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Fusion complex formation protects synaptobrevin against proteolysis by tetanus toxin light chain

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Abstract The clostridial neurotoxin, tetanus toxin, is a Zn^{2+}-dependent protease which inhibits neurotransmitter exocytosis by selective cleavage of the synaptic vesicle protein, synaptobrevin. Synaptobrevin is thought to serve as a receptor for two neuronal plasma membrane proteins, syntaxin and SNAP-25, which in the presence of non-hydrolyzable ATP analogs form a 20 S fusion complex with the soluble fusion proteins NSF and α-SNAP. Here we show that synaptobrevin, when in this 20 S complex, or its 7 S precursor, is protected against proteolysis by the enzymatically active tetanus toxin light chain. Our data define distinct pools of synaptobrevin, which provide markers of different steps of vesicle/plasma membrane interaction.

Key words: Neurotransmitter release; Synaptic vesicle; Synaptobrevin; Tetanus toxin; 20 S fusion complex

1. Introduction

Synaptic release of neurotransmitters is triggered by calcium and occurs via exocytotic fusion of synaptic vesicles with the presynaptic plasma membrane [1]. Recent studies indicate that an evolutionarily conserved set of protein interactions utilized at different stages of vesicle-based intracellular protein transport is also essential for synaptic vesicle/plasma membrane fusion [2]. In particular, the ubiquitous ATPase *N*-methylmaleimide-sensitive fusion protein (NSF) and the soluble NSF attachment proteins (SNAPs) have been implicated in transmitter secretion because of their ability to associate with the vesicle membrane protein, synaptobrevin (or VAMP), and the plasma membrane proteins, syntaxin and SNAP-25 (for synaptosomal associated protein of 25 kDa) [3]. Since these latter proteins bind to α-SNAP, they were named SNAREs (Snap Receptors) [3].

Upon solubilization the SNAREs form a stable complex which sediments at 7 S and is postulated to mediate selective docking of synaptic vesicles at the plasma membrane [4,5]. A sequence of post-docking intermediates involving the SNAREs has also been postulated from in vitro observations. One of the 7 S complexes also contains synaptotagmin, another vesicle protein proposed to constitute the Ca^{2+} receptor that triggers membrane fusion [6,7]. SNARE associated synaptotagmin is displaced from the 7 S complex upon binding of α-SNAP, which in turn allows NSF recruitment into the SNARE complex. In the presence of a non-hydrolyzable ATP analog the interactions between all aforementioned proteins are maintained, and the resulting complex can be identified as an entity that migrates at 20 S. In contrast, ATP hydrolysis by NSF disrupts the 20 S complex, a process that has been postulated to drive vesicle/plasma membrane fusion [3,4,8].

The importance of the protein interactions outlined above is strengthened by independent evidence showing that the synaptosomal SNAREs are the targets of clostridial neurotoxins, the most potent inhibitors of neurotransmitter release known [9]. These toxins are composed of a heavy chain responsible for target cell specificity, and a light chain which acts as a Zn^{2+}-dependent endoprotease. Synaptobrevin is specifically cleaved by tetanus toxin and botulinum toxin B, D and F light chains [10–12], whereas syntaxin and SNAP-25 are substrates for other botulinum toxin serotypes [13,14]. These toxin proteases have been shown to utilize unique sites in the polypeptide sequence [9]. In squid, where the cleavage site of synaptobrevin is conserved, tetanus toxin injection into the presynaptic terminal of the giant synapse leads to disruption of a post-docking step in synaptic vesicle fusion [15]. Thus, the role of the SNAREs in the release mechanism may extend beyond simple membrane recognition. One way to address this issue is to investigate the toxin sensitivity of the SNAREs within distinct fusion complex intermediates produced in vitro. In this study we show that synaptobrevin is protected from tetanus toxin cleavage in both the 7 S and 20 S complex, a result that extends the idea [15] that this SNARE may directly function at multiple points of the vesicle fusion mechanism.

