Mapping of Functional Domains of γ-SNAP*

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γ-Soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein (γ-SNAP) is capable of stabilizing a 20 S complex consisting of NSF, α-SNAP, and SNAP receptors (SNAREs), but its function in vesicular transport is not fully understood. Our two-hybrid analysis revealed that γ-SNAP, unlike α-SNAP, interacts directly with NSF, as well as Gaf-1/Rip11, but not with SNAREs. Gaf-1/Rip11 is a γ-SNAP-associated factor that belongs to the Rab11-interacting protein family. To gain insight into the molecular basis for the interactions of γ-SNAP with NSF and Gaf-1/Rip11, we determined the regions of the three proteins involved in protein-protein interactions. γ-SNAP bound to NSF via its extreme C-terminal region, and the full-length NSF was needed to interact with γ-SNAP. Both the N-terminal and C-terminal regions of γ-SNAP were required for the binding to Gaf-1/Rip11. Gaf-1/Rip11 bound to γ-SNAP via its C-terminal domain comprising a putative coiled-coil region. Although the C-terminal domain of Gaf-1/Rip11 also interacts with Rab11, the binding of γ-SNAP and Rab11 to Gaf-1/Rip11 was not mutually exclusive. Rather, Gaf-1/Rip11 was capable of serving a link between γ-SNAP and Rab11. A complex comprising γ-SNAP and Gaf-1/Rip11 was disassembled in a process coupled to NSF-mediated ATP hydrolysis, suggesting that the interaction between γ-SNAP and Gaf-1/Rip11 is of functional significance.

Many membrane fusion events occurring in membrane trafficking in eukaryotic cells require a common set of proteins such as N-ethylmaleimide-sensitive factor (NSF) and SNAPs and membrane compartment-specific proteins consisting of complementary sets of coiled-coil proteins called SNAREs (1–4). Although the precise molecular mechanism for membrane fusion is not fully understood, one current model predicts that membrane fusion is driven by a tight binding between vesicle-associated SNAREs (the VAMP/synaptobrevin family) and target membrane-associated SNAREs (the syntaxin and SNAP-25 families) (5). SNAREs per se encode compartmental specificity of membrane fusion (6). Following membrane fusion, the cis (same membrane) SNARE complex is disassembled by the chaperone ATPase NSF (2). SNAPs not only serve as a linkage between NSF and the cis SNARE complex (7, 8) but also couple the energy produced by NSF-mediated ATP hydrolysis to a conformational change in syntaxin of the cis SNARE complex (9), which leads to the disassembly of the NSF-SNAPs-SNAREs complex (7, 8).

The SNAP family consists of ubiquitously expressed α and γ isoforms and a brain-specific β isoform (10). So far α-SNAP has been studied most extensively. α-SNAP stimulates the ATPase activity of NSF (11) by interacting with its N-terminal and extreme C-terminal regions (12) and is essential for a wide variety of membrane fusion events including intra-Golgi protein transport (13). Based on the results of the x-ray crystallographic analysis of Sec17p, the yeast ortholog of α-SNAP (13, 14), it has been proposed that α-SNAP (Sec17p) functions as lever arms that transmit forces generated by a conformational change in NSF to SNAREs (15). Given the high sequence similarity between α-SNAP and β-SNAP, it is postulated that the function of β-SNAP is quite similar to that of α-SNAP. On the other hand, the physiological significance of γ-SNAP is much less documented. Although γ-SNAP enhances the secretion of neurotransmitters from several types of cells (16, 17), it exhibits a very low transport activity in an intra-Golgi protein transport assay (13) and is not required for transport from the endoplasmic reticulum to the Golgi (18). γ-SNAP stabilizes the 20 S NSF-α-SNAP-SNAREs complex but cannot substitute for α-SNAP in complex formation (19).

