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Protein–protein interactions and protein modules in the control of neurotransmitter release

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Information transfer among neurons is operated by neurotransmitters stored in synaptic vesicles and released to the extracellular space by an efficient process of regulated exocytosis. Synaptic vesicles are organized into two distinct functional pools, a large reserve pool in which vesicles are restrained by the actin-based cytoskeleton, and a quantitatively smaller releasable pool in which vesicles approach the presynaptic membrane and eventually fuse with it on stimulation. Both synaptic vesicle trafficking and neurotransmitter release depend on a precise sequence of events that include release from the reserve pool, targeting to the active zone, docking, priming, fusion and endocytotic retrieval of synaptic vesicles. These steps are mediated by a series of specific interactions among cytoskeletal, synaptic vesicle, presynaptic membrane and cytosolic proteins that, by acting in concert, promote the spatial and temporal regulation of the exocytotic machinery. The majority of these interactions are mediated by specific protein modules and domains that are found in many proteins and are involved in numerous intracellular processes. In this paper, the possible physiological role of these multiple protein–protein interactions is analysed, with ensuing updating and clarification of the present molecular model of the process of neurotransmitter release.

Keywords: exocytosis; synaptic vesicle proteins; presynaptic proteins; cytoskeleton; SNAREs; protein interaction modules

1. INTRODUCTION

Neurotransmitter release is a fundamental process in intercellular communication among neurons and between neurons and effector cells. Recent results obtained with complementary approaches ranging from molecular biology to biochemistry, genetics and microbiology support the idea that neuronal exocytosis is a particular aspect of the more general processes of vesicular fusion and constitutive secretion shared by all eukaryotic cells. However, neuroexocytosis exhibits remarkable characteristics which make it the most sophisticated and high-performance example of regulated secretion, including the rapidity of excitation–secretion coupling, the resistance to exhaustion during prolonged, high-frequency activity and the ability to change the release efficiency depending on microenvironmental conditions.

These characteristics are accounted for by the presence in the nerve terminal of two functionally distinct pools of synaptic vesicles (SVs) and of an efficient local recycling of exocytosed SVs. The existence of the two pools has been demonstrated by a variety of electrophysiological, functional and morphological studies. The SVs of the releasable, or proximal, pool are docked to the presynaptic membrane at the active zone and possibly already primed for exocytosis; those of the reserve or distal pool constitute an immediate and strategically localized source to replenish the releasable pool on activity-dependent depletion (Greengard *et al.* 1993; Pieribone *et al.* 1995; Brodin *et al.* 1997; Kuromi & Kidokoro 1998; Neher 1998) (figure 1). In general, the releasable pool represents only a small percentage of the total SV content of the terminal (0.5–15% depending on the type of synapse), but its size is functionally very important to define the number of neurotransmitter quanta released per impulse. The size of the reserve pool varies greatly among synapses and is related to the recycling capacity and to the functional requirements of the nerve terminal. In general, terminals with tonic, low-frequency activity possess a relatively small reserve pool, whereas terminals with phasic, high-frequency activity require the presence of large reserve pools (Brodin *et al.* 1997). During repetitive stimulation, the ability of a nerve terminal to release neurotransmitter not only depends on the recycling capacity and the number of SVs in the releasable pool, but it is also proportional to the size of the reserve pool and to the rate of mobilization of SVs from the reserve to the releasable pool. This machinery offers a molecular explanation for the processes of synaptic plasticity involving an increased or decreased responsiveness of the nerve terminal to release neurotransmitter (Greengard *et al.* 1993).

The analysis of the functional characteristics of neurotransmitter release suggests that two sequential processes with different rates and regulatory mechanisms are involved: (i) the transition of SVs from the reserve to the

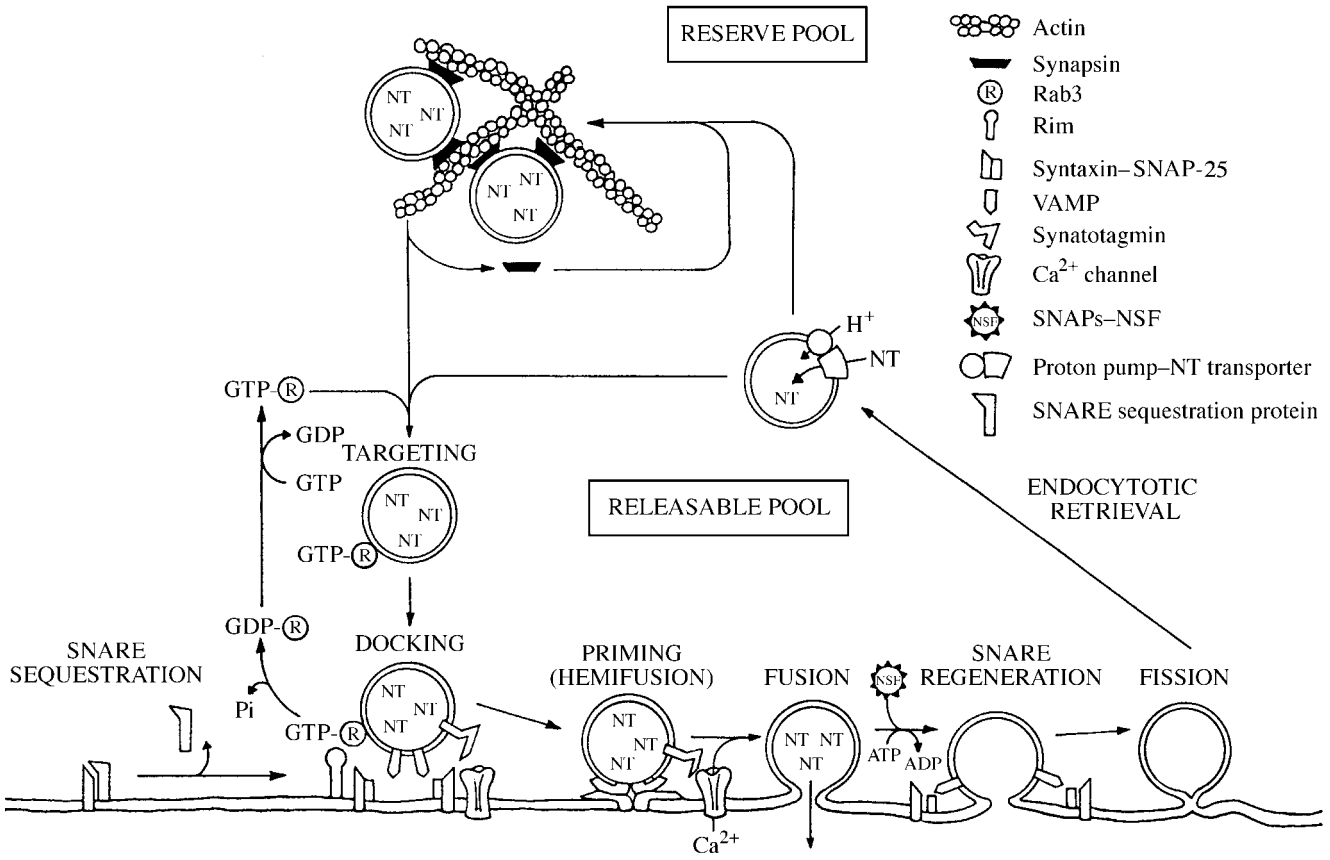

Figure 1. Putative model of the mechanisms of neurotransmitter release. Synaptic vesicles have to undergo a sequence of events that includes release from the reserve pool, targeting to the active zone, docking, priming, fusion and endocytotic retrieval. The main proteins that are likely to play a role in the exocytotic machinery are indicated only in the steps in which they are thought to be involved. NT, neurotransmitter. For further details, see text.

releasable pool; and (ii) the obligatory events of targeting, docking and priming of the releasable vesicles eventually followed by fusion with the presynaptic membrane on stimulation and Ca^{2+} entry (Benfenati & Valtorta 1995). In the last few years, great progress has been made in the elucidation of the molecular mechanisms underlying SV exocytosis and in the identification of the key players of these processes.

