Role of the Guanine Nucleotide Exchange Factor Ost in Negative Regulation of Receptor Endocytosis by the Small GTPase Rac1*

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The Rho family of GTPases has been implicated in the regulation of intracellular vesicle trafficking. Here, we investigated the mechanism underlying the negative regulation of clathrin-mediated endocytosis of cell surface receptors mediated by the Rho family protein Rac1. Contrary to previous reports, only the activated mutant of Rac1, but not other Rho family members including RhoA and Cdc42, suppressed internalization of the transferrin receptor. On the other hand, down-regulation of Rac1 expression by RNA interference resulted in enhanced receptor internalization, suggesting that endogenous Rac1 in fact functions as a negative regulator. We identified a guanine nucleotide exchange factor splice variant designated Ost-III, which contains a unique C-terminal region including an Src homology 3 domain, as a regulator of Rac1 involved in the inhibition of receptor endocytosis. In contrast, other splice variants Ost-I and Ost-II exerted virtually no effect on receptor endocytosis. We also examined subcellular localization of synaptojanin 2, a putative Rac1 effector implicated in negative regulation of receptor endocytosis. Each Ost splice variant induced distinct subcellular localization of synaptojanin 2, depending on Rac1 activation. Furthermore, we isolated γ-aminobutyric acid type A receptor-associated protein (GABARAP) as a protein that binds to the C-terminal region of Ost-III. When ectopically expressed, GABARAP was co-localized with Ost-III and potently suppressed the Ost-III-dependent Rac1 activation and the inhibition of receptor endocytosis. Lipid modification of GABARAP was necessary for the suppression of Ost-III. These results are discussed in terms of subcellular region-specific regulation of the Rac1-dependent signaling pathway that negatively regulates clathrin-mediated endocytosis.

In eukaryotic cells the continuous uptake of essential nutrients such as iron-laden transferrin and the cholesterol-laden low density lipoprotein is carried out through clathrin-mediated endocytosis of their respective receptors. Additionally, clathrin-mediated endocytosis is the main pathway for internalization of various growth factor receptors. It mediates rapid clearance and down-regulation of activated receptors, thereby regulating the levels of cell surface receptors. The receptor-ligand complex is internalized into a clathrin-coated pit that is formed through the action of the adaptor protein complex AP-2 and a variety of accessory proteins, including AP180, Eps15, amphiphysin, and epsin. Subsequently, clathrin-coated pits pinch off to form clathrin-coated vesicles that are encapsulated by a polygonal clathrin coat and carry concentrated receptor-ligand complexes into the cell. Clathrin-coated vesicle fission is driven by the GTPase dynamin. The coat of clathrin-coated vesicles is removed shortly after the vesicle forms, generating an early endosome. This endosome fuses with a late endosome, where the low pH causes the dissociation of the receptor-ligand complex. A receptor-rich region buds off to form a separate vesicle that recycles the receptor to the plasma membrane (for reviews, see Refs. 1 and 2).

Many components of the endocytic machinery, including AP-2, AP180, amphiphysin, epsin, endophilin, and dynamin, have phosphatidylinositol 4,5-bisphosphate (PI4,5P2)2 binding domains. The interaction with PI4,5P2 is essential for many aspects of clathrin-mediated endocytosis, including coated pit assembly, clathrin-coated vesicle formation, and their uncoating (3). The phosphatidylinositol 5-phosphatase synaptojanin 1, which interacts with several clathrin-associated proteins, has an important role in adjusting the inositol lipid composition of membranes. By reducing the PI4,5P2 level, synaptojanin 1 negatively regulates membrane recruitment of dynamin and adaptor proteins, such as AP-2 and AP180, leading to clathrin uncoating. Disruption of the synaptojanin 1 gene resulted in neurological defects and early postnatal death, presumably due to synaptic vesicle recycling disorder (accumulation of clathrin-coated vesicles) in neurons with increased PI4,5P2 levels (4). Synaptojanin 2, an isoform of synaptojanin 1, is ubiquitously present in a wide variety of cells and tissues. Synaptojanin 2, like synaptojanin 1, contains an N-terminal pleckstrin homology (PH) domain, which binds PI4,5P2, and a C-terminal carboxy domain that interacts with various clathrin-coated pit assembly factors. Inhibition of synaptojanin 2 activity by RNA interference suppressed endocytosis of transferrin receptor.

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2 The abbreviations used are: PI4,5P2, phosphatidylinositol 4,5-bisphosphate; GEF, guanine nucleotide exchange factor; HA, hemagglutinin; GABARAP, γ-aminobutyric acid type A receptor-associated protein; EGF, epidermal growth factor; GST, glutathione S-transferase; MBP, maltose-binding protein; RNAi, RNA interference; siRNA, small interfering RNA; GFP, green fluorescent protein; WT, wild type.
expressed in contrast to synaptotagin 1, which is highly enriched in the nerve terminus (5). Possessing a Rac binding domain at its C-terminal portion, synaptotagin 2 has been reported to serve as a specific effector of Rac1 that regulates clathrin-mediated endocytosis (6, 7). For this function, phosphatidylinositol 5-phosphatase activity seems to be required (6).

The GDP/GTP cycle of Rho family GTPases is regulated by DbI family guanine nucleotide exchange factors (GEFs), which enhance release of GDP from the protein-GDP complex. The catalytic DbI homology domain that precedes the adjacent regulatory pleckstrin homology domain is conserved in all 69 DbI family GEFs. Rho family GTPases are implicated in the regulation of actin cytoskeletal rearrangements and subsequent cellular responses, including cell movement and cell division (8). They also regulate gene expression and the production of superoxide (8). Considering such diverse physiological functions, Rho family GTPases must be strictly regulated in a subcellular region-specific manner. Activities of Rho family proteins including RhoA, Rac1, and Cdc42 are indeed spatially coordinated for dynamic regulation of cell adhesion and migration (9).

