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Minimal Residues in Linker Domain of Syntaxin 1A Required for Binding Affinity to Ca^{2+}/Calmodulin-Dependent Protein Kinase II

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The linker domain is important for the conformational change syntaxin 1A, which enables it to act as a SNARE for exocytosis. We found that when applied exogenously, the linker domain is a potent inhibitor of exocytosis through inhibiting interaction between autophosphorylated CaMKII and endogenous syntaxin 1A (Ohyama et al. [2002] J. Neurosci. 22:3342–3351). To identify the simplest and the most potent inhibitor for exocytosis, we further characterized the linker domain and determined the minimal number of residues required for CaMKII binding. The minimal length of the CaMKII-binding site was 145–172 residues and a loss of G172 considerably weakened affinity for CaMKII. The basic amino acid clusters, R151 and K146, were indispensable for binding, whereas R148 was not. A comparison of the CaMKII-binding in several syntaxin isoforms revealed that the substitution of S162 attenuated CaMKII-binding activity. These results suggest that S162 is an important residue as well as the basic amino acid cluster within region 145–172 of the linker domain.

Key words: exocytosis; syntaxin; SNARE mechanism; calmodulin-dependent protein kinase II (CaMKII); linker domain

The most important molecular principle for intracellular vesicular trafficking from yeast to mammals is based on the SNARE mechanism, (Bock et al., 2001; Rizo and Südhof, 2002). This theory states that complexes composed of SNARE proteins mediate vesicular targeting and fusion. Among these, the syntaxin family is the largest and each member plays roles in many vesicular trafficking events through the SNARE mechanism (Bennett et al., 1993; Bock et al., 2001; Dacks and Doolittle, 2002). In neurons, syntaxin 1A is the key component for regulated exocytosis in terms of neurotransmitter release from the synaptic vesicles.

Syntaxin 1A consists of three N-terminus helical domains (Ha, Hb, and Hc), one linker domain, one C-terminus helical domain (H3), and one transmembrane domain (Fernandez et al., 1998). The most important domain in syntaxin is thought to be H3, where more than ten proteins bind, including other SNAREs. The Ha–Hc domains are thought to negatively regulate H3 domain activity. The linker domain (145–184) connects Ha–Hc with H3, and it plays a critical role in conformational changes of syntaxin 1A between closed and open forms (Dulubova et al., 1999; Misura et al., 2000). When closed, the linker domain is folded to prevent exposure of the H3 domain and when open, the linker is released to enable H3 to bind to other proteins. Thus, the linker domain is thought to be involved in the "activation" of syntaxin 1A, although the activation process remains unclear because how the linker domain is released is not known. Although the closed form binds selectively to Munc-18, no linker domain-binding protein was identified until 2001.

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We demonstrated that the autophosphorylated form of CaMKII binds specifically to the linker domain of syntaxin in a Ca^{2+}/ATP-dependent manner, where no other known syntaxin-binding proteins bind at this site (Ohyama et al., 2002). We also showed that the microinjected CaMKII-binding domain of syntaxin specifically decreases the frequency of exocytosis in chromaffin cells and in neurons, probably due to interference with endogenous CaMKII-syntaxin complex. These results indicate that the binding of CaMKII to the linker domain is an important step in the regulation of exocytosis. To change the conformation of syntaxin 1A through the linker domain, the biochemical properties of the domain need to be characterized. Because CaMKII is the only protein yet known to bind the linker domain, we examined the properties of mutant linker domains by evaluating their CaMKII-binding activity. We identified several amino acid residues that are critical for CaMKII-binding. We discuss the importance of these residues in roles of the linker domain in conformational changes of syntaxin 1A.

