Evidence for interaction of the fusion protein α -SNAP with membrane lipid

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α -SNAP [soluble *N*-ethylmaleimide-sensitive fusion protein (NSF)-attachment protein] is required for fusion of transport vesicles with their target membrane. In this study, we have examined the membrane-binding properties of α -SNAP. We have found that in several tissues a much larger amount of α -SNAP per unit weight of protein is bound to membranes than is free in the cytosol. Biochemical analysis shows that a fraction of α -SNAP behaves in ways characteristic of hydrophobic, lipid-

associated proteins. These findings suggest that membrane binding may be accounted for, at least in part, by interaction with membrane lipid. Consistent with this idea, binding of newly synthesized α -SNAP to brain membranes was found to be independent of functional SNAP receptors and could be accounted for by direct binding of α -SNAP to membrane lipid. Furthermore, membrane lipid enhanced the ability of α -SNAP to stimulate NSF-dependent ATPase activity.

INTRODUCTION

Transport of proteins in eukaryotic cells is mediated by vesicles which bud from one compartment and fuse specifically with another. Although the mechanism of vesicle fusion has yet to be defined, several of the proteins required have been identified and some partial reactions have been reconstituted using purified components [1,2]. It is clear from these studies that the fusion machinery is conserved to a remarkable degree. Many transport reactions within the cell use common or closely related components, and these components are found in a wide variety of eukaryotic cells [3].

Most vesicle fusion events described so far require *N*-ethylmaleimide-sensitive fusion protein (NSF), and soluble NSF attachment proteins (α -, β - and γ -SNAPs) [4–7]. NSF–SNAP interaction will not occur in solution, unless proteinaceous receptors for α -SNAP (SNAREs) are provided in the form of detergent-solubilized membrane extracts [8]. Under these conditions a highly specific association occurs which is saturable with regard to α -SNAP and is controlled by ATP. SNAPs are also able to bind to plastics, and when bound are able to bind NSF [9] and significantly stimulate its ATPase activity [10].

The SNAREs present in neuronal tissue are abundant proteins confined to the synaptic vesicle (synaptobrevin; [11,12]) or the presynaptic membrane (syntaxin and SNAP-25 (synaptosome-associated protein of molecular mass 25 kDa) [13,14]), and are consequently termed v (vesicle)- or t (target)-SNAREs. Their functional significance is underlined by the finding that they are specific targets of the *Clostridial* neurotoxins [15–17]. Homologues of v- and t-SNAREs have been identified in non-neuronal cells [18,19], and several examples of each SNARE family have been found in yeast, where they are active in fusion events on a number of transport pathways [3] and form interacting complexes [20].

According to the original ‘SNARE hypothesis’ [2], membrane docking is mediated via the interaction of v-SNAREs with cognate t-SNAREs. These docking complexes, which can be

isolated from detergent-solubilized membrane extracts, recruit NSF and α -SNAP to form a ‘fusion complex’ [21,22]. Rearrangement of the SNAREs upon ATP hydrolysis then leads to membrane fusion and subsequent release from the membranes of the soluble components of the reaction. In support of this model, disassembly of the SNARE complex by α -SNAP and NSF has been reported [22,23], and shown to be accompanied by transient alteration of the conformation of syntaxin such that it can no longer bind synaptobrevin [24].

There is, in fact, considerable uncertainty about the temporal relationship between SNARE complex disassembly and membrane fusion. It has been pointed out that membrane fusion occurs too rapidly for these events to be coupled directly [25]. Moreover, recent evidence regarding fusion between yeast vacuoles is consistent with the release of α -SNAP from membranes before docking [26]. Together, these findings imply a pre-fusion role for α -SNAP. In order to extend our understanding of the role of α -SNAP in membrane fusion, we have examined the nature of the association of α -SNAP with biological membranes and with liposomes. We find that α -SNAP possesses some characteristics expected of integral membrane proteins. Additionally, and in contrast to its binding to Golgi membranes [27], binding of α -SNAP to brain membranes does not require functional SNAREs and can be accounted for by binding to membrane lipid. The potential significance of these findings for SNAP function during membrane fusion is discussed.

EXPERIMENTAL

Preparation of membranes

Porcine brains were homogenized in a Waring blender with two volumes of homogenization buffer (140 mM sucrose/20 mM Hepes/70 mM potassium acetate, pH 7.2) containing 1 mM dithiothreitol and 40 μ g/ml PMSF. Homogenates were centrifuged for 30 min at 5000 g_{av} in a Beckman JA10 rotor. The

Abbreviations used: CAT, chloramphenicol acetyltransferase; ECL, enhanced chemiluminescence; HBS, Hepes-buffered saline; HRP, horseradish peroxidase; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF-attachment protein; SNAP-25, synaptosome-associated protein of molecular mass 25 kDa; SNARE, SNAP receptor; TID, 3-(trifluoromethyl)-3-(*m*-iodophenyl)-3*H*-diazirine.

