

AN ABSTRACT OF THE THESIS OF

Daniel Nevriy for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on December 5, 2001. Title: Cloning and Characterization of GRASP, a Novel Retinoic Acid-Induced Gene from P19 Embryonal Carcinoma Cells.

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✓
Mark E. Leid

Retinoic acid (RA) exerts important effects in the processes of vertebrate development, cellular growth and differentiation, and homeostasis. However, the mechanisms of action of RA in the control of cellular and developmental processes are incompletely understood, as the retinoid target genes have not been fully characterized. The goal of these studies described herein was to contribute towards a greater understanding of the cellular effects of retinoids through the identification and characterization of an RA-induced gene from mouse P19 embryonal carcinoma cells.

The predicted amino acid sequence of GRASP is characterized by several putative protein-protein interaction motifs, suggesting that GRASP may function in cell signaling pathways. Towards the goal of identifying which signaling pathways GRASP may participate in, a yeast two-hybrid screen was performed using GRASP as a bait to

identify protein interaction partners. The general receptor for phosphoinositides 1 (GRP1), a guanine nucleotide exchange factor for the ADP-ribosylation factor 6 (ARF6) GTPase, was identified as a GRASP interaction partner. GRASP was shown to colocalize with endogenous ARFs in cells and enhance GRP1 association with the plasma membrane, suggesting that GRASP may function as a scaffold protein in the recruitment of GRP1 and ARF6 to plasma membrane loci.

Overexpression of GRASP was observed to induce accumulation of GRASP in the endosomal compartment where GTP-binding deficient mutants of ARF6 reside, suggesting that GRASP induced a block in an ARF6 plasma membrane recycling pathway. Coexpression of GRP1, but not a catalytically inactive mutant, dramatically reduced the accumulation of GRASP in this compartment. Furthermore, GRP1 mutants that lack the region of interaction with GRASP failed to prevent accumulation of GRASP in the endosomal compartment, suggesting that GRASP recruits GRP1 to the endosomal compartment where GRP1 stimulates nucleotide exchange on ARF6 and recycling.

Results described herein demonstrate that GRASP functions in the ARF6 regulated plasma membrane recycling pathway, and that upon overexpression, induces a block in recycling. Our results suggest a role for GRASP as an adapter or scaffold

protein that may link cell surface receptors to the ARF6 recycling pathway, resulting in modulation of signal transduction events at the cell surface.

Cloning and Characterization of GRASP, a Novel Retinoic Acid-Induced Gene from

P19 Embryonal Carcinoma Cells

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CONTRIBUTION OF AUTHORS

Chapter 2 is reproduced with the permission of The American Society of for Biochemistry and Molecular Biology and *The Journal of Biological Chemistry*. All of the experiments in this chapter were performed and analyzed by myself in the laboratory of Mark Leid with the following exception: Dorina Avram assisted with the Southern analysis of 36B4 cDNA in Fig. 2.2. Valerie Peterson performed protein purification of GST-GRASP for use as an immunogen in the preparation of GRASP polyclonal antibodies. Paul Dowell assisted in the preparation of GRASP expression vectors. Scott Hansen assisted with various microscopy techniques. All remaining authors assisted with data interpretation and/or manuscript preparation.

All of the experiments described in Chapter 3 were performed by myself in the laboratory of Mark Leid with the following exception: Valerie Peterson performed GST-pull down experiments described in Fig. 3.5 and, in addition to Mark Leid, contributed much appreciated intellectual effort towards devising a working model of GRASP in the ARF6 recycling pathway. Remaining authors assisted with data interpretation and/or manuscript preparation.

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Cloning and Characterization of GRASP, a Novel Retinoic Acid-Induced Gene from P19 Embryonal Carcinoma Cells

Chapter 1

Introduction

Vitamin A is an essential nutrient in the diet for normal growth, differentiation (Wolback and Howe 1925), reproduction (Thompson, Howell et al. 1964), and vision (Wald 1968). The major natural forms of vitamin A include retinol, retinal, and retinoic acid (RA), which contain a conjugated double-bond system rendering them extremely hydrophobic and thus quite water insoluble (Fig. 1.1). Therefore, transport, metabolism, and utilization of retinol and its derivatives requires the actions of a diverse class of retinoid binding proteins, which are present in serum and retinoid target tissues (Soprano 1994).

Vitamin A is absorbed from the diet mainly from animal sources as retinyl esters, although significant amounts are derived from plant carotenoid pigments, such as β -carotene (Soprano 1994). Within intestinal mucosal cells β -carotene is converted via a two-step process to retinol. On the other hand, retinyl esters are hydrolyzed in the intestinal lumen and the resulting retinol is absorbed by the mucosal cells. Thus, retinol is the major form of dietary vitamin A in animals.

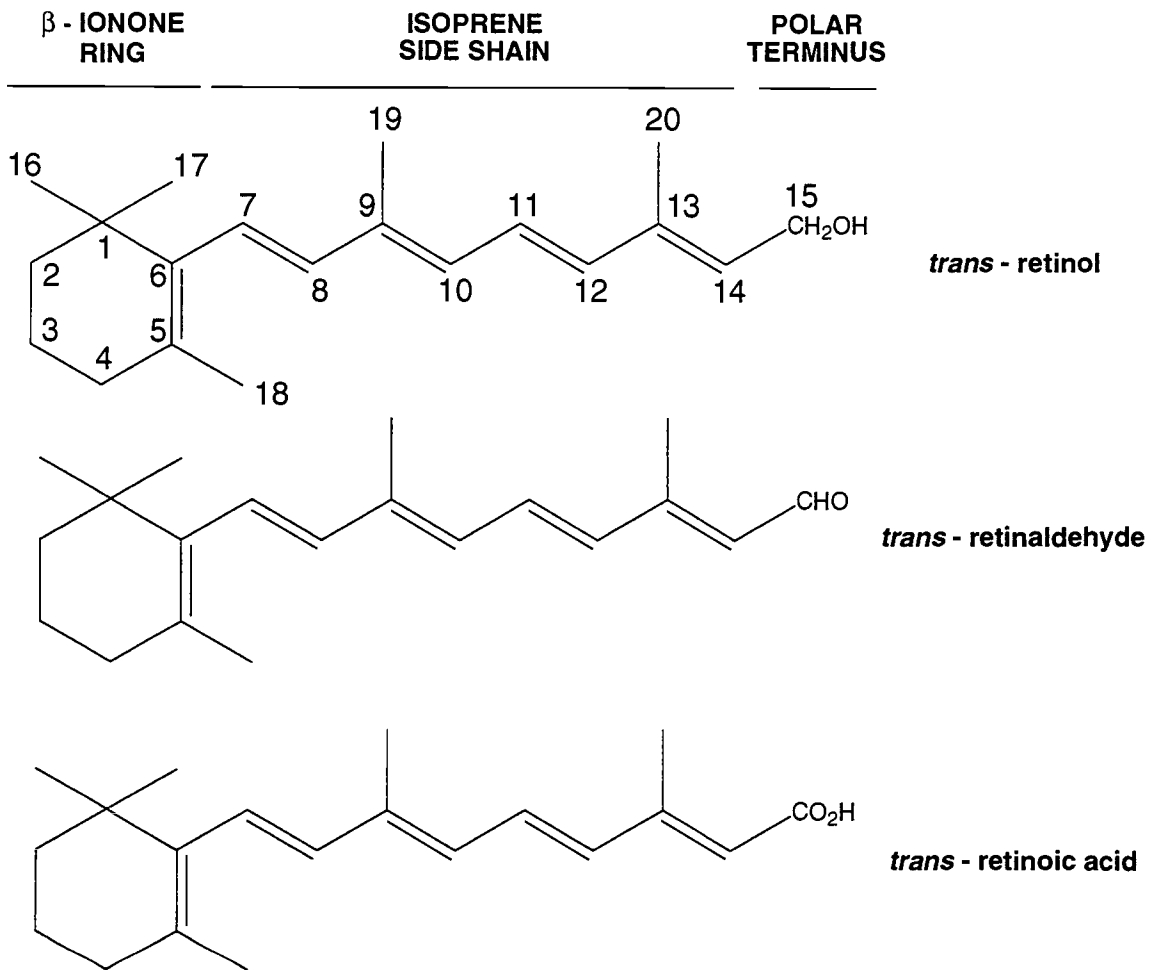


Figure 1.1 Naturally occurring *trans*-retinoids.

In intestinal mucosal cells, retinol is esterified with free fatty acids and incorporated into chylomicrons for transport to the liver (Soprano 1994). Once in the liver, retinyl esters can be stored or converted to retinol and transported to individual target tissues with the aid of serum retinol binding protein (RBP). In the target tissue, retinol is sequestered by a cellular retinol binding protein (CRBP), that facilitates its conversion to the aldehyde, retinal (Leid in press). CRBP-retinal then serves as a substrate for a retinaldehyde dehydrogenase enzyme, that catalyzes the irreversible formation of RA (Leid in press). Vitamin A target tissues include virtually all tissues in the body and, with the exception of the requirement of retinol for vision, and perhaps reproduction, exogenous RA is able to replace vitamin A for all its functions (Giguere 1994). Thus, RA is believed to act as the primary active metabolite of vitamin A in the regulation of development and homeostasis.

1.1 Role of retinoids in growth and development

Retinoids are essential for normal growth as demonstrated by classic experiments performed in the 1920s with vitamin A-deficient rats (Wolback and Howe 1925). Consequently, the occurrence of vitamin A deficiency under natural conditions is mainly restricted to growing periods of the life cycle, i.e., gestation and early life. Clues that retinoids play an important role in development came from observations that

either a deficiency or an excess of retinoids induces severe malformations of the developing embryo. Indeed, retinoids are some of the most potent natural teratogens known, and studies of the teratogenic effects of retinoids have contributed towards a greater understanding of the specific roles of retinoids in development.

Experimental studies in rodents have demonstrated that exposure to high retinoid concentrations during development results in severe craniofacial abnormalities (Roberts and Sporn 1984). These include malformation of the cartilagenous and bony facial tissues, such as the mandible, palate, and maxilla, as well as the outer ear (Roberts and Sporn 1984). These effects have also been observed in human fetuses exposed to 13-*cis*-retinoic acid during gestation (Kraft 1992; Lammer and Armstrong 1992). In addition, malformations have been observed in hindbrain structures as well as the cardiovascular system (Kraft 1992; Lammer and Armstrong 1992). Of particular note is the effect of RA on cells of mesenchymal origin. Treatment of 9-day-old rat embryo explants with RA prevents formation of the pharyngeal arches that later give rise to the mandible and maxilla (Morriss and Steele 1977). In addition, observations that both an excess and deficiency of RA lead to the suppression of yolk sac circulatory tissue in embryo explants suggest that these mesenchymally derived cells require a narrow range of RA concentrations for proper development (Thompson, Howell et al. 1969; Morriss and Steele 1977). The concentrations of retinoids required to exert these effects in vitro are very low, suggesting that the responses are selective and do not arise

due to a generalized toxic action on the embryo as a whole. These studies have prompted speculation that RA may be a true morphogen in development by inducing in a dose-related manner differentiation of cells in the embryo according to their position relative to organizing regions.

Perhaps the most appreciated developmental process influenced by RA is limb morphogenesis. During limb regeneration in urodeles, local application of RA can respecify the positional memory of progenitor cells that arise at the amputation plane, leading to regeneration of duplicated structures (Tabin 1991). For example, if amputation occurs at the wrist, local application of high doses of RA results in the regeneration of the entire arm, producing serial duplication of skeletal structures, whereas lower concentrations of RA result in more proximal regenerates (Tabin 1991). Similarly, RA has also been shown to affect the developing chick limb bud. Application of RA to the limb bud results in a mirror-image duplication of the limb pattern that is identical to the pattern resulting from grafts of posterior ZPA (zone of polarizing activity) tissue, which has been identified as an organizer (Tickle, Lee et al. 1985). It was demonstrated that RA induced the conversion of anterior cells into ZPA cells, which in turn provided the actual pattern-duplicating stimulus (Wanek, Gardiner et al. 1991). These studies have cast doubt on the role of RA as a true morphogen in development, but rather suggest that RA induces formation of the organizer tissue, which may then establish morphogenic gradients.

1.2 Retinoid regulation of cellular differentiation

It has been known for nearly 75 years that vitamin A exerts control over cell differentiation and proliferation. It was noted by Wolback and Howe, in their studies of vitamin A deficiency in the rat, that vitamin A was essential for the normal differentiation of the epithelia (Wolback and Howe 1925). Vitamin A deficiency leads to the conversion of secretory epithelial cells to a squamous, keratinizing phenotype (Wolback and Howe 1925). Importantly, it was observed in organ culture that retinoid treatment could reverse the keratinization of epithelial cells derived from vitamin A-deprived animals (Sporn, Clamon et al. 1975). Later studies in vitamin A-deprived rats fed with retinoic acid demonstrated that the deficiency adversely affects the rates of differentiation of a subclass of mucous-secreting goblet cells (Rojanapo, Lamb et al. 1980). In contrast, epithelial tissues that normally lack goblet cells, such as skin, exhibit a more pronounced keratinization, becoming thickened, dry, and scaly (Matoltzy 1976). In support of these findings, it was shown that retinoids could regulate the terminal differentiation of epidermal cells, a change accompanied by marked alterations in the pattern of keratin genes expressed (Fuchs and Green 1981; Kopan, Traska et al. 1987). Thus, vitamin A and its derivatives exert important control in the maintenance and structure of epithelial tissues, mediated by its effects on cellular differentiation.

In addition to their role in promoting and maintaining epithelial cell differentiation, retinoids have also been shown to induce growth arrest and

differentiation in a number of cultured neoplastic cell lines including neuroblastomas, melanomas, and teratocarcinomas (Roberts and Sporn 1984). Observed effects of retinoids include inhibition of anchorage-independent growth and colony formation in soft agar, induction of cell-cycle arrest, and promotion of terminal differentiation of certain leukemias and embryonal carcinoma cells (Roberts and Sporn 1984). For example, RA has been shown to induce terminal differentiation of the human myeloid leukemia cell line HL60 to morphologically and functionally mature granulocytes (Breitman, Selonick et al. 1980). The differentiating effects of RA in this system are particularly impressive, as the potency of RA is several orders of magnitude greater than that of other known agents (Breitman, Selonick et al. 1980). Retinoids are also potent inducers of embryonal carcinoma cell differentiation. P19 cells (McBurney and Rogers 1982) are a multipotent mouse embryonal carcinoma cell line that have been extensively utilized as a model system to study the effects of RA on differentiation. P19 cells differentiate into all three primitive germ layers depending on the growth conditions and concentrations of RA administered (McBurney 1993). When grown in a monolayer and treated with RA, P19 cells undergo differentiation along endodermal and mesodermal pathways (Roguska and Gudas 1985). In contrast, RA has a biphasic effect on P19 cells grown in aggregates. When treated with low (1-10 nM) concentrations of RA, P19 cell aggregates differentiate primarily via the endodermal pathway while high concentrations (>300 nM) of this retinoid induce differentiation

into neurons, glial, and fibroblast-like cells (Jones-Villeneuve, McBurney et al. 1982). While many of the effects of retinoids on growth of transformed cells are reversible, effects on differentiation represent a stable alteration of the transformed phenotype to a nonneoplastic phenotype. Therefore, the ability of retinoids to induce differentiation of various cancer cells make them particularly attractive therapeutic candidates.

Retinoid therapy has been approved for the treatment a number of human diseases, and is in early and late stage clinical trials for the treatment of many others. In fact, 13-*cis*-retinoic acid is currently the most effective treatment for acne vulgaris and other skin diseases (Schaefer and Reichert 1990). In addition, clinical approval of RA therapy in acute promyelocytic leukemia (APL) was a great advance, as RA treatment leads to complete remission of this disease (Huang, Ye et al. 1988). Retinoid treatment has also shown promise in the treatment of various other cancers of the head and neck, cervix, and most other epithelial tissue-derived cancers (Bollag and Holdener 1992). However, substantial drawbacks to retinoid therapy are the acute teratogenic effects (described above) and other adverse side effects, including severe nausea and skin irritations. Therefore, attempts are currently underway to design novel retinoids with the aim of separating the therapeutic benefits of retinoids from the undesirable side effects (Dawson and Okamura 1990).

1.3 Molecular mechanisms of retinoid action

Although much progress had been made in demonstrating the important role of retinoids in the regulation of development and cellular function, a mechanistic basis of the effects of retinoids was not uncovered until the discovery of nuclear receptors for RA. In 1987, two groups studying the superfamily of steroid hormone receptors identified a novel member of this family as the nuclear receptor of RA, referred to as RAR α (Fig. 1.2; Giguere, Ong et al. 1987; Petkovich, Brand et al. 1987), thus implicating RA as a direct regulator of transcription. A second subtype, RAR β , was isolated shortly thereafter as the gene product encoded by a cDNA cloned from the integration site of the hepatitis B virus in a human hepatocellular carcinoma (de The, Marchio et al. 1987). Subsequent isolations, using low-stringency hybridization techniques, led to the isolation of the third member, RAR γ (Zelent, Krust et al. 1989). It was later demonstrated that all three RAR loci generate multiple isoforms, differing solely in their amino terminal region and arising through alternative splicing or differential promoter usage (Giguere 1994). In agreement with the pleiotropic effects of retinoids on cellular function, RARs are expressed in all tissues of the body, although each RAR subtype exhibits restricted spatial and temporal patterns of expression in the embryo and adult, suggesting that each RAR may perform unique functions (Leroy, Krust et al. 1992).

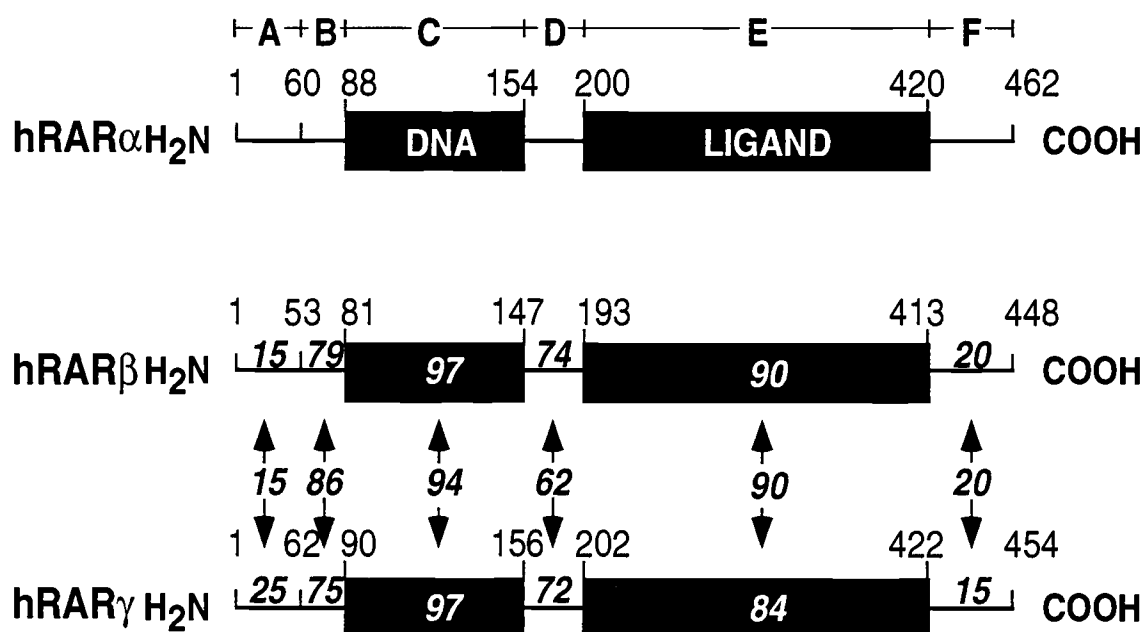


Figure 1.2 **Homology comparison of amino acid sequences of each human RAR subtype.** Amino acid residue positions (top) corresponding to the boundaries between the different regions A-F, and percentage homology are given for each comparison

Shortly after the identification of the RARs, another family of RA-responsive receptors belonging to the superfamily of nuclear receptors was identified, the RXRs (Mangelsdorf, Ong et al. 1990; Mangelsdorf, Borgmeyer et al. 1992). Like the RARs, the RXRs consist of three family members, α , β and γ subtypes (Fig. 1.3), each encoded by separate genes that give rise to multiple isoforms differing in their amino terminal regions (Mangelsdorf, Ong et al. 1990; Mangelsdorf, Borgmeyer et al. 1992). Amino acid sequence comparisons revealing only weak homology with RARs, coupled with observations that RXRs respond to somewhat higher concentrations of retinoic acid, suggested that the natural ligand of the RXRs may not be all-*trans*-RA, but was likely closely related (Mangelsdorf, Ong et al. 1990). These speculations were confirmed when the RXR ligand was identified as the 9-*cis*-RA (9cRA) stereoisomer (Heyman, Mangelsdorf et al. 1992; Levin, Sturzenbecker et al. 1992). 9cRA binds the RXRs with high affinity and is present in vivo at significant levels, suggesting that 9cRA may be the natural ligand (Heyman, Mangelsdorf et al. 1992; Levin, Sturzenbecker et al. 1992).

Mechanistic studies of the DNA binding strategies of steroid hormone receptors revealed that these receptors bind their DNA response element as homodimers (Kumar and Chambon 1988). However, studies of the DNA binding properties of RAR and other non-steroid nuclear receptors revealed that accessory factors present in the nuclear

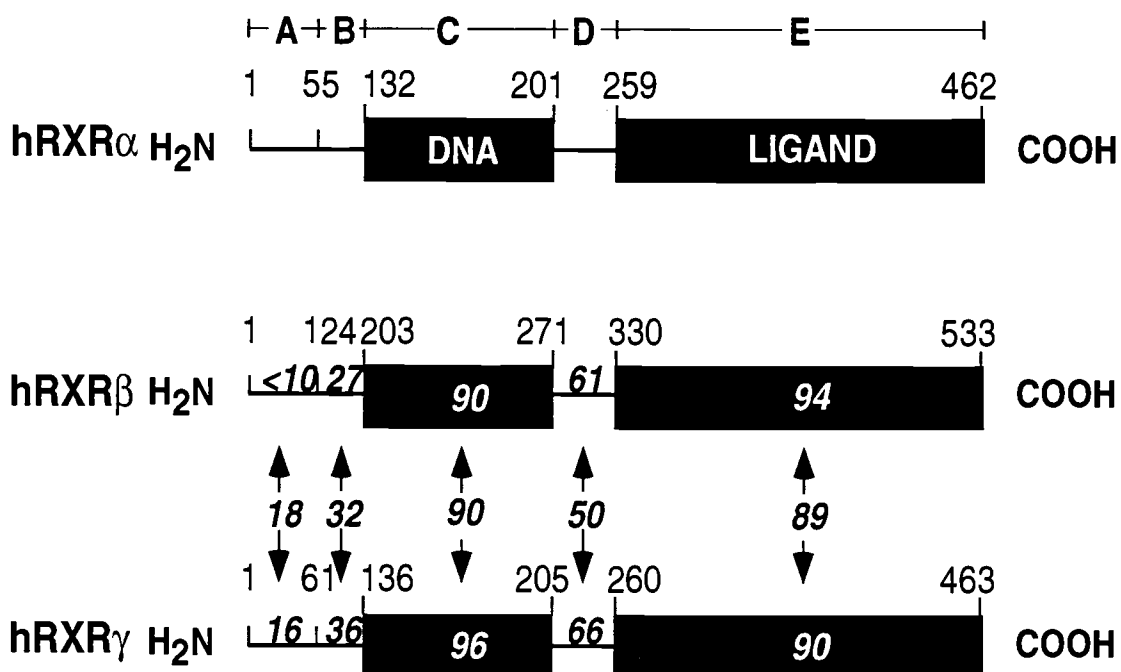


Figure 1.3 **Homology comparison of amino acid sequences of each human RXR subtype.** Amino acid residue positions (top) corresponding to the boundaries between the different regions A-E, and percentage homology are given for each comparison

extracts of cell lysates were required for high affinity binding of these receptors to their response elements. Observations that RAR and thyroid hormone receptor (T_3R) could form heterodimers in solution led to the hypothesis that the accessory factor present in the nuclear extracts might be members of the family of nuclear receptors. Indeed, this hypothesis was confirmed by a number of groups that discovered RAR family members heterodimerize with RXR and bind all known retinoic acid responsive elements as a RAR-RXR heterodimeric complex (Yu, Delsert et al. 1991; Bugge, Pohl et al. 1992; Leid, Kastner et al. 1992; Zhang, Hoffmann et al. 1992). Subsequent gene “knockout” experiments have supported the hypothesis that the RAR-RXR heterodimer is the functional unit transmitting the retinoid signal *in vivo* (Kastner, Grondona et al. 1994), as RAR/RXR double knockout animals exhibit profound developmental defects that resemble syndromes of severe vitamin A deficiency. In addition to binding to RARs, RXRs form heterodimeric complexes with T_3R , vitamin D receptor (VD_3R), peroxisome proliferator activated receptor (PPAR) and other orphan nuclear receptors (Kliewer, Umesono et al. 1992; Kliewer, Umesono et al. 1992; Kliewer, Umesono et al. 1992; Marks, Hallenbeck et al. 1992). Thus, one class of retinoid receptors, the RXRs, function as coregulators of nuclear receptor function in a plethora of hormonal signaling pathways.

The RARs and RXRs, like all nuclear receptors, share common structural and functional properties (Fig 1.4). These receptors exhibit a highly conserved, modular

structure composed of six regions designated A-F (Green and Chambon 1988). The amino terminal A/B regions vary in length and have been shown to harbor a ligand-independent transcriptional activation function. The highly conserved C region comprises the DNA binding domain of these receptors and is composed of approximately 66-68 amino acids that are organized into two C₂H₂ zinc finger motifs (Rastinejad, Perlmann et al. 1995; Rastinejad 2001). Region D comprises a hinge region that harbors a nuclear localization signal, while the E region encompasses the ligand binding domain (LBD) and also harbors an oligomerization domain. The LBD of nuclear receptors is highly structurally conserved despite considerable divergence at the amino acid level. This is highlighted by the fact that the RARs and RXRs, which both bind 9-*cis*-RA with high affinity, exhibit only 28-33% amino acid identity in the LBD between them. The LBD also harbors a ligand-dependent transcriptional activation function, which results from the juxtaposition of several helices within the LBD facilitating efficient interaction with transcriptional coactivator proteins.

