Physical and Functional Interaction of Rabphilin-11 with Mammalian Sec13 Protein

IMPLICATION IN VESICLE TRAFFICKING*

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Rab11a small G protein (Rab11p) is implicated in vesicle trafficking, especially vesicle recycling. We have previously isolated a downstream effector of Rab11p, named rabphilin-11. We found here that rabphilin-11 directly bound the mammalian counterpart of yeast Sec13 protein (mSec13p) in cell-free and intact cell systems. Yeast Sec13p is involved as a component of coat proteins II in the Sar1p-induced vesicle formation from the endoplasmic reticulum, but the precise role of mSec13p is unknown. The interaction of rabphilin-11 with mSec13p was enhanced by GTP-Rab11p. Rabphilin-11 localized on the vesicles in perinuclear regions and along microtubules oriented toward the plasma membrane, whereas mSec13p partly colocalized with rabphilin-11 in the perinuclear regions, most presumably the Golgi complex. Disruption of the rabphilin-11-mSec13p interaction by overexpression of the mSec13p-binding region of rabphilin-11 impaired vesicle trafficking. These results indicate that the rabphilin-11-mSec13p interaction is implicated in vesicle trafficking.

Rab11p is a Rab small G protein family member that localizes to early endosomes, recycling endosomes, the trans-Golgi network membranes, and the post-Golgi secretory vesicles and regulates vesicle trafficking, especially vesicle recycling (1–7). Zeng et al. (8) and we (9) have independently isolated the same downstream effector of Rab11p from bovine and rat, named Rab11BP and rabphilin-11, respectively. Rab11BP/rabphilin-11 (rabphilin-11) consists of 908 aa and contains one proline-rich region and six WD-40 repeats in addition to a Rab11p-binding domain. Free rabphilin-11 localizes in the cytosol, but upon binding to GTP-Rab11p, rabphilin-11 localizes to early endosomes, recycling endosomes, and the Golgi complex, as well as along microtubules oriented toward lamellipodia (9), suggesting that GTP-Rab11p recruits rabphilin-11 to these membranes and exerts its function through rabphilin-11. However, the mode of action of the Rab11p-mSec13p interaction in vesicle trafficking remains to be clarified. We searched here for a rabphilin-11-interacting molecule(s) by the yeast two-hybrid method and identified it as a mammalian counterpart of the yeast Sec13 protein (Sec13p).

Yeast Sec13p is involved as a component of the COPII of the vesicles that bud from the ER in a Sar1p-dependent manner (10–12). When GDP-Sar1p is converted to GTP-Sar1p by the action of Sec12p, a GDP/GTP exchange protein of Sar1p (13–15), Sec23 and Sec24p are recruited to the membranes of the ER followed by recruitment of Sec13p and Sec11p, causing bud formation (12, 16, 17). When GTP-Sar1p is converted to GDP-Sar1p by the action of Sec23p, a GTPase-activating protein of Sar1p (18), the coated vesicles are uncoated (12, 17, 18). The mammalian counterpart of yeast Sec13p (mSec13p) has been isolated (19), but its precise role or mode of action remains unknown.

We describe here the rabphilin-11-mSec13p interaction and its role in vesicle trafficking.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assay—Yeast transformations were performed by the lithium acetate method (20). Yeast transformants were selected in dextrose-containing selection (SD) medium (21). The yeast reporter strain L40 expressing pLexA-rabphilin-11-1 (1–631 aa) was transformed with a human T cell cDNA library (MATCHMAKER human T cell oligo(dT)-primed library in pACT, CLONTECH). About 1.0 × 10⁶ transformants were screened, and library plasmids from 16 positive clones were analyzed by transformation tests and DNA sequencing (22, 23). β-Galactosidase activity was measured by liquid and filter assays (23, 24). Where indicated, cotransformation was performed into yeast strain TAT7.

