Endocytosis of Epidermal Growth Factor Receptor Regulated by Grb2-mediated Recruitment of the Rab5 GTPase-activating Protein RN-tre

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The Grb2 adaptor protein is best known for its role in signaling to the small GTPase p21ras, mediated through its interaction with the SOS guanine nucleotide exchange factor. Here, we demonstrate that Grb2 also signals to Rab5, a small GTPase that plays a key role in early endocytic trafficking. Grb2 functions through association with RN-tre, a GTPase-activating protein for Rab5. Grb2 and RN-tre associate both in vitro and in vivo, with interaction mediated by both SH3 domains of Grb2 and extended proline-rich sequences in RN-tre. Association between Grb2 and RN-tre is constitutive and occurs independently of Eps8, a previously identified binding partner of RN-tre. Epidermal growth factor (EGF) stimulates recruitment of RN-tre to the EGF receptor (EGFR) in a Grb2-dependent manner. Grb2 and the EGFR are internalized and co-localized in endocytic vesicles in response to EGF. Overexpression of RN-tre blocks the internalization of both proteins, consistent with its function as a negative regulator of Rab5 and endocytosis. Strikingly, RN-tre does not block EGF-receptor autophosphorylation, creating docking sites for the SOS guanine nucleotide exchange factor (GEF) for p21ras, to the plasma membrane (4–7). This leads to GTP-loading of p21ras, followed by activation of the Raf/MEK/MAP kinase cascade, which is essential for proliferation (8).

Binding of EGF to the EGFR initiates not only mitogenic signals, but also triggers receptor endocytosis (2). After internalization and delivery to endosomes, receptors are ultimately targeted for degradation to the lysosome, a process referred to as receptor down-regulation (9, 10). Endocytosis of the EGFR occurs through a clathrin-dependent mechanism (11, 12), and requires the small GTPase Rab5 (13–15). Rab5 has multiple functions, including promoting receptor recruitment into clathrin-coated pits, fusion of nascent endocytic vesicles with early endosomes, and homotypic fusion of endosomes (16–22). In its active, GTP-bound form, it recruits cytosolic factors such as EEAs1 and Rabaptin-5 to promote endosome docking and fusion (23–25). However, recycling of Rab5 to the GDP-bound state is essential for normal trafficking, as expression of GTPase-deficient Rab5 leads to the formation of giant early endosomes (21). Thus, the activity of Rab5 GEFs and GTPase-activating proteins (GAPs) must be coordinated for the maintenance of proper trafficking.

RN-tre was recently shown to function as a GAP for Rab5 (14). Originally identified as a binding partner for the EGFR substrate Eps8 (26), RN-tre contains a Rab family GAP homology domain (alternatively referred to as a TrH domain) at its N terminus, and an extended proline-rich C terminus (26, 27). A C-terminally truncated RN-tre mutant was found to be modestly transforming, causing anchorage-independent growth at a low frequency and increasing sensitivity to the proliferative effects of EGF (26). Overexpression of RN-tre in HeLa cells inhibits endocytosis of both the EGF and transferrin receptors, consistent with a requirement for Rab5 activation during receptor endocytosis (13, 14).

Among these proteins, Grb2 has been extensively characterized for its role in proliferative signaling. Grb2 serves to recruit SOS, a guanine nucleotide exchange factor (GEF) for p21ras, to the plasma membrane (4–7). This leads to GTP-loading of p21ras, followed by activation of the Raf/MEK/MAP kinase cascade, which is essential for proliferation (8).

The epidermal growth factor receptor (EGFR) is an evolutionarily conserved transmembrane tyrosine kinase that controls cellular proliferation and differentiation (1, 2). The EGFR is activated by a family of related ligands that includes EGF and transforming growth factor α. Binding of ligand induces receptor autophosphorylation, creating docking sites for the recruitment of SH2 domain-containing signaling molecules, such as Grb2, Shc, Ras GTPase-activating protein (RasGAP), phosphotyrosinositol 3-kinase, and phospholipase Cγ (3).

