Identification of Rab6 as an *N*-ethylmaleimide-sensitive fusion protein-binding protein

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In this study we show the interaction of *N*-ethylmaleimide-sensitive fusion protein (NSF) with a small GTP-binding protein, Rab6. NSF is an ATPase involved in the vesicular transport within eukaryotic cells. Using the yeast two-hybrid system, we have isolated new NSF-binding proteins from the rat lung cDNA library. One of them was Rab6, which is involved in the vesicular transport within the Golgi and *trans*-Golgi network as a Ras-like GTPase. We demonstrated that the N-terminal domain of NSF interacted with the C-terminal domain of Rab6, and these proteins were co-immunoprecipitated from the rat brain extract. This interaction was maintained preferentially in the presence of hydrolysable ATP. Recombinant NSF-His_6 can also bind to C-terminal Rab6–glutathione S-transferase under the conditions to allow the ATP hydrolysis. Surprisingly, Rab6 stimulates the ATPase activity of NSF by approx. 2-fold as does α-soluble NSF attachment protein receptor. Anti-Rab6 polyclonal antibodies significantly inhibited the Rab6-stimulated ATPase activity of NSF. Furthermore, we found that Rab3 and Rab4 can also associate with NSF and stimulate its ATPase activity. Taken together, we propose a model in which Rab can form an ATP hydrolysis-regulated complex with NSF, and function as a signalling molecule to deliver the signal of vesicle fusion through the interaction with NSF.

Key words: NSF, Rab protein, small G-protein, vesicular transport.

INTRODUCTION

The extensive network of intracellular membranous organelles allows the eukaryotic cell to carry out a variety of specialized tasks, such as the biogenesis of cellular organelles, secretion of molecules, nucleocytoplasmic transport and endocytosis. The correct and specific transport of macromolecules to the subcellular organelles is an essential process ensuring the maintenance of life. One of the remarkable properties of protein transport through the secretory or endocytic pathway is that small transport vesicles mediate this process. In the study of protein transport, the finding of *N*-ethylmaleimide-sensitive fusion protein (NSF) was a breakthrough [1]. Since then, subsequent findings of soluble NSF-attachment protein (SNAP) and SNAP receptors (SNAREs) highlighted the mechanisms of protein transport [2,3]. The SNARE hypothesis proposed by Rothman has been considered as a general model to explain the specificity of the intracellular protein transport [4]. Each transport vesicle bears a unique address marker for one or more vesicle SNAREs obtained from its donor membrane during vesicle budding, while each target membrane is identified by one or more target SNAREs. Targeting specificity would thus be achieved by vesicle SNAREs binding to matching target SNAREs. Through its interaction with SNAPs, NSF binds to SNAREs to form a complex known as the '20 S particle'. The ATPase activity of NSF disassembles the 20 S complex and causes the membrane fusion [5].

NSF is a key molecule in the vesicle-mediated protein transport. It was first identified as a factor required to restore the transport activity of Golgi membranes inactivated by *N*-ethylmaleimide [1], and then shown to be implicated in many other vesicle-mediated protein transport events, including intra-Golgi protein transport, the secretory pathway and the endocytic pathways in eukaryotic cells [6,7].

NSF forms a barrel-shaped homohexamer [8], and is composed of three domains: the N-domain, responsible for the SNAP/SNARE complex formation; and two ATP-binding domains (D1 and D2), required for 20 S fusion-complex disassembly and hexamer formation [9,10]. NSF is known to exist both in the cytosol and in the membranes of the subcellular organelles such as the Golgi, endosomes and isolated synaptic vesicles [1,11,12]. However, NSF shows differential association properties with membranes. NSFs found on synaptic vesicles, clathrin-coated vesicles, nuclear membrane and endosomes are not released upon treatment with Mg^{2+}-ATP [13–15], whereas NSF is released from Golgi membranes with the same treatment [14].

Although numerous reports have emphasized the involvement of NSF in vesicular transport, the role of NSF has been argued, especially concerning the time point of NSF involvement. In the original SNARE hypothesis, it was believed that NSF is recruited from the cytosol and plays a role at the very point of membrane fusion by ATP hydrolysis by itself [3,4]. However, several lines of evidence suggested other aspects of NSF function. It has been reported that the rate of ATP hydrolysis by NSF is significantly

Abbreviations used: GST, glutathione S-transferase; ct-Rab6–GST, C-terminal Rab6–GST; Ni-NTA, Ni^{2+}-nitrilotriacetate; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF-attachment protein; SNARE, SNAP receptor; RT, reverse-transcriptase; anti-GCI, anti-Golgi complex; DTT, dithiothreitol.

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The nucleotide sequence data reported will appear in GenBank®, DDBJ, GSDB and EMBL Nucleotide Sequence Databases under the accession numbers AF091834 (for rat NSF) and AF148210 (for Rab6).

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slower than the time required for synaptic vesicle fusion to occur [16]. It was also shown that ATP hydrolysis is not required in the final step of regulated exocytosis [17]. Furthermore, kinetic analysis of intra-Golgi transport indicated that NSF may be required for vesicle formation [18], suggesting the pre-fusion role for NSF. In addition, new NSF-binding proteins, such as GluR2, β-arrestin1, and the Golgi-associated ATPase enhancer of 16 kDa ('GATE-16') have been reported [19–22]. However, much of the data accumulated so far do not elucidate the *in vivo* role of NSF and the regulatory mechanisms of NSF action.

With this in mind, we raise the possibility that NSF has an unrecognized activity via the direct interaction with proteins which are not part of the classical 20 S particle. In the present study, we investigated the role of NSF by screening the new NSF-binding proteins. Using part of NSF as bait, we screened a rat lung cDNA library in the yeast two-hybrid system.

MATERIALS AND METHODS

Materials

GTP and ATP radionucleotides were from Dupont NEN (Boston, MA, U.S.A.). Anti-Rab6 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-NSF antibody was from StressGen (Victoria, BC, Canada). Fluorescently labelled secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA, U.S.A.). Anti-Golgi complex (anti-GCI) was purchased from the A.T.C.C. Brefeldin A and ATP[S] were from Sigma.