Preparation of Recombinant Proteins—Standard molecular biological techniques were used for construction of plasmids, DNA sequencing, and polymerase chain reaction (25). The cDNA fragments encoding several rabphilin-11 deletion mutants were inserted into pBTM116, encoding the DNA-binding domain of LexA: pLexA-rabphilin-11-1 (1–631 aa) was transformed with a human T cell cDNA library (MATCHMAKER human T cell oligo(dT)-primed library in pACT, CLONTECH). About 1.0 × 10⁶ transformants were screened, and library plasmids from 16 positive clones were analyzed by transformation tests and DNA sequencing (22, 23). β-Galactosidase activity was measured by liquid and filter assays (23, 24). Where indicated, cotransformation was performed into yeast strain TAT7.

Assay for Rabphilin-11-mSec13p Interaction in Vitro—Rabphilin-11full and rabphilin-11-1 (1–631 aa) were in vitro translated using the TNT T7-coupled reticulocyte lysate system (Promega Corp.) and incubated with MBP or MBP-tagged mSec13p (100 pmol) prebound to an amylase resin column in buffer A (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1% Nonidet P-40, and 150 mM NaCl) at 4 °C for 90 min. After the beads were washed four times with the same buffer, the bound proteins were eluted by adding 100 µl of buffer A containing 10 mM maltose. The eluates were subjected to SDS-PAGE followed by autoradiography. The mouse brain cytosol was subjected to immunoprecipitation with 10 pmol of the anti-mSec13p antibody or the premi...
mune rabbit polyclonal antibody bound to 20 μl of protein A-Sepharose beads (Amersham Pharmacia Biotech). Comparable amounts of the pellets were subjected to SDS-PAGE followed by immunoblotting with the anti-rabphilin-11 antibody. The antibodies used here were prepared as described below.

**Cell Culture and Transfection—**HeLa cells and BHK cells were supplied by Dr. S. Orita (Discovery Research Laboratory, Shionogi & Co. Ltd., Osaka, Japan). Both HeLa cells and BHK cells were cultured at 37 °C in a humidified atmosphere of 5% CO2 and 95% air in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Life Technologies, Inc.), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. These cells were transiently transfected with the plasmids encoding the indicated proteins by use of Superfect reagent (Qiagen).

**Antibodies and Immunofluorescence Staining—**A rabbit polyclonal antibody against mSec13p was raised against glutathione S-transferase-tagged mSec13p. The antisera was affinity-purified with MBP-tagged mSec13p covalently coupled to CNBr-activated Sepharose beads (Amersham Pharmacia Biotech). An anti-rabphilin-11 antibody was obtained as described (9). Hybridoma cells expressing the mouse monoclonal anti-Myc antibody (9E10) were purchased from American Type Culture Collection (Manassas, VA). An anti-Golgi p58 antibody was purchased from Sigma. An anti-Calnexin antibody was purchased from StressGenn Biotechnologies Corp. (Victoria, Canada). Second antibodies for immunofluorescence microscopy were obtained from Chemicon International, Inc. (Temecula, CA). Immunofluorescence staining was performed as described (9). The cells were examined by use of an Eclipse E800 immunofluorescence microscope (Nikon, Tokyo, Japan).

**Exoyosis of VSV G Proteins—**Equal amounts of the plasmids encoding several rabphilin-11 deletion mutants and GFP-tagged VSV G protein were used for cotransfection experiments. After 17 h, transfected BHK cells were incubated at 20 °C for 2 h. Parallel samples were transferred to 37 °C for 1 h immediately after incubation at 20 °C. Cells were then fixed and examined by immunofluorescence microscopy.

**RESULTS**

We first attempted to isolate a rabphilin-11-interacting protein(s) by use of the yeast two-hybrid method with the N-terminal fragment (rabphilin-11-1, 1–631 aa) as a bait from a human T cell cDNA library. Screening of 1 × 106 transformants yielded 16 positive clones, and 6 of these clones encoded full-length mSec13p. One of the six clones was selected for further analysis. The selected clone (clone 5) indeed interacted with rabphilin-11-1 (Fig. 1A). This clone also interacted with full-length rabphilin-11 (rabphilin-11-full), to a lesser extent, but did not interact with the C-terminal fragment (rabphilin-11-4, 632–908 aa) (Fig. 1A).

