A common mechanism for the regulation of vesicular SNAREs on phospholipid membranes

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The SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) family of proteins is essential for membrane fusion in intracellular traffic in eukaryotic organisms. v-SNAREs (vesicular SNAREs) must engage target SNAREs in the opposing membrane to form the fusogenic SNARE complex. Temporal and spatial control of membrane fusion is important for many aspects of cell physiology and may involve the regulation of the SNAREs resident on intracellular membranes. Here we show that the v-SNARE synaptobrevin 2, also known as VAMP (vesicle-associated membrane protein) 2, is restricted from forming the SNARE complex in chromaffin granules from adrenal medullae to the same degree as in brain-purified synaptic vesicles. Our analysis indicates that the previously reported synaptophysin–

synaptobrevin interaction is not likely to be involved in regulation of the v-SNARE. Indeed, the restriction can be reproduced for two distinct v-SNARE homologues, synaptobrevin 2 and cellubrevin/VAMP3, by reconstituting them in pure liposomal membranes. Overall, our data uncover a common mechanism for the control of SNARE engagement where intact phospholipid membranes rather than proteins down-regulate vesicular SNAREs in different cellular organelles.

Key words: chromaffin granule, phospholipid membrane, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE), synaptobrevin, vesicle-associated membrane protein (VAMP).

INTRODUCTION

A conserved family of SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) proteins is required for fusion in all intracellular compartments [1–3]. SNAREs that are present on vesicles destined for fusion are called v-SNAREs (vesicular SNAREs) whereas SNAREs residing in the target membrane are referred to as t-SNAREs (target SNAREs). One v-SNARE homologue, synaptobrevin 2, also known as VAMP (vesicle-associated membrane protein) 2, functions in the fusion of secretory vesicles with the plasma membrane in neurons and endocrine cells, whereas another homologue, cellubrevin (VAMP3), is found in endocytic compartments in a wide variety of cells [2–4]. Upon their engagement, v- and t-SNAREs form an extremely stable α -helical bundle which probably drives fusion [5]. SNARE complexes that are SDS-resistant are assembled *in vivo*, presumably at the point of membrane fusion [6,7].

It is essential for cell physiology that membrane fusion should take place with high spatial and temporal precision. Prevention of uncontrolled formation of the SNARE complex may be one important point for control of membrane fusion. Recently, we found that synaptobrevin 2 on synaptic vesicles is restricted from forming the SNARE complex due to its properties on the phospholipid membrane [8]. Synaptophysin, a vesicular tetra-span membrane protein, has been also implicated in blocking the entry of synaptobrevin 2 into the SNARE complex [9–12]. To better understand the molecular basis and extent of v-SNARE regulation, we analysed the behaviour of synaptobrevin 2 in chromaffin granules using soluble t-SNAREs as a molecular probe. We now provide the first evidence that synaptobrevin 2 is restricted from SNARE complex formation in chromaffin granules, which have negligible amounts of synaptophysin, indicating that factors other

than synaptophysin are likely to be responsible for the restriction. Importantly, the cytoplasmic part of synaptobrevin 2 has been shown to interact with phospholipid membranes [13–15]. By probing the availability of synaptobrevin 2 and its homologue cellubrevin on reconstituted phospholipid membranes, we now demonstrate that an intact phospholipid membrane serves as a general regulator for the SNARE motif of vesicular SNAREs. Interestingly, restriction of v-SNAREs on the phospholipid membrane can be overcome, to a degree, by either long incubations or through calcium action.

EXPERIMENTAL

Preparation of synaptic vesicles and chromaffin granules

Synaptic vesicles were isolated from rat brains as described previously [8]. Chromaffin granules were purified from bovine adrenal medullae on a sucrose gradient as described previously [16] and visualized by thin-section electron microscopy after embedding using EPON-812 (TAAB, Aldermaston, Berks., U.K.). Electron micrographs were recorded on a Philips EM208 electron microscope. Immunoblotting of secretory vesicles was performed using monoclonal anti-synaptotagmin antibody (clone 41.1), rabbit anti-synaptophysin antibody and monoclonal anti-VAMP antibodies (clones 69.1 and 10.1; all antibodies from Synaptic Systems, Goettingen, Germany).

Preparation of SNARE proteins

SNAP-25 (synapse-associated protein of 25 kDa) and synaptobrevin 2 were isolated from bovine brain as described previously [8]. SNAP-25 was dialysed against 1 litre of buffer A (100 mM

Abbreviations used: SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; v-SNARE, vesicular SNARE; t-SNARE, target SNARE; VAMP, vesicle-associated membrane protein; SNAP-25, synapse-associated protein of 25 kDa.