2. Materials and methods

2.1. Materials

The light chain of tetanus toxin [16] was a gift of Prof. E. Habermann. pQE9 vectors containing the full length coding sequence of NSF [3] and α-SNAP [17], kindly provided by Drs. J. Rothman and T. Söllner, were used to produce recombinant His_6-NSF-myc and His_6-α-SNAP as described [17]. His_6-NSF-myc and His_6-α-SNAP protein concentrations were estimated by Coomassie blue staining of SDS-PAGE samples and compared against known quantities of the marker proteins, bovine serum albumin and glyceraldehyde-6-phosphate dehydrogenase. Prior to use, the His_6-NSF-myc was concentrated over Centricon 30 filters (Amicon).

2.2. Membrane extracts

The solubilization of salt-washed crude rat brain membranes with Triton-X-100 was performed as described [3]. Protein content was estimated by a modified Lowry procedure [18].

2.3. 20 S Fusion complex assembly and disassembly

The distinct intermediates of the SNARE complex were formed from brain membrane extracts essentially as described [3,4]. The Triton X-100 extracts were used directly or after dilution into reaction buffer (20 mM HEPES-KOH, pH 7.0, 100 mM KCl, 1 mM dithiothreitol, 0.5% (w/v) Triton X-100); for immunoprecipitation experiments the reaction

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Abbreviations: NSF, *N*-ethylmaleimide-sensitive fusion protein; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel-electrophoresis; SNAP, soluble NSF attachment protein; SNAP-25, synaptosomal associated protein of 25 kDa; SNARE, SNAP receptor.

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buffer also contained 1% (w/v) polyethyleneglycol 4000 and 1% (w/v) glycerol. In all experiments basic incubations were supplemented with either no additions (7 S); His_6-α-SNAP alone (+α-SNAP); His_6-α-SNAP and His_6-NSF-myc fusion proteins under either ATP non-hydrolizing (20 S) or hydrolizing (Dis20S) conditions. The reactions were incubated for 30 min at 4°C and cleared by centrifugation. Then tetanus toxin light chain was added, and incubations continued at 37°C for 40 min. As extended periods at this temperature are known to compromise NSF function in vitro [19], two controls without toxin were routinely performed at 4°C and 37°C, although no differences were apparent between these two controls (data not shown). Tetanus toxin cleavage of different pools of synaptobrevin in these in vitro incubations was analyzed by either glycerol gradient centrifugation or immunoprecipitation using methods detailed elsewhere [4]. Immunoprecipitations were made with the syntaxin specific mAb 10H5 [20] covalently coupled to fast-flow protein A-Sepharose beads (100 μg/10 μl beads). 0.1 M glycine (pH 2.7) was used to elute the proteins bound to the beads.

2.4. Gel-electrophoresis and Western blotting

Protein samples from glycerol gradients or immunoprecipitations were concentrated by trichloroacetic acid precipitation [21] and subjected to SDS-PAGE on 15% gels [22] before transfer to nitrocellulose. The blots were cut into four horizontal strips corresponding to the apparent molecular weight ranges of 14–20, 21–30, 30–45, and >45 kDa, and immunodecorated with antibodies raised against synaptobrevin [23], SNAP-25 (Affiniti Research Products Ltd.), syntaxin [20] and synaptotagmin (mAb 1D12; see [20]), respectively. Immunoreactivity was detected using horseradish peroxidase-coupled secondary antibody in combination with the ECL detection system (Amersham). Detection of NSF and α-SNAP immunoreactivities [4] was performed by reprobing the 30–45 kDa and >45 kDa strips after inactivating the original secondary antibodies with 0.05% (w/v) sodium azide.

