NSF and SNAP are present on adrenal chromaffin granules

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Abstract ***N*-ethylmaleimide sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs) are involved in many vesicular transport steps. It has been proposed that SNAPs and NSF associate with their membrane receptors only when vesicles dock on the target membrane. Analysis of NSF and α-SNAP distribution in fractionation of organelles from adrenal medulla indicated that a substantial amount of both proteins distributed with chromaffin granules. Further fractionation of intact granules and lysed granule membranes showed exact overlap of NSF and α-SNAP distribution with chromaffin granules. These results suggest that NSF and α-SNAP are associated with chromaffin granules and support the idea that they function prior to docking of the granules on the plasma membrane.**

Key words: Exocytosis; Chromaffin granule; SNAP; NSF; Vesicular traffic; secretion

1. Introduction

Multiple vesicular traffic steps within cells involve the *N*-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs) [1]. NSF was identified as an essential protein for intra-Golgi transport [2] and was found to require α-, β- or γ-SNAPs for its association with Golgi membranes [3,4]. The homologues of these proteins in yeast, sec 18 and sec 17, are essential for the function of the secretory pathway in this organism [5]. NSF and SNAPs were implicated in regulated exocytosis from the finding that they associate, in membrane extracts, with three synaptic proteins VAMP, syntaxin and SNAP-25 [6,7] known from their sensitivity to clostridial neurotoxins to be essential for neurotransmission [8,9]. The ability of these membrane proteins to bind SNAPs led to their description as SNAP receptors (SNAREs).

Based on protein interactions in detergent-solubilised membrane extracts, it was proposed that synaptic vesicle docking could be mediated by the vesicle protein VAMP binding to syntaxin and SNAP-25 which were believed to be solely localised to the plasma membrane [7]. SNAP and NSF would only then associate with this proposed docking complex to form a 20S protein complex [7]. ATP hydrolysis by NSF would then lead to disassembly of the complex as a step leading to exocytosis [7]. Recent work on the localization of these proteins in neurons and neuroendocrine cells has cast doubt on this model. Both syntaxin and SNAP-25 have been found on the synaptic vesicle [10,11] and so has NSF [12]. One potential explanation for these findings is that the synaptic vesicle is formed by recycling from the plasma membrane [13] and syntaxin and SNAP-25 may have been internalized with the vesicle during its initial formation or subsequent recycling.

VAMP, syntaxin and SNAP-25 are expressed by chromaffin cells [14,15] and exocytosis is sensitive to clostridial neurotoxins [16] indicating the importance of these proteins in chromaffin cells. A functional role for α-SNAP and NSF has been suggested from the stimulatory effect on exocytosis in chromaffin cells of exogenous α-SNAP [17–19]. The chromaffin granule membrane has been shown to contain syntaxin [20] and more SNAP-25 [21,22]. The latter findings are significant since the chromaffin granules are not initially formed by recycling from the plasma membrane but by budding from the trans-Golgi network [13]. The presence of these proteins on the chromaffin granules must require an alternative model to explain their function. In this study we have examined whether NSF and α-SNAP are present on the chromaffin granule. The data presented suggest that they are present, indicating that they may be able to exert their function prior to granule docking on the plasma membrane.

2. Materials and methods

2.1. Fractionation of post-nuclear organelles on sucrose gradients

The adrenal medullas were dissected from bovine adrenal glands and homogenised in buffer A (0.3 M sucrose, 5 mM EDTA, 5 mM Hepes, pH 7.3) filtered through muslin to remove any disrupted tissue and twice centrifuged at 800×*g* for 15 min to sediment nuclei. The post-nuclear supernatant was centrifuged at 100 000×*g* for 60 min at 4°C to sediment post-nuclear organelles. The pellet was resuspended in buffer A, loaded on a 0.3–2.0 M sucrose gradient, centrifuged at 100 000×*g* for 90 min at 4°C and twelve 1-ml fractions collected for analysis.

2.2. Preparation of chromaffin granules and fractionation on sucrose gradients of granule or granule membranes

For the preparation and fractionation of a large granule fraction [23], the post-nuclear supernatant from homogenised adrenal medullas was centrifuged at 17 000×*g* for 20 min at 4°C. Mitochondria were carefully washed from the surface of the pellet which was then resuspended in buffer A and overlaid on a cushion of 1.7 M sucrose in 5 mM EDTA, 5 mM Hepes pH 7.3 and centrifuged at 100 000×*g* for 60 min. The pellet of intact granules (large granule fraction) was resuspended in buffer A, loaded on a 0.3–2.0 M sucrose gradient, centrifuged at 140 000×*g* for 3 h and twelve 1-ml fractions collected with the pellet resuspended as fraction 13.