Chen et al. (20) reported that Gaf-1 (γ-SNAP-associated factor-1) encoded by the clone KIAA0857 (21) interacts with γ-SNAP and is localized in the mitochondria. Independently, Prekeris et al. (22) demonstrated that the same protein but differently named Rip11 (Rab11-interacting protein), because of its ability to bind to Rab11, is localized in recycling endosomes and regulates apical membrane trafficking by interacting with Rab11 in polarized epithelial cells. Recent studies revealed that Gaf-1/Rip11 and several other proteins constitute a family of Rab11-interacting proteins (22–27).

In the present study we extensively characterized the interactions of γ-SNAP with NSF and Gaf-1/Rip11. The results suggest that γ-SNAP interacts with NSF via its extreme C-terminal region, whereas the N-terminal and C-terminal regions are required for the interaction with Gaf-1/Rip11. In addition, we showed a complex comprising γ-SNAP and Gaf-1/Rip11 is disassembled in a process coupled to NSF-mediated ATP hydrolysis.

EXPERIMENTAL PROCEDURES

Antibodies—A Gaf-1/Rip11 fragment (amino acids 524–653) and the full-length γ-SNAP were expressed as His$_6$-tagged proteins in Escherichia coli cells and purified using a nickel-nitrioltriacetic acid-agarose column (Qiagen). The isolated proteins were injected into rabbits to produce antisera. Monoclonal antibodies against Rab11 (clone 47; Transduction Laboratories), γ-tubulin (GTU-88; Accurate Chemical and Scientific Co.), FLAG (M2; Sigma), and GST (Clontech) were obtained. A polyclonal antibody against GST was obtained from Santa Cruz Biotechnologies. A monoclonal antibody against c-Myc (clone 9E10) was purified from ascitic fluid and cross-linked to protein G beads.
GST-hybrid analysis. Full-length or mutant γ-SNAP cDNA fragments and full-length NSF cDNA were inserted into pGBT9 and pGAD424, respectively. The interaction of γ-SNAP with NSF, or its fragments in yeast was examined using a β-galactosidase filter assay. B, pull-down experiments. Coomassie-stained gel of isolated Hisγ-tagged NSF (lane 1), GST (lane 2), GST-γ-SNAP (full-length; γ-SNAP/F) (lane 3), GST-γ-SNAP (amino acids 1–152; γ-SNAP/N) (lane 4), and GST-γ-SNAP (amino acids 153–312; γ-SNAP/C) (lane 5). To analyze protein-protein interactions, Hisγ-tagged full-length NSF (10 μg) was mixed with glutathione-Sepharose 4B that had been incubated with 12 μg of GST (lane 6), GST-γ-SNAP (full-length) (lane 7), GST-γ-SNAP (amino acids 1–152) (lane 8), or GST-γ-SNAP (amino acids 153–312) (lane 9). The bound proteins were eluted with glutathione, separated by SDS-PAGE, and stained.

Using dimethyl pimelimidate. A monoclonal antibody against NSF (2E5) was prepared as described (28).

Plasmids—pQE9, pQE30, and pQE31 were purchased from Qiagen and used for the expression of Hisγ-tagged proteins. pGE4-4T-1 was obtained from Amersham Biosciences and used for the expression of GST fusion proteins. The clone KIA0857 was obtained from the Kazusa DNA Research Institute, Kisarazu, Japan. pEBG (29) and pFLAG-CMV2 (Eastman Kodak Co.) were used for the expression of proteins in mammalian cells. The cDNA encoding γ-SNAP was a generous gift from Dr. S. W. Whiteheart (University of Kentucky College of Medicine). The plasmids for Myc-tagged Rab11Q70L and Rab11S20N were kindly donated by Dr. Y. Takai (Osaka University).

Two-hybrid Analysis—The full-length cDNA of γ-SNAP was inserted into pGBT9, and the resultant plasmid was used as bait. A GAL4 DNA activation domain fusion library in pACT2 (MATCHMAKER human kidney cDNA library; Clontech) was used to isolate interacting clones. Yeast colonies that grew in medium lacking histidine were picked up, and their β-galactosidase activity was assayed on filters. To examine protein-protein interactions, cDNAs for γ-SNAP and α-SNAP were inserted into the bait plasmid pGBT9, and cDNAs for NSF and Gaf-1/Rip11 were inserted into the prey plasmid pGAD424. β-Galactosidase activity was measured after transformation of yeast with the indicated combinations of the bait and prey plasmids.

