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Grp1-ASSOCIATED SCAFFOLD PROTEIN (GRASP) IS A REGULATOR OF THE ADP RIBOSYLATION FACTOR 6 (ARF6)-DEPENDENT MEMBRANE TRAFFICKING PATHWAY

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Abstract

GRASP interacts with Grp1 (general receptor for phophoinositides 1; cytohesin 3), which catalyzes nucleotide exchange on and activation of ADP-ribosylation factor-6 (Arf6). Arf6 is a low molecular weight GTPase that regulates key aspects of endocytic recycling pathways. Over-expressed GRASP was found to accumulate in the juxtanuclear endocytic recycling compartment (ERC). GRASP co-localized with a constitutively inactive mutant of Arf6 in the ERC in a manner that was reversed by expression of wild-type Grp1. Co-expression of GRASP and Grp1 promoted membrane ruffling, a cellular hallmark of Arf6 activation. GRASP accumulation in ERC was found to block recycling of the major histocompatibility complex-I (MHC-I), which is trafficked by the Arf6-dependent pathway. In contrast, over-expression of GRASP had no effect on the recycling of transferrin receptors, which are trafficked by a clathrin-dependent pathway. These findings suggest the GRASP regulates the non-clathrin/Arf6-dependent, plasma membrane Cycling and signaling pathways.

Keywords

Arf6; Clathrin-independent; GRASP; receptor-recycling; Scaffold-protein; Trafficking

1. Introduction

GRASP was identified in our laboratory as an all-*trans* retinoic acid-induced gene in P19 cells (Nevrivy et al., 2000). GRASP was subsequently re-cloned and referred to as tamalin (Kitano et al., 2002). GRASP has been shown to interact with numerous neuronal proteins (Kitano et al., 2003) and has been suggested to play a role in the intracellular trafficking of receptors, such as group 1 metabotropic glutamate receptors (mGluRs; Kitano et al., 2002), and a kinase-deficient isoform of neurotropin-3 receptor (TrKCT1; Esteban et al., 2006). Previous work from this laboratory has also demonstrated that GRASP interacts with cytohesin family members Grp1 (also known as cytohesin 3) and Art nucleotide-binding site

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opener (ARNO; Nevrivy et al., 2000). Grp1 and ARNO, are guanine nucleotide exchange factors (GEFs) for small G proteins of the Art family.

Art proteins, like other G-proteins, cycle between inactive, GDP-bound, and active, GTP-bound conformations, which interact differentially with various classes of effector proteins (Donaldson, 2003, Donaldson et al., 2009, Gillingham and Munro, 2007). The only known class III Art, Arf6, localizes to the plasma membrane and endosomal compartments. Arf6 is known to regulate key aspects of vesicular trafficking, actin reorganization, and cellular migration (D'Souza-Schorey and Chavrier, 2006, Donaldson, 2003, Sabe, 2003).

Arf6 has also been described to regulate a novel trafficking pathway that is utilized by membrane proteins lacking cytoplasmic, clathrin-binding motifs, such as major histocompatability complex I (MHC-I; Radhakrishna and Donaldson, 1997). The hit of cargo molecules that traverse through this pathway has since expanded (Arjonen et al., 2012, Delaney et al., 2002, Palacios et al., 2001, Powelka et al., 2004, Radhakrishna and Donaldson, 1997, Sannerud et al., 2011, Scarselli and Donaldson, 2009, Yu et al., 2011). Recent studies have identified components of the Ras-signaling pathway (McKay et al., 2011, Xie et al., 2012), glucose transporters (Li et al., 2012), and enzymes involved in the etiology of the Alzheimer's disease to be trafficked by the Arf6-dependent pathway (Sannerud et al., 2011). One suggestion is that by utilizing this distinct non-clathrin route spatio-temporal compartmentalization, which is crucial in complex signaling cascades, can be achieved

The use of constitutively active (Arf6 Q67L; GTP-bound) and inactive (Arf6 T27N, GDP-bound; Arf6 N122I, nucleotide-free) mutants has been helpful in delineating the mechanistic details of the Arf6 pathway. Arf6 Q67L localizes within invaginations of the plasma membrane, and appears to be responsible for the generation of membrane ruffles, and increased internalization of membrane proteins (Brown et al., 2001, Honda et al., 1999, Naslavsky et al., 2004, Radhakrishna et al., 1999). In contrast, Arf6 T27N accumulates in large aggregates of tubulovesciular structures and its expression reduces the recycling of membrane proteins (Blagoveshchenskaya et al., 2002, Jovanovic et al., 2006, Powelka et al., 2004, Radhakrishna and Donaldson, 1997). Arf6 N122I is a lesser known point mutant of Arf6, which mimics functional and localization characteristics of Arf6 T27N variant (Honda et al., 1999, Riley et al., 2003, Wong and Isberg, 2003), both of which exert similar dominant negative activity.

Small G proteins of the Rab family have been used for subcelullar identification of the various endosomal compartments. Canonical markers include Rab5 for early endosomes, Rab7 for late endosomes, and Rab4 and Rab11 for recycling endosomes (Zerial and McBride, 2001). Arf6 T27N has been demonstrated to reside predominantly in $Rab22^{+}$ and $Rab11^{+}$ recycling endosomal compartments in HeLa cells (Powelka et al., 2004, Weigert et al., 2004). These results have led to the hypothesis that nucleotide exchange and activation of Arf6 occurs within the juxtanuclear endocytic recycling compartment (ERC; D'Souza-Schorey et al., 1998, Radhakrishna et al., 1999, Radhakrishna and Donaldson, 1997). To complete the cycle, GTP hydrolysis by Arf6 at the plasma membrane appears to be required

for internalization and its subsequent localization to the ERC (Cohen et al., 2007, D'Souza-Schorey and Chavrier, 2006, D'Souza-Schorey et al., 1998, Yang et al., 1998)

Nucleotide exchange on and GTP hydrolysis by Arf6 proteins, are facilitated by numerous GEFs and GTPase activating proteins (GAPs), respectively (Gillingham and Munro, 2007, Jackson and Casanova, 2000, Jackson et al., 2000). The cytohesin family of Arf6-GEFs share a common, four-domain structure consisting of an amino terminal coiled-coil (CC) domain, followed by a Sec7 domain that is responsible for GEF activity, a pleckstrin homology (PH) domain that binds phosphoinositides, and a polybasic region at the carboxyl terminus (Jackson and Casanova, 2000). We have previously shown that the Grp1 CC-domain directly interacts with GRASP (Nevrivy et al., 2000), while the PH domain of Grp1 has been shown to have very high selectivity for phosphatidyl 3,4,5-triphosphate (PIP3; Klarlund et al., 1997).

In this study we have identified GRASP as a regulator of the Arf6-dependent trafficking pathway. GRASP localizes in the ERC and along the plasma membrane of HeLa cells. Results of the present study suggest that GRASP recruits Grp1 to endosomal structures and likely facilitates the activation of Arf6, leading to cortical cytoskeletal rearrangements. We also show that GRASP regulates Arf6-dependent recycling in HeLa cells. Thus, GRASP is a component of the carefully orchestrated Arf6-dependent trafficking and signaling pathways.

