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Novel Putative Targets of *N*-ethylmaleimide Sensitive Fusion Protein (NSF) and α/β Soluble NSF Attachment Proteins (SNAPs) Include the Pak-Binding Nucleotide Exchange Factor βPIX

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Abstract

N-ethylmaleimide sensitive fusion protein (NSF) is a chaperone that plays a crucial role in the fusion of vesicles with target membranes. NSF mediates the ATP-consuming dissociation of a core protein complex that assembles during vesicle fusion and it thereby recharges the fusion machinery to perform multiple rounds of fusion. The binding of NSF to the core complex is mediated by co-chaperones named soluble NSF attachment proteins (SNAPs), for which three isoforms (α, β and γ) are known. Here, we sought to identify novel targets of the NSF-SNAP complex. A yeast two-hybrid screen using the brain specific βSNAP isoform as bait revealed, as expected, NSF and several isoforms of the SNARE protein syntaxin as interactors. In addition, three isoforms of the reticulon protein family and two isoforms of BNIP3 interacted with βSNAP. A yeast two-hybrid screen using NSF as bait identified Rab11-FIP3 and the Pak-binding nucleotide exchange factor βPIX as putative binding partners. βPIX interacts with recombinant NSF in co-sedimentation assays and the two proteins may be co-immunoprecipitated. A leucine zipper (LZ) motif within the C-terminus of βPIX mediates binding to NSF; however, this fragment of βPIX does not exhibit dominant negative effects in a cellular assay. In summary, our results support the evolving view that NSF has numerous targets in addition to conventional SNARE complexes.

Keywords

N-ethylmaleimide sensitive fusion protein; chaperone; soluble NSF attachment protein; SNAP receptor; syntaxin

The investigation of the chaperone *N*-ethylmaleimide sensitive fusion protein (NSF) has been instrumental in understanding the molecular mechanism of membrane fusion events. First, it was shown that NSF binds to soluble NSF attachment proteins (SNAPs). Three isoforms of SNAP exist, of which α and γ isoforms are ubiquitously expressed and the β isoform is restricted to the brain [Clary et al., 1990; Whiteheart et al., 1993]. α- and γ-SNAP were subsequently used in biochemical experiments to identify SNAP receptors (SNAREs), the targets of the NSF-SNAP complex [Söllner et al., 1993]. The SNAREs

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purified from brain detergent extracts were identical to the synaptic vesicle protein synaptobrevin-2 and the plasma membrane proteins syntaxin-1A/B and synaptosome-associated protein of 25 kDa (SNAP25). A short coiled-coil forming sequence is shared by these SNAREs and by many homologous SNARE proteins that were subsequently identified from different tissues and organisms [Weimbs et al., 1997]. Next, it was found that coiled-coil motifs from SNAREs located on different membranes (trans-configuration) form a four helix bundle that forces the vesicle membrane near to the plasma membrane and initiates the fusion event. A hallmark of the energetically stable SNARE complexes is their resistance to the detergent sodium dodecyl sulfate (SDS). Complexes of SNAREs on the same membrane (cis-configuration), formed as a result of fusion events, become disassembled in an ATP-consuming process dependent on SNAP and NSF. This disassembly allows the SNAREs to participate in additional rounds of fusion [Mayer et al., 1996; reviewed in Jahn et al., 2003].

Recently, several putative targets have been suggested for the NSF-SNAP complex that do not fall into the structural classification of SNAREs. One group of putative targets has been identified due to direct binding to NSF. This direct contact contrasts with the strictly SNAP-mediated interactions of classical SNAREs with NSF. Direct NSF binding has been demonstrated for the AMPA receptor subunit GluR2 [Nishimune et al., 1998; Osten et al., 1998], for G-protein coupled receptors [Cong et al., 2001; Heydorn et al., 2004], for βarrestin [McDonald et al., 1999], for rab proteins [3, 4 and 6; Han et al., 2000] and for the GABARAP/GATE16/LMA1 protein family (reviewed in Whiteheart and Mateeva, 2004). Furthermore, the yeast homologue of NSF, Sec18p, has been shown to interact with the dynamin-like GTPase Vps1p [Peters et al., 2004]. A second group of novel NSF-SNAP targets has been identified due to binding to the SNAP isoforms. αSNAP binds to BNIP1 [Nakajima et al., 2004], βSNAP has been reported to bind to synaptotagmin [Schiavo et al., 1995], both α and β SNAP bind to Pick1 in complex with GluR2 [Hanley et al., 2002] and γSNAP binds to the Rab11-interacting protein [RIP11; Chen et al., 2001; Tani et al., 2003]. Finally, NSF and SNAPs have been identified as constituents of large multimolecular complexes for which the exact binding sites for NSF/SNAP are not yet known. Early endosomal antigen-1 (EEA-1), rabaptin-5, rabex-5 and syntaxin-13 form such a complex associated with endosomes [McBride et al., 1999].

The discoveries of novel actions of NSF have been most influential in the field of synaptic transmission. The reports on the interaction between NSF and the AMPA-type glutamate receptor subunit GluR2 [Nishimune et al., 1998; Osten et al., 1998] have been the starting point for the recognition of AMPA receptor trafficking as common mechanism for a postsynaptic strengthening of synaptic transmission [Collingridge and Isaac, 2003]. In analogy to core complex disassembly, NSF dissociates a complex of AMPA receptors and the associated protein Pick1 in an ATP-dependent process [Hanley et al., 2002].