Pull-down Experiments Using Recombinant Proteins—Recombinant GST proteins were mixed with 10 μl of glutathione-Sepharose 4B and then the mixture was gently rotated at 4°C for 30 min. After the beads were washed, they were incubated with Hisγ-tagged proteins in incubation buffer comprising 20 mM HEPES/KOH, pH 7.0, 50 mM KCl, 2 mM mercaptoethanol, 0.5 mM ATP, 10% glycerol, and 1 mg/ml bovine serum albumin at 4°C for 1 h with gentle rotation. The beads were washed three times with incubation buffer devoid of bovine serum albumin, and the bound proteins were eluted with 50 mM Tris-HCl, pH 8.0, with 5 mM glutathione. The eluted proteins were concentrated by trichloroacetic acid-deoxycholate precipitation and then subjected to SDS-PAGE on 10% gels. Gels were stained with Coomassie Brilliant Blue R-250

Immunoprecipitation and Pull-down Experiments in a Mammalian Expression System—Transfection of cells and preparation of cell lysates were performed as described (30). For co-transfection, plasmids (1 μg each) were used. At 20 h after transfection, cells plated on 35-mm dishes were washed and then lysed in 300 μl of lysis buffer comprising 25 mM HEPES/KOH, pH 7.2, 1% Triton X-100, 150 mM KCl, 0.5 μg/ml
leupeptin, 2 μM pepstatin, 2 μg/ml aprotinin, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The lysates were clarified by centrifugation. To the supernatants was added 5–5 μg of an antibody. After 30 min on ice, 10 μg of protein G-Sepharose 4B was added to the mixtures and then incubated at 4 °C for 2 h with gentle rotation. The beads were washed with lysis buffer several times and then the bound proteins were eluted by boiling in 10 mM SDS-PAGE sample buffer. For pull-down experiments, cell lysates were mixed with 10 μg of glutathione-Sepharose 4B and then incubated at 4 °C for 2 h with gentle rotation. The beads were washed, and the bound proteins were eluted as described above.

**Immunoblotting**—Proteins were separated by SDS-PAGE and then transferred to nitrocellulose membranes. After incubation with appropriate primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies, the membranes were developed on autoradiographic film by enhanced chemiluminescence (Amersham Biosciences).

**Preparation of Rat Liver Membranes**—Livers were removed from starved rats, rinsed, perfused, and weighed. They were cut into small pieces and homogenized with a Potter-Elvehjem homogenizer (5 strokes) in 4 volumes of homogenization buffer comprising 25 mM HEPES/KOH, pH 7.2, 100 mM KCl, 0.5 mM leupeptin, 2 μM pepstatin, 2 μg/ml aprotinin, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The homogenate was centrifuged at 3,000 rpm (Tomty TA-4 rotor) for 10 min and then at 10,000 rpm for 10 min. The supernatant was centrifuged at 33,000 rpm (Beckman SW50 rotor) for 1 h to recover membranes.

**Velocity Sedimentation**—Velocity sedimentation was performed essentially as described (7) with a slight modification. Rat liver membranes (1.0 mg) were solubilized with 1% Triton X-100, and insoluble materials were removed by centrifugation. The membrane extracts (220 μl) were mixed with His6-tagged NSF (3 μg) and His6-tagged γ-SNAP (0.7 μg), and the mixture was incubated on ice for 30 min. When indicated, 1 mM MgCl2 was added to the mixture at a final concentration of 8 mM. The mixture was layered on the top of glycerol gradients consisting of 0.4 ml each of gradient buffer containing 35, 32.5, 30, 27.5, 25, 22.5, 20, 17.5, 15, and 12.5% glycerol (w/v). After centrifugation at 34,000 rpm (Beckman SW50 rotor) for 13 h, fractions (0.4 ml each) were recovered from the top. The proteins of the gradient fractions were precipitated with trichloroacetic acid and deoxycholate, washed with acetone, and then dissolved in SDS-PAGE sample buffer.

