The GRP1 protein contains a Sec7 homology domain that catalyzes guanine nucleotide exchange on ARF-ribosylation factors (ARF) 1 and 5 as well as a pleckstrin homology domain that binds phosphatidylinositol-(3,4,5)P₃, an intermediate in cell signaling by insulin and other extracellular stimuli (Klarlund, J. K., Guilherme, A., Holik, J. J., Virbasius, J. V., Chawla, A., and Czech, M. P. (1997) Science 275, 1927–1930). Here we show that both endogenous GRP1 and ARF6 rapidly co-localize in plasma membrane ruffles in Chinese hamster ovary (CHO-T) cells expressing human insulin receptors and COS-1 cells in response to insulin and epidermal growth factor, respectively. The pleckstrin homology domain of GRP1 appears to be sufficient for regulated membrane localization. Using a novel method to estimate GTP loading of expressed HA epitope-tagged ARF proteins in intact cells, levels of biologically active, GTP-bound ARF6 as well as GTP-bound ARF1 were elevated when these ARF proteins were co-expressed with GRP1 or the related protein cytohesin-1. GTP loading of ARF6 in both control cells and in response to GRP1 or cytohesin-1 was insensitive to brefeldin A, consistent with previous data on endogenous ARF6 exchange activity. The ability of GRP1 to catalyze GTP/GDP exchange on ARF6 was confirmed using recombinant proteins in a cell-free system. Taken together, these results suggest that phosphatidylinositol-(3,4,5)P₃ may be generated in cell membrane ruffles where receptor tyrosine kinases are concentrated in response to growth factors, causing recruitment of endogenous GRP1. Further, co-localization of GRP1 with ARF6, combined with its demonstrated ability to activate ARF6, suggests a physiological role for GRP1 in regulating ARF6 functions.

Signaling through receptor tyrosine kinases regulates multiple processes critical to cell growth, differentiation, and viability (1–3). A common element of receptor tyrosine kinase signaling that is required to regulate many cellular processes appears to be a class of enzymes, PtdIns 3-kinases (4–6), that catalyze phosphorylation of the 3′-position on phosphatidylinositol (PtdIns) or its phosphorylated derivatives to produce PtdIns(3)P, PtdIns(3,4)P₂, or PtdIns(3,4,5)P₃. We recently identified a protein, GRP1, that binds PtdIns(3,4,5)P₃ with high affinity through its pleckstrin homology (PH) domain and contains a Sec7 homology domain that catalyzes guanine nucleotide exchange of ARF-ribosylation factor (ARF) proteins (7, 8). Two isoforms of GRP1 denoted ARNO (9) and cytohesin-1 (10) with similar domain structures have also been identified. GRP1, ARNO, and cytohesin-1 expressed in cultured cells as fusion proteins of green fluorescent protein are rapidly recruited to the plasma membrane in response to receptor tyrosine kinase activation (11–13). Such recruitment appears to require the PH domains of these proteins. However, neither the cellular localization nor the regulation of endogenous GRP1-like proteins has yet been characterized.

ARF proteins appear to regulate membrane trafficking pathways and are converted from a biologically inactive, GDP-bound state to an active, GTP-bound form by guanine nucleotide exchange factors (14). Six different mammalian isoforms of ARF have been identified and classified based on size and sequence similarities: ARF1, -2, and -3 in class 1; ARF4 and -5 in class 2; and ARF6 in class 3 (14). ARF6 differs from the other five isoforms in that it is located primarily in the plasma membrane and pericentriolar regions of the cell (15–17), functions in endosome cycling (15, 16, 18) and actin polymerization (19, 20), and is brefeldin A-resistant (17, 21). The specificity of Sec7 domains for catalyzing guanine nucleotide exchange on ARF proteins is at present unresolved. Frank et al. (22) observed that GTP loading of ARF6 in vitro was increased by ARNO, while in other studies by two independent laboratories, recombinant GRP1 appeared not to catalyze ARF6 exchange using different assay methods (8, 23). Based on these latter results and the disruption of Golgi function by expression of high levels of human GRP1, Franco et al. (23) have proposed that ARF1 is the major substrate for GRP1 in intact cells. These authors have proposed that ARF6 is not a substrate for GRP1. However, in contrast to this idea, the co-localization of ARNO and the rat homologue of cytohesin-1 with ARF6 rather than ARF1 (12, 22) suggested further evidence of an interrelationship between GRP1-like proteins and ARF6. Thus, the aim of the present study was to clarify whether GRP1 is able to enhance GTP loading of ARF6 in intact cells, and to determine whether PtdIns(3,4,5)P₃ generation by receptor tyrosine kinases actually regulates the co-localization of endogenous GRP1 with ARF6. Here we show the wortmannin-sensitive translocation of endogenous GRP1 from intracellular stores to plasma membrane ruffles and the co-localization of heterologously expressed as well as endogenous GRP1 and ARF6 in membrane ruffles. Further, both GRP1 and the related protein cytohesin-1 cause significantly elevated levels of GTP-bound ARF6 when expressed in COS-1 cells, suggesting that ARF6...
function is indeed regulated by these PH and Sec7 domain-containing proteins.