Yeast two-hybrid screening

Yeast two-hybrid screening was performed using the Matchmaker Two-Hybrid System (ClonTech). The N-terminal domain of rat NSF, subcloned in the *Eco*RI site of DNA-binding-domain vector pGBT9, was used as a bait. The sense primer, 5′-CCGGAATTCCGGTGCCCTACGGATGAA-3′, and the antisenese primer, 5′-CCGGAATTCAGGATGTTGTTCAGCTG-3′, were designed to amplify the N-terminal region of NSF from the rat. The underlined sequences contained *Eco*RI restriction sites for cloning. By reverse-transcriptase (RT)-PCR, NSF primers amplified a product of the expected size (1071 bp) from brain cDNA of the Sprague–Dawley rat. Sequence analysis revealed that the cDNA fragment was rat NSF (GenBank® Accession No. AF091834). A rat lung cDNA library (ClonTech), constructed in the GAL4 activation domain vector pGAD10, was screened using Y190 yeast strain as host. Following colony selection on plates lacking tryptophan, histidine and leucine, and a filter β-galactosidase assay, seven positive clones were isolated and characterized. Later, the specificity of the interaction between NSF and Rab6 was also confirmed by transforming Y190 yeast containing N-terminal NSF-pGBT9 with the various C-terminal fragments of Rab6 in pGAD10.

Immunoprecipitation from whole brain extract

Rat brains from Sprague–Dawley (15 g) were dissected and homogenized with 15 strokes of a type A Dounce homogenizer in 30 ml of homogenization buffer [HB; 25 mM Hepes, pH 7.4, 150 mM NaCl, 1 % Triton X-100, 1 mM dithiothreitol (DTT), 1 mM PMSF]. Tissue extracts were kept on ice for 1 h, and spun at 12000 ***g*** for 10 min. The supernatant was collected and used for immunoprecipitation. Brain extracts (3 mg) were immunoprecipitated with 2 μg of anti-Rab6 antibody (Santa Cruz Biotechnology; sc-310) or non-immune rabbit IgG for 2 h at 4 °C. The immune complex was incubated with Protein A–Sepharose beads (Pharmacia) for 2 h at 4 °C, washed three times with 1 ml of HB buffer, suspended in 2 × SDS/PAGE sample buffer, and then resolved by SDS/PAGE (10 % gel). To examine the ATP-dependence of interaction, rat brain extracts (1 mg) were prepared, immunoprecipitated, and washed with HB buffer supplemented with either 1 mM ATP/4 mM $MgCl_2$ or 1 mM ATP[S]/2 mM EDTA.

Western-blot analysis

Western blotting was performed according to the enhanced chemiluminescence (ECL®) system protocols (Amersham Pharmacia Biotech). The antibodies used were anti-NSF pAb (StressGen; VAP-SV055), anti-glutathione S-transferase (GST) mAb (Santa Cruz Biotechnology; sc-138) and anti-Rab6 pAb (Santa Cruz Biotechnology; sc-310).

GTP blotting

GTP-binding proteins were detected as described by Serafini et al. [23]. After transfer, blots were blocked for 2 h in 30 ml of PBS blocking buffer (containing 1 % gelatin and 0.1 % Tween 20), incubated for 2 h in 10 ml of binding buffer (20 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 0.5 % gelatin, 0.1 % Triton X-100 and [α-^{32}P]GTP, 50 μCi/ml), washed for 1 h with five changes of the binding buffer, air-dried, and autoradiographed using X-ray film with intensifying screens at −80 °C.

Expression and purification of recombinant proteins

To express C-terminal Rab6–GST (ct-Rab6–GST), the Rab6 insert encoding the region from asparagine-99 to cysteine-208 was cloned into pGEX4T-1 bacterial expression vector (Pharmacia) between the *Eco*RI and *Xho*I sites. DH5α *Escherichia coli* cells transformed with ct-Rab6-pGEX4T-1 were grown and induced with 1 mM isopropyl β-D-thiogalactoside for 3 h at 37 °C. Cells were disrupted by sonication in buffer S (50 mM Hepes/KOH, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 1 mM DTT). After the centrifugation at 12000 ***g*** for 20 min, the supernatant was collected and fusion proteins were purified by GSH–Sepharose (Pharmacia). After pooling the purified fractions, recombinant ct-Rab6–GST was dialysed overnight against buffer S supplemented with 10 % glycerol.

His_6-tagged Rab6C, a novel human Rab6 isoform, was expressed and purified as described by Fitzgerald and Reed [24]. It was reported that human Rab6C has approx. 98 % similarity in amino acid sequence with that of human Rab6A, and has almost the same motifs found in human Rab6A, such as three GTP-binding regions and two protein kinase C phosphorylation sites. NSF–His_6 and α-SNAP–His_6 were purified as described in [25,26].

The cDNAs encoding rat Rab3 and Rab4 were obtained by PCR amplification from the first strand cDNA template and reversed-transcribed from the rat brain total RNA using the following primers: Rab3 sense primer, 5′-ATATGGCCTCAG-CCACAGAC-3′; Rab3 antisense primer, 5′-TCAGCAGGCG-CAATCCTG-3′; Rab4 sense primer, 5′-ATATGTCCG AGG-CTTACGAT-3′; Rab4 antisense primer, 5′-CTAGCAGCCAC-ACT CCTG-3′. The resulting fragments were introduced into PCR-II plasmid (Invitrogen, San Diego, CA, U.S.A) and confirmed by nucleotide sequencing, indicating that these cDNAs encode the full open reading frame of the rat *Rab*3 and *Rab*4 genes. *Eco*RI fragments from pCR-II were subcloned into the

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*Eco*RI sites of pGEX4T-1 vector (Pharmacia) to generate Rab3–GST and Rab4–GST fusion proteins.

Immunofluorescence microscopy

L6 cells were seeded on coverslips and incubated either in the absence or presence of 10 μg/ml Brefeldin A for 1 h before fixation. Cells were washed with ice-cold PBS, fixed with 3.7% paraformaldehyde for 1 h and then permeabilized with blocking solution (1% BSA in PBS) containing 0.3% Triton X-100 for 20 min on ice. Cells were incubated with the following primary antibodies for 2 h at room temperature: polyclonal anti-NSF antibody (StressGen), polyclonal anti-Rab6 antibody (Santa Cruz Biotechnology) and monoclonal anti-rat Golgi complex (anti-GCI antibody; A.T.C.C., CRL-1869). Cells were then incubated with rhodamine-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories) for 2 h on ice. Stained cells were analysed by fluorescence microscopy (Nikon Optiphot).