Binding of ligand to RAR and/or RXR induces a substantial conformational change in the receptors, resulting in rearrangement of helices within the LBD and recruitment of a coactivator complex that harbors histone acetyltransferase (HAT) activity (Xu, Glass et al. 1999). The coactivator complex is composed of proteins harboring HAT activity, including p300/CBP, P/CAF, and the p160 family of proteins (Xu, Glass et al. 1999). Transcriptional coactivators are thought to function by binding

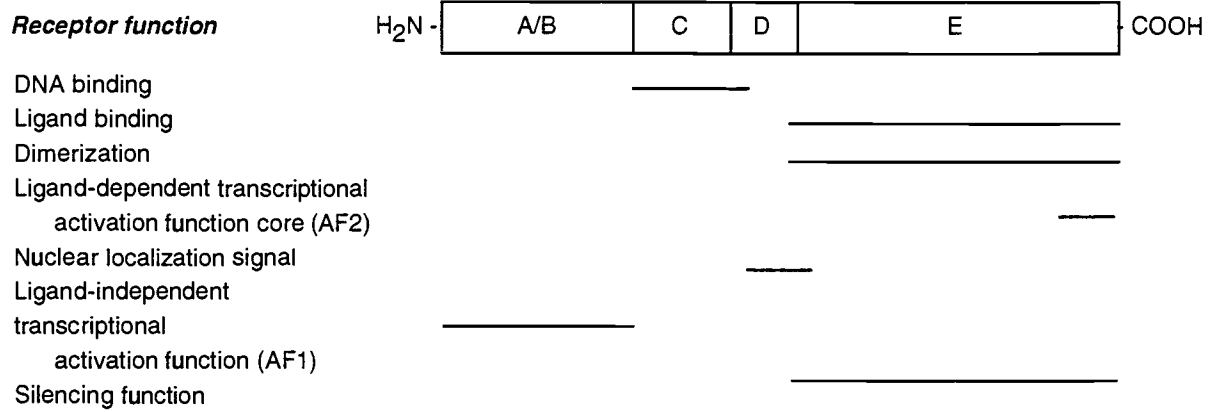


Fig. 1.4 Anatomy of a nuclear receptor.

to the nuclear receptor on the template and inducing a decondensation of chromatin mediated by acetylation of the histones (Xu, Glass et al. 1999). This decondensation is believed to be followed by dissociation of the coactivator complex and recruitment of a second class of coactivator complex to the ligand-bound receptors that harbor components of the Pol II machinery, resulting in preinitiation complexes and transcription (Ito and Roeder 2001).

In contrast to ligand-mediated activation of the receptors, unliganded-RAR-RXR complexes are able to repress transcription through recruitment of corepressor proteins to the template such as nuclear receptor corepressor (NcoR; Horlein, Naar et al. 1995) and silencing mediator of retinoid and thyroid receptors (SMRT; Chen and Evans 1995). NCoR and SMRT, in turn recruit a histone deacetylase (HDAC) complex to the template (Huang, Zhang et al. 2000), resulting in deacetylation of lysine residues on histones. Deacetylated histones are believed to have increased affinity for DNA, resulting in the formation of more densely packed chromatin that is less likely to be transcribed (Struhl, Kadosh et al. 1998).

Although the molecular basis of how retinoids induce changes in the pattern of gene expression has become clearer, a full understanding of these changes as they relate to cellular and developmental processes will require the identification and functional characterization of the retinoid-induced target genes. A few RA-responsive genes have been identified to date. These genes encode transcription factors (La Rosa 1988; Song

and Siu 1989; Kondo, Takahashi et al. 1992; Zwartkruis, Krut et al. 1993; Bouillet, Chazaud et al. 1995; Boudjelal, Taneja et al. 1997; Oulad-Abdelghani, Chazaud et al. 1997), metabolic enzymes (White, Guo et al. 1996), growth factor receptors (Kobayashi, Kurihara et al. 1994), extracellular matrix proteins (Gudas and Wang 1986; Vasios, Gold et al. 1989) and secreted proteins (Smolich and Papkoff 1994; Oulad-Abdelghani, Chazaud et al. 1998), however, as many of these genes are novel and are as yet not fully characterized, the totality of the mechanism of action of retinoids in the control of developmental processes remains to be elucidated.

1.4 ADP-ribosylation factors

In contrast to the retinoid signals, which are generally anti-proliferative and promote cellular differentiation, signaling by members of the Ras superfamily of small guanine nucleotide binding (G) proteins typically promotes cellular proliferation, alteration in cellular morphology, or changes in vesicular trafficking pathways (Moss and Vaughan 1998). ADP-ribosylation factors (ARFs) are members of this family that regulate key aspects of membrane traffic in cells (Moss and Vaughan 1998). ARF proteins are divided into three classes based on phylogenetic analysis, gene structure, and molecular mass. The class I ARFs, which include ARFs 1-3, are the best characterized and function in vesicular trafficking pathways in the Golgi apparatus

(Serafini, Orci et al. 1991; Balch, Kahn et al. 1992), while little is known about the function of class II ARFs (ARFs 4-5). ARF6, the least conserved member of the family, comprises class III and regulates endocytic and actin remodeling pathways (D'Souza-Schorey, Li et al. 1995; Radhakrishna, Klausner et al. 1996).

ARF proteins cycle between inactive GDP and active GTP-bound conformations. The alternative conformations of ARFs, induced by GTP-binding and subsequent hydrolysis of the bound nucleotide to GDP, facilitate interaction with, and activation of various classes of effector proteins. As ARF proteins are poorly able to exchange GDP and hydrolyze GTP on their own, these reactions are catalyzed by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), respectively. An increasing number of proteins exhibiting ARF GAP activity have been described (Donaldson 2000). Interestingly, many of these proteins harbor well defined signaling motifs, such as ankyrin repeats, PH, proline-rich and SH3 domains, which may link the ARF cycle to various signal transduction pathways. ARF GEFs, which all share a conserved catalytic domain of approximately 200 amino acids that is homologous to yeast Sec7 protein, fall into two categories based on size and sensitivity to inhibition by brefeldin A (BFA), a toxic fungal metabolite that blocks protein secretion and Golgi function.

1.5 The cytohesin family of ARF GEFs

Members of the cytohesin family of proteins comprise the low molecular weight BFA-insensitive ARF GEF category. The cytohesin family includes cytohesin-1 (also known as B2-1; Liu and Pohajdak 1992; Kolanus, Nagel et al. 1996), ARNO (ARF nucleotide-binding site opener; also known as cytohesin-2; Chardin, Paris et al. 1996) GRP1 (also known as ARNO3; Klarlund, Guilherme et al. 1997), and cytohesin-4 (Ogasawara, Kim et al. 2000). The cytohesins share a common domain structure consisting of an amino-terminal coiled-coil domain, followed by a Sec7 domain that is responsible for GEF activity, a pleckstrin homology (PH) domain that binds phosphoinositides and aids in membrane recruitment, and a polybasic region at the carboxyl terminus. Several laboratories have demonstrated a phosphatidylinositol 3-kinase-dependent translocation of cytohesins to the plasma membrane of cells (Clodi, Vollenweider et al. 1998; Nagel, Zeitlmann et al. 1998; Venkateswarlu, Gunn-Moore et al. 1998; Venkateswarlu, Oatey et al. 1998; Langille, Patki et al. 1999; Venkateswarlu, Gunn-Moore et al. 1999; Klarlund, Holik et al. 2001). However, the cellular consequences of 3'-phosphoinositide generation as it relates to the ARF cycle are relatively unexplored.

Early studies that demonstrated cytohesins exhibited GEF activity on ARF1 (Paris, Beraud-Dufour et al. 1997), coupled with studies in transfected cells which

showed that overexpression of ARNO and ARNO3 (GRP1) resulted in a disassembly of the Golgi complex (Franco, Boretto et al. 1998; Monier, Chardin et al. 1998), led to the conclusion that cytohesins function on class I ARFs. In support of these findings, a Golgi targeting sequence was identified in the coiled-coil domain of the cytohesins (Lee and Pohajdak 2000). In addition to their possible role in regulating ARF1 function, recent studies have shown that cytohesins are able to activate ARF6 *in vitro* (Frank, Upender et al. 1998; Knorr, Nagel et al. 2000), in cultured cells (Langille, Patki et al. 1999), and colocalize with ARF6 in plasma membrane ruffles (Frank, Upender et al. 1998; Langille, Patki et al. 1999). While it has been demonstrated that cytohesins display GEF activity on a variety of ARF subtypes, these results suggest that the *in vivo* specificity of cytohesins with regard to ARF subtypes is likely mediated by the cellular localization of the cytohesin.

1.6 ARF6 regulation of plasma membrane recycling pathways

Overexpression analyses of GTP binding-defective (dominant negative, GDP-bound) and GTPase-deficient (constitutively active, GTP-bound) ARF6 mutants in cells have greatly contributed towards the functional elucidation of the ARF6 cycle (D'Souza-Schorey, Li et al. 1995). D'Souza-Schorey and coworkers discovered a role for ARF6 in receptor mediated endocytosis and plasma membrane recycling by

analyzing transferrin receptor recycling in TRVb-1 cells expressing ARF6 mutants (D'Souza-Schorey, Li et al. 1995). Endocytic sorting and recycling pathways play fundamental roles in the uptake of ligands at the cell surface and attenuation of extracellular ligand-mediated signal transduction pathways (Waterman and Yarden 2001). It was shown that in cells expressing a GTPase deficient mutant of ARF6, ARF6 Q67L, transferrin receptors were localized exclusively to the plasma membrane along with ARF6 Q67L and cells exhibited increased transferrin cell surface binding (D'Souza-Schorey, Li et al. 1995). In contrast, cells expressing a GTP-binding deficient mutant of ARF6, ARF6 T27N, exhibited a block in plasma membrane recycling as transferrin receptors were internalized on endosomes with the ARF6 mutant, leading to a reduction in transferrin cell surface-binding (D'Souza-Schorey, Li et al. 1995). These results have led to the hypothesis that nucleotide exchange on ARF6 occurs on recycling endosomes, resulting in translocation and fusion of the endosomes with the plasma membrane (Fig. 1.5; D'Souza-Schorey, Li et al. 1995; Peters, Hsu et al. 1995; D'Souza-Schorey, van Donselaar et al. 1998). To complete the cycle, GTP hydrolysis of ARF6 at the plasma membrane appears to be required for ARF6 to become internalized and localize in the endosomal compartment (Fig. 1.5).

Despite evidence suggesting that nucleotide exchange of ARF6 occurs on endosomes, there are limited studies linking the cytohesins to ARF6-mediated

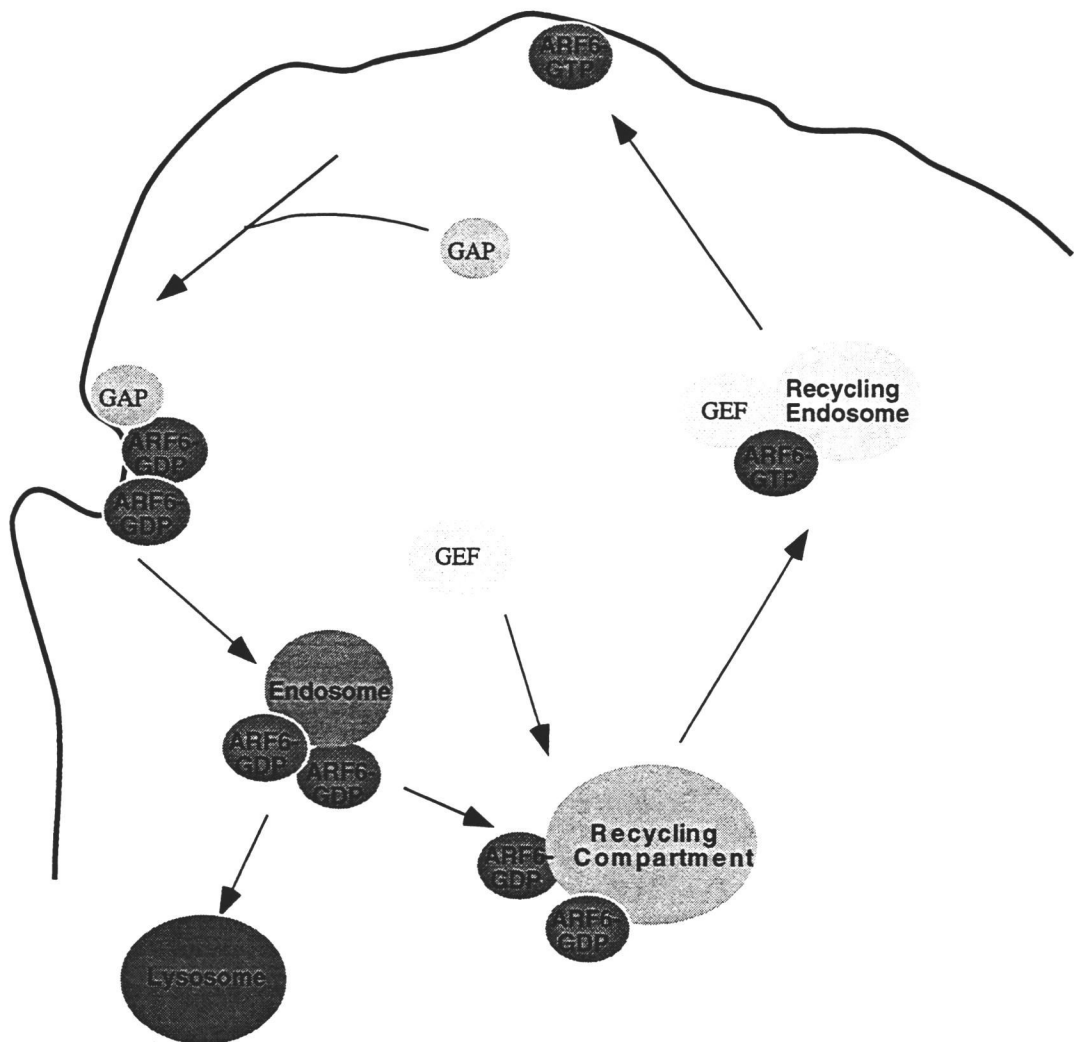


Figure 1.5. Regulation of plasma membrane recycling by ARF6. GTPase activating protein (GAP)-catalyzed hydrolysis of ARF6-GTP results in endocytosis and localization of ARF6-GDP on the endosome. The endosomes are next sorted and components that are destined to recycle back to the plasma membrane may localize in a juxtanuclear recycling compartment. In contrast, endosomal components targeted for degradation are shuttled to the lysosome. Recruitment of an ARF6 guanine nucleotide exchange factor (GEF) to the endosomal compartment results in exchange of the bound GDP for GTP on ARF6 and recycling of the endosome to the plasma membrane.

endosomal recycling. Franco and coworkers demonstrated that overexpression of the ARF6 GEF, EFA6, in TRVb-1 cells resulted in a redistribution of transferrin receptors from intracellular vesicles to the plasma membrane (Franco, Peters et al. 1999), whereas overexpression of ARNO3, the human homolog of GRP1, had no effect on the distribution of transferrin receptors. Additionally, Maranda et al have shown that ARNO and ARF6 are recruited to endosomes in kidney proximal tubule endothelial cells in a pH-dependent manner (Maranda, Brown et al. 2001), suggesting that ARNO may stimulate nucleotide exchange and recycling of the endosome to the plasma membrane.

1.7 Research objectives

The goal of the studies described herein was to contribute towards a greater appreciation of the molecular mechanisms of RA in exerting such profound effects on cellular function. Towards that end, molecular techniques were employed to identify and subsequently characterize a novel RA-induced gene from P19 embryonal carcinoma cells.

Chapter 2 describes the isolation of a novel RA-induced gene, referred to as GRASP, from P19 embryonal carcinoma cells. Experiments were conducted to examine the expression profile of GRASP during RA-induced differentiation of P19

cells, as well as the distribution of GRASP in the adult mouse. Yeast two-hybrid screening was also performed with the aim of identifying interaction partners of GRASP. Preliminary studies were conducted examining the role of GRASP in relation to one of its newly identified interaction partners, the general receptor for phosphoinositides 1 (GRP1).

Chapter 3 describes more detailed studies identifying the role of GRASP in plasma membrane recycling pathways regulated by ARF6 and GRP1. Specifically, the effect of GRASP overexpression on this pathway was examined. Deletion analyses were carried out to identify the regions in GRASP and GRP1 responsible for regulation of the plasma membrane recycling pathway.

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Chapter 2

Interaction of GRASP, a Protein Encoded by a Novel Retinoic Acid-Induced Gene, with Members of the Cytohesin Family of Guanine Nucleotide Exchange Factors

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2.1 Abstract

A novel, retinoic acid (RA)-induced gene, GRP1-Associated Scaffold Protein (GRASP), was isolated from P19 embryonal carcinoma cells using a subtractive screening strategy. GRASP was found to be highly expressed in brain and exhibited lower levels of expression in lung, heart, embryo, kidney and ovary. The predicted amino acid sequence of GRASP is characterized by several putative protein-protein interaction motifs suggesting that GRASP may be a component of a larger protein complex in the cell. Although GRASP does not harbor a predicted membrane spanning domain(s), the protein was observed to be associated with the plasma membrane of transiently transfected mammalian cells. Yeast two-hybrid screening revealed that GRASP interacted strongly with the General Receptor for Phosphoinositides 1 (GRP1), a brefeldin A-insensitive guanine nucleotide exchange factor for the ADP-ribosylation factor (ARF) family of proteins. GRASP•GRP1 interactions were also demonstrated in vitro and in mammalian cells in which GRASP was shown to enhance GRP1 association with the plasma membrane. Furthermore, GRASP colocalized with endogenous ARFs at the plasma membrane in transfected cells suggesting that GRASP may modulate signaling by this family of small GTPases.

2.2 Introduction

Retinoic acid (RA) exerts important effects in the processes of vertebrate development, cellular growth and differentiation, and homeostasis (reviewed in Gudas 1994). Cellular effects of RA are mediated by two families of nuclear receptors, retinoic acid (RAR) and retinoid X (RXR) receptors, both of which are members of the steroid/thyroid hormone receptor superfamily of ligand-dependent transcription factors (reviewed in (Chambon 1996). Retinoid signals induce the expression of a wide array of target genes implicated in mediating the pleiotropic effects of RA on cellular function. These genes encode transcription factors (LaRosa and Gudas 1988; Song and Siu 1989; Kondo, Takahashi et al. 1992; Zwartkruis, Kruyt et al. 1993; Bouillet, Chazaud et al. 1995; Boudjelal, Taneja et al. 1997; Oulad-Abdelghani, Chazaud et al. 1997), metabolic enzymes (White, Guo et al. 1996), growth factor receptors (Kobayashi, Kurihara et al. 1994), extracellular matrix proteins (Gudas and Wang 1986; Vasios, Gold et al. 1989), and secreted proteins (Smolich and Papkoff 1994; Bouillet, Oulad-Abdelghani et al. 1996; Oulad-Abdelghani, Chazaud et al. 1998) postulated to convey positional information in the developing embryo.

P19 embryonal carcinoma (EC) cells (McBurney and Rogers 1982) have been widely used as a model of RA-induction of cellular differentiation as these cells have the capacity to differentiate into all three primitive germ layers under the appropriate cell culture conditions and inducers (McBurney 1993). When grown in a monolayer

and treated with all-trans-retinoic acid (T-RA), P19 cells undergo differentiation along endodermal and mesodermal pathways (Roguska and Gudas 1985). In contrast, T-RA has a biphasic effect on P19 cells grown in aggregates. When treated with low (1-10 nM) concentrations of T-RA, P19 cell aggregates differentiate primarily via the endodermal pathway while high concentrations (>300 nM) of this retinoid induce differentiation into neurons, glial, and fibroblast-like cells (Jones-Villeneuve, McBurney et al. 1982). Additionally, P19 cell aggregates treated with DMSO (0.5-1.0%) differentiate into skeletal and cardiac muscle cells (McBurney, Jones-Villeneuve et al. 1982).

In contrast to the retinoid signals, which are generally anti-proliferative and promote cellular differentiation, signaling by members of the Ras superfamily of small guanine nucleotide binding (G) proteins typically promote cellular proliferation, alteration in cellular morphology, or changes in vesicular trafficking pathways (Moss and Vaughan 1998). ARF1 through ARF6 proteins are members of the Ras superfamily that are implicated in the control of various vesicular trafficking pathways (Moss and Vaughan 1998) and may also be involved in cytoskeletal reorganization and morphological phenotypy (Radhakrishna, Klausner et al. 1996; D'Souza-Schorey, Boshans et al. 1997). Activation of ARF proteins occurs as a result of GDP/GTP exchange, a reaction catalyzed by guanine nucleotide exchange factors (GEFs). ARF

GEFs fall into two broad categories, defined primarily by molecular mass and sensitivity to inhibition by brefeldin A (BFA; (Moss and Vaughan 1998), a toxic fungal metabolite that inhibits ARF nucleotide exchange (Donaldson, Finazzi et al. 1992). Cytohesin-1 (also known as B2-1; (Liu and Pohajdak 1992; Kolanus, Nagel et al. 1996), ARNO (ARF nucleotide-binding-site opener; also known as cytohesin-2; (Chardin, Paris et al. 1996), and GRP1 (Klarlund, Guilherme et al. 1997) represent a recently identified subfamily of ARF GEFs that are insensitive to the effects of BFA. These GEF proteins share a common domain organization and exhibit a high degree of overall sequence similarity (80-90% amino acid identity). The catalytic activity of these GEFs is greatly enhanced by binding of phosphoinositides (Chardin, Paris et al. 1996; Meacci, Tsai et al. 1997; Klarlund, Rameh et al. 1998; Pacheco-Rodriguez, Meacci et al. 1998), which is thought to induce GEF translocation to membranes (Paris, Beraud-Dufour et al. 1997; Klarlund, Rameh et al. 1998) thereby facilitating subsequent interactions with ARFs.

Although the cellular functions of the cytohesin subfamily members are not well understood, conflicting data point to two functional consequences of activation of ARFs by these BFA-insensitive GEFs. Because it was initially shown that these GEFs were able to catalyze GDP/GTP exchange on ARF1, which is known to be involved in ER-Golgi and intra-Golgi transport (Donaldson, Cassel et al. 1992; Robinson and Kreis

1992), attempts were made to analyze the cellular consequences of overexpression of these proteins on the function of the Golgi apparatus. Monier and coworkers (Monier, Chardin et al. 1998) have shown that overexpression of ARNO (cytohesin-2) leads to a disassembly of the Golgi complex in transfected HeLa cells, a phenomenon that has also been observed upon treatment of cells with BFA (Donaldson, Cassel et al. 1992). Similar effects have been observed in BHK-21 cells upon overexpression of ARNO3, the human homologue of GRP1 (Franco, Boretto et al. 1998). However, endogenous ARNO does not cofractionate with Golgi membranes, but rather does so with plasma membrane markers (Frank, Upender et al. 1998). Furthermore, it has been demonstrated that ARNO colocalizes at the cell periphery with ARF6 in transfected cells (Frank, Upender et al. 1998). In a later study, ARNO was demonstrated to function as a GEF for ARF6 in transfected HeLa cells as evidenced by its ability to induce remodeling of the cortical actin cytoskeleton (Frank, Hatfield et al. 1998), a known function of ARF6 (Radhakrishna, Klausner et al. 1996). Similar results implicating GRP1 as a GEF for ARF6 were obtained by other laboratories (Clodi, Vollenweider et al. 1998; Langille, Patki et al. 1999).

With the aim of identifying potential target genes that mediate the effects of tRA on induction of cellular differentiation, we isolated cDNAs corresponding to differentially expressed mRNAs from P19 cells treated with tRA. We report herein the

cloning, expression, and characterization of a novel gene that is induced by tRA in P19 cells. GRASP is strongly upregulated by tRA in P19 cells and the protein encoded by this gene displays a plasma membrane localization. GRP1 was isolated as a GRASP interaction partner and GRASP was shown to colocalize with both GRP1 and ARFs at the plasma membrane of transfected HEK293 cells. Coexpression of GRASP and GRP1 resulted in an increased partitioning of GRP1 into the particulate fraction of cells suggesting that the two proteins are part of a stable complex at the plasma membrane. The presence of putative protein-protein interaction motifs within GRASP is highly suggestive that GRASP may function as an adaptor or scaffolding protein. We propose that GRASP may aid in the localization of GRP1 to discrete signaling networks and facilitate stimulation of ARF mediated events at the plasma membrane.

2.3 Materials and Methods

2.3.1 Cell Culture and Transfections

P19 cells were cultured and treated with tRA essentially as described (Berg and McBurney 1990). Human embryonic kidney (HEK) 293 cells were maintained and transfected as described previously (Avram, Ishmael et al. 1999).

2.3.2 Plasmid Constructs

Vectors for the yeast two hybrid system (pBTM116 and pASV; Le Douarin, Pierrat et al. 1995), the yeast reporter strain L40 (Le Douarin, Pierrat et al. 1995), and the random primed mouse embryo cDNA library inserted into the VP16 activation domain (AD)-encoded pASV3 vector (Le Douarin, Pierrat et al. 1995) were all kind gifts from Drs. R. Losson and P. Chambon (IGBMC, Illkirch, France). The GRP1 construct was kindly provided by Drs. Michael Czech and Jes Klarlund (Klarlund, Guilherme et al. 1997). All constructs were prepared using standard techniques. Supplemental details are available upon request.

2.3.3 Subtractive Hybridization and cDNA Cloning of GRASP

Subtractive hybridization was performed as described (Bouillet, Oulad-Abdelghani et al. 1995). Full-length GRASP was isolated from a mouse embryo cDNA library (10.5 days post-coitum) that was kindly provided by Dr. P. Chambon and J.-M. Garnier.

2.4.9 Isolation of RNA and Northern Blot Analysis

Total RNA was isolated using TRI-REAGENT (Molecular Research Center) according to the manufacturer's protocol. Poly (A)⁺ RNA was isolated by two rounds of purification on oligo(dT) cellulose (Pharmacia Biotech). For northern analyses, 5-10

µg of poly (A)⁺ RNA were separated on a 1% agarose-formaldehyde gel, transferred to a ZetaProbe (Bio-Rad) membrane and probed with α[³²P]dCTP-labeled cDNA fragments. Multiple exposures of the blots were used for quantitative densitometric scanning using the MCID Imaging software as previously described (Leid 1994).