MATERIALS AND METHODS

Recombinant Proteins

We amplified cDNA fragments corresponding to the various regions of syntaxin 1A and those encoding syntaxin-binding proteins by PCR and inserted them into pGEX-6P-1 (Amersham-Pharmacia) in frame with GST (8, 9). Site-directed mutagenesis proceeded as described by Imai et al. (1991). Labeled protein binding was detected as described (Ohyama et al., 2001, 2002). The cDNAs of syntaxin 1A, 2, 3, and 4 were provided by Drs. K. Akagawa (Kyorin University, Mitaka, Tokyo), R.H. Scheller (Stanford University, Stanford, CA), T. Abe (Brain Research Institute, Niigata, Japan), and H. Shibata (Institute of Bioregulation, Gunma University, Maebashi, Gunma, Japan), respectively. CaMKII was autophosphorylated in presence of 0.1 mM $CaCl_2$, 0.2 mM ATP, 2 mM $MgCl_2$, and 10 μM calmodulin at 30°C for 15 min. The binding of CaMKII was assayed using immobilized GST-fusion proteins produced in *E. coli* as described (Ohyama et al., 2002). Usually, the binding reaction proceeded in the presence of 1 mM $CaCl_2$, 0.5 mM ATP, and 2 mM $MgCl_2$. We used brain S_2 fraction to examine the binding of other syntaxin-binding proteins (Ohyama et al., 2002).

RESULTS

We demonstrated that syntaxin 1A binds to CaMKII via its linker domain and found that syntaxin 1A (145–172) was the tentative minimal unit required for the CaMKII binding (Ohyama et al., 2002). We searched for a shorter binding motif, but deleting one to five amino acids from the C-terminus of this fragment attenuated CaMKII-binding affinity (Fig. 1A). We then examined the importance of the basic amino acid cluster residing in 146–151. The affinity of K146G for CaMKII was reduced, and that of K146G-R148G was similar to that of K146G (Fig. 1B). Thus, among the three basic amino acids, K146 was also important as well as R151. In contrast, R148 was less important for this binding (Fig. 1B).

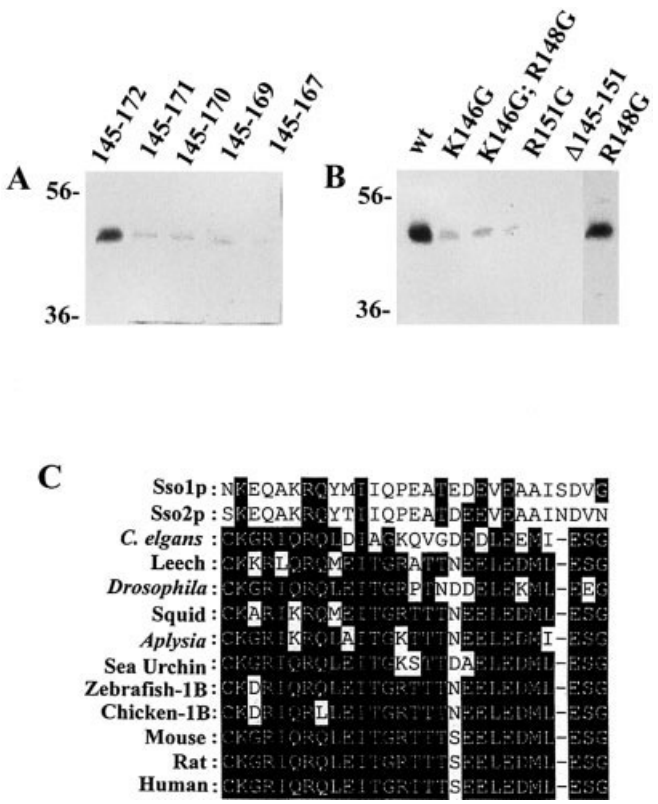

Fig. 1. CaMKII-binding activity derived from linker domain of syntaxin 1A. Fragments were expressed as GST-fusion proteins using pGEX-6P-1 (Amersham-Pharmacia) and immobilized to glutathione-Sepharose. Autophosphorylated CaMKII was incubated with Ca^{2+} and Mg^{2+}-ATP, then bound CaMKII was eluted using PreScission Protease. CaMKII-binding was monitored by immunoblotting using anti-CaMKII antibody. **A:** Linker domain fragment of 145–172 derived from syntaxin 1A is minimal CaMKII-binding site. Three other deletion mutants (145–171, 145–170, and 145–169) of C-terminus portion had less than 20% of CaMKII-binding activity of 145–172, and 145–167 had less than 10%. **B:** Basic amino acid cluster (K146, R148, R151) is important for CaMKII-binding. Each fragment was 145–184 of syntaxin 1A (wt) or a mutant derived from this fragment. K146G mutation remarkably reduced CaMKII-binding; activity of K146G-R148G double mutant was similar to K146G single mutant. Fragments 145–184 (R151G) (represented as R151G) and 152–184 (D145–151) almost completely lost CaMKII-binding. **C:** Alignment of part of linker domain of thirteen syntaxin 1 homologues from many organisms corresponding to 145–172 of syntaxin 1A. SSo1p and Sso2p are syntaxins from *Saccharomyces cerevisiae*. Zebrafish-B and chicken-B represent syntaxin 1B. Sequences of syntaxin 1A from other organisms are shown. Boxed sequences are identical among over seven species.