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Figure 1 Synaptobrevin 2 in chromaffin granules is restricted from SNARE complex formation

(A) Electron micrographs of synaptic vesicles (SV) and chromaffin granules (CG); scale bars, 1 μm . (B) Immunoblotting of vesicles using antibodies against synaptotagmin, synaptophysin and synaptobrevin 2 (Syb). (C) Synaptobrevin 2 resident in the secretory vesicle membranes does not freely enter SNARE complex unless membranes are removed by the addition of *n*-octylglucoside (OG). A 30 min reaction was performed. Samples were immunoblotted using a monoclonal anti-synaptobrevin 2 antibody, clone 69.1. Syx, syntaxin.

NaCl/20 mM Hepes, pH 7.3) overnight at 4 °C prior to use. Syntaxin 1A cytoplasmic domain (amino acids 1–261) and the full-length cellubrevin were expressed as glutathione S-transferase fusion proteins in *Escherichia coli* strain BL21 (DE3) at 37 °C for 3 h [4,17]. After lysis, glutathione S-transferase fusion proteins were isolated on glutathione beads (Amersham Biosciences). Syntaxin or cellubrevin recombinant proteins were released from glutathione S-transferase by incubating the beads with thrombin. Proteins were further purified on a Superdex 200 column (Amersham Biosciences). Protein concentrations were estimated using BCA reagent (Pierce).

Preparation of v-SNARE proteoliposomes

Proteoliposomes were prepared essentially as described previously [8]. Briefly, chloroform solutions of brain phosphatidylcholine, brain phosphatidylethanolamine, brain phosphatidylserine and cholesterol (all from Avanti Polar Lipids) were mixed (12:5:3:5, mol/mol) and dried under a stream of nitrogen. Synaptobrevin or cellubrevin was added in the presence of 0.8% (w/v) *n*-octylglucoside, and the phospholipid–protein mixtures (10:1, w/w, respectively) were dialysed against buffer A. Negative stain microscopy was performed as for the synaptic vesicle preparation. Dialysed sample was mixed 1:1 (v/v) with Optiprep (Invitrogen), overlaid with a 30% Optiprep solution containing buffer A and centrifuged for 1 h at 500 000 *g* at 4 °C in a TLA 100.4 rotor (Beckman). Liposomes were collected from the top of the upper phase, and protein content was estimated using the Coomassie Plus protein kit (Pierce).

Probing of v-SNARE availability for SNARE complex formation

All reactions were performed in buffer A. Syntaxin 1A cytoplasmic domain and SNAP-25 (3 μg each) were mixed with synaptic

vesicles (1 μg of protein), chromaffin granules (10 μg of protein) or v-SNARE proteoliposomes (0.5 μg of protein) and incubated at 37 °C for 30 min in the absence or presence of 0.8% (w/v) *n*-octylglucoside. Assembly of SNARE complex was stopped by addition of SDS-containing sample buffer and Western immunoblotting was carried out as described previously [8].

RESULTS

Synaptophysin has been suggested to inhibit synaptobrevin 2 engagement into the SNARE complex, and thus we sought to find out whether this inhibition correlates with synaptophysin levels in subcellular organelles. We isolated synaptic vesicles and chromaffin granules and assessed synaptophysin and synaptobrevin 2 levels in these organelles (Figure 1A). Figure 1(B) shows that synaptophysin immunoreactivity is almost absent in chromaffin granules when compared with its levels in synaptic vesicles. Synaptobrevin 2 levels, however, correlated well with those of synaptotagmin (Figure 1B), the proposed calcium sensor in exocytosis [8,18].

To probe synaptobrevin 2, we added an excess of purified syntaxin lacking its transmembrane region and SNAP-25. Because the SNARE complex is thermodynamically stable and, once formed, is not disrupted by SDS, its assembly both *in vivo* and *in vitro* can be followed by Western immunoblotting techniques [6,7,17]. Synaptobrevin 2 exhibited resistance to syntaxin and SNAP-25 in chromaffin granules (Figure 1C, left-hand panel), suggesting that synaptophysin may not act as an inhibitor and that some other factors may be responsible for synaptobrevin 2 restriction. As reported previously for synaptic vesicles [8], addition of a membrane-solubilizing detergent efficiently released synaptobrevin 2 for SNARE complex formation (Figure 1C).