**Disassembly of a Complex Containing GST-Gaf-1/Rip11 and γ-SNAP**—Cells expressing GST-Gaf-1/Rip11 and FLAG-tagged γ-SNAP, cultured on 35-mm dishes, were lysed in 300 μl of lysis buffer. The lysates were mixed with an equal volume of cell lysates separately prepared from non-transfected cells. When indicated, 1 mM MgCl2 was added to the mixture at a final concentration of 8 mM. The mixture was incubated at 16 °C for 1 h and then GST-Gaf-1/Rip11 was pulled down with glutathione beads. The proteins pulled down were subjected to SDS-PAGE and analyzed by immunoblotting with antibodies against GST and FLAG.

For the preparation of NEM-treated cell lysates, non-transfected cells were lysed in lysis buffer devoid of dithiothreitol. The cell lysates were incubated with 1 mM NEM on ice for 15 min followed by 15 min treatment with 2 mM dithiothreitol to quench excess NEM.

**RESULTS**

**Screen of γ-SNAP-interacting Proteins**—To identify γ-SNAP-interacting proteins other than Gaf-1/Rip11 (20), we screened a human kidney expression library using the full-length γ-SNAP as bait. Screening two times yielded 68 positive clones. Among

**Table I**

| Syntaxin 4 | Syntaxin 5M2 | Syntaxin 7 | Syntaxin 18 | Celldubrevin | SNAP-23 |
|-----------|-------------|------------|-------------|--------------|---------|
| α-SNAP    | ++          | ++         | +           | ++           | ND      | ND      |
| γ-SNAP    | –           | –          | –           | –            | –       | –       |

* A fragment of syntaxin 18 (amino acids 82–335) was used.
* Not determined.
the C-terminal region common to Gaf-1/Rip11 and its alternative splice variant is involved in the interaction with γ-SNAP. A two-hybrid analysis. Full-length γ-SNAP cDNA and full-length or truncated Gaf-1/Rip11 cDNA fragments were inserted into pGBT9 and pAD424, respectively. The interaction between γ-SNAP and Gaf-1/Rip11 or truncated ones in yeast was examined using a β-galactosidase filter assay. C2 represents the C2-domain. Hatched and filled boxes denote the C-terminal region common to Gaf-1/Rip11 and its alternative splice variant and the putative coiled-coil region, respectively. B, interaction between γ-SNAP and truncated Gaf-1/Rip11 constructs in mammalian cells. 293T cells were transfected with a plasmid encoding GST (lanes 1 and 4) or a Gaf-1/Rip11 fragment corresponding to amino acids 608–640 (lanes 2 and 5) or 590–640 (lanes 3 and 6) fused to GST, in addition to a plasmid for FLAG-tagged γ-SNAP. The GST fusion proteins in cell lysates were pulled down, and the precipitated proteins were analyzed by immunoblotting with antibodies against γ-tubulin, FLAG, and GST (lanes 4–6). Five percent input was also analyzed (lanes 1–3).

Interaction between γ-SNAP and NSF—Because the direct interaction between γ-SNAP and NSF was not reported previously, we decided to analyze the interaction in detail. We first generated γ-SNAP mutants encoding the N-terminal half (amino acids 1–152) and the C-terminal one (amino acids 153–312). A two-hybrid assay revealed that the C-terminal region is responsible for the interaction with NSF (Fig. 1A). We then generated a series of mutants with C-terminal deletions. When two amino acids were removed from the C terminus, the interaction between γ-SNAP and NSF was abolished. To examine whether the two C-terminal residues, Leu-311 and Cys-312, directly contribute to the interaction with NSF, we replaced them individually and both by Ala via site-directed mutagenesis. The results showed that both the residues are important for the interaction with NSF (Fig. 1A).