Recently, a complex of a Pak-binding nucleotide exchange factor for Rac/Cdc42 named βPIX, and a GTPase activating protein for Arf named GIT1 has been implicated in the regulation of postsynaptic mechanisms in Drosophila [Parnas et al., 2001] and mammalian neurons [Ko et al., 2003; Zhang et al., 2003, 2005]. βPIX binds directly to the postsynaptic scaffold protein Shank, which is involved in the regulation of dendritic spine formation [Park et al., 2003]. The molecular action of βPIX in these processes is still unknown.

The aim of this study was the identification of novel targets of the NSF-SNAP chaperone complex and the evaluation of a possible involvement of such targets in postsynaptic processes. The original strategy used by Söllner and colleagues to utilise SNAPs for the identification of putative NSF targets has previously been pursued by yeast two-hybrid screens with α- and γ-isoforms as baits [Hatsuzawa et al., 2000; Chen et al., 2001; Tani et al., 2003]. We have continued this approach by performing yeast two-hybrid screens of rat

brain cDNAs using NSF and βSNAP as baits. We obtained prey clones for NSF itself, classical SNAREs and several novel putative targets of the NSF-SNAP complex.

MATERIALS AND METHODS

Materials

Enzymes for DNA manipulations were from Roche Molecular Biochemicals (Indianapolis, IA) or New England Biolabs (Beverly, MA).

Yeast Two-Hybrid Screens and Related Methods

Yeast two-hybrid screens using a full-length rat βSNAP or rat NSF and a rat brain cDNA prey library in pVP16-3 or pGAD10 respectively were performed essentially as described [Betz et al., 1997]. For small scale yeast transformations, bait and prey plasmids were co-transformed by a lithium acetate method into *Saccharomyces cerevisiae* L40 reporter strain and colonies were tested for activation of the β-galactosidase reporter gene by filter assays [Vojtec et al., 1993].

Molecular Biology

Rat βPIX C-term (aa 524–646) isolated from rat brain cDNA prey library was prepared as a glutathione-*S*-transferase (GST) fusion protein by subcloning into pGEX4.1t (Amersham Biosciences, Piscataway, NJ). GST rat βPIX C-terminal fragments were prepared by PCR from βPIX C-term and subcloned into pGEX4.1t; βPIX-GBD (aa 524–558), βPIX-LZ (aa 579–640) and βPIX-PL (aa 639–646). Full-length human mycβPIX, a kind gift from Wannian Yang, was subcloned into pGEX4.1 and pHA-CMV (Clontech, Palo Alto, CA) to prepare GST-βPIX and HA-βPIX, respectively. For fluorescent fusion protein preparation, the same βPIX constructs were subcloned into pEGFP-C1 (Clontech). All constructs were verified by DNA sequencing (Department of Biochemistry, University of Oxford, UK).

Sindbis Expression System (Invitrogen, Carlsbad, CA) was used for expression of green fluorescent protein (GFP) fusion proteins in neuronal cells. EGFP-βPIX constructs were subcloned into the *Xba*I restriction site of pSinREP5 and in parallel with helper pDS(26S) plasmid, RNA was prepared by in vitro transcription. BHK cells were transfected with helper and structural RNAs by electroporation for sindbis virus production. After 48 h significant cell death was observed and virus particles were then harvested from the culture media.

Co-Sedimentation Assay ‘GST Pulldown’

Recombinant His_6NSF was prepared from M15[pREP4] (Qiagen, Valencia, CA) bacterial cell lysates and His_6T7-Shank1(PDZ) from BL21(DE3) (Stratagene, LaJolla, CA) bacterial cell lysates. Purification was performed essentially as decribed by Hanson et al. [1995]. GST fusion proteins were prepared from BL21 bacterial cell lysates. In co-sedimentation assays, 2 μg of GST fusion protein was immobilized on 20 μl of 50% glutathione-sepharose slurry (Amersham). For NSF co-sedimentation 100 nM of His_6NSF in buffer N (25 mM HEPES, pH 7.3, 150 mM KCl, 1 mM DTT, 1 mM ATP, 2 mM EDTA, 0.5% Triton X-100) was applied to the immobilized GST fusion proteins for 1 h. Complexes were then washed four times with buffer N before solubilisation of sedimented proteins in Laemmeli buffer. Proteins were detected by Western blot using HRP-conjugated monoclonal anti-His antibody (Roche). For co-sedimentation of Shank1(PDZ), 50 nM T7-Shank1(PDZ) in buffer S (25 mM HEPES, pH 7.3, 150 mM KCl, 1 mM DTT, 1 mM ATP, 2 mM EDTA, 1% Triton X-100, 10% Glycerol) was applied to the immobilized GST fusion proteins for 1 h. Complexes were then washed four times in buffer S before solubilisation of sedimented proteins in Laemmeli buffer. Proteins were detected by Western blot using monoclonal anti-

T7 antibody (Merck Biosciences, Germany). Flag-GIT1 (a kind gift of Richard Premont) and mycβPIX were transiently expressed in HEK293 cells for 18 h, before solubilisation in PBS with 1% Triton X-100 supplemented with protease inhibitor cocktail (Roche). Cell lysates were applied to immobilized GST fusion proteins for 1 h before four washes in PBS with 1% Triton X-100. Co-sedimented proteins were solubilised in Laemmeli buffer and detected by Western blot with either 9E10 monoclonal anti-myc (Santa Cruz Biotechnology, CA) or M2 monoclonal anti-flag (Sigma) antibodies.