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supernatant was centrifuged for 3 h at 30000 g_{av} . The pellets were resuspended in homogenization buffer and crude membranes were separated on discontinuous sucrose gradients of 40% (w/v) sucrose (5 ml) and 20% (w/v) sucrose (15 ml) in 10 mM Tris/HCl, pH 7.5. The gradients were centrifuged for 3 h at 100000 g_{av} in a Beckman SW28 rotor, and membranes were isolated from the interface. For the preparation of carbonate-extracted membranes, crude membranes were diluted with 4 vol. of 125 mM Na_2CO_3 , pH 11.5, and incubated for 30 min at 4 °C. The membranes were recovered by centrifugation and resuspended in 20 mM Hepes/NaOH, pH 7.4/100 mM NaCl/2 mM EDTA. For determination of α -SNAP content of different rat tissues, membranes were prepared from heart, adrenal gland, liver, kidney, spleen or skeletal muscle which was homogenized in Hepes-buffered saline (HBS) (20 mM Hepes, pH 7.6/150 mM NaCl, containing 1 mM EDTA, 1 mM PMSF and 1 μ g/ml pepstatin) at 4 °C and centrifuged at 500 g for 5 min to obtain the post-nuclear supernatants. These were then centrifuged at 100000 g for 20 min to yield the membrane and cytosol fractions. The membrane fractions were resuspended in HBS to a protein concentration of 1–2 mg/ml.

Preparation of recombinant proteins

NSF-myc was prepared as described [28] to approximately 70% purity. His $_6$ - α -SNAP was prepared as described [29] to a purity of > 95%. Radiolabelled α -SNAP was synthesized from a plasmid containing the α -SNAP cDNA [27] in a combined transcription/translation kit (Promega). The translated protein was enriched within a 30–50% ammonium sulphate fraction, so that 80–90% of the total radiolabel was protein-associated. Translations typically contained 1 μ g DNA and 60 μ Ci [35 S]methionine. Incorporation of radiolabel specifically into α -SNAP was confirmed by SDS/PAGE and autoradiography. Similar methods were used to prepare other proteins translated *in vitro*.

Binding of α -SNAP

To measure binding of α -SNAP, carbonate-extracted membranes (50 μ g protein) or membrane-derived liposomes were incubated on ice for 5 min with [35 S] α -SNAP translated *in vitro* (2.5 ng) in NB buffer [20 mM Hepes, pH 7.4/2 mM EDTA/100 mM KCl/1 mM dithiothreitol/1% (w/v) PEG 4000 containing 250 μ g/ml of soybean trypsin inhibitor] [27]. After binding was complete, sucrose from an 80% (w/v) stock was added to give a final concentration of 60% (w/v) sucrose. This was overlaid with 100 μ l 50% (w/v) sucrose in NB buffer, followed by 60 μ l NB buffer alone. After centrifugation in a TLS55 rotor (containing inserts for TLA 100 tubes) for 30 min at 140000 g , fractions (10 μ l), taken from the top of the tube, were counted for radioactivity. All tubes were treated with Sigmacote (Sigma) before use.

'Fusion complex' formation

To measure 'fusion complex' formation, carbonate-extracted membranes (50 μ g protein) were incubated on ice with α -SNAP, translated *in vitro* (2.5 ng), and NSF-myc (2 μ g) for 5 min, before the addition of Triton X-100 to a final concentration of 1% (w/v). The incubation was supplemented, after a further 15 min, with 100 μ l of a 10% (v/v) slurry of protein G-agarose beads (Calbiochem) to which had been bound 9E10 anti-myc antibody (10 mg antibody to 1 ml beads, packed volume). After overnight incubation on a rotator at 4 °C, the agarose beads were recovered by centrifugation at 8000 g for 30 s, and washed 3 times with

wash buffer [50 mM Tris/HCl, pH 7.5/250 mM NaCl/5 mM EDTA/1% (w/v) TX-100]. The final pellets were resuspended in scintillation fluid and radioactivity was estimated.

