numbers of residues.

Sequences involved in GEF specificity

Franco et al. (1998) have shown that ARF1 is a much better substrate than ARF6 for B2-1/cytohesin-1, ARNO, and ARNO3. The isolated Sec7 domain of ARNO had identical specificity. This suggests that the Sec7 domain performs the catalytic and substrate specificity of GEFs. The Sec7 domains of B2-1, ARNO, and ARNO3 are 84% identical and 96% conserved, indicating that the small ARF-GEFs prefer class I ARF rather than class III ARF as substrates. If this is true, then GEFs for other classes of ARFs may be from other subfamilies of which the Sec7 domains are divergent from that of the small ARF-GEFs. Indeed this seems to be the case. EFA6 is specific for ARF6 and its Sec7 domain is unique, compared to other Sec7 domains (Franco et al., 1999). However, because the regions that the Sec7 domain of the small ARF-GEF recognized are conserved among ARFs, it suggests that other regions, in addition to the Sec7 domain, are involved in the ARF interaction in order to provide the substrate specificity. In support of this, Pacheco-Rodriguez et al. (1999) showed that a region in addition to the Sec7 domain is involved in the interaction of cytohesin-1 with ARF.

If these three small ARF-GEFs all prefer class I ARF as substrates, then either their functions are redundant or the specific localization of ARF-GEFs in the cell plays a functional role. In other words, the cellular localization is in charge of the specificity of these proteins. This localization can be accomplished by binding to different adaptors. This model fits the hypothesis that the coiled-coil region of the small ARF-GEFs localizes the protein to different adaptors, possibly in different subcompartments in the Golgi complex. Such localization arrangement of the highly similar ARF-GEFs ensures that certain ARF can be activated at the right place. In support of this idea, the similarity

among the coiled-coil domains of the small ARF-GEFs is considerably lower, 48 % identical and 82% conserved, than the rest of the protein.

From another point of view, the mechanism of ARF intracellular localization remains unknown. Because the carboxyl terminus of ARF is the most divergent region, it could be involved in interaction with corresponding GEFs and consequently the distinct localization.

Regulation of ARF activation

The details of the conformation of ARF interaction with the Sec7 domain have been studied(Antonny et al., 1997; Beraud-Dufour et al., 1999). In their experiments the Sec7 domain of ARNO was used. On the premise that the small ARF-GEFs were cytosolic, they proposed a model that nicely explains the interaction sequence between ARF and its GEF. However, one question still remains. If both inactive, GDP bound ARF and ARF-GEF are cytosolic, then when and how are these proteins translocated to the right place? Again, the results in this thesis can add a piece to this puzzle. An adjusted model is summarized as the following steps: 1. GEF localizes to certain membranes via a coiled-coil interaction with its adaptor; 2. ARF recognizes the membrane-associated GEF; 3. the myristate tail of ARF inserts into the membrane phospholipid; 4. Sec7 domain of GEF interacts with ARF switch I and switch II regions; 5. N-terminus of ARF leaves the protein core and becomes membrane attached; 6. conformational change of ARF enables GDP dissociation; 7. free GTP binds to ARF; 8. ARF switch region conformation is changed; 9. Sec7 departs from ARF; 10. ARF is activated (Figure 4.2).