To examine whether rabphilin-11 directly interacts with mSec13p, the binding of in vitro translated [35S]methionine-labeled rabphilin-11-1 (1–631 aa) or rabphilin-11-full to MBP-tagged recombinant mSec13p was examined. Both rabphilin-11-1 and rabphilin-11-full directly interacted with mSec13p (Fig. 1B, a and b). The interaction of rabphilin-11 with mSec13p was confirmed by coimmunoprecipitation from the mouse brain cytosol (Fig. 1C). Moreover, subcellular localization analysis in HeLa cells showed that overexpressed Myc-tagged mSec13p localized at the ER and the Golgi complex (Fig. 2, A and B), whereas endogenous rabphilin-11 localized in perinuclear regions and along microtubules oriented toward the plasma membrane; Myc-tagged mSec13p and rabphilin-11 colocalized in perinuclear regions, most presumably the Golgi complex (Fig. 2C). Essentially equivalent results were obtained in BHK cells and Madin-Darby canine kidney cells (data not shown). These results suggest that rabphilin-11 directly interacts with mSec13p in both cell-free and intact cell systems.

We next studied the mSec13p-binding region of rabphilin-11 by the yeast two-hybrid method. Rabphilin-11-full interacted with mSec13p to some extent, but the region (rabphilin-11-3, 504–631 aa) that contains the second and third WD-40 repeats and is located next to the Rab11p-binding region (500–504 aa), interacted with mSec13p to a greater extent (Fig. 3A). Other regions, excluding this region, did not interact with mSec13p. These results suggest that a region(s) other than the mSec13p-binding region masks this region, thereby hindering the interaction with mSec13p, and that another factor is necessary to open this folded structure.

We next examined the effect of Rab11p on the rabphilin-11-mSec13p interaction by the yeast two-hybrid method. The rab-
 philin-11-full-mSec13p interaction was greater upon coexpression with a dominant active mutant of Rab11p (Rab11pQ70L) than upon coexpression with a dominant negative mutant of Rab11p (Rab11pT25N) or with no expression of these mutants (Fig. 3B), suggesting that the rabphilin-11-full-mSec13p interaction is enhanced by the binding of GTP-Rab11p to rabphilin-11. This enhancement, however, did not reach the level of the rabphilin-11-3-mSec13p interaction.

We attempted to determine the rabphilin-11-binding region of mSec13p by the same method. The N-terminal one-third (1–187 aa), the middle one-third (135–249 aa), or the C-terminal one-third (249–323 aa) of mSec13p did not interact with rabphilin-11-2 (300–631 aa). Myc-tagged rabphilin-11-3 (504–631 aa) or the mSec13p-binding region of rabphilin-11 (Myc-tagged rabphilin-11-5; 300–504 aa) markedly inhibited the transport of GFP-VSV G protein (Fig. 4B, a and b). However, overexpression of Myc-tagged rabphilin-11-full (data not shown) or Myc-tagged rabphilin-11-2 (300–631 aa), which contains both the Rab11p-binding and mSec13p-binding regions, did not affect this transport (Fig. 4Bc). These results, together with the earlier observation that Rab11p regulates Golgi-to-plasma membrane vesicle transport (6), suggest that the rabphilin-11-mSec13p interaction is important in vesicle transport.

DISCUSSION

We have shown here that rabphilin-11 directly interacts with mSec13p by use of three different methods: yeast two-hybrid, affinity column chromatography, and immunoprecipitation. The interaction of full-length rabphilin-11 with mSec13p is weaker than that of the mSec13p-binding region of rabphilin-11 with mSec13p, suggesting that a region(s) other than the mSec13p-binding region masks this region and hinders the interaction with mSec13p. Consistently, the binding of GTP-Rab11p to full-length rabphilin-11 enhances its interaction with mSec13p, whereas GTP-Rab11pQ70L, which activates Rab11p, does not.