To assess whether the observed restriction of synaptobrevin 2 is due to protein factors at all, we analysed the behaviour of

Figure 2 Synaptobrevin 2 is down-regulated when reconstituted into phospholipid membranes

(A) Synaptobrevin 2 (Syb) incorporates efficiently into liposomes. Liposomes (Top) were floated away from unincorporated protein (Bottom). (B) Electron micrograph of dialysed proteoliposomes; scale bar, 0.5 μm . (C) Even distribution of synaptobrevin 2 on the exterior and interior leaflets of liposomes. Proteoliposomes were trypsinized in the absence or presence of detergent as indicated. About 50% of the synaptobrevin 2 resides on the outside of the liposomes. OG, *n*-octylglucoside. (D) Control trypsinolysis of synaptic vesicles (SV) shows protection of the luminal part of synaptotagmin. Immunoblotting was performed using a monoclonal antibody against the luminal part of synaptotagmin 1. (E) Synaptobrevin 2 on phospholipid membranes does not enter the SNARE complex in a 30 min reaction unless membranes are dissolved. Synaptobrevin 2 proteoliposomes were incubated in the absence or presence of excess syntaxin (Syx) and SNAP-25. For membrane dissolution, *n*-octylglucoside was used. Boiling disrupts the SNARE complex.

the v-SNARE in reconstituted proteoliposomes. Brain-purified synaptobrevin 2 was incorporated into liposomes and the efficiency of incorporation was demonstrated by flotation on an Optiprep gradient (Figure 2A). Synaptobrevin 2 proteoliposomes appeared disperse and averaged 50 nm in diameter by negative-stain microscopy (Figure 2B). To estimate the proportion of synaptobrevin 2 on the exterior surface of the proteoliposomes, we treated them with trypsin, which can only cleave exposed proteins. Western immunoblotting of proteoliposomes at different concentrations in parallel with trypsin-treated samples indicated that approx. 50% of synaptobrevin 2 is on the exterior of the proteoliposomes (Figure 2C). To confirm that there had been sufficient time for the protein to be fully digested, trypsinolysis was also performed in the presence of a membrane-solubilizing detergent (Figure 2C). In a control reaction using synaptic vesicles, trypsin was able to cleave only the cytoplasmic part of synaptotagmin and not its luminal part (Figure 2D), demonstrating that the trypsin treatment does not compromise the membrane integrity. This validates our estimate of even synaptobrevin distribution on the exterior and interior of proteoliposomes.

Despite substantial amounts of synaptobrevin 2 being present on the outside of liposomes, the v-SNARE on pure phospholipid membranes did not enter the SNARE complex in a 30 min reaction (Figure 2E), behaving in an identical manner to both synaptic vesicles and chromaffin granules (Figure 1C). Furthermore, dissolution of the phospholipid membrane by the addition of a mild detergent was sufficient to relieve the restriction, enabling complete engagement of synaptobrevin 2 into the SNARE complex (Figure 2E). To test whether the regulation of v-SNAREs is specific to neuroendocrine exocytosis or is a more universal feature of SNARE-mediated membrane fusion, we probed the availability of cellubrevin on phospholipid membranes after reconstituting it into liposomes. Cellubrevin was fully incorporated into proteoliposomes as evidenced by the flotation assay (Figure 3A). We estimated the proportion of the protein accessible on the exterior surface of proteoliposomes by trypsinolysis to be 60–70% (Figure 3B). Finally, we probed cellubrevin proteoliposomes with

an excess of syntaxin and SNAP-25. SNARE complex formation was analysed by Coomassie staining, as the cellubrevin-recognizing antibody (clone 10.1) does not detect the SNARE complex, most probably due to occlusion of the antibody's epitope in the complex. Cellubrevin on liposomes was potentially down-regulated from forming the SNARE complex when on phospholipid membranes, mimicking the behaviour of synaptobrevin 2 in both proteoliposomes and native organelles (Figure 3C).

The potent restriction of vesicular SNAREs by phospholipid membranes is rather unexpected, given that recent reconstitution experiments indicate that SNAREs, by themselves, can drive membrane fusion, albeit with slow kinetics [19,20]. To address this discrepancy we investigated a possible impact of the reaction times. Since our standard reactions were carried out for 30 min, we increased the reaction times to 1 or 2 h. In longer reactions the soluble syntaxin/SNAP-25 could engage synaptobrevin 2 in synaptic vesicles, but this was inefficient, and only limited amounts of the SNARE complex can be detected by long film exposures (Figure 4A). When synaptic vesicles were mixed with t-SNARE liposomes, formation of the SNARE complex was also very slow, but could be stimulated by the presence of 100 μM calcium in the reaction (Figure 4B) as reported previously [8]. Finally, v-SNARE liposomes, containing only synaptobrevin 2, did not exhibit complex formation with syntaxin/SNAP-25 liposomes in 30 min reactions, but addition of millimolar calcium potentiated the SNARE engagement (Figure 4C). As such high calcium concentrations are unlikely in the cytoplasm, stimulus-triggered exocytosis probably requires accessory proteins to overcome the restriction of vesicular SNAREs on phospholipid membranes for faster fusion kinetics.