2.3.5 Yeast Two-Hybrid Screening and β-galactosidase Assays

Yeast two-hybrid screening and β-galactosidase assays were performed essentially as described (Dowell, Ishmael et al. 1997) except that the *Sacchromyces cerevisiae* L40 reporter strain contained the bait plasmid pBTM116/GRASP 180-257.

2.3.6 GST Pull Down Experiments

GST fusion proteins were produced, crude bacterial lysates were prepared and GST pull down experiments were conducted as previously described (Dowell, Ishmael et al. 1997).

2.3.7 Reverse Transcription-Polymerase Chain Reaction Assays

RT-PCR and Southern analysis were performed as described (Avram, Ishmael et al. 1999) using appropriate primers and probes. Detailed information is available upon request.

2.3.8 Production of GRASP Polyclonal Antibodies

Antisera was raised in the goat against purified GST-GRASP by Bethyl Laboratories (Montgomery, TX). The anti-GRASP antisera was affinity purified by passing serum from the immunized goat multiple times over a GST immunosorbent to absorb anti-GST antibodies. The serum was then passed over a GST-GRASP immunosorbent to capture antibodies specific for GRASP and this column was eluted at low pH. The extent of anti-GST contamination in the affinity purified anti-GRASP antisera was found to be less than 5%.

2.3.9 Indirect Immunofluorescence and Confocal Microscopy

Twenty-four hours following transfection, HEK293 cells growing on coverslips were fixed in 4% paraformaldehyde and permeabilized in two changes of PBS + 0.02% Triton-X-100. Antibody incubations were performed using standard techniques with the anti-Myc monoclonal antibody (Invitrogen), anti-GRASP polyclonal antibody, or the monoclonal antibody ID9 (kindly provided by Dr. Richard Kahn), which has been reported to recognize all human ARF proteins (Cavenagh, Whitney et al. 1996). Myc-GRASP immune complexes were detected using fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse antibodies (Southern Biotechnology Associates Inc.). FITC-conjugated rabbit anti-goat and TRITC-conjugated rabbit anti-mouse were used as secondary antibodies for the

detection of anti-GRASP and ID9, respectively. Images were captured on a Leica inverted confocal microscope model TCS4D and processed using Photoshop 5.0 (Adobe Systems, Inc.)

2.3.10 Preparation of HEK293 Extracts and Immunoblotting

Cytoplasmic and membrane extracts of HEK293 cell extracts were prepared essentially as described (Hansen and Casanova 1994). Twenty micrograms of protein from the various extracts were subjected to immunoblot analysis as described previously (Leid 1994) using anti-Myc (Invitrogen) or anti-GFP (Clontech) monoclonal antibodies. For coimmunoprecipitation analysis, HEK293 cells were collected in solubilization buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 1mM EDTA, 1 mM DTT, 10% (v/v) glycerol, 0.1 mM PMSF, 10 μ M pepstatin A, 10 μ M leupeptin, 25 μ g/ml aprotinin, 0.05% (v/v) NP-40 and 0.5% (v/v) Triton-X-100) and incubated at 4°C with rotation for 30 min. The lysate was cleared by centrifugation at 16,000 x g for 20 min. at 4°C. Three hundred micrograms of lysate protein was incubated with the anti-Myc antibody in a volume of 1.5 mL for 30 min. at 4°C, after which a rabbit anti-mouse secondary antibody (Southern Biotechnology Associates, Inc) was added and the incubation continued for an additional 30 min. Protein A-Sepharose (100 μ L, 50 mg/ml in solubilization buffer; Pharmacia) was then added and the incubation was continued at

in solubilization buffer; Pharmacia) was then added and the incubation was continued at 4°C with rotation for an additional 30 min. The beads were collected by centrifugation (800 x g for 1 min. at 4°C) and subsequently washed gently six times with 1.0 mL solubilization buffer. Immunoprecipitates were subjected to immunoblot analysis as described above. Various exposures of the blots were used for quantitative densitometric scanning as previously described (Leid 1994).

2.4 Results

2.4.1 Cloning of GRASP, a tRA-induced gene

Subtractive hybridization was used to identify tRA-induced genes in P19 EC cells grown in monolayers. A 2.1 kb transcript, referred to hereafter as GRASP, was shown to be induced by tRA in a dose-dependent manner by northern analysis of P19 cells grown in monolayer culture (Fig. 2.1A, middle panel). As a control, the blot was also probed for RAR β , a gene known to be strongly induced by RA in P19 cells (Song and Siu 1989) and for 36B4, which encodes the acidic ribosomal phosphoprotein P0, a highly expressed "housekeeping" gene (Krowczynska, Coutts et al. 1989) that is unresponsive to tRA treatment.

2.4.2 Induction of GRASP in the presence of cycloheximide

In order to determine if induction of GRASP occurred independently of protein synthesis, mRNA was isolated from P19 cells treated with 1.0 μ M tRA in the presence and absence of cycloheximide (10 μ g/ml; (Obrig, Culp et al. 1971), and analyzed by northern blotting. GRASP mRNA levels in P19 cells were increased by cycloheximide treatment (Fig. 2.1B, middle panel, lane 3) and this was further augmented by treatment with tRA (compare lanes 3 and 4 of Fig. 2.1B), suggesting that at least part of the induction of GRASP expression by tRA in P19 cells did not require protein synthesis. Induction of RAR β by tRA was not attenuated by cycloheximide treatment under these conditions (Fig. 2.1B, top panel).

2.4.3 Time course of tRA-induced expression of GRASP in P19 cells

Northern analyses were performed to determine the time course of tRA induction of GRASP in P19 cells grown in monolayer and aggregate cultures, as induction of P19 cell differentiation by tRA under these two conditions results in markedly distinct phenotypes (Jones-Villeneuve, McBurney et al. 1982; Roguska and Gudas 1985). Induction of GRASP was observed within 2 hrs. of initiation of tRA treatment in both monolayer and aggregate cultures (compare lanes 1 and 2 with lanes 6 and 7 of Fig. 2.1C, middle panel). Growth of P19 cells in aggregates resulted in a

higher basal expression of GRASP (2-fold induction) relative to cells grown in monolayer culture (compare lanes 1 and 6 of Fig. 2.1C, middle panel) whereas there was no difference in basal RAR β levels when comparing monolayer and aggregate cultures (lanes 1 and 6, respectively, of Fig. 2.1C, top panel). GRASP transcripts increased gradually following tRA treatment of P19 cells grown in a monolayer, reaching a maximum of 18-fold induction at 24 hrs. (Fig. 2.1C, lanes 1-4, middle panel). In contrast, tRA induction of RAR β expression in P19 cells grown in monolayers reached maximal levels at 8 hrs. (56-fold induction) and further induction of this gene was not observed over the remainder of this experiment (Fig. 2.1C, top panel). GRASP and RAR β exhibited similar induction patterns of tRA inducibility in P19 cells grown in aggregate culture with the corresponding mRNA levels continuing to increase during the entire 48 hrs. of RA treatment (Fig. 2.1C, lanes 6-10). Thus, although RAR β and GRASP both appear to be direct targets of tRA in P19 EC cells, the pattern of inducibility of these two genes is subtly distinct suggesting that additional regulatory mechanisms may exist for one or both genes.

2.4.4 GRASP contains multiple sites for protein-protein interactions

The GRASP fragment isolated by subtractive screening was used to isolate a full-length clone from a randomly primed mouse embryo cDNA library. The 1,978 bp

GRASP clone obtained contained an open reading frame (ORF) encoding a 392 amino acid protein with a predicted molecular mass of 42,382 Da. This ORF is preceded by a single, in-frame stop codon (nucleotides 35-37) and is followed by a polyadenylation-like motif (nucleotides 1944-1949, Fig. 2.2A). In addition, the 3' untranslated region of the GRASP transcript contains an AU-rich cluster (nucleotides 1264-1276) similar to those found in and proposed to account for the very low level expression of many immediate-early genes (Shaw and Kamen 1986; Jones and Cole 1987; Kabnick and Housman 1988; Wilson and Treisman 1988; Shyu, Belasco et al. 1991).

A database search revealed that GRASP is a novel gene that displayed very little overall similarity with known sequences. However, analysis of the predicted amino acid sequence revealed that GRASP contains several known protein-protein interaction motifs (see Figs. 2.2A and B), including: (1) a putative SH3 domain binding site (PXXP) within an alanine-/proline-rich region (Feng, Chen et al. 1994), (2) a PDZ domain (see below and Fig. 2.2B; (Kennedy 1995), and (3) a leucine-rich region predicted to be α -helical in nature (data not shown). Amino acid alignment of the PDZ domain of GRASP with those of various other proteins (Fig. 2.2B) revealed that GRASP exhibited substantial similarity to members of the MAGUK family of proteins (Woods and Bryant 1991; Cho, Hunt et al. 1992; Itoh, Nagafuchi et al. 1993; Kim, Cho

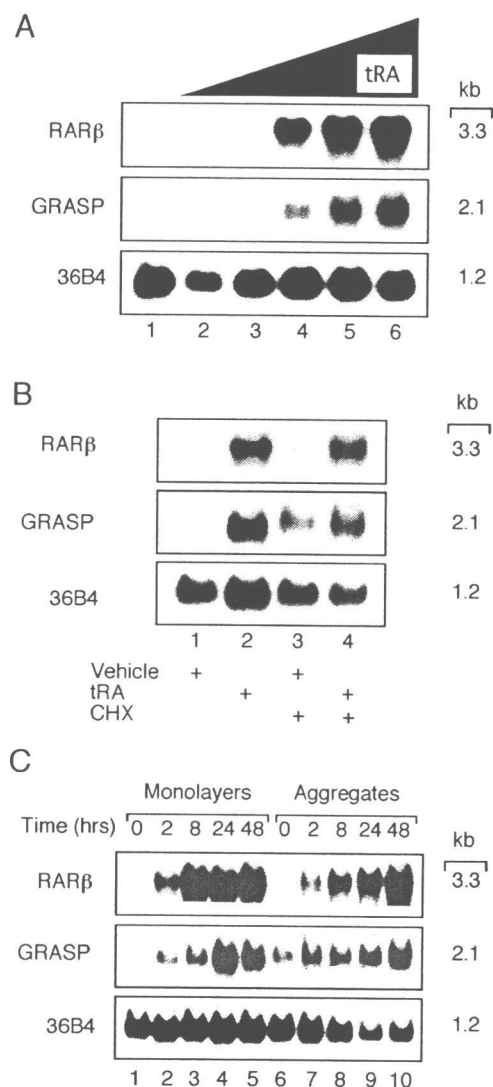


Figure 2.1. Induction of GRASP in P19 cells by tRA. (A) Northern blot of poly (A)+ RNA isolated from P19 EC cells grown in monolayer culture and treated with vehicle (0.1% ethanol; lane 1 of all panels) or increasing concentrations of tRA (1-10,000 nM, in increments of one log unit; lanes 2-6 of all panels) for 24 hrs. (B) Effect of cycloheximide on tRA induction of RAR β and GRASP. P19 cells grown in a monolayer were treated with tRA (1 μ M) and/or cycloheximide (10 μ g/ml) as indicated for 24 hours. Note that treatment of P19 cells with cycloheximide alone resulted in a modest induction of GRASP expression (lane 3) that was further enhanced by tRA treatment (lane 4). (C) Time course of GRASP induction. P19 cells grown in monolayer and aggregate cultures were treated with 1.0 μ M tRA and poly (A)+ RNA was isolated at the various times for analysis. The blots shown in A, B and C are derived from gels containing 5-10 μ g of poly (A)+ RNA per lane and all blots were probed sequentially with 32 P-labeled, randomly primed fragments of GRASP, RAR β , and 36B4.

et al. 1996; Muller, Kistner et al. 1996) and to the regulator of G-protein signaling RGS12 (Snow, Antonio et al. 1997).

2.4.5 Distribution of GRASP in mouse tissues

RT-PCR analyses revealed that GRASP was very highly expressed in brain relative to the other tissues examined, but expression was also detected in lung and heart, and to a lesser extent in embryo (10-12.5 days post coitum), kidney and ovary (Fig. 2.2C). Expression of GRASP was not observed in thymus, spleen, liver or testis.

2.4.6 Subcellular localization of GRASP in HEK293 cells

To study the subcellular localization of GRASP in mammalian cells and sequence determinants thereof, constructs encoding Myc epitope-tagged full-length and deletion mutants of GRASP were used to transfect HEK293 cells transiently and the fusion proteins were visualized by indirect immunofluorescence and confocal microscopy. Although GRASP does not harbor predicted membrane-spanning domains, both Myc-GRASP and Myc-GRASP 1-186 appeared to be localized to the plasma membrane (Figs. 2.3A and B) in addition to some diffuse cytosolic staining. In contrast, Myc-GRASP 189-392, which lacks the amino terminal alanine-/proline-rich region and the PDZ domain, was not localized to the plasma membrane but rather was

associated with large vesicular structures that were distributed diffusely throughout the cytoplasm (Fig. 2.3C). These results suggest that full-length GRASP is associated with the plasma membrane of transiently transfected HEK293 cells and that the amino terminal alanine-/proline-rich region and/or PDZ domain are responsible for this localization.

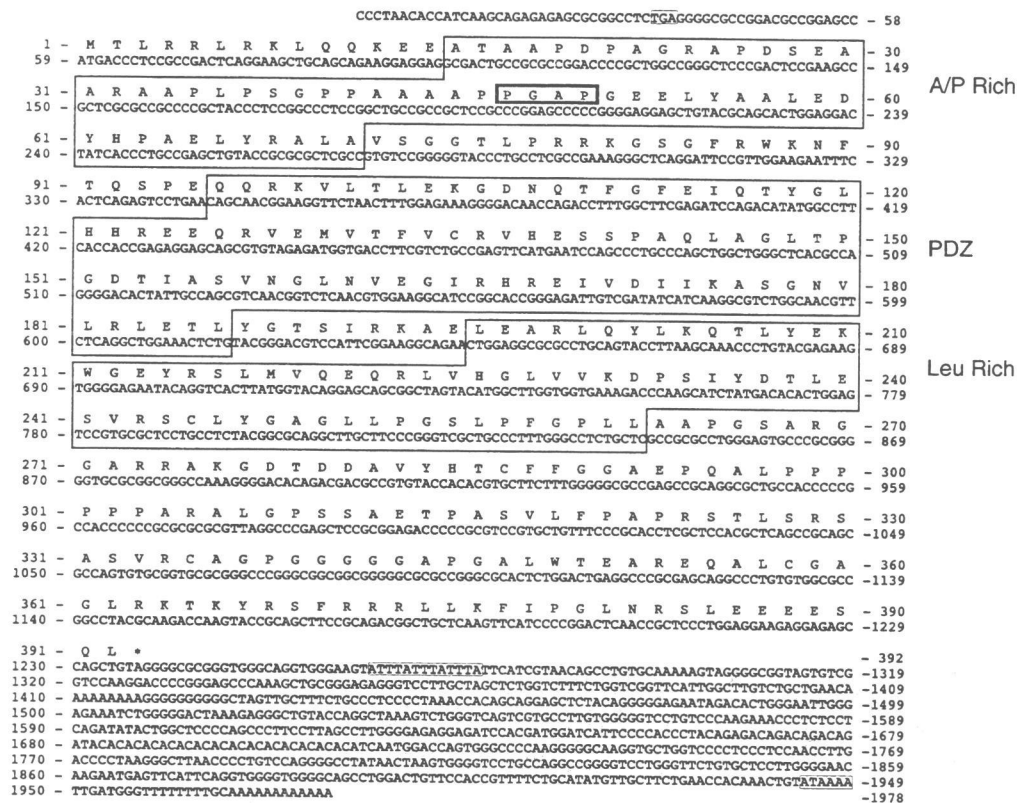
2.4.7 Isolation of GRP1 as an interaction partner of GRASP

The presence of putative protein-protein interaction motifs and the lack of any known catalytic domains suggested that GRASP may function as an adaptor protein. Toward the goal of elucidating the function of this novel protein, we employed a yeast two-hybrid system (Le Douarin, Pierrat et al. 1995; Avram, Ishmael et al. 1999; Dowell, Ishmael et al. 1999) to isolate proteins expressed in the mouse embryo that may interact with GRASP subdomains. From a screen using the leucine-rich region of GRASP (amino acids 180-257) as a bait, a clone was isolated that encoded amino acids 1-156 of GRP1. The leucine rich region of GRASP was observed to interact strongly and specifically with GRP1 1-156 in yeast (Fig. 2.4A).

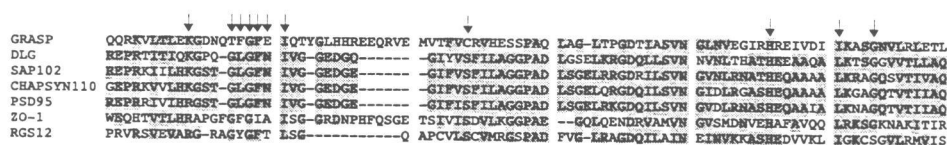
In order to confirm the interactions of GRASP and GRP1 that we observed in yeast, in vitro GST pull-down experiments were performed. GRP1 exhibited a strong interaction with both GST-GRASP and GST-GRASP 1-257 (Figs. 2.4B and C, lanes 3

Figure 2.2. Nucleotide and predicted amino acid sequence and tissue distribution of GRASP transcripts. (A) Nucleotide and predicted protein sequence of GRASP. Numbers in margins correspond to the nucleotide and amino acid positions. The following features are indicated by a box in the sequence: (1) an in-frame stop codon preceding the GRASP open reading frame (nucleotides 35-37), (2) an alanine- and proline-rich region (amino acids 15-71) containing a putative SH3 domain binding site (PGAP; amino acids 47-50), (3) a PDZ domain (amino acids 96-186, see also *B*), (4) a leucine-rich region (amino acids 196-262), (5) an AU-rich sequence (nucleotides 1264-1279), (6) and a polyadenylation signal (nucleotides 1944-1959). (B) Alignment of the PDZ domain of GRASP with PDZ domains from other proteins. Amino acid identities and conserved substitutions are indicated by shaded boxes. Arrows are positioned over residues implicated in peptide binding by some PDZ domain-containing proteins. The sequences are as follows: GRASP, amino acids 96-186; lethal(1)discs-large (DLG), third PDZ domain, amino acids 482-564; SAP102, third PDZ domain, amino acids 400-482; CHAPSYN110, third PDZ domain, amino acids 417-499; PSD95, third PDZ domain, amino acids 309-391; ZO-1, amino acids 19-107; RGS12, amino acids 17-95. This alignment was performed using ClustalW 1.7. (C) Expression of GRASP in the embryo and various adult mouse tissues. RT-PCR and Southern analysis were performed as described in **Materials and Methods**. Shown in *C* is a representative experiment that was replicated three times.

A



B



C

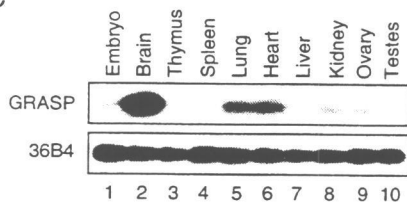


Fig. 2.2

and 4), but not with GST-GRASP 1-186, GST-GRASP 1-82, or GST alone (lanes 5, 6, and 2, respectively). These results demonstrate that the amino acid residues sufficient for interaction with GRP1 are contained within the leucine-rich region of GRASP (residues 180-257) and that full-length GRASP is capable of strong interaction with GRP1 *in vitro*.

2.4.8 Residues in the N-terminal region of GRP1 mediate the interaction with GRASP

In order to determine the region of GRP1 responsible for mediating interaction with GRASP, *in vitro* protein-protein interaction assays were carried out using GST-GRASP and truncation mutants of GRP1 (Fig. 2.5A). Full-length GRP1 (Fig. 2.5B, lane 3) and GRP1 1-95 (lane 9) exhibited a strong interaction with GST-GRASP, but neither protein interacted with GST alone (lanes 2 and 8, respectively). GRP1 1-95 contains a coiled-coil domain (residues 18-59) and a small fragment of the Sec7 domain (residues 72-95; see Fig. 2.5A). In contrast, GRP1 72-399, which lacks the amino terminal coiled-coil domain but contains the entirety of both the Sec7 and PH domains, failed to interact with GST-GRASP (Fig. 2.5B, lane 6), confirming that the latter domains of GRP1 are not required for interaction with GRASP. Because cytohesin-2 is also expressed in P19 cells (see below) and the amino terminal coiled-coil region of GRP1 and cytohesin-2 share considerable identity (see Fig. 2.5C), we next determined

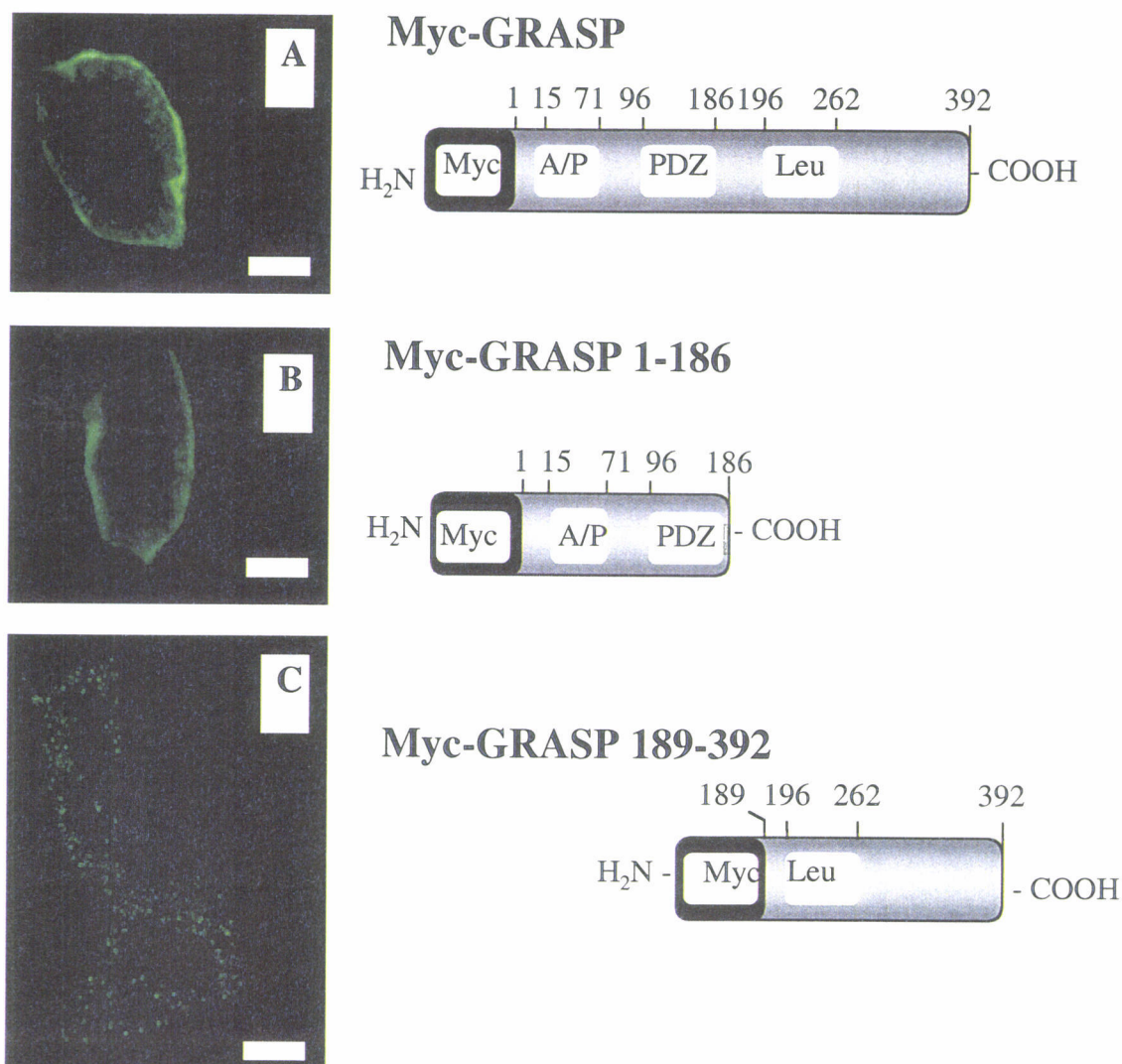


Figure 2.3. Subcellular localization of GRASP protein in transfected HEK293 cells. (A-C) Indirect immunofluorescence of Myc-GRASP and indicated deletion mutants in HEK293 cells examined by confocal microscopy. HEK293 cells were transiently transfected with expression vectors encoding Myc epitope-tagged wild-type and deletion mutants of GRASP and analyzed after 24 hrs. by indirect immunofluorescence and confocal microscopy. Myc-GRASP and the respective deletion mutants were visualized in transfected cells using an anti-Myc monoclonal and FITC-conjugated, goat anti-mouse secondary antibodies. The sizing bar in all panels corresponds to 5 mm. Schematic representations of the Myc-tagged GRASP expression vectors used in these transient transfection experiments are shown to the right of each panel.

if GRASP and cytohesin-2 interact in vitro. Cytohesin-2 1-93 which contains the amino terminal coiled-coil region (see Fig. 2.5C), like GRP1 1-95 exhibited a strong interaction with GST-GRASP, but not GST alone (Fig. 2.5B lanes 12 and 11, respectively). These findings demonstrate that the amino terminal regions of both GRP1 and cytohesin-2 are sufficient to mediate interaction with GRASP.

2.4.9 BFA-insensitive ARF GEFs are differentially expressed in P19 cells

Because GRASP and GRP1 interacted strongly in yeast and in vitro, we next sought to determine if GRP1 and the other closely related members of this subfamily, cytohesin-2 and cytohesin-1, are coexpressed with GRASP during RA-induced differentiation of P19 EC cells, which may facilitate a physiologically relevant interaction. RT-PCR and Southern blotting were employed to determine the relative expression of these transcripts in vehicle- and tRA- (1.0 μ M, 24 hrs.) treated P19 cells. Expression of cytohesin-1 was not detected in either untreated or tRA-treated P19 EC cells whereas cytohesin-2 was highly expressed in both samples (Fig. 2.5D, lanes 1 and 2 and lanes 3 and 4, respectively). GRP1 expression was also detected in P19 cells (Fig. 2.5D, lanes 5 and 6). Neither cytohesin-2 nor GRP1 expression appeared to be responsive to tRA treatment of P19 cells (Fig. 2.5D, lanes 3 and 4 and lanes 5 and 6, respectively).

