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SNAP receptors implicated in vesicle targeting and fusion

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The *N*-ethylmaleimide-sensitive fusion protein (NSF) and the soluble NSF attachment proteins (SNAPs) appear to be essential components of the intracellular membrane fusion apparatus. An affinity purification procedure based on the natural binding of these proteins to their targets was used to isolate SNAP receptors (SNAREs) from bovine brain. Remarkably, the four principal proteins isolated were all proteins associated with the synapse, with one type located in the synaptic vesicle and another in the plasma membrane, suggesting a simple mechanism for vesicle docking. The existence of numerous SNARE-related proteins, each apparently specific for a single kind of vesicle or target membrane, indicates that NSF and SNAPs may be universal components of a vesicle fusion apparatus common to both constitutive and regulated fusion (including neurotransmitter release), in which the SNAREs may help to ensure vesicle-to-target specificity.

TRANSACTIONS among the membrane-bound compartments in the cytoplasm of eukaryotic cells are generally executed by transport vesicles which bud from one membrane and fuse selectively with another¹. The mechanism by which each vesicle chooses its target is currently unknown, but must embody the essence of compartmental specificity. Choice of target is implicit in all vesicular fusion events, from the ubiquitous steps in the constitutive secretory and endocytotic pathways (such as the fusion of a vesicle from the endoplasmic reticulum with the Golgi) to specialized and tightly regulated forms of exocytosis (such as the triggered fusion of a synaptic vesicle containing neurotransmitter with the axonal membrane). Here we describe a fundamental relationship between these processes, suggesting that a general apparatus is used to bring about fusion, and that specificity is established by the pairing of compartment-specific proteins in the transport vesicle and target membrane, respectively.

The *N*-ethylmaleimide-sensitive fusion protein is a soluble tetramer of 76K subunits which was purified^{2,3} on the basis of its ability to restore intercisternal Golgi transport in a cell-free

system^{4,5}. It is required for transport vesicle fusion, as vesicles accumulate at the acceptor membrane in its absence^{6,7}. In yeast, NSF is encoded by the *SEC18* gene⁸, originally shown to be necessary for transport from the endoplasmic reticulum (ER) to the Golgi⁹. The *SEC18* protein can replace NSF in a mammalian system for cell-free Golgi transport⁸ and is required at every discernible step of the secretory pathway *in vivo*¹⁰. NSF thus appears to participate in a variety of intracellular fusion processes.

NSF requires additional cytoplasmic factors to attach to Golgi membranes¹¹. Three species of monomeric soluble NSF attachment proteins, termed α -, β - and γ -SNAP (M_r s of 35, 36 and 39K, respectively), have been purified from brain^{12,13} and SNAP activity is necessary for vesicle fusion *in vitro*¹³. α -SNAP (but not β - or γ -SNAP) can restore animal cell Golgi transport activity to cytosol prepared from *sec17* mutant yeast, implying¹³ that the *SEC17* gene encodes α -SNAP in yeast. *SEC17* is now known to be functionally equivalent to α -SNAP¹⁴, and the two proteins are clearly related¹⁵. In the absence of functional *SEC17* (or *SEC18*), ER to Golgi transport stops in yeast, and transport

vesicles accumulate¹⁶. SNAPs, like NSF, thus appear to be components of a general intracellular membrane fusion apparatus common to all eukaryotic cells.

SNAPs bind to distinct sites in membranes which up until now have only been operationally defined, and NSF will only interact with SNAPs that are already attached to these sites¹⁷. α -SNAP and β -SNAP compete for binding to the same receptor site with low nM affinity. γ -SNAP binds to a noncompetitive site in the same complex¹⁷, and, although not essential for NSF binding, increases the complexes' affinity for NSF¹⁸. Crosslinking studies suggest that the SNAP receptor contains an α -SNAP-binding subunit of 30–40K (ref. 17). When membrane-bound NSF-SNAP-SNAP receptor complexes are solubilized with detergent, they sediment as a distinct multisubunit particle at 20S (ref. 18), which may form the core of a generalized apparatus catalysing bilayer fusion at its point of assembly^{6,13}. The 20S particles are also formed when detergent extracts of Golgi membranes containing SNAP receptors are mixed with SNAPs and NSF. When NSF and SNAPs are added in excess, all SNAP receptor activity in the membrane extract is incorporated into 20S particles¹⁸.

NSF is an ATPase containing two ATP-binding sites in separate domains¹⁹, and the binding and hydrolysis of ATP are critical in determining the stability of NSF³ and its attachment to membranes^{2,18}. Stable 20S particles can be formed in the presence of either Mg-ATP- γ S (a non-hydrolysable analogue of ATP) or ATP without magnesium. In the presence of Mg-ATP, however, particles rapidly dissociate even at 0°C, liberating NSF in a process that requires ATP hydrolysis¹⁸. This disassembly may be an intrinsic step in the fusion mechanism¹⁸.

Purification of SNAP receptors

To purify SNAP receptors, we used the specificity inherent in the assembly and disassembly of 20S particles as the basis of an affinity purification technique (schematized in Fig. 1): 20S particles were formed and attached to a solid matrix, allowing purified SNAP receptor to be released by particle disassembly when ATP was subsequently hydrolysed.

A recombinant form of NSF, epitope-tagged with a Myc peptide²⁰ (EQKLISEEDL) at its carboxy terminus, was expressed in *E. coli* and shown to be functional²¹. The 20S particles were formed in solution by mixing a Triton X-100 extract of membranes with pure, recombinant α - and γ -SNAPs expressed in *E. coli* and NSF-Myc at 0°C (ref. 15) in the presence of ATP- γ S and EDTA (to chelate any magnesium). A monoclonal anti-Myc IgG (9E10; ref. 22) attached to protein G beads was then added to bind the 20S particles through their NSF-Myc subunits. The anti-Myc IgG does not inhibit NSF-Myc function in cell-free transport assays (our unpublished results).