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§ The abbreviations used are: EGFR, epidermal growth factor receptor; WT, wild type; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; HA, hemagglutinin; GST, glutathione S-transferase; SH3-C, C-terminal SH3 domain; SH3-N, N-terminal SH3 domain.
hypothesized that other SH3 domain-containing proteins might function as adaptors to recruit RN-tre to the EGF receptor. In the current study, we identify Grb2 as an in vitro binding partner for RN-tre. RN-tre is recruited to the activated EGF receptor by Grb2, and inhibits internalization of the receptor complex. Interestingly, RN-tre did not inhibit endocytosis of a Grb2 mutant that fails to bind RN-tre, suggesting that inhibition of receptor internalization specifically requires interaction with Grb2. These results identify a novel role for Grb2 in regulating Rab5 and specifying the trafficking fate of activated growth factor receptor complexes.

EXPERIMENTAL PROCEDURES

Tissue Culture—COS cells, HeLa cells, and fibroblasts were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin, and streptomycin. Eps8-expressing and null fibroblasts have been described previously and were generously provided by Drs. Pier Paolo Di Fiore and Letizia Lanzetti (14). COS cells and fibroblasts were transfected using LipofectAMINE (Invitrogen), and HeLa cells were transfected using Fugene 6 (Roche), according to manufacturer’s instructions.

Constructs—Human wild-type and SH3 point mutants of Grb2 in pEBB were generously provided by Dr. Bruce J. Mayer. The Grb2 inserts were excised from pEBB using BamHI and NotI, and subcloned into the corresponding sites of pEBG to generate GST fusions.

The cDNA encoding RN-tre in pEGFP-C1 was provided by Dr. Pier Paolo Di Fiore (14). The entire open reading frame was amplified by polymerase chain reaction (PCR) and subcloned into a modified version of pcDNA3 (Invitrogen) containing an N-terminal HA-Tag; further details are available on request. RN-tre (466) was generated by digesting with PvuII, which removes from amino acid 467 to the C terminus of RN-tre; for RN-tre (720), encoding the N-terminal 720 amino acids, a STOP codon was introduced at amino acid 721. The sequence of all constructs was confirmed by automated sequencing. In the RN-tre(XXXOS) mutant, also provided by Dr. Pier Paolo Di Fiore, amino acids 728 and 729 are mutated to glycine and serine, respectively (14, 29).

In Vitro Translation Studies—In vitro translations were performed using the TNT system (Promega) according to the manufacturer’s instructions. [35S]methionine was purchased from PerkinElmer Life Sciences, according to the manufacturer’s instructions. The TNT system (Promega) according to the manufacturer’s instructions.

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In Vitro Binding Studies—In vitro translations were performed using the TNT system (Promega) according to the manufacturer’s instructions. [35S]methionine was purchased from PerkinElmer Life Sciences. In vitro-translated products were diluted in GPLB Buffer (20 mM Tris pH 7.4, 150 mM NaCl, 5 mM MgCl2, 0.5% Nonidet P-40, 5 mM β-glycerophosphate, 1 mM dithiothreitol) supplemented with protease inhibitors (pepsstatin, leupeptin, aprostatin, and phenylmethylsulfonyl fluoride), and pulled down using various GST-fusion proteins (10 μg) bound to glutathione Sepharose beads or anti-HA antibody as indicated and incubated by pull-down reactions were washed five times in GPLB. Samples were resolved by SDS-PAGE. Gels were stained with Coomassie Blue to confirm the amounts of GST fusion protein, incubated in 1% sodium salicylic acid (pH 6.0), and visualized by autoradiography.

GST fusion proteins encoding Grb2, Nck, p85, p85(SH3), and Src(SH3) were purified from Escherichia coli as previously described. Purified GST fusions of the isolated N- and C-terminal SH3 domains of Grb2 were purchased from Santa Cruz Biotechnology, Inc. (sc-4034AC and sc-4036AC, respectively).