Various Rho family members are also involved in the regulation of intracellular membrane trafficking in eukaryotic cells (10). In particular, roles of RhoA, Rac1, and Cdc42 in endocytic pathways have been well documented. RhoA is reported to be an essential component of a constitutive, presumably clathrin-independent, endocytic pathway in Xenopus oocytes (11). Similarly, activated Rac1 causes production of membrane ruffles and associated pinocytic vesicle formation (12). In macrophages, Rac1 and Cdc42 mediate immunoglobulin receptor-dependent phagocytosis, whereas RhoA mediates complement receptor-dependent phagocytosis (13). Furthermore, Cdc42 regulates endocytic transport to the basolateral plasma membrane in polarized epithelial cells (14). With regard to clathrin-mediated endocytosis, activated mutants of RhoA and Rac1 were reported to potently inhibit transferrin receptor-mediated endocytosis (15). In polarized cells, activated RhoA stimulates the rate of both apical and basolateral endocytosis (16), whereas activated Rac1 inhibits this process (17). TCL, a Cdc42-related GTPase, on the other side, regulates the entry of endocytosed receptors into early/sorting endosomes (18).

Although the effect of overexpressed active mutants was previously reported, it remains unclear whether endogenous Rac1 indeed acts as a regulator of intracellular membrane trafficking. Moreover, the mechanism whereby upstream signals regulate Rac1 remains totally unknown. As a step toward understanding these issues, we herein examined the effect of down-regulation of Rac1 that inhibits receptor endocytosis. Moreover, the mechanism whereby upstream signals regulate Rac1 remains totally unknown. As a step toward understanding these issues, we herein examined the effect of down-regulation of Rac1 that inhibits receptor endocytosis.

**EXPERIMENTAL PROCEDURES**

*Materials—* Antibodies against Myc (mouse monoclonal (sc-40) and rabbit polyclonal (sc-789)) and hemagglutinin (HA) (rat monoclonal (1867423)) epitope tags were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Roche Applied Science, respectively. Antibodies against FLAG (mouse monoclonal (F3165) and rabbit polyclonal (F7425)) and V5 (rabbit polyclonal (V8137)) epitope tags were purchased from Sigma-Aldrich. Antibodies against actin (mouse monoclonal (sc-8432)) and γ-aminobutyric acid type A receptor-associated protein (GABARAP) (goat polyclonal (sc-9190)) were purchased from Santa Cruz Biotechnology. A mouse monoclonal anti-Rac1 antibody (23A8) was purchased from Upstate (Charlottesville, VA). A rabbit polyclonal anti-Ost-III antibody was raised against a synthetic peptide corresponding to a C-terminal sequence of Ost-III (1085CPEPAEILS1093). Alexa Fluor 488/546/647-conjugated anti-mouse IgG, anti-rabbit IgG, and anti-rat IgG antibodies were purchased from Invitrogen. Horseradish peroxidase-conjugated anti-mouse IgG, anti-rabbit IgG, and anti-rat IgG antibodies were purchased from Amersham Biosciences. A horse-radish peroxidase-conjugated anti-goat IgG antibody was purchased from Daiichi Kagaku (Japan). Alexa Fluor 546-conjugated transferrin was purchased from Invitrogen. Human epidermal growth factor (EGF) was purchased from PeproTech (Rocky Hill, NJ).

*Plasmids—* cDNAs encoding N-terminal HA-tagged human small GTPases (Rac1(WT), Rac1(G12V), Cdc42(WT), Cdc42(G12V), RhoA(WT), and RhoA(G14V)) were subcloned into the mammalian expression vector pEF-BOS (19). cDNAs encoding N-terminal Myc-tagged Ost splice variants (NCBI accession numbers AB116075 (Ost-I), AB116074 (Ost-II), and DQ978380 (Ost-III)) were subcloned into the mammalian expression vector pCMV5 (NCBI accession number AF239249). The cDNA encoding an N-terminal Myc-tagged C-terminal portion of Ost-III containing the Src homology 3 domain (amino acids 944–1097) (designated Myc-Ost-III (944–1097)) was also subcloned into pCMV5. cDNAs encoding N-terminal FLAG-tagged human α- and β-PIX were subcloned into the mammalian expression vector pCMV2 (Sigma-Aldrich). Mammalian expression vectors pCIneoFLAG-ALS2_L (20), pcS2-Myc-Tiam1 (21), and pcDNA3-Myc-Vav2 (22) were kindly provided by Shinji Hadano and Joh-E Ikeda (Tokai University), Haruhiko Sugimura (Hamamatsu University School of Medicine), and Tomoko Tominaga (Okazaki National Research Institutes), respectively. cDNAs encoding N-terminal FLAG-tagged GABARAP, N-terminal FLAG- and C-terminal V5-tagged GABARAP (designated GABARAP117), and N-terminal FLAG- and C-terminal V5-tagged GABARAP (1–115) (designated GABARAP115) were subcloned into pCMV2. The cDNA for synaptotagmin 2 was kindly provided by Marc Symons (North Shore-LIJ Research Institute), which was subcloned into pCMV5 after adding the N-terminal V5 tag. cDNAs for Ost-III (944–1097) and GABARAP were subcloned into pGEX6P (Amersham Biosciences) and pMAL-c2 (New England Biolabs) for the expression in Escherichia coli as a fusion with glutathione S-transferase (GST) and maltose-binding protein (MBP), respectively. The cDNA encoding the N-terminal Myc-tagged Cdc42/Rac interactive binding domain (amino acids 67–150) of PAK1 (NCBI accession number Q13153) (designated Myc-PAK1-(67–150)) was subcloned into pGEX2T (Amersham Biosciences). The cDNA encoding the C-terminal triple V5-tagged Cdc42/Rac
interactive binding domain (amino acids 67–150) of PAK1 (designated PAK1-(67–150)-3\times V5) was subcloned into pGEX2T.

Cell Culture and Plasmid Transfection—Human cervical carcinoma HeLa cells were cultivated in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Plasmids were introduced into HeLa cells using the SuperFect transfection reagent (Qiagen) according to the manufacturer’s instructions.