The beads were formed into a column and washed extensively before a first (nonspecific) elution with Mg-ATP- γ S to elute any proteins attached in a Mg²⁺-sensitive fashion, or by Mg-ATP- γ S *per se*. A second (specific) elution was then done with Mg-ATP (replacing Mg-ATP- γ S). Only proteins released as

the immediate consequence of ATP hydrolysis on the column will be recovered in this specific eluate. This scheme is based on that described previously¹⁸ but includes a number of key modifications detailed in the figure legends. As NSF remains on the beads, the specific eluate should consist of a fraction of the added SNAPs, together with additional polypeptides representing SNAP receptors, ideally in stoichiometric amounts.

A detergent extract of a crude, salt-washed total particulate fraction from the grey matter of bovine brain was used as the source of potential SNAP receptors. Membranes were washed with 1 M KCl before use to remove most of their endogenous SNAP supply¹². As recombinant SNAPs were added in great excess for the binding reaction (Fig. 1), these should compete out the remaining endogenous SNAPs, preventing them from forming particles on the beads. Figure 2a shows a Coomassie blue-stained SDS-urea-high Tris-polyacrylamide gel of the specific eluate (lane 3), the nonspecific eluate fraction (lane 2), and the specific eluate from a control experiment omitting NSF-Myc (lane 1). Several bands (labelled A–F) appear only in the specific (Mg-ATP) eluate and depend on the presence of NSF. A set of bands at about M_r 70K are present in all three lanes, but apart from these, bands A–F are substantially pure in the specific eluate fraction. Band F runs as a sharper band in a standard Laemmli gel (Fig. 2b; specific eluate, stained with silver) than in the high Tris-urea gel (Fig. 2a). Bands A and C virtually co-electrophorese with the abundant band D in a Laemmli gel (Fig. 2b; see below).

Identification of SNAP receptors

To identify specific bands, they were excised from blots and digested with trypsin, giving peptides that were then separated by high-pressure liquid chromatography and microsequenced (see Fig. 3 for details). Bands B and D co-electrophoresed with recombinant γ -SNAP and α -SNAP, respectively, both of which were His₆-tagged¹⁵ and thus slightly larger than their endogenous counterparts (not shown). These identifications were confirmed by microsequencing peptides from bands B and D (not shown).

Sequences from six major peptides containing a total of 70 residues were obtained from band A, each precisely matching the sequence of syntaxin B (p35B) from rat brain²³ (Fig. 3b). The sequences of these and peptides from the other specific bands are shown in association with the band from which they were obtained (Fig. 3a). No sequences or fragments attributable to any other protein were found, indicating that the bulk of material in band A is syntaxin B.

Five major peptides containing a total of 53 residues were sequenced from band C (Fig. 3a), and proved to differ at only one position from the published sequence of syntaxin A from rat brain²³; the difference presumably reflects the species involved. Expected sequence differences between the 84% identical syntaxins A and B were found in the peptides from bands C and A (Fig. 3b). The syntaxins run more slowly than expected from their relative molecular masses (about 35K) in the high percentage Tris-SDS-urea-polyacrylamide gels were used,

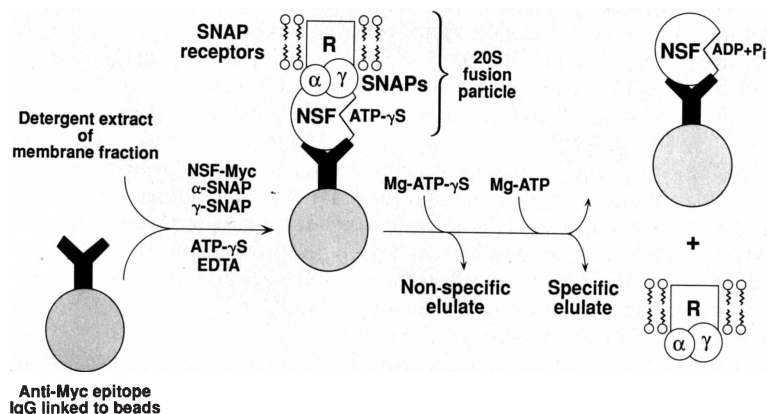


FIG. 1 Procedure used to purify SNAP receptors (SNAREs). Recombinant NSF, α -SNAP, and γ -SNAP are assembled into 20S particles by SNAREs present in a crude detergent extract of membranes. The SNAREs are incorporated stoichiometrically into the particles, which are then bound to beads by means of NSF. For this purpose, the NSF is epitope-tagged with Myc, and an anti-Myc monoclonal IgG is linked to the beads. The beads are washed and then eluted first with Mg-ATP- γ S (nonspecific eluate) and then with Mg-ATP (specific eluate). The bound 20S particles disassemble in the presence of Mg-ATP (but not Mg-ATP- γ S), releasing stoichiometric amounts of SNAPs and SNAREs. NSF remains bound to the beads. Experimental details are given in Fig. 2 legend.

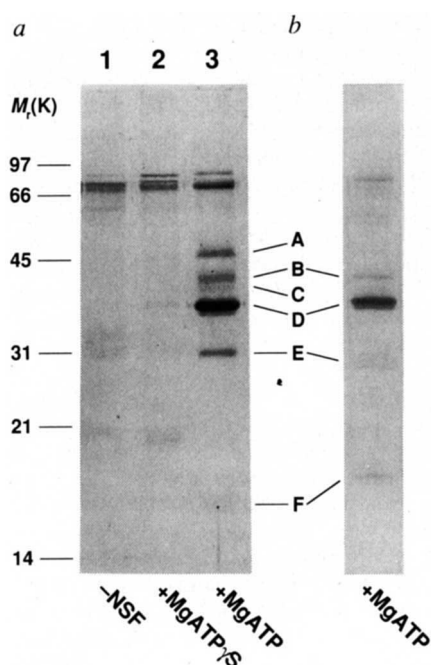


FIG. 2 Identification of proteins released from NSF after ATP hydrolysis. *a*, Polyacrylamide gel stained with Coomassie blue. Lane 1, control, Mg-ATP eluate of control binding reaction in the absence of NSF; lane 2, 'nonspecific' eluate from complete binding reaction with NSF-Myc and Mg-ATP- γ S; lane 3, 'specific' eluate of the same column as for lane 2 following the exchange of ATP for ATP- γ S (in the presence of EDTA) and addition of Mg^{2+} to allow ATP hydrolysis (Fig. 1). *b*, Silver-stained Laemmli gel of the specific (Mg-ATP) eluate.