These two important basic residues are conserved from yeast to mammals in the CaMKII-binding site in the linker domain (corresponding to rat syntaxin 1A [145–172]), as well as E166 (Fig. 1C), an essential residue for structural conversion between the closed and the open forms (Dulubova et al., 1999). We tentatively designated the linker-N domain as corresponding to 145–172 of syntaxin 1A, because it is located two-thirds of the linker domain from the N-terminal.

Among syntaxin family members, syntaxins 2, 3, and 4 are localized in the plasma membranes of many kinds of cells, as well as syntaxin 1A, which is expressed mainly in neurons, and these isoforms have close homology with syntaxin 1A (Bennett et al., 1993). To identify other important residues for CaMKII binding to the linker domain, we compared the CaMKII-binding activity of each syntaxin isoform because the amino acid sequences in their linker domains differ slightly (Bennett et al., 1993; see Fig. 4A). Figure 2A shows that the CaMKII-binding affinity of each syntaxin isoform differed, as did that of Munc-18a (Tamori et al., 1998) or tomosyn (Fujita et al., 1998). Namely the binding affinity was syntaxin 1A > 2 > 3 > 4. Because three N-terminus helical domains regulate H3 domain activity, we constructed chimeric proteins composed of the N-terminus half derived from syntaxin 1A and the C-terminus half derived from other syntaxins. The CaMKII-binding activity of each chimeric protein was similar to that of C-terminus syntaxin isoforms (Fig. 2B). Thee CaMKII-binding affinity of other chimeric proteins, in which the N-terminus half was derived from non-neuronal syntaxin isoforms and the C-terminus was derived from syntaxin 1A, was similar to syntaxin 1A (Fig. 2B). Thus, we concluded that binding affinity was determined by the C-terminus that contains the linker domain. This hypothesis was confirmed by the direct binding of CaMKII to the linker domain of each syntaxin isoform, with which the N-terminus domain did not interfere (Fig. 2C). Syntaxin 2 bound significantly to CaMKII, but with weaker affinity than syntaxin 1A, and the linker-N domain was responsible for this binding (Fig. 2C).

The binding of CaMKII with syntaxins 1A and 2 both required autophosphorylation of CaMKII (Fig. 3A), Ca^{2+}, and ATP (Fig. 3B). In addition, syntaxin 2 bound to CaMKII was displaced in the presence of a high concentration of the linker domain fragment of syntaxin 1A (Fig. 3C). These results indicate that syntaxin 2 bound to CaMKII in the same manner as syntaxin 1A, except with lower affinity.

Although in syntaxin 2, most of the amino acid residues in the linker domain are identical to those of syntaxin 1A, syntaxin 2 has much lower affinity for CaMKII-binding (Fig. 2A–C). Within the amino acid residues corresponding to the linker-N of syntaxin 1A, the most distinct difference was that S162 of 1A was changed to D (D163) in syntaxin 2 (Fig. 4A). To estimate whether this residue is necessary for CaMKII binding, we constructed the S162D mutant of syntaxin 1A and examined the binding. The CaMKII-binding activity of S162D-syntaxin 1A was much weaker than that of the wild-type (Fig. 4B). Similarly, because the most remarkable difference between syntaxins 1A and 3 is the alteration of T159 in 1A–K in syntaxin 3, we constructed mutant syntaxin 1A (T159K). This CaMKII-binding affinity of this mutant and of its linker-N domain was attenuated significantly (Fig. 4B). These mutations did not alter binding to tomosyn or Munc-18 (Fig. 4C), indicating that these residues are specifically important for interaction with CaMKII.

