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Minireview

Cellular functions of NSF: Not just SNAPs and SNAREs

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Abstract ***N*-ethylmaleimide sensitive factor (NSF) is an ATPases associated with various cellular activities protein (AAA), broadly required for intracellular membrane fusion. NSF functions as a SNAP receptor (SNARE) chaperone which binds, through soluble NSF attachment proteins (SNAPs), to SNARE complexes and utilizes the energy of ATP hydrolysis to disassemble them thus facilitating SNARE recycling. While this is a major function of NSF, it does seem to interact with other proteins, such as the AMPA receptor subunit, GluR2, and β2-AR and is thought to affect their trafficking patterns. New data suggest that NSF may be regulated by transient post-translational modifications such as phosphorylation and nitrosylation. These new aspects of NSF function as well as its role in SNARE complex dynamics will be discussed.**

Keywords: NSF; SNARE; α-SNAP; GluR2; β2-AR; Post-translational modification

1. Introduction

Vesicular traffic is essential for cellular homeostasis. Cargo-containing transport vesicles bud from a donor compartment, then fuse with an appropriate acceptor compartment assuring a vectorial flow of membrane proteins/lipids and luminal contents through the cell. At its core, vesicular transport requires cargo selection and vesicle production through a budding process. The subsequent vesicle transport and targeting process concludes with specific membrane fusion of the vesicle to its target membrane. Early molecular analysis of vesicular trafficking between Golgi cisternae identified an essential *N*-ethylmaleimide sensitive factor (NSF) [1]. Subsequent studies identified NSF adaptors, soluble NSF attachment proteins (SNAPs) [2], and SNAP receptors [3] (SNAREs). The characterization of these proteins has lead to a detailed picture of the events leading to membrane fusion. Despite this knowledge, there are many unanswered questions regarding how SNAREs are regulated and how NSF itself might be regulated. In addition, new data suggest that NSF may have other cellular roles that require its ATPase-driven chaperone activity. This review will attempt to give an "NSF-centric" overview of some of these topics.

2. SNAREs

SNARE proteins are the minimal machinery for membrane fusion [4]. SNAREs are classified into vesicle (v) and target-membrane (t)-SNAREs according to their localization [3] or R (arginine) and Q (glutamine)-SNAREs based on a key residue in the center of their SNARE domains [5]. There are two types of t-SNAREs: syntaxin-type and SNAP-25 type. v-SNAREs and syntaxin-like t-SNAREs are type II integral membrane proteins with a single transmembrane domain (TMD). The TMDs not only anchor SNAREs to a membrane, but may contribute to complex assembly and fusion pore formation [6,7]. SNAP-25-like proteins lack a TMD and are generally anchored to the membrane through thioester-linked acyl groups [8]. All SNARE proteins characteristically contain conserved heptad repeats of approximately 60–70 residues termed the SNARE motif. SNAP-25-like SNAREs contain two such motifs. The SNARE motif forms an amphipathic α-helix where the hydrophobic residues are in register on the same face to form the core of the SNARE complexes [9].

In addition to the SNARE motif, many SNARE proteins have divergent N-terminal domains. The N-terminal half of syntaxin-1a contains an autonomously-folded, three-helix bundle, called the Habc-domain [10]. This domain forms a groove that accepts the SNARE motif generating a "closed" conformation of syntaxin-1a. The N-terminal domain of v-SNAREs is highly divergent and divides the family into "brevins" that contain short domains, and "longins" that contain longer

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Abbreviations: NSF, *N*-ethylmaleimide sensitive factor; AAA, ATPases associated with various cellular activities; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; VAMP, vesicle associated membrane protein; TMD, transmembrane domain; AMPAR, α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid receptor; GluR2, glutamate receptor 2; GABA, γ-amino-butyric acid; GBR, $GABA_B$ receptor; β2-AR, β2-adrenergic receptor; GPCR, G-protein coupled receptor; CRLR, calcitonin receptor-like receptor; RAMP, receptor activity-modifying proteins; AM, adrenomedullin; NMJ, neuromuscular junction; PKC, protein kinase C; PTP, protein tyrosine phosphatase; GATE-16, Golgi-associated ATPase enhancer of 16 kDa; VCP, valosin containing protein; Rip11/Gaf-1, Rab interaction protein 11/γ-SNAP associated factor 1; EEA-1, early endosome antigen 1; SRH, second region of homology; LMA1, low molecular weight activity 1; GEC1, glandular epithelial cell 1

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structures [11]. For the brevins, the N-terminus attains a partially folded state when SNARE complexes are assembled and may play a role in controlling the stability of primed fusion complexes [12]. The longin domains appear to regulate v-SNARE sorting and have been shown to be important for spatially directed exocytosis in neurons [13]. From these examples, it seems possible that these N-terminal domains may be key structural elements to allow regulation of SNARE complex assembly and perhaps disassembly.

2.1. Assembly of SNARE complexes

Structural information from neuronal SNARE complexes shows that the SNARE motifs assemble into parallel, twisted, coiled-coil, four-helix bundles by burying the hydrophobic residues inside the core (Fig. 1). Three of the helices are contributed by t-SNAREs (1 from syntaxin and 2 from SNAP-25), with the other helix provided by the v-SNARE. The coiled-coil is composed of 15 hydrophobic layers arranged perpendicular to the axis of the helical bundle, and contains a central hydrophilic zero-layer with one R- and three Q-residues. The surface of the complex is highly grooved with several charged regions [9,14].