EXPERIMENTAL PROCEDURES

Materials—Wild type and mutant ARF cDNA constructs were kindly provided by Dr. V. Hsu, and myristoylated ARF6 protein was provided by Dr. J. Cassanova. The cDNA encoding cytohesin-1 was provided by Dr. B. Seed. Two different GRP1 cDNA constructs were synthesized containing C-terminal MYC (AEEKQLISEEEDLKLG) or HA (YPYDVPDYA) epitope tags. The GRP1 Sec7, PH, and coiled-coil domains were fused at the C terminus with HA epitope tags and cloned into pCMV5 vectors. Affinity-purified GRP1 antibodies were produced by injecting rabbits with a GRP1-glutathione S-transferase (GST) fusion protein attached to glutathione-conjugated agarose beads. Antibodies directed against GST were removed with several incubations with lysates of bacteria expressing GST. Serum was then incubated with GRP1-GST immobilized on Affi-Gel 10 (Bio-Rad) and washed with Tris buffer (10 mM Tris, pH 7.5) and Tris-NaCl buffer (10 mM Tris, 0.5 mM NaCl, pH 7.5). Antibodies were eluted first with 100 mM glycine (pH 2.5) and then 100 mM triethylamine (pH 11.5). Antibodies found in the glycine and triethylamine eluates were pooled and stored at −20 °C in 50% glycerol. The anti-MYC monoclonal antibody MYC1-9E10.2 (American Type Culture Collection) was produced against a human c-MYC polypeptide conjugated to keyhole limpet hemocyanin (24). Anti-HA polyclonal antisemur was produced in rabbits using a polypeptide conjugated to keyhole limpet hemocyanin. Rabbit polyclonal antisemur R-6037 was produced against the carboxyl-terminal 20 amino acids of ARF1 conjugated to keyhole limpet hemocyanin. Rabbit polyclonal antisemur purchased from Sigma.

Culture—COS-1 cells were grown in six- or 12-well tissue culture plates (Falcon) in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Upstate Biotechnology Inc.), 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate (Life Technologies, Inc.). CHO-T cells (Chinese hamster ovary cells stably expressing human insulin receptors) (25) were grown in six- or 12-well tissue culture plates using F-12 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate. All cells were grown at 37 °C in the presence of 5% CO2.

Immunofluorescence Microscopy of HA Epitope-tagged GRP1 and ARF6 Expressed in CHO-T Cells—2×105 CHO-T cells or 1×105 COS-1 cells were seeded on sterile 22-mm glass coverslips in six-well tissue culture plates and transfected the following day with 3–100 ng of the desired cDNA constructs. The precise amount of each cDNA construct to be transfected was optimized to obtain low expression levels using immunofluorescence as an end point. A total of 1 μg of DNA (including appropriate amounts of pCMV5) and of LINcofectAMINE (Life Technologies, Inc.) were used per well in serum-free medium. Five hours after the addition of DNA to the cells, the medium was removed and replaced with medium containing 0.5% bovine serum albumin. CHO-T cells were stimulated with insulin (100 nM for 3 min) 24 h post-transfection and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) (171 mM NaCl, 10.1 mM Na2HPO4, 3.35 mM KCl, 1.84 mM KH2PO4, pH 7.2). COS-1 cells were stimulated with 20% fetal bovine serum or epidermal growth factor (EGF; 50 ng/ml) or left unstimulated (-). Cells were washed with PBS, incubated for 15 min in buffer A, and then incubated for 2 h at room temperature with 25 μg/ml affinity-purified anti-GRP1 antiserum (50 μg/ml protein A-purified anti-GRP1 antiserum diluted in buffer A) or left unstimulated (-). Cells were then fixed, blocked, and immunolabeled with anti-HA antibodies and FITC- or rhodamine-conjugated anti-rabbit IgG antibody as outlined under “Experimental Procedures.” 50–100 cells transfected with each construct were counted on three separate coverslips (150–300 cells total) and scored blindly for the presence of HA-tagged constructs in membrane ruffles. A, insulin stimulated CHO-T cells transfected with HA-tagged constructs of GRP1 (upper panel) or GRP1 Sec7, coiled-coil, or PH domains (lower panel). B, graphic representation of insulin-induced mobilization of GRP1 constructs to membrane ruffles. Data are expressed as the mean ± S.E.