2. Materials and Methods

2.1. Cells and Cell Culture

HeLa cells (obtained from American Tissue Culture Collection; catalog number CCL-2) were grown in DMEM supplemented with 10% fetal bovine serum, 100 g/ml streptomycin, and 100 U/ml penicillin. Glass coverslips were coated with 0.02% gelatin as per standard protocol. Cells were seeded in a 12-well plates and transfected after they reached 80–90% confluency with Lipofectamine 2000 reagent (Invitrogen, USA) as per manufactures protocols.

2.2. Antibodies, Plasmids and Transient Transfection

Arf6 expression vectors were kindly provided by Dr. K. Nakayama (University of Tsukuba, Ibaraki, Japan; Ref. Toda et al., 1999). The Grp1 construct (Klarlund et al., 1997) was kindly provided by Drs. Michael Czech and Jes Klarlund (University of Massachusetts Medical Center). The GFP tagged Rab4, Rab5, Rab7 and Rab11 constructs (Johnson et al., 2005) were kindly provided by Drs. Volker Vogt (Cornell University) and Mark Johnson (University of Missouri). All constructs were subsequently subcloned into pCDNA3.1(+) (Invitrogen, USA) or into pEGFP-C1 (Clontech, USA) vectors using standard molecular biology protocols. Alexa-546-conjugated transferrin (546-Tfr) and Alexa-350-conjugated phalloidin were purchased from Molecular Probes (Invitrogen, USA). Secondary antibodies conjugated to Cy3 flurophores was purchased from Jackson Immunoresearch (USA). Other antibodies used included a goat polyclonal antibody raised against purified GST-GRASP (Bethyl Laboratories; Ref. Nevrivy et al., 2000), mouse anti-HA (12CA5; Roche Applied Sciences, USA), mouse anti-Myc (Ab-1; OP10, Oncogene Research Products), goat anti-Grp1 (N-17; sc 9730; Santa Cruz Biotech), rat anti-GFP (kindly provided by Drs. Chrissa

Kioussi and Michael Gross, Oregon State University) and mouse anti-human MHC-I (#311402, BioLegend, USA).

2.3. Fluorescence Microscopy

Approximately 16–24 hrs. following transfection, HeLa cells were fixed in 4% paraformaldehyde (PFA) for 10 min. and processed for immunofluroscence microscopy. Until otherwise mentioned, all images were captured using a ZEISS LSM 510 confocal microscope with 63x Plan Apo 1.3 NA objective. These images where then processed using Photoshop CS4 (Adobe Systems, Inc., USA). For GRASP endosomal scoring assays, a minimum of 50 cells were counted and scored for the accumulation of $GRASP^{+}$ endosomes. Only cells that exhibited a large accumulation of GRASP in the endosomal compartment were scored as positive. All experiments were conducted in triplicate, and then verified in 3–5 independent transfections.

2.4. Receptor internalization and recycling assays

2.4.1—MHC-I recycling assays were conducted 16 hrs. after transfection. Cells were chilled to 0–4°C for 60 min. to stop all endocytic activity, followed by incubation in a mouse anti-human MHC-I antibody (7.5 μg/ml) for 60 min. at 0–4°C. Surface labeled, MHC-I receptors where allowed to internalize at 37°C in complete medium for 1 hr., followed by stripping of the uninternalized surface receptors by multiple washes in a mild acid-stripping buffer (0.5% acetic acid, 0.5 M NaCl, pH 3.0), followed by three washes of ice-cold 1x PBS. The cells were then chased for indicated time intervals in prewarmed, complete medium at 37°C, followed by a second wash with the stripping buffer, and fixation with 4% PFA for 10 min. Samples were processed for indirect-immunofluroscence to visualize receptors that failed to be recycled (and/or degraded).

2.4.2—The continuous transferrin recycling assay was adapted from previous work (Magadan et al., 2006). Briefly, cells were serum starved in internalization media (DMEM containing 2 mg/ml bovine serum albumin) for 60 min. at 37°C. Subsequently, these cells where incubated with 10 μg/ml Alexa 546-Transferrin (Tfr-546) in prewarmed internalization media for 60 min. These cells where then rapidly washed with prewarmed recycling media (complete medium with 100-fold excess of unlabeled transferrin and 100 μm deferoxamine mesylate to prevent re-internalization of the receptors) and chased in this medium for indicated time intervals. At the end of indicated times, recycled transferrin receptors present on the cell-surface were stripped by multiple washes with a mild acid-stripping buffer (0.5% acetic acid, 0.5 M NaCl, pH 3.0), fixed with 4% PFA for 10 min. and processed for fluorescence microscopy to visualize receptors that failed to be recycled (and/or degraded).

2.5. Quantitation of cellular signal intensity

The fluorescence signal intensity of the receptors at the cell surface was measured according to published techniques (Magadan et al., 2006, Scarselli and Donaldson, 2009). Briefly, 30 cells were randomly selected and imaged using a 510 LSM confocal microscope (Zeiss) with an oil 40x plan Apo objective. The pinhole was completely open, and all the images were taken with identical acquisition parameters, those previously optimized for the

fluorescent signals to be in the dynamic range. Under these conditions the total fluorescence measured is proportional to the amount of receptors present per cell. The total fluorescence of the individual cell was measured by manually demarcating the edges and using the 'measurement tool' in Photoshop CS4 to obtain the integrated pixel density, which was expressed in arbitrary units/cell.

2.6. Coimmunoprecipitation analyses

These studies were conducted as described previously (Nevrivy et al., 2000).

2.7. Statistical analyses

A Student's t-test was performed to determine if the difference between means of two groups were statistically significant. For comparison of three or more groups, values from control and experimental groups were compared using a two-way, repeated measures ANOVA followed by Bonferroni post hoc analyses. All statistical analyzes was carried out using GraphPad Prism 5.0 (GraphPad Software Inc., USA).

3. Results

3.1. GRASP co-localizes with a constitutively inactive point mutant of Arf6 in a Rab11$^+$ recycling endosomal compartment

We have previously reported that GRASP localized predominantly to the plasma membrane of transfected HEK293 cells (Nevrivy et al., 2000). In addition, we have consistently observed endogenous GRASP staining in perinuclear, endosomal-like structures, in HeLa cells (Fig. S1). For the advantages of a strong fluorescent signal and enhanced resolution, we used ectopic expression of either Myc-GRASP or GFP-GRASP to visualize GRASP in all further experiments. The relative levels of over-expressed GRASP and endogenously expressed GRASP in HeLa cells were assessed by immunoblot analysis (Fig. S2). HeLa cells transfected with expression vectors encoding either GFP- or Myc-GRASP localized similarly to endogenous GRASP, i.e., in juxtanuclear, tubular, endosomal like structures and along the plasma membrane (Figs. 1A and S3). Additionally, over 20% of these transfected cells exhibited enrichment of GRASP in punctate, endosomal-like aggregates (Fig. 1B), and this proportion appeared to be a function of GRASP expression levels (Fig. 1C). Arf6 T27N, a constitutively inactive point mutant of Arf6, is known to localize within a sub-population of the Rab11$^+$ endosomal recycling compartment (ERC; Caplan et al., 2002, Donaldson et al., 2009, Powelka et al., 2004, Radhakrishna et al., 1999, Weigert et al., 2004). Arf6 N122I was also found to localize predominantly in Rab4$^+$ and Rab11$^+$ recycling endosomes, and did not appear to co-focalize with either Rab5$^+$ (early endosomal marker) or Rab7$^+$ (late endosomal marker; Fig. 2A–D) endosomes. The punctate aggregation of over-expressed GRASP resembled that of the constitutively inactive Arf6 N122I, localizing predominantly in Rab11$^+$ recycling endosomes (Fig. 2 E–H).