The above-mentioned molecular events involve the participation of SV, cytoskeletal, presynaptic membrane and soluble proteins. Like instruments in an orchestra, each of these proteins plays distinct, yet interrelated, functions and undergo specific interactions with selected partners, highly modulated within the nerve terminal. The key interactions of SV proteins that are directly involved in the execution and/or modulation of neurotransmitter release are with the actin-based cytoskeleton and with presynaptic membrane proteins, the former implicated in the regulation of SV availability for release, the latter involved in the final steps of exocytosis. A large number of recent studies have demonstrated that: (i) these interactions involve binding of relatively large domains of the protein partners; (ii) multiple partners often compete for the same binding site; and (iii) the formation of multimeric protein complexes may uncover new binding sites for other protein partners, adding directionality to the reactions leading to exocytosis. In addition, a series of physiologically relevant interactions among proteins involved in the exo-endocytotic cycle of SVs occur through the intervention of peculiar multipurpose domains (such as the C_2, J, SH3, SH2, PTB, PTZ and WW domains) that are found in many proteins. A limited number of basic modules therefore perform multiple and distinct recognition functions. Although highly conserved, these domains are thought to act in a very specific manner due both to the high specificity of the domain–acceptor binding and to the compartmentalization of both the domain-containing protein and the acceptor protein.

A large number of recent and excellent reviews on the molecular mechanisms involved in exocytosis are available (e.g. Calakos & Scheller 1996; Zucker 1996; Goda 1997; Goda & Südhof 1997; Linial 1997; Hanson *et al.* 1997*a*; Bennett 1997; Geppert & Südhof 1998; Betz & Angleson 1998; Ludger & Galli 1998). Therefore, this paper will deal only with some aspects of these processes, with the aim of giving a survey of the main types of interactions occurring within nerve terminals directly or indirectly involved in mediating neurotransmitter release.

2. INTERACTIONS OF SYNAPTIC VESICLES WITH THE CYTOSKELETON

Morphological and functional data (Landis *et al.* 1988; Hirokawa *et al.* 1989; Bernstein & Bamburg 1989) indicate that SVs in the reserve pool are organized in clusters linked reversibly to the cytoskeleton. Since the nerve terminal cytomatrix mainly consists of actin filaments,

actin and actin-binding proteins associated with SVs are thought to be critical for the accumulation of SVs as well as for the regulation of their trafficking and availability for exocytosis.

The major SV proteins interacting with the actin-based cytoskeleton are the synapsins (De Camilli *et al.* 1990; Greengard *et al.* 1993). Synapsins I and II bind to both actin monomers and filaments and promote the polymerization of actin monomers and the formation of bundles of filaments. These interactions are mediated predominantly by the highly conserved C domain of the synapsins and are modulated by site-specific phosphorylation. As the interactions of the synapsins with actin and SVs are not mutually exclusive, the synapsins have been proposed to cross-link SVs to the actin-based cytoskeleton (Benfenati *et al.* 1992). Indeed, video–microscopy experiments demonstrated that the synapsins are both necessary and sufficient for SVs to bind actin filaments (Ceccaldi *et al.* 1995). Due to the existence of multiple binding sites on SVs, the synapsins may also cross-link adjacent SVs and form SV clusters within the nerve terminal (Benfenati *et al.* 1993). Thus, the synapsins, by clustering SVs, attaching them to preformed actin filaments and nucleating actin filaments from the SV surface, may be directly responsible for the formation and maintenance of the reserve pool of SVs both during synaptogenesis and in the adult neuron (figure 2).

Modulation of SVs and actin binding following synapsin phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (and also, albeit less intensely, by protein kinase A and MAP kinase) can control the transition of SVs from the reserve pool to the releasable pool, thus regulating the efficiency of neurotransmitter release and synaptic plasticity (Greengard *et al.* 1993; Jovanovic *et al.* 1996). The transitions of SVs, triggered by disruption of the synapsin-mediated vesicle–vesicle and vesicle–actin cross-bridges, can be envisaged as a preparatory event for an SV to undergo the final steps of exocytosis (Benfenati & Valtorta 1995). Such a model has been confirmed by the observation that, with respect to SVs in the reserve pool, SVs in the releasable pool (either docked or fused with the presynaptic membrane) are depleted of synapsin I (Torri Tarelli *et al.* 1992; Pieribone *et al.* 1995). The synapsins may therefore play both a structural and a regulatory role in SV trafficking (figure 1).

These functions of the synapsins have been confirmed by experiments in which the levels of synapsins were manipulated. Thus, depletion of synapsins decreases the number of SVs in nerve terminals, depletes the reserve pool of SVs and alters synaptic plasticity phenomena (Ferreira *et al.* 1994; Pieribone *et al.* 1995; Rosahl *et al.* 1995; Li *et al.* 1995; Takei *et al.* 1995; Ryan *et al.* 1996), whereas precocious expression of synapsins promotes an accelerated functional and structural maturation of nerve terminals with an increased number of SVs clustered in proximity of the presynaptic membrane (Lu *et al.* 1992; Schaeffer *et al.* 1994; Valtorta *et al.* 1995). As the interactions of synapsin II with both actin and SVs appear stronger and less sensitive to modulation by phosphorylation than those of synapsin I (Nielander *et al.* 1997), it is possible that synapsin II plays a predominant structural role in the formation and maintenance of the reserve pool, whereas synapsin I has evolved for regulating SV trafficking between the reserve and the releasable pools (see also Hilfiker *et al.*, this issue).

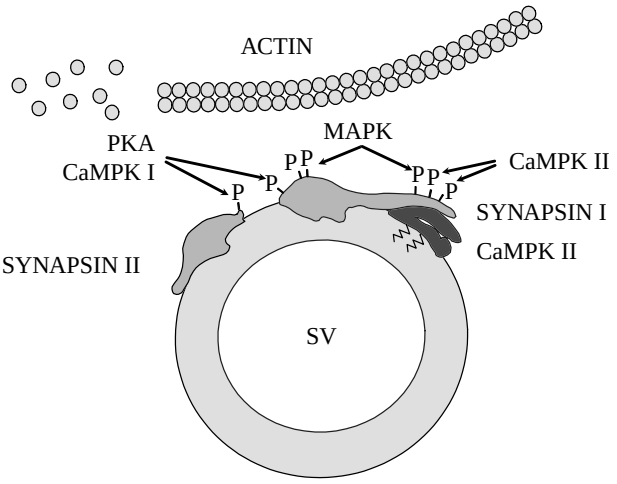

Figure 2. Schematic representation of the molecular interactions of the synapsins with the synaptic vesicle membrane and with actin monomers and filaments. P, phosphorylation site; PKA, protein kinase A; CaMPK, Ca^{2+}/calmodulin-dependent protein kinase; MAPK, MAP kinase.

In addition to synapsins, other proteins may mediate the interactions between SVs and the cytoskeleton. Rabphilin, a GTP-dependent Rab3a binding protein, interacts with the actin binding proteins α-actinin and β-adducin. Such interactions may participate in the Rab3a-dependent cytoskeletal remodelling at the nerve terminal level (Miyazaki *et al.* 1994; Kato *et al.* 1996). Similarly, other proteins permanently or transiently associated with SVs may interact with cytoskeletal proteins. Among these are p115 TAP (transcytosis-associated protein), proposed to anchor vesicles at cytoskeletal sites proximal to active zones (Barroso *et al.* 1995; Calakos & Scheller 1996) and the tyrosine kinase c-Src, involved in cytoskeletal remodelling either through interactions with cell adhesion molecules or by direct phosphorylation of cytoskeletal proteins (Erpel & Courtneidge 1995; Thomas & Brugge 1997). However, the evidence for a defined functional role played by these proteins in exocytosis is still limited.

3. INTERACTIONS OF SYNAPTIC VESICLES WITH THE PRESYNAPTIC MEMBRANE

A large network of interactions between SV proteins and presynaptic membrane proteins have been described, although the precise functional role is clear in only a few cases. These interactions are involved in multiple events that include docking, formation and disassembly of the fusion core complex, and regulation of membrane fusion by Ca^{2+} (figure 1).

(a) *Docking of synaptic vesicles to the presynaptic membrane*

Docking of small SVs storing and releasing classical neurotransmitters is specific for active zones. As voltage-sensitive Ca^{2+} channels are concentrated at active zones (reviewed in Calakos & Scheller 1996), specific docking is responsible both for the spatial regulation of exocytosis and for the highly efficient excitation–secretion coupling.