In Vivo Co-immunoprecipitation Studies—COS cells were transfected using LipofectAMINE for 4–5 h and then were allowed to recover overnight. Cells were either harvested the following day or starved in 0.5% fetal bovine serum in Dulbecco’s modified Eagle’s medium for 24 h as indicated. For examining co-precipitation of HA-RN-tre with GST-tagged Grb2 alleles or the EGFR, cells were lysed in Buffer X (phosphate-buffered saline, 0.5% Triton X-100, 5 mM MgCl2, 5 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM dithiothreitol, plus protease inhibitors) for 10 min on ice and pelleted in a microcentrifuge for 10 min at 4 °C. The clarified supernatant was then precipitated using glutathione Sepharose beads or anti-HA antibody as indicated and incubated for 4 h at 4 °C with constant mixing. Beads were washed three times in Buffer Y (20 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM sodium vanadate, 1 mM dithiothreitol, plus protease inhibitors), twice in Buffer Z (20 mM HEPES (pH 7.4), 2.5 mM MgCl2, 0.05% Triton X-100, 5% glycerol, 1 mM sodium vanadate, 1 mM dithiothreitol, plus protease inhibitors). Samples were eluted by boiling for 5 min in Sample Buffer (125 mM Tris (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 7.5% glycerol, bromphenol blue). Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and visualized using Enhanced Chemiluminescence (ECL).

For examining association of endogenous Grb2 and RN-tre, HeLa cells were lysed in Buffer X. Equal aliquots of lysate were immunoprecipitated with anti-Grb2 (Santa Cruz, sc-8034), anti-RN-tre (generously provided by Dr. Pier Paolo Di Fiore) (26), or nonspecific mouse IgG. After incubation for 4 h, immunoprecipitates were washed three times in Buffer Y and three times in Buffer Z. Samples were immunoblotted with anti-RN-tre antibody.

Confocal Microscopy—HeLa cells were seeded on glass coverslips at 1.7–2.0 × 105 per 35 mm dish. The following day, cells were transfected using Fugene 6. Cells were starved in serum-free Dulbecco’s modified Eagle’s medium the next day for 24 h, and stimulated where indicated with EGF (Invitrogen) at 100 ng/ml for 10–15 min. Cells were fixed in 3.7% formaldehyde, washed, and probed with the indicated antibodies for 1–2 h. Secondary antibodies used were Cy3-conjugated donkey anti-mouse IgG (heavy and light chain) or fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (heavy and light chain) (Jackson Immunoresearch). Coverslips were mounted with SlowFade (Molecular Probes) and viewed on a Zeiss confocal microscope with LSM510 software using excitation wavelengths of 488 nm (for fluorescein isothiocyanate) or 546 nm (for Cy3).

Antibodies—For immunofluorescence, anti-HA antibodies from Roche (Clone 12CA5) or Santa Cruz (sc-805) were utilized. Anti-GST antibody was affinity purified from rabbits immunized with bacterially expressed GST. Anti-SOS antibody (sc-259) was from Santa Cruz Biotechnology, Inc. Anti-EGRF antibody for immunofluorescence was from Upstate Biotechnologies, Inc. (05–104); anti-EGRF antibody for immunoblotting was from NeoMarkers (MS-400).

RESULTS

RN-tre Binds Specifically to the GRB2 Adaptor Protein in Vitro—The C-terminal 376 amino acids of RN-tre contains multiple potential SH3-binding sites. To test whether SH3-domain containing proteins other than Eps8 might bind to RN-tre, SH3 domains from a variety of signaling molecules were expressed as GST fusions and purified from E. coli. The recombinant proteins were incubated with in vitro-translated RN-tre, and the bound RN-tre was detected by autoradiography. As shown in Fig. 1A, RN-tre associated strongly with