Liposome preparation

Carbonate-extracted pig brain membranes (100 mg protein) were homogenized at room temperature in 25 ml chloroform/methanol/butylated hydroxytoluene (2:1:0.0005, by vol.) using a Potter-Elvehjem homogenizer. The mixture was centrifuged (5000 g_{av} for 10 min), the solvent phase (approx. 20 ml) was collected and 4 ml of 0.9% (w/v) NaCl was added. Phase separation to remove residual proteins and non-lipid contaminants was followed by three further extractions with 3 ml of 0.9% (w/v) NaCl/chloroform/methanol (47.5:3:47, by vol.), and 30 mg of the final lipid mixture was used to prepare liposomes. All manipulations were performed under N_2 . Lipids were dissolved in chloroform/methanol (4:1), dried as a lipid film on to the walls of a glass tube, resuspended in NB buffer and sonicated for 30 min in a sonication bath (Deco FS100; Decon Ultrasonics, Hove, Sussex, U.K.). Unilamellar liposomes were then sized (Malvern Autosizer; Malvern Instruments Ltd., South Malvern, Worcs., U.K.). Liposomes were also produced from phosphatidylcholine (30 mg, from egg).

Phospholipid assay

Phospholipids were assayed as described previously [30]. Lipids were extracted from membrane samples after precipitation of proteins with chloroform/methanol/butylated hydroxytoluene (2:1:0.0005, by vol.). The organic phase, containing the lipids, was added to 200 μ l of 0.9% (w/v) NaCl for 5 min and re-extracted. Further extraction was performed using 150 μ l of 0.9% (w/v) NaCl/chloroform/methanol (47.5:3:47, by vol.) and the organic phase was retained for phospholipid analysis. To this phase (approx. 0.5 ml) was added 30 μ l of 10% (w/v) $Mg(NO_3)_2$ in 95% (w/v) ethanol. After mixing, the solvents were evaporated and the precipitate was heated strongly to ash. After cooling, 300 μ l of 0.5 M HCl was added for 15 min at 95 °C followed by 0.7 ml of a solution of 1.42% (w/v) ascorbic acid/0.36% (w/v) ammonium molybdate for 1 h at 37 °C. The absorbance at 820 nm was then measured. Phosphatidylcholine liposomes (1–100 μ g) were used as a standard.

ATPase assay

Assay of ATPase was based on the spectrophotometric determination of phosphate released from ATP [31]. Samples (generally 5 μ g NSF and 5 μ g α -SNAP) were incubated in NSF ATPase buffer [100 mM KCl/25 mM Tris/HCl, pH 9.0, containing 0.5 mM dithiothreitol, 2 mM $MgCl_2$, 0.6 mM ATP and 10% (w/v) glycerol] in a final volume of 200 μ l. After various times at 37 °C, samples (20 μ l) were removed and 250 μ l of the colour reagent (0.034% Malachite Green in 1 M HCl, containing 1.25% ammonium molybdate) was added, followed by 35 μ l of 34% (w/v) sodium citrate. Phosphate content was determined by measuring the absorbance at 630 nm, after allowing colour development at room temperature for $\geq$ 30 min. Under the conditions used the release of phosphate was linear for at least 30 min. For determination of rates of phosphate release, samples were taken over a period of 20 min. To confirm that phosphate release was caused by ATP hydrolysis, some experiments were supplemented with [2- 3 H]ATP (0.1 μ Ci; 15 Ci/mmol; Amersham International). Samples were analysed by TLC on PEI cellulose, using unlabelled ATP and ADP as standards, followed by

detection of radioactivity for each species. Ratios of ADP:ATP were calculated for each sample.

Cross-linking of proteins to 3-(trifluoromethyl)-3-(m -[125 I]iodo-phenyl)-3*H*-diazirine ([125 I]TID)

This was performed according to published procedures [32]. Protein (30 μ g) was diluted with 150 μ l HBS, to which was added 10 μ Ci [125 I]TID (in 4 μ l 80 % EtOH; Amersham International). After 5 min at room temperature, the mixture was exposed to UV irradiation for 3 min with frequent agitation. Protein was precipitated with chloroform/methanol/water (1:3:4, by vol.) [33], recovered by centrifugation at 8000 g for 5 min and the pellet was washed several times with methanol to remove unincorporated radiolabel before analysis by SDS/PAGE and autoradiography.

Immunoblotting

Proteins were separated by SDS/PAGE and transferred to nitrocellulose membranes. Blots were incubated with monoclonal antibodies (ascites fluid) diluted 1:1000 in Blotto (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 % (w/v) powdered non-fat milk and 0.05 % (v/v) Tween 20) overnight at 4 °C. The antibodies used were anti- α -SNAP (CI 77.2; [24]), anti-syntaxin (Sigma Immunochemicals), anti-SNAP-25 (MCA 1308; Serotec), anti-rab 3 (CI 42.1; [34]) and anti-synaptobrevin (MCA 1309; Serotech, or CI 69.1; [35]). Incubation with secondary antibody, 35 S-labelled rabbit anti-mouse IgG (final concentration 5 μ Ci/ml, 1000 Ci/mmol; Amersham) or horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody (diluted 1:1000), was for 1 h at room temperature. Blots were visualized and quantified, where necessary, by phosphorimage analysis, or were visualized by enhanced chemiluminescence (ECL). Extraction with Triton X 114 (Pierce) was performed as described previously [36]. Protein was assayed according to the method of Bradford [37].