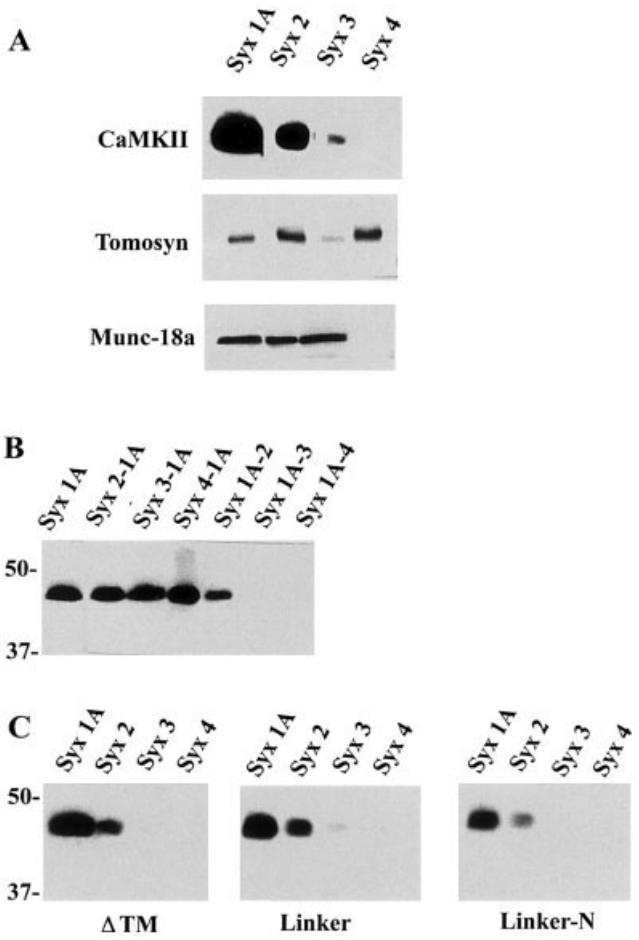

Fig. 2. The CaMKII-binding of mammalian syntaxin isoforms. **A:** Syntaxin isoforms bind differently to syntaxin-binding proteins. Each immobilized syntaxin isoform without its transmembrane domain was incubated with rat brain lysate, then binding proteins were eluted using PreScission protease and detected by immunoblotting. **B:** CaMKII-binding activity of chimeric recombinant proteins composed of two syntaxin isoforms. Syx 2-1A, syntaxin 2 (1–145) and 1A (145–262); syx 3-1, syntaxin 3 (1–144) and 1A (145–262); syx 4-1A, syntaxin 4 (1–151) and 1A (145–262); syx 1A-2, syntaxin 1A (1–144) and 2 (146-); syx 1A-3, syntaxin 1A (1–144) and 3 (145-) and syx 1A-4, syntaxin 1A (1–144) and 4 (152-). CaMKII-binding was monitored by immunoblotting (B and C). **C:** Comparison of CaMKII-binding with syntaxin isoforms. Syntaxin 1A (syx 1A): DTM (1–262), Linker (145–184), and linker-N (145–172); Syntaxin 2 (syx 2): DTM (1–263), linker (146–185), and linker-N (146–173); Syntaxin 3 (syx 3): DTM (1–261), linker (145–184), and linker-N (145–172); Syntaxin 4 (syx 4): DTM (1–270), linker (152–191), and linker-N (152–180).

DISCUSSION

We demonstrated that 1) the linker-N (145–172) is the shortest site that can bind to CaMKII; 2) that among the basic amino acid cluster (K146, R148, R151), K146 but not R148 is important as is R151; and 3) that S162 and T159 are also necessary for the high affinity binding of CaMKII, based on the comparison with syntaxin isoforms. The linker domain where CaMKII binds regulates the