Cell Culture and Immunoprecipitation

HEK293 cells were grown in Dulbecco's modified Eagle's media supplemented with 10% foetal calf serum at 37°C under 5% CO_2. Ten centimetres plates were transfected when 70% confluent with 12 μg total of DNA using Lipofectamine 2000 reagent (Invitrogen). After 48 h, cells were lysed in buffer H (25 mM HEPES, pH 7.3, 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100) supplemented with protease inhibitors in the presence of either 0.5 mM ATP, 2 mM $MgCl_2$, or 0.5 mM AMP-PNP (Sigma), 3 mM EDTA. After clarification by centrifugation, 1–2 mg of lysate was incubated with 1 μg of anti-HA antibody (#1867423, Roche) conjugated to protein A sepharose slurry for 2 h at 4°C. Complexes were washed four times in buffer H with either ATP · $MgCl_2$ or AMP-PNP · EDTA, before solubilisation in Laemmeli buffer. Co-immunoprecipitates were detected by Western blot using anti-NSF monoclonal antibody (ab16681, Abcam, UK)

Confocal Microscopy

Low-density hippocampal cultures were prepared from E18 embryonic tissue as previously described [Terashima et al., 2004] and plated on poly-L-lysine coated coverslips at a cell density between 100 and 500 cells/mm^2. At 14 days in vitro neurons were incubated with Sindbis virus for 18 h, after which cells were fixed in 3.7% paraformaldehyde, 5% sucrose PBS. PSD95 containing synapses were identified with anti-PSD95 monoclonal antibodies (ABR, Golden, CO) after cell permeabilisation with 0.1% Triton X-100. Secondary anti-mouse antibodies (Molecular Probes, Eugene, OR) conjugated to Alexa Fluor 568 were used for revelation. Immunocytochemistry and EFGP fusion proteins were analysed by confocal microscopy with a Leica AOBS system (Nussloch, Germany). Sections of primary dendrite were imaged through a 63 /1.32 oil objective at a digital resolution such that one pixel equals 0.09 μm^2. Images were collected with LCS confocal software (Leica) and quantified using Image J (NIH). For dendritic structural analysis all protrusion >0.5 μm and <9 μm were measured for length and maximum breadth. Data were statistically analysed from five independent experiments by Student's *t*-test as applicable. Data are expressed as the mean ± SEM.

RESULTS

A direct interaction between NSF and GluR2 has been reported independently by three different groups [Nishimune et al., 1998; Osten et al., 1998, Song et al., 1998]. In each case the interaction was detected with yeast two-hybrid prey constructs of NSF lacking a few N-terminal amino acid residues (aa). In a re-screen of a rat brain cDNA library using the GluR2 C-terminus as bait we obtained two independent prey clones of NSF lacking the N-terminal 9 aa or the N-terminal 15 aa. Surprisingly, GluR2 showed no interaction with a full-length NSF construct in yeast two-hybrid β-galactosidase assays (not shown). Full-length NSF constructs are functional in the yeast two-hybrid system as they show strong signals upon dimerisation (see below). Biochemical assays demonstrated that full-length NSF can bind to GluR2 in a SNAP- and ATP-regulated manner [Osten et al., 1998; Hanley et al., 2002], thus, the *N*-terminal truncation might be required only in the yeast two-hybrid system to prevent dissociation of complexes by endogenous SNAP (Sec17 in yeast). The binding

site for SNAPs is localized within the N-terminal region of NSF [Yu et al., 1999]. We reasoned that N-terminal truncated NSF might be a useful tool to identify further targets that directly interact with NSF.

Putative Interaction Partners of NSF

Using the N-terminal 9 aa truncated NSF as bait we screened an adult rat brain cDNA library. We obtained 257 clones with histidine autotrophy of which most of them showed β-galactosidase activity. Those clones were grouped by restriction digest analysis and representatives were sequenced. The majority of clones represented multiple copies of 18 independent clones for NSF itself, all harbouring the C-terminal oligomerisation domain. Interestingly, the borders of partial clones for NSF corresponded well with borders of secondary structure elements described in crystal structure determinations of the N-terminal and C-terminal domains of NSF [Lenzen et al., 1998; Yu et al., 1998; May et al., 1999; Yu et al., 1999]. All full-length and partial NSF clones oligomerised with full-length as well as the 9 aa-truncated NSF.

Beside several clones coding for transcription factors and proteins often obtained from our library (e.g. UBC-9), multiple copies of individual clones coding for four different proteins were obtained (Table I), namely the recycling endosomal protein Rab11-FIP3, the Golgi protein αCOP, the germinal centre kinase Mink2 and the Pak-interacting rho guanine nucleotide exchange factor βPIX. None of these prey clones interacted with lamin as a control, full-length NSF or the GluR2 C-terminus in yeast two-hybrid β-galactosidase assays (not shown).

Putative Interaction Partners of bSNAP

Binding of NSF to its targets is most often mediated by SNAPs, and yeast two-hybrid screens for αSNAP [Hatsuzawa et al., 2000] and γSNAP [Chen et al., 2001; Tani et al., 2003] have been reported. Here, we used full-length βSNAP as bait to screen a juvenile rat brain cDNA library. We obtained 347 clones with histidine autotrophy of which most of them showed β-galactosidase activity. All positive clones were identified by sequencing. Two independent prey clones coded for full-length NSF. The majority of prey clones from the screen represented several isoforms of syntaxin (Table I), with isoform 1A/B being most abundant. Interestingly, all syntaxin clones coded for fragments including both, the SNARE motif and the C-terminal transmembrane region. Deletion of the transmembrane region decreased the signal intensity in β-galactosidase assays of βSNAP and syntaxin-1A (not shown).