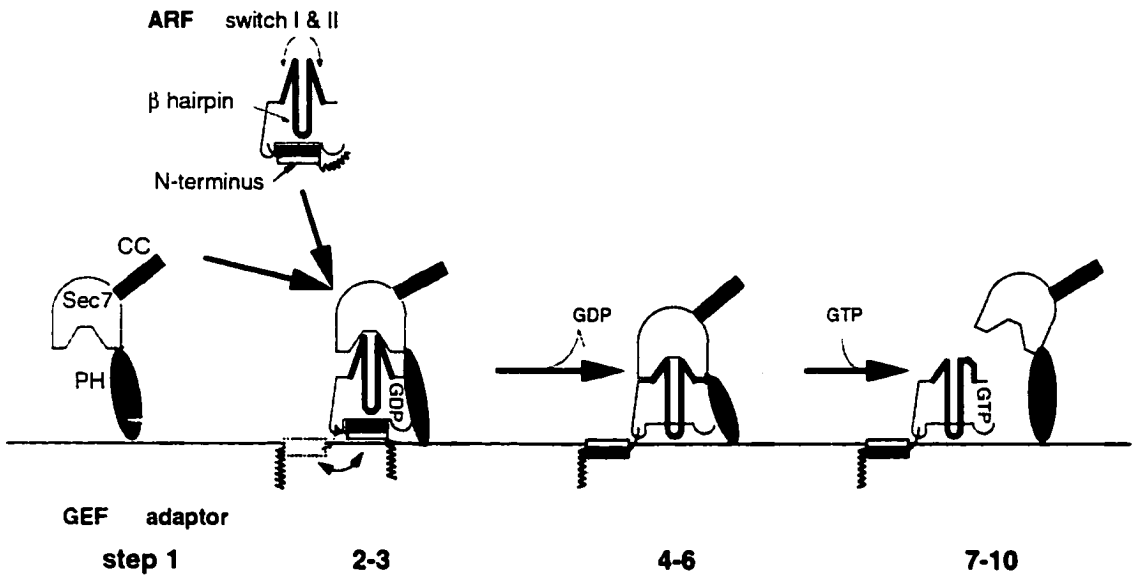

Figure 4.2 Model of the activation of ARF by the small ARF-GEFs. Detail is as described in the text. ARF switch I, switch II, β-hairpin, and N-terminus, which are involved in the activation process, are indicated. The adaptor for the GEF is in light gray. The small GEF contains a coiled-coil region (CC), the Sec7 domain, and a PH domain as indicated.

Future perspectives

Many questions remain to be answered concerning the regulation of ARFs and the corresponding ARF-GEFs. To further study and confirm the targeting mechanism described in this thesis, it is necessary to identify the proteins with which the small ARF-GEFs interact. Whether the PH domain translocates the protein to the plasma membrane or not under physiological conditions, and whether it is involved in the signaling

transduction pathway to regulate the interaction of ARF and the small GEF members, remain to be tested.

It appears that ARFs have more than one major function, vesicle formation, in the cell. Recent findings show that ARF GTPases are involved in recruiting PtdIns 4-kinase-β and PtdIns(4) 5-kinase, in addition to activation of phospholipase D and recruiting coat proteins (Godi et al., 1999). In addition, recently ARF1 was identified as an essential factor for the COPs recruitment in the early endocytic pathway (Gu and Gruenberg, 2000). This function of ARF1 is not via activation of phospholipase D. This implies that B2-1 and other ARF-GEFs are involved in those functions or that various types of GEFs are involved in different functions. The later explanation is tempting because recently the numbers of identified ARF-GEFs and their subfamilies have been increasing. Various ARF-GEF subfamilies may interact and thus regulate distinct ARF functions via different pathways.

Appendix I

Supporting Results

In addition to the results presented in chapters 2 and 3, there were several experiments omitted from the publications. These data are presented here because they support the conclusions of chapters 2 and 3.

Materials and Methods

Most of the materials and methods were described in previous chapters. Different materials and methods employed in this section are as follows.

Cell Culture

Raji and YT cell lines were obtained from the ATCC. YT, a human cytolytic NK cell line, was obtained from Dr. Stephen Anderson, NCI, USA. Cell lines were propagated in RPMI 1640 containing 10% fetal bovine serum and antibiotics at 37 °C in 5% CO_2.

Antibodies

β-COP mouse monoclonal antibody and clathrin goat polyclonal antiserum were purchased from Sigma. LAMP1 mouse monoclonal antibody was purchased from StressGen. Cy3-conjugated donkey anti-rabbit antibodies and Alexa (488)-conjugated donkey anti-goat antibodies were purchased from Jackson Immuno Research and Molecular Probes, respectively.

Immunofluorescence Microscopy

Suspended cells were centrifugated onto polylysine-coated glass slides prior to fixation (700 rpm, 20 sec). After centrifugation, the procedures were the same as for adhesive cells described in previous chapters.

Localization of B2-1

By indirect immunofluorescence, B2-1 was visualized at the perinuclear region in a punctate pattern. Several cell lines were tested. Here we show the localization of B2-1 in the lymphoid cell lines Raji, YT and Jurkat (Fig. A.1). Due to the huge nucleus and their growth in suspension culture, the individual cells are not well spread and extended. Consequently the organelles inside the cell are less clear when employing visible confocal microscopy. Nevertheless, the perinuclear distribution of B2-1 was also found in these cell lines.