Associations of purified recombinant proteins

Recombinant NSF–His_6 (1 μg) was purified and immobilized on Ni^{2+}-nitrilotriacetate (Ni-NTA)–agarose (100 μl; Qiagen). After GST (1 μg), ct-Rab6–GST (1 μg), Rab3–GST (1 μg) or Rab4–GST (1 μg) was added, these mixtures were incubated either in 500 μl of buffer A (25 mM Hepes/KOH, pH 7.4, 100 mM KCl, 2 mM EDTA, 0.5% Triton X-100, 2 mM DTT, 0.5 mM ATP and 0.1 mM PMSF) or 500 μl of buffer D (buffer A supplemented with 8 mM $MgCl_2$) at 4 °C for 1 h. Agarose beads were washed with 1 ml of buffer A or buffer D three times and samples were analysed by Western blotting for the detection of NSF–His_6, GST, ct-Rab6–GST, Rab3–GST and Rab4–GST.

To test the association of rat brain NSF with Rab6C–His_6, recombinant Rab6C–His_6 (2 μg) immobilized on Ni-NTA agarose was incubated with the rat brain extract (500 μg) prepared as described above. After 1 h, Ni-NTA–agarose beads were washed five times with 1.5 ml of buffer A or buffer D and analysed by Western blotting.

ATPase activity assay

The ATPase activity of NSF was measured as described in [25]. NSF–His_6 proteins (2 μg) were incubated in 50 μl of assay solution (20 mM Tris/HCl, pH 9.0, 17 mM NaCl, 83 mM KCl, 10 mM $MgCl_2$ and 1 mM ATP) supplemented with 3 μM of [γ-^{32}P]ATP (6000 Ci/mmol) at 37 °C for various time periods. In some experiments, recombinant proteins were added at the following molar ratios: NSF/α-SNAP, 1:2; NSF/Rab6C, 1:3; NSF/ct-Rab6, 1:3; NSF/GST, 1:3; NSF/Rab3, 1:3; and NSF/Rab4, 1:3. To terminate the reaction, 250 μl of ice-cold, activated charcoal [10% (w/v) in 0.2 M HCl and 1 mM NaH_2PO_4] were added and reaction mixtures were centrifuged at 12000 ***g*** for 10 min at 4 °C. Aliquots of the supernatants (100 μl) containing the released [^{32}P]P_i were counted using a liquid-scintillation counter (Pharmacia). To check the NEM sensitivity, NSF proteins were pretreated with 5 mM NEM for 30 min on ice, followed by 5 mM DTT for 30 min, and used for the ATPase activity assay.

RESULTS

N-terminus of NSF interacts with Rab6 in the yeast two-hybrid system

To identify novel proteins which interact with NSF, we screened a rat lung cDNA library using part of NSF as a bait in the yeast two-hybrid system. From the conserved domains of mouse SKD2, human NSF and Chinese hamster NSF, the specific primers were designed (Figure 1A). The NSF cDNA of mouse was originally identified as a suppressor of K^+ transport growth defect (SKD2) [27]. The bait was composed of the part of rat NSF (corresponding to amino acid residues cysteine-11 to methionine-372) which contains the N-domain and the partial D1 domain (Figure 1B). Several positive clones were isolated through the nutrient marker selections and β-galactosidase assay. After nucleotide sequencing, it was found that two of the seven clones were Rab6 cDNAs (GenBank® Accession No. AF148210).

Rab6 is a small GTP-binding protein, known to be involved in the intra-Golgi protein transport [28,29]. Transformants with NSF/pGBT9 and Rab6/pGAD10 displayed strong histidine autotrophy and β-galactosidase activity (Figure 1C). The isolated rat Rab6 clone was found to encode a protein which started at the 69th threonine residue of the corresponding human Rab6 and contained the rest of the Rab6 sequence (Figure 2A). The isolated region of rat Rab6 clones had only one amino acid difference compared with human Rab6 (asparagine-202 in the rat Rab6 instead of serine-202 in the human Rab6).

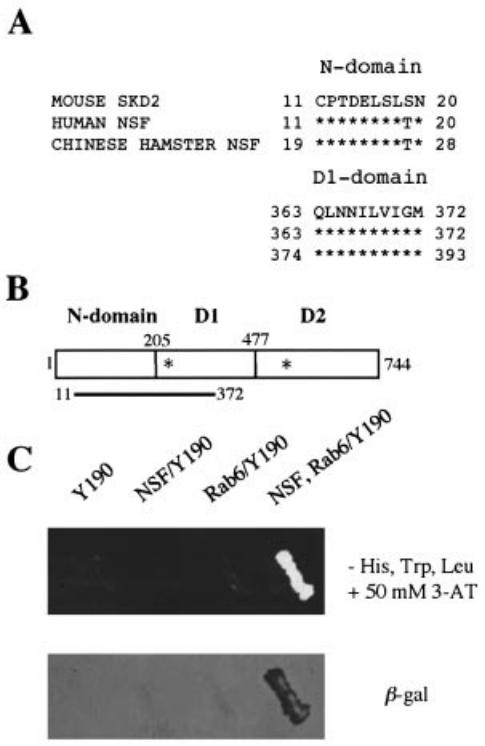

Figure 1 Rab6 binds to the N-terminal domain of NSF in the yeast two-hybrid system

(**A**) The conserved domains of NSF from mouse SKD2, human NSF and Chinese hamster NSF. From the conserved domains which are present within the N-terminal domain and the D1 domain, the specific primers were synthesized and RT-PCR was performed with the total RNA of the rat brain. The generated cDNA fragment of 1071 bp was inserted into pGBT9 vector. Identical amino acids are denoted by asterisks. (**B**) The domains of rat NSF. NSF is composed of three domains, N-domain, D1 ATP-binding domain and D2 ATP-binding domain. The underlined region (amino acid residues 11–372) represents the portion of NSF used as a bait. Asterisks indicate the ATP-binding domains: 260-GPPGCGKT-267 and 543-GPPHSGKT-550. (**C**) Specific interaction between rat NSF and rat Rab6. The nutritional marker selection and filter β-galactosidase assay were carried out to confirm the interaction between NSF and Rab6. Abbreviation: 3-AT, 3-amino-1,2,4-triazole.