Transferrin Incorporation—HeLa cells transfected with expression plasmids were starved in serum-free Dulbecco’s modified Eagle’s medium for 7 h before transferrin treatment. Alexa Fluor 546-conjugated transferrin (50 µg/ml) was then added to serum-starved HeLa cells. After incubation for the indicated periods of time, cells were washed twice with phosphate-buffered saline and fixed with 3.7% (w/v) formaldehyde. Incorporation of transferrin into cells was assessed under confocal laser scanning microscopy as described below.

RNA Interference (RNAi)—The small interfering RNA (siRNA) designed from the human Rac1 cDNA sequence (5’-UGGAGAAUAUACCCUCUGUCUUU-3’) (RAC1 Validated Stealth RNAi, Invitrogen) was used for down-regulation of Rac1. The control siRNA (catalog number 1022076) was purchased from Qiagen. Cells were transfected with siRNAs (100 nm) using the RNAiFect or TransMessenger transfection reagent (Qiagen) according to the manufacturer’s instructions.

Immunofluorescence Microscopy—Immunofluorescence staining was performed as described (23). Images were obtained using a confocal laser scanning microscope (LSM510 META, Carl Zeiss) and processed by the Zeiss LSM Image Browser version 3.5. Color conversion was applied in some images to allocate a single color to each epitope. Fluorescence intensity was quantified by NIH ImageJ (Version 1.36).

In Situ Detection of Rac1-GTP—HA-tagged Rac1(WT) was expressed in HeLa cells (or HeLa cells transfected with pCMV5-Myc-Ost-III) by the use of an adenoviral expression system (catalog number 6173, Takara Bio, Japan). The expression of Rac1(WT) was detected in almost 100% of infected cells (data not shown). After starvation in Dulbecco’s modified Eagle’s medium supplemented with 5% (v/v) fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin for 7 h, cells were stimulated with EGF (100 ng/ml) for 30 min or left unstimulated. Subcellular localization of the activated form of Rac1 (Rac1-GTP) was visualized by using GST-PAK1-(67–150)-3\times V5 as a probe as described previously (23).

Immunoblotting—Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a 0.45-µm pore-size polyvinylidene fluoride membrane (PallCorp., Pensacola, FL). The membrane was stained with respective primary and horseradish peroxidase-conjugated secondary antibodies followed by visualization by enhanced chemiluminescence detection reagents (Amersham Biosciences).

Pulldown Assay for Rac1-GTP—E. coli cells harboring pGEX2T-Myc-PAK1-(67–150) were induced with 0.5 mM isopropyl β-d-thiogalactopyranoside for 5 h at 18°C. Harvested cells were suspended in buffer A (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1.5 µg/ml aprotinin), sonicated, and centrifuged at 100,000 \times g for 30 min at 4°C. Glutathione-Sepharose beads were mixed with an aliquot of the supernatant containing GST-Myc-PAK1-(67–150) for 1 h at 4°C and washed 3 times with buffer B (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 20 mM MgCl₂, 0.5% (w/v) Nonidet P-40). HeLa cells were lysed in buffer B and centrifuged at 20,000 \times g for 5 min at 4°C. HeLa cell lysates (1 \times 10⁶ cells) were incubated with glutathione-Sepharose beads conjugated with GST-Myc-PAK1-(67–150) (10 µg) for 1 h at 4°C. Subsequently, glutathione-Sepharose beads were washed three times with buffer B. Precipitated Rac1 was detected by immunoblotting using an anti-Rac1 antibody.

Yeast Two-Hybrid Screening—A MATCH MAKER yeast two-hybrid human brain library (catalog number HY4004AH, Clontech, 5 \times 10⁶ independent clones) was screened using a C-terminal region (amino acids 944–1097) of Ost-III as bait according to the manufacturer’s instructions. The interaction between isolated clones and Ost-III (944–1097) was confirmed by growth on the selection plate (synthetic dropout medium – Trp/– Leu/– His/– Ade/ + 2.5 mM 3-amino-triazole) and the β-galactosidase assay according to the manufacturer’s instructions.

In Vitro Association between Ost-III and GABARAP—E. coli cells harboring pGEX6P-Ost-III-(944–1097) were induced with 0.5 mM isopropyl β-d-thiogalactopyranoside for 5 h at 18°C. Harvested cells were suspended in buffer A, sonicated, and centrifuged at 100,000 \times g for 30 min at 4°C. Glutathione-Sepharose beads were mixed with an aliquot of the supernatant containing GST-Ost-III-(944–1097) (or GST as a control) for 1 h at 4°C and washed 3 times with buffer B. E. coli cells harboring pMAL-c2-GABARAP were induced with 0.5 mM isopropyl β-d-thiogalactopyranoside for 5 h at 18°C. Harvested cells were suspended in buffer C (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1.5 µg/ml aprotinin), sonicated, and centrifuged at 100,000 \times g for 30 min at 4°C. MBP-GABARAP was purified with amylose-agarose, and the MBP moiety was removed by digestion with factor Xa. Purified recombinant GABARAP (1.25 µg) was dissolved in buffer B and incubated with glutathione-Sepharose beads conjugated with GST-Ost-III-(944–1097) (or GST as a control) (10 µg) for 1 h at 4°C. Subsequently, glutathione-Sepharose beads were washed three times with buffer B. Precipitated GABARAP was detected by immunoblotting using an anti-GABARAP antibody.

