Selective stimulation of the D1 ATPase domain of *N*-ethylmaleimide-sensitive fusion protein (NSF) by soluble NSF attachment proteins

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Abstract ***N*-Ethylmaleimide-sensitive fusion protein (NSF) is required for most intracellular membrane fusion events. NSF is recruited to membranes by soluble NSF attachment proteins (SNAPs) and membrane-resident SNAP receptor (SNARE) proteins. The 20 *S* complex of NSF/SNAPs/SNAREs disassembles when NSF hydrolyses ATP, and this disassembly event is believed to be essential for membrane fusion. SNAPs stimulate NSF ATPase activity, but it is not known which of NSF's two ATPase domains (D1 or D2) is affected. Using recombinant mutant NSFs defective in ATP hydrolysis in one domain only, we found that SNAPs stimulate NSF ATPase activity by a selective action on the D1 domain, yet had no effect on the D2 domain. Since the D1 domain of NSF is implicated in 20 *S* complex disassembly, this supports the idea that SNAP stimulation of NSF ATPase activity is required for membrane fusion.**

Key words: *N*-Ethylmaleimide-sensitive fusion protein; NSF attachment protein; SNAP receptor; AAA ATPase; Membrane fusion; Vesicular traffic

1. Introduction

Considerable advances have been made in the study of the proteins required for vesicular trafficking within cells [1]. Several soluble proteins have been identified by their ability to reconstitute mammalian intra-Golgi transport, including *N*-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs). Subsequently it has been shown that NSF is required for many transport steps in the mammalian secretory and endocytic pathways [1]. NSF and alpha-SNAP are functionally equivalent to the yeast SEC18 and SEC17 gene products respectively, which are required in vivo for yeast secretion [2]. The widespread involvement of NSF and SNAPs in membrane trafficking suggests that they are key general components in the steps leading to membrane fusion.

When incubated with detergent-extracted brain membranes, in the absence of hydrolysable ATP, alpha-SNAP and NSF are associated in a 20 *S* complex with three membrane proteins: syntaxin, SNAP-25 (synaptosomal associated protein of 25 kDa) and VAMP (vesicle-associated membrane protein), collectively termed SNAP receptors (SNAREs) [3]. Homologues of the synaptic SNAREs are involved in many intracellular membrane trafficking steps [4], suggesting that the general requirement for NSF/SNAPs in membrane fusion is due to promiscuous interactions of these key proteins with the various SNAREs. Assembly of the SNARE complex was postulated to allow the correct docking of vesicles to their target membrane [3]. Hydrolysis of ATP, by NSF, causes the 20 *S* complex to disassemble, which was proposed to trigger fusion of vesicle and target membranes by an as yet unknown mechanism [5]. More recently it has been suggested that NSF and SNAPs may act in a priming role before vesicle docking [6–8].

When alpha-SNAP is immobilised on plastic surfaces, it is able to bind NSF and stimulate its ATPase activity [9], yet alpha-SNAP is unable to bind or stimulate NSF in solution. This suggests that stimulation of NSF ATPase activity is likely to occur when alpha-SNAP is correctly bound to the SNARE complex, thus acting as a molecular switch to activate NSF. Direct evidence to support this theory comes from the recent observation that alpha-SNAP mutants able to bind NSF, but unable to stimulate its ATPase activity, are unable to support either 20 *S* complex disassembly or exocytotic membrane fusion [10]. Despite the apparent requirement for SNAP stimulation of NSF ATPase activity in membrane fusion, it is not known how this occurs. NSF has two distinct ATPase domains, D1 and D2 [11,12], which are conserved in the AAA family of ATPases [13]. Hence, SNAP stimulation could potentially occur as a result of stimulation of one or both ATPase domains. Here we report that alpha-SNAP selectively stimulates the D1 ATPase domain of NSF.

2. Materials and methods

Plasmids encoding His_6-tagged NSF and alpha-SNAP were generously donated by Dr. J.E. Rothman (Memorial Sloan Kettering Cancer Centre). Nickel-nitrilotriacetic acid (NTA)-agarose was obtained from Qiagen (Dorking, UK), all other reagents were of analytical grade and obtained from Sigma (Poole, UK).