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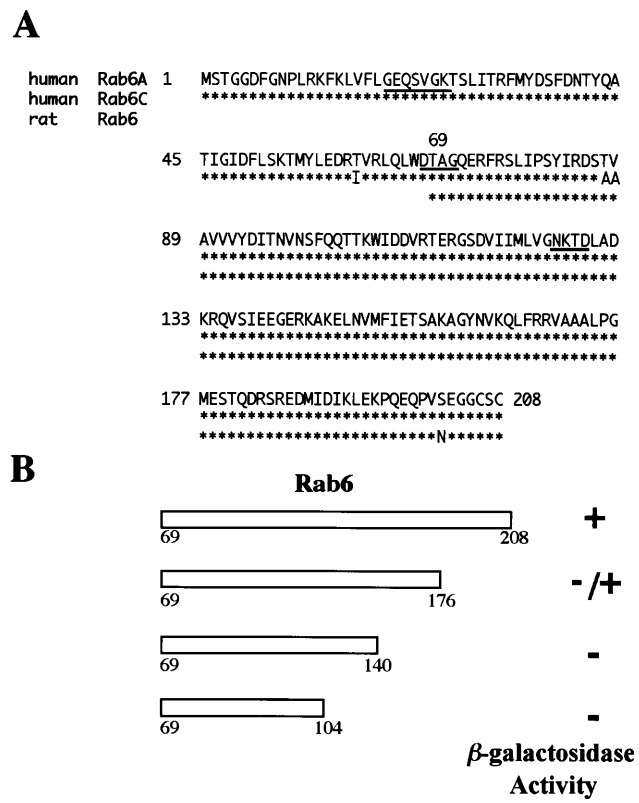

Figure 2 Binding domain within the C-terminus of Rab6

(**A**) Alignments of the sequences of the isolated partial cDNA of rat Rab6, human Rab6A and human Rab6C. The underlined residues are the conserved residues of GTP-binding domains. Identical residues are denoted by asterisks. (**B**) The C-terminus of Rab6 responsible for binding to NSF. The C-terminal deletion mutants of Rab6 were tested for their binding to NSF in the yeast two-hybrid system. β-Galactosidase activity is shown for each construct.

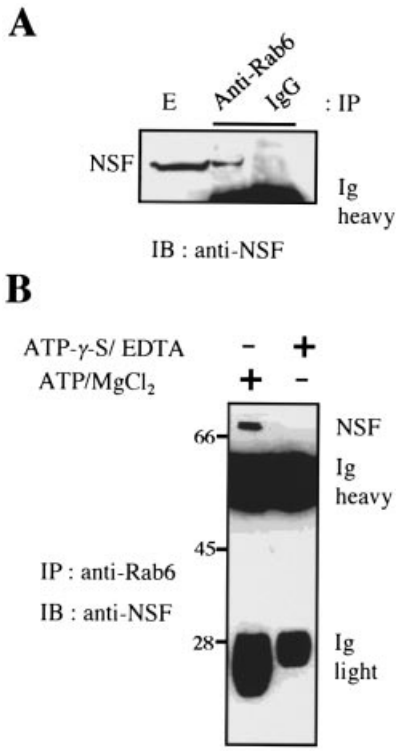

Figure 3 ATP-hydrolysis-regulated co-immunoprecipitation of NSF and Rab6 from rat brain extract

(**A**) Triton X-100 extracts of rat brain were immunoprecipitated with anti-Rab6 antibody or non-immune rabbit IgG. E, brain extract (100 μg). (**B**) Rat brain extracts prepared either in the presence of 1 mM ATP/4 mM $MgCl_2$ or 1 mM ATP[S]/2 mM EDTA were immunoprecipitated with anti-Rab6 antibody. (**A**, **B**) The immunoprecipitates were resolved by SDS/PAGE (10% gel), transferred on to nitrocellulose and probed with anti-NSF antibody. IB, immunoblotting; IP, immunoprecipitation.

C-terminus of Rab6 is responsible for the binding to NSF

To identify the interacting region within the C-terminus of Rab6 in more detail, we constructed a series of deletion mutants of the Rab6 C-terminus and looked at the interaction with NSF by β-galactosidase assay (Figure 2B). Only one mutant containing the 32 residues from methionine-177 to cysteine-208 showed a robust β-galactosidase response, whereas the mutant containing 36 residues from glycine-141 to proline-176 showed a pale blue colour. These data suggest that the NSF-binding motif is located within the C-terminal 68 amino acid residues of Rab6, probably within the last 32 amino acids (residues 177–208).

NSF binds to Rab6 *in vivo*

The interaction between NSF and Rab6 shown by the yeast two-hybrid assay was then confirmed by biochemical approaches, such as co-immunoprecipitation and *in vitro* binding assays using recombinant fusion proteins. We first demonstrated the co-immunoprecipitation of these proteins from 1% Triton X-100 lysates of rat whole brain. Tissue lysates were immunoprecipitated with anti-Rab6 antibody and the resolved immunoprecipitates were probed with anti-NSF antibody. As shown in Figure 3A, NSF was precipitated by anti-Rab6 antibody. In contrast, NSF was not detected in the immunoprecipitates precipitated by non-immune rabbit IgG. However, we failed to detect Rab6 in the immunoprecipitates precipitated by anti-NSF antibody, since the molecular mass of Rab6 (24 kDa) was similar to that of the Ig light chain (24–25 kDa), and may not be resolved by SDS/PAGE analysis (results not shown).

It has been reported that the NSF–SNAP complex exists in a stable association only under the conditions of inhibited ATP hydrolysis [3]. It was also shown that GluR2 and β-arrestin1 were preferentially immunoprecipitated with NSF in the presence of ATP/EDTA [20,21]. To examine whether Rab6 and NSF interaction is also influenced by the nucleotide-binding status of NSF, the immunoprecipitation was carried out either in the presence of ATP/$MgCl_2$ or ATP[S]/EDTA (ATP hydrolysable and non-hydrolysable condition respectively). Unexpectedly, NSF preferentially co-immunoprecipitated with Rab6 in the presence of ATP/$MgCl_2$, whereas NSF and Rab6 interaction was impaired in the presence of ATP[S]/EDTA (Figure 3B). These results showed that the property of the interaction between NSF and Rab6 was different from those between NSF and its previously identified binding proteins, such as SNAP, GluR2, and β-arrestin1.