Great focus has been put on the molecular steps of SNARE complex assembly. It is thought to start at the SNAREs' N-termini and proceed in a zipper-like fashion toward the membrane anchor at the C-termini [15]. Before assembly, syntaxin-1a shows significant α-helicity, but SNAP-25 and synaptobrevin/VAMP-2 are largely unstructured. Upon ternary complex formation, SNAP-25 and synaptobrevin show dramatic increases in α-helicity. Considerable evidence [16] now implies that syntaxin-1a and SNAP-25 form a transient 1:1 complex, which serves as an "on-pathway" intermediate. After establishment of the t-SNARE heterodimer, synaptobrevin, from the opposing membrane, binds to form a high affinity complex, which spans the two fusing bilayers. The energy associated with SNARE complex assembly is thought to drive membranes into close apposition, which then directly or indirectly leads to fusion [16–18]. After membrane fusion, SNAREs remain as a complex in the same membrane. Disassembly of these *cis* complexes is achieved by the concerted action of α-SNAP and NSF.

3. α-SNAP

Clary et al. [2] determined that NSF required a peripheral membrane protein adaptor to bind Golgi membranes. This protein was called Soluble NSF Attachment Protein or SNAP. In mammals, there are three SNAPs, α, β, and γ; in yeast there is one, Sec17p. In mice, α-SNAP is encoded by the *Napa* gene and its deletion is embryonic lethal [19]. A G → A missense mutation in exon 4 of the *Napa* gene results in an M105I substitution that appears to be the cause of hydrocephalus with hop gait (*hyh*) in mice. Affected animals show a dome-shaped head with a small cerebral cortex. They die postnatally from a progressive enlargement of the ventricular system. Binding of the M105I mutant to SNARE complexes, however, was indistinguishable from that of the wild-type α-SNAP. The mutation also had no effect on NSF-mediated, SNARE complex disassembly. Intriguingly, both mRNA and protein levels of α-SNAP were decreased in *hyh* mice. Though the biochemical data do not yield a clear explanation of the defect in *hyh* mice, the animals' phenotype indicates that the reduced availability of α-SNAP is detrimental to apical transport and cell fate regulation in neurons [19].

3.1. α-SNAP binding to SNARE complexes

α-SNAP does not bind synaptobrevin but can associate with the syntaxin/SNAP-25 heterodimer [20]. α-SNAP appears to first bind to syntaxin/SNAP-25. Association of synaptobrevin with this complex generates a third binding site for α-SNAP [20,21]. Consistently, the binding affinity of α-SNAP to syntaxin and SNAP-25 is weak but addition of synaptobrevin dramatically enhances binding [22]. Deletion mutagenesis suggests that the N-terminal 63 and C-terminal 37 residues of α-SNAP are important for binding to SNARE complexes. Binding of α-SNAP involves the C-terminal residues (194–243, part of the SNARE motif) of syntaxin-1 and the N-terminal residues (25–100) of SNAP-25 [20]. However, since there is large sequence variation among the SNARE family, it seems likely that α-SNAP primarily recognizes the overall shape of the coiled-coil structure and not specific residues.

The crystal structure of Sec17p shows that it contains an N-terminal twisted sheet of α-helical hairpins with a protruding C-terminal α-helical globular bundle (Fig. 1). One edge of the twisted sheet is longer than the other forming concave and convex faces in the structure. The concave face has a distribution of negative charges, which is most pronounced at the extreme C-terminus. Both the concave face and the longer edge contain residues that are conserved among all SNAPs [23]. As outlined by Rice and Brunger [23], there are two possible models of SNAP-SNARE interaction: "face-on" and "edge-on". Mutagenesis data show SNAP–SNARE complex interactions involve positively charged α-SNAP residues distributed over the concave surface of its twisted sheet domain [24].

4. NSF

NSF is encoded by the *Sec18* gene in yeast [25] and a neuronal specific version is encoded by the *comatose* gene (dNSF1) in *Drosophila* [26]. NSF is clearly important since conditional mutations in Sec18p and *comatose* both lead to cessation of membrane transport under restrictive temperatures. Each protomer of the homo-hexamer has three domains: the amino-terminal domain (NSF-N, 1–205) is required for SNAP–SNARE binding; the first ATP-binding domain (NSF-D1, 206–477), which provides ATPase activity, is required for SNARE complex disassembly; and the second ATP-binding domain (NSF-D2, 478–744) is required for hexamerization [27,28]. From sequence homologies of NSF-D1 and NSF-D2, NSF is a member of the AAA family of ATPases that generally uses ATP hydrolysis to alter the conformation of a substrate protein [29].

4.1. NSF structure

From several studies using quick-freeze/deep-etch electron microscopy [30] or cryo-electron microscopy (cryo-EM) with single-particle averaging of NSF-α-SNAP–SNARE complexes [31], NSF clearly shows six-fold symmetry. It appears to be arranged like a barrel composed of two rings (presumably NSF-D1 and NSF-D2) with six knobs extending from the barrel