We conducted studies to determine if the localization of GRASP was similar to and/or affected by the co-expression of wild-type Arf6, Arf6 Q67L or Arf6 N122I. The punctate localization of GRASP was not coincident with that of either wild-type Arf6 (Fig. 2l) or Arf6 Q67L (Fig. 2J), but completely co-localized with Arf6 N122I (Fig. 2K). Furthermore,

co-expression of Arf6 N122I with GRASP dramatically increased the proportion of cells exhibiting punctate localization of GRASP, from 22% to 79%, while no such effect was observed with either wild-type Arf6 or Arf6 Q67L (Fig. 2L). We observed a partial overlap of endogenous GRASP with wild-type Arf6$^+$ compartments and the enrichment of endogenous GRASP in Arf6 N122I$^+$ endosomal structures in HeLa cells (Fig. S4). Collectively, these results suggest that over-expression of GRASP mimics the localization pattern of the constitutively inactive Arf6 point mutants, both of which appear to localize predominantly in Rab11$^+$ recycling endosomes.

Arf6 is known to regulate a distinct, clathrin-independent pathway of plasma membrane recycling, which is blocked by the expression of constitutively inactive Arf6 point-mutants (Blagoveshchenskaya et al., 2002, Naslavsky et al., 2004, Radhakrishna and Donaldson, 1997, Yang et al., 1998). The data above suggest that the propensity for GRASP to localize in ERC is increased by the expression of Arf6 N122I, indicating that GRASP might be involved in the Arf6-dependent pathway of plasma membrane recycling under physiological conditions.

3.2. Co-expression of Grp1 prevents accumulation of GRASP in the endosomal compartment

Arf6 activation is required to initiate recycling of cargo from ERC back to the plasma-membrane (Grant and Donaldson, 2009). We hypothesized that over-expression of GRASP may titrate cellular levels of Arf6-GEF(s), leading to a relative scarcity of free Arf6-GEF(s), and compromised Arf6 function, which we would observe as intense localization of GRASP in ERC. If true, co-expression of an Arf6-GEF should reduce GRASP localization in the endosomal compartment. To test this hypothesis, HeLa cells were transfected with expression vectors encoding GFP-GRASP in the presence and absence of wild-type Grp1-HA, an Arf6-GEF, which interacts with GRASP (Nevrivy et al., 2000). All transfections were normalized with empty vector to minimize artifactual interpretations. Membrane ruffles, which are associated with activated Arf6, were not observed in HeLa cells individually transfected with GRASP or Grp1 expression vectors (Fig. 3A–H). In contrast, we observed that the co-expression of Grp1 and GRASP resulted in a striking increase in membrane ruffling, in which both GRASP and Grp1 localized (Fig. 3I–M). Moreover, co-transfection of Grp1 resulted in a dramatic reduction of transfected cells exhibiting punctate localization of GRASP (55% to 25%; Fig. 3M).

The ability of Grp1 to reduce GRASP aggregation in ERC required both the coiled-coil (CC) domain of Grp1, which interacts directly with GRASP (Nevrivy et al., 2000), and the catalytic Sec7 domain (Grp1 Coil-Sec7; Fig. 4). All Grp1 mutants were expressed at levels equivalent to that of wild-type Grp1 (Fig. S5A,B). The isolated CC domain of Grp1 (Grp1 Coil) did not affect localization of GRASP in ERC in any detectable manner (compare Fig. S5C–E and F–H), and the combined Sec7 and PH domains nearly doubled the fraction of transfected cells exhibiting punctate localization of GRASP (Fig. 4). However, none of the Grp1 truncation mutants, including Grp1 Coil-Sec7, promoted membrane ruffling with GRASP in a manner observed for wild-type Grp1 (data not shown). Thus, the coiled-coil region and catalytic activity of Grp1 were found to be necessary to reduce GRASP

accumulation in ERC, while the generation of membrane ruffles required all the known functional domains of Grp1. To confirm these findings, we generated catalytically inactive (Grp1 E161K) and PIP3 binding-deficient (Grp1 R284D) point mutants of Grp1 based on homology with the corresponding mutants of ARNO (Cherfils et al., 1998, Mukherjee et al., 2001, Sato et al., 2003, Varnai et al., 2005). These point mutants interacted with GRASP in a manner that was indistinguishable from that of wild-type Grp1 (data not shown) but neither reduced the aggregation of GRASP in ERC (Fig. 4).

Considered together, these data suggest that once GRASP and Grp1 are recruited to the ERC, Grp1 may stimulate guanine nucleotide exchange and activation of Arf6. However, over-expression of GRASP leads to its accumulation in the ERC, perhaps by sequestering and preventing the recruitment of soluble pools of Grp1 to the ERC. This blockade was reversed by co-transfecting catalytically active forms of Grp1 that are competent to interact with GRASP.

3.3. GRASP localizes in ERC independent of Grp1

In order to define the region(s) of GRASP responsible for endosomal targeting, we examined the subcellular distribution of several GRASP mutants by fluorescence microscopy and found that endosomal localization required the leucine-rich domain (amino acids 180–257) of GRASP (data not shown), which also interacts with the Arf6 GEFs, Grp1 and ARNO (Nevrivy et al., 2000). These findings suggest that interaction with cytohesins may be necessary for endosomal targeting of GRASP. Alternatively, endosomal targeting and interaction with GEFs of the cytohesin family may be separable functions of the leucine-rich region of GRASP. A series of deletion mutants was used to distinguish between these two possibilities. The expression levels of all GRASP mutants were similar as verified by immunoblotting (see Fig. S6).

We sought to identify the regions within the leucine-rich domain of GRASP that specified interaction with Grp1 in HeLa cells. Grp1 co-immunoprecipitated with GFP-GRASP, GFP-GRASP 180–257 and 180–230 (Fig. 5A, lanes 8–10). In contrast, Grp1 did not co-immunoprecipitate with GFP-GRASP 231–392 or 208–392 (Fig. 5A, lanes 11 and 12 respectively). These results suggest that amino acids spanning 180–207 of GRASP might be important for interacting with Grp1. However, a construct encoding GFP-GRASP 191–207 did not co-immunoprecipitate with Grp1 (not shown). Thus, the Grp1 interaction domain of GRASP appears extensive and likely spans amino acids 180–230, residues of the leucine-rich domain.