NSF binds to Rab6 *in vitro*

The interaction between NSF and Rab6 was further confirmed by NSF–His_6 affinity chromatography. Recombinant NSF–His_6 was tested for its ability to retain recombinant ct-Rab6–GST,

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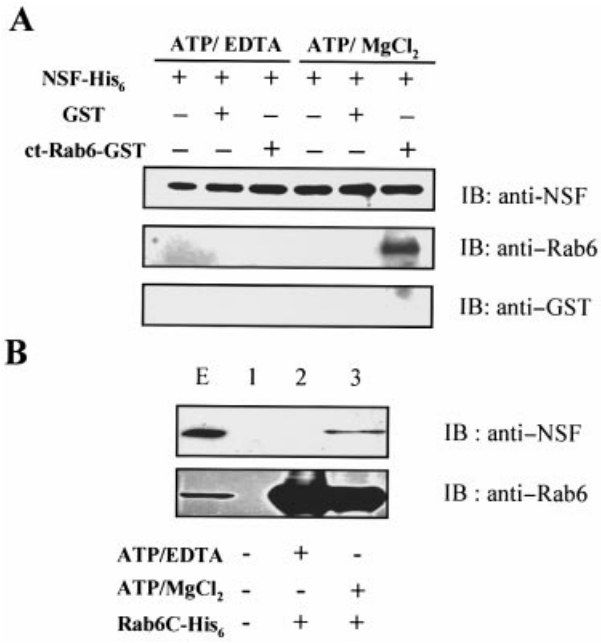

Figure 4 Association of NSF and Rab6 *in vitro*

(**A**) Interaction of NSF–His_6 with ct-Rab6–GST. Ni-NTA–agarose beads coupled with NSF–His_6 (1 μg) were incubated with ct-Rab6–GST (1 μg) or GST (1 μg) in the presence of either 1 mM ATP/4 mM $MgCl_2$ or 1 mM ATP[S]/2 mM EDTA for 1 h at 4 °C. Samples were then resolved by SDS/PAGE (10% gel), transferred on to nitrocellulose and probed by anti-NSF, anti-Rab6 or anti-GST antibodies. (**B**) Western-blot analysis with anti-NSF antibody showing the binding of rat brain native NSF to Rab6C–His_6. Rat brain extracts (E, 200 μg) were incubated with Ni-NTA–agarose only (lane 1) or Rab6C–His_6 (2 μg) coupled to Ni-NTA–agarose for 1 h at 4 °C in the presence of either 1 mM ATP/2 mM EDTA (lane 2) or 1 mM ATP/4 mM $MgCl_2$ (lane 3). The samples were resolved by SDS/PAGE, transferred on to nitrocellulose and probed with anti-NSF or anti-Rab6 antibodies. IB, immunoblotting.

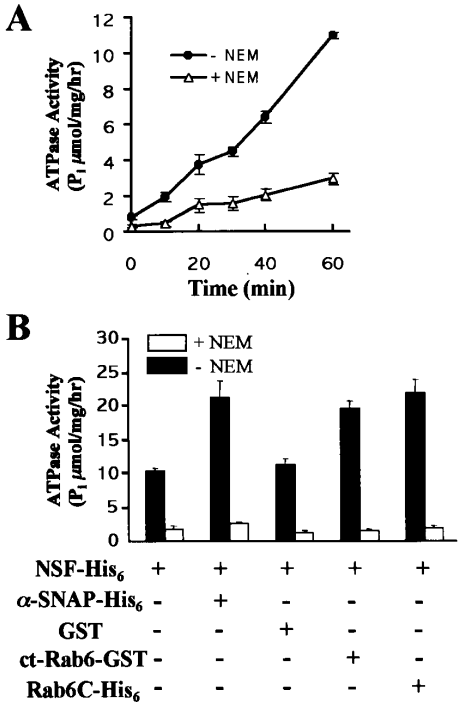

Figure 5 Rab6 can stimulate the ATPase activity of NSF

(**A**) Time course of [^{32}P]P_i release by NSF–His_6 at 37 °C. The ATPase activities of recombinant NSF-His_6 (●) or NEM-treated NSF–His_6 (△) were measured at the indicated time points. (**B**) ATPase activities of NSF–His_6 in the presence of α-SNAP–His_6, GST, ct-Rab6–GST or Rab6C–His_6. The mixed samples were incubated for 1 h at 37 °C and the released [^{32}P]P_i was measured using a liquid-scintillation counter. (**A**, **B**) The results shown are means ± S.D. of three separate experiments. The background signal from reactions with NSF on ice was subtracted.

which comprises C-terminal amino acid residues 99–208 (Figure 2A). We have used the C-terminal Rab6 (ct-Rab6–GST), since it was previously found that the C-terminal domain of Rab6 was responsible for the binding to NSF in the yeast two-hybrid system (Figure 2B). It was found that ct-Rab6–GST could be recognized by both anti-GST and anti-Rab6 antibodies.

Recombinant NSF–His_6 fusion proteins were purified, immobilized on Ni-NTA–agarose and mixed with either GST or ct-Rab6–GST. These mixtures were then incubated either in the presence of ATP/$MgCl_2$ or ATP/EDTA (hydrolysable or non-hydrolysable condition respectively). As seen in Figure 4A, ct-Rab6–GST is retained by NSF–His_6 only in the presence of ATP/$MgCl_2$.

Next, we investigated whether recombinant Rab6C–His_6 binds to the native rat brain NSF. Recombinant Rab6C–His_6 fusion proteins immobilized on Ni-NTA–agarose were mixed with rat brain extracts in the presence of either ATP/$MgCl_2$ or ATP/EDTA, washed, and then subjected to Western-blot analysis with anti-NSF antibody. Figure 4B shows that NSF can bind specifically to Rab6C–His_6 in the presence of ATP/$MgCl_2$. These results indicate that NSF can bind to Rab6 under the conditions which allow ATP hydrolysis.

Rab6 can stimulate the ATPase activity of NSF

We then looked at the functional aspect of this interaction. Since it was reported that α-SNAP stimulates the ATPase activity of NSF [25], we decided to see whether Rab6 might modulate the ATPase activity of NSF. We tested this possibility by measuring the release of [^{32}P]P_i from [γ-^{32}P]ATP in the presence of Rab6. First, we measured the ATPase activity of recombinant NSF. Figure 5(A) shows that the ATPase activity of NSF was time dependent, with a peak activity of 11 ± 0.5 μmol P_i/mg of NSF per h at 37 °C. It seems that this activity is consistent with the previously reported ATPase activity of NSF [25]. We then checked the NEM sensitivity of ATPase activity. As shown in Figure 5(A), NEM significantly inactivated the ATPase activity of NSF. These results support the view that the ATPase activity of NSF was sensitive to NEM [1,9].