METHODS. All manipulations were performed at 0–4 °C. Bovine brain tissue was initially stripped of meninges, and all but the grey matter removed and discarded. The resulting tissue (30g) was homogenized with 30 strokes of a Dounce homogenizer in buffer A (20 mM Tris-HCl, pH 8.0, 1 M KCl, 250 mM

although they virtually co-electrophorese with α -SNAP (35K) in standard Laemmli gels (Fig. 2*b*, and data not shown).

Three major peptides (Fig. 3*a*) from band E gave sequences (Fig. 3*c*) matching a protein predicted from a mouse brain complementary DNA clone which by coincidence has been termed SNAP-25 (for synaptosome-associated protein of 25K; ref. 24). Of 45 residues obtained, 42 were identical to the published sequence of mouse SNAP-25. The discrepancies, two of which are conservative, are probably due to species differences. SNAP-25 migrates at M_r 31K in the SDS-urea gel.

All five peptides (containing 43 identified residues) sequenced from band F (Fig. 3*a*) exactly matched the sequence of VAMP/synaptobrevin-2 from bovine brain²⁵ (Fig. 3*d*). VAMP-2, the major isoform of VAMP/synaptobrevin in brain, differs slightly from VAMP-1 (refs 26, 27); all the sequences obtained from band F correspond to VAMP-2. All HPLC peptide peaks from band F, other than those attributable to trypsin autolysis, were shown to be derived from VAMP/synaptobrevin. As a control, the region corresponding to band F from the blot of the gel of the nonspecific (Mg-ATP- γ S) eluate was subject to tryptic digestion and HPLC analysis in parallel with band F from the Mg-ATP eluate in two separate experiments. None of the VAMP/synaptobrevin-2 peptide peaks from band F were present in the HPLC profile of the control; only the autolytic tryptic peptides were detected.

Two peptides from the digest of band F failed to give any sequence, suggesting that they had blocked N termini. Mass spectroscopy indicated that these components had m/z values of 2,680.90 and 2,838.36 respectively, differing by 157.46, or approximately the molecular mass of Arg (156.19 average isotopic mass). The tryptic cleavage site closest to the N terminus in bovine VAMP/synaptobrevin-2 consists of an Arg-Arg sequence (at positions 30–31), so the expected partial cleavage

sucrose, 2 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride (PMSF). A total particulate fraction was isolated by centrifugation in a Ti45 rotor (Beckman) for 60 min at 33,000 r.p.m. The pellet was then resuspended in buffer A by Dounce homogenization and the membranes collected by centrifugation. The resulting pellet was washed once in buffer B (10 mM HEPES/KOH, pH 7.8, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT) and then resuspended in 100 ml buffer B. Triton X-100 was added slowly with mixing to a final concentration of 4% (v/v), and the suspension incubated on ice with frequent mixing. After 45 min, the suspension was clarified by centrifugation (Ti-45 rotor) for 60 min at 33,000 r.p.m. and the supernatant dialysed overnight against 100 vol 25 mM Tris-HCl, pH 7.8, 50 mM KCl, 1 mM DTT, 1% (v/v) Triton X-100. After dialysis, the material was clarified by centrifugation (Ti-45 rotor for 60 min at 33,000 r.p.m.), aliquoted, and stored at –80 °C. Protein concentration was measured using the BCA reaction (Pierce) with ovalbumin as standard. This bovine brain extract (2mg protein) was preincubated in the presence of His $_6$ - α -SNAP (12 μ g protein), His $_6$ - γ -SNAP (4 μ g protein) and NSF-Myc (12 μ g protein) in buffer C (25 mM HEPES/KOH, pH 7.0, 0.75% (w/v) Triton X-100, 75 mM KCl, 1 mM DTT, 2 mM EDTA, 0.5 mM ATP (for lane 1) or 0.5 mM ATP- γ S (for lanes 2 and 3), 1% (w/v) polyethylene-glycol (PEG) 4,000, 0.4 mM PMSF) for 30 min at 4 °C in a final volume of 2 ml. Mouse anti-Myc monoclonal antibody (200 μ g protein, termed 9E10; ref. 22) covalently coupled to protein G-Sepharose 4 fast-flow (Pharmacia) was added and the incubation continued for 2 h with constant agitation. (The anti-Myc IgG was covalently coupled to the protein G-Sepharose using 20 mM dimethylsuberimide as described⁴⁷.) The beads were packed into a column, washed with 10 vol buffer D (20 mM HEPES/KOH, pH 7.0, 0.5% (w/v) Triton X-100, 100 mM KCl, 1 mM DTT, 2 mM EDTA, 0.5 mM ATP (lane 1) or 0.5 mM ATP- γ S (lanes 2 and 3), 0.4 mM PMSF) and the proteins eluted with 6 column-volumes of buffer D containing 8 mM $MgCl_2$ (resulting in a concentration of free Mg^{2+} of 4 mM). The column containing ATP- γ S (lanes 2 and 3) was washed with an additional 2 column-volumes of buffer D containing 4 mM EDTA to complex the free Mg^{2+} , 3 column-volumes of buffer D containing ATP to exchange for bound ATP- γ S, and then eluted with 6 column-volumes of buffer D containing 8 mM $MgCl_2$ to allow ATP hydrolysis. The appropriate fractions containing the eluate were pooled, precipitated with trichloroacetic acid, boiled in sample buffer⁴⁸ and analysed by electrophoresis on Tris-urea/SDS-polyacrylamide (18% acrylamide, 6M urea, 750 mM Tris-HCl, pH 8.85, 50 mM NaCl, 0.1% SDS⁴⁹) and stained with Coomassie blue R-250. For sequencing, the reaction was scaled up 25-fold.

would yield two N-terminal-derived peptides. If it is assumed that the initiator Met is removed, and that the newly generated N-terminal Ser is acetylated, adding 42.04 to the M_r , the two masses correspond almost perfectly with the predicted M_r s [MH^+] of the bovine VAMP/synaptobrevin-2 peptides spanning residues 2–30 and 2–31 (2,681.90 and 2,838.09, respectively). The N terminus of VAMP/synaptobrevin-2 thus appears to be processed and acetylated.