We next determined the region of the leucine-rich domain of GRASP that was responsible for targeting GRASP to endosomes. We transfected HeLa cells with expression constructs encoding GRASP truncation mutants, GFP-GRASP 180–257, 180–230, 231–392 or 208–392. We observed that the propensity with which GFP-GRASP 180–257 accumulated in ERC was similar to that of wild-type GRASP (~55%; Figs. 5B and S7A,B), while the GFP-GRASP 180–230, exhibited reduced ability to form punctate endosomal aggregates (~30%; Figs. 5B and S7C,D). We next analyzed truncation mutants that failed to interact with Grp1 (see Fig. 5B). GFP-GRASP 231–392, localized in ERC but lost the ability to aggregate in these ERC structures efficiently (Figs. 5B and S7E,F), while GFP-GRASP 208–392,

exhibited localization in Arf6 N122I^{+} ERC in a manner indistinguishable from wild-type GRASP (54%; Figs. 5B and S7G,H). However, co-expression of wild-type Grp1 did not prevent accumulation of GFP-GRASP 208–392 in the ERC (compare Fig. 5C and 5D). Interestingly, co-expression of wild-type Grp1 led to redistribution of GRASP mutants GFP-GRASP 180–257 and 180–231 to the plasma membrane and into the nucleus (Fig. S8).

These results define two separable regions within the leucine-rich domain of GRASP, one conferring interaction with Grp1, and the other responsible for endosomal targeting of GRASP. GRASP residues 180–230 were sufficient to interact with Grp1, while GRASP truncation mutants GFP-GRASP 208–392 and 231–392 were unable to interact with Grp1 and yet successfully localized in endosomal structures. This revealed that endosomal localization of GRASP was independent of its binding with Grp1 while the release of GRASP from ERC required its interaction with Grp1 (see Fig. 5B–D). Further, GRASP residues between amino acids 208 and 230 of GRASP seem to harbor a minimal endosomal localization signal, without which efficient targeting of GRASP to endosomes was lost (see Fig. 5B and Fig. S7E,F). Amino acids flanking this minimal endosomal targeting sequence, i.e., amino acids 231–392, also affected the frequency with which GRASP was targeted to the endosomes. Addition of residues 231–257 to GRASP 180–230 (i.e., GRASP residues 180–257) resulted in a two-fold increase in endosomal targeting (see Fig. 5B). Therefore, in addition to the minimal endosomal localization signal of GRASP (residues 208–230), and the Grp1-interacting residues of leucine-rich domain, other domains of GRASP and/or unknown factors interacting with those domains may collectively fine tune the subcellular localization characteristics of GRASP in HeLa cells. In summary, these results demonstrate the presence of an endogenous endosomal targeting signal within GRASP that is separable from the Grp1-interaction residues in the leucine-rich domain of the protein.

3.4. GRASP regulates the Arf6-dependent recycling of membrane proteins

The GTP-binding deficient mutant of Arf6, Arf6 T27N, has been reported to block plasma membrane recycling of Arf6-dependent cargo, such as interleukin-2α receptor (IL-2αR) and major histocompatiblity complex I (MHC-I), but not the recycling of clathrin-dependent cargo, such as transferrin receptors (TfrR; Blagoveshchenskaya et al., 2002, Naslavsky et al., 2004, Radhakrishna and Donaldson, 1997, Yang et al., 1998). Based on the similarities between localization characteristics of constitutively inactive Arf6 dominant negative mutants (Arf6 T27N and N122I; collectively referred to as dnArf6), and the punctuate aggregates formed in cells expressing high levels of GRASP, we hypothesized that over-expression of GRASP would phenocopy dnArf6, with respect to the recycling of MHC-I receptors. To determine this we carried out a MHC-I recycling assay and a continuous TfrR recycling assay in cells transfected with vectors encoding either GFP or GFP-GRASP. A fluorescent signal corresponding to internalized MHC-I receptors was lost rapidly in the absence of transfected GRASP (not shown) or in cells expressing low levels of GRASP (compare Fig. 6A–C and D–F), most likely indicating that these receptors had been recycled back to the cell surface (and/or degraded). In contrast, internalized MHC-I receptors co-localized with the punctate aggregates of GRASP (Fig. 6G–I) in cells expressing high levels of GRASP, and these MHC-I receptors were unable to recycle out of the cell efficiently (compare Fig. 6G–I and J–L). Internalized MHC-I receptors are known to recycle back to

the cell-surface rapidly in HeLa cells (Blagoveshchenskaya et al., 2002). In agreement, we found that about 50% of the internalized receptor population was either recycled back to the plasma membrane and/or degraded after a 10 min. chase in cells expressing either GFP or low levels of GRASP. In contrast, appreciable amounts of internalized MHC-I receptors remained in ERC-like structures even after a 60 min. chase in cells expressing high levels of GRASP (~70%; Fig. 6M).

High levels of GRASP did not block recycling of clathrin-dependent cargo, such as TfrR. We observed that internalized TfrR partially co-localized with GRASP$^+$ endosomal structures (Fig. 7A–C and G–I). However, internalized TfrRs were recycled back to the plasma membrane (and/or degraded) equivalently and efficiently in cells expressing both low and high levels of GRASP (compare Fig. 7A–C and D–F; and Fig. 7G–I and J–L respectively; Fig. 7M). These findings suggest that GRASP, like dnArf6, does not likely play a role in TfrR recycling.

Collectively, our data suggest that high levels of GRASP recapitulate the activity of dnArf6 in blocking the release of MHC-I receptor, but not that of TfrR, from ERC in HeLa cells, suggesting a potential role of GRASP in the Arf6-dependent pathway.

4. Discussion

GRASP has been shown to facilitate the cell-surface expression of group 1 metatropic glutamate receptors (mGluRs; Kitano et al., 2002) and kinase-deficient neurotrophin receptor (TrKCT1; Esteban et al., 2006); however, in both cases the precise mechanisms for these effects remain unknown. In the present study we established GRASP as a novel regulator of the Arf6-dependent, receptor trafficking pathway. GRASP was shown to localize in juxtanuclear endosomal recycling compartment (ERC), and GRASP•Grp1-mediated regulation of the Arf6 pathway was crucial for Arf6-dependent recycling of receptors from this compartment. A proposed model for GRASP action in the Arf6 pathway of receptor trafficking is shown in Fig. 8.