3. Results

3.1. Tetanus toxin cleavage of synaptobrevin in membrane extracts

Incubation of Triton X-100 extracts prepared from rat brain membranes with tetanus toxin light chain indicated that efficient and specific cleavage of synaptobrevin described for more intact preparations [10] was retained in the presence of detergent, although both isoforms of synaptobrevin were cleaved (Fig. 1). A minor proportion of the synaptobrevin present was, however, resistant to toxin cleavage even after prolonged incubation (>2 h; see Fig. 1, and results not shown). This prompted us to investigate whether an association of synaptobrevin with the SNAREs SNAP-25 and syntaxin could account for this protection. Upon the addition of recombinant α-SNAP alone or together with His_6-NSF-myc and ATP-γ-S, synaptobrevin that appeared insensitive to tetanus toxin cleavage was still observed. In contrast, when the α-SNAP and NSF recombinant proteins were added in the presence of ATP, synaptobrevin was completely digested (Fig. 1). Since these conditions favour 20 S complex disassembly [4], only uncomplexed synaptobrevin appeared to be digested by the toxin.

3.2. Investigation of synaptobrevin cleavage by gradient sedimentation

To extend the observations made with crude membrane extracts, the latter were incubated under conditions promoting distinct stages of complex formation and then separated by glycerol gradient centrifugation [3,4]. This analysis revealed the expected distribution of SNAREs. With no additions to the crude extract, syntaxin, SNAP-25 and synaptobrevin co-sedimented in a peak of about 7 S (Fig. 2A, 7S). Under these conditions, synaptobrevin in addition exhibited a peak of immunoreactivity in the lightest fractions, which is indicative of a pool of solubilized synaptobrevin that is distinct from that co-distributing with syntaxin and SNAP-25. 20 S complex conditions caused a re-distribution into denser fractions of all SNARE proteins, with a peak at >15 S (Fig. 2A, 20S). The positions of His_6-α-SNAP and His_6-NSF-myc immunoreactivities in these gradients were consistent with the formation of a multimeric α-SNAP/NSF/SNARE complex (data not shown). Under the conditions used, approximately 50% of the synaptobrevin immunoreactivity was found in these denser fractions, which confirms original observations of Söllner et al. [4]. Under complex disassembly conditions (Fig. 2A, Dis20S), there was a small but discernable shift in the distribution of SNAREs to lighter fractions, this being most marked for synaptobrevin (see also [5]). In contrast, the distribution of synaptotagmin immunoreactivity was unchanged by the incubation conditions; the small fraction of α-SNAP displaceable synaptotagmin that associates with the 7 S complex [4] could therefore not be adequately tracked by glycerol gradient centrifugation.

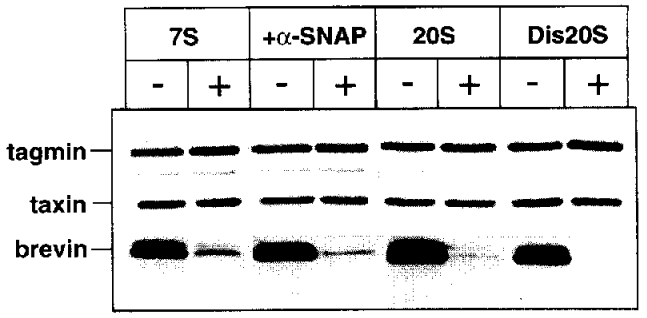

Fig. 1. Tetanus toxin cleavage of detergent-solubilized synaptobrevin. Salt-washed rat brain membranes (40 μg of protein) were incubated for 30 min at 4°C in a final volume of 15 μl either alone (7S), in the presence of 3 μg of His_6-α-SNAP (+α-SNAP), or in the presence of 3 μg of His_6-NSF-myc and 1.25 μg His_6-α-SNAP in buffer supplemented with either 2 mM $MgCl_2$ and 0.5 mM ATP-γ-S (20S) or ATP (Dis20S). Incubation was then continued at 37°C for 40 min in the absence (−) or presence (+) of 600 ng of tetanus toxin light chain.

The distinct pools of synaptobrevin fractionated on the gycerol gradients were analyzed for their susceptibility to cleavage by tetanus toxin. For this purpose fractions enriched in either associated or uncomplexed synaptobrevin were divided and incubated with or without tetanus toxin light chain (Fig. 2B). In all cases the fractions enriched in 7 S or 20 S complex showed an almost complete protection of synaptobrevin against toxin cleavage. In contrast, light fractions containing non-associated synaptobrevin showed very marked degradation. This strengthens the view that complexed synaptobrevin is protected against tetanus toxin action.