The material in the 70K region of all three eluates (specific, nonspecific and control) (Fig. 2) was microsequenced (from the specific eluate) in an attempt to determine if any synaptotagmin/p65 was present, as this protein can be immunoprecipitated together with syntaxins²³. No evidence for its presence was found, although it cannot be excluded. Of three peptides sequenced, two were from Hsp70, an ATP-binding protein²⁸, and one was from a protein not found in the Genbank database.

Scanning and integrating the Coomassie blue stain in the gel shown in Fig. 2*a* gives the following ratios when staining is corrected for molecular weight, expressed as a fraction of α -SNAP: syntaxin B, 0.18; SNAP-25, 0.15; VAMP/synaptobrevin-2, 0.23. The syntaxin A band (band C) is weak compared with syntaxin B and could not readily be quantitated. The sum of all of the SNAP receptor species is 0.56 mol per mol α -SNAP, and so is approximately stoichiometric, given that proteins vary in staining by Coomassie blue, and the stoichiometry of SNAP and receptor in complexes does not need to be 1:1. The relative amounts of the different SNAP receptors in such a co-purified mixture may not reflect their relative abundance in the starting material, as their affinities for SNAPs may differ. The ratio of γ -SNAP to α -SNAP was ~0.2.

The role of syntaxins, SNAP-25, and synaptobrevin as SNAP receptors is supported by (1) their absence when NSF was omitted from the purification; (2) the need for ATP hydrolysis,

rather than the mere presence of ATP, to elute them from the beads; (3) the approximately stoichiometric amounts purified relative to SNAP itself; and (4) the substantial purity of the proteins obtained. The fact that all of these proteins originate from the synapse further strengthens this conclusion.

As further confirmation, we attempted to follow the incorporation of α -SNAP receptor into 20S particles formed from crude extracts of bovine brain membranes using an antibody raised

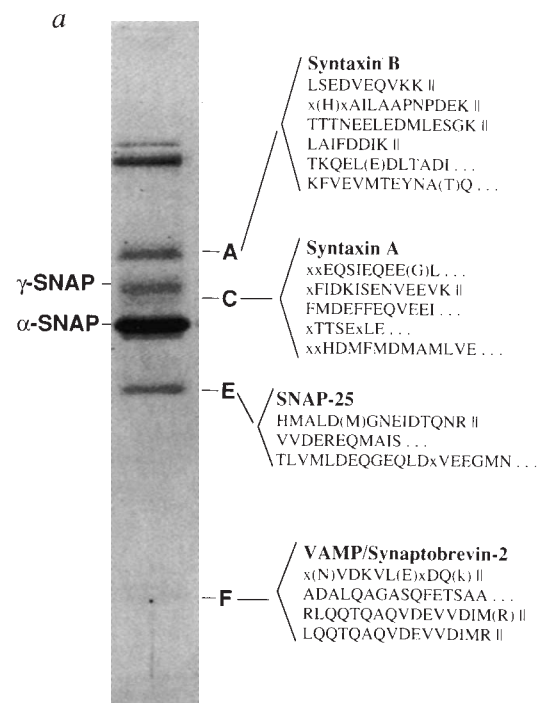


FIG. 3 Identification of SNAP receptors by amino-acid sequencing. Each of bands A–F was excised from an electroblot onto a nitrocellulose membrane after staining with Ponceau S, digested with trypsin, and the fragments separated by reverse-phase HPLC. *a*, Peptide sequences from each of the indicated bands are shown on the right (one-letter code). An 'X' means that no residue could be identified at this position. A capital letter in parentheses means that the residue was identified with a lower degree of confidence; a lower-case letter in parentheses means that the residue was present but in very small amounts. The symbol || indicates that the C terminus of the peptide was known. A dotted line means no more interpretable signals were obtained, but that this was unlikely to be the C terminus. The identify of bands B and D (Fig. 2) as γ -SNAP and α -SNAP, respectively, was confirmed by peptide sequencing. In the case of the syntaxin A peptide XTTSExLE... (from band C), only this limited sequence was obtained because of instrument failure. However, mass analysis of this peptide (on 1/25th of the sample) gave a value of $m/z=3,274.8$; this is in good agreement with the value ($MH^+=3,276.67$) calculated for the predicted tryptic peptide (residues 156–186 of syntaxin A) which contains the limited sequence, so confirming the identity of the entire peptide. Mass analysis was also critical in the identification of band F as VAMP/synaptobrevin-2, confirming the presence of an *N*-acetylated peptide corresponding to residues 2–31 in the digest of band F (see text for details). *b*, Amino-acid sequences of syntaxins A (top) and B (below) from rat brain²³. Shown above syntaxin A are the peptide sequences obtained from band C, and below syntaxin B are the peptide sequences obtained from band A. *c*, Complete amino-acid sequence of synaptosome-associated protein 25 (SNAP-25) from mouse brain²⁴. Above are the peptide sequences obtained from band E. Underlined sequences in *d* and *b* are peptides identified by mass analysis.