Endogenous B2-1's perinuclear localization was identified within the Golgi complex. Ectopic GFP-tagged B2-1 also partially overlapped with the Golgi markers, giantin, ERGIC-53, and TGN46 in Cos-1 cells. In addition to Golgi markers, other subcellular compartment protein markers were used to examine B2-1's localization. The B2-1 distribution pattern was distinct from GRP78 (an ER marker), LAMP1 (a lysosomal protein), and clathrin (an endosomal protein) (Fig. A.2). Clathrin-coated vesicles travel between the plasma membrane and the *trans*-Golgi network and are directly involved in endocytosis. LAMP1 (lysosome-associated membrane protein 1), on the other hand, is primarily localized in lysosomes and late endosomes. Compared to these ER,

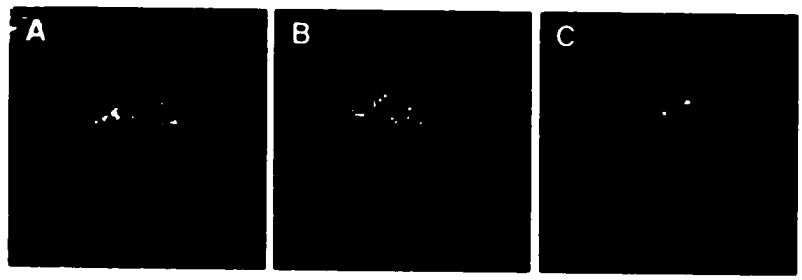

Figure A.1 Subcellular localization of B2-1. Immunofluorescent detection of B2-1 using B2-1 antiserum in Raji (A), YT (B), and Jurkat (C) cell lines. FITC-conjugated goat anti-rabbit secondary antibody was used. The cell size ranges normally from 20 to 30 μm.

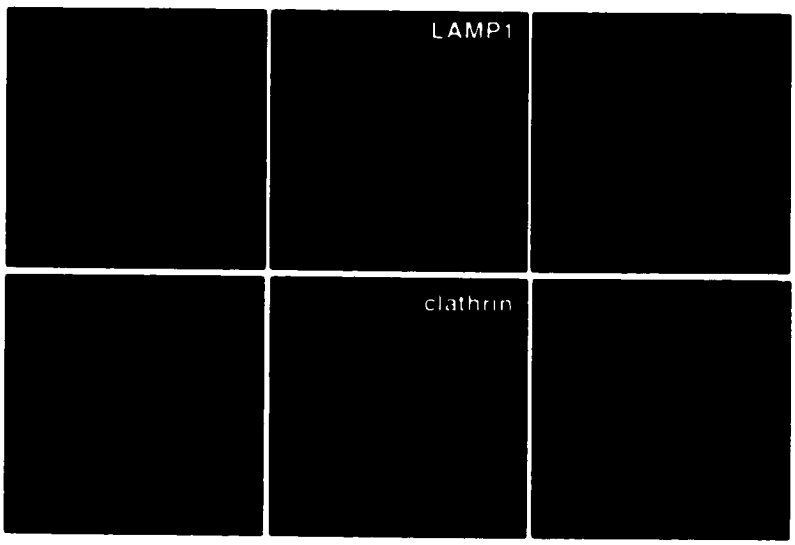

Figure A.2 Double labeling of B2-1 and marker proteins. Cos-1 cells were double labeled with B2-1 antiserum and LAMP1 monoclonal antibody (StressGen) or clathrin goat polyclonal antiserum (Sigma) as indicated. B2-1 (left panels) was visualized by Cy3-conjugated donkey anti-rabbit antibodies (Jackson ImmunoResearch). LAMP1 and clathrin (middle panels) were visualized by Alexa (488)-conjugated goat anti-mouse and Alexa (488)-conjugated donkey anti-goat antibodies (Molecular Probes), respectively. Right panels are superimposed images.

endosome, and lysosome resident proteins, it is evident that B2-1 is predominantly associated with the Golgi network.