Amylose-agarose beads were mixed with an aliquot of the supernatant containing MBP-GABARAP (or MBP as a control) for 1 h at 4°C and washed 3 times with buffer C. GST-Ost-III-(944–1097) was purified with glutathione-Sepharose. Purified recombinant GST-Ost-III-(944–1097) (10 µg) was dissolved in buffer C and incubated with amylose-agarose beads conjugated with MBP-GABARAP (or MBP as a control) (10 µg) for 1 h at 4°C. Subsequently, amylose-agarose beads were washed 3 times with buffer C. Precipitated GST-Ost-III-(944–1097) was detected by immunoblotting using an anti-Ost-III antibody.
Modulation of Receptor Endocytosis by Ost

Role of Rac1 in Transferrin Receptor Endocytosis—Lamaze et al. (15) has shown that the ectopic overexpression of activated forms of RhoA and Rac1 inhibited clathrin-mediated endocytosis of the transferrin receptor. As a first step to understanding the role of Rho family GTPases in clathrin-mediated receptor endocytosis, we also examined the effect of the activated mutants on transferrin receptor endocytosis by detecting the incorporation of fluorescent transferrin (24). The activated mutant of Rac1, but not Cdc42, potently inhibited endocytosis of the transferrin receptor (Fig. 1A) as Lamaze et al. (15) reported. However, we did not observe any reduction in transferrin receptor endocytosis when activated RhoA was expressed (Fig. 1A). Currently, we do not know the reason for this discrepancy, but our RhoA construct was indeed effective in stress fiber formation in various types of cells (data not shown). Fluorescence intensity per μm² was quantified, and relative transferrin incorporation compared with vector-transfected cells was illustrated in Fig. 1B. We also observed an inhibitory effect of activated Rac1 but neither RhoA nor Cdc42 on endocytosis of the EGF receptor (data not shown). In addition, we estimated the expression level of the transferrin receptor by immunoblotting, detecting no significant difference among cells harboring Rho family GTPases (data not shown).

We then asked whether physiological activation of Rac1 exerts a negative effect like mutational activated Rac1. EGF is known to induce Rac1 activation in various cell types including HeLa cells (25, 26), and thus, we examined the effect of EGF pretreatment on transferrin receptor endocytosis. Transferrin receptor endocytosis was in fact inhibited by 30-min pretreatment with EGF (Fig. 1, C and D). Upon EGF treatment, Rac1 activation occurred in both the plasma membrane and the perinuclear region as demonstrated by in situ detection of Rac1-GTP (Fig. 1E). To further confirm the involvement of Rac1, the expression of endogenous Rac1 was abrogated by RNAi, and its effect on EGF-dependent negative regulation of transferrin receptor endocytosis was examined. Rac1 knockdown indeed canceled the effect of EGF, implicating Rac1 as a downstream component (Fig. 1, C and D).

Although EGF is a possible extracellular factor that regulates receptor endocytosis via Rac1 as described above, it remains unclear whether Rac1 is indeed responsible for a negative regulation when transferrin receptor endocytosis occurs in response to transferrin stimulation alone. Therefore, we next simply compared transferrin receptor endocytosis after the stimulation with transferrin in control and Rac1 knock-down cells. Incorporation of siRNA was monitored by the expression of green fluorescent protein (GFP), and transferrin incorporation in GFP-positive cells was quantified. In Rac1 knock-down cells, transferrin receptor incorporation was significantly enhanced (Fig. 2), suggesting that Rac1-dependent negative regulation also operates downstream of the transferrin receptor itself. Transferrin incorporation upon EGF pretreatment in Rac1 knock-down cells was comparable with that in untreated Rac1-GTP in the plasma membrane is indicated by white triangles. Rac1-GTP in the perinuclear region is indicated by a white arrow. Scale bar, 10 μm.

FIGURE 1. Suppression of transferrin receptor endocytosis by activated Rac1. A, transferrin receptor endocytosis in HeLa cells expressing Rho family GTPases. Indicated Rho family proteins were ectopically expressed (green), and incorporation of Alexa Fluor 546-conjugated transferrin after incubation at 37 °C for 10 min was visualized (red). Scale bar, 10 μm. B, quantification of transferrin incorporation after incubation at 37 °C for 10 min. Fluorescence intensity of Alexa Fluor 546-conjugated transferrin per μm² of cells expressing the indicated proteins was quantified, and relative values compared with vector-transfected cells were shown. The values are expressed as the means ± S.D. of three independent experiments where at least 50 cells were quantified in each experiment. C, transferrin receptor endocytosis in HeLa cells after EGF stimulation. HeLa cells were transfected with the control (−) or Rac1-specific (+) siRNA and pretreated with (+) or without (−) EGF (100 ng/ml) at 37 °C for 30 min. Incorporation of Alexa Fluor 546-conjugated transferrin after incubation at 37 °C for 10 min was visualized (red). Scale bar, 10 μm. D, quantification of transferrin incorporation after incubation at 37 °C for 10 min. HeLa cells were transfected with the control (−) or Rac1-specific (+) siRNA and pretreated with (+) or without (−) EGF (100 ng/ml) at 37 °C for 30 min. Fluorescence intensity of Alexa Fluor 546-conjugated transferrin per μm² of cells was quantified, and relative values compared with control siRNA-transfected cells without EGF pretreatment were shown. The values are expressed as the means ± S.D. of three independent experiments where at least 30 cells were quantified in each experiment. E, in situ detection of Rac1-GTP in HeLa cells after EGF stimulation. Rac1-GTP was visualized after treatment with (+) or without (−) EGF (100 ng/ml) at 37 °C for 30 min.
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wild-type cells (Fig. 1D) in contrast to transferrin incorporation in Rac1 knock-down cells without EGF pretreatment, which was increased over twice (Fig. 2B). This difference is presumably due to different assay conditions; fluorescence intensity of transferrin incorporated into GFP-positive cells was quantified in Fig. 2B, whereas in Fig. 1D fluorescence intensity of transferrin in all siRNA-transfected cells was quantified. Nevertheless, it remains possible that EGF suppression of transferrin receptor endocytosis is mediated also by Rac1-independent mechanisms.