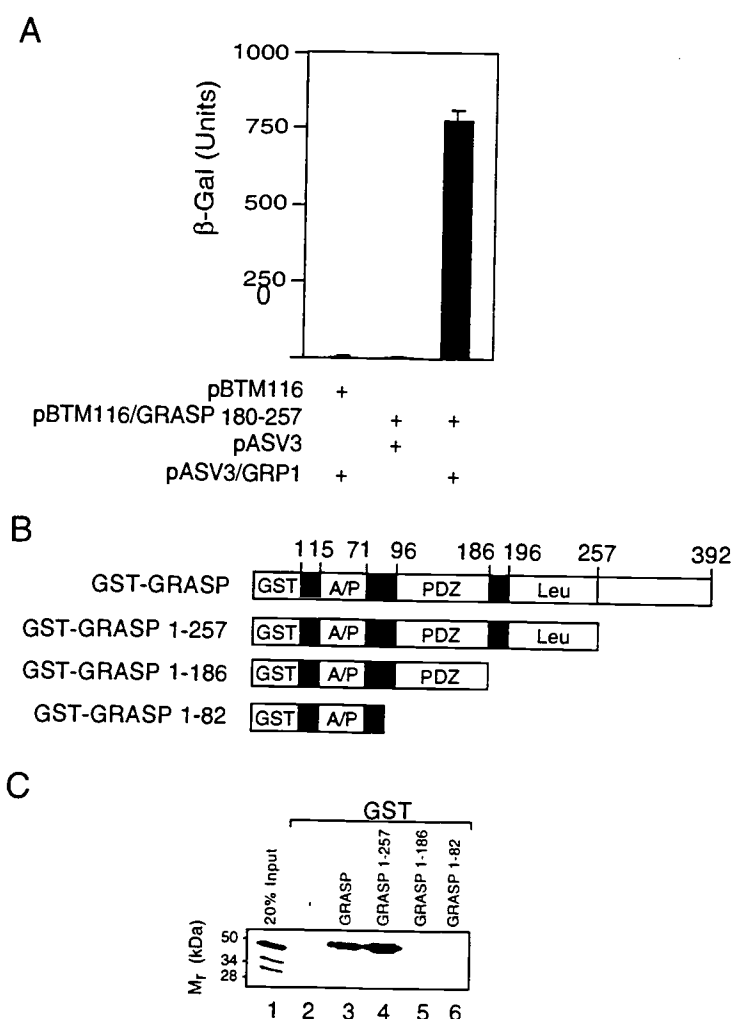


Figure 2.4. Interactions between GRP1 and GRASP. (A) Interactions between GRP1 and GRASP in yeast. A bait consisting of the LexA DBD fused to GRASP 180-257 and a prey consisting of the VP16 activation domain fused to GRP1 1-156 were used to cotransform the L40 strain of *S. cerevisiae*. β -galactosidase assays were performed in liquid yeast culture as described in *Materials and Methods*. Each β -galactosidase value represents the mean \pm standard error of 4-6 independent determinations. (B) Schematic of the GST-GRASP fusion constructs used for *in vitro* protein-protein interaction assays. (C) *In vitro* protein-protein interaction between full-length GRP1 and GRASP truncation mutants. GST, GST-GRASP or the corresponding deletion mutants of GRASP were bound to glutathione sepharose and used as affinity matrices to examine interactions with full-length, [35 S]methionine-labeled GRP1. Shown is a gel from a representative experiment that was replicated three times.

2.4.10 Coimmunoprecipitation of GRASP and GRP1 from HEK293 cell extracts

Coimmunoprecipitation analyses were performed using extracts of HEK293 cells transfected with expression vectors encoding both Myc-GRASP and GFP-GRP1 to determine if GRASP and GRP1 were capable of interaction in a cellular context. Myc-GRASP was immunoprecipitated from whole cell extracts using an anti-Myc monoclonal antibody and immunoprecipitates were then analyzed by immunoblotting using a monoclonal antibody directed against GFP. The anti-Myc antibody coimmunoprecipitated GFP-GRP1 from cells transfected with Myc-GRASP and GFP-GRP1 (Fig. 2.6, lane 6) and coimmunoprecipitation of GFP-GRP1 was dependent on the presence of Myc-GRASP (compare lanes 5 and 6). However, the anti-Myc antibody did not coimmunoprecipitate GFP in control cells expressing both Myc-GRASP and GFP (Fig. 2.6, lane 4). These results clearly demonstrate that GRP1 and GRASP interact in extracts of transiently cotransfected mammalian cells.

2.4.11 GRASP colocalizes with GRP1 at the plasma membrane

As we have shown that GRASP is associated with the plasma membrane of transfected cells (Fig. 2.3A), we employed immunofluorescence and laser-scanning

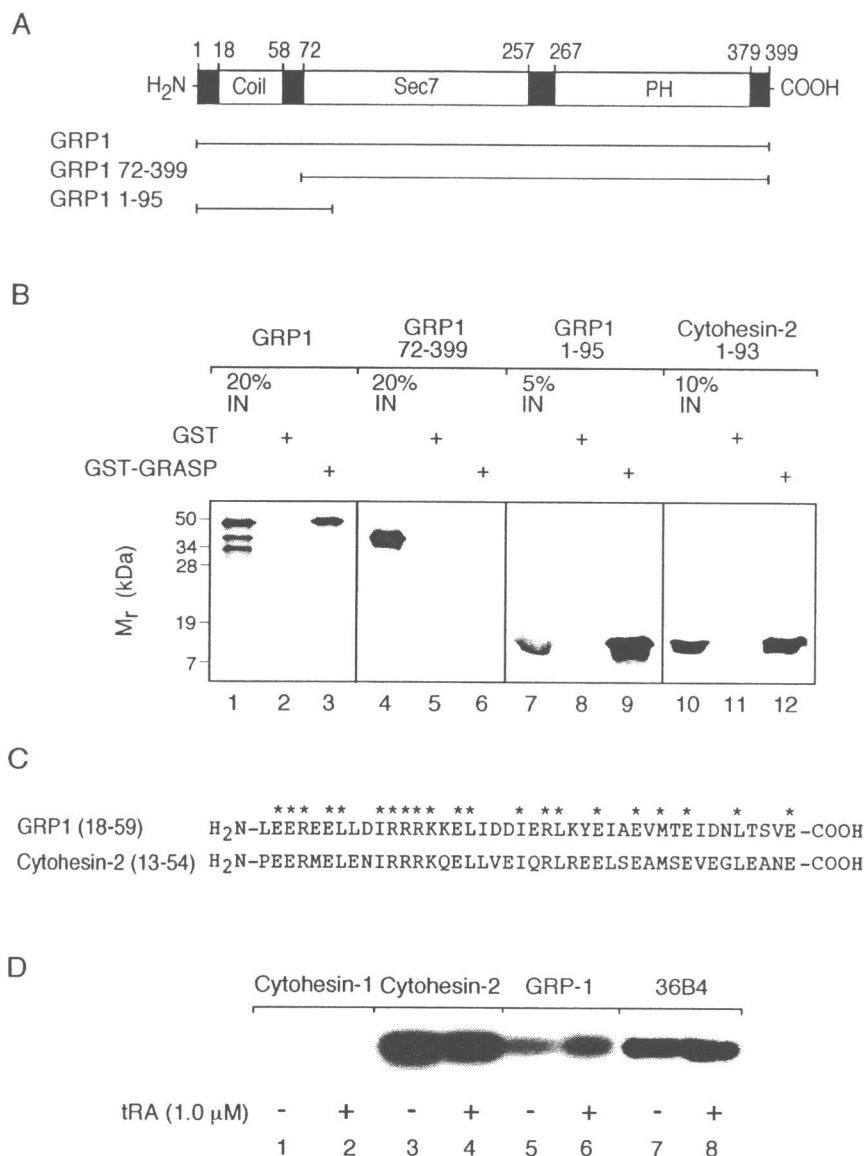


Figure 2.5. Interactions of GRP1 and cytohesin-2 with GRASP and relative expression of BFA-insensitive ARF GEFs during RA-induced differentiation of P19 EC cells. (A) Schematic of the GRP1 truncation mutants. (B) *In vitro* protein-protein interactions between GST-GRASP and GRP1 and cytohesin-2 truncation mutants. (C) Sequence alignment of the coiled-coil regions of GRP1 and cytohesin-2. Asterisks indicate amino acid identities. (D) Relative expression of ARF exchange factors in P19 cells treated with tRA. RT-PCR was performed using 1.0 μg of total RNA isolated from P19 cells grown in a monolayer and treated with 1.0 μM tRA (or 0.1% ethanol) for 24 hrs. The blots were probed with an end-labeled oligonucleotide that exhibited identity to all three BFA-insensitive ARF GEFs. Shown in B and D are representative experiments that were replicated four and three times, respectively.

confocal microscopy to determine if GRASP colocalizes at the cell periphery with GFP-tagged GRP1. In the absence of GFP-GRP1 expression, Myc-GRASP was detected at the cell periphery and plasma membrane of HEK293 cells (Fig. 2.7A; see also Fig. 2.3A). Consistent with these data, Myc-GRASP was found to be stably associated with the membrane fraction of transiently transfected HEK293 cells as detected by immunoblot analysis (data not shown).

In the absence of Myc-GRASP expression, GFP-GRP1 displayed a diffuse cellular staining that included substantial plasma membrane localization as detected by confocal microscopy (Fig. 2.7B). However, GFP-GRP1 did not appear to be stably associated with the membrane fraction as detected by immunoblot analysis of cell fractions prepared from these cells (Fig. 2.7F). For example, the ratio of GFP-GRP1 immunoreactivity present in the cytosolic versus the membrane fraction was approximately 10:1 (Fig. 2.7F, lanes 9-11). This finding, when considered with the results of confocal microscopy, suggests that GRP1 may be loosely associated with the plasma membrane in the absence of Myc-GRASP expression.

Coexpression of Myc-GRASP and GFP-GRP1 and staining with the corresponding antibodies revealed a precise overlap in the localization of the two proteins at the plasma membrane of HEK 293 cells (Figs. 2.7C and D, respectively; see also overlay, Fig. 2.7E). Moreover, coexpression of Myc-GRASP appeared to stabilize

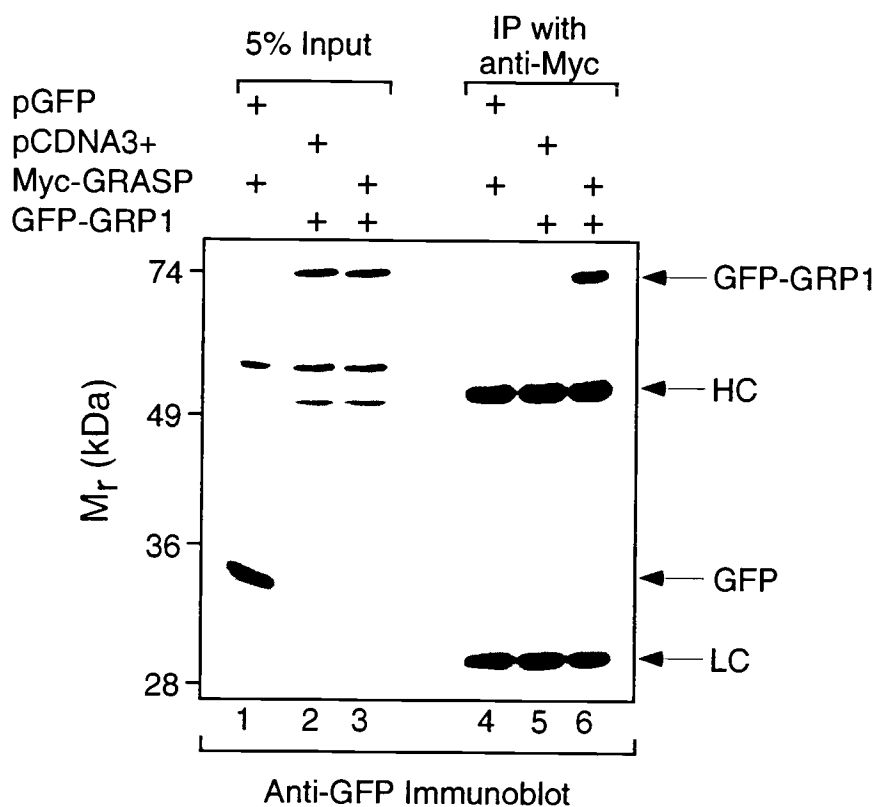


Figure 2.6. Coimmunoprecipitation of GFP-GRP1 and Myc-GRASP from mammalian cell extracts. Whole cell lysates from HEK 293 cells transfected with the indicated expression vectors were immunoprecipitated using an anti-Myc monoclonal antibody. Immunoprecipitates were then subjected to immunoblot analysis using an antibody directed against GFP. Shown is a gel from a representative experiment that was replicated twice. The immunoglobulin heavy (HC) and light (LC) chains are indicated.

association of GFP-GRP1 with plasma membrane loci as indicated by immunoblotting. In contrast to results obtained when GFP-GRP1 was expressed with increasing amounts of an empty expression vector (Fig. 2.7F lanes 9-11; see also above), cotransfection with increasing amounts of a Myc-GRASP altered the subcellular distribution of GFP-GRP1 such that the cytoplasmic:membrane ratio of GFP-GRP1 immunoreactivity approached or exceeded unity (Fig. 2.7F, lanes 12 and 13). The distribution of GFP was unaffected by cotransfection with a Myc-GRASP expression vector (Fig. 2.7F, compare lanes 4-6 with 7 and 8). Collectively, these results demonstrate an increased association of GRP1 with plasma membrane loci in the presence of Myc-GRASP. These findings also lend further support to the hypothesis that GRASP•GRP1 complexes exist in intact, transfected cells.

2.4.12 GRASP colocalizes with ARFs at the plasma membrane

It is hypothesized that recruitment of GRP1 to the plasma membrane leads to its association with and subsequent activation of ARF proteins (Klarlund, Rameh et al. 1998; Langille, Patki et al. 1999). To determine if GRASP localizes to cellular ARF loci, HEK293 cells were transfected with Myc-GRASP and double-labeled with affinity-purified, anti-GRASP antiserum and a monoclonal antibody that recognizes all human ARF subtypes (ID9; kindly provided by Dr. Richard Kahn; Cavenagh, Whitney

et al. 1996). In agreement with previous results, Myc-GRASP was found to associate with the plasma membrane of transfected HEK293 cells and, to a lesser extent, was diffusely distributed in the cytoplasm (Figs. 2.8B, E and H). Endogenous ARFs appeared to be distributed mainly in the cytosol, however, staining was also apparent at the plasma membrane (Figs. 2.8A, D and G). An overlay of the GRASP and ARF confocal images reveals a precise colocalization of the two proteins at the plasma membrane (Figs. 2.8C, F, and I). Interestingly, colocalization of ARFs and GRASP was also observed in various surface protrusions of the plasma membrane (Figs. 2.8I), the formation of which have been observed upon activation of ARF6 (Radhakrishna, Klausner et al. 1996). These results suggest that GRASP colocalizes with endogenous ARFs and lends support to the hypothesis that GRASP may influence ARF signaling.

2.5 Discussion

We report the molecular cloning of GRASP, a novel gene that is upregulated in P19 EC cells treated with tRA. Induction of GRASP by tRA in P19 cells was not completely inhibited by cycloheximide, suggesting that this gene may be a direct target of tRA. However, induction of GRASP by tRA differed qualitatively and quantitatively in P19 cells grown as monolayers versus aggregates suggesting that GRASP may be subject to additional regulatory controls. GRASP is selectively expressed in a number

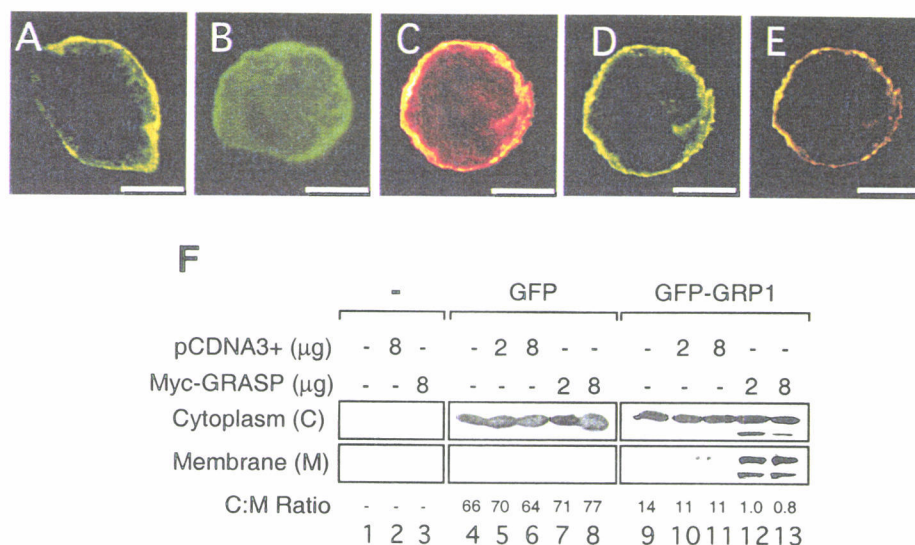


Figure 2.7. Colocalization of GRASP and GRP1 at the plasma membrane. HEK293 cells were transiently transfected with expression vectors encoding Myc-GRASP and GFP-GRP1. The corresponding proteins were localized 24 hrs. after transfection by indirect immunofluorescence (Myc-GRASP) and fluorescence (GFP-GRP1) confocal microscopy. Transfected cells were stained with an anti-Myc monoclonal (A and C) and FITC- or TRITC-conjugated goat anti-mouse secondary antibodies (panels A and C, respectively). (A) HEK293 cells expressing Myc-GRASP and stained with the anti-Myc antibody. (B) HEK293 cells expressing GFP-GRP1 and examined by fluorescence confocal microscopy. (C) Cells expressing both Myc-GRASP and GFP-GRP1 stained with the anti-Myc monoclonal and TRITC-conjugated goat anti-mouse secondary antibodies detecting only Myc-GRASP. (D) HEK293 cells expressing both Myc-GRASP and GFP-GRP1 and examined by fluorescence confocal microscopy detecting only GFP-GRP1. (E) An overlap of the fluorescence from Figs. C and D. Note that only areas of coincident localization are shown in E (yellow). The sizing bar corresponds to 5 μm. The images shown in A – D are from a single transfection experiment that was replicated 4-6 times. (F) Increased association of GRP1 with the membrane fraction of cells in the presence of GRASP. HEK293 cells were cotransfected with either 1.0 μg of GFP or GFP-GRP1 expression vectors along with the indicated amounts of either a Myc-GRASP expression vector or an empty vector encoding the Myc epitope (pCDNA3+). Cells were harvested for extract preparation 24 hrs after transfection. Twenty micrograms of protein derived from the indicated cell extracts were analyzed by immunoblotting using the anti-GFP antibody. Expression of Myc-GRASP was confirmed by immunoblotting (data not shown). Cytoplasmic to membrane (C:M) ratios were generated by densitometric scanning as described in *Materials and Methods*. Shown is a representative experiment that was replicated three times.

of mouse tissues with the highest level of expression observed in brain, heart and lung. GRASP transcripts were also detected in 10-12.5 day mouse embryos.

Analysis of the coding sequence revealed that GRASP harbors multiple sites for protein-protein interactions, which raises the possibility that GRASP may function as an adaptor or scaffolding protein. A search of the protein databases revealed that GRASP displayed partial sequence similarity with clone B3-1, a gene that was originally isolated from a Natural Killer (NK)/T cell subtracted library (Dixon, Sahely et al. 1993). The regions of similarity include the PDZ domain, the leucine rich region, and residues within the carboxyl terminal region (data not shown). Interestingly, B2-1 (Liu and Pohajdak 1992), later referred to as cytohesin-1 (Kolanus, Nagel et al. 1996), was cloned from the same subtracted NK/T cell library which, in light of the results presented herein, raises the possibility that B3-1 and B2-1 may interact and coordinate some NK cell functions. The function of B3-1 remains unknown, although Dixon and colleagues (Dixon, Sahely et al. 1993) speculated that B3-1 may be a transcription factor because of the presence of a leucine zipper domain and prevalence of negatively charged residues. However, the results of indirect immunofluorescence and confocal microscopy studies reported herein demonstrated that Myc-GRASP was excluded from the nucleus and appeared to be localized at the plasma membrane. Because there are no predicted membrane spanning domains within its sequence, it seems likely that GRASP

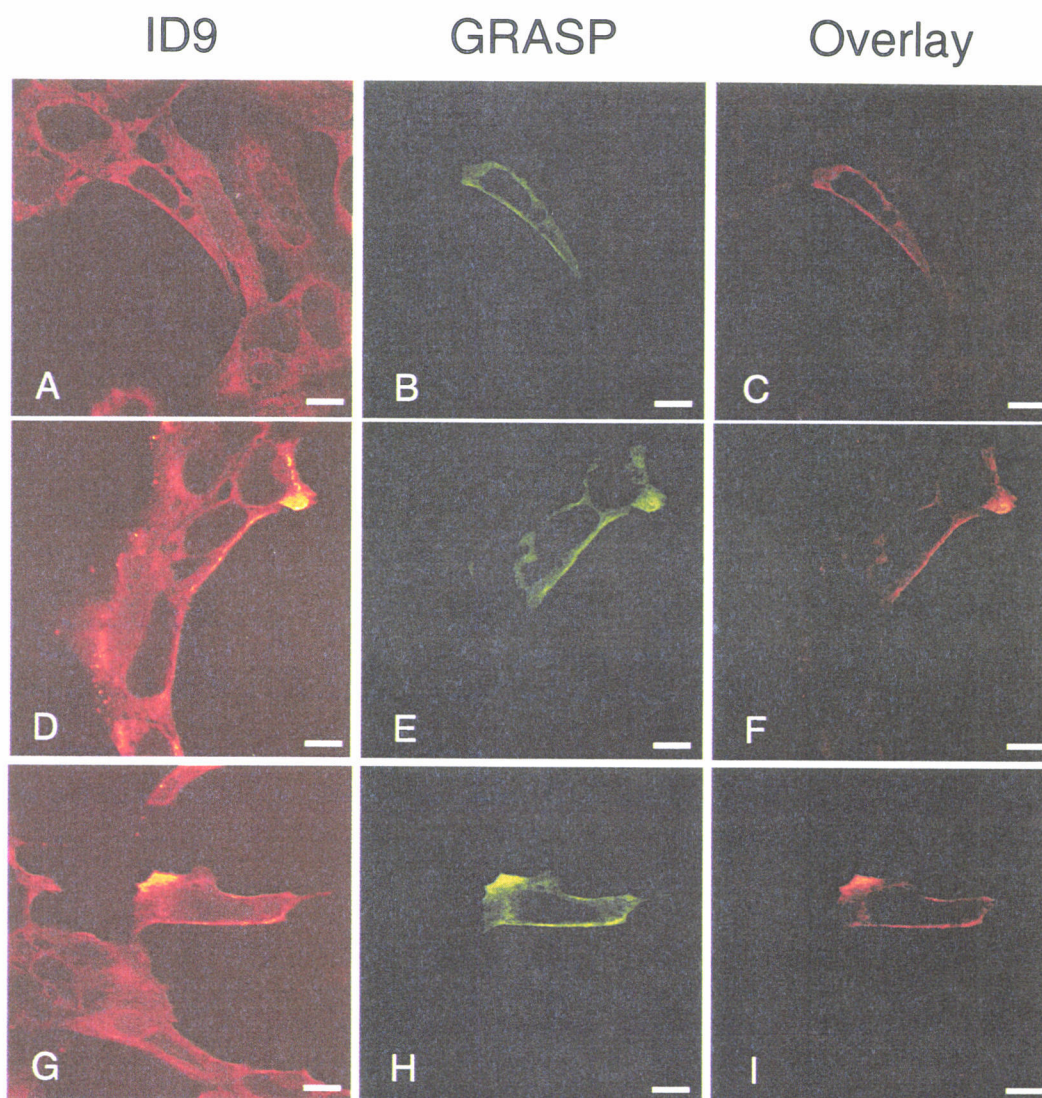


Figure 2.8. **GRASP colocalizes with endogenous ARFs.** HEK293 cells were transiently transfected with an expression vector encoding Myc-GRASP and analyzed by indirect immunofluorescence and confocal microscopy after 24 hrs. Transfected cells were stained with anti-GRASP antiserum (*B*, *E*, and *H*) and ID9 (*A*, *D* and *G*), a monoclonal antibody that recognizes all human ARF proteins (Cavenagh, Whitney et al. 1996), along with the appropriately labeled secondary antibodies as described in *Materials and Methods*. Shown in *C*, *F*, and *I* is an overlap of GRASP and ID9 staining from the representative cells. Note that the staining shown in Figs. *C*, *F*, and *I* represents only areas of coincident localization. *A-C*, *D-F*, and *G-I* were generated from independent experiments that were replicated four times. The sizing bar corresponds to 5 μm .

is a peripheral membrane protein. Localization of GRASP at the plasma membrane appeared to be dictated by the amino terminal region of the protein which harbors both the alanine-/proline-rich region and the PDZ domain. A deletion mutant that lacks the alanine-/proline-rich region and PDZ domain, Myc-GRASP 189-392, was observed to be associated with large vesicular structures located diffusely throughout the cell. It is not known whether Myc-GRASP 189-392 associates with these structures simply as a result of mislocalization, or whether these structures arise as a result of Myc-GRASP 189-392 expression, which would suggest a dominant inhibitory effect. A more thorough analysis of the nature of these vesicles will be required to clarify this issue. Nevertheless, our data clearly implicate amino terminal residues, including the alanine-/proline-rich region and PDZ domain, as important determinants of GRASP subcellular localization. These results are consistent with reports that PDZ domains play a role in the proper cellular localization of many proteins (Ponting, Phillips et al. 1997).

