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Association of *N*-ethylmaleimide-sensitive factor with synaptic vesicles

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Abstract

N-Ethylmaleimide-sensitive factor (NSF) mediates docking and/or fusion of transport vesicles in the multi-pathways of vesicular transport. NSF was highly expressed in brain and adrenal gland. Immunostaining of cerebellum with an anti-NSF monoclonal antibody showed that NSF is predominantly localized in the molecular layers and the glomeruli of the granule cell layers. This distribution coincided well with that of synaptophysin, a marker protein of synaptic vesicles. Purification and immunoprecipitation revealed that NSF is associated with brain synaptic vesicles. The present results suggest that NSF is associated with synaptic vesicles without Ca^{2+} influx.

Key words: Synaptic vesicle; Vesicular transport; Neurotransmitter

1. Introduction

N-Ethylmaleimide-sensitive factor (NSF) was first characterized as a protein that mediates intra-Golgi protein transport reconstituted in a cell-free system [1,2]. This protein is an *N*-ethylmaleimide-sensitive ATPase [3] and contains two homologous nucleotide-binding domains [3,4]. Site-directed mutagenesis study revealed that the two homologous regions are important for both ATPase and protein transport activities [5]. Several lines of evidence suggest that NSF most likely promotes fusion of freshly uncoated non-clathrin-coated vesicles with the target Golgi cisternae [6,7].

NSF forms a 20 S complex, which comprises, other than NSF, soluble NSF attachment proteins (SNAPs) and SNAP receptors (SNAREs) [8]. By using a reconstitution system of the 20 S NSF complex, Söllner et al. [9] recently identified that syntaxin, SNAP-25, and synaptobrevin are components of the SNARE complex. The localization of the three SNAREs (syntaxin and SNAP-25 are present on the plasma membrane and synaptobrevin is on synaptic vesicles) made Söllner et al. [9] propose a docking model of synaptic vesicles with the plasma membrane via the 20 S NSF complex. According to their model, Ca^{2+} promotes the dissociation of synaptotagmin, a Ca^{2+}-binding protein in the synaptic vesicles, from the SNARE complex, which allows the association of NSF with the SNARE complex via SNAP. Subsequent hydrolysis of ATP by NSF causes the disassembly of the 20 S complex and triggers the fusion of synaptic vesicles with the plasma membrane [10]. This hypothesis is quite an attractive explanation of the mechanism of Ca^{2+}-regulated neurotransmitter secretion, but remains to be elucidated. It is known that NSF is present in membranes as well as in cytosol [2]. NSF may form the 20 S complex on synaptic vesicles without Ca^{2+} influx. This idea was supported by the present observation that NSF is associated with synaptic vesicles.

2. Materials and methods

2.1. Materials

An anti-NSF monoclonal antibody (MAb 2E5) was prepared as described previously [3]. A monoclonal antibody against synaptophysin (SY38) was purchased from Progen. Avidin–biotin–peroxidase complex (ABC) kits for immunostaining were purchased from Vector Laboratories.

2.2. Light microscopy

Adult male Sprague–Dawley rats were successively perfused from the left ventricle with the following solutions: Hanks' solution (pH 7.4) for 2 min, Hanks' solution (pH 7.4) containing 4% paraformaldehyde and 0.1% glutaraldehyde for 10 min, and PBS containing 50 mM NH_4Cl for 5 min. The small brains were removed, cut, and frozen in liquid nitrogen. Frozen sections were prepared with a cryostat and mounted on gelatin-coated glass slides. The sections were preincubated for 30 min in phosphate-buffered saline (PBS) containing 0.3% H_2O_2 to inactivate endogenous peroxidase activity. For the staining of synaptophysin, this step was omitted because the H_2O_2 treatment significantly reduced immunoreactivity. The preincubated sections were immersed for 15 min in PBS containing 20% normal goat serum, 0.5% bovine serum albumin, and 0.3% Triton X-100, and then incubated for 2 h with MAb 2E5, anti-synaptophysin antibody, or control mouse IgG. After washing with PBS containing 0.3% Triton X-100, the sections were incubated for 30 min with 5 μg/ml biotinylated goat anti-mouse IgG in PBS containing 0.5% bovine serum albumin and 0.3% Triton X-100 for 30

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Abbreviations: NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.

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min. After washing with PBS, the biotinylated sections were incubated for 30 min with ABC in PBS [11,12] and developed with 0.1% 3,3-diamino-benzidine in 50 mM Tris-HCl (pH 7.4) containing 0.02% H_2O_2. The reaction was enhanced by the treatment of 0.1% aqueous OsO_4 for 1 min.