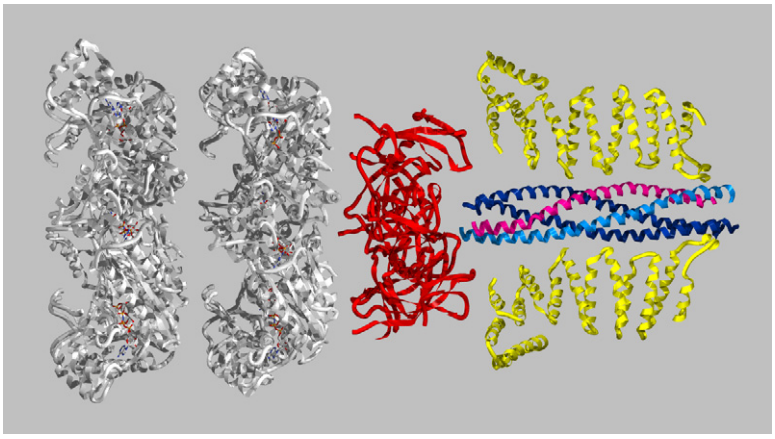

Fig. 1. 20S Particle. Depicted are the crystal structures for each element of the 20S particle. The two ATP-binding domains of NSF (D1 and D2) in white are modeled from the NSF-D2 structure (1D2N). A trimer of NSF-N domains, in red, is based on the three-in-three-out model of May et al. [33] (1QDN). Only two yeast α-SNAPs (Sec17p, 1QQE) are depicted in yellow. The coiled-coil SNARE complex is depicted with syntaxin-1a in light blue, synaptobrevin/VAMP-2, in magenta, and SNAP-25 in dark blue (2BUO). The images were created with Swiss PDB viewer and rendered with Pov-Ray.

(presumably NSF-N). The conformation depends on the nucleotide bound. ATP-charged NSF is a hollow cylinder: NSF-D1 and NSF-D2 form hexameric rings that are arranged in a double-layered barrel, while NSF-N domains emerge from the sides of the ring. The ADP-bound morphology is slightly wider, and does not possess outwardly flared NSF-N domains [30,31]. These nucleotide-state-dependent conformations may hold the secret to how NSF uses ATP hydrolysis to disassemble SNARE complexes.

NSF-N is a kidney-shaped domain composed of two subdomains: N_A (a.a. 1–83) and N_B (a.a. 87–201), which are joined by a short linker [32,33] (Fig. 2A). N_A is made of six β strands arranged in a barrel with two "ψ loops" containing short α helices (α1 and α2) extending over the top. N_B is an α/β roll, where four β strands wrap around a single amphipathic α-helix. The interface between N_A and N_B is predominantly hydrophilic with two small hydrophobic clusters at the edges of the interface. The surface of NSF-N possesses an overall positive charge and contains three grooves, all large enough to accommodate an α-helix [32,33]. At this stage, each could be important for SNAP–SNARE complex binding, though mutagenesis experiments suggest that groove 3 is the likely binding site. Groove 3 contains the first N_A ψ loop in the double ψ loop beta barrel motif, which consists of strand β5 and the loop between strands α1 and α2 [32]. Mutation of one highly conserved residue (R67E) in this surface completely eliminates binding [34]. Sec18p-N (yeast NSF) has the same overall fold [35] with a conserved groove 3.

The structure of NSF-D2 was the first AAA domain to be determined [36,37] (Fig. 2B). Overall, it consists of two subdomains: an N-terminal nucleotide-binding subdomain (residues 505–676), and a C-terminal α-helical subdomain (residues 677–750). The N-terminal subdomain (which contains the Walker A and B box motifs) has the wedge shape with a central five-stranded parallel β-sheet. The C-terminal subdomain is composed of four α helices. This subdomain lies above the nucleotide-binding domain and contributes several residues to nucleotide binding.

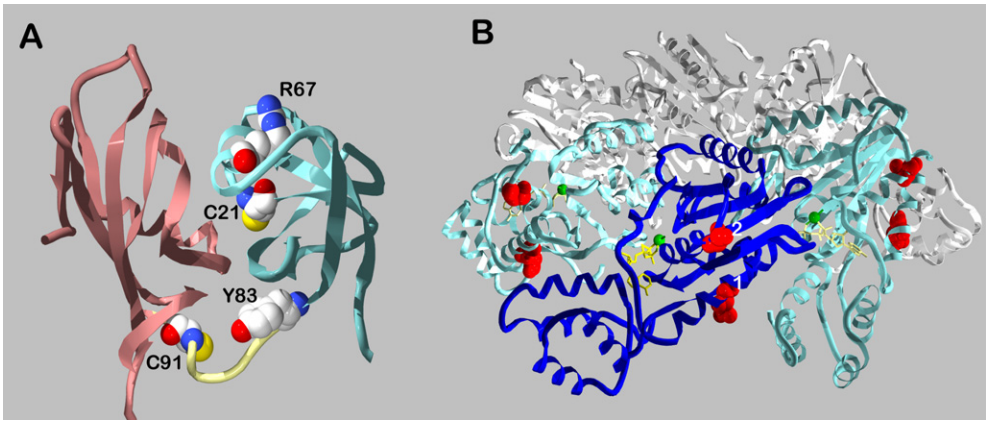

Fig. 2. Post-translational modifications of NSF. Panel A, crystal structure of the N-terminal domain of NSF, showing the locations of the modified cysteine and tyrosine residues. The N_A subdomain is in rose and the N_B subdomain is in aqua. The image is based on 1QDN and was generated using Swiss PDB viewer and rendered with Pov-Ray. Panel B, crystal structure of the NSF-D2 hexamer. Three subunits are shown in white, two in aqua and one in blue. The modified residues are indicated by red. Position 1 is predicted to be equivalent to the phosphorylated Ser237 in the NSF-D1 domain. Position 2 is Ser569, phosphorylated by Pctaire1. The image is based on 1D2N and was created with Swiss PDB viewer and rendered with Pov-Ray.