RESULTS

A significant proportion of cellular α -SNAP is membrane-associated

To gain an idea of the steady-state distribution of the protein between membrane-bound and soluble pools, crude membrane and cytosol fractions of several tissues from rat were immunoblotted for α -SNAP, and for two proteins that are known to be

Figure 1 α -SNAP is membrane-associated in various tissues

Crude membrane (m) and cytosolic (c) fractions (10 μ g protein) from rat heart (H), adrenal gland (A), liver (L), kidney (K), spleen (S) and skeletal muscle (SM) were immunoblotted for α -SNAP, rab 3 and synaptobrevin II (syb. II). Bands were visualized using an HRP-labelled secondary antibody and ECL.

Figure 2 α -SNAP has hydrophobic characteristics

Upper panel: Extraction of α -SNAP in Triton X-114. Membranes (100 μ g protein) and recombinant His $_6$ - α -SNAP (10 μ g) were extracted with 1 % Triton X-114. Aqueous (A) and detergent (D) phases were analysed for α -SNAP content by either Western blot, using a 35 S-labelled secondary antibody followed by phosphorimaging (for membranes), or by Coomassie Blue staining followed by scanning densitometry (for recombinant protein). As a control for detergent-dependent aggregation, His $_6$ - α -SNAP was also extracted with 1 % Triton X-100. The lower panels show labelling of α -SNAP by [125 I]TID. Recombinant His $_6$ - α -SNAP (30 μ g) was incubated with [125 I]TID. Cross-linking to protein was assessed by SDS/PAGE followed by Coomassie Blue staining (middle panel) and autoradiography (lower panel). As positive and negative controls respectively, equal amounts of BSA and transferrin (Tf) were also incubated with [125 I]TID. Molecular-mass markers (kDa) are shown on the left.

membrane-associated, the isoprenylated rab 3 [38] and the integral membrane protein synaptobrevin-II [11,12]. As expected from previous reports [29], α -SNAP was detected in all tissue samples (Figure 1). In contrast, rab 3 and synaptobrevin-II were expressed at significant levels only in a restricted group of tissues. Where detected, the amounts of rab 3 and synaptobrevin-II per unit weight of protein were much larger in the membrane fraction than in the cytosol. Strikingly, this was also the case for α -SNAP, indicating that, at any given time, a significant proportion of this protein is membrane-associated.

α -SNAP has hydrophobic characteristics

To assess whether α -SNAP contains hydrophobic regions capable of interacting with membrane lipids two approaches were taken, phase separation in Triton X-114 [32] and labelling with the UV-activated hydrophobic cross-linking reagent [125 I]TID [36]. When brain-derived membranes were extracted in Triton X-114 and the phases separated, α -SNAP partitioned into both the aqueous (68 %) and the detergent (32 %) phases (Figure 2, upper panel),

Figure 3 α-SNAP binds to membranes

Upper panel: brain membranes (100 μg protein) were incubated with either 100 mM NaCl/20 mM Hepes, pH 7.4, or Na₂CO₃ and then pelleted as described in the Experimental section. The supernatants (S1) were removed and the membrane pellet was resuspended in fresh buffer or Na₂CO₃. After further centrifugation, the final pellet (P) and supernatant (S2), together with S1, were analysed for α-SNAP by immunoblotting and phosphorimaging. For comparison, a sample from the original membrane preparation was also analysed (T). Lower panel: pig brain membranes (50 μg phospholipid) were incubated with [³⁵S]α-SNAP and re-isolated on a discontinuous sucrose gradient. Fractions were analysed for protein (○) and radiolabel (●). A sample lacking membranes was also analysed for radioactivity (■). These results are from a representative experiment (of seven experiments).

indicating a significant degree of hydrophobicity. In contrast, transferrin (a hydrophilic protein) partitioned exclusively into the aqueous phase, when added to the membrane preparation before extraction (results not shown). It might be argued that partitioning of α-SNAP into the detergent phase is a result of tight interaction between α-SNAP and integral membrane proteins, rather than a characteristic of α-SNAP itself. However, purified recombinant α-SNAP also partitioned to a significant extent into the detergent phase (Figure 2, upper panel). This is unlikely to have been caused by detergent-dependent aggregation of the protein, which might have led to subsequent pelleting, since all of the protein remained soluble in Triton X-100. [¹²⁵I]TID will cross-link soluble proteins that contain exposed hydrophobic domains, with the fatty acid binding protein BSA being a particularly good substrate [32]. In the experiment illustrated in Figure 2 (lower panels), both BSA and recombinant His₆-α-SNAP were labelled efficiently with [¹²⁵I]TID, again indicating the presence of hydrophobic regions in α-SNAP. In contrast, virtually no reagent was incorporated into transferrin.