2.3. Preparation of organelles

Rat brain synaptosomal fractions and synaptic vesicles were prepared according to the method of Huttner et al. [13]. For the final purification step of synaptic vesicles, the permeation chromatography on controlled-pore glass was replaced by Sephacryl S-1000 chromatography. For experiments to characterize synaptic vesicle-associated NSF, peak fractions of synaptophysin on sucrose density gradient centrifugation were used. Golgi membranes from Chinese hamster ovary (CHO) cells were prepared as described by Balch et al. [14]. Crude synaptic vesicles were prepared from bovine brain as described by Kishi and Ueda [15].

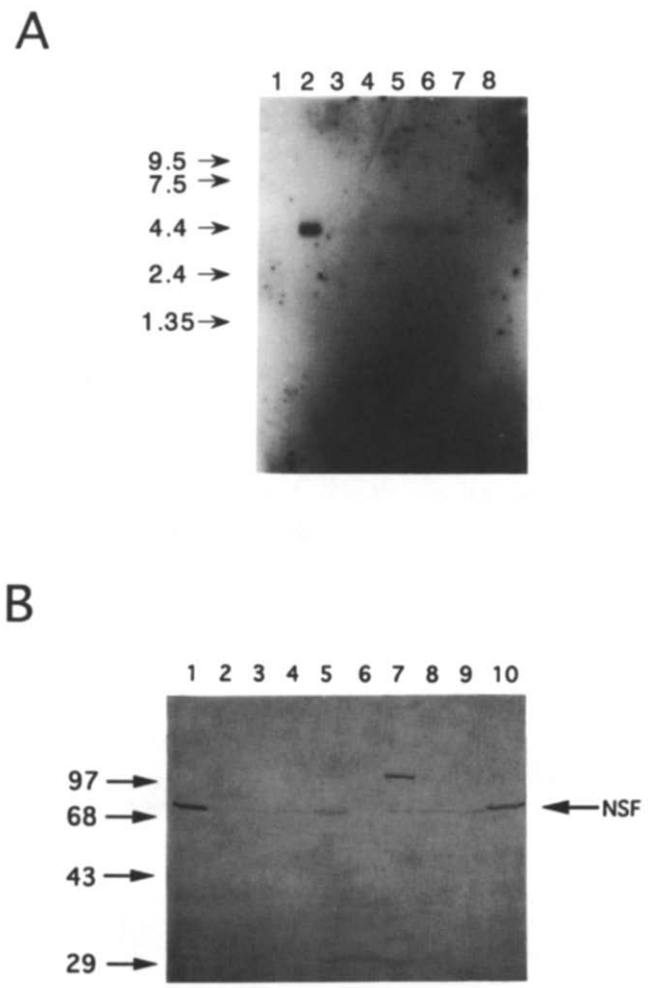

Fig. 1. Expression of NSF in various tissues. (A) Expression of mRNA for NSF in various human tissues. Each lane contained 2 μg of highly pure poly(A^+) RNA from heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and pancreas (lane 8). Arrows indicate the positions of markers. (B) Expression of NSF protein in various rat tissues. Rat tissue homogenates in 10 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose and 1 mM phenylmethylsulfonyl fluoride were centrifuged at 1,000 × *g* for 10 min. The supernatants (100 μg each) were subjected to SDS-PAGE with a 10% gel and immunodetected with MAb 2E5. Brain (lane 1), heart (lane 2), kidney (lane 3), lung (lane 4), liver (lane 5), muscle (lane 6), pancreas (lane 7), spleen (lane 8), testis (lane 9), and adrenal gland (lane 10). Arrows indicate the positions of molecular weight markers.