Docking involves interactions between SV proteins and presynaptic membrane proteins whose distribution does overlap with that of the active zones. As the SNAP receptor (SNARE) proteins, VAMP–synaptobrevin, syntaxin and SNAP-25, are not likely to be directly involved in SV docking (see below), search for additional interactions and protein partners has been carried out, with identification of a few other potential candidates for the docking reaction. At the present time, a number of potential interactions between SV proteins and presynaptic membrane proteins, through which SVs may dock to the active zone been demonstrated *in vitro*. However, with few exceptions (such as voltage-dependent Ca^{2+} channels and Rim), an exclusive or enriched location of these presynaptic proteins at active zones has not been clearly demonstrated. Vesicles may dock to the presynaptic membrane via a single interaction or via multiple interactions. In the latter case, one interaction may be responsible for the spatial selectivity of docking, with the others playing a facilitatory role on the docking reaction by increasing the apparent affinity of the SV binding. In addition, functional heterogeneity among docked vesicles may exist and vesicles appearing morphologically docked may interact with the presynaptic membrane more or less tightly, being fusion competent ('primed') or fusion incompetent.

Promising candidates for SV docking are the interactions of the SV protein synaptotagmin with presynaptic membrane proteins. Synaptotagmin interacts directly with syntaxin, SNAP-25, neurexin I and Muncl3 (Südhof & Rizo 1996; Schiavo *et al.* 1997), as well as with the cytoplasmic domains of the αlA and αlB subunits of the N- and P/Q-type Ca^{2+} channels, respectively (Kim & Catterall 1997; Charvin *et al.* 1997; see also Seagar *et al.*, this issue). All these interactions may contribute to the specific docking of SVs to the presynaptic membrane. In addition, some of them such as that with syntaxin, are Ca^{2+}-sensitive and may play a role in triggering Ca^{2+}-dependent fusion (see below).

Other potential candidates for SV docking are the interactions of Rab3 with Rim, Csp with Ca^{2+} channels and DOC2 with Muncl8/n-Secl. Although the precise role of the SV-specific small G protein Rab3 in exocytosis has not been completely elucidated yet, a putative involvement of Rab and Rab effector proteins has been proposed in various steps, including targeting, docking and fusion (for reviews, see Bean & Scheller 1997; Südhof 1997; Geppert & Südhof 1998). Two specific Rab3 effectors have been identified, namely Rabphilin and Rim. Both proteins contain a Zn-finger domain essential for binding to GTP-Rab3, and a tandem of C_2 domains (see below). While Rabphilin is recruited to SVs through its binding to GTP-Rab3 (Stahl *et al.* 1996), Rim possesses an additional PTZ domain that is probably involved in its preferential localization at active zones in conventional synapses, and at presynaptic ribbons in ribbon synapses (Wang *et al.* 1997). The different location of the two known Rab effectors suggests distinct functions in the series of events leading from SV targeting to SV fusion. While the physiological role of Rabphilin is still unclear, Rim may function as a presynaptic signal of correct docking for the GTP-Rab-bearing SVs. The interaction between Rim and Rab3, occurring only when SVs approach the presynaptic membrane, can play an important role in the docking process (or at least in the first specific step initiating docking), as well as in the subsequent steps of exocytosis.

A functional interaction of the cysteine string protein Csp with presynaptic Ca^{2+} channels has been suggested by molecular biology and genetic approaches. The protein was originally cloned as a Ca^{2+} channel subunit and successively found to be specifically associated with SVs (Buchner & Gundersen 1997). Although Csp null mutants exhibit an impairment of the excitation–secretion coupling (Umbach *et al.* 1994), no proof of a direct and stable interaction between Csp and Ca^{2+} channels is available thus far. The most likely role of Csp in exocytosis is as a DnaJ-like molecular chaperone stabilizing, or disrupting, specific protein–protein complexes (see below). A potential target of Csp action is the interaction between syntaxin and the α subunit of P/Q-type and N-type Ca^{2+} channels that is associated with a decrease in availability of channels due to the slowing of their recovery from inactivation (Bezprozvanny *et al.* 1995; Kim & Catterall 1997). The interaction of Csp with the syntaxin–Ca^{2+} channel complex following SV docking may induce dissociation of the complex and relief of the channel inhibition. Csp would then have an indirect stimulatory effect on Ca^{2+} channel activity with the dual role of docking vesicles to active zones and making Ca^{2+} entry restricted to the only active zones bearing docked vesicles.

DOC2, an SV-associated, C_2 domain-containing protein, has recently been found to interact in a Ca^{2+}-independent manner with the presynaptic membrane-associated, syntaxin-binding protein Muncl8/n-Secl (Verhage *et al.* 1997). Although the latter protein is not exclusively localized at active zones, this interaction may have the dual role of contributing to SV docking in conjunction with other SV and presynaptic membrane components and of regulating syntaxin availability for the formation of the fusion core complex (see below).

Docking can potentially involve binding of proteins to inositol phospholipids (PIns) enriched in specific membrane compartments. One such example is represented by Mint (Okamoto & Südhof 1997), a protein that associates with the presynaptic membrane through PDZ domains and that binds with high affinity to phosphatidylinositol phosphates transiently produced during exocytosis (Martin 1997). Mint also binds to Muncl8/n-Secl and to the Muncl8/n-Secl-syntaxin complex. Therefore, the binding of the complex Mint–Muncl8/n-Secl on the presynaptic membrane to DOC2 and PIns on the SV membrane could be an efficient docking system, at the same time delivering free syntaxin for the subsequent formation of the fusion core complexes.

(b) *Formation of the fusion core complex and role of coiled-coil structures*

The pathway leading to SV exocytosis is aimed at the formation of a heterotrimeric 7S complex between the SV protein VAMP/synaptobrevin and the presynaptic membrane proteins syntaxin and SNAP-25 that eventually proceeds to achieve fusion of the two juxtaposed membranes. The three proteins, identified by two independent laboratories for being both receptors for

soluble NSF attachment proteins (SNAPs) and for the N-ethylmaleimide-sensitive factor (NSF) (and therefore named SNAP receptors or SNAREs), and specific targets for tetanus and botulinum neurotoxins were initially implicated in docking SVs to the presynaptic membrane and in recruiting SNAPs and NSF to the docking complex to elicit fusion (Schiavo *et al.* 1992, 1993; Sollner *et al.* 1993*a*,*b*; Huttner 1993).

In the last few years, the initial hypothesis has changed due to the following series of experimental evidence: (i) SVs still appear morphologically docked to the presynaptic membrane after cleavage of either SNARE protein in synapses treated with clostridial neurotoxins. Rather, the number of docked vesicles often appears increased with respect to control terminals, suggesting that cleavage might have impaired a crucial event in SV exocytosis downstream of the docking step (Hunt *et al.* 1994; Marsal *et al.* 1997); (ii) SVs are still able to dock to active zones in *Drosophila* mutants lacking syntaxin (Broadie *et al.* 1995); (iii) in PC12 cells, SNAP-25 does not appear to be engaged in the core complex until a stage that immediately precedes Ca^{2+}-triggered fusion (Banerjee *et al.* 1996); (iv) v- and t-SNAREs reconstituted into distinct liposome populations can form ternary complexes and elicit membrane fusion, demonstrating that they are both necessary and sufficient for fusion to occur (Weber *et al.* 1998); (v) in yeast, SNAPs, NSF and ATP are not required for docking or fusion, but for a predocking stage in which SVs and the presynaptic membrane have to become competent again for fusion with SNARE proteins in a monomeric state and located in the appropriate membrane compartment (Mayer *et al.* 1996; Nichols *et al.* 1997).

Based on the new evidence available, a model has been proposed in which the SNARE proteins are not necessary for specific docking of SVs to the active zones, but rather represent the true fusion proteins. *In vitro*, the SNAREs spontaneously assemble in a complex that is SDS-resistant and that can be dissociated by SNAPs/NSF on consumption of ATP (Sollner *et al.* 1993*b*; Pellegrini *et al.* 1995; Hayashi *et al.* 1995; Fasshauer *et al.* 1997). This suggests that monomeric SNAREs are thermodynamically metastable, representing a reservoir of potential energy that is released on formation of the ternary complex. In other words, the SNARE proteins can operate energy transitions that depend on their association state with the other SNAREs, reaching the lowest energy state when they are assembled into the fusion core complex. These assumptions imply that the energy released by SNARE binding can potentially be used to trigger fusion or to force the juxtaposed vesicle and presynaptic membranes into close contact until an intermediate, metastable state of hemifusion is reached. On the other hand, regeneration of the system, consisting of complex disruption and recovery of monomeric SNAREs, requires energy from ATP hydrolysis.