FIG. 1. RN-tre binds specifically to the Grb2 adaptor protein through extended proline-rich sequences. A, HA-tagged RN-tre was translated in vitro in the presence of [35S]methionine. Products were incubated with the indicated GST-SH3 domain-containing recombinant proteins. Complexes were washed, and the associated RN-tre was visualized by autoradiography. Nck, p85, and Grb2 represent the full-length molecules; p85 SH3 and Src SH3 represent the isolated SH3 domains of those molecules. B, the indicated RN-tre constructs were translated in vitro, then subjected to pulldown assays using either GST or GST-Grb2. RN-tre (466) and RN-tre (720) encode the N-terminal 466 and 720 amino acids of RN-tre, respectively.
GRB2 LINKS RN-TRE TO THE EGF RECEPTOR

**Association of RN-Tre and Grb2 Occurs in Vivo and Is Ligand-independent**—We next examined whether endogenous RN-tre and Grb2 are associated. Extracts prepared from HeLa cells were immunoprecipitated with anti-Grb2 antibody or isotype-matched non-immune control. As shown in Fig. 3A, RN-tre specifically co-precipitated with Grb2, confirming that these proteins are associated in vivo in the absence of overexpression.

Most SH3 domain/proline-rich peptide interactions are constitutive, occurring in a mitogen-independent manner. For example, Grb2 and SOS associate in quiescent cells, and binding is not further enhanced by mitogen (7). However, ligand-dependent SH3 domain interactions have also been described. For example, Grb2 association with dynamin is stimulated by EGF (32). To characterize the association between RN-tre and

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**Fig. 2. Optimal binding of RN-tre requires both SH3 domains of Grb2.** A, HA-tagged RN-tre was translated in vitro and subjected to pull-down assays using GST fusion proteins encoding FL Grb2, the isolated N-terminal SH3 domain (SH3-N), or the C-terminal SH3 domain (SH3-C). B, COS cells were co-transfected with HA-RN-tre and the indicated GST-tagged allele of Grb2. Grb2 was precipitated using glutathione Sepharose beads, and the associated RN-tre was detected by immunoblotting with anti-HA antibody. As a control, same precipitates were immunoblotted for the presence SOS, which binds to the N-terminal SH3 domain of Grb2. SH3-N, point mutant in N-terminal SH3 domain; SH3-C, point mutant in C-terminal SH3 domain; SH3-N (-), point mutant in both SH3 domains. WCL, whole-cell lysates; pulldown, glutathione Sepharose pulldowns. Whole-cell lysates were also probed with anti-GST to confirm equal expression of the Grb2 mutants.

**Fig. 3. Endogenous RN-tre and Grb2 associate in an EGF-independent manner in vitro.** A, HeLa cell extracts were immunoprecipitated with anti-RN-tre, anti-Grb2, or non-immune (non-imm) antibody. Washed immunocomplexes were immunoblotted with anti-RN-tre antibody. B, COS cells were co-transfected with HA-RN-tre and GST-tagged Grb2 (G2), Nck, or control vector (-). Cells were starved for 24 h and stimulated for the indicated time with EGF (100 ng/ml). Cells were lysed, and the Grb2 or Nck was precipitated using glutathione Sepharose beads. Washed pull-down reactions were analyzed by immunoblotting with anti-HA, anti-SOS, or anti-GST antibodies.
Grb2, COS cells were co-transfected with RN-tre and Grb2. Cells were serum-starved for 24 h and then stimulated with EGF for various times. Grb2 was precipitated and the associated RN-tre was monitored by immunoblotting. As seen in Fig. 3B, RN-tre was present in anti-Grb2 precipitates in serum-starved cells, and no increase was observed upon EGF stimulation. Anti-phosphotyrosine blots confirmed appropriate stimulation of cells (data not shown). Conversely, Grb2 was constitutively present in RN-tre immunoprecipitates (Fig. 4). This interaction was specific, because Nck, which bound weakly to RN-tre in vitro (Fig. 1), failed to associate with RN-tre in vivo (Figs. 3 and 4). Thus, Grb2 and RN-tre associate specifically in a ligand-independent manner in vivo.