3.3. Immunoprecipitation analysis

To better define the pool of synaptobrevin that resists tetanus toxin cleavage, we immunoprecipitated distinct fusion complex intermediates [4]. In a first set of experiments, extracts were incubated to produce different fusion complex intermediates, subjected to tetanus toxin light chain treatment, and immunoprecipitated on syntaxin immunobeads before analysis of the resulting pellets and supernatants. Under 7 S conditions, all characterized components of this 'precursor' complex were efficiently precipitated (Fig. 3, 7S). The specificity of our

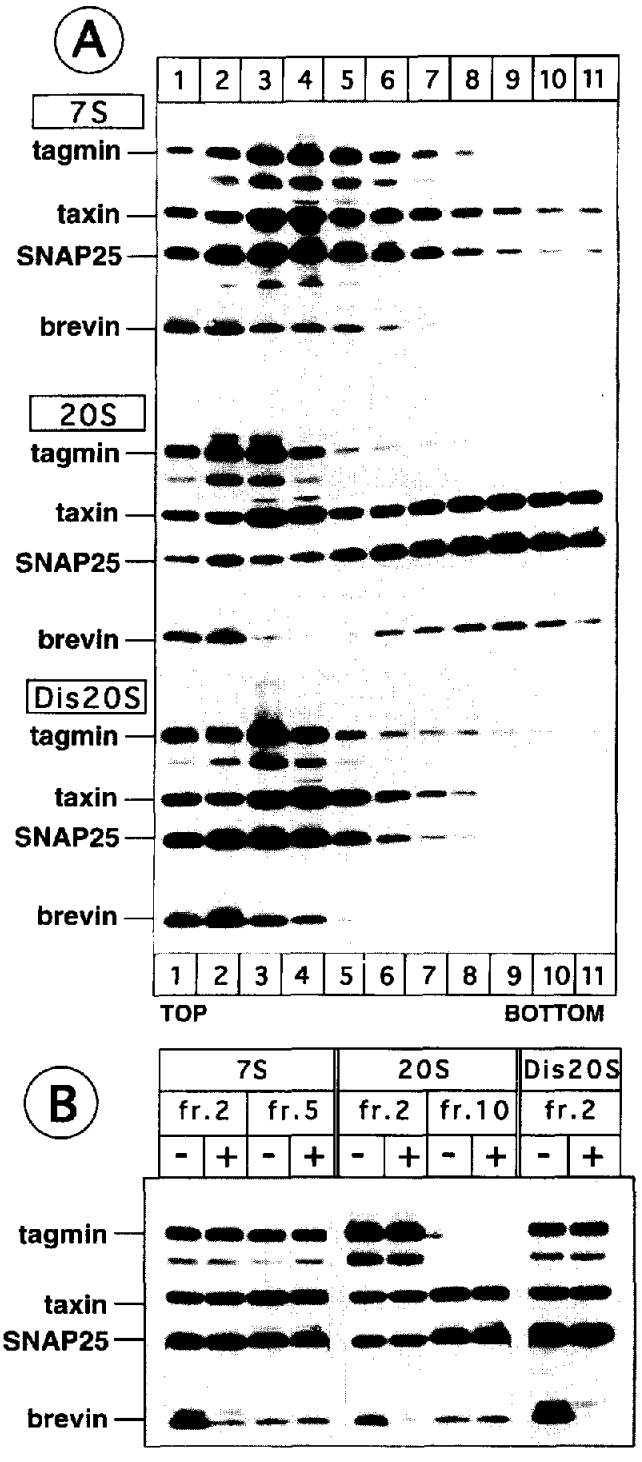

Fig. 2. Effect of tetanus toxin light chain on fusion complexes enriched by glycerol gradient centrifugation. Salt-washed rat brain membranes (200 μg of protein) were incubated at 4°C for 30 min in reaction buffer without addition (7S), in the presence of 15 μg of His_6-α-SNAP without (+α-SNAP), or with 15 μg of His_6-NSF-Myc supplemented with 0.5 mM ATP and either 2 mM EDTA (20S), or 2 mM $MgCl_2$ (Dis20S). The samples were then subjected to centrifugation over glycerol gradients made up in buffers appropriate for the complex intermediate investigated. Aliquots (300 μl) of gradient fractions were (A) precipitated and analyzed by SDS-PAGE and immunoblotting, and (B) in parallel incubated at 37°C for 40 min under agitation in the absence (−) or presence (+) of 1.5 μg of tetanus toxin light chain prior to protein precipitation, SDS-PAGE and immunoblotting.

immunoprecipitation conditions was apparent from parallel incubations made with anti-synaptophysin (SY38; see [24]) immunobeads, which failed to co-isolate synaptophysin with any of the SNAREs (data not shown). The amounts of synaptobrevin precipitated from either untreated or toxin treated 7 S extracts were very similar. However, efficient cleavage of synaptobrevin is indicated by a marked decrease of synaptobrevin immunoreactivity in the supernatants of the extracts incubated with tetanus toxin light chain; a small fraction of uncleaved synaptobrevin in these supernatants probably reflects incomplete immunoprecipitation of the solubilized 7 S complex. Very similar results were obtained upon addition of α-SNAP and under 20 S conditions (Fig. 3, +α-SNAP and 20S). Again, the synaptobrevin immunoprecipitated by the syntaxin antibodies was resistant to cleavage by tetanus toxin light chain, although the amount of associated synaptotagmin decreased as α-SNAP bound to the complex. When