When a cell undergoes division, the Golgi apparatus disappears. In this study, the loss of B2-1's Golgi staining was observed in dividing cells. As shown in Fig. 2.6A (giantin panels), a dividing cell did not have either giantin staining or the GFP-B2-1 fluorescent signal. In the YT cell line, the vesicular perinuclear staining pattern of endogenous B2-1 disappeared in dividing cells (Fig. A.3). Microtubule staining clearly showed that the dividing cell contained two microtubule-organizing centers.

Sensitivity to BFA treatment

Although the small Sec7-domain-containing proteins are BFA insensitive *in vitro*, B2-1's distribution was altered by BFA treatment of Cos-1 cells. Various cell lines have different degrees of response to BFA treatment. The Raji cell line appears to be less sensitive to BFA treatment under the experimental conditions of this study. In comparison with the Cos-1 cell line (Fig. 2.4), the Golgi marker ERGIC-53 was less affected after a 30 min treatment with BFA in Raji (Fig. A.4). Another Golgi marker β-COP was dispersed to the cytoplasm after a 10 min treatment. B2-1's redistribution in Raji was not as obvious as that seen with the Cos-1 cells.

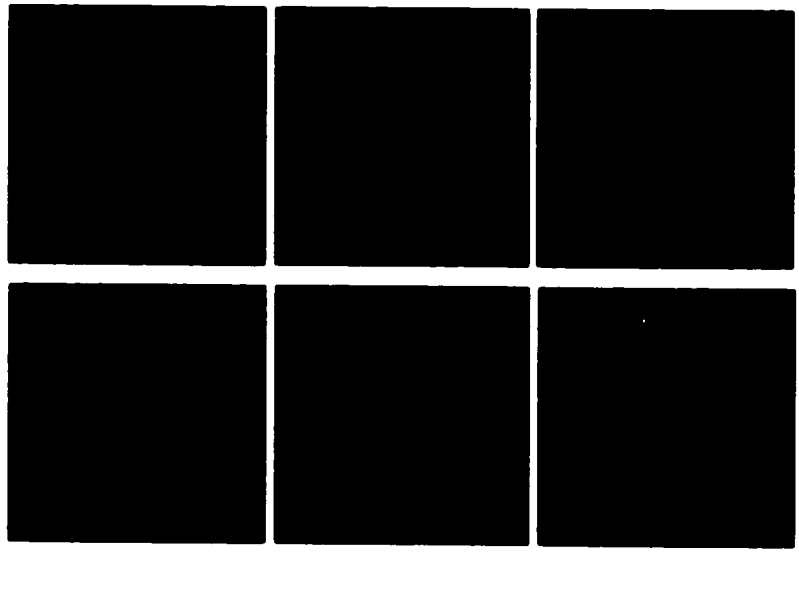

Figure A.3 B2-1 localization in a dividing cell. YT cells were double labeled with B2-1 antiserum and tubulin antibody. B2-1 (left panels) was visualized by FITC-conjugated goat anti-rabbit antibody. Microtubules (middle panels) were visualized by Cy3-conjugated goat anti-mouse antibody. Two sets of images (top and bottom) with one dividing cell in each are shown.