To confirm the results of the two-hybrid analysis, we performed an in vitro binding assay using isolated bacterial proteins. To facilitate purification, NSF was expressed as a His6-tagged protein, and γ-SNAP was expressed as a GST fusion protein. Consistent with the results of the two-hybrid analysis, His6-tagged NSF was pulled down with GST-γ-SNAP (full-length) (Fig. 1B, lane 7) and GST-γ-SNAP (amino acids 153–312) (lane 9) but not with GST-γ-SNAP (amino acids 1–152) (lane 8). In addition, His6-tagged NSF was not pulled down with GST-γ-SNAP in which Leu-311 was replaced by Ala (L311A) (Fig. 1C, lane 8). Although His6-tagged NSF was pulled down with GST-γ-SNAP in which Cys-312 was replaced by Ala (C312A) (lane 9), the amount was smaller than that pulled down with wild-type GST-γ-SNAP. These results clearly showed that the extreme C-terminal region, especially Leu-311, is important for the interaction with NSF.

We next determined the binding site for γ-SNAP on NSF. NSF can be divided into three domains, N-terminal and two ATP-binding domains referred to as D1 and D2 (28, 31, 32). Two-hybrid analysis and in vitro binding experiments revealed that the full-length NSF, but neither the N-terminal domain (amino acids 1–205) nor the D1D2 domain (amino acids 206–744), binds to γ-SNAP (Fig. 2). These results are consistent with the fact that only full-length NSF clones were obtained in the two-hybrid screen.

γ-SNAP Does Not Bind to SNAREs—a-SNAP interacts primarily with syntaxin and weakly with SNAP-25 (9, 33). Our previous results (34) demonstrated that SNAREs can interact directly with NSF, primarily with syntaxin and weakly with SNAP-25 (9, 33). Our previous results (34) demonstrated that SNAREs can interact directly with NSF, primarily with syntaxin and weakly with SNAP-25 (9, 33). Consistent with the results of the two-hybrid analysis, His6-tagged NSF was pulled down with GST-γ-SNAP (full-length) (Fig. 1B, lane 7) and GST-γ-SNAP (amino acids 153–312) (lane 9) but not with GST-γ-SNAP (amino acids 1–152) (lane 8). In addition, His6-tagged NSF was not pulled down with GST-γ-SNAP in which Leu-311 was replaced by Ala (L311A) (Fig. 1C, lane 8). Although His6-tagged NSF was pulled down with GST-γ-SNAP in which Cys-312 was replaced by Ala (C312A) (lane 9), the amount was smaller than that pulled down with wild-type GST-γ-SNAP. These results clearly showed that the extreme C-terminal region, especially Leu-311, is important for the interaction with NSF.

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essential for γ-SNAP to be incorporated into the 20 S complex. **Interaction between γ-SNAP and Gaf-1/Rip11**—To gain insight into the mechanism governing the interaction between γ-SNAP and Gaf-1/Rip11, we determined the region of Gaf-1/Rip11 involved in the association with γ-SNAP. The binding site for γ-SNAP on Gaf-1/Rip11 must be within its C-terminal 131 amino acids, because the C-terminal region common to Gaf-1/Rip11 and its putative alternative splice variant interacts with γ-SNAP in the two-hybrid system. We constructed a series of C-terminal fragments of Gaf-1/Rip11 and performed a yeast two-hybrid assay using the full-length γ-SNAP as bait (Fig. 4A). The results confined the region responsible for the interaction with γ-SNAP to amino acids 590–640 of Gaf-1/Rip11. This region comprises a putative coiled-coil region (amino acids 608–640), but the coiled-coil region alone was not sufficient to interact with γ-SNAP. To confirm this observation, we constructed plasmids to express amino acids 590–640 and 608–640 of Gaf-1/Rip11 as GST fusion proteins and γ-SNAP as a FLAG-tagged protein and performed pull-down experiments after cotransfection of the plasmids into 293T cells (Fig. 4B). Consistent with the results of the yeast two-hybrid analysis, FLAG-tagged γ-SNAP was pulled down with the GST fusion protein comprising amino acids 590–640 of Gaf-1/Rip11 (lane 6) but not with that comprising amino acids 608–640 (lane 5).