extracts are supplemented with α-SNAP, NSF and ATP, the complex immunoprecipitated by syntaxin antibodies is disrupted [3,4]. This was demonstrated here by a decrease in immunoprecipitated synaptobrevin and SNAP-25 (Fig. 3, Dis20S). Under these conditions, synaptobrevin previously associated with the 7 S complex became sensitive to cleavage. It is interesting that immunoprecipitates from the tetanus toxin light chain treated extracts showed a complete absence of synaptobrevin immunoreactivity, although in untreated extracts some remained. This might indicate that the toxin also acts on partially disassembled fusion complexes. Additional immunoblots with an antibody specific for the amino acid sequence 36–57 of synaptobrevin [5] showed that the cleaved N-terminal fragment of synaptobrevin was released into the supernatant (data not shown).

To ensure that the above observations were not due to a preferential re-equilibration of uncleaved synaptobrevin into the 7 S complex, this intermediate was separated from the solubilized synaptobrevin and used as a precursor for further manipulations. This was achieved by binding the 7 S complex to syntaxin immunobeads and incubating this with components as described for the crude membrane extract. The resulting immunoisolates were then incubated with or without tetanus toxin light chain. The comparatively poor displacement of synaptotagmin and the weak release of synaptobrevin into the supernatant seen under Dis20S conditions suggest inefficient complex disassembly on the immunobeads. Despite this limitation, the bead approach clearly revealed that the resistance of synaptobrevin to tetanus toxin cleavage is due to its interaction with the other SNAREs and, secondly, that this resistance appears unaffected by the protein–protein interactions that characterize the formation of a 20 S complex from the 7 S intermediate (Fig. 4). In contrast, conditions that induced disruption of the 20 S complex caused release of associated synaptobrevin which then was completely degraded by the toxin's light chain. Control experiments with the protease ficin showed that synaptobrevin in the bead-bound 20 S complex is in principle accessible to proteases (data not shown).

4. Discussion

The discovery that the light chain of tetanus toxin is a Zn^{2+}-dependent protease which exhibits a marked substrate specificity for the synaptic vesicle protein synaptobrevin, afforded an elegant explanation of this protein's potent neurotoxicity [10].