DISCUSSION

Eukaryotic cells contain numerous, often closely aligned, membranous organelles whose traffic and SNARE-dependent fusion are, by necessity, strictly regulated. It is very likely that SNAREs

Figure 3 Cellubrevin on phospholipid membranes exhibits identical restriction to its homologue, synaptobrevin 2

(A) Cellubrevin incorporates efficiently into liposomes. Liposomes (Top) were floated away from unincorporated protein (Bottom). (B) Distribution of cellubrevin in liposomes. Proteoliposomes were trypsinized in the absence or presence of *n*-octylglucoside (OG) as indicated. 60–70 % of the cellubrevin is on the outside of the liposomes. (C) Cellubrevin is inhibited from SNARE complex formation by phospholipid membranes. Cellubrevin proteoliposomes were incubated for 30 min with excess syntaxin (Syx) and SNAP-25 in the absence or presence of *n*-octylglucoside. Samples were analysed by SDS/PAGE followed by Coomassie staining (upper panel) or immunoblotting using a monoclonal antibody against the SNARE motif, clone 10.1 (lower panel).

in cellular membranes are under strict control to avoid aberrant membrane fusion. For example, syntaxin in target membranes could be regulated through its interaction with ubiquitous cytosolic Munc18/nSec1 [21]. By analogy, association of synaptophysin with synaptobrevin has been proposed in several studies to play a similar inhibitory role in the formation of the SNARE complex [9–12], suggesting a neuron-specific type of SNARE regulation. Since Munc18 regulates syntaxin in membrane traffic throughout evolution, one would expect that mechanisms controlling vesicular SNAREs may also be conserved. Together our data indicate that v-SNAREs indeed share one control mechanism: restriction by resident phospholipid membranes.

Previous observations that synaptobrevin 2 associates with either t-SNAREs or synaptophysin in brain extracts [9–12] can be explained by insufficient levels of endogenous active syntaxin and SNAP-25 to drive all of the synaptobrevin 2 into the SNARE complex. Indeed, addition of an excess of syntaxin and SNAP-25 to synaptic vesicle extracts, even though they have large amounts of synaptophysin, is sufficient to enable all synaptobrevin 2 to enter the SNARE complex. The role for synaptophysin in regulating SNARE-mediated membrane fusion is also undermined by the fact that synaptic vesicle fusion appears to be normal in the absence of synaptophysin, although it is difficult to eliminate the possibility of compensatory mechanisms in these knockout studies [22,23]. However, using native organelles with markedly

Figure 4 Engagement of vesicular synaptobrevin 2 by the target SNAREs requires long reaction times

(A) Synaptobrevin 2 in synaptic vesicles (SV) forms the SNARE complex with soluble target SNAREs with prolonged incubation times. Syx, syntaxin. (B) Formation of the SNARE complex between synaptic vesicles and syntaxin/SNAP-25 liposomes is accelerated by 100 μM calcium. (C) Significant formation of the SNARE complex between synaptobrevin 2 liposomes and syntaxin/SNAP-25 liposomes occurs only at calcium concentrations of 10 mM. A 30 min reaction was performed.

different levels of synaptophysin we showed that synaptobrevin 2 restriction does not correlate with synaptophysin levels (Figure 1). Together, both biochemical and genetic approaches point to roles for synaptophysin other than direct regulation of SNARE complex formation. These roles likely include synaptic vesicle recycling and synapse formation [24,25].

The cytoplasmic part of synaptobrevin 2 consists of two parts: a characteristic proline-rich N-terminal sequence and the SNARE motif necessary for SNARE complex formation [5,26]. A recent EPR study mapped amino acids responsible for synaptobrevin 2 restriction to the SNARE motif [27], suggesting that other v-SNAREs may possess similar properties on phospholipid membranes. Interestingly, replacement of synaptobrevin with cellubrevin *in vivo* does not compromise the strict control of vesicle fusion or the calcium requirement in exocytosis [28]. Our data now show that reconstitution of ubiquitous cellubrevin into pure phospholipid membranes is sufficient to restrict this v-SNARE from syntaxin/SNAP-25 (Figure 3). Since cellubrevin is found in endosomal compartments and functions in membrane traffic in a wide

variety of cells [4], v-SNARE regulation by phospholipid membranes most likely represents a general phenomenon. Uncovering of Munc18 [29] and the phospholipid membrane as widespread regulatory factors of syntaxins [30] and v-SNAREs, respectively, provides us with a rational basis to decipher the molecular mechanisms underlying membrane fusion. Identification of these mechanisms becomes the next major challenge for our understanding of membrane trafficking in eukaryotic cells.

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