Binding of α-SNAP to brain membranes does not require proteins

The predominant membrane localization of α-SNAP and its hydrophobic nature suggested that membrane-binding might be accounted for, at least in part, by protein-lipid interactions.

Table 1 Formation of 20 S 'fusion complexes'

Detergent-extracted membranes (50 μg phospholipid) were incubated with [³⁵S]-α-SNAP and NSF-myc, and analysed for 'fusion complex' formation as described in the Experimental section. The results of control samples lacking membrane or NSF-myc, or immunoprecipitates which had been washed in buffer containing Mg-ATP are also shown. The values are the means ± S.E.M. (n = 3).

Incubation conditions	Immunoprecipitated [³⁵ S]α-SNAP (d.p.m.)
Complete	7732 ± 245
No membranes	839 ± 280
No NSF-myc	543 ± 83
Mg-ATP wash	816 ± 54

Brain membranes were used to examine this possibility, since they are a rich source of SNAREs [11–14,21] and can be prepared in large quantity. Membranes were first washed in Na₂CO₃ to remove peripherally-bound proteins, including α-SNAP. As shown in Figure 3 (upper panel), two successive washes removed much α-SNAP, though a significant fraction (approximately 30%) remained membrane-bound. After incubation with radiolabelled α-SNAP, translated *in vitro*, carbonate-washed membranes were re-isolated using a bottom-loaded discontinuous sucrose density gradients. Fractions were then analysed for α-SNAP content.

After flotation on sucrose gradients, most of the membrane migrated to the 50%/0% sucrose interface (Figure 3, lower panel). Though some [³⁵S]α-SNAP remained in the loading zone a significant proportion (an average of 31.3% ± 0.8%, n = 7) migrated with the membranes. Protein precipitation and autoradiography indicated that all of the radioactivity migrating with the membranes was in α-SNAP (results not shown). Since we employed flotation gradients to remove the possibility of artefacts caused by membrane-dependent aggregation of α-SNAP, it appeared that migration of α-SNAP to the interface was dependent on its binding to membranes. Furthermore, virtually no [³⁵S]α-SNAP migrated to the interface when membranes were omitted from the incubation (Figure 3, lower panel; 0.8% ± 0.1% of total radioactivity, n = 3).

The same membranes were also analysed for their ability to form 20 S 'fusion complexes' with NSF and α-SNAP in detergent extracts, since this is a convenient assay for SNARE activity [28]. When detergent extracts were incubated with [³⁵S]α-SNAP and myc-tagged NSF, radiolabel could be recovered on protein G-agarose beads using a monoclonal antibody directed against the myc epitope (Table 1). Binding of [³⁵S]α-SNAP was due to formation of *bona fide* 'fusion complexes' since it required membrane extracts and NSF, and was released by inclusion of Mg-ATP during washing [21,22].

Binding of α-SNAP to Golgi membranes has been reported to depend on protease-sensitive receptors [27]. In the experiment illustrated in Figure 4 (upper panel), trypsin treatment of brain-derived membranes led to the proteolysis of more than 95% of the SNAREs synaptobrevin, syntaxin and SNAP-25. However, binding of [³⁵S]α-SNAP to control and trypsin-treated membranes was virtually identical (Figure 4, lower panel). In contrast, protease treatment abolished the incorporation of [³⁵S]α-SNAP into 'fusion complexes' (Table 2), as expected from digestion of the neuronal SNAREs. Abolition of complex formation could not be ascribed to digestion of radiolabelled α-SNAP by residual protease activity, since subsequent SDS/PAGE analysis showed that all of the α-SNAP remained intact (results not shown).

Figure 4 Protease sensitivity of α -SNAP binding to membranes

Upper panel: membranes (1 mg/ml protein) were incubated without (lane 1) or with (lane 2) 1 mg/ml trypsin for 20 min at 37 °C, followed by 1 mM PMSF for a further 10 min on ice, to quench protease activity. Membranes were pelleted and resuspended in fresh buffer. After SDS/PAGE, samples were analysed by immunoblotting for the proteins indicated on the left. Bands were visualized using an HRP-labelled secondary antibody and ECL. Molecular-mass markers (kDa) are shown on the right. Lower panel: pig brain membranes (50 μ g phospholipid; 1 mg/ml protein) were incubated with 1 mg/ml trypsin for 20 min at 37 °C. The protease activity was then quenched by the addition of 1 mM PMSF, and the membranes were pelleted and resuspended in fresh buffer (□). Alternatively, membranes were treated with trypsin that had been pre-quenched with PMSF (●). Binding of [³⁵S]α-SNAP to each membrane preparation was monitored on sucrose gradients. Values are means $\pm$ S.E.M. ($n = 4$).