2.1. Purification of NSF and its mutants

Point mutations in the NSF sequence to produce the ATP-hydrolysis mutants described by Whiteheart et al. [12] were created using 'Quickchange' site-directed mutagenesis kit (Stratagene). Mutagenic primers used were as follows: E329Q: sense 5′-CATCATCTTTGATCAAATCGATGCCATC-3′, antisense 5′-GATGGCATCGATTTGATCAAAGATGATG-3′; D604Q: sense 5′-GTGTGGTGGTTGATCAGATCGAAAGGC-3′, antisense 5′-GCCTTTCGATCTGATCAACCACCACAC-3′.

Recombinant His_6-tagged proteins were purified from the cytosolic fraction of XL-1 Blue *Escherichia coli* (Stratagene), using Ni-NTA chromatography, as described previously [12]. Additional purification was achieved by gel filtration chromatography on a Superdex 200 column (Pharmacia, 16/60; 120 ml bed volume), in buffer A (20 mM HEPES, pH 7.0; 200 mM KCl; 2 mM 2-mercaptoethanol; 0.5 mM ATP; 10% (v/v) glycerol; 50 mM Imidazole). Point mutations were confirmed by sequence analysis (Oswel, UK).

2.2. ATPase assay

ATPase assays were carried out as previously described [9], with the following modifications. Assays were carried out in flat-bottomed microtitre plates. Wells were preincubated with either 50 μl of alpha-

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SNAP (250 μg/ml in buffer A) or buffer A alone for 30 min at room temperature. Preincubation solutions were removed and replaced with 0.4 μg of wild-type or mutant NSF in a final volume of 40 μl in ATPase assay buffer (25 mM Tris, pH 9.0; 100 mM KCl; 10% (v/v) glycerol; 2 mM $MgCl_2$; 1 mM DTT; 0.6 mM ATP or as indicated in the figure legends), for 2 h at 37°C. Hydrolysed phosphate was determined by a spectrophotometric assay [14]. Values were corrected for non-enzymic breakdown of ATP by running duplicate assays in the absence of NSF protein and subtracting these values (and also the values for pre-existing phosphate in the added proteins) from the raw data.

3. Results and discussion

In order to address the question which of NSF's two ATPase domains is stimulated by SNAPs, site-directed mutagenesis was used to construct mutant NSF proteins defective in ATP hydrolysis in either the D1 domain only (E329Q) or the D2 domain only (D604Q). The recombinant His-tagged proteins thus produced behaved similarly to wild-type, His-tagged NSF in gel filtration chromatography (Fig. 1), as previously reported [12]. From comparison with molecular mass standards, the major peaks of all three proteins corresponded to a molecular mass of around 550 kDa. This value is consistent with the most recent structural analysis of NSF [15], and so it seems likely that the quaternary structure of native NSF is hexameric rather than trimeric. Although all three NSFs display similar major peak elutions, there are differences between them in terms of secondary peaks and expression levels. The E329Q mutant was expressed at very low levels relative to wild-type, whereas the D604Q mutant was surprisingly found to be purified in larger quantities than wild-type (Fig. 1). In addition, whereas NSF and E329Q displayed a single peak of putative hexamer with no detected higher molecular mass material, D604Q exhibited a distinct peak close to the void volume of the Superdex 200 column used (Fig. 1). It is not clear whether this material represents higher order oligomeric NSF or simply NSF aggregates. Nevertheless, the observation

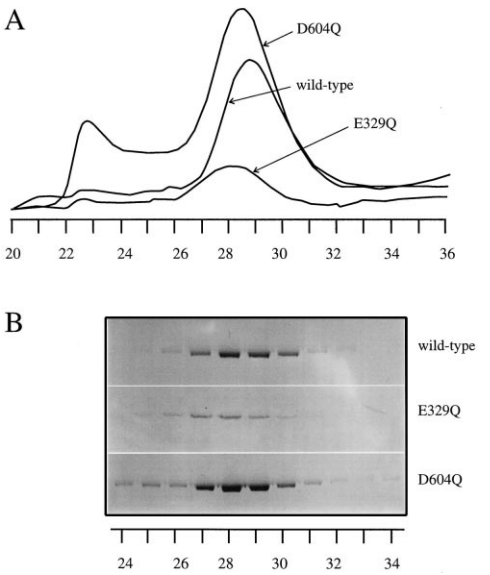

Fig. 1. Superdex 200 purification of wild-type NSF, E329Q, and D604Q. A: Absorbance traces (A_{280}, using the same scale) obtained during the purification of wild-type NSF, E329Q, and D604Q; 2-ml fractions were collected during each run. B: Samples of each of the fractions were separated by SDS-PAGE and stained with Coomassie blue. Material in fractions 27–30 was pooled and concentrated.