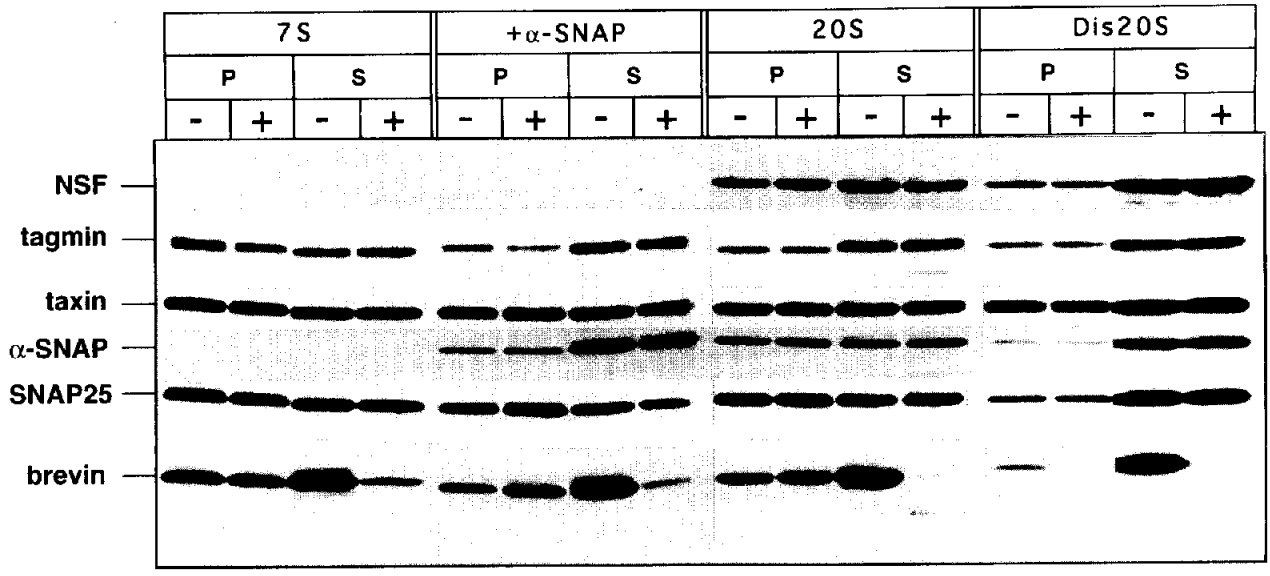

Fig. 3. Immunoprecipitation of fusion complexes from extracts treated with tetanus toxin light chain. Salt-washed rat brain membranes (40 μg of protein) were incubated in immunoprecipitation buffer with additions as shown in Fig. 1, before further incubation at 37°C for 40 min, in the absence (−) or presence (+) of 600 μg of tetanus toxin light chain. The samples were then immunoprecipitated to give pellets (P) and supernatants (S) for further analysis.

It also provided strong evidence that synaptobrevin plays an essential role in the neurotransmitter release process. Biochemical experiments have defined synaptobrevin as a component of a synaptosomal protein complex that is assumed to mediate vesicle docking and fusion [3–5]. In particular synaptobrevin's in vitro association with the presynaptic plasma membrane SNAREs, SNAP-25 and syntaxin, has been postulated to constitute the cellular correlate of docked synaptic vesicles [5]. This synaptobrevin containing 7 S complex that recruits α-SNAP and NSF provides a plausible model of how vesicle fusion at specialized cellular locations, like the nerve terminal, can be catalyzed by an ubiquitous set of cytosolic proteins. Our data showing that synaptobrevin complexed with its cognate SNAREs is insensitive to tetanus toxin cleavage indicates that upon docking synaptobrevin becomes resistant to tetanus toxin. The finding that only a small fraction of solubilized synaptobrevin is resistant to cleavage by tetanus toxin is consistent with morphological data showing that only a small fraction of the total vesicle pool is docked [25]. If the tetanus toxin-insensitive pool of synaptobrevin is indeed representative of docked vesicles, the toxin can provide a sensitive tool to measure the biogenesis of the docked state. Notably, although the pool of docked vesicles is small, it represents the physiologically most active population, being the pool used in the triggered release of neurotransmitter [25]. Therefore, the slow action of toxin seen when it is directly introduced into nerve terminals [10,15] may relate, partly, to the inaccessibility of toxin to the SNARE complexed synaptobrevin.