Table 2 20 S complex formation is protease-sensitive

The incorporation of [³⁵S]α-SNAP into 'fusion complexes' was measured using untreated membranes (50 μ g phospholipid), or membranes treated with 1 mg/ml trypsin for 20 min at 37 °C as described in the Experimental section. Incubations with untreated membranes which lacked NSF or which lacked membranes, were included as controls. The values are the means $\pm$ S.E.M. ($n = 3$).

Incubation conditions	Immunoprecipitated [³⁵ S]α-SNAP (d.p.m.)
Untreated	14045 $\pm$ 815
With trypsin	1608 $\pm$ 399
No NSF-myc	1563 $\pm$ 83
No membranes	1073 $\pm$ 404

Figure 5 Saturation of α -SNAP binding to membranes and SNARE complexes

Binding of [³⁵S]α-SNAP to pig brain membranes (50 μ g phospholipid) in the presence of the indicated amounts of unlabelled recombinant His₆-α-SNAP was assessed by measuring the amount of radiolabel migrating with membranes (fractions 1–10) after separation on bottom-loaded sucrose density gradients (□). Incorporation of [³⁵S]α-SNAP into 'fusion complexes' formed using membrane extracts (50 μ g phospholipid) in the presence of unlabelled recombinant α-SNAP was also determined (●). Values are means $\pm$ S.E.M. (binding, $n = 3$; complex formation, $n = 4$).

Additionally, treatment of membranes at 95 °C for 20 min destroyed SNARE function completely, yet reduced α-SNAP binding to membranes by only 50 % (results not shown).

Further evidence that binding of [³⁵S]α-SNAP to membranes differed from its interaction with SNAREs came from competition experiments. Addition of increasing amounts of unlabelled His₆-α-SNAP resulted in a small loss of binding of [³⁵S]α-SNAP to membranes (Figure 5), but significant binding was observed even when 10 μ g unlabelled α-SNAP was included. In contrast, unlabelled α-SNAP competed effectively with [³⁵S]α-SNAP for incorporation into 'fusion complexes', with labelled complex formation being almost completely abolished by 1 μ g α-SNAP.

Although the SNARE-independent binding of [³⁵S]α-SNAP to membranes might be non-specific, this seems unlikely since incubations contained significant quantities of carrier protein. Moreover, binding of [³⁵S]α-SNAP to membranes was compared with the binding of other ³⁵S-labelled proteins translated *in vitro*. Unlike α-SNAP, [³⁵S]chloramphenicol acetyl transferase ([³⁵S]CAT), a soluble protein, did not readily associate with membranes. Only 17 % of [³⁵S]CAT, relative to [³⁵S]α-SNAP, bound to the same preparations of membranes ($n = 4$). In contrast, luciferase, a phospholipid binding protein [39], bound to membranes almost as well as [³⁵S]α-SNAP (89 % relative to [³⁵S]α-SNAP, $n = 3$).

α-SNAP binds to liposomes

Lipids were extracted from porcine brain membranes and re-constituted into unilamellar liposomes. Binding of α-SNAP to these liposomes was then compared with binding to membranes. Significant binding of α-SNAP to both brain membranes and

Figure 6 α -SNAP binds to liposomes

[³⁵S] α -SNAP was incubated with brain membranes (●) or brain membrane-derived liposomes (50 μ g phospholipid; ■). Binding was assessed as described in the legend to Figure 5. The migration of brain-membrane-derived liposomes is also indicated (○). The results are from a representative experiment (of 13 experiments).

Table 3 Lipid-bound α -SNAP bound is functional

Fractions from the peaks of liposome-bound or free α -SNAP, separated by discontinuous sucrose-density gradient, were combined and equal amounts of trichloroacetic-acid-precipitable radioactivity from each pool were incubated with NSF-myc and membrane extracts to monitor incorporation of [³⁵S] α -SNAP into 20 S complexes. Control samples (using liposome-bound [³⁵S] α -SNAP) lacked NSF-myc. The values are the means $\pm$ S.E.M. ($n = 3$).

Incubation conditions	Immunoprecipitated [³⁵ S] α -SNAP (d.p.m.)
Lipid-associated α -SNAP	4104 $\pm$ 181
Unbound α -SNAP	3946 $\pm$ 156
No NSF-myc	489 $\pm$ 102

brain-membrane-derived liposomes was detected (Figure 6). Membranes migrated to a sharper peak than reconstituted liposomes (see also Figure 3, lower panel), so that the profile of radiolabel migration also differed. However, integration of the upper peak of radioactivity showed that the efficiency of α -SNAP binding to brain-membrane-derived liposomes was 119% compared with binding to brain membranes. On average, 31.9 $\pm$ 6.5% ($n = 13$) of total radioactivity migrated with liposomes.