METHODS. Tryptic digestions and peptide separation. Ponceau S staining bands were excised, digested in situ with trypsin (1 μ g sequencing grade trypsin; Boehringer-Mannheim), and the resulting fragments separated by RP-HPLC (ref. 50). Generally, HPLC systems were as described⁵⁰ and equipped with a 2.1 $\times$ 250 mm Vydac C4 (214TP54) column from the Separations Group (Hesperia, CA). Peptides resulting from digests of band F were

against a C-terminal peptide from SNAP-25. Detergent extract from salt-washed membranes was incubated with excess recombinant α - and γ -SNAPs with (Fig. 4c) or without (Fig. 4b) excess recombinant NSF, and the products were separated by velocity centrifugation in a linear glycerol gradient. In the absence of NSF, SNAP-25 sedimented at about 5S, but in its presence, almost all SNAP-25 cosedimented with NSF, α -SNAP, and γ -SNAP in the 20S particle (Fig. 4d). Assembly of NSF and SNAPs into the 20S particle required bovine brain membrane extract (Fig. 4a), as expected from earlier studies with Golgi membranes¹⁸.

Discussion

In this study, we have purified SNAP receptors from a crude brain membrane fraction on the basis of their ability to assemble

b

		FM DEFFEQVEEI	FIDKISEN	
Rat Syntaxin A	---RTQELRT AKSDDDDDV TVTVDRDRFM	DEFFEQVEEI	RGFIDKIAEN	47
Rat Syntaxin B	MKDRQTQLRS AKSDDEEEV -VHVDROHFM	DEFFEQVEEI	RGCEKLSLSD	49
			LSED	
	VEEVK		EQSI	
	VEEVKRGHSA ILASPNDEK TKEELEELS	DIKKTANKVR	SKLSIEQSI	97
	VEQVKQKHS ILAAPNDEK TQKELEDLIA	DIKKTANKVR	SKLKATIEQSI	99
	VEQVKQKHS ILAAPNDEK TQKELEDLIA	DI		
	EQEGL			
	EQEGLNRSS ADLRIRKQH STLSRKFEV	MSEYNATQSD	YRERCKGRIG	147
	EQEGLNRSS ADLRIRKQH STLSRKFEV	MTEYNATQSK	YRDRCKDRIG	149
		KEFEV	MTEYNATQ	
	T TSExLE			
	RQLEITGRIT TSEELEDMLE SQNPATFASG	LIMSSLSKQ	ALSEIETRHS	197
	RQLEITGRIT TSEELEDMLE SQGLAIFDD	TKMDSQMTKQ	ALNEIETRHN	199
	TT TSEELEDMLE SQGLAIFDD	IK		
	HMEDMDA MIVE			
	EIIKLENSIR ELHDMEDMA MIVESQGEMI	DRIEYNVEHA	VDYVERAVSD	247
	EIIKLETSIR ELHDMEDMA MIVESQGEMI	DRIEYNVEHS	VDYVERAVSD	249
	TKKAVKYQSK ARKKIMIII CCVILGIIIA	STIGGIFG-		285
	TKKAVKYQSK ARKKIMIII CCVILGVIA	SSIGGTGL		288

c

Mouse SNAP-25

				TLVML	DEQGEQLDxV	
MAEDADMNE	LEEMQRRADQ	LADESLESTR	RMLQVLEESK	DAGIRTLVML	DEQGEQLERI	60
EEGMN					V	
EEGMQDINKD	MKEAEKNLTD	LKFGCLCVC	PONKLSKSSA	YKKAQGNQD	GVVASQPARV	120
VDEREQMAIS				HMALDMGNE	IDTQNR	
VDEREQMAIS	GGFIRRVIND	ARENEMDENL	EQVSGIIGNL	HMALDMGNE	IDTQNRQIDR	180
IMEKADSNKT	RIDEANORAT	KMLGSG	206			

d

Bovine
VAMP/Synaptobrevin 2

		RLQQTQAQVD	EVVDIMR	NV	DKVLExDQK	
MSATAATAPF	AAPAGEGGRP	APPNLTNR	RLQQTQAQVD	EVVDIMRVNV	DKVLEFDQKL	60
ADAL	QAGASQFET	SAA				
SELDORADAL	QAGASQFET	SAKLKRYW	WKNLMIMIL	GVCAIILII	IIVYFSS	117

fractionated on a 1 $\times$ 100 mm Inertsil 100GL-I-ODS-110/5 C18 column from SGE (Ringwood, Australia). The 1-mm column was operated in a system consisting of a model 140B syringe pump (ABI, Foster City, CA) and an ABI model 783 detector, fitted with a LC-Packings Kratos-compatible capillary flow cell which was directly connected to the column outlet (system assembly by C. Elicone); gradient slope was 1% B per min at a flow of 30 μ l min⁻¹. Fractions were stored at -70 °C before sequence or mass analysis. Peptide sequencing: Peptides were sequenced with the aid of an ABI model 477A automated sequencer, optimized for femtomol phenylthiohydantoin analysis as described^{51,52}. Fractions were always acidified (20% TFA final concentration) before application on the sequencer disc. Mass analysis: Aliquots (typically 1/50–1/25) of selected peak fractions were analysed by matrix-assisted laser desorption time-of-flight mass spectrometry using a Vestec (Houston, TX) LaserTec instrument with a 337 nm output nitrogen laser operated according to ref. 53. The matrix was α -cyano-4-hydroxy cinnamic acid and a 25 kV ion acceleration and 3 kV multiplier voltage were used. Laser power was adjusted manually as judged from optimal deflections of specific maxima, using a Tektronix TDA 520 digitizing oscilloscope.

20S particles from SNAPs and NSF, and their release from NSF upon ATP hydrolysis (studies using Golgi membranes as source material will be described elsewhere). The simplest explanation for the fact that multiple polypeptides are obtained is that each is a distinct SNAP receptor. In particular, there is no evidence that any of these polypeptides associate, for example, as heterodimers⁵⁴. All of them must thus have either the capacity to assemble a 20S particle, or a very high affinity for one that has been assembled. As our method is biased to isolating receptors of the highest abundance and affinity, we have probably isolated only some of the receptor types that are present.