A previous study revealed that Gaf-1/Rip11 is associated with γ-tubulin (20). Interestingly, endogenous γ-tubulin was efficiently pulled down with the construct comprising amino acids 608–640, which could not interact with γ-SNAP, suggesting that this region is sufficient to interact with γ-tubulin. The fact that the amount of γ-tubulin bound to the construct containing amino acids 590–640 of Gaf-1/Rip11 was trivial compared with that bound to the construct containing amino acids 608–640 may imply that expressed FLAG-tagged γ-SNAP competed with endogenous γ-tubulin for the binding to the construct containing amino acids 590–640 of Gaf-1/Rip11.

To determine the region of γ-SNAP involved in the interaction with Gaf-1/Rip11, we performed a two-hybrid assay using γ-SNAP or its deletion mutants as bait. The results are summarized in Fig. 5A. Deletion of N-terminal 23 amino acids or C-terminal 89 amino acids eliminated the interaction with Gaf-1/Rip11. Similar results were obtained when pull-down experiments were conducted using a mammalian expression system (Fig. 5B).

**Gaf-1/Rip11 Is Able to Link γ-SNAP to Rab11**—Members of the Rab11-interacting protein family share a highly conserved region of 20 amino acids at the C terminus (22–26). Prekeris et al. (23) showed that a peptide corresponding to amino acids 628–645 of Gaf-1/Rip11 competes with Gaf-1/Rip11 protein for the binding to Rab11. Because the Rab11-binding domain on Gaf-1/Rip11 markedly overlaps with the γ-SNAP-binding domain, we wondered whether the γ-SNAP-binding domain can interact with Rab11. Myc-tagged Rab11 proteins and the full-length Gaf-1/Rip11 or its fragments fused to GST were expressed in 293T cells, and immunoprecipitation was performed by using an anti-Myc antibody. As shown in Fig. 6A, the γ-SNAP-binding domain (amino acids 590–640 of Gaf-1/Rip11) can interact with the constitutively active Rab11, Rab11Q70L (lane 9), although its interaction was markedly weaker than the interaction of the full-length Gaf-1/Rip11 (lane 10). Perhaps the C-terminal sequence (amino acids 641–645) of the Rab11-binding domain significantly contributes to the binding to Rab11. No interaction was observed with the inactive Rab11, Rab11S25N (lane 12). The coiled-coil region responsible for the interaction with γ-tubulin (amino acids 608–640 of Gaf-1/Rip11) did not bind to Rab11Q70L (lane 8).
Functional Regions of γ-SNAP

Fig. 6. Interaction between Gaf-1/Rip11 and Rab11. A, the C-terminal region of Gaf-1/Rip11 is involved in the interaction with Rab11. 293T cells were transfected with a plasmid encoding GST (lane 1), Gaf-1/Rip11 fragment corresponding to amino acids 608–640 (lanes 2 and 5), 590–640 (lanes 3 and 6), or full-length Gaf-1/Rip11 (lane 4) fused to GST, in addition to a plasmid encoding Rab11Q70L (lanes 1–4) or Rab11S25N (lanes 5 and 6) tagged with Myc. The expression levels of Myc-tagged Rab proteins were comparable (data not shown). Myc-tagged Rab proteins were immunoprecipitated, and the precipitated proteins were analyzed by immunoblotting with an anti-GST antibody (lanes 7–12). Five percent input was also analyzed (lanes 1–6). HC and LC denote immunoglobulin heavy chain and light chain, respectively. B, Gaf-1/Rip11 can link Rab11 to γ-SNAP. 293T cells were co-transfected with plasmids for Gaf-1/Rip11 (lanes 1, 2, 5, and 6), Myc-tagged Rab11Q70L (lanes 1, 3, 5, and 7) or Rab11S25N (lanes 2, 4, 6, and 8), and FLAG-tagged γ-SNAP (lanes 1–8). Myc-tagged Rab proteins were immunoprecipitated with an anti-Myc antibody cross-linked to protein G beads, and the precipitated proteins were analyzed by immunoblotting with antibodies against Gaf-1/Rip11, FLAG, and Myc (lanes 5–8). Five percent input was also analyzed (lanes 1–4).