In HeLa cells, both endogenously and exogenously expressed GRASP (at low levels) localized in endosomal-like compartments and along the plasma membrane (see Figs. 1, 2, S1 and S3). However, when expressed exogenously at higher levels, GRASP aggregated selectively in the endosomal compartment and its localization characteristics were reminiscent of dnArf6 mutants (Caplan et al., 2002, Wong and Isberg, 2003). In contrast to a previous report (Toda et al., 1999), we found little or no co-localization of Arf6 N122I with late-endosomal marker Rab7. Rather, we consistently found that the subcellular localization of Arf6 N122I mimicked that of Arf6 T27N, predominantly in Rab11$^+$ and Rab4$^+$, juxtanuclear ERC (see Fig. 2A–D). Furthermore, co-expression of Arf6 N122I with low levels of GRASP resulted in enhanced accumulation of GRASP in ERC, indicating that GRASP and Arf6 likely function in similar plasma membrane trafficking pathway(s).

Our studies with GRASP truncation mutants suggested that GRASP may harbor an endogenous endosomal localization signal(s) and likely recruits Grp1 to ERC. We hypothesize that GRASP aggregation in these ERC structures is a consequence of disrupting the GRASP:Grp1 ratio, thereby preventing the entry of Grp1 in the endosomal trafficking

pathway. This hypothesis was supported by our finding that co-expression of Grp1 dramatically reduced the accumulation of GRASP in ERC (see Fig. 3M). However, our results cannot rule out an alternative hypothesis in which a third, unknown protein competes with Grp1 to bind to the coil region of GRASP. Regardless of the nuances of this mechanism, it is clear that GRASP can localize within the ERC independent of Grp1 but in a manner that is prevented by over-expression of Grp1 (see Figs. 3,4,5, S7 and S8). Co-expression of Grp1 with GRASP also led to a striking increase in cortical membrane ruffling, and GRASP and Grp1 co-localized in these structures along the plasma membrane. However, neither the catalytically inactive Grp1 nor a Grp1 mutant lacking the GRASP-interaction domain affected the aggregation of GRASP in ERC and/or resulted in the generation of membrane ruffles (see Figs. 4 and data not shown). Membrane ruffling is a well-documented, downstream consequence of Arf6 activation (Boshans et al., 2000, D'Souza-Schorey and Chavrier, 2006, Donaldson, 2003, Miao et al., 2011, Sabe, 2003, Schweitzer et al., 2011). These findings therefore suggest the existence of a GRASP-Grp1-Arf6 pathway, which minimally involves: (1) GRASP recruitment of Grp1 to ERC (2) Grp1-catalyzed nucleotide exchange on Arf6, leading to activation of the latter, and (3) translocation of the GRASP•Grp1 complex into membrane ruffles.

Two confounding results arose from our studies involving the Grp1 mutants. First, co-expression of Grp1-mutant, Grp1 Sec7-PH, lacking the GRASP-interaction domain increased the frequency of cells in which GRASP localized as aggregates in ERC (see Fig. 4). Second, the Grp1 mutant lacking a PH domain, Grp1 Coil-Sec7, prevented aggregation of GRASP in ERC, while the point mutant that is defective for PIPS binding, Grp1 R284D, did not (see Fig. 4). Based on the studies from Cohen and coworkers (Cohen et al., 2007), it is clear that the interaction of the Grp1 PH domain with Arf6 facilitates efficient targeting of activated Arf6 to cortical structures. We observed that the Grp1 Sec7-PH fragment localized along the plasma membrane (data not shown), and it is conceivable that this mutant may facilitate cortical localization of basally activated Arf6, leading to increased internalization of $GRASP^+$ endosomes, and an increased proportion of cells exhibiting $GRASP^+$ ERC. Furthermore, in addition to the PIPS sensing abilities, the PH domain of Grp1 was recently shown to inhibit cell spreading when overexpressed in COS-7 cells (Varnai et al., 2005). In contrast, other mutants of the Grp1 PH domain have been shown to bind PIPS in vitro but were unable to inhibit cell spreading in COS-7 cells (Varnai et al., 2005), suggesting a putative protein partner that is necessary for the dominant negative activity of the Grp1 PH domain. Such putative protein partners might also provide the dominant negative effect of R284D, which prevents release of GRASP from ERC.

The physical interaction of GRASP and Grp1, along with the catalytic and PIPS-sensing activities of the latter was found to be collectively required for generation of membrane ruffles (see Figs. 3, S8 and data not shown). Acute activation of Arf6 by treatment with AlF_4 (Radhakrishna et al., 1999) or by expression of Arf6-GEFs (Franco et al., 1999, Langille et al., 1999, Santy and Casanova, 2001) results in generation of ruffling protrusions along the plasma membrane. Our studies now show the presence of GRASP in these cortical actin structures (Fig. 3), suggesting a potential role of GRASP in facilitating the Arf6-mediated cytoskeletal rearrangements. Arf6-mediated cytoskeletal remodeling is thought to be required for the formation of pseudopods and membrane ruffles, neurite outgrowth, cell

spreading, cell migration and phagocytosis (D'Souza-Schorey and Chavrier, 2006, Miao et al., 2011, Schweitzer et al., 2011). By extension, these results also suggest a potential role of GRASP in the repositioning of receptors, such as mGluRs and TrKCT1, to specific regions of the plasma membrane during neurite outgrowth and cellular migration.

Interestingly, HeLa cells singly transfected with GRASP mutants GFP-GRASP 180–257 or 180–230, recapitulate the localization pattern of wild-type GRASP (see Fig. S7), however, these mutants predominantly exhibit a nuclear localization when co-expressed with wild-type Grp1 (Fig. 1,2 and S8). This result suggests the involvement of the GRASP amino terminal region and/or the PDZ domain in the efficient targeting of GRASP to cortical structures. Further studies are clearly needed to verify these observations and hypothesis.

5. Conclusion

Our studies provide insight into the role of GRASP in intracellular trafficking of receptors and its potential role in vivo. These results provide direct evidence for predominant involvement of GRASP in the regulation of Arf6-dependent cargo. Hence, it may be productive to focus future efforts on the study of the trafficking of Arf6-dependent cargo, including membrane-bound receptors, in *Grasp*$^{-/-}$ mice (Ogawa et al., 2007, Yanpallewar et al., 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Grp1	general receptor for phosphoinositides-1 (cytohesin 3)
GRASP	Grp1-associated scaffold protein
ARNO	Art nucleotide-binding site opener
Art	ADP ribosylation factor
ERC	endosomal recycling compartment
MHC-I	major histocompatability complex I
PIP2	phosphatidylinositol 4,5 bisphosphate
ANOVA	analysis of variance
GEF	guanine nucleotide exchange factor
GAP	GTPase activating protein

CC	coiled-coil
PH	pleckstrin homology
PIP3	phosphatidylinositol 3,4,5 triphosphate
HEK	human embryonic kidney
Tfr	transferrin
TfrR	transferrin receptor
s.e.m	standard error of the mean

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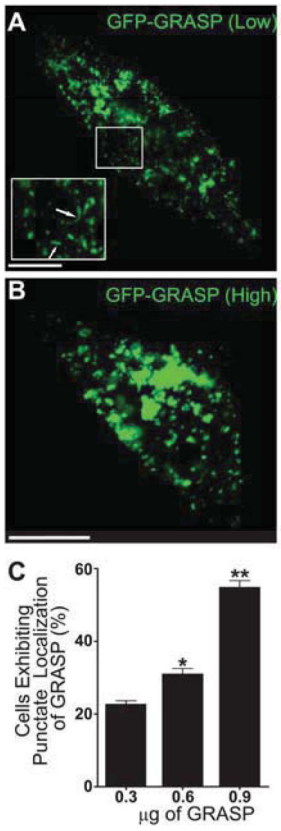