By exploiting the same enzyme cycle used in the vesicle fusion process to offer two layers of biological specificity, we have purified SNAP receptors on the basis of their function, giving a largely pure preparation of four proteins from the crudest possible extract of brain, all of which originate from synapses. This striking selection for synaptic components probably reflects the degree to which the brain is specialized for synaptic vesicle fusion. The fact that each SNAP receptor purified corresponds to a previously cloned and sequenced gene no doubt reflects the exhaustive structural characterization of synaptic components by molecular biologists who have focused on the synapse because of its central importance in neuronal development and function^{29,30,54}. Despite this effort, however, the roles of these proteins are unknown because of the lack of functional assays.

Of the SNAP receptors we have isolated, SNAP-25 is found in the presynaptic terminals of neurons²⁴ but has not been more precisely localized. Although its sequence suggests it is hydrophilic, it behaves as an integral membrane protein²⁴ and is palmitoylated at one or more of the four cysteine residues between positions 85 and 92 (ref. 55). Fatty acyl-CoA is required for NSF-dependent fusion⁵⁶, and SNAP-25 or related proteins may serve as the relevant acyl acceptors. Multiple acylations of such proteins could create a hydrophobic surface as a trigger for fusion.

VAMP/synaptobrevin is inserted into synaptic vesicles by a single hydrophobic C-terminal segment, and the remainder of

the protein is cytoplasmic. Both tetanus and botulinum B toxins, potent inhibitors of neurotransmitter release, are proteases specific for VAMP/synaptobrevin-2 (ref. 31), suggesting that this SNAP receptor is essential for synaptic vesicle fusion *in vivo*. Syntaxin²³ has the same topography as VAMP/synaptobrevin, but is concentrated in 'active zones' of the presynaptic membrane²³ at which a subpopulation of synaptic vesicles is docked³² to allow fusion within 200 μ s of the calcium influx induced by an action potential³³. Components of the fusion machinery may have to be largely preassembled at these sites to allow such a rapid response.

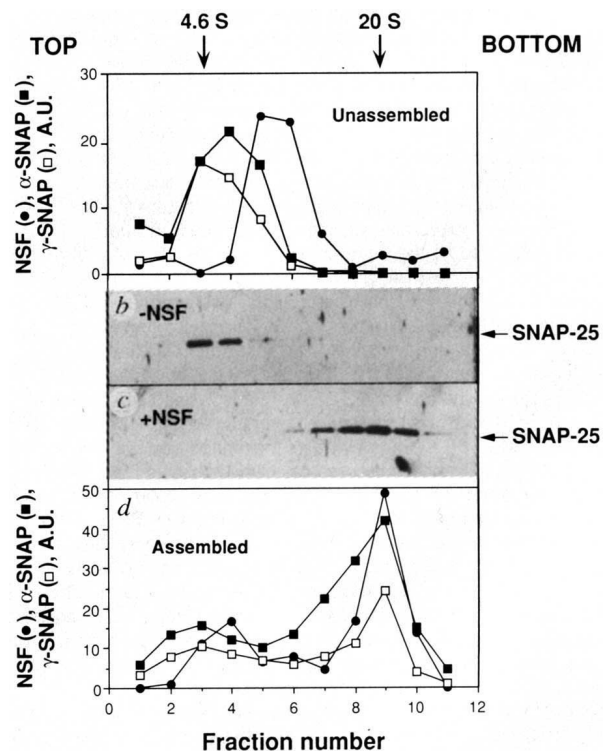
In this light, the fact that 20S particles can contain VAMP/synaptobrevin (from the synaptic vesicle) and syntaxin (from the plasma membrane) is of interest, because these complexes might form part of the fusion apparatus docking the vesicle to its target in the active zone. It is not yet possible to say whether syntaxin and VAMP/synaptobrevin exist together in a single 20S particle (Fig. 5a), or whether separately assembled particles join together to form an attachment site (Fig. 5b), perhaps with the aid of additional proteins.

The fact that molecules previously implicated in regulated exocytosis at the synapse are SNAP receptors implies that the same NSF- and SNAP-dependent machinery necessary for many constitutive fusion events also underlies triggered release of neurotransmitters at synapses. By extension, we would expect that NSF and SNAP are similarly employed in other forms of regulated exocytosis³⁴.

How can constitutive fusion machinery also be used in triggered exocytosis? An inhibitory component(s) must apparently prevent the 20S fusion particle from engaging, or from completely assembling in the first place. Such a fusion clamp could either cover an active site in NSF, SNAP or the SNAP receptors, or could prevent any of the additional cytoplasmic subunits of the fusion machinery³⁵ from binding. In the case of the synapse, a calcium trigger would remove the clamp. A strong candidate for such a clamp is the synaptic vesicle membrane protein synaptotagmin^{36,37}. Synaptotagmin co-immunoprecipitates with syntaxin²³, and the recombinant pro-

FIG. 4 Incorporation of SNAP-25 and other components into 20S fusion particles. *a, b*, Conditions in which 20S particles do not form (for example, controls); *c, d*, conditions in which 20S particles assemble. Components of the 20S fusion particle were incubated and then fractionated by glycerol gradient centrifugation, and the fractions analysed by SDS-PAGE to determine the location of α -SNAP and γ -SNAP, and to determine the locations of NSF and SNAP-25. Bovine serum albumin (4.6S) and α_2 -macroglobulin (20S) were used as standards.