Strikingly, Rab6 stimulated the ATPase activity of NSF by approx. 2-fold, as did α-SNAP (Figure 5B). Furthermore, C-terminal Rab6 also had a stimulatory effect on the ATPase activity of NSF. This stimulated level was similar to the level enhanced by α-SNAP. In contrast, GST alone had no stimulatory effect on the ATPase activity of NSF.

We then examined the effect of the anti-Rab6 antibody on the stimulatory effect of Rab6. Polyclonal antibodies against Rab6 can recognize the amino acids within the C-terminus of Rab6. Figure 6 shows that anti-Rab6 antibody inhibited the Rab6-stimulated ATP hydrolysis of NSF up to 60%. Specificity of inhibition by anti-Rab6 antibody was confirmed by the use of non-immune rabbit IgG. Taken together, these results indicate that Rab6 can specifically bind to NSF and stimulate the ATPase activity of NSF.

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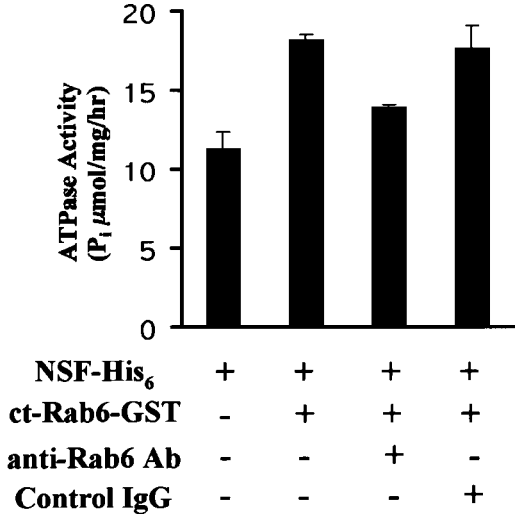

Figure 6 Polyclonal antibodies against Rab6 can significantly block the stimulatory effect of Rab6 on the ATPase activity of NSF

Either anti-Rab6 antibody or non-immune rabbit IgG (2 μg each) was incubated with the mixtures of recombinant NSF and Rab6 (1 μg each) for 1 h at 37 °C. The released [^{32}P]P_i was then measured using a liquid-scintillation counter. The results shown are means ± S.D. of three separate experiments. The background signal from reactions with NSF on ice was subtracted.

Localizations of NSF and Rab6 on the Golgi apparatus

We then investigated the distribution of NSF and Rab6 in the L6 rat myoblasts by immunofluorescence analysis. A clear and significant staining pattern was observed on one side of the perinuclear region, typical of the Golgi stacks, with anti-NSF antibody, even though the faint staining was also seen throughout the cell (Figure 7a). Double-labelling of L6 cells with Golgi-specific monoclonal anti-rat Golgi complex (anti-GCI) [30] and polyclonal anti-NSF antibody showed almost identical staining patterns, indicating that NSF is mainly localized in the Golgi stacks (Figure 7a and 7b).

We next performed a double-labelling experiment using monoclonal anti-GCI antibody and polyclonal anti-Rab6 antibody. We showed that the staining pattern of anti-Rab6 antibody overlapped with that of anti-GCI (Figure 7c and 7d). It is known that Brefeldin A specifically disassembles the Golgi complex [31]. In the present study we show that Brefeldin A completely disintegrated the Golgi stainings by anti-NSF, anti-Rab6 and anti-GCI antibodies (Figure 7e–7h). Taken together, these results clearly demonstrate that Rab6 is co-localized with NSF predominantly in the Golgi apparatus.

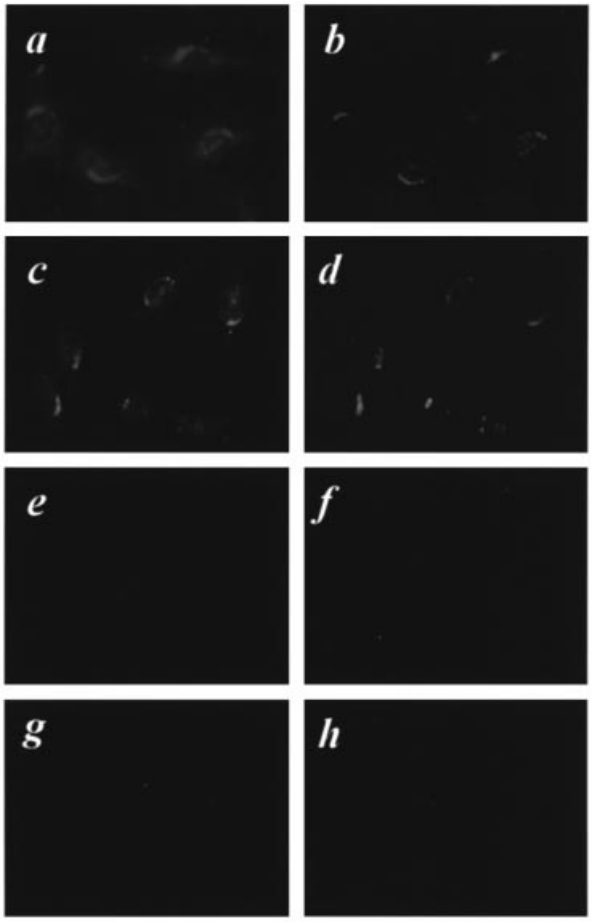

Figure 7 Co-localization of NSF and Rab6 on the Golgi stacks in L6 rat skeletal myoblasts

L6 cells grown on coverslips were incubated in the absence (**a–c**, **d**) or presence (**e–g**, **h**) of Brefeldin A (10 μg/ml) for 1 h. After treatment with Brefeldin A, cells were washed with ice-cold PBS, fixed with paraformaldehyde and permeabilized with Triton X-100. Cells were incubated with both anti-NSF antibody (**a**, **e**) and mouse monoclonal anti-GCI antibody (**b**, **f**), or with both anti-Rab6 antibody (**c**, **g**) and mouse monoclonal anti-GCI antibody (**d**, **h**). Rhodamine-conjugated goat anti-rabbit Igs were used to detect NSF and Rab6, and FITC-conjugated goat anti-mouse Igs were used to detect GCI. Magnification × 100.

Both Rab3 and Rab4 interact with NSF and stimulate its ATPase activity

Considering that NSF has a general role in the intracellular protein transport events, it might be assumed that NSF interacts also with other Rab proteins. We investigated whether other Rabs can interact with NSF and stimulate its ATPase activity. We chose Rab3 and Rab4 as candidates.