The transition from largely unstructured monomers to a tightly packed ternary complex dramatically increases α-helicity and thermal stability of the SNAREs (Fasshauer *et al.* 1997). The remarkable stability of the fusion core complex indicates that the proteins bind to each other through relatively large portions of their structure. The COOH-terminal region of syntaxin (residues 199–243) is required for binding to both VAMP/synaptobrevin and SNAP-25; a large central region of VAMP/synaptobrevin (residues 27–96) binds to SNAP-25 and syntaxin and both the NH_2- and the COOH-terminal regions of SNAP-25 are required for VAMP/synaptobrevin and syntaxin binding. Each of the regions involved in SNARE binding displays strong propensity for α-helical formation and contains sets of heptad repeats in register (reviewed in Calakos & Scheller 1996; Hanson *et al.* 1997*a*).

Heptad repeats consist in amphipathic α-helices that contain a repeated pattern of hydrophobic residues. These repeats exhibit high propensity to bind to each other in a coiled-coil quaternary structure in which the hydrophobic residues are found at the interface between the protein–protein complexes. Coiled-coils are formed when two or more right handed α-helices wrap around to each other, leading to the formation of a left-handed superhelical twist. Many examples of coiled-coil structures are found in nature, including the dimerization of DNA-binding proteins through the leucine zipper motif, the assembly of fibrous proteins, such as keratin, myosin or tropomyosin, and the multimerization of the influenza virus haemagglutinin. Coiled-coil structures fulfil a series of functions: they are physically resistant and rigid structures that provide a scaffold for structural or regulatory components; they allow dynamic arrangements and rearrangements of biochemical factors, such as activators of transcription; and, interestingly, drive conformational changes in fusogenic proteins (such as the influenza virus haemagglutinin) that trigger exposure of fusion peptides (reviewed in Lupas 1996).

Coiled coils can assemble in parallel or antiparallel fashion. While initially the v- and t-SNAREs binding was thought to be antiparallel (Sollner *et al.* 1993*a*), recent data from independent laboratories have demonstrated that syntaxin and VAMP/synaptobrevin assemble by the formation of a tight coiled-coil in which their α-helices are aligned in a parallel fashion, with the NH_2-terminal cytoplasmic regions and the COOH-terminal membrane anchors located at the same ends of the coiled-coil (Lin & Scheller 1997; Hanson *et al.* 1997*b*). By quick-freeze–deep-etch electron microscopy the 7S ternary SNARE complex appears as a 14 nm × 4 nm rod consisting of four bundled α-helices (one from VAMP/synaptobrevin and syntaxin, respectively, and two from SNAP-25) (Hanson *et al.* 1997*b*). These data have recently been confirmed by the analysis of the crystal structure of the core complex (Sutton *et al.* 1998).

The data reported so far suggest a model in which the free energy released by the 7S complex formation, sufficient to overcome the electrostatic repulsion and to remove the hydration barrier between the two facing membranes, is coupled with membrane fusion or prefusion. Formation of the complex from the free NH_2-terminal regions of VAMP/synaptobrevin and syntaxin may pull the SV and presynaptic membranes close together (nucleation of the 7S complex) (figure 1). As the process proceeds towards the COOH-terminal membrane anchors of the SNAREs, the facing membranes are zipped up together and forced into an extremely close apposition (zippering of the 7S complex). Under these conditions, the facing membranes can fuse their

cytoplasmic leaflets and form a metastable hemifusion diaphragm separating the synaptic cleft from the SV interior. The model partially resembles the mechanism of fusion induced by viral fusion proteins, such as influenza virus haemagglutinin. After activation, haemagglutinin assembles into homotrimers through the formation of a coiled-coil structure that acts as a spring-loaded hinge, propelling a previously buried hydrophobic peptide into the target membrane (White 1992). This similarity is particularly interesting when considering that one of the SNAREs, VAMP/synaptobrevin, has been predicted to contain a peptide with potentially fusogenic properties (Jahn & Südhof 1994).

The model, although still hypothetical, indicates that it is the assembly, and not the NSF-mediated disassembly of the SNARE complex that drives fusion. Thus, what could be the function of SNAPs/NSF in the process of exocytosis? After fusion, the stable ternary complexes, associated with the single fused membrane, may recruit SNAPs and NSF from the cytoplasm. NSF is an ATPase that acts as a molecular chaperone and its function is likely to be that of regenerating the components of the core complex before the endocytotic recycling of fused SVs takes place. The energy released from ATP hydrolysis by NSF can be used for the active, energy-dependent process of disruption of the 7S complex to regenerate monomeric SNAREs and reactivate them for a new reaction cycle. In order to recover v- and t-SNAREs associated with their respective membrane compartments, this process should occur before the fission step of endocytosis (figure 1). However, the observation of heterotopic recovery of t-SNAREs in the SV membrane (Walch-Solimena *et al.* 1995) indicates that this process may not always keep pace with the exo-endocytotic cycle of SVs. Although ATP hydrolysis by NSF is likely to occur after the fusion event, there are no clear proofs of whether the 7S complex recruits SNAPs/NSF before or after fusion. Moreover, a role in the temporal regulation of SNAP/NSF recruitment can be played by complexins, a family of proteins that compete with SNAPs for their binding to the SNARE complex (McMahon *et al.* 1995).

In addition to the regeneration of monomeric SNAREs, SNAPs and NSF may also be involved in priming SVs for fusion, by facilitating exchange of SNARE binding partners and by promoting the formation of the fusion core complex. In fact, SNAPs/NSF may serve to disassemble parallel v-SNARE/t-SNARE complexes formed on the SV membrane, making VAMP/synaptobrevin available for successive rounds of exocytosis.

(c) *Regulation of the fusion core complex formation*

(i) *Spatial regulation: sequestration of binding partners*

Binding of some identified proteins to v- and t-SNAREs has been found to play a regulatory role in SNARE function by preventing the formation of the fusion core complex. Among these proteins, Muncl18/n-Secl and Muncl3 bind to syntaxin, the ATPase Hrs-2 binds to SNAP-25, synaptophysin binds to VAMP/synaptobrevin and SV2 binds to synaptotagmin (if we can consider the latter protein a v-SNARE) (Calakos & Scheller 1996; Schivell *et al.* 1996; Betz *et al.* 1997; Bean *et al.* 1997).

As the t-SNAREs syntaxin and SNAP-25 are widely distributed in the axonal membrane well beyond the active zones (Galli *et al.* 1995), the t-SNARE sequestration proteins may play the primary role of preventing an indiscriminate formation of fusion core complexes outside the active zones. Both Muncl18/n-Secl and Muncl3 bind to the same site of the NH_2-terminal domain of syntaxin and may have similar roles in sequestering the protein. However, the severe deficits in neurotransmitter release observed in Muncl18 and Muncl3 mutants (Hosono *et al.* 1992; Hosono & Kamiyo 1991) suggest that these proteins may also play an important role in delivering syntaxin to the docking complex and, possibly, in capturing and regenerating syntaxin on the presynaptic membrane after disassembly of the fusion complex.

If the sequestration hypothesis holds true, what could be the role of the v-SNARE sequestration? VAMP/synaptobrevin is complexed with synaptophysin (partly in association with myosin V and V-ATPase), although the functional regulation of this interaction has not been clarified (Ludger & Galli 1998; Prekeris & Terrian 1997). As SVs are committed for exocytosis, there should be no need to sequester their SNARE. However, since significant amounts of t-SNAREs are found on SVs (Walch-Solimena *et al.* 1995), VAMP/synaptobrevin sequestration could have the role of preventing it from associating with syntaxin and SNAP-25 on the SVs membrane. In fact, parallel binding of v- to t-SNAREs on SVs would prevent the interaction with presynaptic t-SNAREs that makes the docked vesicle 'fusion competent'. If this is the case, dissociation of the synaptophysin–VAMP/synaptobrevin complex should occur just after the docking step, to preserve SV reactivity for the formation of the fusion core complex.

Another, not alternative, function of the SNARE sequestration proteins could be that of stabilizing SNARE proteins in the monomeric state. As mentioned above, monomeric SNAREs are thermodynamically metastable and possess a high level of free energy. Although SNARE sequestration proteins are not classical chaperones, their binding to SNAREs may keep them monomeric and thereby reactive for the formation of the fusion core complex.

(ii) *Temporal regulation: mutually exclusive interactions*

SNARE sequestration proteins may also function in the temporal regulation and synchronization of the v- and t-SNARE interactions at the appropriate stage of the SV cycle, provided that a SV or presynaptic membrane protein displaces them from their respective SNARE partner. The putative t-SNARE unlocking proteins DOC2, Mint and Tomosyn have been described.