PBS, incubated for 15 min in buffer A, and then incubated for 2 h at room temperature with 25 μg/ml affinity-purified anti-GRP1 antiserum (50 μg/ml protein A-purified anti-GRP1 antiserum diluted in buffer A) or left unstimulated (-). Cells were washed with buffer A, incubated with a 1:1000 dilution of FITC-conjugated goat anti-rabbit antibody for 30 min, washed again with buffer A, postfixed with 4% formaldehyde for 10 min, and then mounted on slides with 1,4-diazabicyclo-(2,2,2)-octane. Four sets of approximately 40–75 cells per condition (160–300 cells total) were scored with regard to GRP1 in membrane ruffles or disruption of the Golgi caused by brefeldin A.

GTP Loading of ARF Proteins—In vitro guanine nucleotide exchange assays were done according to the protocol of Frank et al. (22). Briefly, myristoylated ARF6 protein (1.0 μM) was combined with 1.5 mg/ml azolecin vesicles and 4 μM [35S]GTP-S in assay buffer (50 mM HEPS, pH 7.5, 1 mM MgCl2, 100 mM KCl, and 1.0 mM dithiothreitol). 50 nM GRP1-GST fusion protein was added to experimental tubes or with or without 50 μM brefeldin A. Reactions were stopped at 2, 4, 7, 10, 20, and 40 min by adding ice-cold assay buffer to each reaction tube. Samples were then filtered through nitrocellulose membranes using a Bio-Rad BIO-DOT apparatus and washed extensively with cold assay buffer to remove unbound [35S]GTP-S from the ARF6 retained on the membranes. ARF6-bound [35S]GTP-S was quantitated by counting washed nitrocellulose membranes in a Beckman LS 5900 scintillation counter.

To investigate GTP loading of ARF proteins in intact cells, COS-1 cells were seeded in six-well tissue culture plates at a concentration of 5×104 cells/well. The following day, cells were transfected with varying amounts of HA-tagged ARF1 or ARF6 and with GRP1, cytohesin-1, or pCMV5 vectors using the calcium phosphate precipitation technique. 24–48 h post-transfection, cells were transfected to phosphate and
serum-free Dulbecco’s modified Eagle’s medium supplemented with 25 mM HEPES (pH 7.2), 2 mM pyruvate, and 375 μCi/ml [32P]orthophosphate for 16 h. Brefeldin A (50 μM, 5 min) was added directly to the tissue culture medium. Spent medium was aspirated from each well and replaced with 500 μl of lysis buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM MgCl2, 1% Triton X-100, 0.05% cholate, 0.005% SDS, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM NaF, 1 mM vanadate). Cell lysates were scraped from each well and cleared by centrifugation. Each supernatant (50 μl) was analyzed by Western blotting, and the remainder was transferred to fresh tubes containing 5 μl of anti-HA polyclonal antiserum and 10 μl of protein A-Sepharose beads. Immunoprecipitations were conducted at 4 °C for 2 h and followed by extensive washing of the protein A beads with ice cold wash buffer (50 mM HEPES, pH 7.4, 0.5 M NaCl, 5 mM MgCl2, 0.1% Triton X-100, 0.05% cholate, 0.005% SDS). 20 μl of elution buffer (75 mM KH2PO4, pH 3.4, 5 mM EDTA, 0.5 mM GTP, 0.5 mM GDP) was added to each tube, heated to 85 °C for 3 min, and spotted on polyethyleneimine-cellulose thin layer chromatography plates (Merck). Chromatography was conducted for approximately 1 h using 0.65 M KH2PO4 buffer (pH 3.4). After autoradiography, separated guanine nucleotides were visualized with UV light, cut from the chromatography plates, and counted in a Beckman LS 5000 b-counter. Western blots were performed on cell lysates according to established methods (26) using anti-HA antiserum.