It has been proposed that the PDZ domains of several proteins facilitate synaptic neurotransmission by physically linking various components of a signal transduction cascade at specific, subcellular loci (Kornau, Schenker et al. 1995; Gomperts 1996; Kim, Cho et al. 1996; Niethammer, Kim et al. 1996). For example, InaD contains five PDZ domains each of which binds a specific protein component in the *Drosophila* phototransduction signaling pathway facilitating their juxtaposition and

enhancing the efficiency and specificity of signal transmission (Tsunoda, Sierralta et al. 1997). The possibility that GRASP may perform a similar cellular function within the context of ARF signaling pathways is suggested by the present findings but this remains to be established.

Members of the cytohesin subfamily of ARF GEFs can be recruited to the plasma membrane of cells in response to growth factor stimulation (Nagel, Zeitlmann et al. 1998; Venkateswarlu, Gunn-Moore et al. 1998; Venkateswarlu, Oatey et al. 1998). Activation of many growth factor receptors at the cell surface results in stimulation of PI 3-kinase, and subsequent phosphorylation of the D3 position on phosphatidylinositol and its phosphorylated derivatives (Hawkins, Jackson et al. 1992). Generation of 3'-phosphorylated derivatives, specifically phosphatidylinositol (3,4,5)-trisphosphate, is thought to promote the association of cytohesin subfamily members with membranes through interaction of phosphatidylinositol (3,4,5)-trisphosphate with the GEF PH domain (Nagel, Schilcher et al. 1998; Venkateswarlu, Gunn-Moore et al. 1998; Venkateswarlu, Oatey et al. 1998). Conversely, inhibitors of PI 3-kinase, such as wortmannin, have been shown to block translocation of these proteins to the plasma membrane, resulting in cytosolic localization (Venkateswarlu, Gunn-Moore et al. 1998; Venkateswarlu, Oatey et al. 1998). It is thought that recruitment of these proteins to the membrane creates a favorable interface for the interaction of GEF and ARF proteins,

leading to stimulation of GDP/GTP exchange and subsequent activation of ARFs (Chardin, Paris et al. 1996; Frank, Upender et al. 1998; Klarlund, Rameh et al. 1998; Langille, Patki et al. 1999). This is supported by in vitro experiments that demonstrated enhanced GEF stimulated GTP γ S binding by various myristoylated ARFs in the presence of lipid vesicles and phosphorylated derivatives of phosphatidylinositol (Chardin, Paris et al. 1996; Frank, Upender et al. 1998; Klarlund, Rameh et al. 1998; Langille, Patki et al. 1999). Enhanced GDP/GTP exchange was completely abolished by addition of the inositol headgroup moiety, inositol 1,3,4,5-tetrakisphosphate, to the reaction, indicating that enhanced GEF activity results from its association with membranes and not an allosteric change that promoted its catalytic activity (Klarlund, Rameh et al. 1998). Taken together, these results are consistent with a model that binding of phosphatidylinositol (3,4,5)-trisphosphate to the PH domain of a particular GEF results in the translocation of that GEF to the plasma membrane, at which the protein is able to promote GDP/GTP exchange on ARF proteins.

The results of the present study identify GRASP as a novel interaction partner for GRP1 and suggest that cellular localization of GRP1 may be influenced by the interaction of the GRP1 coiled-coil domain with GRASP. Similar results have been obtained by Neeb and coworkers who demonstrated an interaction of the coiled-coil region of msec7-1, the rat homologue of cytohesin-1, and the presynaptic protein

Munc13-1, which serves to target msec7-1 to the active zone at the presynapse (Neeb, Koch et al. 1999). It may be speculated that the presence of a PDZ domain in GRASP could allow the selective subcellular targeting of GRP1 or cytohesin-2 to discrete plasma membrane domains, resulting in subsequent activation of distinct ARFs. This is an attractive possibility that could result in the juxtaposition of GEF(s) and components of specific signaling pathways. Other motifs present in GRASP could also facilitate interactions with possible downstream effectors, implicating GRASP as a putative scaffold protein. A parallel example of this type of subcellular targeting may exist in the Rho family of GTPases. Tiam1, a GEF for Rac1 (Habets, Scholtes et al. 1994; Michiels, Habets et al. 1995), has been implicated in regulating neuronal morphology (Leeuwen, Kain et al. 1997). In addition to a PH and a Dbl homology domain, Tiam1 contains a PDZ domain (Habets, Scholtes et al. 1994) that may target this GEF to a particular subcellular locus thus facilitating specificity in mediating Rac1-induced events.

Interaction of GRP1 with GRASP or GRASP-like proteins may also be speculated to promote adhesion of the α L β 2 integrin (LFA-1) to its extracellular ligand, ICAM-1. Kolanus and coworkers (Kolanus, Nagel et al. 1996) have shown that cytohesin-1 interacts with carboxyl terminal residues of the β 2 integrin receptor subunit, increasing the avidity of LFA-1 for ICAM-1. Because of the strong sequence

similarity, it seems likely that other members of the cytohesin subfamily may be able to function in this capacity. If GRASP, or a GRASP-like protein, acting through its alanine-/proline-rich region and/or PDZ domain, associates with integrin receptors and/or associated proteins, it is conceivable that recruitment of cytohesin family members to this locus may similarly enhance the avidity of LFA-1 for ICAM-1. Such speculation would be consistent with previous studies demonstrating that treatment of various cell types with RA results in modulation of integrin adhesion (Dedhar, Robertson et al. 1991; Rossino, Defilippi et al. 1991; Elias, Chammas et al. 1994; Gaetano, Melchiori et al. 1994; Loeser 1994; Ross, Ahrens et al. 1994). For example, Katagiri and coworkers have shown that retinoic acid potentiates cellular adhesion and aggregation mediated by LFA-1 and ICAM-1 in HL-60 cells undergoing neutrophilic differentiation (Katagiri, Kinashi et al. 1996). This alteration in cellular adhesiveness does not result from increased levels of expression of either LFA-1 or ICAM-1, but rather is induced by a change in the avidity state of LFA-1 (Katagiri, Kinashi et al. 1996). This change in LFA-1 avidity was shown to require de novo protein synthesis and to occur within 24 hrs. of initiation of RA treatment (Katagiri, Kinashi et al. 1996), consistent with the time course of maximal induction of GRASP mRNA in P19 cells reported herein.

In summary, RA-induced expression of GRASP may represent a point of convergence between the nuclear receptor signaling pathways and activation of plasma membrane-associated ARF proteins. Induction of GRASP by RA may influence cellular phenotype via one or more of the multiple pathways described above that may underlie, at least in part, the pleiotropic effects of RA during cellular differentiation and/or in cellular function.

2.6 Abbreviations

The abbreviations used are RA, retinoic acid; RAR, retinoic acid receptor; tRA, all-*trans*-retinoic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; GST, glutathione *S*-transferase; BFA, brefeldin A; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; PH, pleckstrin homology; HEK, human embryonic kidney; ARNO, ARF nucleotide-binding site opener; EC, embryonal carcinoma; GEF, guanine nucleotide exchange factor.

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Chapter 3

Overexpression of GRASP Results in a Disruption of the ARF6-Mediated Plasma Membrane Recycling Pathway

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3.1 Abstract

GRASP is a retinoic acid-induced gene product from P19 embryonal carcinoma cells that we have previously shown interacts with the ADP-ribosylation factor 6 (ARF6) guanine nucleotide exchange factors general receptor for phosphoinositides 1 (GRP1) and cytohesin-2. ARF6, a member of the Ras superfamily of low molecular weight GTPases, regulates key aspects of endocytic recycling pathways. Here we demonstrate that overexpression of GRASP leads to the accumulation of GRASP in the endosomal compartment where GTP-binding deficient mutants of ARF6 reside, suggesting that GRASP may induce a block in an ARF6 recycling pathway. Coexpression of GRP1, but not a catalytically inactive mutant dramatically reduced the accumulation of GRASP in this compartment. Furthermore, GRP1 mutants that lacked the region of interaction with GRASP failed to prevent the accumulation of GRASP in the endosomal compartment, suggesting that GRASP recruits GRP1 to the endosomal compartment where GRP1 stimulates nucleotide exchange of ARF6 and recycling. We show that residues within amino acids 180-257 of GRASP are necessary and sufficient to induce the accumulation of GRASP in the endosomal compartment. We identify residues within this region of GRASP that are required for efficient interaction with GRP1 and also dictate GRASP localization to actin-based structures at the cell periphery, suggesting that GRASP may enter the recycling pathway at these sites through recruitment of GRP1 and ARF6.

3.2 Introduction

ADP ribosylation factors (ARFs) are small GTPases of the Ras superfamily that regulate key aspects of membrane traffic in cells (Moss and Vaughan 1998). ARF proteins were originally identified as cofactors of the cholera toxin-catalyzed ADP-ribosylation of $G_s\alpha$ and have also been shown to be activators of phospholipase D (Brown, Gutowski et al. 1993). ARF proteins are divided into three classes based on phylogenetic analysis and size. The class I ARFs, which include ARFs 1-3, are the best characterized and function in vesicular trafficking pathways in the Golgi apparatus (Serafini, Orci et al. 1991; Balch, Kahn et al. 1992), while little is known about the function of class II ARFs (ARFs 4-5). ARF6, the least conserved member of the family, comprises class III and regulates endocytic and actin remodeling pathways (D'Souza-Schorey, Li et al. 1995; Radhakrishna and Donaldson 1997).

ARF proteins cycle between inactive GDP and active GTP-bound conformations. The alternative conformations of ARFs, induced by GTP-binding and subsequent hydrolysis of the bound nucleotide to GDP, facilitate interaction with, and activation of various classes of effector proteins. As ARF proteins are poorly able to exchange GDP and hydrolyze GTP on their own, these reactions are catalyzed by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), respectively. An increasing number of proteins exhibiting ARF GAP activity have been described (Donaldson 2000). Interestingly, many of these proteins harbor well defined

signaling motifs, such as ankyrin repeats, PH, proline-rich and SH3 domains, which may link the ARF cycle to various signal transduction pathways. ARF GEFs, which all share a conserved catalytic domain of approximately 200 amino acids that is homologous to yeast Sec7 protein, fall into two categories based on size and sensitivity to inhibition by brefeldin A (BFA), a toxic fungal metabolite that blocks protein secretion and Golgi function. Members of the cytohesin family of proteins comprise the low molecular weight BFA-insensitive ARF GEF category. The cytohesin family includes cytohesin-1 (also known as B2-1; (Liu and Pohajdak 1992; Kolanus, Nagel et al. 1996), ARNO (ARF nucleotide-binding site opener; also known as cytohesin-2; (Chardin, Paris et al. 1996), GRP1 (Klarlund, Guilherme et al. 1997), and cytohesin-4 (Ogasawara, Kim et al. 2000). The cytohesins share a common domain structure consisting of an amino-terminal coiled-coil domain, followed by a Sec7 domain that is responsible for GEF activity, a pleckstrin homology (PH) domain that binds phosphoinositides and aids in membrane recruitment, and a polybasic region at the carboxyl terminus. Several laboratories have demonstrated a phosphatidylinositol 3-kinase-dependent translocation of cytohesins to the plasma membrane of cells (Clodi, Vollenweider et al. 1998; Nagel, Zeitlmann et al. 1998; Venkateswarlu, Gunn-Moore et al. 1998; Venkateswarlu, Oatey et al. 1998; Langille, Patki et al. 1999; Venkateswarlu, Gunn-Moore et al. 1999; Klarlund, Holik et al. 2001), however the cellular

consequences of 3'-phosphoinositide generation as it relates to the ARF cycle is relatively unexplored.

Early studies that demonstrated cytohesins exhibited GEF activity on ARF1 (Paris, Beraud-Dufour et al. 1997), coupled with studies in transfected cells which showed that overexpression of ARNO and ARNO3 (GRP1) resulted in a disassembly of the Golgi complex (Franco, Boretto et al. 1998; Monier, Chardin et al. 1998), led to the conclusion that cytohesins function on class I ARFs. In addition to their possible role in regulating ARF1 function, recent studies have shown that cytohesins are able to activate ARF6 *in vitro* (Frank, Upender et al. 1998; Knorr, Nagel et al. 2000), in cultured cells (Langille, Patki et al. 1999), and colocalize with ARF6 in plasma membrane ruffles (Frank, Upender et al. 1998; Langille, Patki et al. 1999). In further support for the role of cytohesins as ARF6 GEFs, Caumont and coworkers have shown that ARNO activates secretion in chromaffin cells in an ARF6-dependent manner (Caumont, Vitale et al. 2000), while Mukherjee et al have shown that the ARF6-dependent beta-arrestin release necessary for luteinizing hormone/choriogonadotropin receptor desensitization requires ARNO catalytic activity (Mukherjee, Casanova et al. 2001).

Overexpression analyses of GTP binding-defective (dominant negative, GDP-bound) and GTPase deficient (constitutively active, GTP-bound) ARF6 mutants in cells have greatly contributed towards the functional elucidation of the ARF6 cycle (D'Souza-Schorey, Li et al. 1995). D'Souza-Schorey and coworkers discovered a role

for ARF6 in receptor-mediated endocytosis by analyzing transferrin receptor recycling in TRVb-1 cells expressing ARF6 mutants (D'Souza-Schorey, Li et al. 1995). They showed that in cells expressing a GTPase-deficient mutant of ARF6, ARF6 Q67L, transferrin receptors were localized exclusively to the plasma membrane along with ARF6 Q67L and cells exhibited increased transferrin cell surface binding (D'Souza-Schorey, Li et al. 1995). In contrast, cells expressing a GTP-binding deficient mutant of ARF6, ARF6 T27N, exhibited a block in plasma membrane recycling as transferrin receptors were internalized on endosomes with the ARF6 mutant, leading to a reduction in transferrin cell surface-binding (D'Souza-Schorey, Li et al. 1995). Studies in HeLa cells, however, revealed that ARF6 cycles between the plasma membrane and a recycling compartment that is distinct from the transferrin receptor recycling compartment, indicating heterogeneity in these compartments depending on the cell type (Radhakrishna and Donaldson 1997). In addition, Peters et al demonstrated that overexpression of these ARF6 mutants in cells resulted in profound changes of the peripheral membrane system (Peters, Hsu et al. 1995). Expression of ARF6 Q67L resulted in localization of the protein to plasma membrane invaginations and protrusive structures, whereas ARF6 T27N expression resulted in the massive accumulation of large aggregates of tubulovesicular structures where the mutant resided (Peters, Hsu et al. 1995). These results have led to the hypothesis that nucleotide exchange on ARF6 occurs on recycling endosomes, resulting in translocation and fusion of the endosomes

with the plasma membrane (D'Souza-Schorey, Li et al. 1995; Peters, Hsu et al. 1995; D'Souza-Schorey, van Donselaar et al. 1998). To complete the cycle, GTP hydrolysis of ARF6 at the plasma membrane appears to be required for ARF6 to become internalized and localize in the endosomal compartment.

Despite evidence suggesting that nucleotide exchange of ARF6 occurs on endosomes, there are limited studies linking GEF function to ARF6-mediated endosomal recycling. Franco and coworkers demonstrated that overexpression of the ARF6 GEF, EFA6, in TRVb-1 cells resulted in a redistribution of transferrin receptors from intracellular vesicles to the plasma membrane (Franco, Peters et al. 1999), whereas overexpression of ARNO3, the human homolog of GRP1, had no effect on the distribution of transferrin receptors. Additionally, Maranda et al have shown that ARNO and ARF6 are recruited to endosomes in kidney proximal tubule endothelial cells in a pH- dependent manner (Maranda, Brown et al. 2001).

Recent work in this laboratory identified GRASP, a novel retinoic acid-induced gene product that interacts with the cytohesin family members GRP1 and cytohesin-2 (Nevrivy, Peterson et al. 2000). Here we report that the localization of overexpressed GRASP in HEK293 cells mimics the endosomal localization of ARF6 GTP-binding deficient mutants, that is indicative of a block in a plasma membrane recycling pathway. Coexpression of ARF6 N122I, a GTP-binding defective mutant, increased the extent of GRASP localization in the endosomal compartment. Interestingly,

coexpression of GRP1 dramatically reduced the accumulation of GRASP in the endosomal compartment, whereas coexpression of a catalytically inactive mutant of GRP1 did not. In addition to a functional Sec7 domain, the presence of the coiled-coil domain of GRP1 was required to prevent GRASP localization in the endosomal compartment, suggesting that GRASP recruits GRP1 to endosomes where GRP1 stimulates guanine nucleotide exchange on ARF6.

3.3 Materials and Methods

3.3.1 Cell Culture and Transfections

Human embryonic kidney (HEK) 293 cells were maintained as described previously (Nevrivy, Peterson et al. 2000). Transfections were carried out as described previously (Nevrivy, Peterson et al. 2000) in 6-well plates using 0.5 µg of each expression vector (1.0 µg total) for fluorescence microscopy studies or in 100-mm plates using 3.0 µg of each expression vector (6.0 µg total) for coimmunoprecipitation analyses.

3.3.2 Plasmid Constructs

ARF6 expression vectors were kindly provided by Dr. K. Nakayama (Toda, Nogami et al. 1999). The GRP1 construct was kindly provided by Drs. Michael Czech and Jes Klarlund (Klarlund, Guilherme et al. 1997). All constructs were prepared using standard techniques. Supplemental details are available upon request.

3.3.3 Fluorescence Microscopy

Twenty-four hours following transfection, HEK293 cells growing on coverslips were fixed in 4% paraformaldehyde and permeabilized in two changes of PBS + 0.02% Triton-X-100. Antibody incubations were performed using standard techniques with the anti-Myc monoclonal antibody (Invitrogen), anti-HA polyclonal antibody (Santa Cruz), or anti-GRP1 polyclonal antibody (Santa Cruz). Images were captured on a Leica fluorescence microscope using Openlab software (Improvision) and processed using Photoshop 5.0 (Adobe Systems, Inc.). For GRASP endosomal scoring assays, a minimum of 50 cells were counted and scored for the accumulation of endosomes that stained positively for GRASP. Only cells that exhibited a large accumulation of GRASP in the endosomal compartment were scored as positive.

3.3.4 Coimmunoprecipitation Analyses

For coimmunoprecipitation analysis, transfected HEK293 cells were collected in solubilization buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 1mM EDTA, 1 mM DTT, 10% (v/v) glycerol, 0.1 mM PMSF, 10 μ M pepstatin A, 10 μ M leupeptin, 25 μ g/ml aprotinin, 0.05% (v/v) NP-40 and 0.5% (v/v) Triton-X-100) and incubated at 4°C with rotation for 30 min. The lysate was cleared by centrifugation at 16,000 x g for 20 min. at 4°C. Three hundred micrograms of lysate protein was incubated with the anti-GFP antibody (Research Diagnostics, Inc.) in a volume of 0.5 mL for 2 hr. at 4°C, after which Protein A-Sepharose (100 μ L, 50 mg/ml in solubilization buffer; Pharmacia) was added and the incubation was continued at 4°C with rotation for an additional 60 min. The beads were collected by centrifugation (800 x g for 1 min. at 4°C) and subsequently washed gently four times with 1.0 mL solubilization buffer. Immunoprecipitates were subjected to immunoblot analysis using goat anti-GRP1 antibody (Santa Cruz).

3.3.5 GST Pull-down Experiments

GST fusion proteins were produced, crude bacterial lysates were prepared, and GST pull-down experiments were conducted as described previously (Nevrivy, Peterson et al. 2000).

3.4 Results

3.4.1 GRASP colocalizes with a GTP-binding deficient mutant of ARF6

We previously reported that GRASP localized predominantly to the plasma membrane of transfected HEK293 cells (Nevrivy, Peterson et al. 2000). In addition, we have consistently observed GRASP staining in punctate, perinuclear structures that appeared to be vesicular in origin (Fig. 3.1A). This pattern of GRASP staining, which has been observed in approximately 20% of transfected cells across a number of experiments, resembles the endosomal staining pattern of cells expressing GTP-binding deficient mutants of ARF6, ARF6 T27N and ARF6 N122I, that induce a block in plasma membrane recycling (D'Souza-Schorey, Li et al. 1995; Toda, Nogami et al. 1999). Thus, we have investigated the possibility that GRASP and ARF6 may function in identical or related endosomal pathways of plasma membrane recycling.

To determine if wild-type GRASP localized in the same endosomal compartment as ARF6 N122I, HEK293 cells were transiently cotransfected with constructs encoding both proteins. These cells were subjected to indirect immunofluorescence microscopy to define the subcellular localization of each protein. Again, we found that GRASP staining was localized to punctate, cytoplasmic structures in transfected cells (Fig. 3.1B) and this staining was coincident with that of ARF6

N122I (Fig. 3.1C, D). As ARF6 N122I has been previously demonstrated to localize in an endosomal compartment and induce a block in plasma membrane recycling (Toda, Nogami et al. 1999), we conclude that the punctate, cytoplasmic structures stained by the GRASP antibody correspond to one or more endosomal compartment(s). Moreover, our data suggest that in a manner similar to that of ARF6 N122I, overexpression of GRASP may induce a block in a plasma membrane recycling pathway.

3.4.2 Coexpression of wild-type or constitutively active ARF6 does not affect GRASP localization in the endosomal compartment

Our results demonstrating colocalization of GRASP with ARF6 N122I suggest a possible involvement of GRASP in an ARF6 plasma membrane recycling pathway. To investigate this possibility further, we determined the effect of expression of wild-type or constitutively active ARF6, as well as ARF6 N122I, on the endosomal localization of GRASP. Neither wild-type nor constitutively active ARF6, ARF6 Q67L, affected the endosomal localization of GRASP, as 18% and 22% of cotransfected cells exhibited GRASP endosomal staining, respectively, compared with 20% of cells transfected with GRASP alone (Figs. 3.2A-C and D-F, see also Fig. 3.2J). In addition, wild-type ARF6 (Fig. 3.2B) and ARF6 Q67L (Fig. 3.2E) did not colocalize with GRASP in the endosomal compartment, however, substantial colocalization of GRASP with both proteins was observed at the plasma membrane of cells in which GRASP staining was

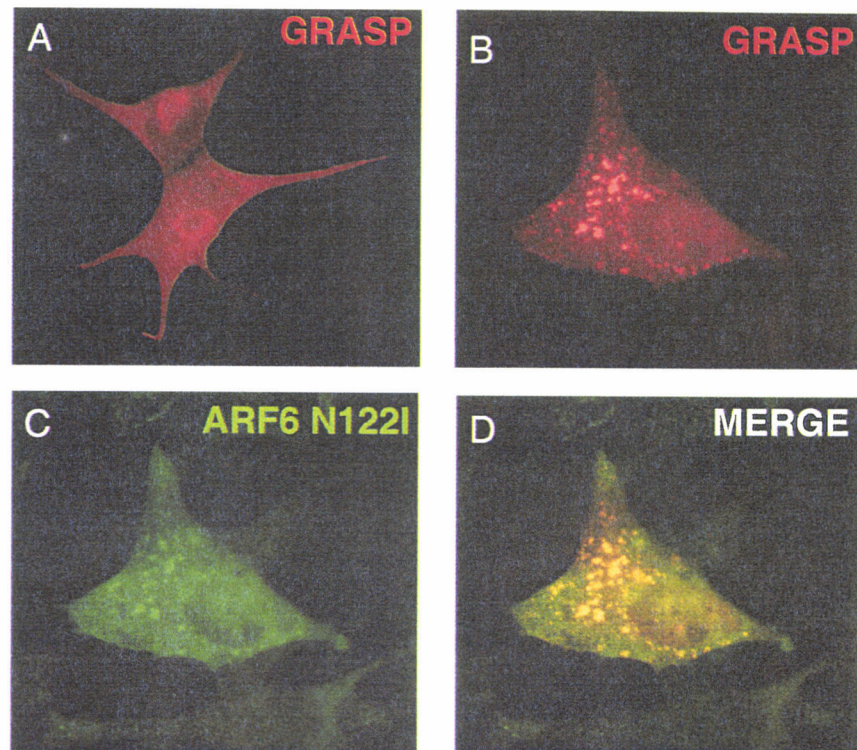


Figure 3.1. **Colocalization of GRASP and a GTP-binding deficient mutant ARF6 in HEK293 cells.** HEK293 cells were transiently cotransfected with an expression vector encoding Myc-GRASP in the absence and in the presence of an expression vector encoding ARF6 N122I-HA, followed by visualization of transfected cells by indirect immunofluorescence microscopy. Transfected cells were fixed in 2% paraformaldehyde and stained with mouse anti-Myc monoclonal or rabbit polyclonal anti-HA antibodies followed by incubation with fluorescently coupled secondary antibodies to localize Myc-GRASP and ARF6 N122I-HA, respectively. *A*, HEK293 cells expressing Myc-GRASP. *B*, HEK293 cells expressing both Myc-GRASP and ARF6 N122I-HA, detecting only Myc-GRASP. *C*, HEK293 cells expressing both Myc-GRASP and ARF6 N122I-HA, detecting only ARF6 N122I-HA. *D*, an overlay of the fluorescence from panels *B* and *C*.

not observed in the endosomal compartment (data not shown). In contrast, coexpression of ARF6 N122I resulted in a dramatic increase in the fraction of cells with an accumulation of GRASP-positive endosomes (52%; Figs. 3.2G-I and J). Moreover, ARF6 N122I and GRASP staining was found to be coincident in the endosomal compartment of these cells (Fig. 3.2I). In cotransfected cells that did not exhibit a block in plasma membrane recycling, both GRASP and ARF6 N122I staining appeared diffusely distributed in the cytoplasm and/or at the plasma membrane (data not shown). These results suggest that overexpression of GRASP, like that of ARF6 N122I, may induce a block at one or more of the endosomal intermediates in a plasma membrane recycling pathway.

3.4.3 High expression levels of wild-type GRASP contribute to GRASP localization in the endosomal compartment

We hypothesized that the possible block in plasma membrane recycling that was observed in a subpopulation of GRASP-transfected cells may be due to overexpression of the protein. To test this hypothesis, HEK293 cells were transfected with increasing amounts of an expression vector encoding GRASP followed by indirect immunofluorescence microscopy. The percentage of cells that exhibited an accumulation of endosomes staining positively for GRASP increased from 20 to 42% with increasing amounts of transfected GRASP expression vector (Fig. 3.3), suggesting

Figure 3.2. Expression of a GTP-binding deficient mutant ARF6, but not wild-type or a constitutively active mutant of ARF6 enhances localization of GRASP in the endosomal compartment. HEK293 cells were transiently cotransfected with a combination of expression vectors encoding Myc-GRASP and either ARF6-HA (*A-C*), ARF6 Q67L-HA (*D-F*), or ARF6 N122I-HA (*G-I*), followed by indirect immunofluorescence microscopy as described in the legend of Figure 1 and scoring of transfected cells for the presence of GRASP endosomal localization. Cotransfected cells shown in *A*, *D*, and *G* are representative cells stained with the Myc antibody that indicate Myc-GRASP fluorescence while those shown in *B*, *E*, and *H* correspond to cells stained with the HA antibody which represent ARF6-HA, ARF6 Q67L-HA, and ARF6 N122I-HA fluorescence, respectively. Overlays of the representative Myc-GRASP and ARF6-HA fluorescence are shown in *C*, *F*, and *I*. *J*, Quantification of cotransfected HEK293 cells that exhibit Myc-GRASP endosomal localization. Statistical significance at the 99% confidence level ($p < 0.01$) is indicated by ** symbols for comparison with values from cells transfected with GRASP alone. The values represent the mean of three independent experiments in which a minimum of 50 transfected cells were scored.