The resistance of liposome-bound α -SNAP to high sucrose concentrations suggested that binding was tight. This was underlined further by experiments to determine the resistance of the interaction to high pH. Standard binding assays were compared with assays in which binding was followed by incubation in 100 mM Na₂CO₃ for 20 min. Liposomes were recovered from density gradients which contained Na₂CO₃ throughout. Recovery of α -SNAP in liposome-containing fractions was actually somewhat higher after Na₂CO₃ treatment (28028 $\pm$ 5122 d.p.m. compared with 20164 $\pm$ 4606 d.p.m. in control incubations, $n = 5$). Lipid-bound α -SNAP was active, when judged by its incorporation into 'fusion complexes' subsequent to solubilization of the liposomes in detergent (Table 3).

Figure 7 Stimulation of NSF-dependent ATPase activity by α -SNAP and lipid

The rate of phosphate release from ATP was determined from reactions containing NSF alone (0.5 μ g; ○), NSF + α -SNAP (0.5 μ g of each protein; □), or NSF + α -SNAP + brain-derived liposomes (0.5 μ g of each protein, 5 μ g phospholipid; △). The values are means $\pm$ S.E.M. ($n = 3$).

Table 4 Kinetic analysis of NSF-dependent ATPase activity

Initial rates of ATP hydrolysis were determined for different ATP concentrations in incubations containing NSF alone, NSF + α -SNAP (0.5 μ g of each protein) or NSF + α -SNAP + phospholipid (5 μ g). For each set of results, data were fitted to one- or two-site models using linear regression of transformed data in a double-reciprocal plot. The kinetic parameters (K_m and V_{max}) for each ATP binding site are given ($n = 3$).

Incubation conditions	K_m (mM)	V_{max} (nmol phosphate/h per μ g of NSF)
NSF alone (K_m 1)	0.11	0.23
NSF alone (K_m 2)	3.40	0.85
NSF + α -SNAP	0.09	0.65
NSF + α -SNAP + phospholipid	0.11	1.22

Thus binding was not restricted to partially folded or denatured protein.

Lipids enhance the ability of α -SNAP to stimulate NSF-dependent ATPase activity

NSF had a basal ATPase activity of approximately 0.19 $\pm$ 0.04 nmol phosphate released/h per μ g of protein (Figure 7). This was increased approximately 2–3-fold by inclusion of a 2-fold molar excess of α -SNAP, in agreement with results published previously [10]. Inclusion of membrane-derived lipid led to a further 2–3-fold stimulation of the ATPase activity. The effect of lipid was saturable, and depended on the presence of α -SNAP (results not shown). Previous kinetic analysis of the ATPase activity of NSF is consistent with a two-site model, and the stimulation by α -SNAP was explained by a substantial reduction of the K_m of the low-affinity site, yielding a one-site kinetic fit [10]. We were able to confirm this result and also to

show that the primary effect of lipid on the stimulation of the ATPase activity of NSF was via a doubling of the $V_{\max}$ (Table 4).

DISCUSSION

Current models of membrane fusion [2] regard α -SNAP as a cytosolic factor which is recruited to membranes to form part of a transient complex upon vesicle–target membrane docking. In this study we have shown that, at steady state, the majority of α -SNAP is bound to cellular membranes. Biochemical analysis of α -SNAP indicates that the protein has hydrophobic characteristics, which may contribute towards membrane binding. The fact that α -SNAP partitions into both the detergent and aqueous phases during TX-114 extraction raises the possibility that α -SNAP exists in two conformations, only one of which exposes hydrophobic regions of the protein to the solvent. The efficient labelling of recombinant α -SNAP by [125 I]TID represents further evidence for the presence of hydrophobic regions on the surface of the protein. Protein substrates for TID include the lipid binding protein, BSA, and the b subunit of the F_1 moiety of the *Escherichia coli* ATP synthase. Furthermore, labelling of this latter protein is known to reflect its ability to interact directly with lipid bilayers [40].