RESULTS AND DISCUSSION

To investigate the effect of PI 3-kinase activation on the subcellular localization of GRP1, CHO-T and COS-1 cells were transfected with HA-tagged GRP1 and analyzed by immunofluorescence microscopy using anti-HA antibodies. Insulin stimulation of transfected CHO-T cells caused enhanced plasma membrane ruffling, as previously reported (27). Strikingly, insulin also caused translocation of expressed GRP1 from a mainly cytosolic distribution to plasma membrane ruffles (Fig. 1A), where phalloidin staining revealed the presence of polymerized actin. Serum and EGF had a similar effect on GRP1 and actin in transfected COS-1 cells (see Fig. 3 and data not shown). Agonist-stimulated GRP1 translocation was abrogated by the addition of 100 nM wortmannin to cells (data not shown), demonstrating the importance of PI 3-kinase activity for GRP1 recruitment to the plasma membrane. Taken together, these data are consistent with the hypothesis that GRP1 is recruited to sites of insulin-mediated PtdIns(3,4,5)P3 synthesis, which are predominantly in plasma membrane ruffles in CHO-T cells.

In order to test whether the PH domain of GRP1 is responsible for the agonist-stimulated translocation of GRP1 to the plasma membrane, CHO-T cells were transfected with HA-tagged constructs of each GRP1 domain, stimulated with insulin, and subjected to immunofluorescence microscopy using anti-HA antibodies. In the absence of insulin, all expressed proteins exhibited a diffuse staining pattern, indicating a cytosolic or cytoplasmic membrane distribution (Fig. 1A). Insulin stimulation resulted in the translocation of GRP1 and the GRP1 PH domain to the plasma membrane, where they were particularly prevalent in membrane ruffles (Fig. 1, A and B). However, the Sec7 and coiled-coil domains remained cytosolic in insulin-stimulated cells. These results are consistent with those reported by Venkateswarlu et al. (11) showing the PH domain-dependent translocation of ARNO to the plasma membrane of 3T3-L1 adipocytes in response to insulin.
ARF6 as a Target of GRP1

To determine if endogenous GRP1 behaves similarly to the HA-tagged construct, we used affinity-purified anti-GRP1 polyclonal antiserum to label endogenous GRP1 in COS-1 and CHO-T cells. Endogenous GRP1 displayed a slightly more punctate and perinuclear pattern than did the overexpressed GRP1 but was similarly translocated to plasma membrane ruffles in response to insulin or EGF (Figs. 2A and 3, A and B). Like expressed HA-tagged GRP1, endogenous GRP1 translocation was inhibited by wortmannin (data not shown). In order to determine if GRP1 is associated with Golgi membranes, cells were treated with brefeldin A prior to stimulation and labeling with anti-GRP1 antibodies. Brefeldin A had no effect on the localization or translocation of endogenous GRP1 in resting or stimulated cells (Figs. 2A and 3, A and B). In contrast, cells labeled with an antibody preparation that recognizes ARF1, -5, and -6 show that brefeldin A effectively disrupted Golgi structure (Figs. 2B and 3C).

Figs. 1–3 show that both heterologously expressed and endogenous GRP1 are translocated to the plasma membrane of CHO-T and COS-1 cells in response to insulin and EGF, respectively, suggesting that ARF proteins located at the plasma membrane might be physiologically relevant substrates for GRP1. CHO-T cells were co-transfected with MYC-tagged GRP1 and HA-tagged ARF6 constructs and then stimulated with insulin. In unstimulated cells under these conditions, GRP1 was located primarily in the cytosol or intracellular membranes, while ARF6 was detectable throughout the plasma membrane (Fig. 4). Upon insulin stimulation, however, ARF6 and GRP1 were strikingly co-localized in plasma membrane ruffles. ARF6 has been observed to reside predominantly in the plasma membrane as well as intracellular vesicles (16–18, 20). Using a polyclonal anti-ARF6 antibody (gift of Dr. J. Donaldson), we also find that endogenous ARF6 is concentrated in the membrane ruffles of CHO-T cells following insulin stimulation (data not shown). Similar results were seen in COS-1 cells co-transfected with GRP1 and ARF6 and stimulated with EGF or fetal bovine serum (data not shown).