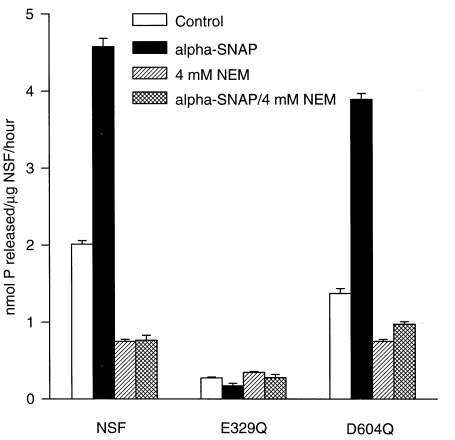

Fig. 2. ATPase activity of wild-type NSF, E329Q and D604Q. Standard ATPase reactions were carried out using 0.4 μg of NSFs per well in ATPase assay buffer. Reactions were carried out using NSFs following pre-incubation in the presence (closed bars) or absence (open bars) of alpha-SNAP; and also using NSFs which had been pre-treated with 4 mM NEM (15 min on ice) in the presence (crosshatched bars) or absence (hatched bars) of alpha-SNAP. The data shown are means ± S.E.M. ($n = 8$) from a representative experiment.

of quaternary structural aberrations in the D604Q mutant supports the idea that the D2 domain of NSF is important for oligomerisation [12]. Only the putative hexameric pools (fractions 27–30) of gel-filtered NSFs were used for subsequent experiments.

Wild-type recombinant NSF has very low, but measurable, intrinsic ATPase activity [9,12,16]. Over a series of experiments, the intrinsic ATPase activities of E329Q and D604Q were found to be 38 ± 9% ($n = 7$) and 67 ± 6% ($n = 8$) of wild-type levels, respectively. These values are consistent with published values for the intrinsic ATPase activities of E329Q (31%) and D604Q (72%) [12]. It should be noted, however, that the activity of E329Q is close to the limit of detectability in our ATPase assay. Hence, it is possible that the D2 domain has negligible ATPase activity. Indeed, a recent study of the ATP/ADP affinities of NSF reached the same conclusion and speculated that nucleotide binding by the D2 domain, rather than hydrolysis, is functionally important [17]. Nevertheless, regardless of whether D2 has low or no ATPase activity, it is clear that the lion's share of NSF's intrinsic ATPase activity resides in the D1 domain, as previously reported [12].

It has previously been shown that plastic-immobilised SNAPs are able to stimulate the intrinsic ATPase activity of NSF [9,18,19]. It can be seen that alpha-SNAP stimulated NSF ATPase activity several-fold and that this SNAP stimulation was completely blocked by *N*-ethylmaleimide (NEM), even though the intrinsic ATPase activity of NSF was only partially blocked by NEM (Fig. 2), as we previously reported [9]. Similar results were obtained for D604Q, whereas E329Q exhibited no SNAP stimulation and was not affected by NEM (Fig. 2). Over a series of 45 experiments using six different batches of wild-type NSF, its intrinsic ATPase activity (100%) was stimulated to 285 ± 19% by alpha-SNAP. Using two different batches of both mutants, the intrinsic ATPase activity of D604Q (100%) was stimulated by alpha-SNAP to 196 ± 16% (eight experiments), whereas that of E329Q (100%) was 86 ± 15% (seven experiments). It should be noted