In order to isolate *Rab*3 and *Rab*4 genes from the rat, RT-PCR was performed using the specific primers complementary to sequences of the rat Rab3 and Rab4. Sequence analysis confirmed that the cloned cDNAs encoded the full open reading frames of Rab3 and Rab4. The full open reading frames were subcloned into pGEX4T-1 vector to produce GST fusion proteins, which were expressed and purified on GSH–Sepharose beads. The purified Rab3–GST and Rab4–GST proteins were estimated to be > 95% pure by Coomassie Brilliant Blue staining (Figure 8A, lanes 5 and 6) recognized by GST antibody, and bound GTP as assessed by GTP overlay assay (Figure 8B, lanes 5 and 6). However, both ct-Rab6–GST and GST could not bind GTP (lanes 2 and 3).

To investigate whether Rab3 and Rab4 can interact with NSF, we performed an *in vitro* binding assay. Ni-NTA–agarose beads coupled to NSF–His_6 were mixed with recombinant Rab3–GST, Rab4–GST or GST, either in the presence of ATP/$MgCl_2$ or ATP/EDTA. The presence of NSF, Rab3–GST and Rab4–GST recombinant proteins were then tested by the specific anti-NSF and anti-GST antibodies (Figure 8C). NSF–His_6 did not interact with GST proteins. However, Rab3–GST preferentially associated with NSF–His_6 in the presence of ATP/$MgCl_2$, indicating that Rab3 interacts with NSF directly under the conditions to allow ATP hydrolysis. Similarly, Rab4–GST bound

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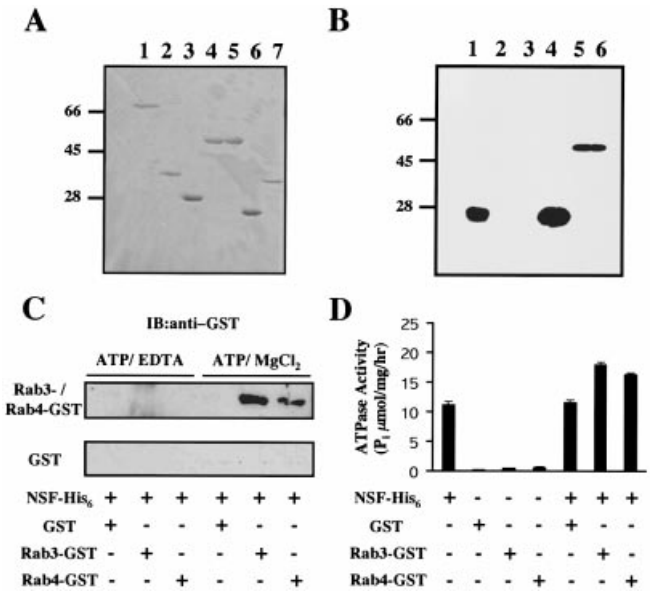

Figure 8 Both recombinant Rab3–GST and Rab4–GST can interact with NSF–His6 and stimulate its ATPase activity

(**A**) Recombinant proteins used in this study. NSF–His_6 (lane 1), α-SNAP–His_6 (lane 2), GST (lane 3), Rab3–GST (lane 4), Rab4–GST (lane 5), Rab6C–His_6 (lane 6), and ct-Rab6–GST (lane 7). Proteins were subjected to SDS/PAGE and detected by Coomassie Brilliant Blue staining. (**B**) GTP-blot analysis of the recombinant proteins. Rat brain extracts (200 μg, lane 1), GST (lane 2), ct-Rab6–GST (lane 3), Rab6–His_6 (lane 4), Rab3–GST (lane 5), and Rab4–GST (lane 6). Proteins were subjected to SDS/PAGE, transferred on to nitrocellulose and probed with [α-^{32}P]GTP. (**C**) Both recombinant Rab3 and Rab4 associate with NSF *in vitro*. Ni-NTA–agarose beads coupled with NSF–His_6 were incubated with GST, Rab3-GST or Rab4–GST in the presence of either 1 mM ATP/4 mM $MgCl_2$ or 1 mM ATP/2 mM EDTA for 1 h at 4 °C. The samples were then subjected to SDS/PAGE, transferred on to nitrocellulose and probed with anti-GST antibody. (**D**) Both recombinant Rab3 and Rab4 stimulate the ATPase activity of NSF. Recombinant proteins were mixed as indicated and incubated for 1 h at 37 °C. The released [^{32}P]P_i was then measured using a liquid-scintillation counter. The results shown are means $\pm$ S.D. of six separate experiments. The background signal from reactions with NSF on ice was subtracted.

to NSF–His_6 in the presence of ATP/$MgCl_2$, although the interaction was found to be minimal. These results support the direct and specific interactions between NSF and Rab proteins.

As shown previously, Rab6 stimulated the ATPase activity of NSF (see Figure 5B). To check whether Rab3 and Rab4 have the same stimulatory effects, we looked at the influences of Rab3 and Rab4 on the ATPase activity of NSF. As shown in Figure 8(D), Rab3 and Rab4, which had no ATPase activity by themselves, stimulated the ATPase activity of NSF by up to 2-fold, whereas GST had no effect on the ATPase activity of NSF. These results suggest that the direct interactions between NSF and Rab3/4 have the meaningful roles in the cell.

DISCUSSION

In this study we have identified Rab6 as an NSF-binding protein and examined its interactions. Moreover, we demonstrated that recombinant Rab3 and Rab4 could interact with NSF and stimulate its ATPase activity. These results suggested the possible role(s) of Rab proteins in protein trafficking processes.

It has been proposed that NSF plays a role in concert with SNAPs and membrane-associated SNAREs to form a '20S fusion complex'. ATP hydrolysis by NSF triggers the conformational change of the SNAREs, leading to the dissociation of the complex and finally to the fusion of the membranes. In this original model, NSF is considered as a driving force for the fusion of transport vesicle and its target membrane [3].

However, some reports have suggested different roles of NSF. It was reported that NSF might have the possible pre-docking attachment site and bind to membranes before the docking and the fusion occur [32,33]. In addition, NSF can interact not only with SNAPs, but also with GluR2, β-arrestin1 and the recently identified GATE-16 [20–22]. These results suggested that NSF acts not only as a simple catalyst in the membrane fusion event, but also as a chaperone which is related to the conformational change of proteins including SNAREs and other proteins [34]. Furthermore, it was recently reported that NSF and ATP are sufficient to evoke membrane fusion without any proteins such as SNAREs in an *in vitro* liposome-fusion assay [35], and that an NSF mutant without ATPase activity and the ability to induce SNARE dissociation can nonetheless promote post-mitotic Golgi membrane fusion [25]. However, in spite of these accumulating results, the exact physiological role(s) of NSF *in vivo* is not yet revealed.