DOC2 is a SV protein that binds to Muncl18/n-Secl and weakens its binding to syntaxin through a competitive interaction (Verhage *et al.* 1997). Although the interaction between DOC2 and Muncl18/n-Secl is weaker than that between Muncl18/n-Secl and syntaxin *in vitro*, other components of the exocytotic machinery (e.g. SNAP-25) may modulate it effectively, making it possible that the interaction between DOC2 and Muncl18/n-Secl acts as a docking signal that releases syntaxin for binding to the v-SNARE VAMP/synaptobrevin. Thus, if a vesicle component is responsible for the removal of the t-SNARE blockade, SVs may behave as keys unlocking presynaptic

t-SNAREs following docking to the active zone. In this condition, only active zones bearing docked vesicles will have reactive t-SNAREs available for the core complex formation.

A recently described presynaptic membrane-associated protein, Mint, binds Munc18/n-Sec1 through a mutually exclusive interaction with respect to syntaxin and thereby may deliver syntaxin to vesicle VAMP/synaptobrevin (Okamoto & Südhof 1997). Although the kinetics of the formation of the Mint–Munc18/n-Sec1 complex have not yet been elucidated, it is possible that binding of Mints to SV PIns (see above) triggers its binding to Munc18/n-Sec1 and the subsequent release of syntaxin from sequestration.

Tomosyn is a recently identified component of the docking–fusion machinery that binds syntaxin and dissociates it from Munc18/n-Sec1. Tomosyn is much more potent than DOC2 and Mint in dissociating the syntaxin–Munc18/n-Sec1 complex and this dissociation is followed by the formation of a 10S Tomosyn complex with syntaxin, SNAP-25 and synaptotagmin. On binding of VAMP/synaptobrevin, the 10S complex may be converted into the 7S fusion core complex, a reaction in which Tomosyn may play a catalytic role (Fujita *et al.* 1998).

(d) *Regulation of fusion by calcium: role of C_2 domain-containing proteins*

When an action potential reaches the nerve terminal, Ca^{2+} enters through voltage-dependent channels and the Ca^{2+} influx abruptly elevates the probability of release of neurotransmitter quanta from the very low level of the resting state, triggering exocytosis in a few hundreds of microseconds. This very efficient coupling of the electrical activity with neurotransmitter release is achieved by three major factors: (i) SVs are docked in close vicinity to the cytoplasmic mouth of the Ca^{2+} channel and therefore are flooded by very high Ca^{2+} concentrations; (ii) SVs possess multiple low-affinity calcium sensors; and (iii) the fraction of docked vesicles primed prior to the Ca^{2+} trigger are likely to be in a metastable intermediate state of hemifusion between the vesicle and the presynaptic membranes. Such a state is temporarily blocked under resting Ca^{2+} levels and proceeds very rapidly to completion on stimulation of the Ca^{2+} sensor(s).

Some presynaptic component(s) should stabilize the prefusion intermediate, preventing its transition to the fusion state. Three synaptic vesicle proteins are potential candidates as SNARE complex stabilizers, namely: (i) synaptotagmin, a member of the SNARE complex (reviewed in Calakos & Scheller 1996; Ludger & Galli 1998), may, under resting conditions, stabilize the SNARE complex-induced hemifusion diaphragm; (ii) Rab3, that also associates to some extent with the SNARE complex, may prevent exocytosis of primed SV adjacent to fused SV (Horikawa *et al.* 1993; Johannes *et al.* 1996; Geppert *et al.* 1997); and (iii) Csp may stabilize the priming state of SV by acting as a molecular chaperone (see below).

The ultimate effect of Ca^{2+} entry may be that of either removing the putative negative clamp or actively triggering fusion by destabilizing the prefusion state. According to the current view, Ca^{2+} induces a very rapid electrostatic and/or conformational change in the fusion core complex through binding to a Ca^{2+} sensor that is most likely represented by the SV protein synaptotagmin. The cytoplasmic region of synaptotagmin contains two tandem domains homologous to the regulatory Ca^{2+} and phospholipid binding domain of protein kinase C, named C_2A and C_2B domains (reviewed in Südhof & Rizo 1996). A large body of experimental evidence indicates that both C_2 domains of synaptotagmin operate as Ca^{2+}-regulated independent modules of interaction with protein partners and phospholipids. C_2A and C_2B bind Ca^{2+} with dissociation constants of 60 and 300–400 μM, respectively. The functional specialization of the two domains is further demonstrated by the distinct interactions in which they are involved. The C_2A domain binds to acidic phospholipids and to syntaxin with a Ca^{2+}-dependence that is comparable with that required to stimulate neurotransmitter release, whereas the C_2B domain mediates the Ca^{2+}-dependent binding to specific phosphoinositides, the Ca^{2+}-dependent interaction with homologous C_2B domains of other synaptotagmin molecules and the Ca^{2+}-independent interaction with the SV protein SV2 (Schivell *et al.* 1996).

The crystal structure of the C_2A domain of synaptotagmin has recently been clarified and shown to consist of a sandwich of two β-sheets each containing four β-strands. This arrangement brings together two connecting loops containing, respectively, three and two aspartate residues that together coordinate the binding of two Ca^{2+} ions (Shao *et al.* 1996). Although Ca^{2+} was found to induce only a minimal structural rearrangement, it increased the stability of the C_2 domain and brought about a noticeable alteration in the electrostatic potential due to the insertion of positive charges into an overall acidic environment (Shao *et al.* 1997). The latter change appears to act as an electrostatic switch turning on the interaction with syntaxin, that is unfavoured at basal Ca^{2+} by electrostatic repulsion. The rapid binding of synaptotagmin to syntaxin at a primed vesicle, triggered by Ca^{2+} influx, may be followed by a structural rearrangement sufficient to destabilize the metastable hemifusion intermediate and to let it proceed to complete fusion. Synaptotagmin can therefore function as a fusion clamp at basal Ca^{2+} levels and, at the same time, act as fusion trigger following stimulation-induced Ca^{2+} influx.

The hypothesis of a dual negative and positive role of synaptotagmin in SV fusion is also supported by the observations that, in mutant mice lacking synaptotagmin I, as well as in *Drosophila* or *Caenorhabditis elegans* bearing null mutations in the synaptotagmin gene, the evoked neurotransmitter release is markedly depressed, while the frequency of spontaneous release is often increased (Nonet *et al.* 1993; Geppert *et al.* 1994; Littleton *et al.* 1994). Therefore, synaptotagmin can: (i) act as a docking protein, positioning SVs to the appropriate presynaptic sites; (ii) participate in the SNARE complex, binding to syntaxin and SNAP-25 and contributing to recruitment of βSNAP and NSF to the core complex (Schiavo *et al.* 1995); (iii) represent a fusion clamp at resting Ca^{2+} concentrations, by preventing the transition from hemifusion to fusion within the SNARE complex; and (iv) trigger Ca^{2+}-dependent fusion, as the major Ca^{2+} sensor at the active zone, by acting either directly as a 'fusion protein' (see below) or indirectly through effector proteins such as syntaxin.

In addition to the predominant role played by synaptotagmin, other proteins may be involved in regulating the fusion machinery and the probability of neurotransmitter release in response to Ca^{2+} influx. One such candidate is Rab3a, which has been proposed to have a role after docking and priming by decreasing the probability of release of primed SVs adjacent to a fused SV (Geppert *et al.* 1997; Bean & Scheller 1997; Geppert & Südhof 1998). This sort of 'lateral inhibition' would limit release to single quanta per action potential, a common feature of central synapses. However, a final answer on this putative function of Rab3 in the regulation of fusion has not been given thus far.

Other C_2 domain-containing proteins associated with the exocytotic machinery have been identified. These proteins include the Rab3 effectors Rabphilin and Rim, DOC2 and Muncl3. Although some of the C_2 domains in these proteins appear to be physiologically important, such as the first C_2 domain of DOC2 for binding to Muncl8/n-Sec1 or the middle C_2 domain of Muncl3 for binding to syntaxin, the Ca^{2+}/phospholipid binding properties of these multiple domains have not been investigated in detail (reviewed in Bennett 1997). In addition, the above-mentioned interactions are not Ca^{2+}-dependent and some of these C_2 domains, such as those of Rim and Muncl3, are not likely to bind Ca^{2+}.