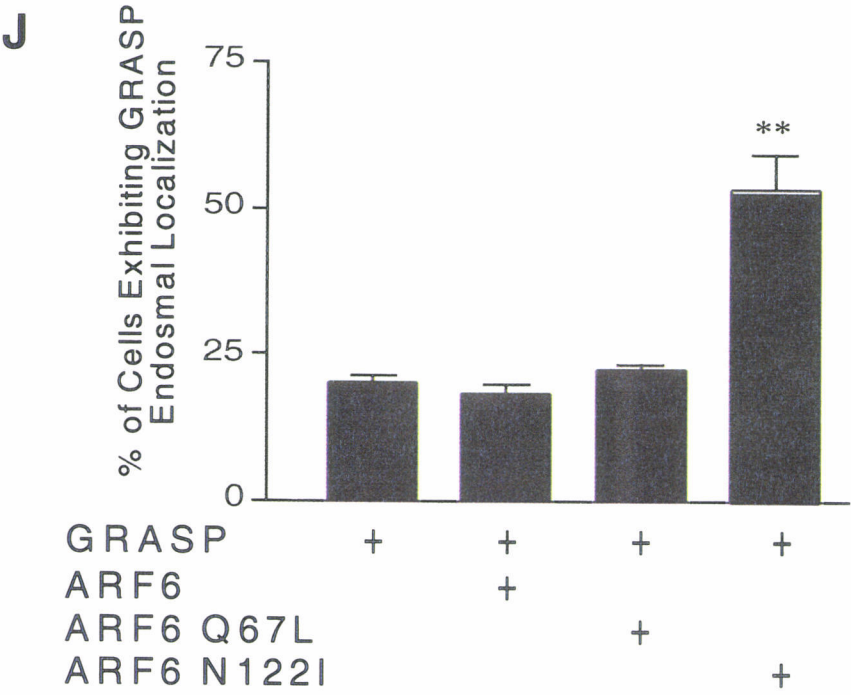
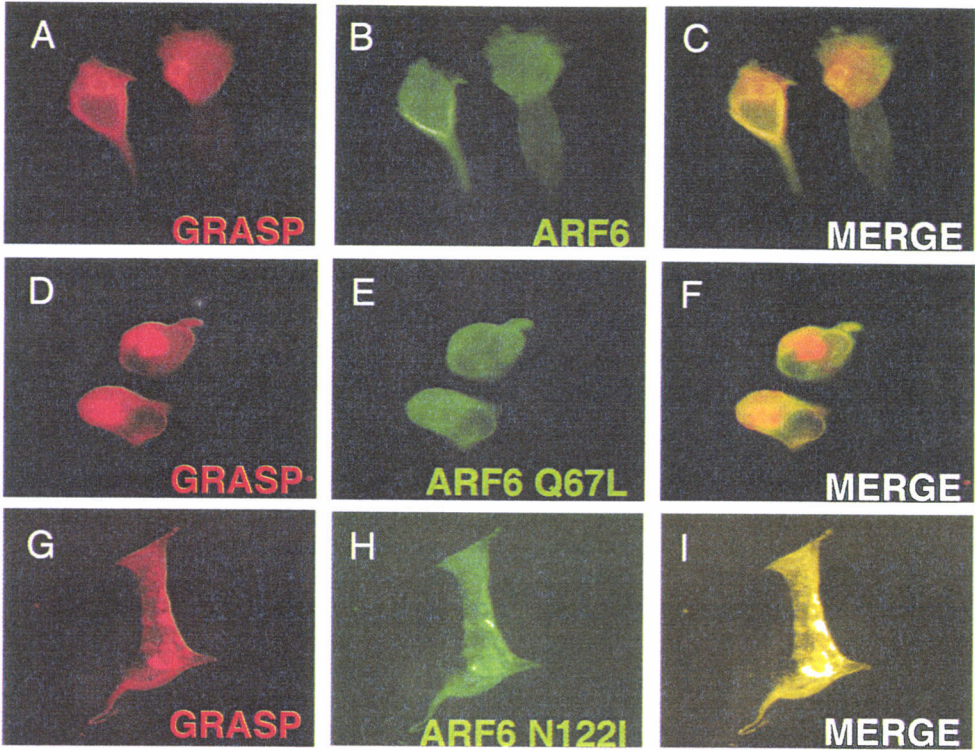


Fig. 3.2

that the frequency of GRASP localization in the endosomal compartment is indeed related to the level of expression of the protein.

3.4.4 Coexpression of GRP1 prevents GRASP localization in the endosomal compartment

ARF6 nucleotide exchange has been hypothesized to occur on recycling endosomes (D'Souza-Schorey, Li et al. 1995; Radhakrishna and Donaldson 1997; Al-Awar, Radhakrishna et al. 2000; Donaldson and Radhakrishna 2001). As we have shown that GRASP localization in the endosomal compartment is proportional to the expression level of the protein, we hypothesized that this resulted from an inability of the ARF6 GEF to be recruited to the endosomes to stimulate nucleotide exchange and recycling. This would occur if endogenous levels of the ARF6 GEF were low and/or an abundance of soluble GRASP sequestered the endogenous GEF in the cytosol, preventing recruitment of the GEF to the endosome. To test this hypothesis, HEK293 cells were transfected with expression vectors encoding GRASP in the presence and absence of an equivalent amount of GRP1, a GEF for ARF6 (Langille, Patki et al. 1999) that we have previously shown interacts with GRASP (Nevrivy, Peterson et al. 2000). Twenty percent of the cells transfected with GRASP alone exhibited a high degree of GRASP endosomal staining (Figs. 3.4A and H). In contrast, only 2% of the cells exhibited an accumulation of GRASP-positive endosomes when cotransfected with

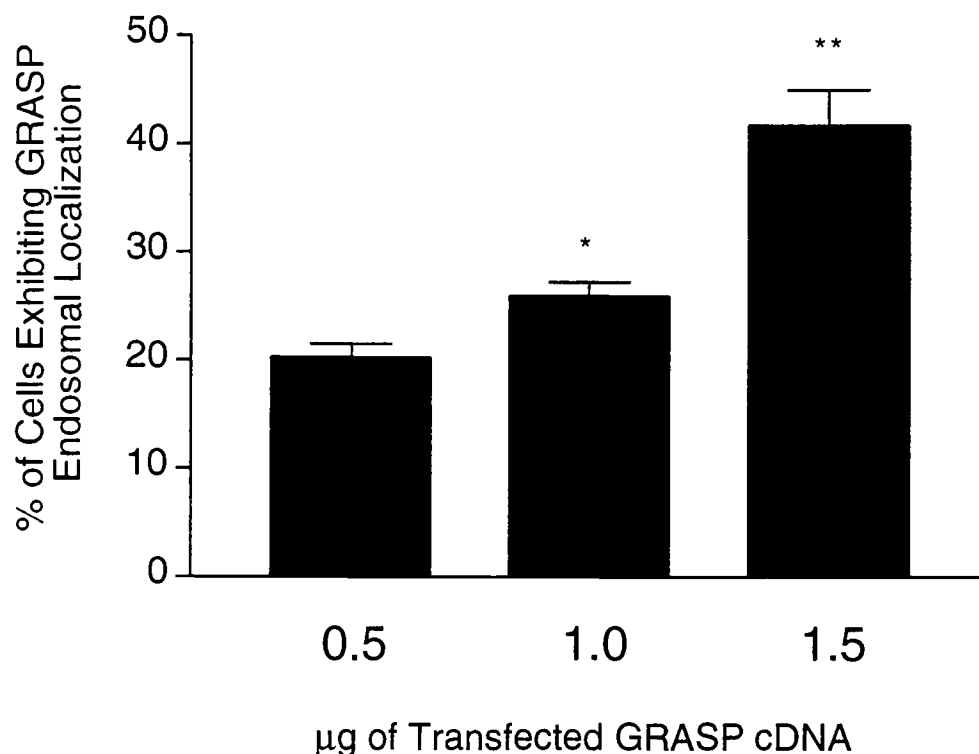


Figure 3.3. High expression levels of GRASP contribute to GRASP localization in the endosomal compartment. HEK293 cells were transfected with increasing concentrations of an expression vector encoding Myc-GRASP followed by indirect immunofluorescence microscopy as described in the legend of Figure 1 and scoring of transfected cells for the presence of an accumulation of endosomes staining positively for Myc-GRASP. Statistical significance at the 95% ($p < 0.05$) and 99% confidence level ($p < 0.01$) is indicated by * and **, respectively, for comparison with 0.5 µg GRASP cDNA. The values represent the mean of three independent experiments in which a minimum of 50 transfected cells were scored.

GRP1 (Fig 3.4H), suggesting that overexpression of GRP1 prevents an accumulation of GRASP in the endosomal compartment. In the 2% of cells that exhibited an endosomal localization of GRASP, a partial endosomal colocalization of GRP1 and GRASP was observed (Figs. 3.4E-G), suggesting that GRASP recruits GRP1 to endosomes where GRP1 may stimulate guanine nucleotide exchange on ARF6. Endosomal localization of GRP1 was not observed in the absence of GRASP under these experimental conditions (data not shown).

3.4.5 GRP1 catalytic activity is required to prevent the accumulation of GRASP in the endosomal compartment

As activated ARF6 is required for plasma membrane recycling (D'Souza-Schorey, Li et al. 1995; D'Souza-Schorey, van Donselaar et al. 1998; Al-Awar, Radhakrishna et al. 2000) and GRP1 catalyzes nucleotide exchange on and activation of ARF6 (Langille, Patki et al. 1999), we hypothesized that the catalytic activity of GRP1 may be required to prevent the accumulation of GRASP in the endosomal compartment. To test this hypothesis, we examined the effect of coexpression of a catalytically inactive mutant of GRP1, GRP1 E161K, on the subcellular distribution of GRASP. The catalytically inactive mutant of GRP1, GRP1 E161K, is based on homology to the catalytically inactive mutant of ARNO, ARNO E156K (Beraud-Dufour, Robineau et al. 1998), an ARF6 GEF that is highly related to GRP1. HEK293 cells were transfected

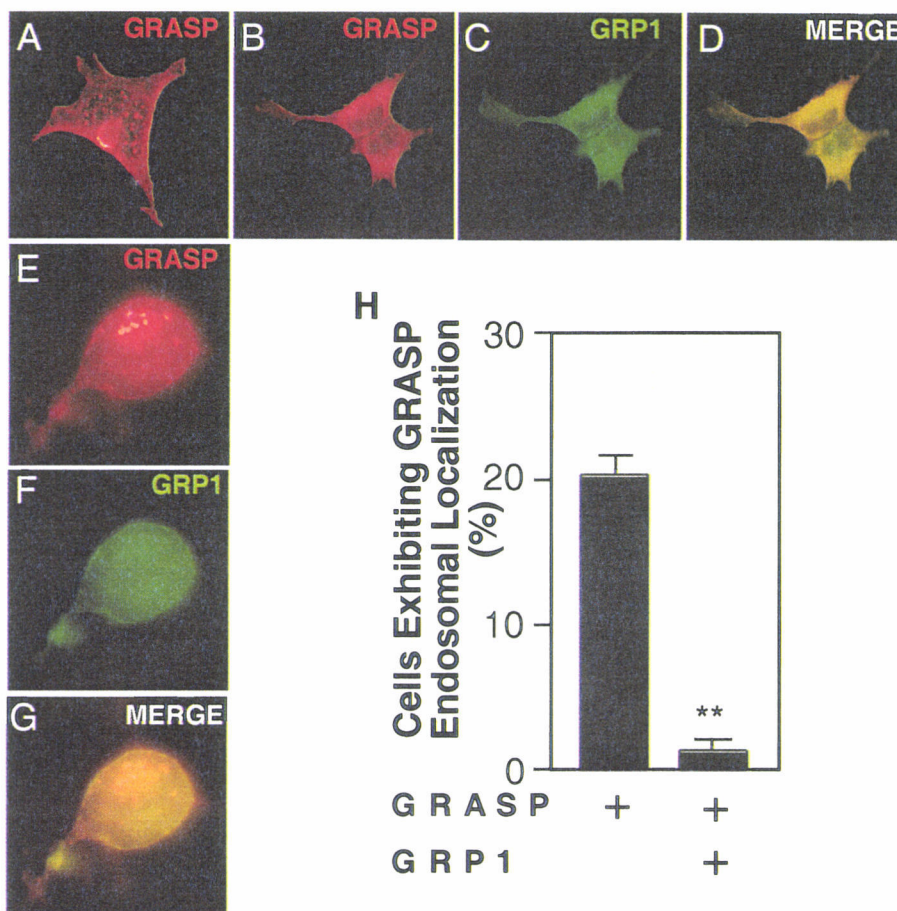


Figure 3.4. Expression of GRP1 prevents GRASP accumulation in the endosomal compartment HEK293 cells were transiently cotransfected with an expression vector encoding Myc-GRASP in the absence (A) and in the presence (B-G) of an equivalent amount of expression vector encoding GRP1-HA, followed by visualization and scoring of transfected cells for GRASP endosomal localization as described in the legend of Figure 1. A, HEK293 cells expressing Myc-GRASP alone. Cotransfected cells shown in B and E are representative cells stained with the Myc antibody that indicate Myc-GRASP fluorescence while those shown in C and F correspond to cells stained with the HA antibody representing GRP1-HA fluorescence. Overlays of the respective Myc-GRASP and GRP1-HA fluorescence are shown in D and G. Note the endosomal colocalization of Myc-GRASP and GRP1-HA in panels E-G. H, quantification of cotransfected HEK293 cells that exhibit Myc-GRASP endosomal localization. Statistical significance at the 99% confidence level ($p < 0.01$) is indicated by **. The values represent the mean of three independent experiments in which a minimum of 50 transfected cells were scored.

with expression vectors encoding GRASP in the presence and in the absence of an equivalent amount of expression vector encoding GRP1 E161K. Cells were visualized by indirect immunofluorescence microscopy and scored for an accumulation of GRASP in the endosomal compartment as described above. As observed previously, 20% of the cells transfected with GRASP exhibited a high degree of endosomal staining (Figs. 3.5A and E) and co-expression of GRP1 E161K had no effect on the degree of endosomal GRASP staining (18%, Fig. 3.5E), even though GRP1 E161K partially colocalized with GRASP on endosomes (Fig. 3.5D). Control experiments revealed that GRP1 E161K interacted with GRASP to a similar degree as wild-type GRP1 in vitro (Fig. 3.5F). These findings demonstrate that the guanine nucleotide exchange catalytic activity of GRP1 is required to prevent GRASP accumulation in the endosomal compartment.

3.4.6 The coiled-coil domain of GRP1 is required to prevent GRASP accumulation in the endosomal compartment

HEK293 cells were transfected with expression vectors encoding truncation mutants of GRP1 (Fig. 3.6A) to determine which domain(s) of GRP1 was required to prevent GRASP accumulation in the endosomal compartment. Cells transfected with GRASP alone exhibited GRASP endosomal staining to a degree observed previously (approximately 20%; Figs. 3.6B and L) and coexpression with a GRP1 mutant lacking

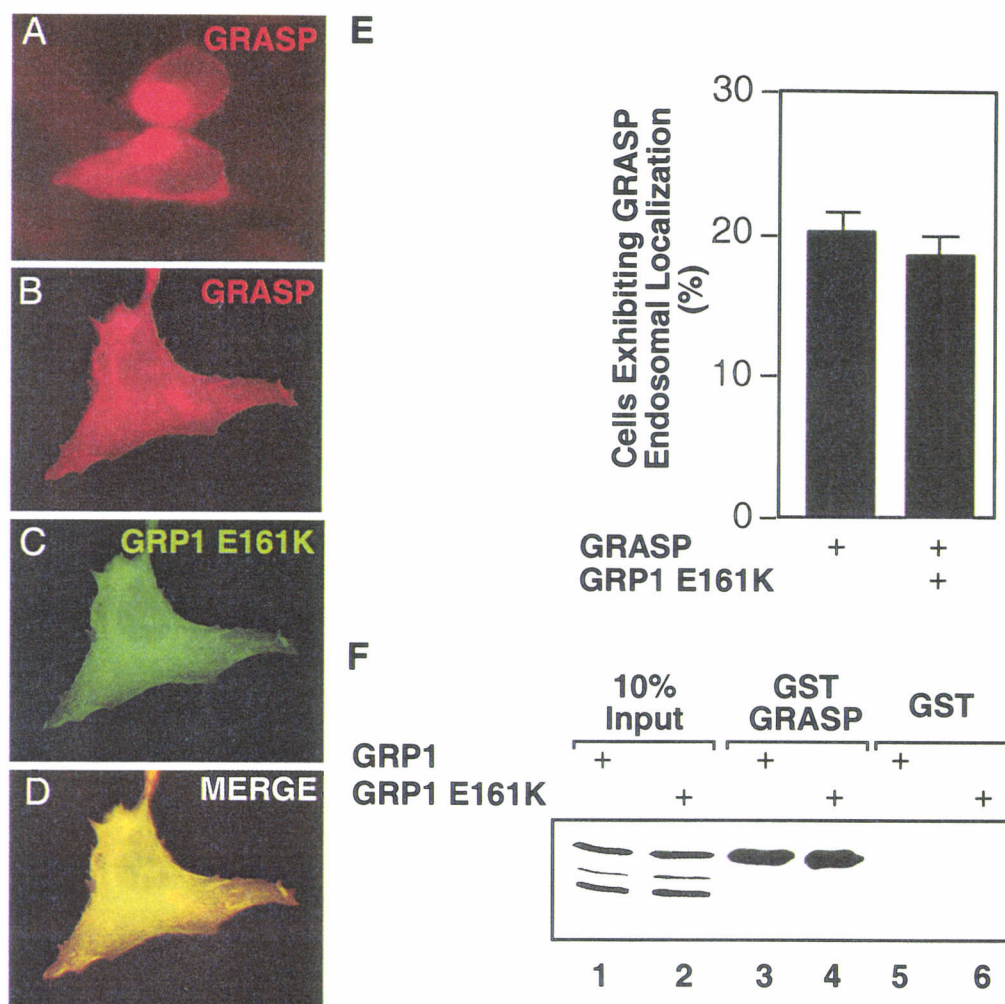


Figure 3.5. Guanine nucleotide exchange activity of GRP1 is required to prevent GRASP accumulation in the endosomal compartment. HEK293 cells were transiently cotransfected with an expression vector encoding Myc-GRASP in the absence (A) and in the presence (B-D) of an equivalent amount of expression vector encoding GRP1 E161K-HA, followed by visualization and scoring of transfected cells for GRASP endosomal localization as described in the legend of Figure 1. A, HEK293 cells expressing Myc-GRASP alone. B, HEK293 cells expressing both Myc-GRASP and GRP1 E161K-HA, detecting only Myc-GRASP. C, HEK293 cells expressing both Myc-GRASP and GRP1 E161K-HA, detecting only GRP1 E161K-HA. D, an overlay of the fluorescence from panels B and C. E, quantification of cotransfected HEK293 cells that exhibit Myc-GRASP endosomal localization. The values represent three independent experiments in which a minimum of 50 transfected cells were scored. F, *in vitro* protein-protein interactions between GST-GRASP and either GRP1 or GRP1 E161K.

the coiled-coil region, GRP1-Sec7-PH, did not alter the percentage of cotransfected cells in which GRASP-positive endosomes were observed (20 and 23%, respectively; Figs. 3.6C-E and L), suggesting that the coiled-coil domain is necessary to rescue the GRASP-mediated block in endosomal recycling. In contrast, coexpression of a GRP1 mutant containing the coiled-coil and Sec7 domain, GRP1 Coil-Sec7, significantly reduced the extent of GRASP localization in the endosomal compartment (2% of cells; Figs. 3.6F-H and L), as did coexpression of a GRP1 point mutant predicted to be defective in PIP₃ binding, GRP1 R284D (Figs. 3.6I-K and L), based on homology to a PIP₃ binding-deficient mutant ARNO (Mukherjee, Casanova et al. 2001), suggesting that neither the PH domain nor PIP₃ binding by GRP1 is required to prevent GRASP localization in the endosomal compartment.

3.4.7 Expression of the coiled-coil domain of GRP1 prevents GRASP localization in the endosomal compartment

As we had observed that expression of GRP1 prevented GRASP from accumulating in the endosomal compartment, we hypothesized that this occurred as a result of the recruitment of GRP1 to recycling endosomes by GRASP, resulting in nucleotide exchange on ARF6 and recycling of the endosomes to the plasma membrane. This hypothesis is supported by our data demonstrating that the catalytic activity of GRP1 is required to prevent GRASP from accumulating in the compartment and that

Figure 3.6. The coiled-coil domain of GRP1 is required to prevent GRASP accumulation in the endosomal compartment. HEK293 cells were transiently cotransfected with an expression vector encoding Myc-GRASP alone (*B*) or in combination with expression vectors encoding either GRP1 Coil-Sec7 (*F-H*) or GRP1-Sec7-PH-HA (*C-E*), followed by visualization and scoring of transfected cells for GRASP endosomal localization as described in the legend of Figure 1. GRP1 Coil-Sec7 was detected with a goat anti-GRP1 polyclonal antibody (Santa Cruz). (*A*) schematic of the GRP1 truncation mutants. *B*, HEK293 cells expressing Myc-GRASP alone. Cotransfected cells shown in *C*, *F*, and *I* are representative cells stained with the Myc antibody that indicate Myc-GRASP fluorescence while those shown in *D*, *G*, and *J* correspond to cells stained with the HA or GRP1 antibodies representing GRP1-Sec7-PH-HA, GRP1 Coil-Sec7, and GRP1 R284D fluorescence, respectively. Overlays of the respective Myc-GRASP and GRP1 fluorescence are shown in *E*, *H* and *K*. *L*, Quantification of cotransfected HEK293 cells that exhibit Myc-GRASP endosomal localization. Statistical significance at the 99% confidence level ($p < 0.01$) is indicated by ** symbols for comparison with values from cells transfected with GRASP alone. The values represent the mean of three independent experiments in which a minimum of 50 transfected cells were scored.

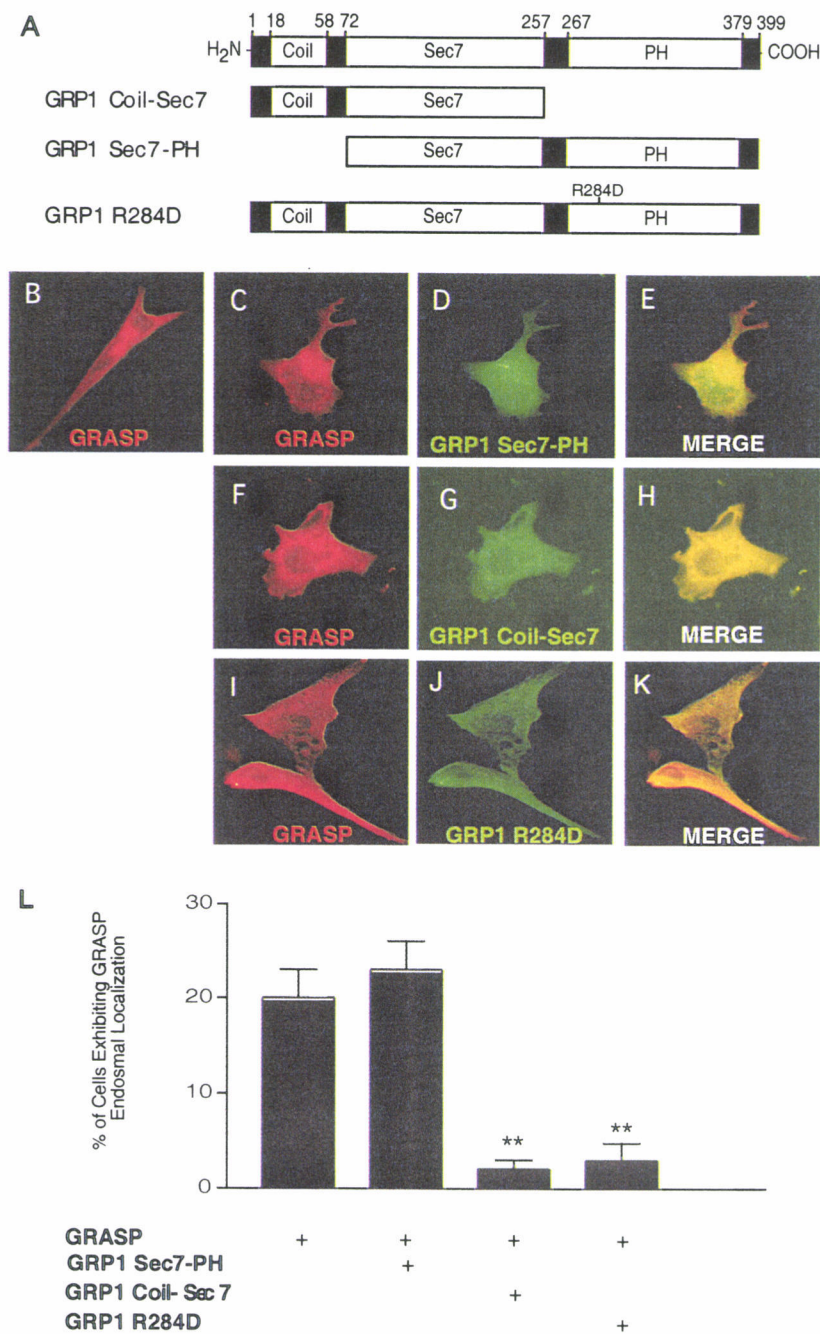


Fig. 3.6

GRP1-Sec7-PH, a mutant that lacks the coiled-coil responsible for interaction with GRASP (Nevrivy, Peterson et al. 2000), failed to prevent GRASP from accumulating in the endosomal compartment. Therefore, we anticipated that coexpression of the isolated coiled-coil region of GRP1 may lead to an enhancement of GRASP-positive endosomes as the coiled-coil fragment of GRP1 would prevent endogenous ARF6 GEFs from being recruited to endosomes that contained GRASP. To test this hypothesis, HEK293 cells were transfected with an expression vector encoding GRASP in the presence and absence of an expression vector encoding the GRP1 coiled-coil region (Fig. 3.7A), followed by indirect immunofluorescence and scoring of transfected cells as described above. GRASP exhibited an endosomal localization to a degree observed previously (Figs. 3.7B and I). Surprisingly, coexpression of the coiled-coil domain of GRP1 with GRASP strongly inhibited GRASP localization in the endosomal compartment (Figs. 3.7C-E) as only 2% of cotransfected cells exhibited an accumulation of endosomes staining positively for GRASP (Fig. 3.7I). In transfected cells that exhibited GRASP accumulation in the endosomal compartment, colocalization of GRP1-coiled-coil and GRASP was observed (Figs. 3.7F-H).