**Grb2 Mediates Recruitment of RN-tre to the EGF Receptor**—Because Grb2 binds to the EGFR in response to ligand, we examined whether this leads to co-recruitment of RN-tre. COS cells were co-transfected with cDNAs encoding HA-RN-tre and the EGFR, either in the absence or presence of Grb2. After serum starvation, cells were stimulated with EGF for 10 min. RN-tre was immunoprecipitated, and the associated EGFR was detected by immunoblotting with anti-EGFR or anti-phosphotyrosine antibody. When co-transfected with control vector, no co-immunoprecipitation of RN-tre and the EGFR was observed, regardless of the presence of EGF (Fig. 4). However, co-expression of Grb2 dramatically induced co-precipitation of the two proteins in a ligand-dependent manner (Fig. 4). Grb2 was also co-precipitated with RN-tre in a ligand-independent manner, as shown in Fig. 3. Grb2-mediated recruitment of RN-tre to the EGFR was specific, as co-expression with Nck had no effect (Fig. 4). Together, these results indicate that Grb2 can mediate recruitment of RN-tre to the EGFR in response to ligand.

**Grb2 Is Internalized with the EGFR in Response to Ligand**—We next examined the subcellular localization of Grb2 and the EGFR after EGF addition. Because several different anti-Grb2 antibodies yielded poor staining of endogenous Grb2, GST-tagged Grb2 was transfected into HeLa cells, and its localization was compared with that of the endogenous EGFR by confocal microscopy. In serum-starved cells, the EGFR exhibited diffuse, uniform staining on the cell surface (Fig. 5A), as previously described (14, 28, 32–34). However, after addition of EGF for 10 min, the receptor was internalized into endosomes, which appeared as discrete punctate structures (Fig. 5B), as previously reported (14, 28, 32–34). Similarly, Grb2 diffusely stained the cytoplasm in the absence of ligand (Fig. 5A), but became internalized into endosomes that co-localized with the EGFR upon addition of EGF (Fig. 5B), as shown previously (33–35). These results confirm that Grb2 is recruited to and internalized with the EGFR.

**RN-tre Specifically Inhibits Internalization of Grb2-associated EGFR**—Previous studies have shown that overexpression of RN-tre blocks internalization of the EGFR because of its ability to serve as a GAP for Rab5 (14). Because RN-tre is recruited to the EGFR via Grb2 (Fig. 4), it is expected that RN-tre might also block internalization of Grb2. To test this, RN-tre and Grb2 were co-transfected into HeLa cells, and their localization was examined by confocal microscopy. As previously reported, RN-tre effectively inhibited internalization of the EGFR (Fig. 6A). RN-tre also abrogated the internalization of WT Grb2 (Fig. 6B). However, internalization of the Grb2 mutant SH3-C, which fails to bind RN-tre, was not inhibited (Fig. 6C). These results indicate that Grb2-mediated recruitment of RN-tre to the EGFR prevents internalization of the receptor/Grb2 complex. Furthermore, inhibition of internalization requires direct interaction between Grb2 and RN-tre.

**RN-tre Binds to Grb2 and Inhibits Its Internalization Independently of Eps8**—Because Eps8 has previously been shown to bind RN-tre, we wished to determine whether it mediates interaction between Grb2 and RN-tre or whether the latter two proteins bind directly. To distinguish between these possibilities, we utilized a double point mutant of RN-tre, denoted PXXGS, which fails to bind Eps8 (14, 29). As shown in Fig. 7, A
SH3-C

noblotting, respectively.

were detected by anti-phosphotyrosine (anti-PY) and anti-GST immu-

noprecipitated with anti-HA antibody, and associated EGFR and Grb2

in Fig. 1. Samples were subjected to autoradiography.