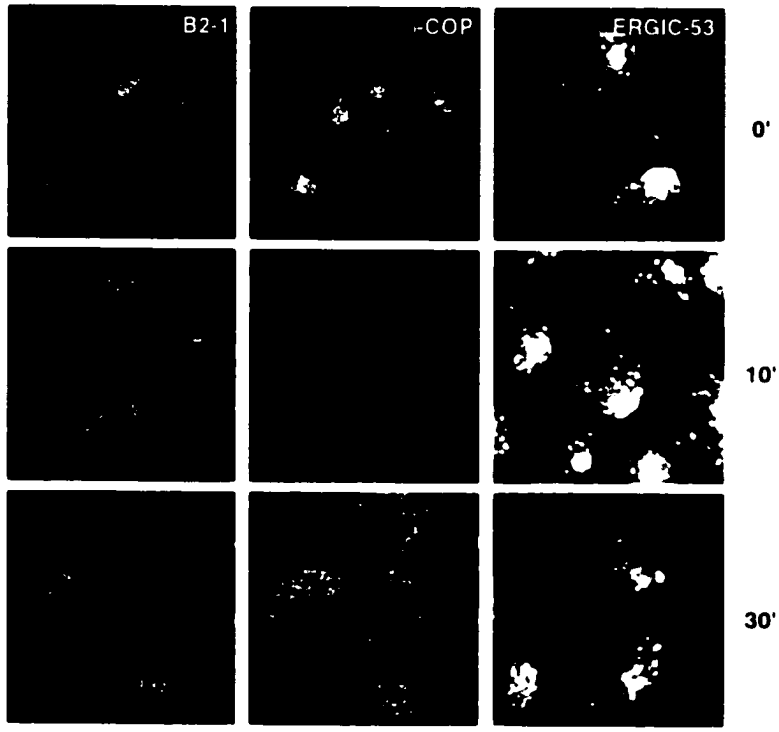

Figure A.4 Effects of brefeldin A treatment on B2-1 subcellular localization. Raji cells were treated with 5 μg/ml brefeldin A for 10 or 30 min as indicated. Cells were probed with B2-1 antiserum, β-COP, or ERGIC-53 antibodies, respectively. Top panels (0') are control cells without brefeldin A treatment. B2-1 (left) was visualized by FITC-conjugated goat anti-rabbit antibody. β-COP (middle) and ERGIC-53 (right) were visualized by Cy3-conjugated goat anti-mouse antibody.

ARF-GEF localizes to the Golgi complex via its amino-terminus

In chapter 3, the Cos-1 cell line was used to demonstrate that B2-1 is targeted to the Golgi complex by its N-terminus. The first 55 amino acids of B2-1 are necessary and sufficient for targeting. The same experiments were performed in the Jurkat cell line and similar results were obtained. In Figure A.5, full length B2-1 (NSP), the amino-terminus (N), and the Sec7+PH region (SP) were transiently expressed, either with a GFP tag (Fig. A.5A) or a myc tag (Fig. A.5B). The GFP-SP construct localized to the nucleus as did the GFP alone construct, whereas the myc-SP distributed mostly in the cytoplasm.
To confirm that the Golgi localization observed is not a coincidental distribution, sectioning images of single cells were collected. Nine sections of one cell were collected as shown in Fig. A.6. It is clear that only one concentrated area representing the Golgi apparatus appeared using the full-length B2-1 (Fig. A.6 A and B) and N-terminus (Fig. A.6 C). No pattern resembling the Golgi was observed using the Sec7+PH construct in all sections (Fig. A.6 D).

The other two ARF-GEFs of the same family, ARNO and ARNO3 contain a coiled-coil amino-terminus. Again, these N-termini specifically target to the Golgi in the Jurkat cell line (Fig. A.7 and Fig. 3.2). In myc-tag constructs the distinct ribbon-like Golgi structure was observed (Fig. A.7).