For the fractionation of granule membranes the pellet containing the intact granules from the large granule fraction above was lysed by resuspension in buffer B (5 mM EDTA, 20 mM Hepes pH 7.3) and freezing and thawing. The membranes were washed once by centrifugation in buffer B, resuspended in 0.3 M sucrose, loaded on a 0.3–1.2 M sucrose gradient and centrifuged at 115 000×*g* for 90 min. Twelve 1-ml fractions were collected with the pellet as fraction 13.

2.3. SDS-PAGE and immunoblotting analysis

The various fractions were separated on 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose and samples analysed by immunoblotting with anti-α-SNAP (1:1000), anti-NSF (1:1000) [17], anti-dop-

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Abbreviations: DBH, dopamine-β-hydroxylase; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; VAMP, vesicle associated membrane protein

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amine β-hydroxylase monoclonal antibody (HBT 4214 at 1:100) or anti-p38 monoclonal (Sigma at 1:1000) as described previously [14]. Anti-α-SNAP was a gift from Dr T. Levine (ICRF, London) and HBT 4214 from Dr D. Apps (Department of Biochemistry, University of Edinburgh). Blots were developed using enhanced chemiluminescence (Amersham plc, Bucks, UK) and quantified by densitometry using Image Quant software (Molecular Dynamics).

3. Results

Antisera raised against NSF and α-SNAP were initially used to probe adrenal cytosol and chromaffin granule membrane fractions. Both NSF and α-SNAP were readily detected in the granule membrane fraction but little was detected in cytosol. To examine the relative distribution of NSF and α-SNAP between organelles in adrenal chromaffin cells and in particular to ensure that the NSF and α-SNAP in the granule membrane fraction did not result from contamination with synaptic-like vesicles, post-nuclear organelles were fractionated on a 0.3–2.0 M sucrose gradient. The distribution of organelle markers on such gradients has been well characterised for adrenal medulla and it has been established that intact chromaffin granules sediment to higher density than any other post-nuclear organelles [24–26]. Fig. 1A shows samples from the gradient probed with anti-DBH, -p38, -NSF or -α-SNAP. For comparison the blots were quantified and the data plotted normalised to the most intense lane (Fig. 1B, C). A peak of the granule membrane marker dopamine-β-hydroxylase (DBH) in fraction 9 represents the position of intact granules (Fig. 1B). A second peak of DBH in the less dense fractions (peak at fraction 6) is likely to represent lysed granule membranes [24]. The peak of intact granules was well separated from the peak of synaptic-like vesicles detected by the vesicle marker p38 (synaptophysin). A low but detectable level of p38 in fraction 9 is consistent with previous reports of the presence of low amounts of p38 in the chromaffin granule membrane [25]. From immunoblotting, the majority of NSF was distributed in two peaks (Fig. 1C) which overlapped with the two DBH positive peaks. α-SNAP was also detected in a peak corresponding to intact granules (fraction 9). A larger proportion of α-SNAP was distributed in a broad peak over the lighter fractions of the gradient (fractions 1–6) where many other cell organelles sediment [25,26]. The most significant result from the analysis of these gradients is that a dense fraction of intact chromaffin granules is well separated from synaptic-like vesicles and contains substantial amounts of the total NSF and α-SNAP.

Further analysis of chromaffin granule membranes was carried out to establish that NSF and α-SNAP were indeed present on the granules and not a contaminating membrane. First, intact granules within the so-called 'large granule frac-