The co-localization of endogenous GRP1 and ARF6 in CHO-T cell plasma membrane ruffles is consistent with the hypothesis that GRP1 can catalyze guanine nucleotide exchange on ARF6 in intact cells. To test this, we first attempted to confirm the results of Frank et al. (22), using their in vitro guanine nucleotide exchange assay that combines recombinant myristoylated ARF6 with azolectin lipid vesicles, [35S]GTPγS, and exchange factor (GRP1). GTP loading of ARF6 was estimated by the amount of radioactive GTPγS associated with ARF6 at various time points. In contrast to negative results we previously obtained under different assay conditions (8), GRP1 enhanced the rate of ARF6 binding to labeled GTPγS approximately 4-fold as compared with ARF6 incubated without GRP1 (Fig. 5). The fact that this enhanced guanine nucleotide exchange on ARF6 was not sensitive to brefeldin A is consistent with previous reports that ARF6 activation both in vivo and in vitro is brefeldin A-resistant (17, 21).

We next developed a novel method to assess GTP loading of ARF proteins in intact cells. COS-1 cells were grown in six-well tissue culture plates and transfected with 1.25 μg/well pCMV5, or HA-tagged constructs of wild type (WT) ARF6, ARF6 T27N, or ARF6 Q67L. Transfected cells were grown for 24–48 h and then transferred to phosphate- and serum-free medium containing 375 μCi/ml [35S]orthophosphate for 16 h. Tissue culture medium was aspirated from each well, and cells were lysed and cleared by centrifugation. HA-tagged ARF proteins were immunoprecipitated with anti-HA antisera. Guanine nucleotides were then eluted from the immunoprecipitated ARF proteins, separated on chromatography plates, and subjected to autoradiography.
tions of ARF1 or ARF6 to ensure comparable expression levels in the presence of GRP1 and cytohesin-1. The amounts of labeled guanine nucleotides (GTP and GDP) bound to ARF proteins were detected on chromatography plates (Fig. 7A), and the levels of ARF proteins detected by Western blots of cell lysates (Fig. 7B) indicated adequate ARF protein expression levels for all three transfection concentrations. Both ARF1 and ARF6 showed a significant increase in labeled GTP loading when co-transfected with GRP1 or cytohesin-1 as compared with control vector (Fig. 7C). Generally, a small decrease in the amount of ARF activation was seen as the amount of transfected ARF was increased, probably due to a decreasing ratio of endogenous and transfected guanine nucleotide exchange factor to ARF protein as amounts of transfected ARF increased.

The ability of GRP1 and cytohesin-1 to activate ARF1 and ARF6 in intact cells in the presence or absence of brefeldin A was then examined using this assay (Fig. 8). In the absence of transfected GRP1 or cytohesin-1, brefeldin A inhibited GTP loading of ARF1 by about 50% (Fig. 8B), suggesting that the endogenous exchange factor or factors for ARF1 in COS-1 cells are brefeldin A-sensitive. In contrast, ARF6 showed no significant decrease in labeled GTP binding in the presence of brefeldin A, indicating that endogenous ARF6 guanine nucleotide exchange factors are not sensitive to brefeldin A. ARF1 co-transfected with GRP1 or cytohesin-1 showed a slight decrease in activation when treated with brefeldin A. This is probably due to the inhibition of endogenous guanine nucleotide exchange factor activity but not the transfected GRP1 or cytohesin-1, because the magnitude of the decreases matched that observed in the absence of transfected GRP1 or cytohesin-1.