4. PHOSPHOLIPID INTERACTING PROTEINS

Albeit not yet completely elucidated, the role of the interactions between protein and phospholipids is likely to be of paramount importance in exocytosis. From a functional point of view, three types of protein–phospholipid interactions can be distinguished: (i) interactions that stabilize the phospholipid bilayer and prevent its fusion with other membranes; (ii) Ca^{2+}-dependent interactions that may potentially trigger membrane fusion; and (iii) interactions with phosphoinositides involved in signal transduction and regulation of vesicle trafficking.

An example of the first mechanism is represented by the synapsins that bind with high affinity to acidic phospholipids of the cytoplasmic leaflet of the SV membrane (Benfenati *et al.* 1989*a*,*b*; Stefani *et al.* 1997). The binding involves both electrostatic and hydrophobic interactions mediated by the synapsin C domain, which is both hydrophobic and highly charged. Hydrophobic labelling has shown that synapsin I penetrates the hydrophobic core of the SV membrane with multiple amphiphatic stretches assuming α-helix or β-sheet secondary structures (J. J. Cheetam, T. Weber, S. Hilfiker-Rothenfluh, F. Benfenati, A. J. Czernik & P. Greengard, unpublished data). Indeed, synapsin I possesses high surface activity and a very high limiting surface at amphiphatic interfaces suggesting that, at the estimated concentrations physiologically associated with SVs, it may cover a significant portion of the vesicle surface and thereby mediate the interactions of SVs with other nerve terminal partners (Ho *et al.* 1991). Phosphorus NMR has also indicated that binding of synapsin I to phospholipid membranes is followed by the stabilization of the phospholipid bilayer, probably achieved through an expansion of the headgroup region of the membrane that makes the transitions toward non-bilayer arrangements more difficult (figure 3; Benfenati *et al.* 1993). These effects

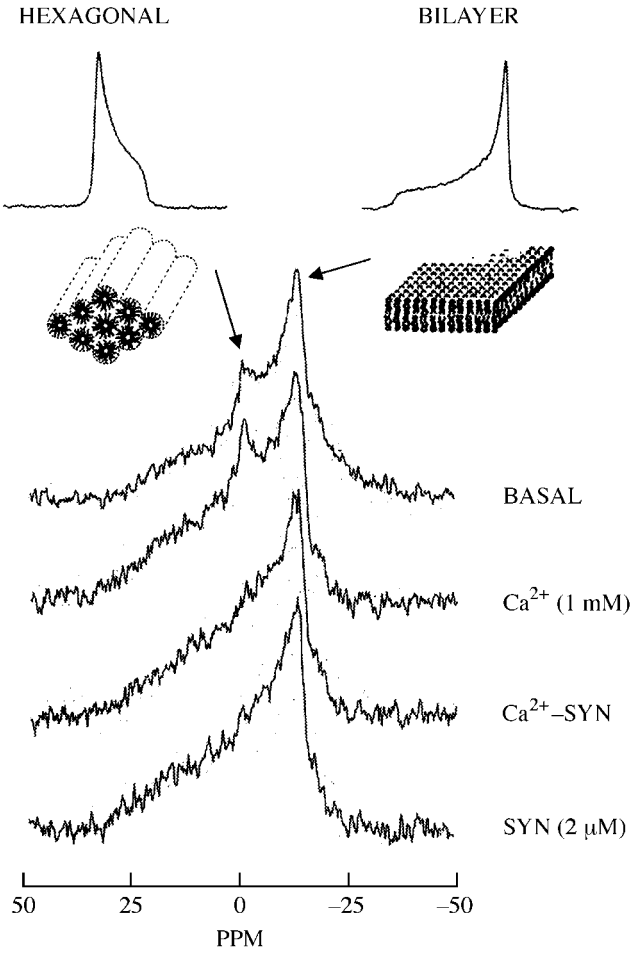

Figure 3. Stabilizing effect of synapsin I on phospholipid bilayers as evaluated by proton decoupled ^{31}P-NMR. Phospholipids (phosphatidylethanolamine:phosphatidylserine, 7:3) organized in extended bilayers exhibit a ^{31}P-NMR powder pattern with a high-field peak and a broad low-field shoulder, whereas a low-field peak and a narrower high-field shoulder are observed when phospholipids are arranged in the unstable hexagonal phase (upper panel). The NMR spectra reported in the lower panel show that synapsin I is capable of preventing the transition of membrane phospholipids from the bilayer to the inverted hexagonal phase induced by the addition of Ca^{2+}, thereby increasing the probability for vesicle phospholipids to assume a bilayer organization both under basal conditions and in the presence of destabilizing stimuli (modified from Benfenati *et al.* 1993).

may play an important functional role in vesicle biogenesis and trafficking. In fact, SVs are among the smallest organelles within cells but, notwithstanding the curvature-induced intrinsic instability, they exhibit a remarkable uniformity in size and a substantial refractoriness to spontaneous fusion events. Since synapsin I undergoes cycles of association with and dissociation from the SV membrane, its stabilizing effect can be transiently lost in synapsin I-depleted SVs that, released from the reserve pool, undergo the final steps of exocytosis with an increased propensity for fusion with the target membrane.

A class of homologous proteins, the annexins, share with the synapsins the ability to associate with acidic membrane phospholipids and to promote the aggregation of liposomes and secretory granules (Creutz 1992). Most of the annexins, including synexin (annexin VII) and calpactin (annexin

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II), enhance the Ca^{2+}-dependent fusion of phospholipid vesicles by accelerating the rate-limiting membrane aggregation process. However, unlike synapsin I, these proteins induce membrane destabilization and their activity is strictly Ca^{2+}-dependent (Creutz *et al.* 1978; Meers *et al.* 1988; Drust & Creutz 1988). The overall contribution of these proteins to neuroexocytosis is still unclear.

Calcium-dependent protein–phospholipid interactions implicated in membrane fusion may involve C_2 domain-containing nerve terminal proteins, such as synaptotagmin. Via its C_2A domain, synaptotagmin binds with very high affinity in a Ca^{2+}-dependent manner to acidic phospholipids enriched in the cytoplasmic leaflet of the plasma membrane (reviewed in Südhof & Rizo 1996). The Ca^{2+}-dependent interaction with phospholipids, together with the similarly Ca^{2+}-dependent oligomerization and syntaxin binding, have the potential to play a direct role in triggering the transition from the hemifusion state of primed vesicles to full fusion at micromolar concentrations of Ca^{2+}.

Phosphoinositides have been proposed to play specific roles in the regulation of actin assembly, membrane fusion and SV trafficking as site-specific second messengers. Phosphoinositide-transforming enzymes such as PIns 4-kinase or PIns4P 5-kinase, have been localized to SV and plasma membranes, raising the possibility that the reversible phosphorylation of phosphatidylinositol may generate compartment-specific signals recruiting and/or activating proteins (De Camilli *et al.* 1996; Martin 1997; Wiedemann *et al.* 1998). Various proteins involved in secretion have been reported to specifically bind PIns phosphates through C_2, PH and PTB domains (Pawson 1995; De Camilli *et al.* 1996). PH domains have a positively charged 'variable loop' face that binds negatively charged phosphate groups of phospholipids, particularly PIns4,5 P_2. PTB domains, first described in the protein Shc as phosphotyrosine-binding domains poorly related to SH2 domains, also bind to the polar group of PIns through a structure related to that of PH domains. PIns-binding proteins involved in SV exocytosis include synaptotagmin, whose C_2B domain binds to PIns3,4,5 P_3 at resting Ca^{2+} levels and to PIns4,5 P_2 at micromolar Ca^{2+} concentrations (Schiavo *et al.* 1996), and Mint, whose PTB domain binds, similarly to the Shc PTB domain, to PIns4,5 P_2 (Okamoto & Südhof 1997). If the activity of PIns kinases is regulated during exocytosis, the transient production of PIns in the SV and/or presynaptic membranes may serve to recruit, activate or modulate SV and/or presynaptic membrane proteins, with the role of regulating SV trafficking. For example, an SV can become susceptible to docking to presynaptic membrane-associated Mint–Munc18/n-Sec1 complex only on formation of specific PIns on its membrane, or the C_2B domain of synaptotagmin may be folded back to bind a SV PIns (e.g. Pins4 P) at resting Ca^{2+} levels and extend to interact with a presynaptic membrane PIns (e.g. PIns4,5 P_2) on Ca^{2+} entry.