Figure 1. GRASP localizes in tubular perinuclear endosomal compartments

HeLa cells were transfected with increasing concentrations of an expression vector encoding GFP-GRASP, and visualized by fluorescence microscopy. (**A and inset**) Cells transfected with low levels of GRASP, exhibits GRASP localization in tubular, perinuclear endosomal-like compartments and along the plasma membrane. (B) High expression levels of GRASP result predominantly in a punctate, aggregated endosomal-like localization of the protein. (C) Quantification of cells exhibiting punctuate aggregates of GRASP represented as a percentage of the total number of transfected cells. The values represent the mean +/− S.E.M. of three independent experiments in which a minimum of 50 transfected cells were scored. Statistical significance is indicated by * and ** symbols for ($p < 0.05$) and ($p < 0.01$), respectively. Scale bars = 10 µM.

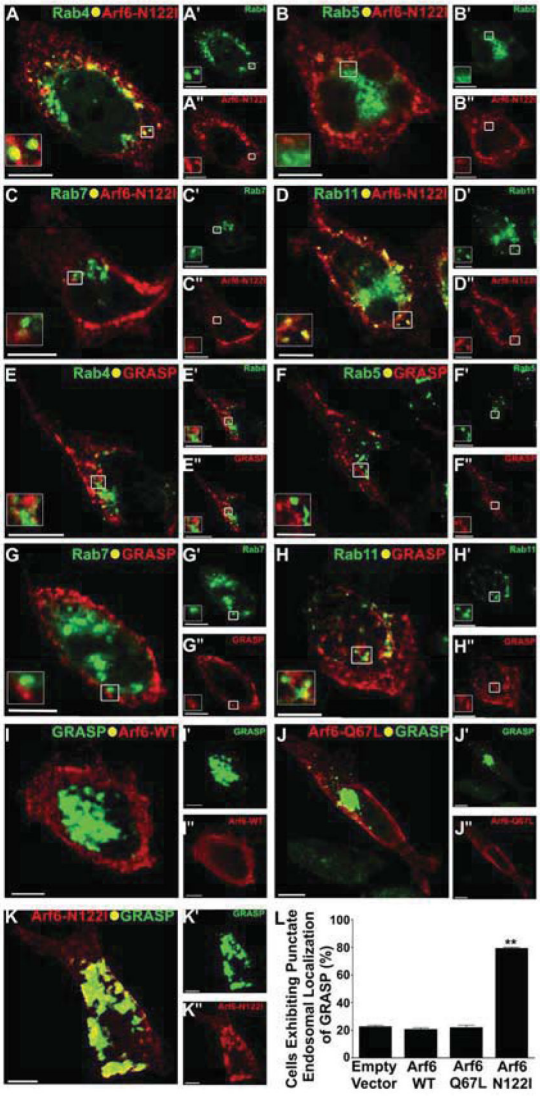

Figure 2. Arf6 N122I co-localizes with and enhances the punctate localization of GRASP in Rab11$^+$ endosomes

HeLa cells were transiently co-transfected with GFP-Rab4, -Rab5, -Rab7 or-Rab11 and either HA-Arf6 N122I (A–D) or Myc-GRASP (E–H) as indicated; the punctate localization of both Arf6 N122I and GRASP strongly co-localized with Rab11$^+$ recycling endosomal structures. (I–K) HeLa cells were transiently transfected with GFP-GRASP and either HA-Arf6 wild-type (WT), HA-Arf6 Q67L, or HA-Arf6 N122I as indicated; the punctate localization of GRASP again strongly and specifically co-localized with the constitutively inactive Arf6 point mutant, Arf6 N122I. All images were captured using the 63x plan Apo objective with optical slice < 0.1 μm. (L) Quantification of cells exhibiting punctate, aggregated endosomal-like localization of GRASP, represented as a percentage of the total number of transfected cells. Values represent the mean +/μ S.E.M. of three independent experiments in which a minimum of 50 transfected cells were scored. The double asterisk denotes a statistically significant ($p < 0.01$) difference in punctate localization of Myc-

GRASP between cells singly transfected with Myc-GRASP vs. cells co-transfected with Myc-GRASP and Arf6 N122I. Scale bars = 10 μM.