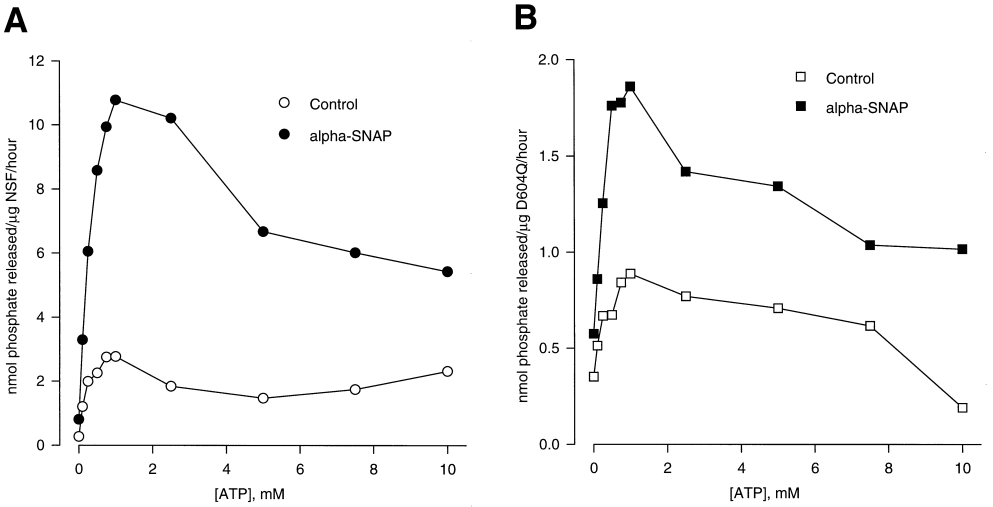

Fig. 3. Effect of increasing ATP concentration on ATPase activity. A: Standard ATPase reactions were carried out with (closed circles), or without (open circles), pre-incubation with alpha-SNAP using 0.4 μg of NSF per well in ATPase assay buffer supplemented with varying concentrations of ATP. The data shown are means of three separate experiments (total $n = 24$ for each data point). B: Identical reactions with (closed squares), or without (open squares), pre-incubation of alpha-SNAP using 0.4 μg of D604Q per well. The data shown are means of three separate experiments (total $n = 24$ for each data point).

that the standard ATPase assay buffer contains 0.6 mM ATP [16], which appears to be a submaximal concentration in our assay, as 1 mM can provide up to five-fold SNAP-mediated stimulation (see Fig. 3). In summary, the observation that the ATPase activity of D604Q, but not E329Q, is stimulated by alpha-SNAP provides compelling evidence that SNAPs stimulate wild-type NSF ATPase activity by activating the D1 domain only.

In our previous work using only wild-type NSF, we analysed ATPase activity over a wide range of ATP concentrations [9]. In Fig. 3, it can be seen that both the intrinsic and SNAP-stimulated ATPase activity of wild-type NSF and D604Q increased with increasing substrate concentration up to around 1 mM, but then decreased as substrate concentration increased beyond this point. The likely reason for this decrease in ATPase activity at high ATP concentrations is product inhibition by ADP, since when assays were performed on wild-type and D604Q using a constant level of ATP (0.6 mM), increasing concentrations of ADP produced a progressive decrease in ATPase activity (Fig. 4). In our previous study [9], we reported that NSF displayed complex enzyme kinetics and concluded that this was due to the action of two distinct catalytic domains, of high (K_m1) and low (K_m2) ATP affinity. However, subsequent analysis has revealed that the low affinity ATPase site we reported was due to non-enzymic breakdown of ATP, which we have controlled for in this study. Therefore, the true situation is likely to be that the intrinsic ATPase activity of NSF displays classical Michaelis-Menten kinetics – as originally concluded by Tagaya et al. [16]. Certainly, this interpretation is consistent with the apparent inactivity of the D2 domain and its lack of activation by SNAPs (Fig. 2). If this is accepted, it follows that