Characterization of a GEF Responsible for the Activation of Rac1 That Negatively Modulates Receptor Endocytosis—It is believed that signal-transducing GTPases including Rac1 are regulated by GEFs specific to each function in a subcellular region-specific manner. In fact, we previously reported that splice variants of the GEF Ost activate Cdc42 in different subcellular regions, leading to different types of cytoskeletal rearrangements (23). Furthermore, we have shown specific coupling of Ost splice variants to receptor-mediated upstream signals (23). Thus, to gain insights into the mechanism by which Rac1 is activated to modulate receptor endocytosis, Ost splice variants were compared for their ability to suppress transferrin receptor endocytosis. Ost-II contains the N-terminal SEC14-like domain, whereas Ost-I lacks this domain as previously described (Ref. 23 and Fig. 3A). Another splice variant Ost-III, which has a unique C-terminal sequence containing an Src homology 3 domain, is identical to Ost-γ (Ref. 27 and Fig. 3A). When transiently expressed in HeLa cells, Ost-III inhibited transferrin receptor endocytosis-like activated Rac1, whereas the other splice variants Ost-I and Ost-II exhibited virtually no significant effects (Fig. 3, B and C). We also observed Ost-III-specific suppression of EGF receptor endocytosis similarly to the transferrin receptor (data not shown). The C-terminal portion of Ost-III (amino acids 944–1097), which contains the Src homology 3 domain but not Dbl homology and pleckstrin homology domains, was ineffective in suppressing transferrin receptor endocytosis (Fig. 3C). Time course experiments revealed that the rate of transferrin receptor internalization was reduced, but the internalization ultimately reached a maximum after 90 min in Ost-III-expressing cells (Fig. 3D). Most of incorporated transferrin was distributed to the perinuclear region after 120 min in all of Ost-I-, Ost-II-, and Ost-III-expressing cells (data not shown). As a step to clarify the mechanism underlying Ost-III-dependent suppression of receptor endocytosis, subcellular regions where Rac1 is activated by Ost-III were visualized (Fig. 3E). In contrast to EGF stimulation, Ost-III activated perinuclear-localized Rac1 but not plasma membrane-localized Rac1, suggesting that Rac1 activated in...
We also tested several other GEFs for Rac1 for their ability to stop codon, which is inserted into the Ost-III mRNA sequence. Namely, Ost-I and Ost-II mRNAs have a single exon-derived sequence containing an in-frame unique to the Ost-III mRNA. Namely, Ost-I and Ost-II mRNAs down Ost-III expression by RNAi because there is no sequence that selectively targets Rac1 that is involved in the regulation of knock-down cells (Fig. 4). Taken together, Ost-III may be a GEF transferrin receptor internalization was canceled in Rac1 was tested. As anticipated, Ost-III-dependent inhibition of Rac1 was diminished by specific siRNA, and the effect of Ost-III on Rac1 (6). Thus, we tested subcellular localization of synaptojanin 2 when co-expressed with Ost splice variants. When co-expressed with wild-type Rac1 and Ost-III, synaptojanin 2 was co-localized with Ost-III in the perinuclear region, where Rac1 was indeed activated as shown in Fig. 3E (Fig. 5). In contrast, synaptojanin 2 was localized uniformly in the cytoplasm (Fig. 5).

Identification of GABARAP as a Binding Partner for Ost-III—To further clarify the mechanism for Ost-III/Rac1-dependent down-regulation of endocytosis, we attempted to isolate a protein that associates with the C-terminal region unique to Ost-III. Through screening of a human adult brain library by using the C-terminal region of Ost-III as bait, we isolated GABARAP as a specific binding protein. Two-hybrid interaction of the bait and the isolated GABARAP clone as assessed by growth on the selection plate is shown in Fig. 6A. Two-hybrid interaction was also confirmed by β-galactosidase assay (data not shown). Moreover, the in vitro interaction between recombinant purified GABARAP and the Ost-III C-terminal region was demonstrated by pulldown assays, implying a direct interaction (Fig. 6, B and C). In addition, Ost-III and GABARAP were partly co-localized when co-expressed in the cell (Fig. 6D).

Regulation of the Ost-III/Rac1 Pathway by GABARAP—Binding of GABARAP to the Ost-III-specific C-terminal region suggests a role of GABARAP in Rac1-dependent negative regulation of transferrin receptor endocytosis. To clarify this issue, we ectopically expressed GABARAP, and its effect on Ost-III or suppress transferrin receptor endocytosis but found no significant effects; -fold increases of transferrin incorporation (mean values of two independent measurements) were 0.93, 0.84, 1.14, 1.01, and 0.97 in ALS2-, α-PIX-, β-PIX-, Tiam1-, and Vav2-expressing cells, respectively. Therefore, at least these GEFs may not be involved in the Rac1-dependent negative regulation.

The perinuclear region may specifically involved in down-regulation of Rac1 by sRNA, the expression level of endogenous Rac1 and (endogenous actin as a control) was detected by immunoblotting. B, transferrin receptor endocytosis in HeLa cells co-transfected with the Rac1 sRNA and the Ost-I expression vector. Cells were co-transfected with the control or Rac1-specific sRNA and the Ost-I expression vector (green), and incorporation of Alexa Fluor 546-conjugated transferrin after incubation at 37 °C for 10 min was visualized (red). Scale bar, 10 μm. C, quantification of transferrin incorporation after incubation at 37 °C for 10 min. Fluorescence intensity of Alexa Fluor 546-conjugated transferrin per μm² of cells was quantified, and relative values compared with control sRNA- and vector-transfected cells were shown. The values are expressed as the means ± S.D. of three or four independent experiments where at least 30 cells were quantified in each experiment.

FIGURE 4. Effect of Rac1 down-regulation on Ost-III inhibition of transferrin receptor endocytosis. A, down-regulation of endogenous Rac1 by sRNA. The expression level of endogenous Rac1 (and endogenous actin as a control) was detected by immunoblotting. B, transferrin receptor endocytosis in HeLa cells co-transfected with the Rac1 sRNA and the Ost-I expression vector. Cells were co-transfected with the control or Rac1-specific sRNA and the Ost-I expression vector (green), and incorporation of Alexa Fluor 546-conjugated transferrin after incubation at 37 °C for 10 min was visualized (red). Scale bar, 10 μm. C, quantification of transferrin incorporation after incubation at 37 °C for 10 min. Fluorescence intensity of Alexa Fluor 546-conjugated transferrin per μm² of cells was quantified, and relative values compared with control sRNA- and vector-transfected cells were shown. The values are expressed as the means ± S.D. of three or four independent experiments where at least 30 cells were quantified in each experiment.
Rac1 function was examined. GABARAP, when co-expressed with activated Rac1, did not significantly affect activated Rac1-dependent inhibition of transferrin receptor internalization (Fig. 7, A and B). In marked contrast, co-expressed GABARAP remarkably suppressed Ost-III-dependent inhibition of transferrin receptor internalization (Fig. 7, A and B). Therefore, GABARAP does not interfere with signaling downstream of Rac1, but instead may negatively regulate GEF activity of Ost-III through direct interaction, thereby canceling Rac1-dependent inhibition. In fact, Ost-III-dependent Rac1 activation as determined by pulldown assays was significantly diminished when GABARAP was co-expressed (Fig. 7C). In contrast, GABARAP did not affect Ost-I- or Ost-II-dependent Rac1 activation as expected (Fig. 7C).