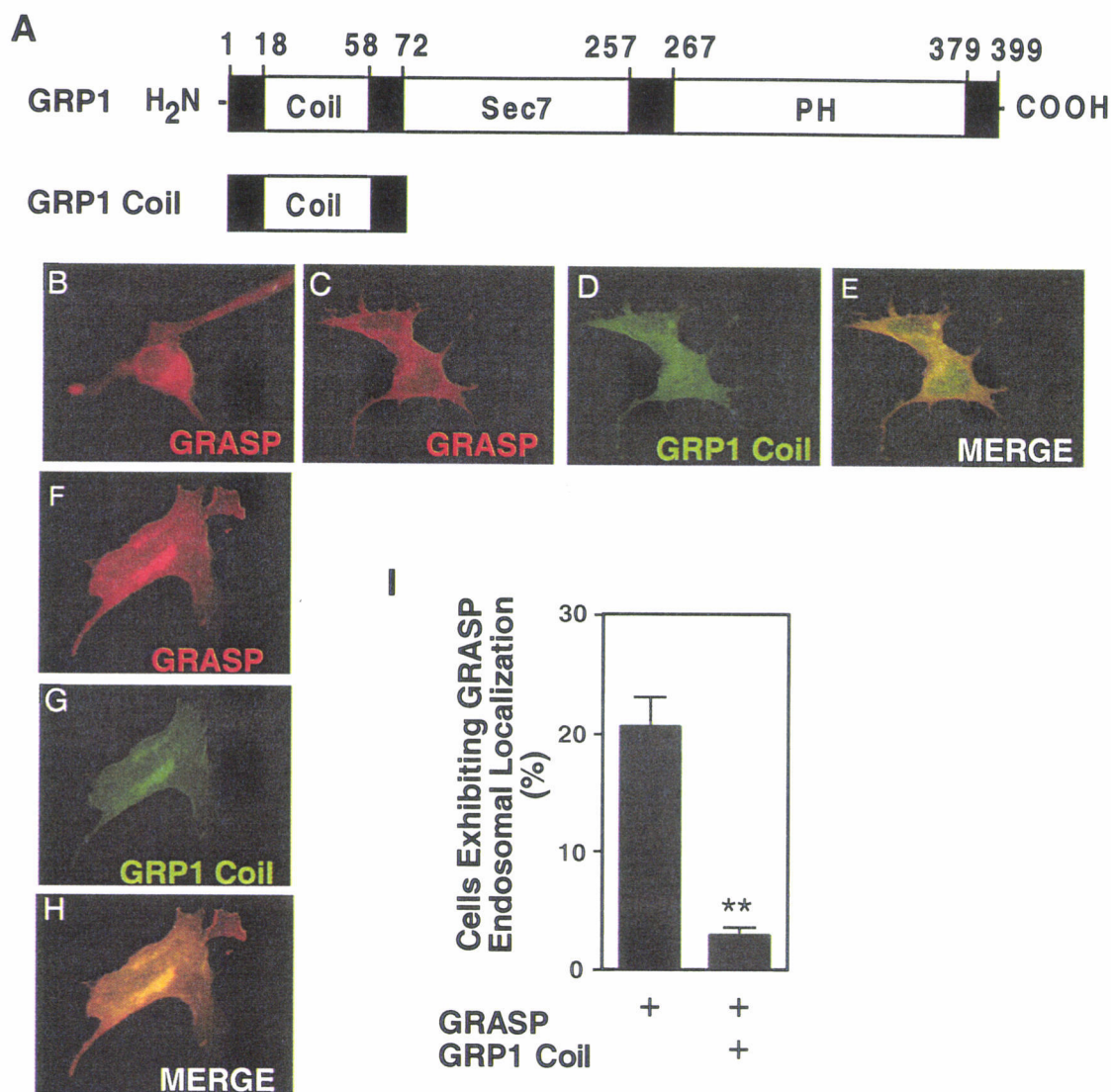


Figure 3.7. Expression of the coiled-coil domain of GRP1 prevents GRASP accumulation in the endosomal compartment. HEK293 cells were transiently cotransfected with an expression vector encoding Myc-GRASP in the absence (*B*) and in the presence (*C-H*) of an equivalent amount of expression vector encoding GRP1-Coil, followed by visualization and scoring of transfected cells for GRASP endosomal localization. *A*, schematic of the GRP1-Coil truncation mutant. *B*, HEK293 cells expressing Myc-GRASP alone. Cotransfected cells shown in *C* and *F* are representative cells stained with the Myc antibody that indicate Myc-GRASP fluorescence while those shown in *D* and *G* correspond to cells stained with the GRP1 antibody representing GRP1-Coil fluorescence. Overlays of the respective Myc-GRASP and GRP1-Coil fluorescence are shown in *E* and *H*. *I*, quantification of cotransfected HEK293 cells that exhibit Myc-GRASP endosomal localization. Statistical significance at the 99% ($p < 0.01$) confidence level is indicated by **. The values represent the mean of three independent experiments in which a minimum of 50 transfected cells were scored.

3.4.8 Residues within the leucine rich region of GRASP specify endosomal targeting

The above results suggest that GRASP targeting to the endosomal compartment occurs independently of interaction with GRP1, and that GRASP is able to recruit GRP1 to the endosome. In order to define the region(s) of GRASP responsible for endosomal targeting, we examined the subcellular distribution of several GRASP mutants by indirect immunofluorescence microscopy and found that only constructs harboring the leucine rich region localized in endosomes (data not shown). Additionally, a construct corresponding to the leucine rich region of GRASP (amino acids 180-257) fused to GFP (Fig. 3.8A) accumulated in the endosomal compartment in a manner similar to that of wild-type GRASP (Fig. 3.8B and F). Moreover, the localization of GFP-GRASP 180-257 was coincident with that of ARF6 N122I (Fig. 3.8C-E), suggesting that residues within the leucine rich domain are necessary and sufficient to induce a block in the ARF6 recycling pathway. Coexpression of either wild-type GRP1 or GRP1 mutants (Fig. 3.8F) prevented GFP-GRASP 180-257 from accumulating in the endosomal compartment as we previously observed in the case of wild-type GRASP (Compare Figs. 3.4-7 with Fig. 3.8F). These results demonstrate that residues within the leucine rich region (amino acids 180-257) of GRASP are necessary and sufficient to target GRASP in the endosomal compartment.

Figure 3.8. Residues within the leucine rich region of GRASP specify endosomal targeting. HEK293 cells were transiently cotransfected with an expression vector encoding GFP-GRASP 180-257 in the absence and in the presence of an expression vector encoding ARF6 N122I-HA or GRP1 and mutants, followed by visualization of transfected cells by fluorescence microscopy. *A*, Schematic of GFP-GRASP 180-257. *B*, HEK293 cells expressing GFP-GRASP 180-257. *C*, HEK293 cells expressing both GFP-GRASP 180-257 and ARF6 N122I-HA, detecting only GFP-GRASP 180-257. *D*, HEK293 cells expressing both GFP-GRASP 180-257 and ARF6 N122I-HA, detecting only ARF6 N122I-HA. *E*, an overlay of the fluorescence from panels *B* and *C*. *F*, Quantification of cotransfected HEK293 cells that exhibit an accumulation of GFP-GRASP 180-257 in the endosomal compartment. Statistical significance at the 99% confidence level ($p < 0.01$) is indicated by ** symbols for comparison with values from cells transfected with GFP-GRASP 180-257 alone. The values represent the mean of three independent experiments in which a minimum of 50 transfected cells were scored

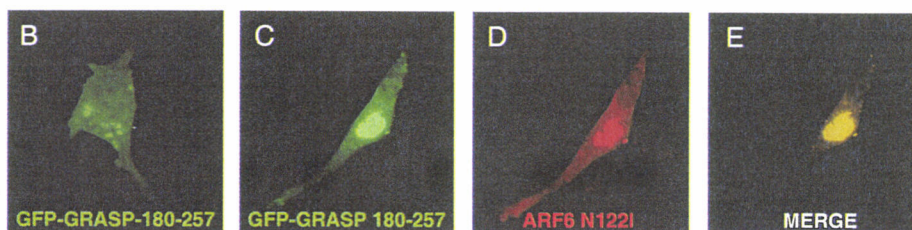
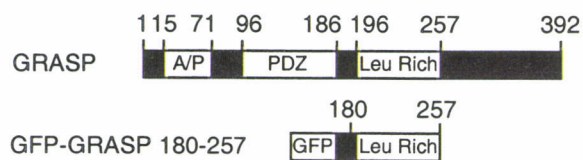
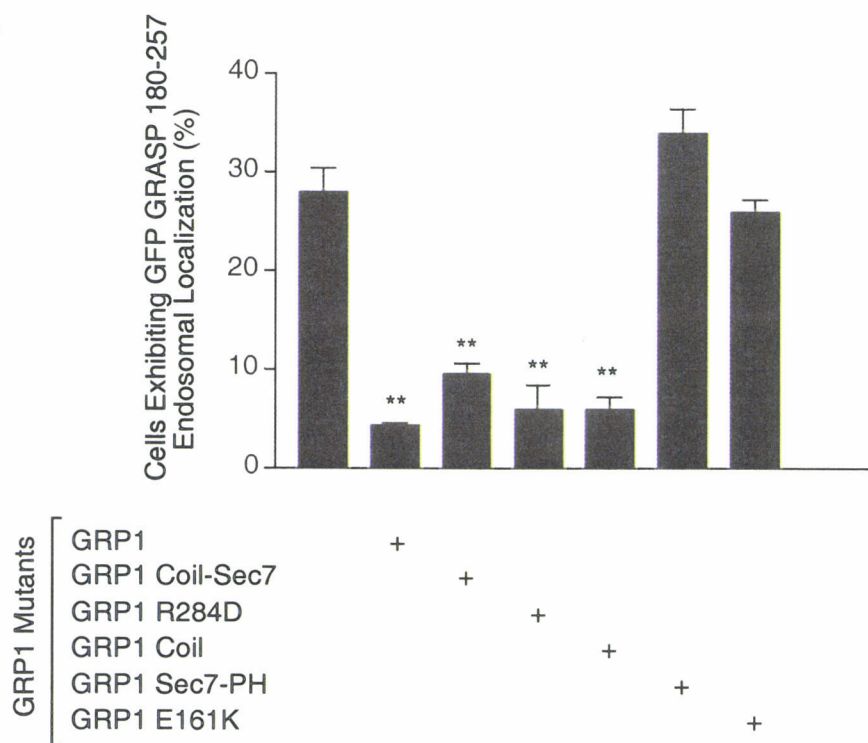
A**F**

Fig. 3.8

3.4.9 Residues within amino acids 180-230 of GRASP are required to mediate interaction with GRP1

Our results indicated that residues within the leucine rich region were responsible for the endosomal localization of GRASP. This finding was somewhat unexpected, as we have shown previously that this region interacts with the ARF6 exchange factors GRP1 and ARNO (Nevrivy, Peterson et al. 2000). These findings suggest the existence of at least two functional domains within the leucine rich region: one specifying interaction with cytohesins, and the other conferring GRASP localization in the endosomal compartment. Alternatively, interaction with cytohesins may be required for endosomal targeting of GRASP. Thus, we conducted experiments to determine if these two functional domains were separable. First, we sought to identify the regions within the leucine rich domain of GRASP (see Fig. 3.9A) that specified interaction with GRP1 by coimmunoprecipitation analyses. GRP1 coimmunoprecipitated with GFP-GRASP 180-230 to a degree at least equal to that of GFP-GRASP 180-257, indicating that residues within amino acids 180-230 are sufficient for interaction with GRP1 (Fig. 3.9B; compare lanes 13 and 12). In contrast, GRP1 did not coimmunoprecipitate with GFP-GRASP 231-392 (Fig 3.9B, lane 14). These results demonstrate that GRASP residues 180-230 are necessary and sufficient for interaction with GRP1.

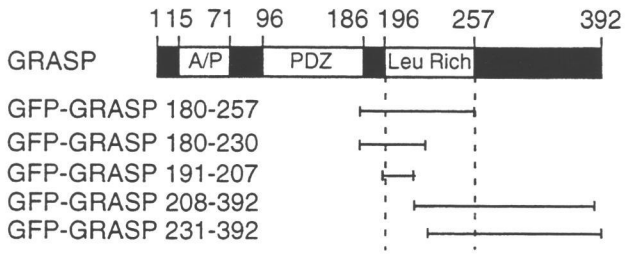
We next attempted to define more precisely which residues within amino acids 180-230 of GRASP mediated the interaction with GRP1. Secondary structural predictions predicted the presence of a coiled-coil motif spanning residues 191-207 (data not shown). Because coiled-coil motifs are common oligomerization motifs, and because we have shown previously that GRASP interacts with residues within the coiled-coil region of GRP1 (Nevrivy, Peterson et al. 2000), we determined if this region was sufficient to interact with GRP1. In addition, a construct encoding the other half of the GRASP leucine rich region was tested for the ability to interact with GRP1 in transfected cells. GRP1 did not coimmunoprecipitate with either GFP-GRASP 191-207, or GFP-GRASP 208-392 (Fig. 3.9C, lane 7 and 8, respectively), however, GRP1 did coimmunoprecipitate with GFP-GRASP 180-230 to a degree observed previously (Fig. 3.9C, lane 6). Control experiments demonstrated expression levels of the GFP-GRASP fusion proteins were equivalent (data not shown). These results suggest that the GRP1 interaction domain of GRASP is extensive and likely spans a large part of the leucine rich region, amino acids 191-207 and 208-230.

3.4.10 Residues within amino acids 208-230 of GRASP are necessary for the endosomal targeting of GRASP

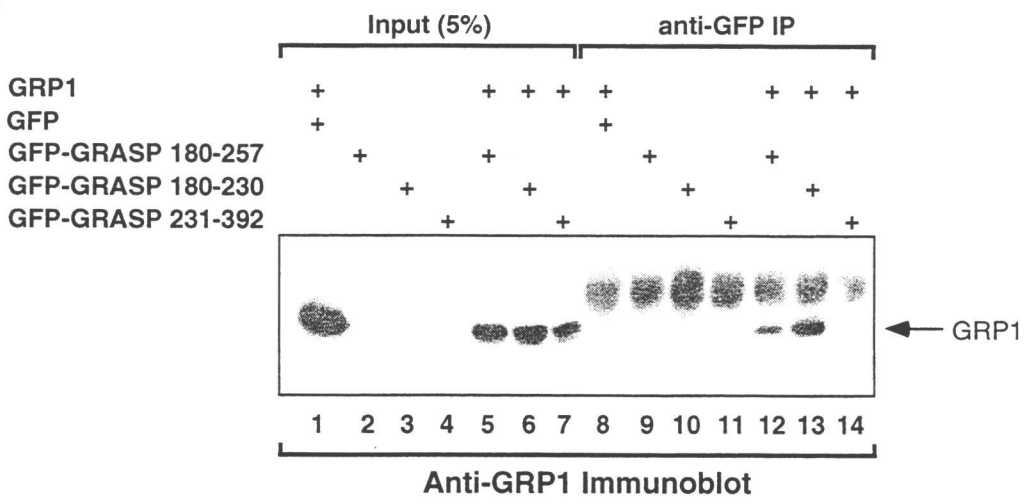
We next determined which region within the leucine rich region of GRASP (residues 180-257) was responsible for targeting GRASP to the endosome. Three

Figure 3.9. Residues within amino acids 180-230 of GRASP are required to mediate interaction with GRP1. Whole cell lysates from HEK293 cells transfected with the indicated expression vectors (3.0 μ g each) were immunoprecipitated using a rabbit anti-GFP polyclonal antibody. Immunoprecipitates were then subjected to immunoblot analysis using an antibody directed against GRP1. *A*, Schematic of the GFP-GRASP truncation mutants. *B*, Coimmunoprecipitation analysis of GRP1 and GFP-GRASP 180-230 and 231-392 truncation mutants. *C*, Coimmunoprecipitation analysis of GRP1 and GFP-GRASP 180-230, 191-207, and 208-392.

A



B



C

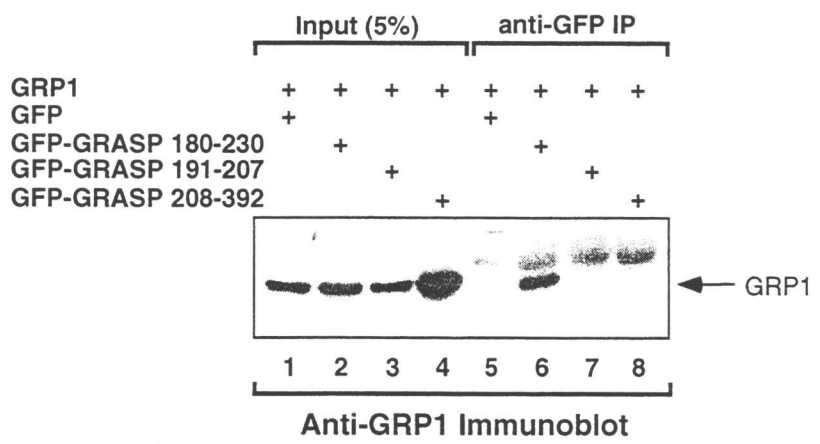


Fig. 3.9

GRASP mutants encoding residues 180-257, 180-230, and 208-392 localized in the endosomal compartment to varying degrees. GRASP 180-257 exhibited endosomal localization in approximately 25% of cells (Figs. 3.10A and F), consistent with previous results (see Figs. 3.8B and F) and to an extent similar to that of wild-type GRASP (Figs. 3.1-7). In contrast, while both GRASP 180-230 (Figs. 3.10B and F), and GRASP 208-392 (Figs. 3.10D and F) localized in endosomes, these mutants did so much less frequently, 5 and 3%, respectively, than either wild-type GRASP or GRASP 180-257. Two GRASP mutants harboring different subregions of the leucine-rich region, GRASP 191-207 (Figs. 3.10C and F) and GRASP 231-392 (Figs. 3.10E and F) did not detectably localize in endosomes. The expression level of all GRASP mutants was similar as verified by immunoblotting (data not shown).

Considered together, these results imply that GRASP amino acids 208-230, which are required for interaction with GRP1 (Fig. 3.9B), harbor an endosomal targeting signal. However, there are two caveats to this interpretation. First, it is clear that amino acids flanking this minimal endosomal targeting signal, i.e., GRASP amino acids 180-207 and 231-257, affect the frequency with which GRASP is targeted to the endosomal compartment. This is most obvious in the latter case as addition of residues 231-257 to GRASP 180-230 resulted in a 5-fold increase in endosomal targeting (Fig. 3.10F). However, the contribution of GRASP amino acids 180-207 was also implied by the reduced endosomal targeting efficiency of GRASP 208-392 (3% frequency; Figs.

3.10D and F) compared to GRASP 180-257 (25% efficiency; Figs. 3.10A and F). Second, the use of these mutants revealed that it was possible to separate the ability of GRASP to interact with GRP1 from endosomal targeting of GRASP, at least partially. This interpretation is based on two observations: (1) GRASP 180-230 interacted with GRP1 at least as efficiently as GRASP 180-257 (Fig. 3.9B), but was targeted to endosomes at a frequency 5-fold less than that of wild-type GRASP (Figs. 3.1-7) or GRASP 180-257 (Figs. 3.10A and F), and (2) GRASP 208-392, which did not interact with GRP1 in cells (Fig. 3.9C), was targeted to endosomes (Figs. 3.10D and F), albeit with a reduced frequency than that of either wild-type GRASP or GRASP 180-257. Thus, it appears that efficient endosomal targeting of GRASP requires interaction with GRP1, or other cytohesin family members, that is conferred by GRASP amino acids 180-230, and GRASP amino acids 231-257 which serve an unknown function.

3.4.11 Amino acids 231-262 target GRASP to the cell periphery rich in cortical actin

We observed that GFP-GRASP 180-230 did not localize in the endosomal compartment at the same frequency compared with that of GFP-GRASP 180-257, even though this mutant interacted with GRP1 to a similar degree. We hypothesized that the reduced frequency may be due to an inefficiency of this mutant in entering the ARF6 recycling pathway compared with that of GFP-GRASP 180-257. This would

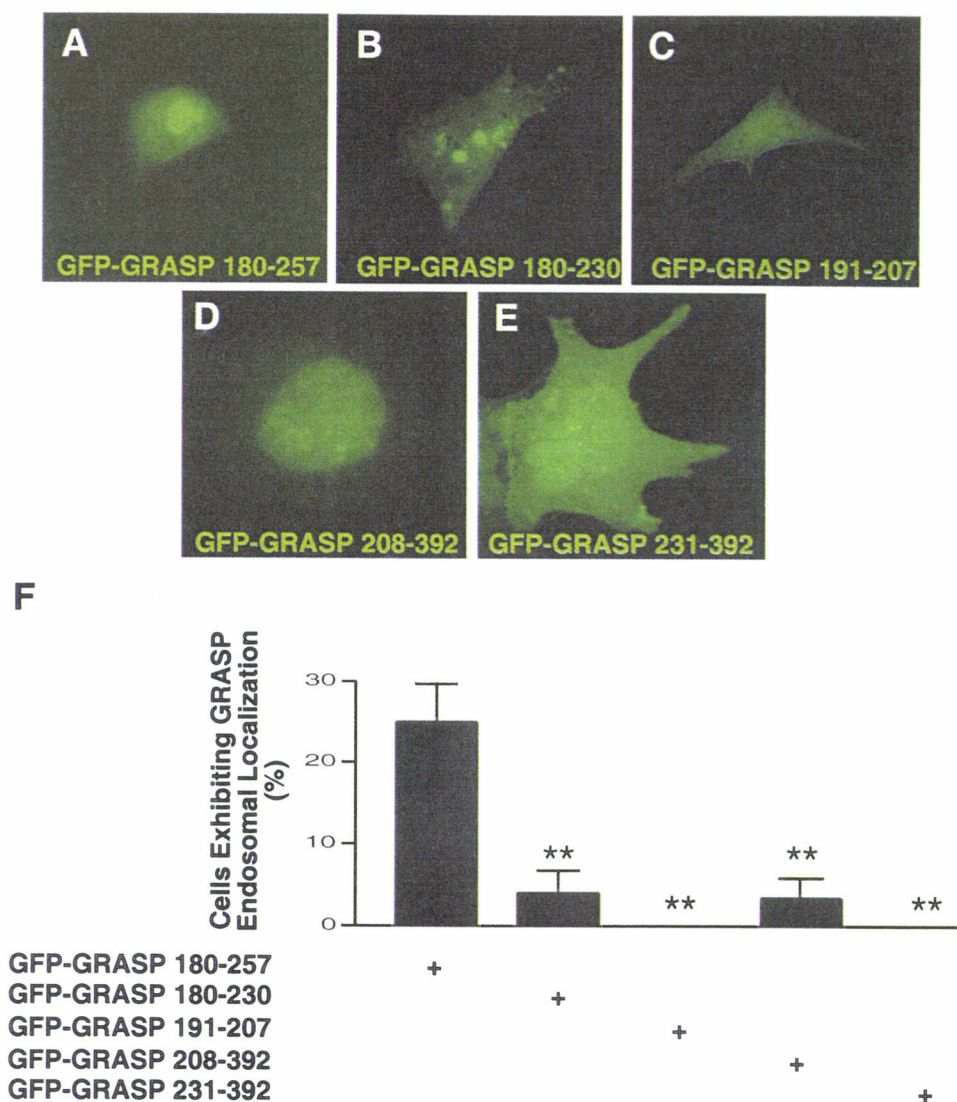


Figure 3.10. Residues within amino acids 208-230 of GRASP are necessary for to target GRASP in the endosomal compartment. HEK293 cells were transfected with GFP-GRASP truncation mutants and visualized after 24 hours by fluorescence microscopy. *A*, HEK293 cell expressing GFP-GRASP 180-257. *B*, HEK293 cell expressing GFP-GRASP 180-230. *C*, HEK293 cell expressing GFP-GRASP 191-207. *D*, HEK 293 cell expressing GFP-GRASP 208-392. *E*, HEK 293 cell expressing GFP-GRASP 231-392. *F*, quantification of transfected HEK293 cells that exhibit accumulation of the GFP-GRASP truncation mutants in the endosomal compartment. Statistical significance at the 99% confidence interval ($P < 0.01$) is indicated by ** symbols comparing values with GFP-GRASP 180-257. The values represent the mean of three independent experiments in which a minimum of 50 transfected cells were scored

necessitate that residues 231-257 are involved in facilitating GRASP entry into the recycling pathway. Curiously, we observed that GFP-GRASP 231-392, a mutant that was not observed in the endosomal compartment, localized predominantly at the cell periphery in what appeared to be cortical actin-based structures (Fig. 3.10E). A similar localization has been observed with full-length GRASP, although to a lesser degree (data not shown), suggesting that the localization of the mutant is not artifactual and may arise out of an inability to become internalized on endosomes. Therefore, we determined if residues within amino acids 231-262 were required to target GRASP to the cell periphery. HEK293 cells were transfected with expression vectors encoding GFP-GRASP 231-392 or GFP-GRASP 263-392 (Fig 3.11A) and visualized by fluorescence microscopy. Cells were counterstained with rhodamine-phalloidin to visualize filamentous actin. As observed previously, GFP-GRASP 231-392 localized at the cell periphery (Fig. 3.11B) and GFP fluorescence was coincident with cortical actin (Figs. 3.11B-D). In addition, the fusion protein exhibited substantial colocalization with short actin filaments that appeared to be distributed at the cell surface (Fig. 3.11D). In contrast, GFP-GRASP 263-392 did not localize at the cell periphery or with actin-based structures, but appeared to be restricted to the cytosol and the nucleus, where large globular aggregates of the fusion protein were observed (Fig. 3.11 E-G). The molecular basis for the aberrant, nuclear distribution of GRASP 263-392 is unknown but may be artifactual arising only when all other targeting signals are removed. These

results demonstrate that residues within amino acids 231-262 of GRASP are required for localization at the cell periphery and actin-based structures, and suggest that localization to these areas may be an important determinant in facilitating entry of GRASP into an ARF6-mediated recycling pathway.