METHODS. Reaction conditions were arranged so that the protein of interest would be maximally incorporated into 20S particles; thus, results in *a* and *d* are composites of several experiments. In the case in which the SNAPs were examined, *in vitro*-translated ³⁵S-labelled α -SNAP (■) and γ -SNAP (□) (ref. 17) ($\sim 2.1 \times 10^6$ c.p.m. for α - and 1.4×10^5 c.p.m. for γ -SNAP) were incubated on ice with bovine brain extract (1.2 mg protein), in the absence (*a*) or presence (*d*) of His₆-NSF (100 μ g protein) in 20 mM HEPES-KOH, pH 7.4, 100 mM KCl, 2 mM DTT, 2 mM EDTA, 0.5 mM ATP, 0.5% (v/v) Triton X-100 in a final volume of 0.5 ml. After 15 min, reactions were loaded onto a 10–35% (w/v) glycerol gradient¹⁸ and centrifuged in an SW41 rotor (Beckman) for 18 h at 40,000 r.p.m. Gradients were fractionated from the bottom at 1 ml per min, and an aliquot of each fraction analysed by SDS-PAGE and autofluorography. Recovery of both radiolabelled SNAPs exceeded 90%. Autofluorographs were scanned with a ScanJet Plus (Hewlett Packard) and the images integrated using Scan Analysis software (BioSoft, Cambridge). To examine NSF (●), His₆-NSF (ref. 15) (10 μ g protein) was incubated with each of the His₆-SNAPs (20 μ g each protein), either in the absence (*a*) or presence (*d*) of bovine brain extract (1.5 mg protein). Samples were incubated and fractionated as described. Aliquots were analysed by western blotting using an anti-His₆ antibody and blots were scanned as described¹⁷. To determine the extent to which SNAP-25 is incorporated into the 20S particle, bovine brain extract was added in limiting amounts (300 μ g protein) and incubated either in the absence (*b*) or presence (*c*) of both His₆-SNAPs and His₆-NSF (30 μ g each protein). Samples were processed and aliquots of each fraction analysed by western blotting using an antibody generated against the C-terminal peptide of SNAP-25. For this purpose, a peptide of



the sequence used in ref. 24 was synthesized but with an additional cysteine at the N terminus, and affinity-purified antibodies generated. Blots were visualized using the ECL System (Amersham). AU, arbitrary unit.

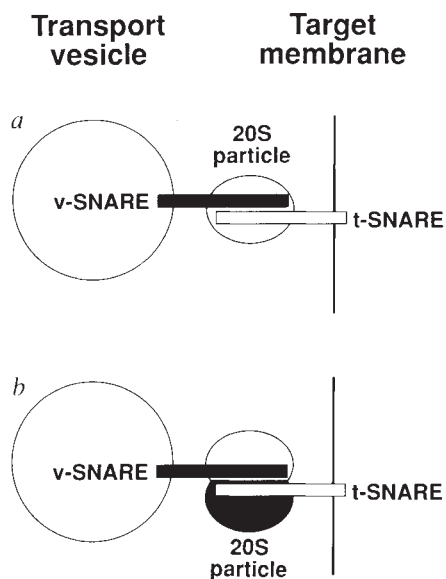


FIG. 5 Models to explain vesicle targeting based on the finding that SNAREs isolated in 20S fusion particles can originate from either the transport vesicle (v-SNAREs) or from the target membrane (t-SNAREs). A 20S particle (containing NSF and SNAPs) that simultaneously binds a v-SNARE and a t-SNARE (a) would attach a vesicle to its target. Alternatively (b), 20S particles, each capable of binding only one SNARE at a time, could interact to attach vesicle to target, a process that perhaps requires other proteins to assemble together.

teins can bind each other²³. It also undergoes a calcium-dependent conformational change³⁸. Although synaptotagmin is not one of the major components of the 20S particles characterized here, this is not surprising, as the interaction between syntaxin and synaptotagmin is disrupted by 0.5 M salt²³, and we used a 1 M salt wash.

Rab3A, a Ras-related GTP-binding protein, is another candidate for a clamp, as this protein dissociates from synaptic vesicles upon stimulation of fusion³⁹. Fusion clamps may respond to a variety of second messengers such as cyclic AMP, activated G proteins, protein phosphorylation, and so on, in addition to calcium, depending on cell type and physiological context.

SNAREs may encode specificity

Both syntaxin and VAMP/synaptobrevin have homologues in yeast^{40,42}. The yeast protein SED5 is related to syntaxin⁴⁰, and its absence blocks ER-to-Golgi transport, leading to the accumulation of transport vesicles; it appears to reside in the Golgi⁴⁰. Another homologue, PEP12, is found primarily on the vacuole membrane, and is required for Golgi-to-vacuole transport⁴¹. The yeast *SEC22*, *BET1* and *BOS1* gene products are related to VAMP/synaptobrevin, and are required for fusion with the Golgi *in vivo*. *BOS1* at least is a component of ER-derived transport vesicles^{16,42-45}. Other recent examples are discussed in ref. 46. SNAP-25 is distantly related to SED5, PEP12, and the syntaxins.

The synaptic SNAP receptors we have identified are thus members of a family of proteins which appear to be distributed in a compartmentally specific fashion, with one set attached to the transport vesicles (v-SNAREs) and another set attached to target membranes (t-SNAREs). This suggests a model in which each transport vesicle contains one or more members of the v-SNARE superfamily obtained on budding from a corresponding donor compartment, and every target compartment in a cell contains one or more members of the t-SNARE superfamily. Specificity in membrane transactions would be assured by the unique and non-overlapping distribution of v-SNAREs and t-SNAREs among the different vesicles and target compartments. In the simplest view, that is, if there were no other source of

specificity, only when complementary v-SNARE and t-SNARE pairs engage would a productive fusion event be initiated. Thus, a v-SNARE from the ER (possibly SEC22) could engage a t-SNARE from the Golgi (possibly SED5), but not one from the lysosome (possibly PEP12). For the proposed mechanism to work (again, assuming no other source of specificity), non-complementary SNAREs would have to be excluded from the same 20S particle (Fig. 5a) or multi-particle assemblies (Fig. 5b), although any one SNARE might be able to form a 20S particle by itself while waiting for a partner to arrive. If this is correct, the 20S fusion particle assembly reaction might be used as a cell-free 'read-out' system to test whether candidate proteins are SNAREs (as shown here for SNAP-25), and to establish which SNAREs, if any, form specific cognate pairs. Localization of the SNARE proteins *in situ* would then establish which are v-SNAREs and which are t-SNAREs and which compartments are involved in each pairing.