Thirty-two copies of 13 independent prey clones coded for isoforms 1, 3 and 4 of reticulon, a protein family of unknown function that localizes to the endoplasmic reticulum and cell surface. Twelve copies coded for the Bcl2-related proteins BNIP3, BNIP3-like protein and Bcl2-like protein-13, apoptosis-inducing proteins that localize to the cytoplasm and mitochondria. As was the case for the syntaxins, all reticulon or Bcl2-related preys coded for fragments that included C-terminal transmembrane regions. Several clones coded for protein tyrosine phosphatases and receptor protein tyrosine phosphatases, however, some of these prey clones have also been obtained in unrelated yeast two-hybrid library screens. Finally, some preys were found as single clones in multiple copies (Table I).

In β-galactosidase assays all βSNAP-interacting prey clones also bound to αSNAP, but not to lamin, full-length NSF, N-terminal 9aa truncated NSF or GluR2 C-terminus (not shown).

NSF Binding to bPIX

From the novel putative NSF-SNAP targets that have been identified in yeast two-hybrid library screenings (Table I) we were particularly interested in βPIX, because work in flies and in mammalian neuronal cultures had implicated this protein as regulator of the postsynaptic structure.

The prey clone obtained from the yeast two-hybrid screen coded a fragment of βPIX covering the region from aa 524 to the C-terminus. First, we expressed this region of βPIX as a fusion protein with GST and tested for the binding of recombinant 6× His-tagged NSF from bacteria. For comparison, we tested in parallel binding of NSF to its binding partner GluR2 by expression of a GST fusion of the GluR2 C-terminus [Nishimune et al., 1998; Osten et al., 1998]. In this assay system, βPIX binds NSF with an efficiency comparable to GluR2 C-terminus (Fig. 1A). GST-full-length βPIX bound NSF with similar efficiency to the prey fragment (Fig. 1B).

To confirm that the association of βPIX and NSF could also occur in vivo, we performed co-immunoprecipitations from transfected HEK293 T cells. Complexes from cells co-expressing N-terminal HA-tagged βPIX and NSF immunoprecipitated with an HA antibody contained NSF, as did immunoprecipitated HA-tagged GluR2, but not HA-tagged GluR1 (Fig. 1C). The association of GluR2 with NSF is reduced under conditions that allow ATP hydrolysis in a similar manner to the SNARE-NSF-SNAP complex [Osten et al., 1998]. To test if the βPIX-NSF interaction had the same characteristics, we performed co-immunoprecipitations in conditions that either permitted ATP hydrolysis (0.5 mM ATP and 2 mM $MgCl_2$) or prevented it with the presence of a non-hydrolysable ATP analogue (0.5 mM AMP-PNP and 3 mM EDTA). NSF only weakly associated with βPIX in conditions that permitted ATP hydrolysis (Fig. 1D).

The fragment of βPIX encoded by the prey clone contains three parts that differ in the complement of binding partners. The N-terminal portion of the fragment (GBD) contains the binding site for the Arf-GAP GIT1 [Zhao et al., 2000]. The middle part contains a leucine zipper region (LZ) that mediates dimerisation or oligomerisation of βPIX [Koh et al., 2001; Kim et al., 2001; Premont et al., 2004]. The C-terminus contains a PDZ-binding motif (PL) that binds to Shank and Scribble [Park et al., 2003; Audebert et al., 2004]. To determine the binding region on βPIX for NSF, GST-fusion proteins of each individual fragment were probed in co-sedimentation assays for binding to HEK293 expressed Flag-GIT1 (Fig. 2A), COS7 cell expressed myc-βPIX (Fig. 2B), bacterially expressed T7-Shank-PDZ-domain (Fig. 2C), and bacterially expressed His_6-NSF (Fig. 2D). In line with previous results, GIT1 bound exclusively to the GBD, βPIX bound exclusively to the LZ and the PDZ domain bound preferentially to the PL, but also to the LZ (as previously reported by Park et al. [2003]) and slightly to the GBD. We found that the binding site on βPIX for NSF is restricted to the LZ (Fig. 2D).

βPIX Overexpression in Cultured Neurons

It was recently reported that overexpression of βPIX into dissociated neuronal cultures causes the formation of numerous thin protrusions [Zhang et al., 2003]. Therefore, we generated GFP-fusion proteins of full-length βPIX and fragments encoding GBD, LZ or PL with the aim of further investigating this process. Delivery of GFP, GFP-βPIX or GFP-βPIX-fragments by recombinant sindbis virus infection of dissociated mature neurons led to strong expression of all proteins within 24 h, as detected by Western blotting using an anti-GFP antibody (Fig. 3A). GFP-βPIX showed a punctate distribution (Fig. 3B.2), and was also present within the axons (Fig. 3B.3). In older neurons which had been maintained for 3 weeks in vitro GFP-βPIX puncta co-localized with PSD95 immunocytochemistry (Fig. 3C).

In contrast, all GFP-βPIX-fragments distributed diffusely within neuronal dendrites and somata similar to GFP (Fig. 3B.4–6). A fraction of GFP-βPIX-LZ expressing cells showed large aggregates of the recombinant protein within the somata (Fig. 3B.7). At higher magnifications thin or stubby protrusions of the dendrites were visible that are likely to represent filopodia and spines, respectively (Fig. 4A). Quantification of the number of filopodia per μm of dendrite showed an increase for GFP-βPIX overexpressing cells, and a decrease for GFP-βPIX-GBD expressing cells (Fig. 4B). However, the number of filopodia in dendrites from GFP-βPIX-LZ expressing neurons was unaltered.