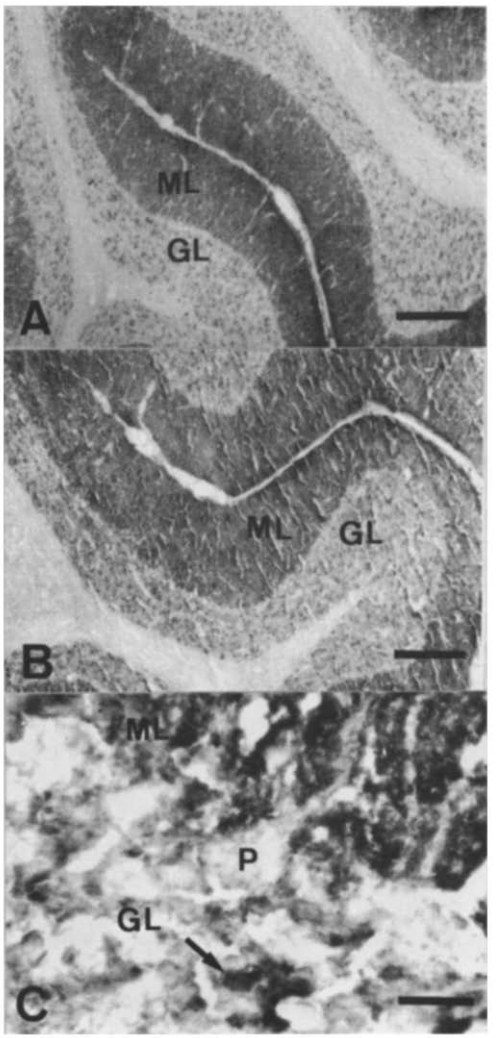

Fig. 2. Distribution of NSF in rat cerebellum. Rat cerebellum was immunostained by using a monoclonal antibody against synaptophysin (A) or NSF (2E5) (B and C). A and B, bar = 1 mm; C, bar = 0.1 mm. P, ML, and GL represent a Purkinje cell, the molecular layer, and the granule cell layer, respectively. An arrow indicates the location of a glomerulus in the granule cell layer.

2.4. Northern blot analysis

A human multiple Northern blot (Clontech) was prehybridized at 65°C for 3 h in 5 × SSPE, 10 × Denhardt's solution, 0.1 mg/ml freshly denatured salmon sperm DNA, and 2% SDS. Hybridization was conducted at 65°C overnight in the same solution plus a randomly ^{32}P-labeled probe (position 734-1228 of CHO NSF). The blot was washed twice at room temperature for 20 min in 2 × SSC containing 0.05% SDS and twice at 50°C for 20 min in the same solution, and then exposed to X-ray film.

3. Results

Northern blot analysis of human tissues showed that a 4.4-kb NSF mRNA is highly expressed in brain (Fig. 1A). This length coincided well with that of CHO NSF [4]. Cloning and sequencing revealed that human brain NSF (2232 bp; GenBank accession number U03985)

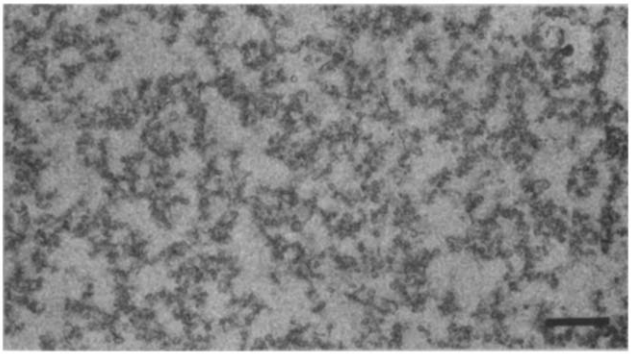

Fig. 3. Electron micrograph illustrating the morphology of the synaptic vesicle fractions on sucrose density gradient centrifugation. Bar = 400 nm.

shares 90% and 97% identity with CHO NSF in DNA and amino acid sequence, respectively [4]. Immunoblotting of rat tissues with MAb 2E5 confirmed the high expression of NSF protein in brain (Fig. 1b). Adrenal gland also highly expressed NSF (Fig. 1B).

We examined the distribution of NSF in rat cerebellum and compared it with that of synaptophysin, a marker protein of synaptic vesicles. Synaptophysin was predominantly localized in molecular layers where nerve endings are concentrated (Fig. 2A). The distribution of NSF (Fig. 2B) was quite similar to that of synaptophysin. At high magnification, immunoreactivity was observed in glomeruli of granule cell layers and presynaptic terminals around the dendrites of Purkinje cells (Fig. 2C). The presence of NSF in the soma of Purkinje cells was not significant, although the Golgi apparatus is located in the cell body. This may reflect the lower density of NSF in the Golgi regions compared with the nerve endings.

To investigate whether NSF is associated with synaptic vesicles, the vesicles were purified from rat brain by the method of Huttner et al. [13] and immunodetected with MAb 2E5. It is known that synaptic vesicles from rat brain can be purified to near homogeneity by this method. First, a synaptosomal fraction was obtained by differential centrifugation of the brain homogenate. Synaptic vesicles were further purified by differential centrifugation and sucrose density gradient centrifugation after hypoosmotic lysis of the synaptosomes. Electron microscopic analyses revealed that the synaptic vesicles thus prepared are highly homogeneous (Fig. 3). Minor contaminants in this preparation were removed by gel-permeation on Sepharcryl S-1000. As expected, this step gave essentially one protein peak (Fig. 4A). Immunoblot analysis revealed that NSF was co-purified with synaptophysin (Fig. 4B). The content of NSF in rat synaptic vesicles was about 0.5% of total synaptic vesicle proteins. This content was about 2-fold higher than that of NSF in CHO Golgi membranes (data not shown). The enrichment of the vesicle marker protein synaptophysin was estimated by immunoblotting to be approximately 14-fold over the homogenate (data not shown), which is comparable to the values reported previously [16,17].