and pulled down using the indicated amount of GST-Grb2 as described

in vitro and pulled down using the indicated amount of GST-Grb2 as described

in Fig. 1. Samples were subjected to autoradiography. B, COS cells were

cotransfected with GST-Grb2 or GST control vector (--), together with

WT or PXGS RN-tre as indicated. Samples were precipitated with

glutathione Sepharose beads, and associated RN-tre was detected by

GS RN-tre as indicated. Samples were precipitated with

XX

WT or P

XX

were co-transfected with GST-Grb2 or GST control vector (---)

for Grb2 and either RN-tre WT (top panels) or PXGS (bottom panels). After

starvation for 24 h, cells were stimulated with EGF for 15 min and then

analyzed by confocal microscopy. Grb2 (left panels) was visualized using

anti-GST antibody; RN-tre (right panels) was visualized using anti-HA

antibody.

and B, PXGS bound as well as WT RN-tre to Grb2 both in vitro and in vivo. These results indicate that Grb2 associates

with RN-tre independently of Eps8.

We further wished to examine whether the ability of RN-tre to inhibit internalization of Grb2-associated EGFR was also

independent of Eps8. First, we confirmed that like WT RN-tre, PXGS was recruited to the EGFR by Grb2 (Fig. 7C). Co-

expression of PXGS effectively inhibited EGF-induced internalization of Grb2 in HeLa cells (Fig. 8). Furthermore, both WT

and PXGS RN-tre inhibited EGF-induced internalization of Grb2 in Eps8-null fibroblasts (Fig. 9). Together, these data

considerably demonstrate that the effect of RN-tre on trafficking of Grb2-associated receptors does not involve Eps8 as an

intermediate.

DISCUSSION

In this study we establish a novel role for Grb2 in endocytic trafficking via regulation of the Rab5 GTPase. This effect is

mediated through interaction with RN-tre, a Rab5-specific GAP. The association between these two proteins entails mul-
tiple contacts between the SH3 domains of Grb2 and proline-rich sequences dispersed over the C-terminal half of RN-tre.

Grb2 recruitment of RN-tre to the EGFR occurs in a ligand-dependent manner and inhibits internalization of Grb2/EGFR

complexes. This inhibition requires direct binding of RN-tre to Grb2, as the SH3-C' mutant of Grb2 was internalized

normally in the presence of RN-tre. Furthermore, the effect of RN-tre on trafficking of Grb2-associated EGFR is independent

of Eps8. Together, these results demonstrate a unique and novel role for Grb2 in dictating the trafficking destiny of recep-
tor complexes.
Ligand-mediated endocytosis of growth factor receptors functions in part to attenuate downstream signaling. Indeed, certain signaling molecules are dissociated from receptors on internalization into endosomes. We speculate that Grb2/RN-tre may participate in regulating the kinetics of signaling downstream of receptors. For example, EGF activates multiple signaling pathways that are together required to induce proliferation. Recruitment of Grb2/RN-tre to the receptor might allow the appropriate signaling events to occur by preventing premature internalization through inactivation of Rab5. Once such signaling events have occurred, Rab5 activation could proceed to direct receptor internalization. This activation might result from inactivation or dissociation of RN-tre, activation of a Rab5 GEF, or both. In this context, it is interesting to note that EGF stimulates the inactivation of RN-tre with kinetics that correlate with its phosphorylation (14).

Recent work by Lanzetti and co-workers showed that inhibition of EGFR endocytosis by RN-tre requires Eps8 (14). This was demonstrated in part through use of the PXXGXS mutant of RN-tre, which neither binds to Eps8 nor inhibits EGFR internalization (14). In contrast, we show here that PXXGS is still able to inhibit internalization of Grb2. The most likely explanation for this apparent discrepancy is that in monitoring EGFR internalization, the entire population of receptors is analyzed. However, only a small fraction might be associated with endogenous Grb2 and still be sensitive to PXXGS. In contrast, by monitoring Grb2 internalization, only those receptors associated with Grb2 are analyzed, allowing the effects of PXXGS to be unmasked.