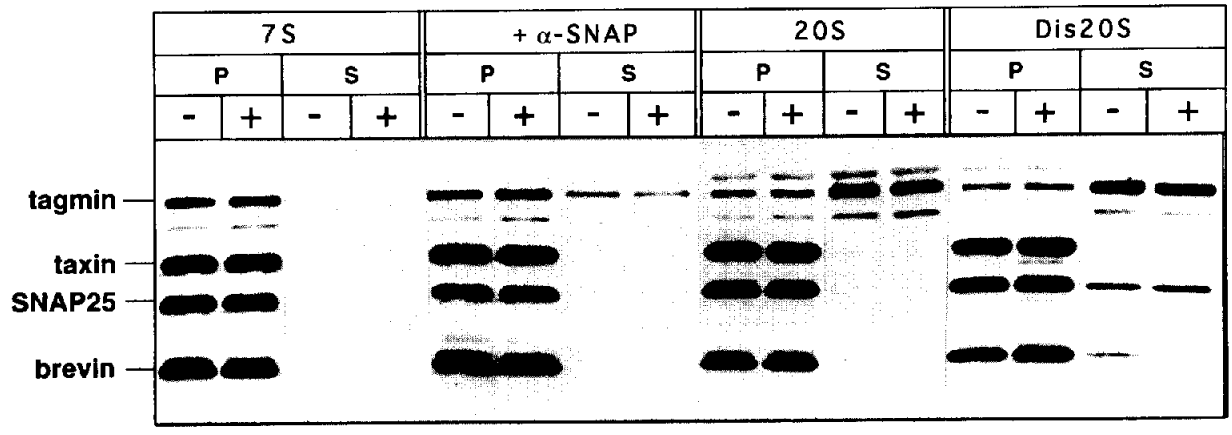

Fig. 4. Tetanus toxin cleavage of immobilized fusion complexes. Salt-washed rat brain membranes (40 μg of protein) were incubated for 30 min at 4°C in immunoprecipitation buffer in a final volume of 100 μl and immunoprecipitated with an equal volume of antisyntaxin-coupled protein A beads. After 5 washes in immunoprecipitation buffer, the beads were gently mixed for 60 min at 4°C in a final volume of 100 μl without addition (7S), in the presence of 7.5 μg of His_6-α-SNAP (+α-SNAP), or in the presence of 2 mM $MgCl_2$, 3 μg of His_6-α-SNAP and 7.5 μg His_6-NSF-myc with 5 mM of either ATP-γ-S (20S) or ATP (Dis20S). After incubation for 40 min at 37°C in the absence (−) or presence (+) of 900 ng of tetanus toxin light chain and 5 washes in immunoprecipitation buffer containing ATP and ATP-γ-S as required, the supernatants and subsequent washes were pooled (S) before analysis in parallel with proteins eluted from the washed immunoprecipitates (P).

The protection of synaptobrevin in all intermediates of 20 S complex assembly is difficult to reconcile with it serving simply to ensure compartment-specific membrane recognition. As mentioned above, terminals exhibiting tetanus toxin perturbed neurotransmitter release show an increase in the number of docked vesicles [15]. Assuming that these morphologically defined organelles represent fusion competent vesicles, either formation of the 7 S complex is not the biochemical correlate of docking, or synaptobrevin has multiple functions in the release process. If the latter is true, the membrane anchored synaptobrevin fragment obtained upon cleavage may be sufficient to mediate vesicle docking, whereas post-docking functions of synaptobrevin may be destroyed by loss of the N-terminal cleavage fragment [10]. Possible post-docking functions of synaptobrevin may include the SNAP-25 mediated increase in the affinity of SNARE interactions [5], accomodation of SNAP and NSF mediated interactions, or a direct role of synaptobrevin in the membrane re-arrangements that underlie exocytotic vesicle fusion [4].

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