Post-translational Modification of GABARAP Is Required for Inhibition of Ost-III—GABARAP belongs to a family of mammalian orthologs of the yeast autophagy-related protein Atg8, and like yeast Atg8, GABARAP is modified through ubiquitylation-like modification steps (28). After cleavage of the C terminus, Gly-116 is exposed as the new C terminus of GABARAP, which is subsequently lipidated. To examine whether this lipid modification is necessary for suppression of Ost-III, two GABARAP constructs tagged at both the N and C termini were generated. GABARAP117 is the wild-type GABARAP construct whose N and C termini are tagged with FLAG and V5, respectively. GABARAP115 lacks C-terminal two amino acids and is tagged similarly to GABARAP117. When ectopically expressed, the C terminus of GABARAP117 was almost completely removed, because the C-terminal V5 tag was not detected by both immunoblotting and immunofluorescence microscopy (Fig. 8, A and B). In contrast, the C terminus of GABARAP115 remained intact as detected by the C-terminal V5 tag (Fig. 8, A and B). Notably, GABARAP115 was totally incapable of suppressing the Ost-III-dependent inhibition of transferrin receptor incorporation, whereas GABARAP117, like the similar construct without the C-terminal tag used in Fig. 7, sup-

FIGURE 6. The interaction between Ost-III and GABARAP. A, yeast two-hybrid interaction. cDNAs for Ost-III-(944–1097) and GABARAP were subcloned into yeast two-hybrid vectors pGBK7 and pACT2, respectively, and two-hybrid interaction was assessed by growth on the selection plate (synthetic dropout medium –Trp–/–Leu–/–His–/–Ade–/2.5 mM 3-aminotriazole), pGBK7-p53 and pACT2-T antigen were used as a positive control. B, pull down of purified recombinant GABARAP with GST-Ost-III-(944–1097). GABARAP associated with GST-Ost-III-(944–1097) (or GST alone as a control), and inputs were subjected to immunoblotting (IB) using an anti-GABARAP antibody. C, pull down of recombinant GST-Ost-III-(944–1097) with MBP-GABARAP. GST-Ost-III-(944–1097) associated with MBP-GABARAP (or MBP alone as a control) was subjected to immunoblotting using an anti-Ost-III antibody. Inputs were stained with Coomassie Brilliant Blue. D, co-localization of Ost-III and GABARAP. Ost-III and GABARAP were ectopically expressed and detected with anti-Myc tag (for Ost-III, red) and anti-FLAG tag (for GABARAP, green) antibodies, respectively. Scale bar, 10 μm.
pressed the effect of Ost-III. Hence, the post-translational modification of GABARAP, at least the cleavage of the C-terminal amino acid, is required for the suppression of Ost-III.

**DISCUSSION**

Rac1 has been implicated in various aspects of intracellular membrane trafficking including clathrin-mediated receptor endocytosis. The first evidence demonstrating a role for Rac1 in clathrin-mediated endocytosis emerged from studies by Lamaze et al. (15) in which constitutively activated forms of Rac1 and RhoA inhibited transferrin and EGF receptor internalization. We obtained similar results although activated RhoA exhibited virtually no significant effect on internalization of these receptors (Fig. 1 and data not shown). It remained unclear, however, whether endogenous Rac1 is indeed involved in the negative regulation of receptor endocytosis. In this paper we show that ligand-induced endocytosis of the transferrin receptor is significantly enhanced when the expression of endogenous Rac1 is reduced, indicating that Rac1 in fact plays a negative role in clathrin-mediated endocytosis (Fig. 2). Further verification of physiological significance using a mouse model may be important. However, Rac1 exerts pleiotropic functions, including the regulation of cytoskeletal rearrangements and gene expression, and as predicted, disruption of the rac1 gene in the whole body resulted in embryonic lethality of the mouse (29). Therefore, knock-out of the upstream regulator, such as Ost-III described in this study, rather than Rac1 itself may cause defects specifically in clathrin-mediated endocytosis.

Upstream signals that trigger Ost-III/Rac1-dependent negative regulation of receptor endocytosis remain incompletely understood. There are three possible mechanisms for triggering the Rac1-dependent down-regulation (Fig. 9). First, some still unidentified extracellular stimuli that activate the Ost-III/Rac1 pathway may be commonly responsible for down-regulation of clathrin-mediated endocytosis of virtually all cell surface

**FIGURE 7.** Effect of GABARAP on Ost-III inhibition of transferrin receptor endocytosis. A, suppression of Ost-III-dependent, but not Rac1(G12V)-dependent inhibition of transferrin receptor endocytosis by GABARAP in HeLa cells. Ost-III or Rac1(G12V) was ectopically expressed (blue) with or without GABARAP (green), and incorporation of Alexa Fluor 546-conjugated transferrin after incubation at 37 °C for 10 min was visualized (red). Scale bar, 10 μm. B, quantification of transferrin incorporation after incubation at 37 °C for 10 min. The percentage of transferrin-containing cells in cells expressing the indicated proteins is shown. The values are expressed as the means ± S.D. of three independent experiments where at least 30 cells were quantified in each experiment. C, suppression of Ost-III-dependent inhibition of transferrin receptor endocytosis by GABARAP117, but not GABARAP115, in HeLa cells. Ost-III was ectopically expressed with GABARAP117 or GABARAP115, and incorporation of Alexa Fluor 546-conjugated transferrin after incubation at 37 °C for 10 min was visualized. The percentage of transferrin-containing cells in cells expressing indicated proteins is shown. The values are expressed as the means ± S.D. of three independent experiments where at least 30 cells were quantified in each experiment.