Although the structure of NSF-D1 has not been determined, its sequence homology to NSF-D2 indicates that the NSF-D2's structure can be used as a guide to understand the mechanism of the catalytically active NSF-D1. Both domains contain nucleotide-binding sites with the classical Walker A and B box motifs. The conserved lysine residues (266, 549) in the two Walker A boxes are crucial to ATP-binding [38,39]. The aspartic acid in the DEXX sequence of the Walker B box is thought to coordinate a Mg^{2+} ion that is needed for ATP hydrolysis, whereas the glutamate is required to activate water for the hydrolysis reaction. Mutation of Glu329 (E329Q) in NSF-D1 results in a dominant negative form of NSF that can bind but not disassemble SNAP–SNARE complexes [28,40].

Within the N-terminal subdomain of both ATP-binding domains, there is a highly conserved region called Second Region of Homology (SRH), which is unique to AAA proteins. At the C-terminus of SRH are two highly conserved arginine residues called Arginine Fingers. Both are important for nucleotide hydrolysis in other AAA proteins and are thought to allow for inter-subunit communication between the protomers of the hexameric ATPases [41]. Mutations of these residues in NSF only have a limited effect on ATPase activity but almost eliminate SNARE complex disassembly [34]. Sensor 1 is present at the N-terminus of the SRH. A conserved polar residue in Sensor 1 is in close proximity to the phosphates of the bound nucleotide. Mutation of this residue (T394P) in Sec18p eliminates ATPase activity [42]. The Sensor 2 comes from the C-terminal helical subdomain. The residues in Sensor 2 are in close proximity to the bound nucleotide and have been proposed to monitor the state of the bound nucleotide. This could propagate nucleotide-dependent conformational change to the outer regions of NSF [41].

4.2. NSF binding to the α-SNAP–SNARE complexes

NSF and α-SNAP do not interact in solution. The interaction of NSF with α-SNAP occurs when α-SNAP binds to membranes, to a plastic surface, or alternatively when trimerized [43]. Cross-linking between α-SNAP and NSF in the membrane-bound complex further confirms this interaction [44]. The deletion mutants show that the C-terminal 45 amino acids of α-SNAP are crucial for interaction with NSF [45]. Consistently when this domain was grafted onto the trimerization domain of thrombospondin, the resulting recombinant protein bound NSF in the absence of SNAREs [43]. Point mutations in this domain, specifically of the penultimate leucine residue (Leu294), yield an α-SNAP that can bind NSF but cannot activate its ATPase activity [46].

NSF-N is required for binding SNAP–SNARE complexes [28,39,43]. Consistently, the Sec17p-(yeast α-SNAP)-binding-defective Sec18-1 allele maps to a 120-amino acid region in the N domain (G89D) [47,48]. Only ATP-charged NSF is binding competent. The ATP-binding mutant, K266A, shows weak binding to the SNAP–SNARE complexes, suggesting that binding of ATP by NSF-D1 most likely induces conformational changes in the D1 and N domains that are crucial to the interaction of NSF with SNAP–SNARE complexes. The neighboring D1 or D2 also possibly participates in binding to α-SNAP because the monomeric mutant N-D1 and hexameric N-D2 can bind but monomeric N domain cannot [28]. This implies that two elements are potentially important to the binding interaction: oligomerization of NSF-N and proximity to an ATP binding domain.

NSF does not bind to SNARE complexes in the absence of α-SNAP [21,49]. Based on this, NSF and α-SNAP are thought to bind in a sequential manner, giving rise to the 20S particle, named for its sedimentation coefficient [50]. In the presence of non-hydrolysable ATP, the 20S particle is stable and can be purified. The SNARE complex in this particle is perhaps reflective of the *cis* configuration that would occur post-fusion. The 20S particle is disassembled in a process coupled to ATP hydrolysis, which probably reflects a major role of NSF in vivo [3,50]. NSF, however, can dissociate NSF-α-SNAP-syntaxin and NSF-α-SNAP-syntaxin-SNAP-25 complexes in vitro, suggesting that it could be a general SNARE chaperone affecting the conformations of multiple SNARE-containing complexes [51].

Electron microscopy studies of the 20S particle show that it has a spark-plug shape [30], with the SNARE complex at the thinner end and the two rings of NSF clearly visible at the wider end (Fig. 1). Three α-SNAP molecules coat the rod-like SNARE complex along its length. While NSF binds to one end, the membrane-spanning regions of VAMP and syntaxin appear located at the other end, adjacent to the N-terminus of α-SNAP [52]. A cyro-EM structure of the 20S particle, suggests that the rings of the two ATP binding domains, NSF-D1 and NSF-D2, form a double-layered barrel, arranged in an anti-parallel orientation (A similar conclusion was made about the p97/valosin containing protein (VCP) until crystallography proved it incorrect [53,54]). Six protrusions, thought to be the NSF N domains, appear to extend sideways from one end of the barrel. Near these protrusions, a cap-like density corresponding to α-SNAP and SNAREs is clearly visible [31]. These data suggest a mechanism by which NSF could untwist the SNARE complexes.

4.3. NSF's ATPase activity

The intrinsic ATPase activity of NSF is very low [27]. NSF-N has been proposed to exert some control over NSF's ATPase activity because antibodies to it cause a 2-fold increase in hydrolytic activity [55]. Binding to immobilized α-SNAP stimulates the ATPase activity [56]; however, maximal stimulation of ATPase activity is achieved when both α-SNAP and SNARE complexes are included [51]. The penultimate leucine of α-SNAP is critical for this activity [45,46]. Consistently, the L294A mutation of α-SNAP is unable to mediate 20S particle disassembly. A direct interaction between NSF and Leu294 (or adjacent residues) seems likely but is as yet unproven.