Myc-tagged Rab11Q70L, Gaf-1/Rip11, and FLAG-tagged γ-SNAP were co-expressed, Gaf-1/Rip11 was still co-precipitated with an anti-Myc antibody (Fig. 6B, lane 5). Surprisingly, a small but significant amount of FLAG-tagged γ-SNAP was also co-precipitated with Myc-Rab11Q70L. On the other hand, no co-precipitation of FLAG-tagged γ-SNAP was observed with Myc-Rab11S25N even in the presence of Gaf-1/Rip11 (lane 6). It should be noted that expression of Gaf-1/Rip11 is prerequisite for the co-precipitation of γ-SNAP with Rab11Q70L (lane 7). These results suggest that Gaf-1/Rip11 is able to link between Rab11 and γ-SNAP, although the linkage efficiency is not high.

A Complex Containing γ-SNAP and Gaf-1/Rip11 Is Disassembled in a Mg\(^{2+}\)-ATP-Dependent Manner—The 20 S NSF-SNAPs-SNAREs complex is disassembled in a process coupled to NSF-mediated ATP hydrolysis (7). This most likely reflects disassembly of the cis SNARE complex after membrane fusion (1–4). We were interested in whether the binding between γ-SNAP and Gaf-1/Rip11 is also affected by NSF and α-SNAP in a Mg\(^{2+}\)-ATP-dependent manner. To address this question, Triton X-100 extracts prepared from cells expressing FLAG-tagged γ-SNAP and GST-Gaf-1/Rip11 were mixed with lysates separately prepared from non-transfected cells, and the mixtures were incubated in the presence of EDTA-ATP or Mg\(^{2+}\)-ATP. GST-Gaf-1/Rip11 in the mixtures was pulled down, and the precipitated proteins were analyzed by immunoblotting. As shown in Fig. 7, addition of Mg\(^{2+}\)-ATP caused the dissociation of FLAG-tagged γ-SNAP from GST-Gaf-1/Rip11 (lane 9), implying the disassembly of the γ-SNAP-Gaf-1/Rip11 complex. Addition of cell lysates from non-transfected cells was necessary for efficient disassembly. It should be noted that FLAG-tagged γ-SNAP and GST-Gaf-1/Rip11 were overexpressed. Perhaps the cell lysates may supply membrane and cytosolic proteins required for the disassembly reaction under conditions in which γ-SNAP and Gaf-1/Rip11 were present at high levels. When cell lysates were pretreated with 1 mM NEM, no dissociation of FLAG-tagged γ-SNAP from GST-Gaf-1/Rip11 occurred (lane 13), indicating the requirement of NEM-sensitive component(s) for this process. Addition of His\(_6\)-tagged NSF and His\(_6\)-tagged α-SNAP restored the dissociation reaction (lane 15). These results unequivocally showed the requirement of NSF for the disassembly of the γ-SNAP-Gaf-1/Rip11 complex.

DISCUSSION

In the present study we analyzed the interactions of γ-SNAP with NSF and Gaf-1/Rip11 in detail using the yeast two-hybrid system and isolated recombinant proteins or a mammalian expression system. Our results demonstrated that the extreme C-terminal region of γ-SNAP, especially Leu-311, is required for the interaction with NSF, and the N-terminal 23 amino acids and C-terminal 89 amino acids of γ-SNAP contribute to the binding to Gaf-1/Rip11. For the interaction with γ-SNAP, on the other hand, the entire coding region of NSF and the C-terminal region (amino acids 590–640) of Gaf-1/Rip11 are required.