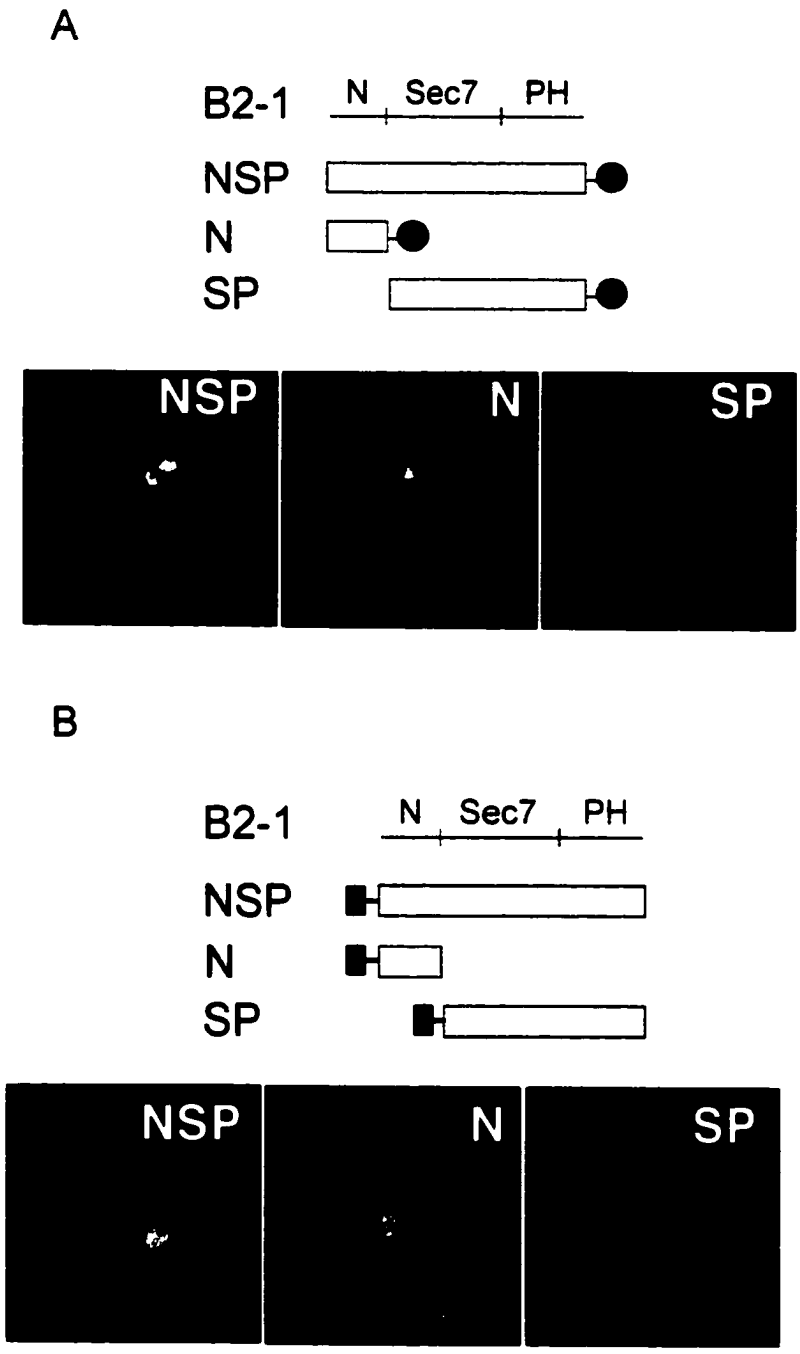

Figure A.5 Subcellular localization of the transient transfected B2-1 constructs in Jurkat cells. B2-1 constructs were either tagged with GFP (A) or myc (B). N, N-terminus, amino acid 1 to 54; SP, Sec7+PH domain, amino acid 55 to 398. NSP represents the full-length B2-1, residues 1-398. The schematic representation of the constructs used for transfection are also shown. Black circle, GFP tag; black rectangle, myc tag.