NSF-D1 accounts for the majority of basal and SNAP-stimulated ATPase activity [38,57]. Mutations in the ATP-binding site of domain D1 (K266A and E329Q) cause a 70–80% decrease in ATPase activity relative to wild-type NSF [38]. The ATP-binding mutant (K266A) disrupts NSF ability to bind to the SNAP–SNARE complexes. The hydrolysis mutant (E329Q) fails to dissociate the SNAP–SNARE complexes [28]. Mutations in the NSF-D2 decrease ATPase activity but only minimally [38]. The ATPase activity of Sec18p is stimulated by Sec17p. The hydrolysis mutant, E350Q in Sec18p-D1, shows no basal or stimulated ATPase activity [58]. The temperature-sensitive, paralytic, mutation comatose^{st17} (G274E) in *Drosophila* NSF1 is near the D1 ATP-binding site [59]. When this mutation was engineered into mammalian NSF

Table 1
Non-SNARE proteins that associate with NSF

NSF interaction	Reference
NSF/Receptor	
GluR2	Osten et al. [62], Nishimune et al. [63], and Song et al. [64]
β2-AR	Cong et al. [65]
CRLR–RAMP3	Bomberger et al. [67]
$GABA_A$ receptor β subunit	Goto et al. [97]
$GABA_B$ receptor 2	Pontier et al. [68]
D1-like dopamine receptor	Heydorn et al. [66]
Dopamine D2 receptor	Zou et al. [98]
Muscarinic M1, M3, M4 and M5 receptors	Heydorn et al. [99]
Tachykinin NK1 and NK2 receptors	
Somatostatin SST1 receptor	
DOP receptor	
Chemokine receptors US27 and US28	
NSF/Rab family	
Rab 3, 4, 6	Han et al. [75]
Rab 11-containing complex (γ-SNAP/Rip11/Gaf-1)	Tani et al. [76]
Rab 11-FIP3	Martin et al. [77]
Rab 5 containing complex (EEA-1, Rabaptin-5, Rabex-5, syntaxin 13)	McBride et al. [78]
NSF/others	
β-Arrestin 1	McDonald et al. [73]
GATE-16	Sagiv et al. [82]
LMA1	Xu et al. [100]
βPIX	Martin et al. [77]
GABARAP	Kittler et al. [101]
PTP-MTG2	Huynh et al. [90]
Pctaire 1	Liu et al. [89]
GEC1	Chen et al. [102]

(G282E), the resulting mutant protein had no ATPase activity [60]. These data demonstrate the importance of the D1 domain to NSF's ATPase activity and thus to its function in membrane trafficking events.

5. Binding of NSF to other substrates

The well-documented role of NSF in vesicular transport is logically based on its interactions with SNAREs and SNAPs; however, several studies point to additional roles for NSF [61]. Specifically, NSF has been shown to be associated with a number of non-SNARE proteins, which can be divided into two classes (Table 1). The first are the C-terminal, cytoplasmic domains of a number of cell-surface signaling receptors. The second class of interactions is more diverse and includes peripheral and soluble cellular proteins as well as cytoskeletal elements. Examples of these interactions will be discussed below.

5.1. NSF binding to membrane receptors

The direct interactions between NSF and the cytoplasmic tails of several cell surface receptors have been reported: *e.g.* the α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid receptor (AMPAR), the β2-AR receptor, the dopaminergic receptor, the adrenomedulin (AM) receptor and the γ-aminobutyric acid (GABA) receptor [62–68]. NSF binding is proposed to modulate the trafficking of these receptors between the plasma membrane and the endosome.

NSF is shown to directly bind to the C-terminal tail of the GluR2 subunit of the AMPA receptor in a SNAP-independent manner. The stable binding is nucleotide-dependent and requires all three domains of NSF. The minimal NSF binding domain of GluR2 is located between Lys844 and Gln853 [62–64]. Further mutagenesis of this segment showed that deletion of the last five amino acids abolishes NSF binding [69]. The NSF–GluR2 interaction is proposed to play a role in the stabilization of surface AMPA receptors on the post-synaptic membrane since a peptide that disrupts the NSF/GluR2-interaction causes a rapid decrease in the size of synaptic currents [70].

A two-hybrid approach was used to demonstrate that the C-terminus of β2-AR mediates binding to NSF. Mutations at any of the last three residues (S411A, L412A, or L413A) ablate NSF binding [65]. The addition of a single alanine residue at the end of the β2-AR tail also abrogates NSF binding. Binding to the β2-AR, however, is not affected by the addition of α-SNAP [71]. The binding of NSF to the β2-AR is critical to allow the receptor to undergo rapid recycling, which might also involve another interaction of NSF with β-arrestin1. β-Arrestins play an important role of desensitization of many G-protein coupled receptors (GPCRs) [72]. Over-expression of NSF in HEK 293 cells significantly enhances agonist-induced β2-AR clearance and rescues the inhibition of β2-AR internalization mediated by the phosphomimetic mutant of β-arrestin 1(βarr1S412D) [73].

There are five dopamine receptor subtypes, which are divided into two classes: D1-like and D2-like. NSF is found to bind to C-terminal tails of the D1 and D5 receptors fused to glutathione *S*-transferase (GST), suggesting that NSF could possibly be involved in the recycling of D1-like receptors [66].