DISCUSSION

Recent reports on novel targets of the chaperone NSF suggest that its function may extend beyond dissociation of core complexes derived from membrane fusion events. Following two conceptual different approaches we searched for additional targets of NSF-SNAP chaperone complexes using the yeast two-hybrid system. First, an N-terminal truncated version of NSF capable of directly interacting with putative targets, and second, a brain specific β isoform of SNAP were used as baits in yeast two-hybrid library screens. Several of the novel candidates that we obtained have already been discussed in the context of NSF dependent processes.

Syntaxin

Syntaxins form the largest class of conventional SNAREs. The detection of seven different syntaxin isoforms as α/βSNAP interactors (Table I) supports the notion that NSF-SNAP chaperone complexes act universally on SNARE complexes. Importantly, αSNAP and βSNAP showed no differences in the binding to syntaxin isoforms, supporting the original suggestion by Clary et al. [1990] that both isoforms share a common binding site on SNAREs. Evidence for a βSNAP-specific function, as previously suggested [Schiavo et al., 1995], was not obtained in our yeast two-hybrid study.

BNIP3

A role of NSF in the regulation of apoptosis was recently suggested by the suppression of apoptosis upon overexpression of αSNAP [Nakajima et al., 2004]. A large family of anti- and pro-apoptotic proteins share structural similarity to Bcl2, most prominently in a domain named BH3. The pro-apoptotic BH3-only protein BNIP1 was shown to bind directly to αSNAP [Nakajima et al., 2004]. In analogy to core complex disassembly NSF-αSNAP dissociates a complex of BNIP1 and syntaxin isoforms in an ATP-dependent manner [Nakajima et al., 2004]. However, BNIP1 is only a weak promoter of apoptosis and localizes to the endoplasmic reticulum. Thus, Nakajima et al. [2004] suggested that the effect of αSNAP on apoptosis could be mediated by the mitochondrial protein BNIP3. Our findings that BNIP3 and BNIP3-like protein interact with αSNAP and βSNAP in the yeast two-hybrid system support this view.

Reticulon

Reticulon isoforms constitute a protein family that is of special interest in respect to neurological diseases. First, several family members have been identified (under the name of Nogo-A) as inhibitors of neurite outgrowth and re-growth after injury [Oertle and Schwab, 2003]. Second, other family members appeared recently as modulators in the process of β-amyloid formation in Alzheimer disease [He et al., 2004]. However, how reticulons affect these disease states remains relatively unknown. A relation of NSF to reticulons is suggested by the finding that reticulon-1C binds to several syntaxin isoforms [Steiner et al., 2004], but a possible sensitivity of reticulon-syntaxin complexes to NSF-mediated dissociation has not been studied. Reticulons are mainly localized in the endoplasmic reticulum where they have

recently been reported to aid the formation of the complex tubular morphology of the peripheral endoplasmic reticulum [Voeltz et al., 2006]. Surprisingly, the endoplasmic reticulum shaping function of the reticulons was identified from a pharmacological sensitivity to *N*-ethylmaleimide (NEM). NEM is also a potent inhibitor of NSF, raising the possibility that NSF may have a role in processing the reticulons during endoplasmic reticulum network formation. A particular structural feature of reticulons is that the C-terminal hydrophobic region has the potential to form alternatively one or two transmembrane spanning regions, resulting in opposite topologies of the N-termini [Oertle and Schwab, 2003]. Intriguingly, several NSF-α/βSNAP targets (syntaxin, BNIP3) belong to a subclass of membrane proteins classified as tail-anchored proteins [Borgese et al., 2003], and binding of βSNAP to syntaxins, BNIP3s and reticulons involved the transmembrane domains [see also Lewis et al., 2001]. Thus, NSF-SNAP chaperone complexes may be more generally involved in the handling of C-terminal hydrophobic tails.

Rab11-FIP

Recognition of a conserved binding motif for the endosomal small GTPase Rab11 in several proteins allowed the characterisation of a Rab11 family of interacting proteins (Rab11-FIP) that regulates the trafficking of proteins and membrane through the endosome [Prekeris et al., 2000, 2001; Hales et al., 2001]. The family member RIP11 has been identified as binding partner of γSNAP [Chen et al., 2001; Tani et al., 2003], and RIP11-γSNAP complexes undergo NSF-mediated disassembly [Tani et al., 2003]. The family member Rab11-FIP3 identified here as putative direct binding partner of NSF shares the feature of several coiled-coil motifs in the C-terminal region with RIP11. The coiledcoil motifs mediate oligomerisation of Rab11-FIPs [Wallace et al., 2002; Junutula et al., 2004] and form the binding site for γSNAP on RIP11 [Tani et al., 2003], suggesting that NSFSNAP may regulate the oligomerisation state of Rab11-FIPs. Interestingly, Rab11-FIP3 dimers seem to be SDS resistant [Horgan et al., 2004]. Rab11-FIP3 determines the asymmetry of cell divisions, thus the mechanisms regulating its activity may be important in this processes [Emery et al., 2005].