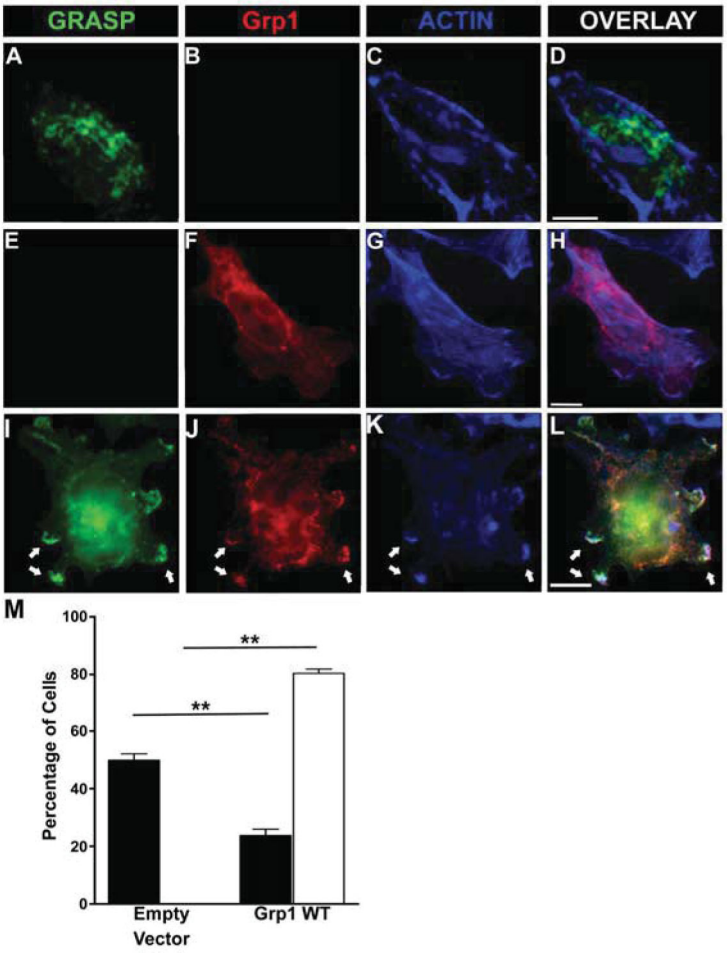

Figure 3. Co-expression of wild-type Grp1 prevents accumulation of GRASP in ERC

HeLa cells were transiently transfected with an expression vector encoding GFP-GRASP with or without Grp1 WT-HA. F-actin was stained using Alexa-350 Phalloidin and cells were scored for percentage of transfected cells exhibiting accumulation of GRASP as aggregates and for membrane ruffles. (A–H) Cells transfected with GFP-GRASP or HA-GRP1 WT exhibit F-actin staining predominantly in stress fibers with only minimal cortical F-actin seen in membrane ruffles.(I–L) Loss of punctate localization of GRASP and its translocation into membrane ruffles (see arrows and dense accumulation of F-actin in cortical protrusive structures) when co-transfected with Grp1 WT. Images were captured using Zeiss Imager.Z1 microscope with a 40x oil Plan-Neofluar objective (M) Quantification and comparison of percentage of transfected cells exhibiting punctate localization of GRASP (solid-black bars) or localization in membrane ruffles (unfilled-bars) in presence or absence of co-transfected Grp1. All values represent the mean +/− S.E.M. of three independent experiments in which a minimum of 50 transfected cells were scored. The double asterisk denotes a statistically significant ($p < 0.01$) difference in punctate localization of GFP-GRASP (black bars) or localization in membrane ruffles (white bars) in the indicated comparisons. Scale bars = 10 μM.

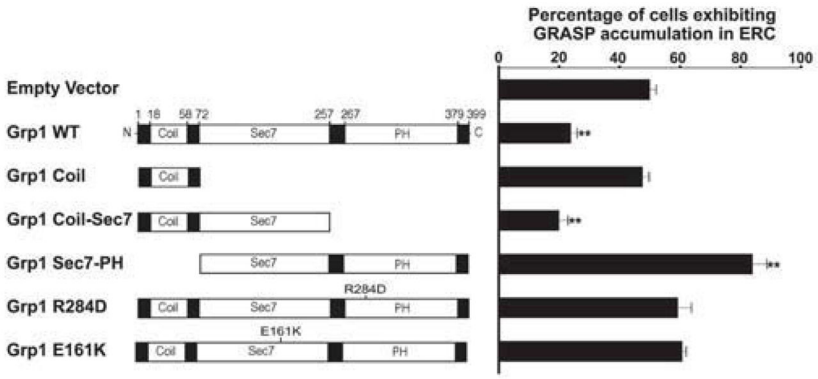

Figure 4. All known functional domains of Grp1 are collectively required to prevent GRASP accumulation in ERC

HeLa cells were transiently co-transfected with an expression vector encoding GFP-GRASP with either WT Grp1 or Grp1 mutants as indicated. Cells were scored for percentage transfected cells exhibiting accumulation of GRASP in ERC aggregates. Values represent the mean +/− S.E.M. of three independent experiments in which a minimum of 50 transfected cells were scored. The double asterisk denotes a statistically significant ($p < 0.01$) difference in ERC localization of Myc-GRASP between cells singly transfected with Myc-GRASP vs. cells co-transfected with Myc-GRASP and WT Grp1 or Grp1 mutants.

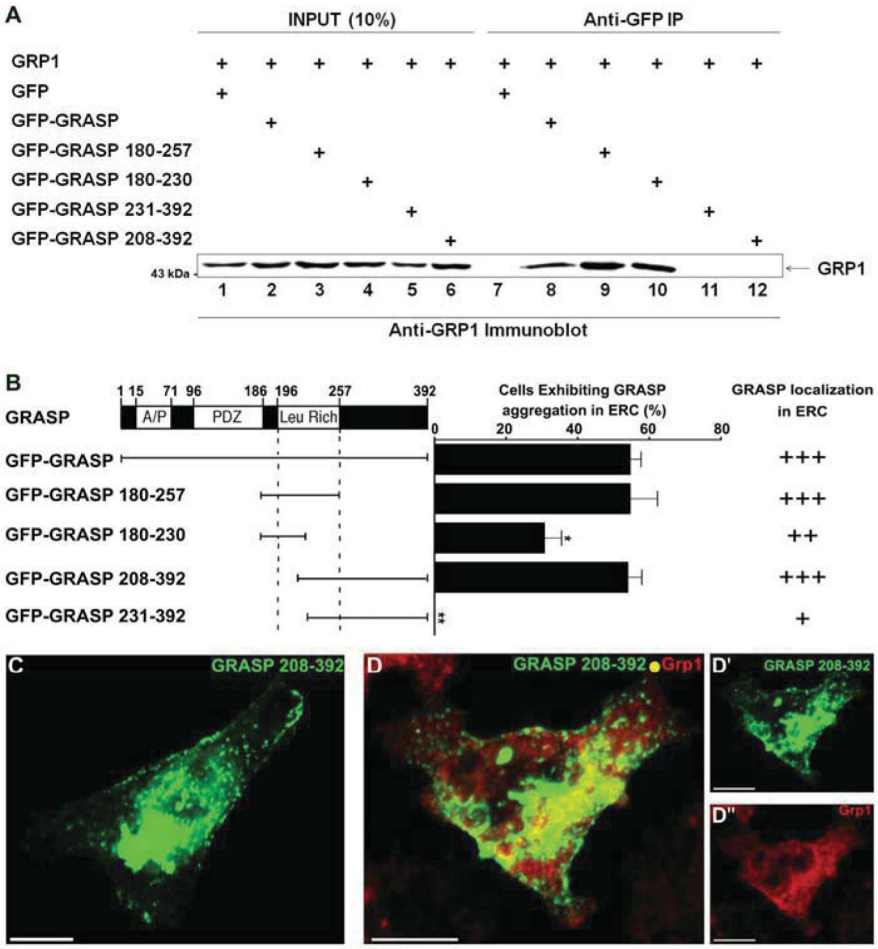

Figure 5. GRASP localizes in ERC independent of Grp1

(A) Co-immunoprecipitation analyses of Grp1 and GFP-GRASP and indicated truncation mutants. (B) Schematic representation of the GRASP truncation mutants and their corresponding propensity/efficiency to aggregate in ERC. Values represent the mean +/− S.E.M. of three independent experiments in which a minimum of 50 transfected cells were scored. Statistical significance at $p < 0.05$ and $p < 0.01$ is indicated by * and ** symbols, respectively. (C) HeLa cells expressing GFP-GRASP 208–392 (D) HeLa cells co-expressing GFP-GRASP 208–392 and Grp1-WT. Scale bars = 10 μM.