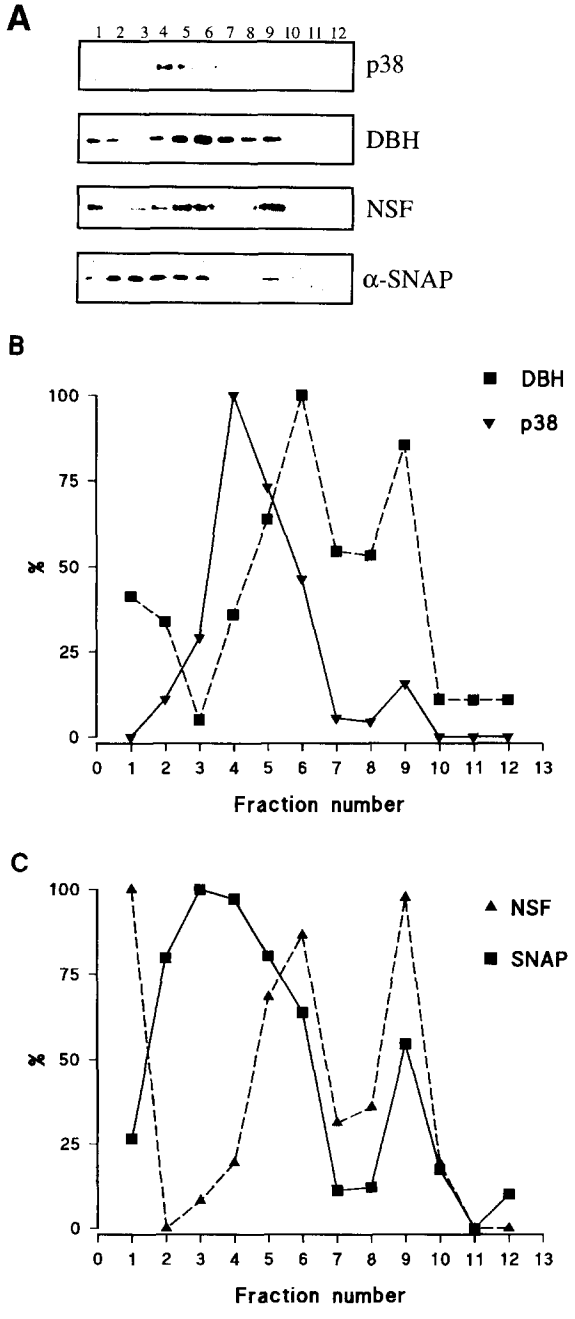

Fig. 1. Fractionation of post-nuclear organelles from adrenal medulla. A. Fractions were probed by immunoblotting with antisera against p38, DBH, NSF and α-SNAP as indicated. B, C. Distribution of DBH, p38, NSF and α-SNAP across the gradient shown normalised to the fraction with the highest signal for each protein.

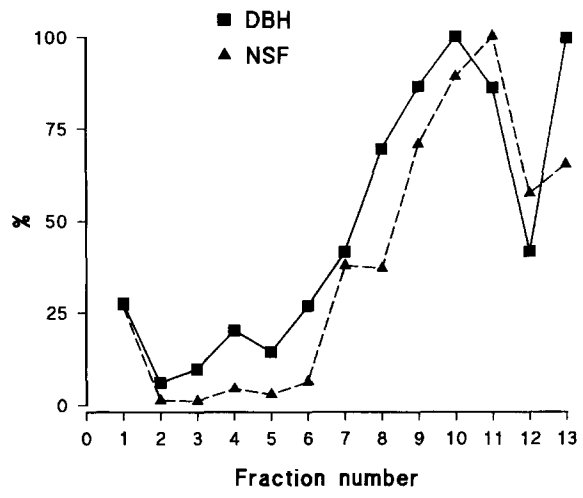

Fig. 2. Fractionation of a large granule fraction from adrenal medulla. A large granule fraction was separated on a 0.3–2.0 M sucrose gradient and the distribution of DBH and NSF determined by immunoblotting and quantification. The data are shown normalised to the fraction with the highest signal for each protein. Fraction 13 corresponds to the pellet from the gradient.

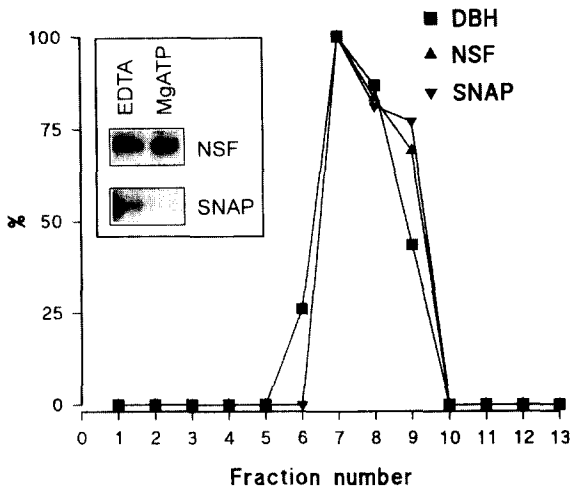

Fig. 3. Fractionation of lysed granule membranes and dissociation of α-SNAP but not NSF in the presence of MgATP. A large granule fraction was lysed, washed and the granule membrane fractionated on a 0.3–1.2 M sucrose gradient. The distribution of DBH, NSF and α-SNAP was determined by immoblotting and quantification and the data normalised to the fraction with the highest signal for each protein. Fraction 13 corresponds to the pellet from the gradient. Inset. Washed membranes from a large granule fraction were incubated for 10 min in 5 mM EDTA, 20 mM HEPES pH 7.3 or 2 mM MgATP, 20 mM HEPES, pH 7.3 at 20°C as indicated. The membranes were washed once by centrifugation in the appropriate buffer and analysed by SDS-PAGE and immunoblotting.