Adrenomedullin receptors are comprised of receptor activity-modifying proteins (RAMP2 or RAMP3) and calcitonin receptor-like receptor (CRLR), which is the GPCR. RAMPs (1–3) are single transmembrane accessory proteins indispensable to the determination of receptor phenotype. Co-expression of RAMP1 with CRLR generates a calcitonin gene-related peptide-1 (CGRP-1) receptor, while co-expression of RAMP2 or RAMP3 with CRLR yields adrenomedullin receptors, AM-1 and AM-2 receptors, respectively [74]. Upon stimulation with AM, the CRLR-RAMP receptor complex is internalized and undergoes degradation or recycling dependent on the cell type. NSF might be involved in altering the intracellular trafficking of the CRLR–RAMP3 receptor complex. When NSF is co-expressed with CRLR–RAMP3 complexes in HEK 293 cells, CRLR–RAMP3 is sorted for recycling rather than undergoing degradation. *N*-ethylmaleimide (NEM) treatment blocks the resensitization/recycling of the receptor after agonist-stimulated desensitization in the rat mesangial cells. RAMP3 is thought to interact with NSF through its C-terminal, type-I PDZ motif (-DTLL). Deletion of the PDZ motif significantly affects the resensitization and recycling of the CRLR–RAMP3 receptor complex in the presence of NSF. Mutagenesis of the DTLL sequence indicates that Asp145, Thr146 and Leu148 are the critical amino acids in the PDZ motif that regulate the RAMP3/NSF interaction [67].

Using full-length C-terminal tails of the metabotropic $GABA_B$ receptor 1 (GBR1) and GBR2 as bait in the yeast two-hy-

brid screen, NSF is revealed as a binding partner of GBR2. This was confirmed by using the GST-GBR2 fusion protein and purified recombinant NSF. The interaction of GBR2 and NSF also occurs in CHO cells and is disrupted upon agonist stimulation. The binding region has been narrowed to the 27 amino acids (residues 799–825; Pep-27) at the C-terminus of GBR2. Inhibition of NSF binding to GBR2 with a TAT-Pep27 fusion peptide blocks the agonist-promoted desensitization of GBR in hippocampal slices and CHO cells. TAT-Pep27 also blocks the GABA-induced protein kinase C (PKC) recruitment and GBR phosphorylation in CHO cells. Given that GBR does not undergo agonist-stimulated internalization in any of the systems tested, the author suggested that NSF regulates $GABA_B$ receptor signaling efficacy in a different way from other receptors. The preassociation of NSF with GBR2 primes the receptor and promotes PKC recruitment and phosphorylation of the GBR2 receptor, resulting in agonist-promoted desensitization of the receptor [68].

5.2. Other NSF-binding interactions

NSF has also been shown to interact with a diverse array of proteins including small GTP-binding proteins of the Rab family, GABA receptor associated protein (GABARAP)/Golgi-associated ATPase enhancer of 16 kDa (GATE-16)/ low molecular weight activity 1 (LMA1) family members, βPIX, protein tyrosine phosphatase (PTP)-MEG2, and Pctaire1. The functional significance of most of these interactions is still largely speculative though provocative. In many cases, the chaperone function of NSF may play a role in the assembly/disassembly cycle of complexes containing these proteins.

Han et al. [75] used the NSF-N-D1 truncation as bait and recovered Rab 6 as a binding partner by two-hybrid screen. This interaction requires the C-terminal 30 amino acids of Rab 6 and can stimulate the ATPase of NSF [75]. Two other Rabs (3 and 4) appear to bind and to stimulate NSF's ATPase activity. NSF also interacts with Rab-containing complexes. NSF binds to a Rab 11-containing complex made up of γ-SNAP and Rab interacting protein 11/γ-SNAP associated factor 1 (Rip11/Gaf-1) [76]. Two-hybrid analysis revealed that γ-SNAP interacts directly with NSF *via* its extreme C-terminus and requires the penultimate leucine (Leu312). Intact NSF is required to interact with γ-SNAP. Both the N-terminal and C-terminal regions of γ-SNAP also mediate binding to the C-terminal domain of Gaf-1/Rip11, a Rab 11 effector. The complex comprising γ-SNAP and Gaf-1/Rip11 is disassembled by NSF in an ATPase-dependent manner. A yeast two-hybrid screen identified Rab 11-FIP3, another Rab 11-binding protein, as a NSF binding partner [77]. NSF has been detected in a complex with Rab 5 effectors: early endosome antigen 1 (EEA-1), Rabaptin-5, Rabex-5, and the SNARE syntaxin-13 [78]. Despite this wide range of interactions, their physiological relevance is still not certain. NSF could be involved in control of the assembly/disassembly cycles of these Rab-containing complexes. Alternatively, these complexes could play a role in targeting NSF to membrane subdomains involved in high levels of membrane fusion. Further experiments are clearly required.

GATE-16 functions in the secretory system [79] and is a member of a ubiquitin-like family of proteins that contain GABARAP and LMA1 [80]. GATE-16 binds to NSF as demonstrated by in vitro studies and by co-immunoprecipitation from cell extracts. Since ubiquitin binds to the N-terminal domain of p97/VCP (a domain similar to NSF-N)[81], GATE-16's similarity to ubiquitin may be useful in analyzing the NSF–GATE-16 interactions. GATE-16 stimulates NSF's ATPase activity. It also interacts with Golgi v-SNARE GOS28 in an NSF- and SNAP-dependent manner leading the authors to propose that GATE-16 is transferred to GOS28 from NSF, stabilizing GOS28 in a "primed" conformation [82].