**FIGURE 8.** Effect of the C-terminal lipid modification of GABARAP on Ost-III inhibition of transferrin receptor endocytosis. A, elimination of the C terminus of GABARAP expressed in HeLa cells as detected by immunoblotting. N-terminal FLAG- and C-terminal V5-tagged full-length GABARAP (designated GABARAP117) and GABARAP(1–115) (designated GABARAP115) were ectopically expressed and detected by immunoblotting using anti-FLAG tag and anti-V5 tag antibodies. Scale bar, 10 μm. C, suppression of Ost-III-dependent inhibition of transferrin receptor endocytosis by GABARAP117, but not GABARAP115, in HeLa cells. Ost-III was ectopically expressed with GABARAP117 or GABARAP115, and incorporation of Alexa Fluor 546-conjugated transferrin after incubation at 37 °C for 10 min was visualized. The percentage of transferrin-containing cells in cells expressing indicated proteins is shown. The values are expressed as the means ± S.D. of three independent experiments where at least 30 cells were quantified in each experiment.
receptors. In this paper we demonstrate that EGF activates Rac1 in the perinuclear region as well as the plasma membrane and in fact suppresses transferrin receptor endocytosis (Fig. 1, C–E). Thus, EGF may be a candidate for an extracellular factor that activates the Ost-III/Rac1 pathway, although a physiological role of EGF in the negative regulation of transferrin receptor endocytosis remains unclear. Second, each ligand may evoke signals that selectively down-regulate internalization of its own receptor in addition to internalization-inducing signals. Taking into consideration that endocytosis of the transferrin receptor upon transferrin stimulation alone was suppressed when Rac1 expression was down-regulated by RNAi (Fig. 2), at least transferrin receptor endocytosis may be controlled by this mechanism. However, it remains obscure whether this is the prevailing mechanism for many cell surface receptors. Third, both receptor-specific and common signals may be required for the Rac1-dependent negative regulation. Further studies that clarify the molecular basis of these signals will provide insights into detailed mechanisms for Ost-III/Rac1-dependent negative regulation.

The phosphatidylinositol 5-phosphatase synaptojanin 2 has been implicated as a Rac1 effector that regulates clathrin-mediated receptor endocytosis (6). Synaptojanin 2 specifically bound to the GTP-bound form of Rac1 and was co-localized with the activated Rac1 mutant at the cell periphery when co-expressed (6). Furthermore, enforced targeting of the synaptojanin 2 catalytic domain to the plasma membrane, like the overexpression of activated Rac1, impaired receptor endocytosis, which is ascribed to aberrantly increased phosphatidylinositol 5-phosphatase activity in the plasma membrane (6). On the other hand, down-regulation of synaptojanin 2 expression by RNAi caused a decrease in the formation of clathrin-coated pits and vesicles, leading to a defect in receptor internalization (30). Therefore, synaptojanin 2 seems to play an important role in the plasma membrane to form clathrin-coated vesicles in a ligand-dependent manner. Collectively, the PI4,5P2 level in the plasma membrane may diminish the PI4,5P2 level and may produce phosphatidylinositol 4-phosphate excessively. This possibly causes disorders of vesicular trafficking between the trans-Golgi network and endosomes because diverse PI4,5P2- and phosphatidylinositol 4-phosphate-binding proteins, such as AP-1, are known to exert critical roles in this process (31). In consequence, recycling of proteins involved in endocytosis including clathrin may be disturbed, leading to the inhibition of ligand-induced receptor endocytosis.

Of course, we should consider the possibility that Rac1 effectors other than synaptojanin 2 may play an important role in Rac1-dependent negative regulation of receptor endocytosis. Considering our data, the downstream effector should be Rac1-specific, like synaptojanin 2, because suppression of receptor endocytosis was observed in activated Rac1-expressing cells but not in cells expressing other GTPases (Fig. 1, A and B). Because Rac1 has been implicated in the regulation of actin cytoskeletal dynamics, it is tempting to speculate that the effect of Rac1 may be ascribed to cytoskeletal rearrangements. This possibility has not been fully ruled out although neither cytochalasin D nor phalloidin, which inhibits actin assembly and stabilizes actin filaments, respectively, showed significant effects on Rac1-dependent inhibition of endocytosis (15).

GABARAP was first identified as a protein that interacts with the γ2 subunit of γ-aminobutyric acid type A receptors (32). GABARAP also interacts with microtubules, suggesting a role in intracellular trafficking and clustering of the γ-aminobutyric acid type A receptor (32). Indeed, GABARAP stimulates γ-aminobutyric acid type A receptor clustering in microtubule-dependent manner and thereby modulates the channel kinetics (33). On the other hand, GABARAP belongs to a family of mammalian orthologs of the yeast autophagy-related ubiquitin-like protein Atg8 (34). However, the exact role of Atg8 in autophagosomal membrane biogenesis is not yet fully understood. Here we found that GABARAP interacts with and thereby inhibits Ost-III, abrogating Rac1-dependent negative regulation of endocytosis. This function critically depends on C-terminal modification of GABARAP (Fig. 8). Therefore, attachment of the lipid moiety at the C terminus may determine specific membrane localization of GABARAP that is required for modulation of endocytosis. In fact, localization of GABARAP to the autophagosomal membrane also requires its C-terminal modification (35). It is also possible that the functional interaction between GABARAP and its binding partners including Ost-III may be augmented upon the modification of GABARAP, considering that lipid modification of the small GTPase Ras is important for the interaction with its effector (36).