The studies by Lanzetti et al. further showed that RN-tre was unable to inhibit internalization of the EGFR in Eps8+/– fibroblasts. Introduction of wild-type Eps8, but not a mutant that fails to bind RN-tre, restored the ability of RN-tre to inhibit EGFR internalization in Eps8+/– fibroblasts. One way to reconcile our findings with these previous results is that Eps8 and Grb2 are both required to elicit efficient recruitment of RN-tre to the EGFR. Thus, in Eps8-deficient fibroblasts, endogenous Grb2 would be insufficient by itself to recruit RN-tre to the receptor and inhibit its internalization. However, upon overexpression, Grb2 would be able to bypass the requirement for Eps8. This might be possible because Grb2 binds multiple sites on the EGFR: it binds directly (4), as well as indirectly though Shc (36, 37). Another possibility is that Eps8 and Grb2 may function at distinct subcellular locations. This is supported by a recent study that analyzed EGFR signaling complexes at the cell surface versus internalized compartments. This work showed that whereas Grb2 was associated with both surface and internalized receptors, Eps8 was predominantly associated with internalized receptors (28). Thus, Grb2 may have a more dominant role in regulating RN-tre early in the endocytic process, whereas Eps8 may predominate later. Indeed, Rab5 has multiple distinct functions during endocytosis. In addition to stimulating sequestration of receptors into coated pits, Rab5 is required for fusion of nascent endocytic vesicles with endosomes, as well as docking and homotypic fusion of early and late endosomes (16–19, 21, 22). It also stimulates association of endosomes with microtubules and promotes endosome movement along microtubules (20). The cycling of Rab5 mediated by its GEFs and GAPs is most likely required during all of these processes.

Our results broaden the role of Grb2 in vesicular trafficking. In addition to RN-tre, Grb2 binds to several other proteins that regulate trafficking. First, Grb2 associates with the large GTPase dynamin, which functions in the excision of clathrin-coated vesicles from the plasma membrane (38–40). GTPase-deficient mutants of dynamin prevent pinching off of vesicles from the membrane, indicating that GTP hydrolysis is required for this process. Binding of Grb2 stimulates the GTPase activity of dynamin, suggesting a positive role for this adaptor in vesicle formation (38). Indeed, one study showed that binding of Grb2 to the EGFR was essential for receptor endocytosis and further suggested that dynamin was the relevant effector (32). However, this interpretation is complicated by the finding that although the C-terminal SH3 domain was important for Grb2's role in promoting endocytosis (32), dynamin binds predominantly through the N-terminal SH3 domain (41, 42). Another Grb2 binding partner is the Cbl proto-oncogene product, which functions as a negative regulator of EGFR signaling. Studies of mammalian Cbl and its Caenorhabditis elegans ortholog, Sli-1, reveal that this protein functions by targeting the EGFR to lysosomes by directly catalyzing its ubiquitination (43–46). Recent work demonstrates that Grb2 plays an essential role in Cbl-mediated receptor degradation (33). In addition, Grb2 binds to synaptojanin and synapsin, two proteins with established roles in synaptic vesicle recycling in neurons (47, 48). However, the functional consequences of these interactions remain to be defined. Nevertheless, these studies together indicate that Grb2 can function at multiple levels to regulate vesicular trafficking.

This notion is further reinforced by studies of Rin1. Rin1 is a p21ras effector that was recently shown to encode a GEF for Rab5 (49). Binding of p21ras stimulates the exchange activity of Rin1, suggesting a possible mechanism for the observation that p21ras stimulates endocytosis. Thus, Grb2 can potentially contribute both to the activation of Rab5 (via SOS-mediated activation of p21ras, leading to activation of Rin1), as well as its inactivation (via recruitment of RN-tre). Receptor recruitment of both the GAP and GEF for a given GTPase has been described previously. For example, Grb2/SOS and RasGAP are recruited to multiple growth factor receptors, allowing the appropriate temporal regulation of p21ras (3). Such findings underscore the importance of cycling of G proteins for their normal cellular functions. Thus, although RN-tre is a GAP for Rab5, it is important not to simply consider it a negative regulator. Rather, it likely functions in a coordinated manner with GEFs such as Rin1 to cycle Rab5 between its GTP- and GDP-bound states to promote complex processes such as endosome fusion and motility.

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