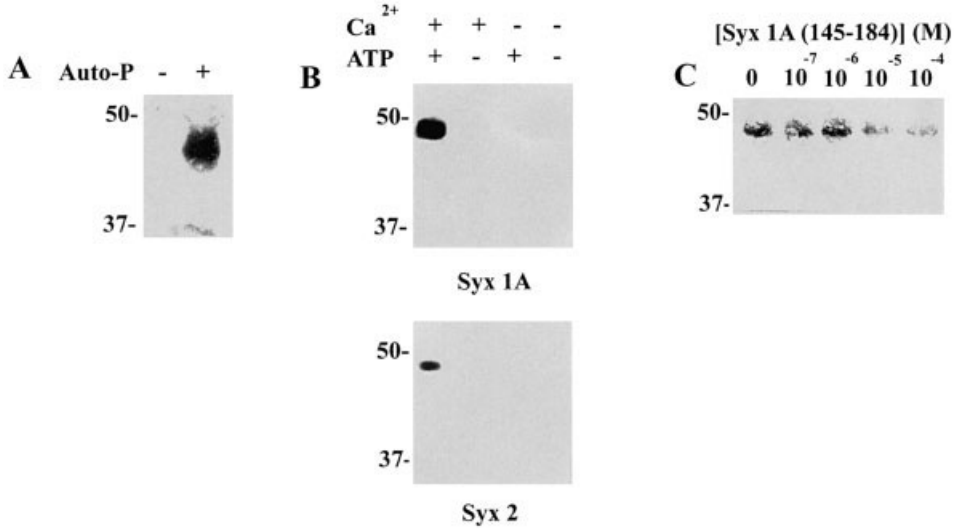

Fig. 3. Syntaxin 2 binds to CaMKII in a manner similar to syntaxin 1A. **A:** Autophosphorylated and non-phosphorylated recombinant CaMKII were incubated with immobilized GST-syntaxin 2 (1–262). Bound CaMKII was eluted using PreScission protease and monitored with anti-CaMKII antibody. Recombinant CaMKIIα (gift from Dr. N.M. Waxham) was produced in Sf9 cells using baculovirus system (Kolb et al., 1998). **B:** CaMKII-binding to syntaxin 2 is Ca^{2+}/ATP-dependent as for syntaxin 1A. Binding proceeded without ATP and with 1 mM Ca^{2+} and 0.5 mM ATP; in some experiments, Ca^{2+} was substituted with 1 mM EGTA. **C:** The binding of CaMKII to syntaxin 2 is released in presence of the linker domain of syntaxin 1A in a dose-dependent manner. The autophosphorylated CaMKII was incubated with the immobilized GST-syntaxin 2. After rinsing, the syntaxin 2-CaMKII complex was incubated with various concentration of the linker domain of syntaxin 1A (145–184) for 1 hr at 4°C. Bound CaMKII was eluted by SDS-sample buffer, electrophoresed, and immunoblotted against anti-CaMKII antibody (Ohyama et al., 2002).

conversion of syntaxin 1A from the closed to the open form, which is a key step in the priming of exocytosis (Richmond et al., 2001). According to X-ray structural analysis (Misura et al., 2000), the basic amino acid cluster belongs to part of the Hc helix and resides inside the molecule when syntaxin 1A assumes the closed conformation in complex with Munc-18 (Dulubova et al., 1999). When syntaxin 1A is free, these residues belong to the linker domain. This finding explains the importance of K146 and R151 for CaMKII-binding, which is thought to be a protein that fixes syntaxin 1A in the open form (Ohyama et al., 2002). We also showed that CaMKII and Munc-18 bind alternatively to syntaxin (Ohyama et al., 2002), and this is probably because these basic amino acids cannot be exposed when syntaxin binds to Munc-18. S162 and T159 belong to the constant linker even in the presence of Munc-18, and S162 forms an α-helix composed of 162–170 (Misura et al., 2000). Mammalian syntaxin 1A has S162. In contrast, *C. elegans* and *D. melanogaster* have D162, as do mammalian nonneuronal syntaxins (Figs. 1C, 4A). The powerful interaction between CaMKII and syntaxin might be required for the higher processing of information in the nervous system of mammals.

Judging from the result that the recombinant syntaxin 1A (1–262), and its mutants (i.e., S162D and T159K) were able to bind to both Munc-18 and tomosyn (Fig. 4C), an H3 domain-binding protein (Fujita et al., 1998), each of them is thought to be composed of a mixture of both the closed and the open forms. These two mutants, however, bound to CaMKII only a less amount than the wild type (Fig. 4B), suggesting that the autophosphorylated CaMKII recognizes the configuration of the syntaxin open form more strictly than Munc-18 or tomosyn, since these mutants appear to take incomplete open forms, to which tomosyn is accessible but CaMKII is not.