Using a truncated form of NSF (Δ-1–9) as bait, Martin et al. [77] detected four NSF interacting proteins in a yeast two-hybrid screen: Rab 11-FIP3 (discussed above), αCOP, Mink2 and βPIX. βPIX, a Pak-binding Rho guanine exchange factor, was of specific interest due to its apparent role in the control of post-synaptic structure. Co-immunoprecipitation experiments confirmed the interaction and GST pull-down assays with βPIX fragments showed that the leucine zipper motif in the C-terminus of βPIX was important for binding. Subsequent studies failed to demonstrate a physiological role for the interaction but the authors suggest that, since βPIX can form higher molecular weight complexes, NSF may play a role in their assembly/disassembly cycle.

6. NSF and the cytoskeleton

Genetic studies in *Drosophila* have linked NSF to cytoskeletal dynamics [83]. *Drosophila* expresses two NSF isoforms: dNSF1, which is the dominant isoform in the adult central nervous system and dNSF2, which shows a much broader distribution [84]. dNSF1 null flies die as pharate adults but dNSF2 deletion is lethal at or before the first instar [85]. Over-expression of the dominant negative mutant of dNSF2 (E326Q) in neurons results in a very interesting phenotype: the overgrowth and hypersprouting of neuromuscular junctions (NMJ). Reversal of this phenotype served as the basis of a screen which identified a number of different classes of proteins involved in such varied processes as transcription and ubiquitin-mediated degradation. Interestingly, several actin-binding proteins were identified (*i.e.* moesin, *jaguar* (a myosin VI), and *quail* (a villin-like protein) [83,86]). β-Tubulin was also found to suppress the $dNSF2^{E/Q}$-induced phenotype. Such data suggest that NSF could play a role in controlling synaptic structure through an effect on the cytoskeleton. Such an effect would be consistent with the loss of cell polarity seen in *Dictyostelium* amoebae expressing defective NSF mutants [87]. However, what is the nature of this connection between NSF and the cytoskeleton? The authors of the *Drosophila* studies suggest that neurotransmitter release (or lack thereof) is not responsible since NMJ overgrowth is not seen in the syntaxin or synaptobrevin null mutants. However, these SNAREs represent only one membrane trafficking pathway in neurons. Additionally, over-expression of α-SNAP reverses the phenotype, suggesting some connection between NMJ overgrowth and membrane trafficking [83]. Perhaps, the effect of the dominant-negative $dNSF2^{E/Q}$ mutant is through disruption of a membrane trafficking pathway that is required to localize cytoskeletal binding proteins to specific regions of the presynaptic or post-synaptic plasma membrane. Further analysis will be required to dissect the mechanistic basis of this interesting effect.

7. NSF regulation

Initially, NSF was thought to be a "house-keeping protein"; constitutively active and not subjected to any regulation. Perhaps this misconception grew out of the fact that regulation of secretion appears to be largely prefusion and directed to SNARE complex assembly. However, recent studies show that NSF activity is not uniform and can be regulated by several different mechanisms, including the reversible inactivation by *S*-nitrosylation and phosphorylation.

7.1. Phosphorylation

NSF has been shown to be phosphorylated in rat brain synaptosomes in a depolarization-induced, calcium-dependent manner and this event correlates with glutamate release from synaptosomes. PKC appears to be responsible and it phosphorylates NSF on Ser237 in NSF-D1. Mutation of this residue to alanine eliminates in vitro phosphorylation and mutation to glutamic acid attenuates NSF binding to SNAP–SNARE complexes [88]. Structurally, the effect of phosphorylation at Ser237 can only be discussed based on the structure of NSF-D2 (Fig. 2B). Ser237 would be in the middle of α1 and well within the reach of the adjacent subunit, particularly the loop between the α8 and α9. The phosphorylation of this residue could restrict the movement of the α8 by charge–charge interaction and thereby affect the conformational changes in Sensor 2 associated with ATP hydrolysis.

NSF also can be phosphorylated by the serine/threonine kinase, Pctaire1. Pctaire1 phosphorylates NSF on Ser569 in NSF-D2 and affects NSF oligomerization [89] (Fig. 2B). Mutation of Ser569 to alanine (S569A) abolishes phosphorylation and stabilizes the NSF oligomer. The S569E mutant caused a defect in oligomerization. Inhibition of Pctaire1 activity by over-expression of its kinase-inactive mutant (Pctaire1-KD) also enhances the ability of NSF to hexamerize. Consistently, overexpression of Pctaire1-KD or NSF-S569A in PC12 cells significantly enhances high potassium-stimulated growth hormone release from dense core vesicles, suggesting phosphorylation of NSF by Pctaire1 plays a role in regulating the calcium-dependent exocytosis [89]. In the crystal structure of NSF-D2, Ser569 is located on the interface between monomers. Phosphorylation of this residue could impact the hexamerization of NSF.

Tyrosine phosphorylation of NSF was shown by Huynh et al. [90]. Tyrosine kinases Fes and Fer phosphorylate NSF on Tyr83 and the tyrosine phosphatase, PTP-MEG2, specifically removes the phosphate (Fig. 2A). Phosphorylation at Tyr83 increases NSF's ATPase activity but prevents α-SNAP binding. This suggests that tyrosine phosphorylated-NSF would be functionally inactive and lead to an accumulation of dead-end *cis*-SNARE complexes, thereby inhibiting membrane fusion. The hypothesis is supported in Jurkat T cells transfected with a NSF Y83F mutant or with PTP-MEG2. These cells contained enlarged secretory vesicles which were fragmented into smaller vesicles when the cells were treated with the PTP inhibitor, pervanadate. This indicates that tyrosine phosphorylation/dephosporylation of NSF can regulate a dynamic cycle of vesicle fusion. In such a model, PTP-MEG2 functions as a positive regulator of NSF to promote secretory vesicle fusion.