Although α-SNAP mediates the attachment of NSF to SNAREs (7, 8), it appears that α-SNAP interacts with NSF only when it binds to SNAREs or plastic (12, 13, 37). Binding of α-SNAP to plastic may cause a conformational change in α-SNAP similar to that occurring upon binding to SNAREs. Consistent with the idea that SNAREs are required for the interaction of α-SNAP with NSF, no clones encoding NSF were obtained in our previous two-hybrid screen using α-SNAP as bait (34). Indeed, our yeast two-hybrid assay showed that α-SNAP hardly interacts with NSF (data not shown). In contrast, γ-SNAP interacts with NSF in the absence of SNAREs.
Despite this difference in the interactions between the two species of SNAPs and NSF, the penultimate leucine residues of the SNAPs (Leu-294 in α-SNAP and Leu-311 in γ-SNAP) are important for the binding to NSF. In comparison with α-SNAP, γ-SNAP appears to have a 22-amino acid insertion between the putative C-terminal α-helix, which corresponds to loop α14 in Sec17p (15), and the penultimate leucine. This extra sequence may shift the position of Leu-311 such that it can interact with NSF in the absence of SNAREs. Interestingly, replacements of the penultimate leucine residues cause opposite effects in terms of the binding to NSF. An α-SNAP mutant in which Leu-294 is replaced by Ala is able to interact with NSF in a 20 S complex but unable to fully disassemble the complex in the presence of Mg^{2+}-ATP (12). In contrast, the corresponding γ-SNAP mutant is unable to bind to NSF.

Gaf-1/Rip11 is a member of the recently identified Rab11-interacting protein family (22–27). The highly conserved C-terminal region of Rab11-interacting protein family members is responsible for the binding to Rab11. In the present study we revealed that the same or markedly overlapping region is capable of interacting with γ-SNAP. Despite the fact that Rab11 and γ-SNAP bind to the overlapping site on Gaf-1/Rip11, overexpression of γ-SNAP did not inhibit the binding of expressed Rab11 to Gaf-1/Rip11. Rather, a significant amount of γ-SNAP was co-precipitated with Rab11 in the presence of Gaf-1/Rip11, suggesting that Gaf-1/Rip11 is able to serve as a link between γ-SNAP and Rab11. How can Gaf-1/Rip11 connect γ-SNAP to Rab11? Members of the Rab11-interacting protein family including Gaf-1/Rip11 are known to possess extensive homo- and hetero-interacting abilities (27). Using these abilities Gaf-1/Rip11 may constitute a scaffold or a hydrophobic patch (38) that can accommodate Rab11 and γ-SNAP. Obviously, members of the Rab11-interacting protein family bind many proteins. Rab11-FIP2, a member of the family, interacts not only with Rab11 but also with proteins involved in recycling and endocytosis such as myosin Vb, Reps1, and α-adaptin (24, 39). A previous study (20), as well as the present one, showed that Gaf-1/Rip11 binds γ-tubulin.

Both N-terminal and C-terminal regions of γ-SNAP contribute to the binding to Gaf-1/Rip11. Interestingly, the corresponding regions of α-SNAP are known to interact with SNAREs (9, 40). Because γ-SNAP does not bind SNAREs, one plausible possibility is that Gaf-1/Rip11 may provide a docking site for γ-SNAP as SNAREs do for α-SNAP. If this were the case, a complex comprising γ-SNAP and Gaf-1/Rip11 would be disassembled in a manner coupled to NSF-mediated ATP hydrolysis. Our present results clearly showed the requirement of NSF for the disassembly of the γ-SNAP-Gaf-1/Rip11. It is possible that γ-SNAP, in cooperation with NSF, may regulate the function of members of the Rab11-interacting protein family.

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