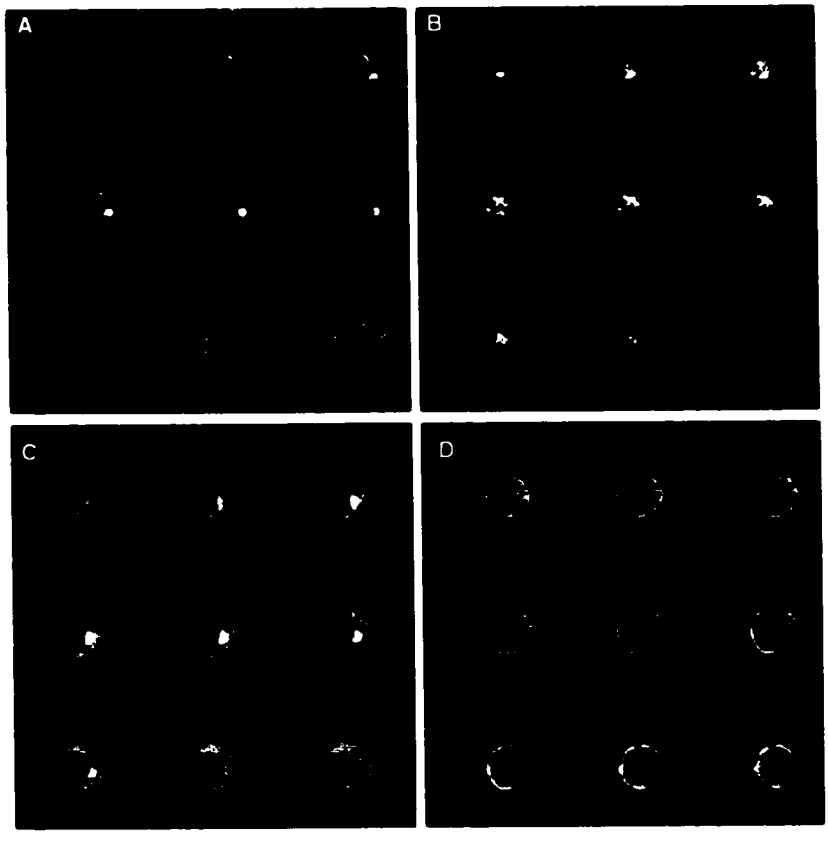

Figure A.6 Sectioning images of single Jurkat cells transfected with myc-tagged B2-1. Nine continuous sectioning images (from left to right, top to bottom) are shown. Each section is 1 μm. (A) full-length B2-1, (B) a cell with high levels of full-length B2-1 overexpression. (C) N-terminus of B2-1 (amino acid 1 to 55), and (D) Sec7+PH domain of B2-1 (residues 55-398).

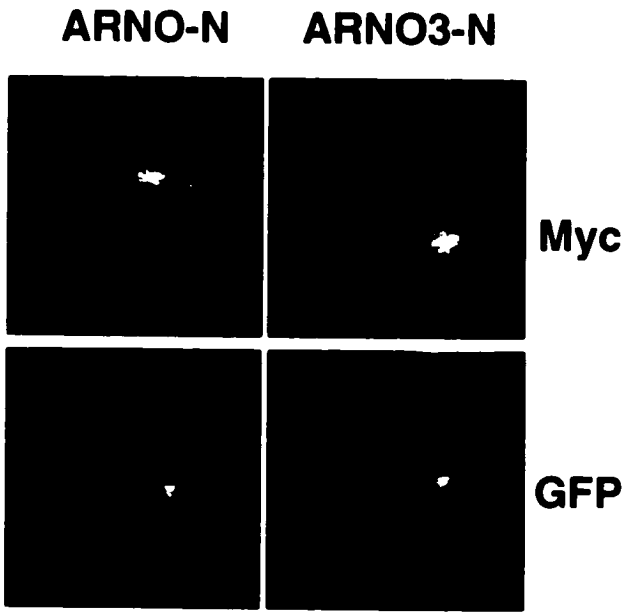

Figure A.7 Subcellular localization of transfectd N-terminus of ARNO and ARNO3 in Jurkat cells. Cells were transient transfected with either myc (top panels) or GFP (bottom panels) tagged N-terminus of ARNO (ARNO-N) and of ARNO3 (ARNO3-N) as indicated.

Western Blotting of proteins expressed from various constructs

All constructs were sequenced to verify their identities. In addition, the ectopic protein expressions were confirmed by Western blotting. The N-terminus fusion proteins are shown in Fig. A.8. The molecular mass of the GFP control is ~27 kDa. Due to the small fragment insertion at the amino-terminus of GFP, some constructs expressed not only the designed GFP-tagged protein but the GFP protein as well. In ARNO-N and ARNO3-N, smaller sized products were also detected, and were possibly made from internal start codon. This result can explain the minor signal which appeared in the nucleus with some of the N constructs even when the proper coiled-coil region was present (Fig. 3.4; Fig. A.5, GFP-N; Fig. A.7, GFP-ARNO-N and GFP-ARNO3-N).

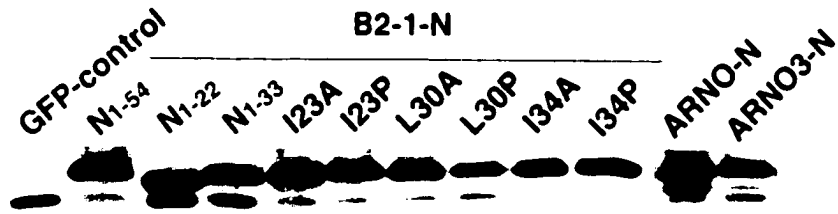

Figure A.8 Western blotting of protein expression of various GFP-tagged N-terminal constructs. The blot was probed with GFP monoclonal antibody (Clontech). The various constructs used in this study are as indicated. The lanes under the B2-1-N labeling are discussed in chapter 3.

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