As mentioned previously, NSF-N contains two subdomains: N_A and N_B. Tyr83 is located on the loop connecting these two subdomains and is involved in stabilization of this loop by forming a hydrogen bond with an adjacent amino acid (Gln90). Gln90 also forms a hydrogen bond with Lys87, further stabilizing the loop. Addition of a negative charge to Tyr83 might affect these interactions and disrupt the interface between the subdomains thus perturbing α-SNAP binding.

7.2. S-Nitrosylation and oxidation

Nitric oxide (NO) is a second messenger in the cardiovascular system that limits vascular inflammation and thrombosis by, in part, affecting endothelial cell and platelet exocytosis. Given that the inhibition of NSF by NEM is based on the sensitivity of specific cysteines to alkylation, NSF would seem a viable target for regulation by *S*-nitrosylation. The nitrosylation of NSF was first demonstrated, in endothelial cells, by Lowenstein and colleagues [91]. They showed that exogenous NSF could rescue the NO-induced inhibition of von Willebrand Factor release from permeabilized endothelial cells. Surprisingly, treatment of NSF with the NO donor 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA-NONOate) did not inhibit NSF's ATPase activity but did block NSF's ability to disassemble SNARE complexes. DTT restored the activity of nitrosylated-NSF, consistent with the reversible nature of the modification. Mutagenesis of NSF's nine cysteines indicates that NSF-N domain Cys21 and 91 (Fig. 2A) and D1-domain Cys264 are likely sites of *S*-nitrosylation. Mutations of those residues to alanine all partially decreased NSF's ATPase activity. Mutation of Cys21 blocks the ability of NSF to interact with SNARE complexes and mutations of Cys91 and Cys264 act as dominant negatives. Cys21 is located on the interface between the two subdomains of NSF-N, which is proposed to be the binding sites between NSF and α-SNAP. Mutation of this residue could affect α-SNAP binding and abolish the interaction between NSF and SNARE complexes. Cys91 is located on the similar position as Tyr83. Its mutation may restrict the conformational change of N domain induced by ATP hydrolysis in NSF-D1 and affect the ability of NSF to disassemble the SNARE complexes. Cys264 is located in the Walker A motif of NSF-D1, which is critical to ATP binding. Mutation of this residue may affect the nucleotide binding and inactivate the NSF.

The nitrosylation of NSF not only occurs in endothelial cells [91], but is also seen in platelets [103]. Lowenstein and colleagues demonstrated that NO inhibits platelet granule exocytosis by *S*-nitrosylation of NSF and adding NSF to the platelets restores secretion. *S*-Nitrosylation of NSF on Cys91 also regulates its binding to the AMPAR GluR2 subunit [92]. Substantial augmentation of NSF–GluR2 binding is observed by treatment with the NO donors. NSF–GluR2 binding is diminished in eNOS null mice. NO donors elicit a rapid, time-dependent surface insertion of GluR2, which is markedly reduced by disrupting the interaction between GluR2 and NSF. It appears NSF *S*-nitrosylation could be a physiologic mediator of the receptor's surface expression during NO-induced synaptic plasticity. It should be noted that there are many potentially nitrosylatable proteins in a cell and it is perhaps overly optimistic to believe the NSF is the only substrate that counts.

Recently, it has been shown that NSF can be regulated by hydrogen peroxide. H_2O_2 inhibits exocytosis from thrombin-stimulated endothelial cells. This inhibition can be reversed by adding NSF. H_2O_2 is thought to inactivate NSF through

oxidation of the Cys264 in NSF-D1. Consistently, mutation of Cys264 to threonine eliminates the sensitivity of NSF to H_2O_2 [93]. While this might suggest that NSF could be a redox sensor in the cell, whose activity is decreased when the oxidation state of the cytosol increases, it seems that, as with nitrosylation, NSF may not be the only relevant target of this level of regulation.

8. Summary

Since its discovery, NSF has been linked to the secretory pathway. Subsequent work has established a role for NSF in the assembly/disassembly cycle of the SNARE proteins, a function essential for cell survival. More recent work suggests that NSF may serve as a structural chaperone for other, non-SNARE complexes. As work progresses on these interactions, one should be guarded to discriminate between truly new functions for NSF and logical sequellae of defective membrane trafficking.

The appreciation of NSF regulation has also been a recent development and reflects our increased understanding of the complexity of membrane trafficking mechanisms. Phosphorylation, nitrosylation, and oxidation may all play roles in controlling NSF activity and thus membrane trafficking. These modifications are generally transient and offer an ideal mechanism for acute but reversible regulation of membrane trafficking. However, care should be taken to establish the uniqueness of the effect on NSF. Since multiple proteins can be modified by nitrosylation or oxidation, the challenge is to establish that their inactivating effects on NSF are truly the cause of the defect in membrane trafficking observed.

In the years since NSF's discovery, much has been learned, however several questions still remain and new ones have surfaced. It is still unclear, at a molecular level, how NSF uses ATP hydrolysis to disassemble the SNARE complexes. It is now clear that NSF is regulated in novel ways, but what are the physiological ramifications of these events? This is a particularly interesting question given the reduction in NSF levels seen associated with epilepsy [94–96]. Finally, what role(s) does NSF play in the non-SNARE complexes? Is it a chaperone affecting their disassembly or is it along for the ride to where it is needed for membrane trafficking? Future studies will address these and many more questions.

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