3.5 Discussion

Overexpressed GRASP was found to exhibit a punctate juxtanuclear localization in HEK293 cells that resembled the recycling endosomal compartment in which GTP-binding mutants of ARF6 reside (D'Souza-Schorey, Li et al. 1995). Coexpression of GRASP and ARF6 N122I increased the degree to which GRASP localized in the endosomal compartment, indicating that GRASP and ARF6 likely function in identical or highly related plasma membrane recycling pathway(s).

Numerous studies have demonstrated that overexpression of ARF6 T27N, a GTP-binding mutant similar to ARF6 N122I, results in an accumulation of recycling endosomes and a block in endosomal recycling (D'Souza-Schorey, Li et al. 1995; Radhakrishna and Donaldson 1997; D'Souza-Schorey, van Donselaar et al. 1998). We propose that GRASP may induce a similar block in recycling based on the following observations: (1) in cells that exhibited an endosomal localization of GRASP, the morphology of the endosomal compartment was indistinguishable from that of cells

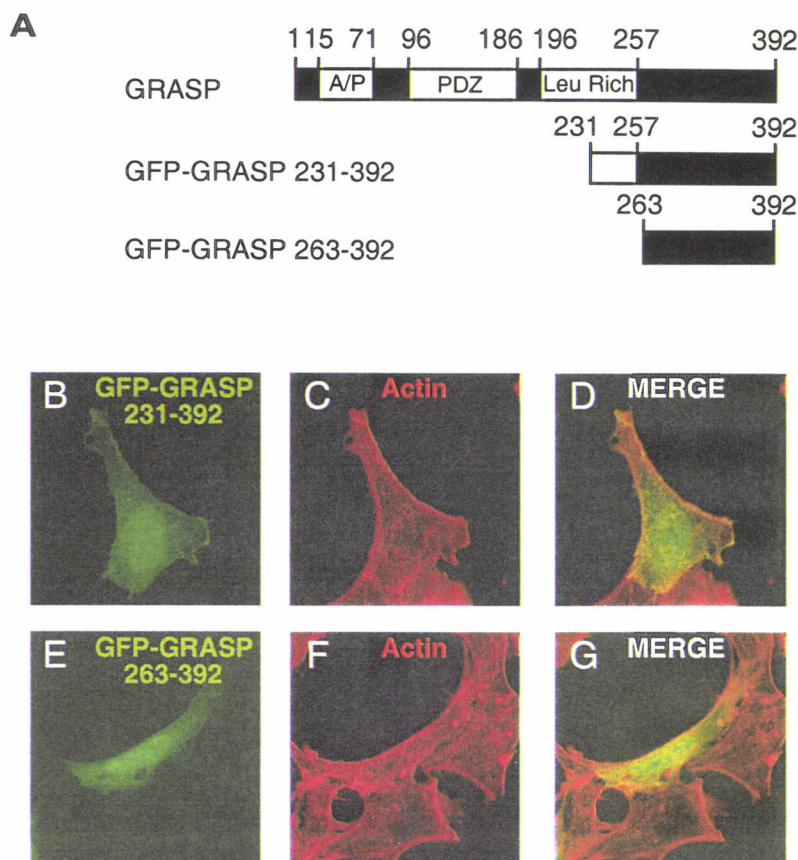


Figure 3.11. Amino acids 231-262 target GRASP to areas of the cell periphery rich in cortical actin. HEK293 cells were transfected with GFP-GRASP truncation mutants and were visualized after 24 hours by fluorescence microscopy. Cells were labeled with rhodamine-phalloidin to visualize filamentous actin. **A**, Schematic of the GFP-GRASP truncation mutants. Transfected cells shown in **B** and **E** are representative cells expressing GFP-GRASP 231-392 and GFP-GRASP 263-392, respectively, while those shown in **C** and **F** correspond to cells stained with rhodamine-phalloidin. Overlays of the respective GFP-GRASP and rhodamine-phalloidin fluorescence are shown in **D** and **G**.

expressing ARF6 N122I, (2) the accumulation of GRASP-positive endosomes was a function of the level of GRASP expression, and (3) coexpression of GRP1, but not a catalytically inactive mutant, dramatically reduced the accumulation of GRASP in the endosomal compartment.

Coexpression of constitutively active ARF6, ARF6 Q67L, with GRASP did not affect the endosomal localization of GRASP. This was unexpected considering that in TRVb-1 cells expressing ARF6 Q67L, transferrin receptors constitutively localize at the cell membrane and are neither internalized nor exhibit endosomal localization (D'Souza-Schorey, Li et al. 1995). Furthermore, an ultrastructural analysis of cells expressing ARF6 Q67L revealed a virtual absence of endosomes in the cell (Peters, Hsu et al. 1995). In contrast, in the presence of overexpressed GRASP, ARF6 Q67L does not appear to affect the endosomal system to the same degree, as structures indicative of endosomes were clearly both evident and abundant. One explanation for this apparent discrepancy would involve GRASP acting in concert with the endogenous ARF6 in promoting endocytosis. Alternatively, this observation would also be consistent with GRASP overexpression exerting a dominant negative effect on ARF6 signaling in the cell, in a manner that is similar to overexpression of GTP binding-deficient mutants of ARF6. ARF6 GTP binding deficient mutants, such as ARF6 T27N and ARF6 N122I, are thought to exert their dominant negative effects by binding to and sequestering GEFs (Boguski and McCormick 1993). As we observed that the endosomal

localization of GRASP was dependent on high expression levels of the protein, we hypothesized that this occurred due to limiting GEF concentrations in the cell and a failure of the GEF to be recruited to endosomes and stimulate nucleotide exchange, resulting in an accumulation of endosomes and a block in recycling. Under these circumstances, accessibility of the GEF to the ARF6 Q67L mutant would be reduced and effects of the mutant diminished. Also, it is noted that interpretation of these results may be further complicated by the use of epitope-tagged versions of ARF6 and ARF6 mutants, which may function inefficiently relative to native proteins and this may have affected the ability of ARF6 mutants to exert a dominant phenotype (Radhakrishna, Al-Awar et al. 1999; Al-Awar, Radhakrishna et al. 2000).

Numerous studies have supported the view that the ARF6 cycle may be unique amongst ARFs in that ARF6 does not cycle between membrane and soluble forms, but rather is constitutively bound to membranes regardless of its GTP status (Cavenagh, Whitney et al. 1996). ARF6 is thought to cycle between a plasma membrane and GTP-bound state and intracellular vesicle and GDP-bound state. However, we did not observe localization of wild-type ARF6 in the endosomal compartment in the presence or absence of GRASP. In contrast, colocalization of ARF6 N122I and GRASP was clearly observed in the endosomal compartment. These observations may suggest that ARF6 does not remain constitutively bound to membranes throughout its GTP/GDP cycle, and may require recruitment by the ARF6 GEF to recycling endosomes to become

activated. In support of this notion, it has been demonstrated that ARF6 is recruited along with ARNO from the cytosol to proximal tubule endosomes in a pH-dependent manner (Maranda, Brown et al. 2001), which has been speculated to result in nucleotide exchange of ARF6 and endosomal fusion with the plasma membrane (Maranda, Brown et al. 2001). In addition, it has been shown that membrane-bound ARF6 could be released into the cytosol upon incubation in buffers containing physiologic concentrations of magnesium, supporting a mechanism of membrane to cytosol shuttling of ARF6 (Gaschet and Hsu 1999). As our results suggest that the accumulation of GRASP in the endosomal compartment arises due to limiting ARF6 GEF concentrations in the cell, the absence of ARF6 on the endosomal compartment would be consistent with a mechanism of ARF6 recruitment to recycling endosomes by an ARF6 GEF. However, it is important to consider that ARF6 mechanisms of plasma membrane recycling may vary depending on the nature of internalized cell surface proteins, the cell type, and the presence/absence of regulatory proteins. This is highlighted by the fact that ARF6 regulates the transferrin receptor recycling pathway in CHO (D'Souza-Schorey, Li et al. 1995), but not HeLa cells (Radhakrishna, Klausner et al. 1996; Radhakrishna and Donaldson 1997). In addition, it is possible that our failure to visualize ARF6 in the endosomal compartment with GRASP may be due to the use of an epitope-tagged ARF6, which may internalize less efficiently than the endogenous ARF6. Although we did not observe ARF6 costaining on the endosomal compartment

with GRASP, it is also possible that ARF6 localization in the endosomal compartment was beyond our limits of detection in this experiment.

Our hypothesis that the endosomal localization of GRASP results from titration of ARF6 GEFs and/or sequestration of endogenous ARF6 GEFs in the cytosol is supported by our finding that coexpression of an ARF6 GEF, GRP1, dramatically reduced the accumulation of GRASP-positive endosomes. However, neither catalytically inactive GRP1 nor a GRP1 mutant lacking the GRASP-interaction domain rescued the GRASP-positive endosomal phenotype. These findings suggest that ARF6-mediated endosomal recycling requires at least: (1) GRASP-GRP1 complex formation on endosomes and (2) GRP1-catalyzed nucleotide exchange on ARF6.

Overexpression of ARNO3, the human homologue of GRP1, did not result in redistribution of transferrin receptors from the recycling endosomal compartment to the plasma membrane of TRNb-1 cells (Franco, Peters et al. 1999), suggesting that ARNO3 does not function as an ARF6 GEF in transferrin receptor recycling. It would be of interest to determine if ARNO3 regulates transferrin receptor recycling in TRNb-1 cells when GRASP is present as our results in HEK293 cells clearly implicate a role for GRP1 in a recycling pathway regulated by ARF6. Coupling of GRASP and GRP1, on the other hand, may result in the targeting of specific classes of cell surface proteins for the ARF6-regulated recycling pathway.

We did not expect that expression of the coiled-coil fragment of GRP1 would prevent the accumulation of GRASP in the endosomal compartment. Rather, we anticipated that the coexpression of the isolated GRP1 coiled-coil would lead to an enhancement of GRASP localization in the endosomal compartment. This was based on our assumption that the coiled-coil region of GRP1 would function as a dominant negative inhibitor of endogenous cytohesins in HEK293 cells. Thus, we expected that the GRP1 coiled-coil region would bind to GRASP on endosomes and block access of endogenous cytohesins at this locus resulting in decreased GEF-catalyzed nucleotide exchange on ARF6, compromised endosomal recycling to the plasma membrane, and accumulation of GRASP-positive endosomes. The lack of GRASP localization in the endosomal compartment in the presence of GRP1-coiled-coil appears to be in conflict with our hypothesis that GRASP recruits GRP1 to the endosomal compartment, and rather suggests that GRP1, or perhaps other cytohesins recruit GRASP to endosomes. However, our data clearly indicate that GRASP recruits GRP1 to the endosomal compartment because: (1) localization of GRP1 and fragments of GRP1 containing the coil-coil domain were observed on endosomes in the presence, but not in the absence of GRASP, (2) a GRASP mutant, GFP-GRASP 208-392, was unable to interact with GRP1 in extracts of transfected cells yet this mutant localized in the endosomal compartment, at least partially, and (3) we were unable to detect endogenous GRP1 or ARNO localization in the endosomal compartment when GRASP was present (data not

shown). Taken together, our data indicate that GRASP localizes in the endosomal compartment independently of GRP1 and is able to recruit GRP1 to the endosomal compartment.

Because overexpression of the coiled-coil fragment of GRP1 would be predicted to function in a dominant negative manner by preventing GRASP from interacting with endogenous cytohesins, our data therefore suggest that interaction of GRASP with endogenous cytohesins at a stage in the ARF6 recycling pathway prior to trafficking to the endosomal compartment appears to facilitate GRASP localization in this compartment. Indeed, our results demonstrate that GFP-GRASP 208-392, which failed to interact with GRP1 by coimmunoprecipitation analyses, localized in the endosomal compartment at a reduced frequency compared with that of GFP-GRASP 180-257, which we have shown interacts with GRP1. These findings support our hypothesis that GRASP interaction with GRP1, or other cytohesins, at a stage in the recycling pathway upstream of trafficking to the endosomal compartment facilitates the localization of GRASP in this compartment.

A key question that arose from these results is how GRASP enters the recycling pathway. We observed that GFP-GRASP 180-230, which interacted with GRP1 to a similar degree as GFP-GRASP 180-257, localized in the endosomal compartment at a reduced frequency (5% versus 25%; Fig. 3.10F). These results suggested that amino acids 231-257 of GRASP may play a role in increasing the frequency at which GRASP

localized in the endosomal compartment. Our results indicated these residues (amino acids 231-262 of GRASP) were required to target GRASP to the cell periphery and cortical actin-containing structures. As endosomes are derived from the plasma membrane, it seems reasonable to speculate that localization of GRASP to cortical areas, mediated by amino acids 231-262, may represent an early event regulating entry of GRASP into the ARF6 recycling pathway, ultimately leading to internalization of GRASP at the plasma membrane and localization in the endosomal compartment. Our results demonstrating that GFP-GRASP 180-230 exhibited a reduced frequency of localization in the endosomal compartment, may therefore be due to an inability of this mutant to be targeted to the cell periphery and cortical areas, resulting in a reduced capacity to enter the ARF6 recycling pathway.

Our results, therefore, suggest that in addition to interaction with cytohesins, proper targeting of GRASP to the cell periphery is required to facilitate GRASP entry into the ARF6 recycling pathway. We envision that the targeting of GRASP to cortical areas results in the recruitment of a cytohesin and, ultimately, ARF6 (Fig. 3.12) to these loci. This juxtaposition of GRASP and ARF6 may ultimately lead to the internalization of GRASP on ARF6-containing endosomes during GTPase activating protein (GAP)-catalyzed hydrolysis of ARF6-GTP. Because we observe that GRASP localizes in the endosomal compartment independently of GRP1, we propose that, at some stage in the ARF6 recycling pathway prior to trafficking to the endosomal compartment, there is an

uncoupling of the GRASP-GRP1 interaction. Interestingly, there exist putative phosphorylation sites on serine, threonine, and tyrosine within residues 189-230 of GRASP (data not shown), which encompasses the region of interaction with GRP1. Such an uncoupling would allow the newly liberated GRP1 to bind additional GRASP proteins, perhaps at the plasma membrane to begin a new round of ARF6 recruitment and internalization. Multiple rounds of uncoupling and internalization, may lead to an accumulation of GRASP in the endosomal compartment. If GRASP expression levels are high due to transient transfection or, perhaps RA treatment, soluble GRASP may sequester GRP1 at cytoplasmic loci. This may compromise the function of GRP1 in catalyzing nucleotide exchange on and activation of ARF6 at the level of the endosome, resulting in a block in endosomal recycling to the plasma membrane.

Although our results do not support a role for either the GRP1 PH domain, or PIP₃ binding by GRP1 in the regulation of the ARF6 recycling pathway in the presence of GRASP, it is important to note that our results were generated in cells under non-stimulated conditions. Therefore, we cannot rule out the possibility that under conditions of PI 3-kinase activation, GRASP may enter the recycling pathway via recruitment by a cytohesin at the plasma membrane. Indeed, we observed that GFP-GRASP 180-230 localized in the endosomal compartment at a reduced frequency, presumably because this mutant lacked residues necessary for proper targeting to the cell periphery. It will be of interest to determine if this mutant enters the recycling

pathway through PIP_3 -dependent recruitment of cytohesin-GRASP complexes to the plasma membrane.

A broader question relates to the central function of GRASP in the ARF6 cycle. GRASP harbors a PDZ domain in the amino terminal region of the protein, and these domains have been shown to bind short C-terminal sequences present in numerous transmembrane receptors (Harris and Lim 2001). Interestingly, the PDZ domain-mediated interaction of NHERF/EBP50 with the $\beta 2$ adrenergic receptor ($\beta 2$ -AR) has been shown to be essential for recycling of the receptor following agonist-induced endocytosis (Cao, Deacon et al. 1999). Disruption of the interaction, likely through phosphorylation of the C-terminal tail of the receptor, directs the receptor to the lysosome for degradation (Cao, Deacon et al. 1999). Furthermore, incorporation of the PDZ binding motif of the $\beta 2$ -AR into the nonrecycling delta opioid receptor, redirects that receptor to recycle, implicating the PDZ binding motif of the $\beta 2$ -AR as a bona fide sorting signal (Gage, Kim et al. 2001). The presence of a PDZ domain in GRASP, coupled with our observations demonstrating GRASP participates in the ARF6 recycling pathway, would make GRASP an ideal candidate to target receptors for the recycling pathway.

GRASP was originally identified as a retinoic acid-induced gene product (Nevrivy, Peterson et al. 2000). We consistently observed 15-20-fold induction of

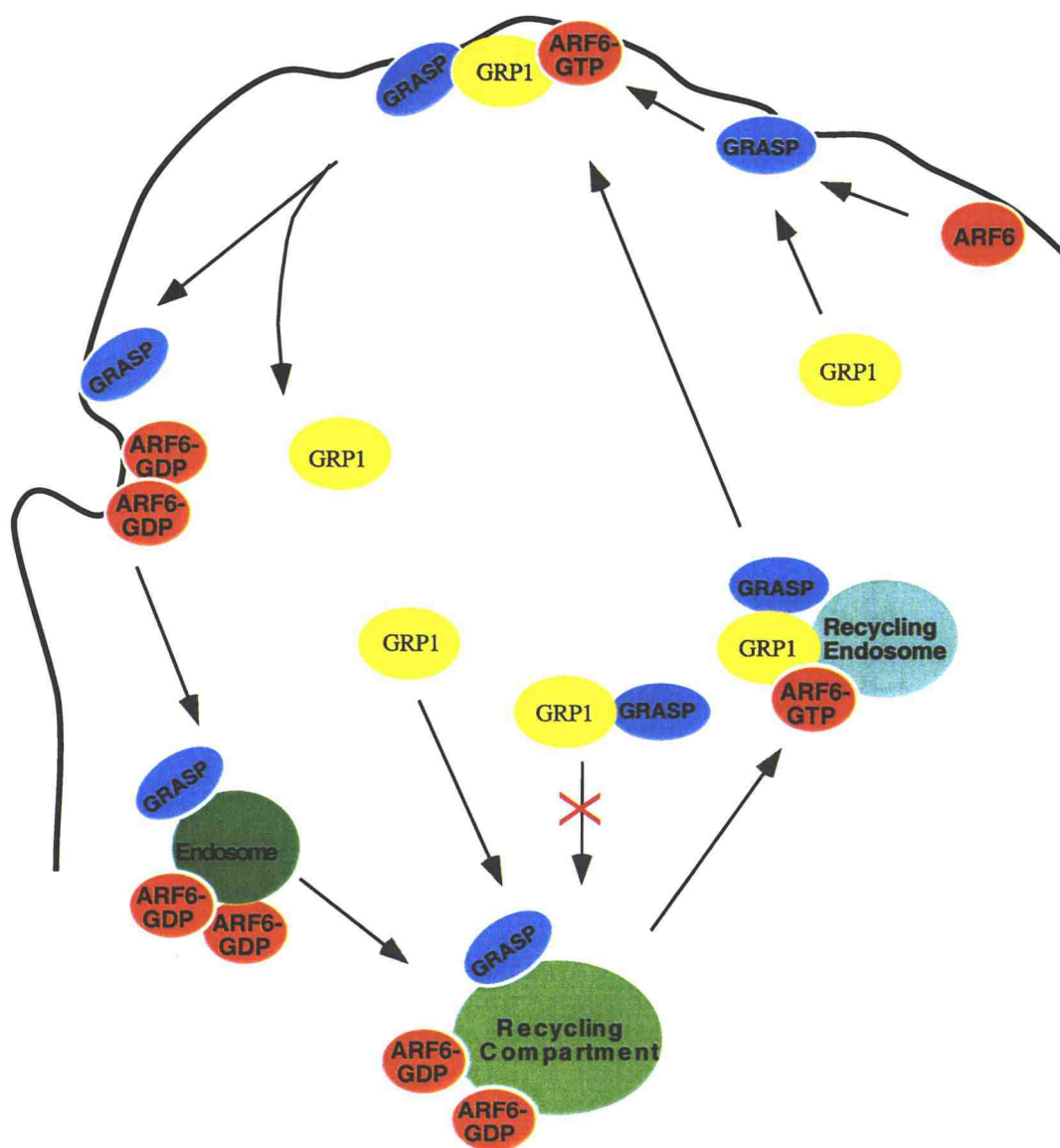


Figure 3.12. **Proposed mechanism of GRASP action in regulating the ARF6 plasma membrane recycling pathway.** GRASP is predicted to enter the pathway by recruitment of GRP1 (or other cytohesins), followed by ARF6 to plasma membrane loci. Following hydrolysis of ARF6-GTP, GRASP along with ARF6-GDP is internalized and localizes in the endosomal compartment. GRASP accumulates in this compartment when levels of GRP1 are low and/or GRP1 is sequestered in the cytosol by GRASP. Recruitment of GRP1 to the GRASP-positive endosomes is predicted to result in nucleotide exchange on ARF6-GDP and trafficking of the endosome to the plasma membrane.

GRASP transcripts during RA-induced differentiation of P19 cells (Nevrivy, Peterson et al. 2000). As our data suggest that the block in recycling induced by GRASP is due to a stoichiometric imbalance of GRASP and an ARF6 exchange factor, it will also be of interest to determine if GRASP induction by RA results in a similar imbalance and effect on the ARF6 cycle during RA-induced cellular differentiation.

3.6 Abbreviations

The abbreviations used are: GRP1, general receptor for phosphoinositides 1; ARF, ADP-ribosylation factor; ARNO, ARF nucleotide-binding site openner; GST, glutathione *S*-transferase; GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; PH, pleckstrin homology; SH3, Src homology 3; BFA, brefeldin A; HEK, human embryonic kidney; GFP, green fluorescent protein.

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Chapter 4

Summary

Retinoic acid is known to exert profound effects on development and differentiation in a wide variety of systems (Gudas 1994) and is also used therapeutically in the treatment of various skin diseases (Schaefer and Reichert 1990) and acute promyelocytic leukemia (Huang, Ye et al. 1988). RA has also shown effectiveness in the prevention and treatment of many other cancers mediated by its effects on growth inhibition and induction of differentiation to nonneoplastic phenotypes (Bollag and Holdener 1992) although the teratogenic effects of retinoids and acquisition of resistance greatly limit the efficacy of retinoids in many of these systems. Therefore, a greater understanding of the cellular and molecular mechanisms of action of RA may aid in the development of more effective treatments, and contribute additional knowledge towards an understanding of the role of retinoids in the control of cellular and developmental processes. The studies described herein were designed to contribute additional knowledge of the cellular mechanisms of action of RA through the identification and functional characterization of a novel retinoic acid-induced gene from P19 embryonal carcinoma cells.

GRASP was identified as a novel RA-induced gene and induction of GRASP transcripts were observed within 2 hrs. of RA treatment. GRASP was also induced by RA in the presence of cycloheximide, albeit to a reduced degree, suggesting that GRASP may be a direct target gene of RA in P19 cells. Analysis of the predicted amino acid sequence of GRASP revealed that GRASP contained several known protein-protein interaction motifs, suggesting that GRASP may function as an adapter protein in signal transduction pathways. Therefore, towards the goal of identifying which signaling pathways GRASP may be involved in, we utilized a two-hybrid screen and identified GRP1 as a GRASP interaction partner. GRASP was found to enhance GRP1 association with the plasma membrane and colocalize with endogenous ARFs.

Upon overexpression, GRASP was observed to accumulate in an intracellular endosomal compartment where GTP-binding deficient mutants of ARF6 reside, suggesting that GRASP induced a block in the ARF6 plasma membrane recycling pathway. Coexpression of GRP1 dramatically reduced the capacity of GRASP to accumulate in this compartment, whereas neither a catalytically inactive mutant of GRP1 nor a GRP1 mutant that lacked the region of interaction with GRASP affected the accumulation of GRASP in this compartment. These results support the hypothesis that the accumulation of GRASP in the endosomal compartment was due to an inability of GRP1 to be recruited to the endosome to stimulate nucleotide exchange on ARF6 and recycling. Regions within GRASP were identified that dictated efficient accumulation

of GRASP in the endosomal compartment, and these residues also dictated interaction with GRP1 and localization of GRASP to cortical actin-based structures at the cell periphery.

These results support a model of GRASP mediated recruitment of GRP1 and ARF6 to plasma membrane loci to initiate endocytosis and recycling. Furthermore, as GRASP contains common protein-protein interaction motifs that are known to bind receptors at the cell surface, our results support a role for GRASP in targeting cell surface receptors for the ARF6 recycling pathway. In light of results demonstrating that the expression level of GRASP influences endosomal recycling, GRASP may be predicted to exert a biphasic effect on this pathway. At concentrations of GRASP that do not exceed that of GRP1 (or other cytohesins) in the cell, GRASP expression would be predicted to direct endosomal components for the recycling pathway, as GRASP would recruit GRP1 to the endosome, resulting in nucleotide exchange of ARF6 and recycling to the plasma membrane. In contrast, at high concentrations of GRASP (relative to GRP1), GRASP would accumulate in the endosomal compartment and endosomal components would not recycle to the plasma membrane. In either scenario, RA induction of GRASP would be predicted to significantly modulate both the amplitude and kinetics of signaling pathways regulated by plasma membrane receptors to which GRASP binds, mediated by changes in occupancy of the receptors at the cell surface. Such alterations, as have been demonstrated for a number of cell surface

receptors (Waterman and Yarden 2001), may have significance in the regulation of cellular and developmental processes by RA.

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