In syntaxin 2, the corresponding residue is substituted to D, as well as in syntaxin 3, explaining partly why syntaxin 2 has weaker affinity for CaMKII than syntaxin 1A, although these isoforms differ by only a few residues in their linker domains Although interactions between SNARE proteins seem somewhat promiscuous in vitro (Yang et al., 1999), investigators have concluded that the binding of CaMKII to syntaxin isoforms is selective. Our results also indicated that syntaxin 2 binds to autophosphorylated CaMKII in a Ca^{2+}/ATP-dependent manner with weaker affinity than syntaxin 1A. Syntaxin 2, also known as epimorphin, a mesenchymal protein essential to epithelial morphogenesis, (Hirai et al., 1992) is localized in the heart, spleen, liver, and testis (Bennett et al., 1993). This protein is also involved in exocytosis from platelets through the SNARE mechanism (Chen et al., 2000; Lemons et al., 2000). Thus, binding to any CaMKII isoform might be important for the regulation of intracellular trafficking involving syntaxin 2. Because CaMKIIγ and δ are ubiquitous among four CaMKII isoforms (Soderling et al., 2001), these isoforms may bind to syntaxin 2 when available, and this interaction might be involved in the functions of these cells.

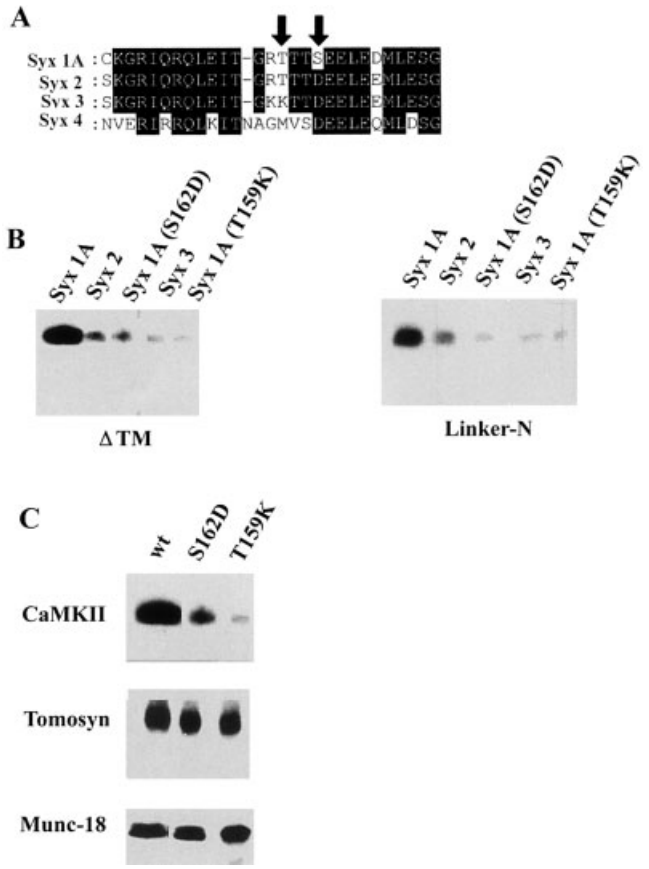

Fig. 4. S162 and T159 in syntaxin 1A are important for CaMKII-binding. **A:**The alignment of linker-N domain in syntaxins 1A, 2, 3 and 4. S162 of 1A is substituted to D in other isoforms (arrow) and T159 of 1A was altered to K of syntaxin 3 (arrow). Boxed residues are identical in more than three isoforms. **B:** S162D and T159K mutants of syntaxin 1A, as well as syntaxins 2 and 3 have reduced CaMKII binding activity. **C:** S162D and T159K have reduced CaMKII-binding activity but do not bind at all to tomosyn or Munc-18.

In conclusion, we identified several important residues in the linker domain for CaMKII-binding, which may be involved in enabling syntaxin 1A to unfold. Currently, we are investigating the essential role of CaMKII-binding in this structural conversion.