βPIX

For the maintenance of synaptic transmission, neurons require active NSF-SNAP chaperone function in the postsynapse [Nishimune et al., 1998]. The postsynaptic actions of NSF may include SNARE-dependent [Luscher et al., 1999] and SNARE-independent [Hanley et al., 2002] processes. The latter involves the dissociation of a complex between the AMPA-type glutamate receptor subunit GluR2 and the coiled-coil and PDZ domain-containing protein Pick1 [Hanley et al., 2002]. Because of our interest in the postsynaptic actions of NSF we focused our work on βPIX as putative target of NSF. Deletion mutants of βPIX in Drosophila have severe defects in the postsynaptic structure [Parnas et al., 2001] while synaptic defects due to the functional loss of NSF2 may be rescued by the βPIX-effector Pak3 [Laviolette et al., 2005]. In the mammalian CNS a complex of βPIX and GIT1 is involved in the regulation of postsynaptic AMPA receptor localization and in the formation of dendritic spines, a specialized structure at glutamatergic postsynapses [Ko et al., 2003; Zhang et al., 2003, 2005]. Binding of NSF is mediated by a C-terminal LZ-type coiledcoil region within βPIX and in line with other NSF substrates is sensitive to ATP hydrolysis. Guided by the similarity between the complex of GluR2 and Pick1 [Hanley et al., 2002] and the complex of Shank and βPIX (Fig. 2; Park et al., 2003], both mediated by dual coiled-coil and PDZ domain mediated interactions, we investigated the influence of NSF on the binding between Shank and βPIX. Although pre-association of a Shank fragment to βPIX increased the amount of bound NSF in co-sedimentation assays, an ATP-dependent dissociation of the complex could not be observed (H.M., unpublished observation). In line with a previous report [Zhang et al., 2003], overexpression of βPIX was found to cause the formation of

numerous thin dendritic protrusions, resembling immature dendritic spines. Extending this reported work, we demonstrated that a fragment of βPIX corresponding to the binding region for GIT1 exerts a dominant negative effect on the formation of protrusions. This finding supports the view that βPIX and GIT1 need to act in conjunction [Zhao et al., 2000; Zhang et al., 2003; Premont et al., 2004] and demonstrates that the process of protrusion formation can be disturbed by dominant negative mutants. A peptide corresponding to the NSF-binding region of GluR2 (pep2m) has successfully been used to elucidate the role of NSF in GluR2 regulation [Nishimune et al., 1998]. We reasoned expressing the NSF-binding region of βPIX might permit a similar illumination of βPIX function. However, in contrast to the GIT1-binding region, overexpression of the NSF-binding region of βPIX could not influence the formation of protrusions (Fig. 4). The lack of an effect could be due to differences in the mechanisms and affinities that mediate the competition between the GIT1-binding region or the LZ region and βPIX binding partners, or to the expression levels reached at the synapse for the different GFP-βPIX-fragments. We noted that neurons expressing high levels of GFP-βPIX-LZ formed large somatic aggregates, suggesting that high concentrations of soluble fusion proteins cannot be reached for this construct. Thus, our dominant negative approach appeared not suitable to demonstrate an involvement of NSF in βPIX-mediated postsynaptic processes.

Recently, it has been reported that βPIX forms SDS-resistant oligomers in the absence of GIT1, whereas in the presence of GIT1 a large (1–2 million Dalton) and stable heteromeric complex forms [Premont et al., 2004]. Thus, NSF could be involved in the arrangement of heteromeric βPIX-GIT1 complexes.

In summary, we present a limited number of novel putative targets for NSF-SNAP chaperone complexes that could be involved in a variety of cellular processes, ranging from apoptosis to synaptic transmission. On a molecular level, NSF-SNAP complexes may be involved in the disassembly of coiled-coil-mediated interactions underlying not only SNARE complexes but also other forms of stable oligomers, and the C-terminal membrane anchoring of NSF-SNAP targets may be mechanistically involved in the dissociation process.

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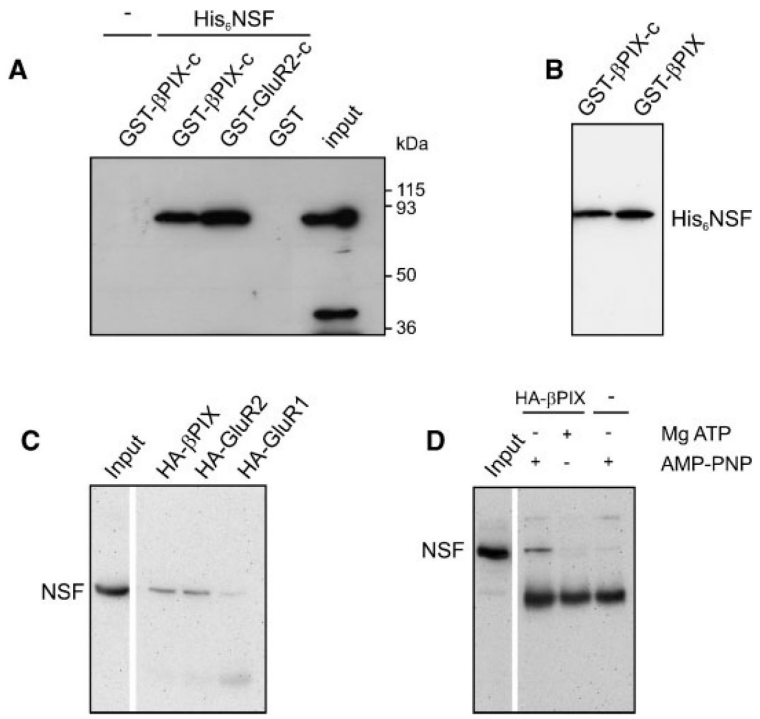

Fig. 1.