A related mechanistic question is whether the SNAPs (and NSF) are associated primarily with a v-SNARE or with a t-SNARE, or whether an essentially symmetrical structure is formed at the junction of vesicle and target. NSF and SNAPs can associate with Golgi membranes to form 20S particles in the absence of transport vesicles¹⁸, implying that 20S particles can be formed with only one SNARE partner. As native NSF is a tetramer of subunits with two ATPase domains each, it is easy to imagine several ways in which a 20S particle containing one NSF could form a symmetrical vesicle-target junction by simultaneously binding a v-SNARE and a t-SNARE (Fig. 5a).

Although many important details need to be established, our findings imply a general role for NSF and SNAP in regulated and constitutive intracellular membrane fusion processes, and in synaptic transmission in particular. The SNAREs we have identified appear to be members of compartment-specific membrane protein mutigene families which may form attachment sites between specific vesicles and their correct target membranes together with NSF and SNAPs. □

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LETTERS TO NATURE

A young source of optical emission from distant radio galaxies

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DISTANT radio galaxies provide valuable insights into the properties of the young Universe—they are the only known extended optical sources at high redshift and might represent an early stage in the formation and evolution of galaxies in general. This extended optical emission often has very complex morphologies, but the origin of the light is still unclear. Here we report spectroscopic observations for several distant radio galaxies ($0.75 \leq z \leq 1.1$) in which the rest-frame spectra exhibit featureless continua between 2,500 Å and 5,000 Å. We see no evidence for the break in the spectrum at 4,000 Å expected for an old stellar population^{1–3}, and suggest that young stars or scattered emissions from the active nuclei are responsible for most of the observed light. In either case, this implies that the source of the optical emission is comparable in age to the associated radio source, namely 10^7 years or less.

One of the keys to understanding the nature of distant radio galaxies is the study of their optical continuum, and multiple broad-band photometry has been extensively used to examine their spectral energy distribution, often in conjunction with stellar population synthesis models. Contamination from various sources, however, can hide the true properties, and obviously affects the interpretation of the data. For example, distant radio galaxies generally have very strong emission lines, which can contribute significantly to broad-band photometric measurements both at visible and near-infrared wavelengths^{4,5} (equivalent widths larger than several hundred angstroms); foreground galaxies⁶ and even galactic stars⁷ can project on the lines of sight. This is especially true when the broad-band photometry is performed on high redshift radio galaxies whose physical origin is still unclear and for which the multiple broad-band photometric data are at least as well fitted by simple power laws. To circumvent the problems inherent in such photometry^{6–8}, our goal here is to test the stellar contribution to the optical emission of distant radio galaxies from deep spectroscopic data.

The most convincing (stellar) model was presented by Lilly¹ and developed by Rigler *et al.*². It assumes that the red continuum emission is due to a symmetric giant elliptical galaxy which becomes dominant at observed infrared wavelengths, while the ultraviolet emission consists of a flat component responsible for the alignment with the radio axis. Rigler *et al.*² have called this interpretation the 'old star hypothesis', and named another stellar scheme developed by Chambers and

Charlot³ the 'range of ages model'. The latter assumes a significant contribution of red supergiant stars (asymptotic giant branch) to the initial mass function, and that the optical colours of distant radio galaxies are an indication of their ages. Both models are apparently compatible with the small scatter of the K-band-magnitude-redshift ($K-z$) diagram, and seem adequately to fit the multiple broad-band photometric properties of distant radio galaxies. With the blue and red populations contributing roughly equally to the flux at 4,000 Å (ref. 2) in the 'old star' hypothesis, the continuum should exhibit a 4,000 Å break (from the old stars (>1 Gyr)) with a slope flattening due to the contribution of the blue population. Similarly, the 'range of ages' model predicts a significant 4,000 Å break for all sources, and especially for the reddest ones. Another model, put forward by Bithell and Rees⁹, assumes a single and extremely young stellar component for distant radio galaxies and expects spectral signatures from young stars between 3,500 and 4,500 Å.

We have obtained deep red spectroscopy of a sample of distant radio galaxies with redshifts larger than 0.7 (beyond which the alignment effect becomes significant), and smaller than 1.1 (beyond which features above 4,700 Å enter the infrared window). One can successfully extract spectra of galaxies with red-magnitude (R) = 21.5 with a signal to noise ratio (S/N) ≥ 10 around 9,000 Å on 4-m class telescopes in 3 hours, with an excellent uniformity of the sky correction (that is, an average pixel-to-pixel deviation less than 10% of the corrected $R = 21.5$ continuum, or less than 0.1% of the sky background (O.L.F., manuscript in preparation)). The ability to detect features in the continuum of faint galaxies beyond $z = 1$ was demonstrated from our spectroscopy of the quasar 3CR245 companion¹⁰. This is a radio-quiet galaxy at $z = 1.013$, which has optical magnitudes more than 1 mag less than distant 3CR galaxies (visible magnitude $V = 22.4$), and for which the 4,000 Å break and the Ca I, H and K bands are remarkably well detected in only two integrations of 45 min each ($S/N > 10$). The galaxy morphology and the amplitude of the break ($D(4,000 \text{ Å}) = 2$; see Bruzual¹¹ for the definition) are characteristic of an elliptical galaxy, as is the detection of relatively strong absorption lines (Ca II, G band). For 3CR galaxies which have brighter magnitudes at optical wavelengths, one would therefore expect to identify spectral absorption features in the continuum in only a few hours of integration under similar observing conditions.

We observed 10 distant 3CR galaxies with redshifts ranging from 0.75 to 1.1. Their optical emission is aligned within 30° of the radio axis, and their infrared-visual colours range from $R-K = 2.5$ to 4, typical of distant radio galaxies. Some of our objects are red enough to be classified by Chambers and Charlot³ as older galaxies (≥ 1 Gyr), and although we miss some of the reddest objects such as 3CR65, we expect this sample adequately to describe the properties of most of the $z > 0.7$ powerful radio galaxies. Figure 1 shows the spectra of 3CR54, 265 and 343.1, compared to the galaxy companion of 3C245 and the residual noise extracted from the two-dimensional data. The S/N on the continuum at 4,000 Å is 9, 8, 12, 12 for these four objects respectively. As can be seen in the examples in Fig. 1, despite