tion' [23] were separated on a 0.3–2.0 M sucrose gradient run for a longer time than above to sediment the dense granules further down the gradient. As seen in Fig. 2 the dense granules marked by DBH sedimented to a peak extending over fractions 7–11 which overlapped with NSF distribution. The high bouyant density of chromaffin granules is due to the dense proteinaceous granule core and so, in order to shift the granule membranes away from any contaminating dense material, the granules can be lysed and washed and the membranes will then sediment at much lower densities. Lysed and washed granule membranes were fractionated on a 0.3–1.2 M gradient and DBH was detected in a peak over fractions 7–9 (Fig. 3). Both α-SNAP and NSF distribution exactly overlapped that of DBH. No NSF or α-SNAP were detected in the pellet from the gradient (fraction 13) indicating that essentially all of the NSF and α-SNAP shifted density with the granule membrane following granule lysis and thus confirming their presence on the granule membrane.

NSF associated with Golgi membrane dissociates in the presence of MgATP whereas synaptic vesicle NSF does not. In order to compare the behaviour of NSF and α-SNAP on granule membranes from a lysed large granule fraction, these were washed with an EDTA-containing buffer or a buffer containing 2 mM MgATP. In the presence of MgATP the majority of α-SNAP was lost from the granule membranes but in contrast NSF remained bound (Fig. 3, inset).

4. Discussion

From analysis of the distribution of α-SNAP and NSF in post-nuclear organelles fractionated by sucrose density gradient centrifugation, it is clear that a significant proportion of adrenal medullary α-SNAP and NSF co-distribute with the chromaffin granule marker DBH. The presence of α-SNAP and NSF on chromaffin granules was confirmed by first showing co-distribution of DBH during sucrose-gradient fractionation of a large granule fraction but also with chromaffin granule membranes on a sucrose gradient after the density shift due to granule lysis. The latter procedure would rule out the possibility that the presence of α-SNAP and NSF co-distributing with DBH was due to an α-SNAP and NSF-rich contaminant with a similar density to the intact chromaffin granules. It was also noted that light membrane fractions contained high levels of α-SNAP but little NSF. The reason for this different distribution of the two proteins in these fractions is unknown.

One important issue is whether the presence of α-SNAP and NSF on chromaffin granules was due to redistribution following homogenisation. This is unlikely for two reasons. First, little α-SNAP or NSF were detected in a cytosol fraction indicating that there is not a substantial soluble pool of the proteins that could be recruited to membranes. Second, α-SNAP and NSF that are membrane associated are removed from Golgi membranes in the presence of MgATP due to ATP hydrolysis by NSF [27]. In order to prevent dissociation and possible re-distribution of α-SNAP and NSF all of the fractionation procedures were carried out in the presence of 5 mM EDTA to chelate Mg^{2+} and prevent hydrolysis of endogenous ATP. When isolated chromaffin granule membranes were treated with EDTA or MgATP containing buffers it was found that α-SNAP dissociated from the membranes in the presence of MgATP. In contrast, NSF remained membrane bound as previously reported for synaptic vesicle NSF [12].

The analysis presented here suggests that α-SNAP and NSF are present on chromaffin granules and is consistent with the finding of NSF on synaptic vesicles [12] and clathrin-coated vesicles [28]. Synaptic vesicles also possess all three of the SNAP receptors (SNAREs) [10,11] and it is now clear apparent that chromaffin granules possess all components that make up the 20S complex, VAMP [14,24] syntaxin [20], SNAP-25 [21,22] and now α-SNAP and NSF. It is not known whether the 20S complex is assembled on the undocked chromaffin granule within intact cells. Despite the fact that few chromaffin granules are docked at the plasma membrane, VAMP can be co-immunoprecipitated with syntaxin and SNAP-25 from detergent extracts of chromaffin cell membranes [14] but it is known that components of this complex can associate follow detergent solubilisation of membranes [29]. Nevertheless, the findings that all components of the 20S complex are present on isolated chromaffin granules raises the possibility that it can assemble on the undocked granule and this would be consistent with growing evidence, for exocytosis [18,30] and other fusion events [32,33], that α-SNAP and NSF have pre-docking roles in a chaperone-like manner [31] rather than only associating with the SNAREs when vesicles dock on their target membrane. An action of SNAP and NSF on undocked secretory granules could not exclude additional actions of SNAP and NSF such as on plasma membrane syntaxin [34] or recycling of the SNARE complex [33].

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