NSF interacts with the PAK binding exchange factor βPIX in vitro and in vivo. GST fusion proteins of isolated yeast two-hybrid βPIX clone (GST-βPIX-c) and the C-terminus of GluR2 (GST-GluR2-c) were immobilized on glutathione-sepharose for 'pulldown' assays. **A**: With anti-his antibody a band corresponding to the size of NSF is detected from the lysates of his_6NSF expressing bacteria, which is precipitated by GST-GluR2-c and GST-βPIX-c. No such band is seen with GST alone and no protein is detected with GST-βPIX-c pulldown of bacterial lysates not expressing his_6NSF (−). **B**: Purified recombinant his_6NSF is also susceptible to retention by immobilized GST-βPIX-c. Expression of the entire βPIX as a GST fusion protein (GST-βPIX) is equally able to precipitate purified his_6NSF. **C**: Expressed NSF is co-immunoprecipitated by HA-tagged βPIX and HA-tagged GluR2 from the lysates of HEK293 T cells, but not by HA-tagged GluR1, detected with NSF antibody. This interaction is susceptible to ATP hydrolysis. **D**: In similar experiments, NSF is co-immunoprecipitated by HA-tagged βPIX in the presence of 0.5 mM AMP-PNP and 3 mM EDTA, but only weakly interacts in the presence of 0.5 mM ATP and 3 mM $MgCl_2$. In control lysate lacking HA-βPIX expression NSF is not co-immunoprecipitated (−). Inputs are of 20 μg of total protein.

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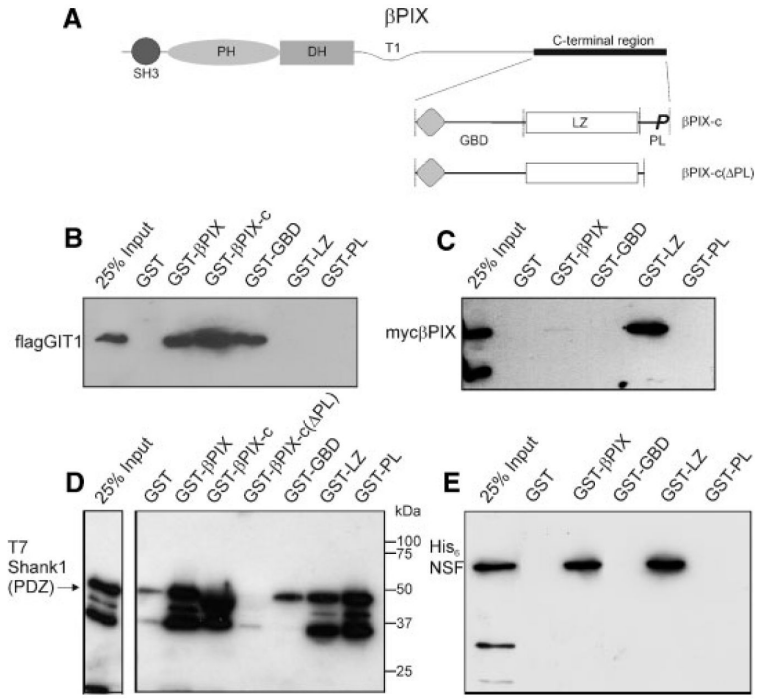

Fig. 2.

NSF binds to βPIX via its LZ domain. **A**: Schematic diagram of βPIX indicating the arrangements of the src-homology 3 (SH3), pleckstrin-homology (PH), Dbl-homology (DH) and regulatory (T1) domains. The C-terminal region of βPIX may be divided into three regions; the GIT-binding domain (GBD), coiled-coil leucine zipper domain (LZ) and PDZ ligand domain (PL). **B**: Immobilized GST fusion proteins containing the GBD are able to retain recombinant Flag-GIT1 from HEK293 cell lysates, detected by anti-flag Western blot. **C**: βPIX is reported to dimerise through its LZ domain. Consistent with this an immobilized GST fusion protein comprising the LZ region retains recombinant mycβPIX from HEK293 cell lysates. **D**: βPIX interacts with the PDZ domain protein Shank1 through its C-terminal PDZ ligand. A bacterially expressed and purified PDZ domain of Shank1 is retained by immobilized GST fusion proteins of the PL, and also the LZ and more weakly the GBD of βPIX. However, deletion of the final leucine residue of βPIX-c (βPIX-c(ΔL)) ablates the interaction with the Shank1 PDZ domain. Co-sedimented proteins were detected by anti-T7 Western blot. **E**: Bacterially expressed and purified his_6NSF co-sediments with GST-LZ. A 100 nM solution of his_6NSF was applied to immobilized GST-βPIX fragments and detected by anti-his_6 Western blot.

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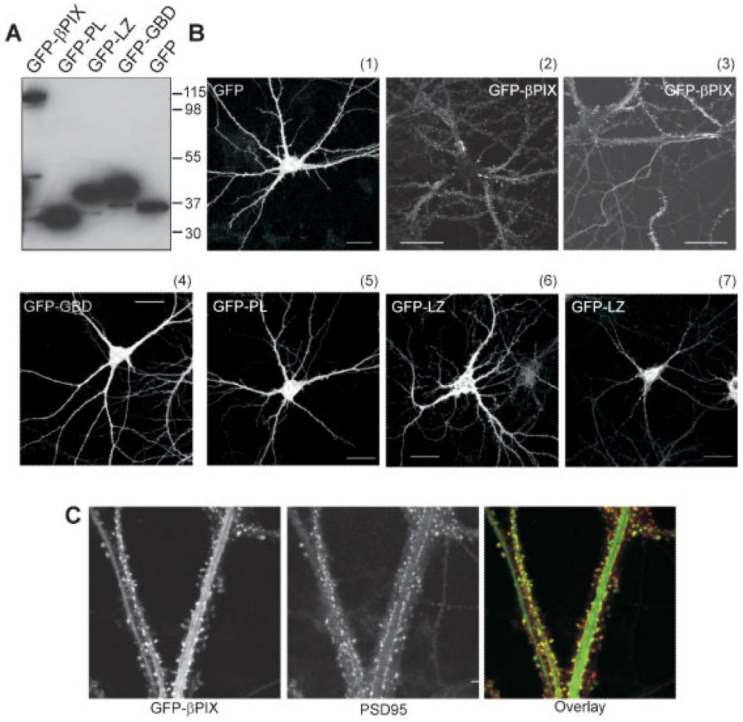

Fig. 3.