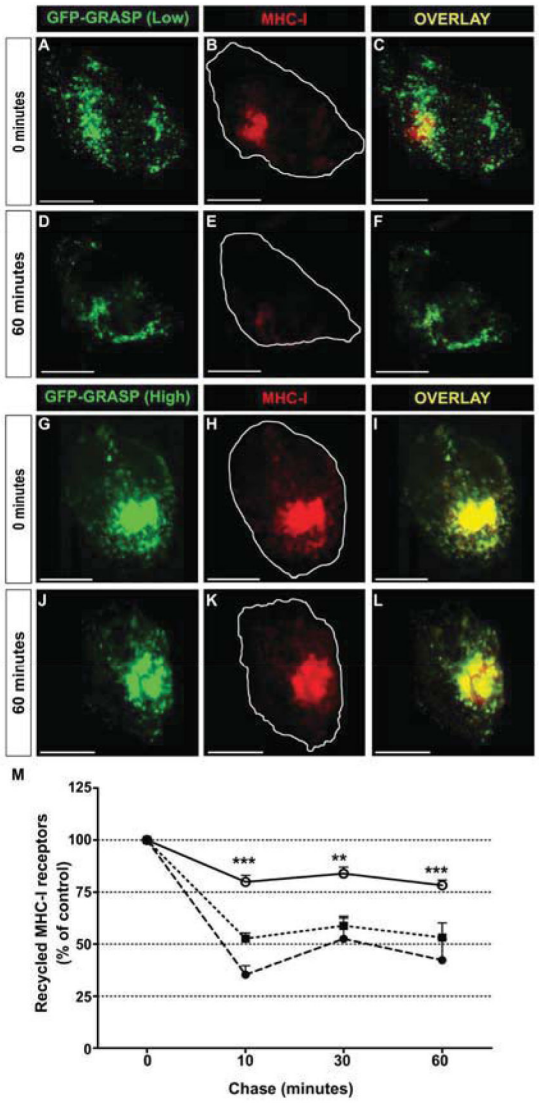

Figure 6. Overexpression of GRASP blocked the release of MHC-I receptors from ERC

(A–F) MHC-I receptor recycling assays in HeLa cells expressing low (A–F) or high (G–L) levels of GRASP. (M) Quantification and comparison of the MHC-I receptors that remained within the cell at varying chase intervals, in cells expressing either GFP (filled squares), low levels of GRASP (filled circles) or high levels of GRASP (open circles). Values in (M) represent the mean +/− S.E.M. of three independent experiments, and statistical significance at $p < 0.01$ and $p < 0.001$ is indicated by double and triple asterisks, respectively. Scale bars in (A–L) = 10 μM.

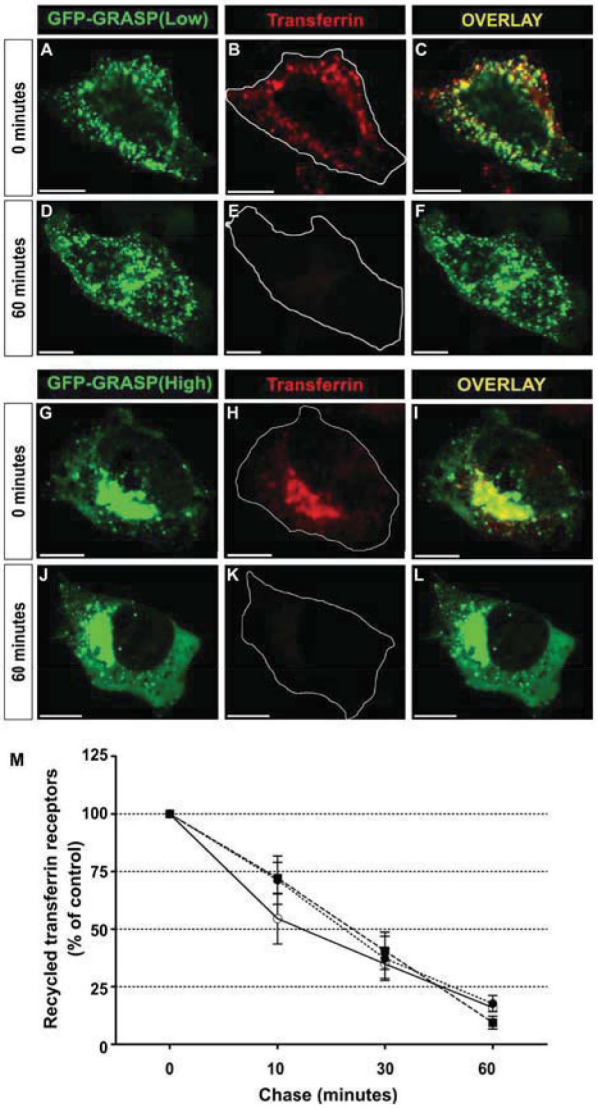

Figure 7. Overexpression of GRASP did not block the release of transferrin receptors from ERC

(A–F) HeLa cells expressing low levels of GRASP lost fluorescence signal from internalized transferrin receptors (TfrR) after a 60 min. chase. (G–I) The internalized transferrin receptors co-localized with the punctuate aggregates of GRASP in HeLa cells. (J–L) Internalized TfrR recycled out of cells expressing high levels of GRASP after a 60 min. chase; (M) Quantification and comparison of TfrR that remained within the cell at varying chase intervals in cells expressing either GFP (filled squares), or low (filled circles) or high levels (unfilled circles) of GRASP. Values in (M) represent the mean +/− S.E.M. of three independent experiments. Scale bars = 10 μM.

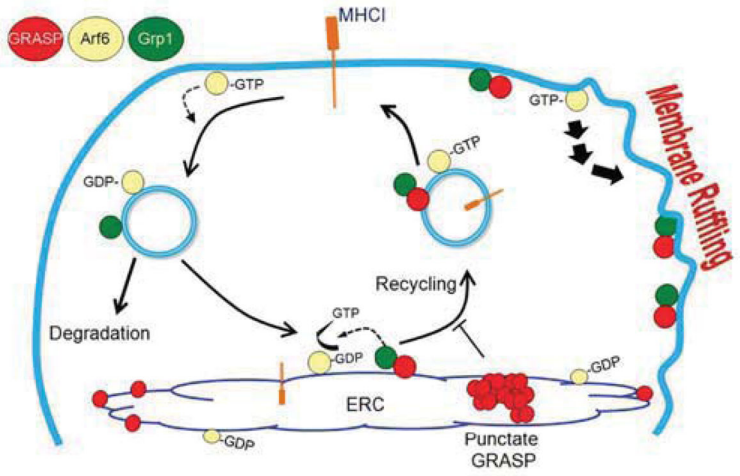

Figure 8. Proposed model for GRASP mediated regulation of the Arf6-dependent trafficking pathway

Following GAP-catalyzed hydrolysis of Arf6-GTP, GRASP andArf6-GDP are internalized and localize in the ERC compartment, in which GRASP is predicted to recruit Grp1 (and/or other cytohesins) to ERC, facilitating Grp1-mediated nucleotide exchange on and activation of Arf6. Following activation of Arf6, GRASP•Grp1•Arf6 translocate to cortical structures, in which the complex initiates Arf6-dependent signaling pathways that lead to membrane ruffling. Elevated levels of Arf6-GTP in cortical structures can then initiate a fresh round of Arf6-dependent internalization to complete the trafficking circuit. GRASP accumulates in ERC when levels of Grp1 are low and/or Grp1 is sequestered away from the ERC compartment by over-expressed GRASP. GRASP aggregates in ERC mimic constitutively dnArf6 mutants and block Arf6-dependent receptor recycling.