The lack of sensitivity of membrane binding of α -SNAP to protease treatment of the membranes, to heat treatment or to competition with unlabelled protein, indicates that α -SNAP can bind to membranes independently of SNAREs. We cannot, of course, rule out the possibility that membranes contain other proteinaceous α -SNAP receptors that are more abundant than SNAREs and are resistant to proteolysis. This seems unlikely, however, since the SNAREs are amongst the most abundant of synaptic proteins. A more likely explanation is that we have detected α -SNAP binding to membrane lipid. This idea is supported by the finding that α -SNAP binds at least as well to brain-membrane-derived liposomes as to carbonate-extracted brain membranes. The demonstration that lipids are able to modulate α -SNAP-stimulated, NSF-dependent ATPase activity indicates that the interaction of α -SNAP with membrane lipids may be functionally relevant. It could be argued that this effect is due simply to the fact that only a small proportion of α -SNAP might be bound to plastic and thus able to interact with NSF, and that addition of lipid would provide further sites for SNAP attachment. However, such a scenario would not be consistent with the simple, single-site kinetics of the ATPase reaction in the presence of α -SNAP alone. More likely, interaction of α -SNAP with lipid, or perhaps other hydrophobic moieties, determines the strength of its interaction with NSF. There are indications that protein transport requires a specific lipid balance, and the activity of phospholipases or other lipid-modifying enzymes [41]. Whether α -SNAP binding shows a preference for specific lipids is not yet clear.

How can we reconcile the apparently protein receptor-independent binding of α -SNAP to membranes with the abundance of α -SNAP receptors in these membrane preparations? SNAREs are known to act as high-affinity α -SNAP receptors only when they are complexed to each other [23,42], although syntaxin alone will bind α -SNAP in solution, albeit with a low affinity [23,42,43]. Complex formation occurs readily in detergent extracts [22], but should occur in intact membranes only when vesicle docking occurs [21]. Indeed, efficient formation *in vivo* of SNARE complexes in yeast requires conditions that favour arrest of vesicular transport at a post-docking stage [20]. Similarly, only a small proportion of the neuronal t-SNAREs syntaxin and SNAP-25 are found complexed to synaptobrevin under normal conditions [44], and it appears that virtually all synaptobrevin II

is complexed to synaptophysin in brain membranes [45] when they are isolated under normal conditions. It may be, therefore, that only specialized membranes such as the Golgi complex, which form stable membrane–membrane contacts [2], retain sufficient v-SNARE/t-SNARE interactions in isolated membranes to allow detection of α -SNAP binding to SNAREs [27]. The membranes we have described here, and others we have used (results not shown), might have too low a concentration of docked SNAREs to support significant SNARE-dependent binding of α -SNAP.

Results from other investigations of α -SNAP binding to membranes also point to some degree of SNARE-independent binding. For example, only about half of the total α -SNAP is released from permeabilized chromaffin cells in the presence of ATP, and release is slow [25]. Further, Sec17p is predominantly membrane-associated in wild-type yeast cells and released from membranes inefficiently by Na_2CO_3 [46]. It has been shown that Sec17p is efficiently removed from yeast membranes by incubation with Mg-ATP [26], suggesting that ATP hydrolysis induces a transient change in α -SNAP conformation which allows it to dissociate from membranes. However, in cells, re-binding of α -SNAP to membranes must be rapid compared with formation of 20 S complexes and re-release to account for the steady-state distribution of α -SNAP. In extracts or broken cells conditions might prevent efficient re-binding of released α -SNAP, thus accounting for the ATP-dependent release that is observed. NSF is released inefficiently from many cellular membranes or purified clathrin-coated vesicles under conditions that permit ‘fusion complex’ disassembly [47,48] (again, however, it is striking that the Golgi complex behaves differently from other cellular membranes, since NSF is efficiently released from this structure in the presence of Mg-ATP [48]). Together, these findings raise the possibility that α -SNAP and NSF bind to SNAREs by moving laterally in the plane of the lipid bilayer, rather than being recruited from the soluble phase, an idea that is also consistent with observations that both proteins are incorporated into transport vesicles before docking occurs [47,49].

Membrane fusion is likely to be mediated by proteins which enable the membranes to become tightly apposed, and also by proteins that displace lipids during formation of a fusion pore [50]. The demonstration of a direct interaction of the fusion-promoting protein α -SNAP with membrane lipids could therefore be significant. α -SNAP is an amphipathic protein which will bind to plastic surfaces [9], is purified by hydrophobic interaction chromatography [51] and yet is highly soluble in aqueous solvents. The ability of amphipathic peptides to destabilize lipid bilayers is well documented. Melittin, for example, can induce channel formation, membrane fusion or bilayer micellization according to ionic conditions [52]. These activities are associated with changes in the conformation of the helix, including oligomerization, and changes in the position of the helix relative to the lipid bilayer. At this stage we cannot be sure that lipid binding plays an important role in α -SNAP function. This is especially true in the light of debate about the point at which α -SNAP operates during fusion [26]. However, we believe that further investigations into the nature of α -SNAP–membrane interactions will help to determine their biological importance and to elucidate the mechanism of membrane fusion.

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