GFP-βPIX is concentrated in spines and axons in cultured hippocampal neurons. **A**: Full-length βPIX and the three C-terminal interaction domains of βPIX were expressed as GFP fusion proteins in primary hippocampal cultures through Sindbis virus infection. Cell lysates from infected neurons were probed for expression of GFP fusion protein expression by Western blotting with anti-GFP antibody 24 h after infection. **B**: GFP signals from 2 to 3 weeks in vitro infected neurons. In comparison to GFP, GFP-βPIX is found in a punctate distribution particularly concentrated in spine-like structures (1–2). GFP-βPIX is also targeted to axons (3). Expression of GFP-GBD, GFP-PL and GFP-LZ results in a distribution similar to GFP without definite subcellular localization (4–5); however, a subset of GFP-LZ-infected neurons form aggregates of GFP signal (6). Scale bar 40 μm. **C**: GFP-βPIX co-localizes with PSD95 (red) in dendrites and spines. Three weeks in vitro neurons were infected for 18 h before fixation and PSD95 immunocytochemistry. Scale bar 10 μm.

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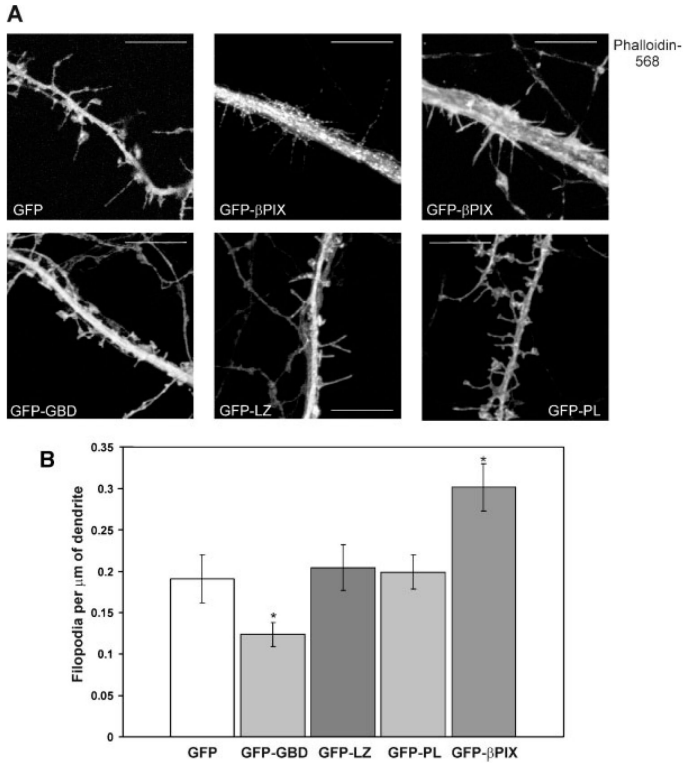

Fig. 4.

The formation of dendritic filopodia is promoted by βPIX expression, but is insensitive to expression of NSF interaction domain. **A**: Cultured hippocampal neurons 11–14 days in vitro infected for 18 h with GFP-βPIX or GFP-βPIX C-terminal interaction domains. Fluorescent images are of short sections of dendrites from infected neurons. Protrusions are seen projecting off the dendrite with all GFP fusion constructs, however GFP-βPIX-induced protrusions may be better seen using the actin stain phaloidin-Alexa 568 (**top right panel**). Scale bar 10 μm. **B**: Quantification of filopodia determined as protrusions >1.5 μm in length without discernable head in sections of dendrites. Whereas βPIX-LZ and βPIX-PL have no effect on the number of filopodia, expression of GPF-βPIX causes a significant increase in the frequency of filopodia (n = 24 dendrites; $P < 0.05$; *t*-test), while expression of GFP-GBD reduces the frequency of filopodia (n = 21; $P < 0.05$). Error bars SEM.

TABLE I

Yeast Two-Hybrid Interacting Partners of NSF and iSNAP

Identity of prey clones derived from a screen using NSF as bait	
NSF	NP_068516
βPIX	AAC39971
Rab11-FIP3	AAH81997
αCOP	NP_034068
Mink2	NP_795712
Identity of prey clones derived from a screen using βSNAP as bait	
NSF	NP_068516
Syntaxin-1A	NP_446240
Syntaxin-1B	NP_077725
Syntaxin-3	NP_112386
Syntaxin-5	NP_113892
Syntaxin-7	NP_068641
Syntaxin-12/13	NP_075228
Syntaxin-17	NP_663775
Reticulon-1	NP_446317
Reticulon-3	AAH62068
Reticulon-4	NP_114019
BNIP3	NP_445872
BNIP3-like	NP_543164
Bcl2-like-13	NP_705736
PTP IV	NP_033000
RPTP N2 (Phogrin)	NP_113788
RPTP sigma	NP_035348
RPTP zeta	NP_037212
VAP-B	NP_068619
Unknown protein	AAH56480/AAH40264