In an attempt to look for the role of NSF, we set out to find new proteins which may interact with NSF. We employed the yeast two-hybrid technique, using part of the NSF as a bait which contained the N-terminal domain and the partial D1 domain, the first ATP-binding domain (Figure 1). It is known that the N-terminal domain of NSF can interact with α-SNAP and recently identified β-arrestin1, whereas the whole sequence of NSF was required for the binding to GluR2 [20,21]. Together with our data, these results strongly imply that the N-terminal domain of NSF is responsible for the interactions with multiple proteins.

By screening the rat lung cDNA library, we identified some putative clones whose gene products had NSF-binding properties. One of the identified clones was Rab6 (Figures 1 and 2). Rab6 is a small GTP-binding protein, which is localized in the membranes of the Golgi apparatus and *trans*-Golgi network as a regulator of protein transport within these organelles. Considering the previous reports that both NSF and Rab6 mainly localize and function in the Golgi stacks, it seemed that the interaction of the two proteins identified in the present report is both true and reasonable. It is reported that Rab6 has three GTP-binding domains. However, the isolated Rab6 cDNA fragment in this report contained only the disrupted second GTP-binding domain and the third GTP-binding domain (Figure 2A). This C-terminal region of Rab6 was responsible for the binding to NSF (Figure 2B). Moreover, we demonstrated that the identified C-terminal region containing only the third GTP-binding domain is enough to stimulate the ATPase activity of NSF (Figure 5B). These results suggest that the C-terminal of Rab6 has the potential for the interaction and the activation of the ATPase activity of NSF. However, we found that this ct-Rab6–GST could not bind GTP by itself (Figure 8B). Also, it seemed that the GDP/GTP-binding status of the full-length Rab6 does not show any difference on the stimulation of ATPase activity of NSF (results not shown). These results suggest that Rab6 interaction with NSF has no GTP-dependency.

The interaction between NSF and α-SNAP is maintained under the inhibited ATP hydrolysis condition [5]. And this binding property was also observed in the interactions between NSF with GluR2 and β-arrestin1 [20,21]. However, we demonstrated that the binding property of NSF and Rab6 was different from those between NSF and α-SNAP, GluR2 or β-arrestin1. It seemed that Rab6 and NSF interaction was impaired under the conditions of inhibited ATP hydrolysis; that is, in the presence of

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ATP/EDTA or ATP[S]/EDTA (Figure 3B and Figure 4). These differences in binding properties suggest that NSF may interact with different proteins depending on the ADP/ATP binding status and play unexpected role(s) in the physiological conditions.

In addition to the biochemical verification of interactions between Rab6 with NSF, we further demonstrated that Rab6 could stimulate the ATPase activity of NSF (Figure 5), and this stimulation effect was significantly inhibited by anti-Rab6 antibody (Figure 6). Considering that both α-SNAP and GATE-16 can also stimulate the ATPase activity of NSF [22,25], we speculate that the regulatory mechanism modulating the activity of NSF may be present in the cell.

To look at the possibility that Rab proteins in general can interact with NSF and stimulate its ATPase activity, we examined the effect of other Rab proteins on NSF. To our surprise, it was demonstrated that both Rab3 and Rab4 could associate with NSF and stimulate its ATPase activity (Figure 8). These results about the stimulatory effects of Rab3/4/6 on the ATPase activity of NSF may suggest the *in vivo* functions of Rab proteins in the protein transport. Until now the direct interaction between NSF and Rabs has not been reported. Recently, McBride et al. [36] suggested that Rab5 effectors can associate with NSF. However, in that report, the direct interaction between NSF and Rab5 was not confirmed. Although we did not yet test the direct interaction between Rab5 and NSF, it is possible that this affinity of native interaction is likely weak and transient, and could be missed. This idea can be supported by the fact that Rab6 and NSF interaction in our study was first identified by the yeast two-hybrid technique which can detect the weak interactions present in the cell.

Although it has been known that NSF is present in the membranes of endosomes, the clathrin-coated vesicles and the synaptic vesicles, immunofluorescence analysis in this report suggests that NSF predominantly localizes in the Golgi stacks (Figure 7, see also [14]). It is thus feasible that the majority of NSF localizes and functions in the Golgi stacks in concert with many factors, regulating the massive protein transport in the Golgi stacks. We speculate that NSF may have different binding affinities towards other Rabs. This idea can be supported by the fact that they have different amino acid sequences which confer the specificity of localizations and functions in the cell [37], although at least 30 Rab proteins belong to the one family.

In the present study, we propose that Rab protein can exert its action as one of the putative signalling molecules to deliver the signal of vesicle fusion to NSFl. That is, upon receiving the up-stream signal, Rab may interact with NSF and stimulate the ATPase activity of NSF, delivering the signal to initiate and permit vesicle fusion with the target membranes. Of course the involvement of the other Rab-interacting proteins, such as GDP-dissociation inhibitors, GTPase activation proteins and effector molecules, should be considered in this process. Thus Rab can catalyse the fusion event by stimulating the ATP hydrolysis of NSF, thereby speeding up the rate of membrane fusion by stimulating the disassembly of 20 S particles.

However, we cannot bring out all the Rab proteins in a simple unified model. Each Rab protein has a unique set of effector molecules. For example, it was reported that Rab3 binds to Rim and Rabphilin [38], Rab4 to Rabaptin 4 [39], Rab6 to Rabkinesin 6 [40], and Rab5 to Rabaptin-5 and phosphoinositide 3-kinase [41,42]. These results imply the functional differences of Rab proteins. Although a large body of evidence suggests that Rab may regulate the assembly of SNARE complex and facilitate vesicle docking, it seemed that Rab proteins may interact with multiple proteins which are not related to one another and play a more complex role(s) in the cell.

Taken together, in contrast with the classical SNARE theory, we demonstrated that NSF binds directly to Rab proteins and that its ATPase activity is stimulated by Rab proteins. However, we cannot also rule out the presence of the other regulatory mechanisms to modulate NSF activity. Although several proteins have been identified as an NSF-binding protein, the mechanism which regulates the enzymic activity and physiological role of NSF remains unknown. The potential role of the interaction between NSF and Rab proteins may be found in this report.

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