The activation of ARF6 co-transfected with GRP1 or cytohesin-1

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**Fig. 7. ARF activation by GRP1 and cytohesin-1 in intact cells.** COS-1 cells were grown in six-well tissue culture plates and transfected with 0.375, 1.25, or 3.75 μg of HA-tagged ARF1 or ARF6 and 1.25 μg of HA-tagged GRP1, cytohesin-1 (CH-1), or pCMV5 per well. Cells were serum-starved and labeled with [32P]orthophosphate for 16 h. A guanine nucleotide exchange assay was then performed on each of the transfectants. Following autoradiography, guanine nucleotides were cut from the chromatography plates and counted with a Beckman LS 5000 β-counter. A, autoradiograph of chromatography plates. B, ARF1 and ARF6 expression as determined by Western blots of cell lysates probed with anti-HA antisera. C, ARF activation expressed as the ratio of [32P]GTP/[32P]GTP + [32P]GDP. Data are expressed as the mean ± S.E.; n = 3.

**Fig. 8. Effect of brefeldin A on GTP loading of ARF proteins in intact cells.** COS-1 cells were transfected with 0.8 μg of HA-tagged ARF1 or ARF6 and 1.25 μg of HA-tagged GRP1, cytohesin-1, or pCMV5 and then serum-starved and radiolabeled with [32P]orthophosphate for 16 h. Brefeldin A (50 μM for 10 min) was added directly to the tissue culture medium. A, autoradiograph of chromatography plates. B, ARF activation expressed as the ratio of [32P]GTP/[32P]GTP + [32P]GDP. Data are expressed as the mean ± S.E.; n = 3–6.
was not affected by brefeldin A. Western blots conducted on cell lysates showed comparable expression levels of ARF6 for each condition (data not shown).

The data presented in Fig. 8, indicating that GRP1 can cause GTP loading of ARF6 expressed in COS-1 cells, is consistent with the results of Fig. 4 indicating that both GRP1 and ARF6 are present in the same cellular compartment. These data are also consistent with the demonstration by Frank et al. (22) and with our data (Fig. 5) showing that recombinant ARNO, GRP1, and cytohesin-1 proteins can actively cause GTP loading of recombinant ARF6 protein in cell-free conditions. ARF1 binding to GTP is also enhanced by GRP1 in both cell-free (8) and intact cell (Figs. 7 and 8) assays. However, little or no ARF1 is normally found in plasma membranes (17) and thus may not be a target of GRP1 in untransfected cells. We have observed that high expression levels of ARF1 in COS-1 cells result in the mistargeting of ARF1 to the plasma membrane as well as the Golgi and cytosol, where it normally resides. This may allow GRP1 and cytohesin-1 access to ARF1, allowing guanine nucleotide exchange to occur. Conversely, high expression levels of GRP1 may artificially affect endogenous ARF1 in intact cells, which raises concerns about the recent claim that GRP1 regulates Golgi function (23). The endogenous proteins may be sufficiently compartmentalized so that little, if any, interaction between GRP1 and ARF1 takes place. Consistent with this hypothesis, brefeldin A did not affect the localization or translocation of ARF1 in resting or stimulated cells but did have a dramatic effect on Golgi structure (Figs. 2B and 3C). Brefeldin A inhibited GTP loading of expressed ARF1 in COS-1 cells not expressing GRP1 (Fig. 8), indicating its endogenous exchange factor is not a GRP1-like protein. In contrast, levels of GTP-

ARF6 as a Target of GRP1

ARF6-induced membrane protrusions appear morphologically distinct from membrane ruffles induced by insulin and other growth factors (20, 27, 31). Recently, ARF6 has been implicated as a necessary element in macrophage phagocytosis (32). In addition, Radhakrishna and Donaldson (18) reported that an inhibitor of actin polymerization, cytochalasin D, inhibits the ARF6-dependent cycling of the human interleukin-2 receptor subunit to the plasma membrane and the formation of aluminum fluoride-induced surface protrusions in ARF6-expressing cells. Thus, actin polymerization through GRP1 and ARF6 may represent a mechanism by which insulin regulates membrane structure or the cycling of proteins to and from the plasma membrane. Recent evidence that microinjected anti-GRP1 antibody modestly inhibits insulin-mediated membrane ruffling in 3T3-L1 cells (33) indicates such a role for GRP1-like proteins in plasma membrane cytoskeletal interactions. Taken together, these considerations suggest the hypothesis that GRP1 and related proteins may regulate cellular processes controlled by ARF6.

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