Finally, we examined whether the rabphilin-11-mSec13p interaction is important in vesicle trafficking. For this purpose, we took advantage of the VSV G protein transport system. VSV G protein is a membrane protein encoded by the vesicular somatitis virus (which is transported to the cell surface along the exocytic pathway) and can be utilized as an ideal marker of vesicle trafficking (6, 26–28). As described (6), in BHK cells, exogenously expressed GFP-tagged VSV G (GFP-VSV G) protein accumulated in perinuclear regions, which presumably correspond to the Golgi complex, by incubation at 20 °C for 2 h and was transported to the plasma membrane by incubation at 37 °C for 1 h (Fig. 4A, a and b). Overexpression of a Myc-tagged dominant negative mutant of Rab11p (Rab11pT25N) markedly inhibited transport of GFP-VSV G protein to the cell surface as compared with control cells (data not shown), consistent with earlier observations (6). Similarly, overexpression of the Rab11p-binding region of rabphilin-11 (Myc-tagged rabphilin-11-5; 300–504 aa) or the mSec13p-binding region of rabphilin-11 full (Fig. 4B, a and b). Overexpression of a Myc-tagged dominant negative mutant of Rab11p (Rab11pT25N) markedly inhibited transport of GFP-VSV G protein to the cell surface as compared with control cells (data not shown), consistent with earlier observations (6). Similarly, overexpression of the Rab11p-binding region of rabphilin-11 (Myc-tagged rabphilin-11-5; 300–504 aa) or the mSec13p-binding region of rabphilin-11 was transformed into strain TAT7 expressing pLexA-Rab11p-rabphilin-11-mSec13p interaction.

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with mSec13p, suggesting that the GTP-Rab11p-binding region may be such a region and that the binding of GTP-Rab11p to this region may directly or indirectly unfold the mSec13p-binding region. However, the enhancement by GTP-Rab11p does not reach the level of interaction of the mSec13p-binding region of rabphilin-11 with mSec13p. It has previously been proposed that there is some factor that promotes the unmasking of the GTP-Rab11p-binding region of rabphilin-11 (8), suggesting that a region(s) other than the GTP-Rab11p-binding region may also be involved in this masking and unfolding mechanism.

We have previously shown that rabphilin-11 is associated with membrane structures, presumably vesicles, in a GTP-Rab11p-dependent manner (9). The precise localization of mSec13p is not known, but by analogy with yeast Sec13p (10, 11), mSec13p may also localize on vesicles. If this is the case, one possible mechanism of the GTP-Rab11p-rabphilin-11-full-mSec13p interaction on vesicles is that GTP-Rab11p first binds to rabphilin-11 to make the folding structure open, eventually leading to the association of the GTP-Rab11p-rabphilin-11-mSec13p complex on the vesicles. The prenyl moiety of GTP-Rab11p may also be involved in the association of the complex with the vesicles, but the specific binding of the GTP-Rab11p-rabphilin-11-mSec13p complex may be determined by the interaction with mSec13p.

It has been reported that yeast Sec13p participates in the formation of two different types of vesicles: the vesicles that bud from the late-Golgi complex (29) and COPII coated vesicles that bud from the ER (10–12, 17). In the former case, Sec13p may be involved in protein transport from the Golgi complex to the plasma membrane (29). We have shown here that mSec13p localizes in perinuclear regions, presumably the ER and the Golgi complex, where the GTP-Rab11p-rabphilin-11 complex localizes in perinuclear regions, presumably the ER and the Golgi complex. By analogy with yeast Sec13p, mSec13p may also be involved in the budding of vesicles from the Golgi complex, and this process may be regulated by Arf small G proteins (30–33). Once the coated vesicles are produced from the Golgi complex, they are uncoated and transported to the plasma membrane along microtubules. Rab family members are involved in the targeting/docking/fusion processes of vesicles to the acceptor membranes (34–37). If Rab11p is also involved in these processes, the rabphilin-11-mSec13p interaction may functionally link two different types of small G proteins, Arf and Rab proteins. Further studies are necessary for our understanding of the mode of action of the rabphilin-11-mSec13p interaction in vesicle trafficking.

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