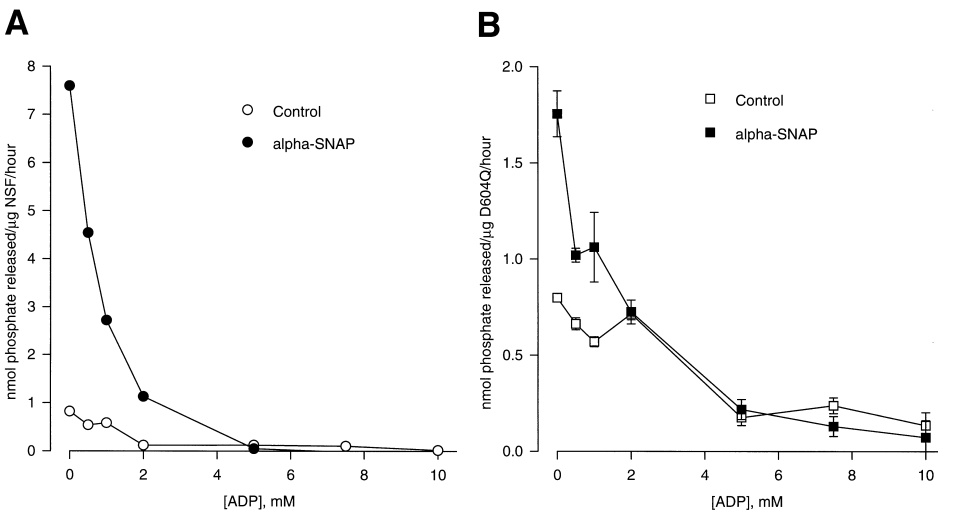

Fig. 4. Effect of increasing ADP concentration on ATPase activity. A: Standard ATPase reactions were carried out with (closed circles), or without (open circles), pre-incubation with alpha-SNAP using 0.4 μg of NSF per well in ATPase assay buffer (0.6 mM ATP) supplemented with varying concentrations of ADP. The data shown are means of two separate experiments (total $n = 16$ for each data point). B: Identical reactions with (closed squares), or without (open squares), pre-incubation of alpha-SNAP using 0.4 μg of D604Q per well. The data shown are means ± S.E.M. ($n = 8$) from a representative experiment.

SNAPs stimulate NSF ATPase activity by increasing the V_{max} of the D1 domain ([9]; this study).

It has been shown previously that ATP hydrolysis by NSF is required to disassemble the 20 *S* complex of NSF, alpha-SNAP and the SNAREs syntaxin, SNAP-25, and VAMP [3]. In addition, by immunoprecipitating myc-tagged NSFs, Nagiec et al. [20] found that D604Q was as effective as wild-type NSF in removal of alpha-SNAP and syntaxin from the immunoprecipitate, whereas E329Q was completely inactive; despite both mutants being equally able to bind these proteins. Using His-tagged NSFs in immunoprecipitation of native syntaxin, we similarly found that wild-type NSF and D604Q exhibited ATP-hydrolysis-dependent removal of alpha-SNAP and NSF from the immunoprecipitate, whereas E329Q was ineffective; although in our hands neither mutant was as efficient as wild-type NSF in binding to the immunoprecipitate (data not shown). Thus, ATP hydrolysis by the D1 domain of NSF is necessary for 20 *S* complex disassembly [20], although its intrinsic activity is not sufficient since wild-type NSF cannot disassemble the complex in the presence of a mutant alpha-SNAP which is unable to stimulate its ATPase activity [10]. Therefore, SNAP-mediated stimulation of the D1 ATPase domain of NSF appears to be essential for disassembly of the 20 *S* complex. In addition, it has been shown that phospholipids can enhance SNAP-mediated stimulation of NSF ATPase activity [18], and due to the position of alpha-SNAP within the 20 *S* complex (binding to the C-terminal domain of syntaxin, adjacent to the membrane [21]), alpha-SNAP would be ideally situated, in the cellular context, to optimally stimulate the ATPase activity of the D1 domain of NSF.

It is now becoming accepted that NSF-mediated disassembly of the 20 *S* complex is the biochemical correlate of vesicle 'priming' for subsequent membrane fusion [8]. Since such disassembly is critically dependent on the D1 domain of NSF [20, this study] this suggests that the ATPase activity of this domain – and more importantly its stimulation by SNAPs – is crucial for most intracellular membrane fusion events. This idea is supported by the ability of nucleotide binding or hydrolysis mutants in the D1 domain of NSF, but not in the D2 domain, to potently inhibit intra-Golgi transport [12,22] or endocytic vesicle fusion [23]; and the inability of SNAP mutants which are unable to stimulate NSF ATPase activity to support regulated exocytosis [10]. The observation here that SNAPs selectively stimulate the D1 ATPase domain of NSF underlines the importance of the SNAP stimulation of NSF to the membrane fusion process and adds to our detailed understanding of SNARE complex disassembly. Although the precise function of this disassembly as it relates to 'priming' of membrane fusion in cells is still the subject of much conjecture, we believe that further study of sub-reactions in the 20 *S* complex assembly/disassembly process using defined mutations within NSF, SNAPs and the SNAREs will resolve the current controversies and ultimately lead to an understanding of vesicle docking and fusion at the molecular level.

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