GABARAP may lie immediately downstream of the transferrin receptor because binding of GABARAP to the transferrin receptor was reported (37). However, GABARAP was co-localized with Ost-III in endomembranes rather than the plasma membrane (Fig. 6D). Therefore, the interaction of GABARAP with the transferrin receptor in the plasma membrane may not be necessary for GABARAP-mediated Ost-III regulation of endocytosis. Moreover, it remains obscure whether the
involvement of GABARAP in the regulation of endocytosis is specific to the transferrin receptor. Future studies will reveal the precise mechanism and physiological significance of GABARAP-mediated modulation of receptor endocytosis.

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REFERENCES

1. Conner, S. D., and Schmid, S. L. (2003) Nature 422, 37–44
2. Marsh, M., and McMahon, H. T. (1999) Science 285, 215–220
3. Cremona, O., and De Camilli, P. (2001) J. Cell Sci. 114, 1041–1052
4. Cremona, O., Di Paolo, G., Wenk, M. R., Luthi, A., Kim, W. T., Takei, K., Danielli, L., Nemoto, Y., Shears, S. B., Flavell, R. A., McCormick, D. A., and De Camilli, P. (1999) Cell 99, 179–188
5. Nemoto, Y., Arribas, M., Haffner, C., and De Camilli, P. (1997) J. Biol. Chem. 272, 30817–30821
6. Malecz, N., McCabe, P. C., Spaargaren, C., Qiu, R., Chuang, Y., and Syed, N., and Rusk, N. (2003) Proc. Natl. Acad. Sci. U. S. A. 98, 4385–4390
7. Nemoto, Y., Arribas, M., Haffner, C., and De Camilli, P. (1997) J. Biol. Chem. 272, 30817–30821
8. Malecz, N., McCabe, P. C., Spaargaren, C., Qiu, R., Chuang, Y., and Syed, N., and Rusk, N. (2003) Proc. Natl. Acad. Sci. U. S. A. 98, 4385–4390
9. Raftopoulou, M., and Hall, A. (2004) Nature 420, 629–635
10. Raftopoulou, M., and Hall, A. (2004) Dev. Biol. 265, 23–32
11. Symsons, M., and Rusk, N. (2003) Curr. Biol. 13, 409–418
12. Schmalzing, G., Richter, H. P., Hansen, A., Schwarz, W., Just, I., and Akertories, K. (1995) J. Cell Biol. 130, 1319–1332
13. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) Cell 70, 401–410
14. Caron, E., and Hall, A. (1998) Science 28, 1717–1721
15. Kroschewski, R., Hall, A., and Mellman, I. (1999) Nat. Cell Biol. 1, 8–13
16. Lamaze, C., Chuang, T. H., Terlecky, L., Bokoch, G. M., and Schmid, S. L. (1996) Nature 382, 177–179
17. Leung, S. M., Rojas, R., Maples, C., Flynn, C., Ruiz, W. G., Jou, T. S., and Apodaca, G. (1999) Mol. Biol. Cell 10, 4369–4384
18. Jou, T. S., Leung, S. M., Fung, L. M., Ruiz, W. G., Nelson, W. J., and Apodaca, G. (2000) Mol. Biol. Cell 11, 287–304
19. de Toledo, M., Senic-Matuglia, F., Salamero, J., Uze, G., Comunale, F., Fort, P., and Blangy, A. (2003) Mol. Biol. Cell 14, 4846–4856
20. Mizushima, S., and Nagata, S. (1990) Nucleic Acids Res. 18, 5322
21. Otomo, A., Hadano, S., Okada, T., Mizumura, H., Kunita, R., Nishi, K., Showguchi-Miyata, J., Yanagisawa, Y., Kohiki, E., Suga, E., Yasuda, M., Osuga, H., Nishimoto, T., Narumiya, S., and Ikeda, J.-E. (2003) Hum. Mol. Genet. 12, 1671–1687
22. Otsumi, Y., Tanaka, M., Yoshii, S., Kawaozoe, N., Nakaya, K., and Sugimura, H. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4385–4390
23. Meng, W., Numazaki, M., Takeuchi, K., Uchibori, Y., Ando-Akatsu, Y., Tominaga, M., and Tominaga, T. (2004) EMBO J. 23, 760–771
24. Ueda, S., Katoaka, T., and Satoh, T. (2004) Cell. Signal. 16, 899–906
25. Sunaguchi, M., Nishi, M., Mizobe, T., and Kawata, M. (2003) Brain Res. 984, 21–52
26. Yamaguchi, K., Hata, K., Wada, T., Moriya, S., and Miyagi, T. (2006) Biochem. Biophys. Res. Commun. 346, 484–490
27. Ray, R. M., Vaidya, R. J., and Johnson, L. R. (2007) Cell Motil. Cytoskeleton 64, 143–156
28. Lorenzi, M. V., Castagnino, P., Chen, Q., Hori, Y., and Miki, T. (1999) Oncogene 18, 4742–4755
29. Tanida, I., Ueno, T., and Kominami, E. (2004) Int. J. Biochem. Cell Biol. 36, 2503–2518
30. Sugihara, K., Nakatsuii, N., Nakamura, K., Nakao, K., Hashimoto, R., Otani, H., Sakagami, H., Kondo, H., Nozawa, S., Aiba, A., and Katsuki, M. (1998) Oncogene 17, 3427–3433
31. Rusk, N., Le, P. U., Mariggiò, S., Guay, G., Lurisci, C., Nabi, I. R., Corda, D., and Symsons, M. (2003) Curr. Biol. 13, 659–663
32. Robinson, M. S. (2004) Trends Cell Biol. 14, 167–174
33. Wang, H., Bedford, F. K., Brandon, N. J., Moss, S. J., and Olsen, R. W. (1999) Nature 397, 69–72
34. Chen, L., Wang, H., Vicini, S., and Olsen, R. W. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11557–11562
35. Ohsumi, Y. (2001) Nat. Rev. Mol. Cell Biol. 2, 211–216
36. Kabeya, Y., Mizushima, N., Yamamoto, A., Oshitani-Okamoto, S., Ohsumi, Y., and Yoshimori, T. (2004) J. Cell Sci. 117, 2805–2812
37. Kuroda, Y., Suzuki, N., and Kataoka, T. (1993) Oncogene 3427–3433