5. OTHER PROTEIN MOTIFS POTENTIALLY INVOLVED IN EXOCYTOSIS

As mentioned in § 1, only a few intracellular modules that mediate protein–protein interactions are involved in a multitude of intracellular processes that are highly specific and compartmentalized. These domains exhibit conserved and distinctive features that are embedded in variable sequences responsible for the affinity and specificity with which each individual domain binds to its specific partners.

(a) *J domains*

J domains consist of stretches of 70 amino acids, typically found in the NH_2-terminus of all members of the DnaJ chaperone–heat shock protein family, composed of two regions of predicted α-helical structure separated by a nonhelical region (Silver & Way 1993; Hendrick & Hartl 1993). The archetypal J domain-containing protein is the bacterial DnaJ, that interacts with the ATPase DnaK (the procaryotic homologue of Hsp70) and with the ADP–ATP exchange factor GrpE, acting as chaperones in protein folding and unfolding reactions. The eukaryotic homologues of DnaJ interact with the chaperone protein Hsp70 or with one of its homologues, such as Hsc70, through a conserved HPD tripeptide and stimulate its intrinsic ATPase activity. The complex can interact with substrate proteins either in native or unfolded conformation and alternatively induce, on ATP hydrolysis, unfolding or refolding of the substrate proteins as well as assembly or disassembly of protein complexes.

A family of SV-associated cysteine string proteins (Csp) exhibit a significant homology with the J domain in the NH_2-terminal region and associate with the external leaflet of the SV membrane through palmitoylation of the unusual string of cysteine residues present in the central region of the molecule (Buchner & Gundersen 1997). The homology with DnaJ proteins suggests a role for Csp as a molecular chaperone, possibly in conjunction with proteins of the Hsp70 family. Csp can interact with unfolded proteins or with proteins in an energetically unstable conformation and favour their correct folding, association with other proteins and stabilization of the protein complexes (Braun & Scheller 1995; Chamberlain & Burgoyne 1997; Buchner & Gundersen 1997). This can be particularly relevant in regulated exocytosis, in which sequential protein–protein interactions and energy level transitions occur.

Although a unequivocal protein partner has not been identified yet, Csp is thought to operate at the SV-active zone interface by stabilizing docking and SNARE complexes and/or by activating voltage-dependent Ca^{2+} channels either directly or by dissociating the syntaxin–Ca^{2+} channel complex. The latter putative role, although not complemented by the experimental evidence of a direct interaction between Csp and Ca^{2+} channels, is supported by the uncoupling of membrane depolarization to neurotransmitter release observed in *Drosophila* Csp mutants (Umbach *et al.* 1994).

(b) *SH3 domains*

Src homology-3 (SH3) domains are ubiquitous intracellular protein modules that interact with proline-rich protein ligands and promote the formation of functionally active protein complexes. Together with Src homology-2 (SH2) domains (see below), SH3 domains were first identified in the Src family of protein tyrosine kinases and

subsequently found to be present in proteins involved in the transduction of signals originating from growth factor receptors, in cytoskeletal components (e.g. spectrin and myosin I), in adapter proteins (e.g. Grb2 and Csk) and in signal transduction enzymes (e.g. non-receptor tyrosine kinases and phospholipase Cγ) (Pawson & Schlessinger 1993; Morton & Campbell 1994; Pawson 1995).

SH3 domains consist of approximately 60 amino acids that, despite differences in the primary structure, exhibit the same three-dimensional topology, consisting of two β-sheets packed against each other at right angles and of three variable loops, namely the RT, n-Src and distal loops. The ligand-binding surface of the domain consists of three sites containing patches of aromatic amino acids (sites 1 and 2) and an acidic residue provided by the RT loop (site 3) (Pawson 1995; Mayer & Eck 1995).

As proline-rich regions are a relatively common finding in many proteins, they should fulfil specific requirements in order to function as SH3 binding sites. The core ligand seems to consist of a seven-residue peptide containing the minimal consensus sequence xPxxP, where Ps represent the two conserved prolines crucial for high-affinity binding and x any residue. The two xP dipeptides fit into the hydrophobic pockets of sites 1 and 2 of the SH3 domain, whereas the third pocket of site 3 requires a basic residue, most frequently an arginine. Since SH3 domain-binding peptides can bind SH3 domains in either parallel or anti-parallel fashion, the basic residue can be located at either the NH_2-terminal or COOH-terminal with respect to the xPxxP motif. The former case conforms to the so-called class I ligand, with the minimal consensus RxxPxxP, and the latter constitutes the so-called class II ligand, with minimal consensus xPxxPxR (Pawson 1995; Mayer & Eck 1995; Simon & Schreiber 1995). In spite of the presence of these core motifs in many proteins, protein–protein interactions mediated by SH3 domains show a high degree of specificity that depends on the x residues included in the minimal consensus as well as on adjacent residues.

An involvement of SH3 domains in the exo-endocytotic cycle of SV was originally suggested by the demonstration that, in nerve terminals, three major proteins are excellent ligands for the SH3 domains of the adapter protein Grb2, namely dynamin, synaptojanin and synapsin I (McPherson *et al.* 1994). As Grb2 is not likely to play a major role in exo-endocytosis, the search of endogenous SH3 domain-containing proteins identified amphiphysin, an AP2-binding protein interacting with dynamin and synaptojanin and involved in SV endocytosis (Cremona & De Camilli 1997), and the tyrosine kinase c-Src interacting with synapsin I (Onofri *et al.* 1997).

Synapsin I is the major SV protein binding the SH3 domain of c-Src and can be affinity purified using the c-Src SH3 domain as ligand. The binding of synapsin I to the SH3 domain of c-Src is followed by a marked activation of the Src tyrosine kinase activity that is reversed by the presence of an excess of Src SH3 domain (figure 4*a*,*b*; Onofri *et al.* 1997). This stimulatory effect supports the functional importance of the interactions involving the SH3 domain in the regulation of Src kinase activity (reviewed in Mayer 1997). Synapsin I is therefore at the crossroads of multiple phosphorylation pathways implicated in the response of neurons to extracellular stimuli, being phosphorylated by Ca^{2+}/calmodulin-dependent kinases, protein kinase A and MAP kinase and acting as an endogenous activator of Src and of tyrosine phosphorylation processes within the nerve terminal (figure 5).