Immunoprecipitation of synaptic vesicles confirmed the association of NSF with synaptic vesicles (Fig. 5). The precipitate by anti-synaptophysin antibody-attached beads contained NSF as well as synaptophysin. Control antibody-attached beads precipitated a small amount of synaptic vesicles, but the amount was as much as that of the precipitate by uncoated beads.

NSF is dissociated from Golgi membranes by a process coupled with ATP hydrolysis [1]. This reflects the disassembly of the 20 S complex consisting of NSF, SNAPs, and SNAREs. We examined the effect of Mg^{2+}-

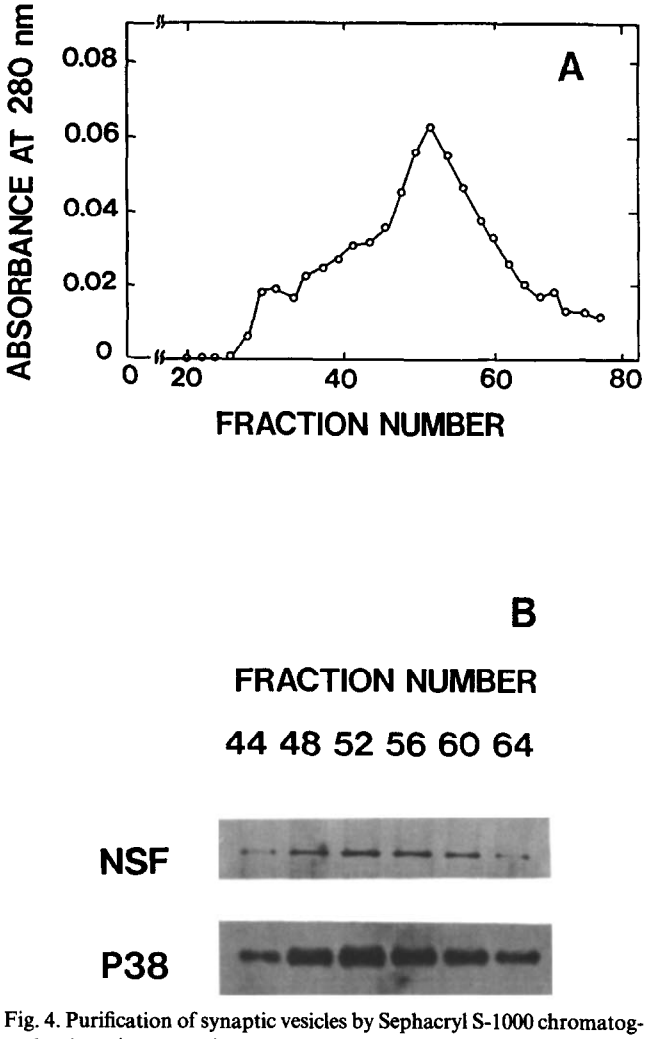

Fig. 4. Purification of synaptic vesicles by Sephacryl S-1000 chromatography. Fractions containing synaptic vesicles on sucrose density gradient centrifugation were applied to a Sephacryl S-1000 column (1.6 × 85 cm) equilibrated with 5 mM HEPES (pH 7.2) containing 300 mM glycine. The column was developed by the same buffer at a flow rate of 10 ml/h, and fractions (1.7 ml each) were collected. (A) Elution profile. (B) Aliquots of fractions were subjected to trichloroacetic acid precipitation followed by SDS-PAGE with a 10% gel. NSF and synaptophysin (p38) were immunodetected by MAb 2E5 and an anti-synaptophysin antibody, respectively.

ATP on the binding of NSF to synaptic vesicles. Fig. 6 shows that NSF in synaptic vesicles is not released by Mg^{2+}-ATP. In a control experiment using CHO Golgi membranes, NSF was released by ATP in a Mg^{2+}-dependent manner. These results suggest that the binding mode of NSF in synaptic vesicles is different from that of NSF in Golgi membranes.