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REFERENCES

Bock JB, Matern HT, Peden AA, Scheller R.H. 2001. A genomic perspective on membrane compartment organization. Nature (London) 409: 839–841.

Bennett MK, García-Arrarás JE, Elferink LA, Peterson K, Fleming AM, Hazuka CD, Scheller RH. 1993. The syntaxin family of vesicular transport receptors. Cell 74:863–873.

Chen D, Bernstein AM, Lemons PP, Whiteheart SW. 2000. Molecular mechanisms of platelet exocytosis: role of SNAP-23 and syntaxin 2 in dense core granule release. Blood 95:921–929.

Dacks JB Doolittle WF. 2002. Novel syntaxin gene sequences from *Giardia*, *Trypanosoma* and algae: implications for the ancient evolution of the eukaryotic endomembrane system. J Cell Sci 115:1635–1642.

Dulubova I, Sugita S, Hill S, Hosaka M, Fernandez I, Südhof TC, Rizo J. 1999. A conformational switch in syntaxin during exocytosis: role of munc 18. EMBO J 18:4372–4382.

Fernandez I, Ubach J, Dulubova I, Zhang X, Südhof TC, Rizo J. 1998. Three-dimensional structure of an evolutionarily conserved N-terminal domain of syntaxin 1A. Cell 94:841–849.

Fujita Y, Shirataki H, Sakisaka T, Asakura T, Ohya T, Kotani H, Yokoyama S, Mizoguchi A, Scheller RH, Takai Y. 1998. Tomosyn: a syntaxin-1-binding protein that forms a novel complex in the neurotransmitter release process. Neuron 20:905–915.

Hirai Y, Takebe K, Takashina M, Kobayashi S, Takeichi M. 1992. Epimorphin: a mesenchymal protein essential for epithelial morphogenesis. Cell 69:471–481.

Imai Y, Matsushima Y, Sugimura T, Terada M. 1991. A simple and rapid method for generating a deletion by PCR. Nucleic Acid Res 19:2785.

Kolb SJ, Hudmon A, Ginsberg TR, Waxham MN. 1998. Identification of domains essential for the assembly of calcium/calmodulin-dependent protein kinase II holoenzymes. J Biol Chem 273:31555–31564.

Lemons PP, Chen D, Whiteheart SW. 2000. Molecular mechanisms of platelet exocytosis: requirements for alpha-granule release. Biochem Biophys Res Commun 267:875–880.

Misura KMS, Scheller RH, Weis WI. 2000. Three-dimensional structure of the neuronal-Sec1- syntaxin 1a complex. Nature (London) 404:355–362.

Ohyama A, Hosaka K, Komiya Y, Akagawa K, Yamauchi E, Taniguchi H, Sasakawa N, Kumakura K, Mochida S, Yamauchi T, Igarashi M. 2002. Regulation of exocytosis through Ca2+/ATP-dependent binding of autophosphorylated CaMKII to syntaxin 1A. J Neurosci 22:3342–3351.

Ohyama A, Komiya Y, Igarashi M. 2001. Globular tail of myosin-V is bound to VAMP/synaptobrevin. Biochem Biophys Res Commun 280: 988–991.

Rizo J Südhof TC. 2002. SNAREs and Munc-18 in synaptic vesicle fusion. Nat Rev Neurosci 3:641–653.

Tamori Y, Kawanishi M, Niki T, Shinoda H, Arakai S, Okazawa H, Kasuga M. 1998. Inhibition of insulin-induced GLUT4 translocation by Munc18c through interactions with syntaxin4 in 3T3-L1 adipocytes. J Biol Chem 273:19740–19746.

Richmond JE, Welmer RM, Jorgensen EM. 2001. An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. Nature (London) 412:338–341.

Soderling TR, Chang B, Brickey D. 2001. Cellular signaling through multifunctional Ca2+/calmodulin-dependent protein kinase II. J Biol Chem 276:3719–3722.

Yang B, Gonzalez Jr, L, Prekeris R, Steegmaier M, Advani RJ, Scheller RH. 1999. SNARE interactions are not selective. Implications for membrane fusion specificity. J Biol Chem 274:5649–5653.