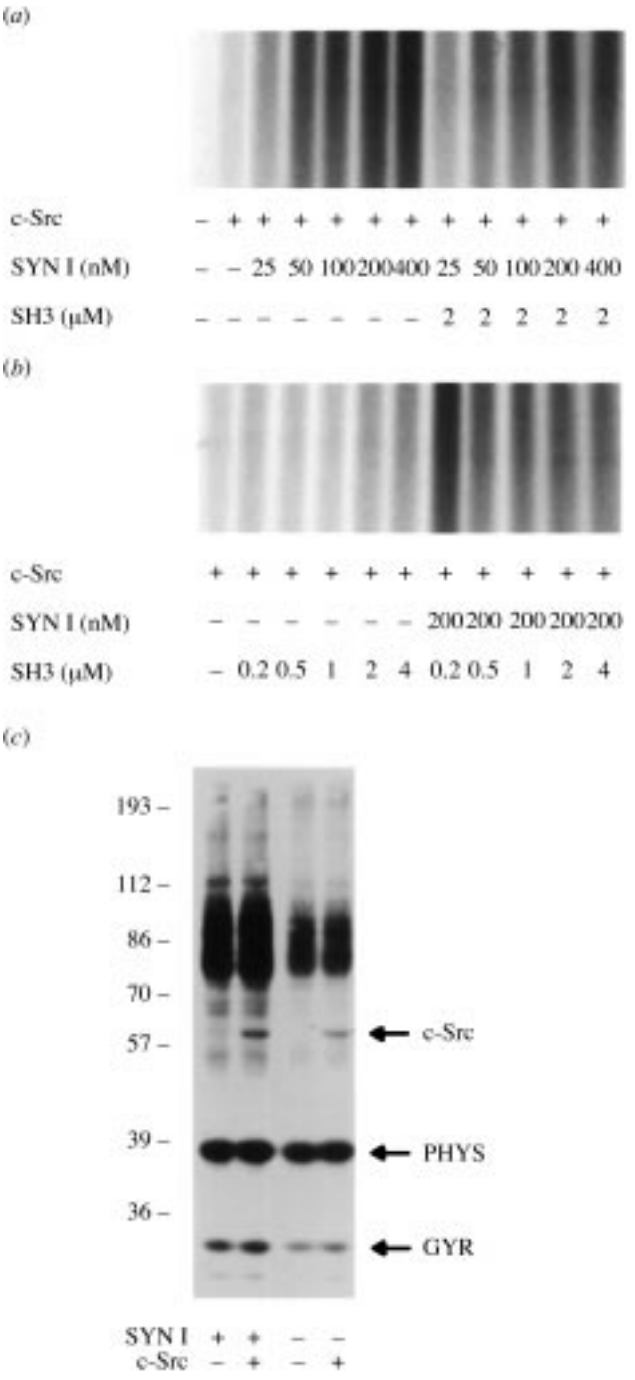

Figure 4. Stimulation of c-Src activity by synapsin I. (*a*) and (*b*) Purified Src kinase was incubated with synapsin I in the absence or presence of the Src SH3 domain as indicated. Tyrosine kinase activity is shown as radioactive phosphate incorporation into the substrate peptide poly(Glu^{80},Tyr^{20}) after SDS-PAGE and alkali autoradiography. (*c*) The endogenous Src activity was evaluated in purified SVs in the presence or absence of endogenous synapsin I and of exogenous purified c-Src by analysing tyrosine phosphorylation of SV substrates by immunoblotting with antiphosphotyrosine antibodies. PHYS, synaptophysin; GYR, synaptogyrin. For further details, see text and Onofri *et al.* (1997).

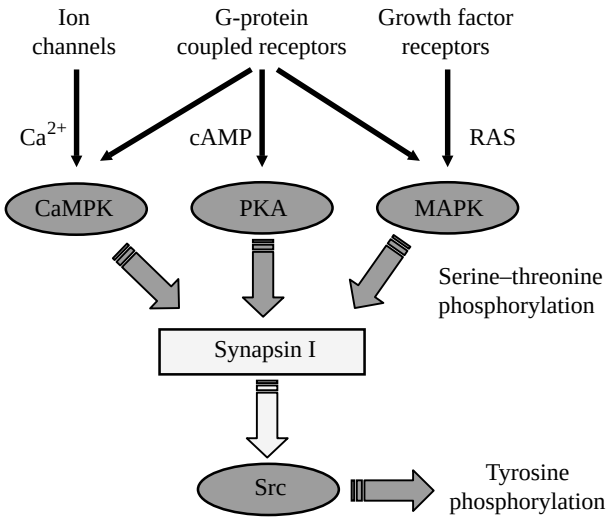

Figure 5. Flowchart of the interrelated signal transduction pathways that involve synapsin I. Synapsin I is phosphorylated on serine residues by multiple kinases in response to a variety of extracellular stimuli and, in turn, acts as an endogenous activator of c-Src tyrosine kinase activity.

Synaptic vesicles possess an endogenous tyrosine kinase activity, most of which is represented by a c-Src pool permanently associated with the SV membrane (Pang *et al.* 1988; Barnekow *et al.* 1990). The demonstration that synapsin I-depleted SVs have a markedly depressed endogenous tyrosine kinase activity (figure 4*c*; Onofri *et al.* 1997) indicates that the level of tyrosine phosphorylation on SVs may depend on the extent of association of synapsin I with the SV membrane. As phosphorylation of synapsin I by Ca^{2+}/calmodulin-dependent protein kinase II promotes its dissociation from SVs (Greengard *et al.* 1993; Stefani *et al.* 1997), the possibility exists that, at resting Ca^{2+} concentrations, synapsin I is bound to SVs and stimulates the endogenous Src activity, whereas at higher Ca^{2+} levels the SV-associated Src activity is depressed as a consequence of the synapsin I loss. By the same token, it is possible that SVs in the reserve pool, being saturated with synapsin I, have a higher tyrosine kinase activity than SVs in the releasable pool that are relatively depleted of synapsin I (Torri Tarelli *et al.* 1992; Pieribone *et al.* 1995).

Although the precise role of tyrosine phosphorylation in the regulation of exocytosis has not yet been clarified (Boxall & Lancaster 1998), the presence of tyrosine phosphoproteins and tyrosine kinases in association with SVs and with the presynaptic membrane suggests a potential modulatory role of tyrosine phosphorylation in the regulation of SV trafficking. Synaptophysin, an abundant integral protein of SVs that forms a complex with the v-SNARE VAMP/synaptobrevin in undocked vesicles (see above), has multiple potential tyrosine phosphorylation sites in its cytoplasmic tail and is effectively phosphorylated by c-Src (Pang *et al.* 1988; Barnekow *et al.* 1990; Onofri *et al.* 1997). It is tempting to speculate that tyrosine phosphorylation of synaptophysin regulates VAMP/synaptobrevin sequestration and/or that tyrosine phosphorylation of SV substrates recruits SH2 domains, expanding the array of possible interactions with proteins involved in nerve terminal signal transduction.

(c) *Other domains potentially involved in exocytosis*

Besides the C_2, J and SH3 domains described above, other domains may potentially be involved in specific recognition events modulating various steps of exocytosis.

PDZ domains consist of 80–90 amino acid motifs present in a variety of unrelated proteins, the majority of which are associated with the cytoskeleton of the cell mantle and with the cytoplasmic side of the plasma membrane. PDZ domains can either bind to specific recognition stretches found at the COOH-terminus of certain proteins or can associate with other PDZ domains. PDZ domains are involved in clustering transmembrane proteins and in targeting signalling proteins to the plasma membrane by recruiting them from the cytosol (Fanning & Anderson 1996). This activity may be particularly important in the assembly of protein networks among SV proteins, presynaptic membrane proteins and cytosolic factors that play a role in docking and fusion. Among the proteins involved in exocytosis identified thus far, Mint and the Rab3 effector Rim contain PDZ domains that presumably mediate their specific association with the presynaptic membrane (Okamoto & Südhof 1997; Wang *et al.* 1997).

Src homology-2 (SH2) domains recognize and bind with high affinity to phosphotyrosine-containing peptides through a bipartite binding site in which one pocket, containing a highly conserved arginine residue, binds the phosphotyrosine, while a second binding surface allows specific recognition of the surrounding residues (Pawson & Schlessinger 1993; Pawson 1995). Synaptic vesicles contain SH2 domain-bearing protein kinases such as c-Src as well as Src substrates such as the integral membrane proteins synaptophysin and synaptogyrin (Pang *et al.* 1988; Barnekow *et al.* 1990; Stenius *et al.* 1995; Onofri *et al.* 1997) that may exhibit SH2 domain-binding regions on their phosphorylation on tyrosine. Although no extensive investigations have yet been carried out, these observations suggest that SH2 domain-mediated interactions may be triggered by activation of tyrosine phosphorylation processes. In addition to SH2 domain-containing proteins, nerve terminal proteins containing the related PTB domain, such as Mint (Okamoto & Südhof 1997), may be involved both in binding phosphotyrosine-containing peptides and in binding specific PIns as discussed above.

WW domains are recently identified protein modules consisting of 38 amino acids with the distinguishing feature of the presence of two highly conserved tryptophans spaced 22 residues apart. They bind with high affinity to a minimal xPPxY consensus sequence similar to, but distinct from, the minimal xPxxP binding motif of SH3 domains (Sudol 1996). Like SH3 domains, WW domains can bind to proline-rich proteins and the possibility exists that they act in concert with SH3 domains or that they compete with SH3 domains for the same polyproline stretches. The precise role of these domains in neurotransmitter release has not yet been demonstrated, but it is easy to predict that they will soon become involved in the complex machinery of the exo-endocytotic cycle of SV.

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6. CONCLUSIONS

In the last ten years, an astonishing convergence of studies from the fields of genetics, biochemistry, molecular biology, cell biology and microbiology has greatly contributed to the elucidation of many previously uncovered aspects of neurotransmitter release mechanisms. The outcome of this mass of investigations has produced a large number of papers describing a multitude of candidate proteins mediating the various molecular steps of the exo-endocytotic cycle of SVs. The large number of SV, plasma membrane and soluble proteins, the multiple interactions among them, the exact timing of the sequential reactions define an incredibly complex network of protein–protein and protein–phospholipid interactions in which it is still difficult to distinguish the interactions that play a pivotal role from those that have only a modulatory role. Although most of the molecular components of the neuroexocytotic machinery are shared by other secretory cells, the remarkable efficiency and sophistication of neurotransmitter release relies on the participation of neuron-specific proteins or of neuron-specific isoforms of ubiquitous proteins involved in secretion that make exocytosis in neurons temporally efficient, resistant to exhaustion and highly regulated.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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