4. Discussion

Northern blot and immunoblot analyses showed that NSF is highly expressed in brain. This observation is essentially the same as recent results by Püschel et al. [18]. Immunocytochemical and biochemical analyses revealed that NSF is associated with synaptic vesicles. NSF associated with the vesicles was not released by Mg^{2+}-ATP, the condition which favors the release of NSF from Golgi membranes [1]. This suggestes that the binding modes of NSF to the two membranes are different. Based on the kinetic experiments using a cell-free intra-Golgi protein transport assay, Wattenberg et al. [19] suggested that NSF is incorporated into transport vesicles in a way not to exchange with exogenously added NSF. Similar results were obtained by using mutant NSF proteins that significantly inhibit intra-Golgi protein transport [5]. There seems to be some mechanism to make NSF tightly bind to transport vesicles.

By using a reconstitution system of the 20 S NSF complex, Söllner et al. [9] demonstrated that syntaxin, SNAP-25, and synaptobrevin are SNAREs. Based on the finding that α-SNAP can displace synaptotagmin from the SNARE complex, they hypothesized that Ca^{2+} allows the interaction between SNAP and NSF by promoting the dissociation of synaptotagmin from the SNARE complex. Subsequent hydrolysis of ATP by NSF results in the disassembly of the 20 S complex, which promotes membrane fusion [10]. O'Connor et al. [20] argued that this model cannot explain the rapid Ca^{2+}-triggered exocytosis. In addition, a Ca^{2+} requirement was not demonstrated for the interaction between synaptotagmin and α-SNAP [10]. O'Connor et al. [20] proposed an alternative model in which NSF and SNAP play a role in docking of synaptic vesicles into a prefusion state upon ATP hydrolysis by NSF, and then synaptotagmin binds to the 20 S complex in a conformation that can be regulated by Ca^{2+}. According to their model, NSF should be present in the 20 S complex without Ca^{2+} influx. The present observation that NSF is associated with synaptic vesicles favors the latter model.

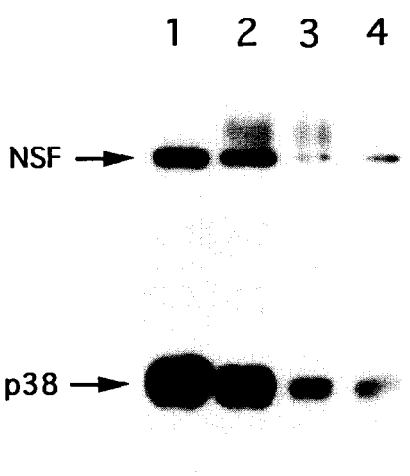

Fig. 5. Immunoprecipitation of synaptic vesicles with an anti-synaptophysin antibody. 1 ml of crude synaptic vesicle fractions (5 mg/ml) from bovine brain was gently stirred at 4°C overnight in the presence of 10 μg of IgG (anti-synaptophysin antibody (lane 2) or control mouse IgG (lane 3)) or absence of IgG (lane 4). 50 μl of anti-mouse IgG-attached Dynabeads M-280 was added to the vesicle fractions and incubated at 4°C for 2 h with stirring. The beads were magnetically separated from the solutions, washed with PBS, suspended in sample buffer, and subjected to SDS-PAGE with a 10% gel. NSF and synaptophysin (p38) were immunodetected by a mixture of MAb 2E5 and anti-synaptophysin antibody. As a marker, rat brain synaptic vesicles (10 μg) were immunodetected (lane 1).

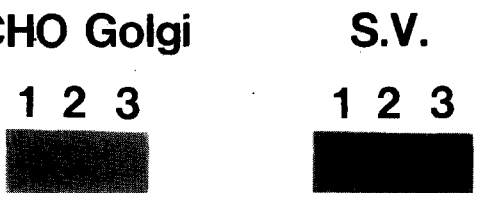

Fig. 6. Retention of NSF on synaptic vesicles in the presence of Mg^{2+}-ATP. CHO Golgi membranes or synaptic vesicles (100 μl) in 25 mM HEPES (pH 7.2) containing 1 mM dithiothreitol, 0.1 M KCl, 0.3 M sucrose, and 2% polyethylene glycol 4000 plus 5 mM $MgCl_2$-0.5 mM ATP or 2 mM EDTA-0.5 mM ATP were incubated on ice for 5 min. The mixtures were then centrifuged at 400,000 × g for 30 min. The supernatants, concentrated by trichloroacetic acid precipitation, were subjected to SDS-PAGE with a 10% gel and analyzed by immunoblotting with MAb 2E5. The whole incubation mixture as a control (lane 1), the Mg^{2+}-ATP-supernatant (lane 2), and the EDTA-ATP-supernatant (lane 3).

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