The Ca\(^{2+}\)-dependent oligomerization activity of the second C2 (C2B) domain of synaptotagmin I (Syt I) has been hypothesized to regulate neurotransmitter release. We previously showed that the cytoplasmic domains of several other Syt isoforms also show Ca\(^{2+}\)-dependent oligomerization activity (Fukuda, M., and Mikoshiba, K. (2000) J. Biol. Chem. 275, 28180–28185), but little is known about the involvement of their C2 domains in Ca\(^{2+}\)-dependent oligomerization. In this study, we analyzed the Ca\(^{2+}\)-dependent oligomerization properties of the first (C2A) and the second C2 (C2B) domains of Syt VII. Unlike Syt I, both C2 domains of Syt VII contribute to Ca\(^{2+}\)-dependent homo- and hetero-oligomerization with other isoforms. For instance, the Syt VII C2A domain Ca\(^{2+}\)-dependently binds itself and the C2A domain of Syt VI but not its C2B domain, whereas the Syt VII C2B domain Ca\(^{2+}\)-dependently binds itself and the C2B domain of Syt II but not its C2A domain. In addition, we showed by gel filtration that a single Syt VII C2 domain is sufficient to form a Ca\(^{2+}\)-dependent multimer of very high molecular weight. Because of this “two handed” structure, the Syt VII cytoplasmic domain has been found to show the strongest Ca\(^{2+}\)-dependent multimerization activity in the Syt family. We also identified Asn-328 in the C2B domain as a crucial residue for the efficient Ca\(^{2+}\)-dependent switch for multimerization by site-directed mutagenesis. Our results suggest that Syt VII is a specific isoform that can cluster different Syt isoforms with two hands in response to Ca\(^{2+}\).

Neurotransmitter release is achieved by a fusion of synaptic vesicles to presynaptic plasma membranes in response to a rapid increase in Ca\(^{2+}\) ions (up to 200 \(\mu\)M) entering through voltage-gated Ca\(^{2+}\) channels (1, 2). Synaptic vesicle proteins are then recycled from the plasma membrane by endocytosis to prepare for the next round of synaptic vesicle exocytosis. The synaptic vesicle cycle is now known to consist of at least four distinct steps: (i) docking of synaptic vesicles to active zones (docking step), (ii) an ATP-dependent priming step, (iii) actual fusion of synaptic vesicles to the presynaptic plasma membrane (fusion step), and (iv) recycling of synaptic vesicles by endocytosis (reviewed in Refs. 3 and 4). A variety of proteins involved in the regulation of each step of the synaptic vesicle cycle have already been identified (3, 4). One of them, synaptotagmin I (Syt I),\(^{1}\) an integral membrane protein of synaptic vesicles, is very interesting, because it regulates three distinct steps in the synaptic vesicle cycle (i.e. docking, fusion, and recycling).

Synaptotagmins constitute a large family, both in vertebrates and invertebrates (5–7), and each member consist of four functional domains: a single transmembrane domain, a first C2 (C2A) domain, a second (C2B) domain, and a short carboxyl terminus containing a WHXL motif (reviewed in Refs. 6, 8–10). In Syt I, these domains have distinct roles in the synaptic vesicle cycle. The WHXL motif in the carboxyl terminus of Syt I is involved in docking of synaptic vesicles to active zones in the squid giant synapse (11). The C2B domain is involved in the synaptic vesicle fusion step (12–14), and the C2B domain is involved in synaptic vesicle recycling, probably by binding to the clathrin assembly protein, AP2 (15–20). Genetic analysis of Drosophila syt mutants (21) and biochemical studies (22–28) have suggested that the C2B domain of Syt I also regulates synaptic vesicle exocytosis. Indeed, mutation (Tyr-312 to Asn substitution) in the C2B domain of mouse Syt II, which corresponds to the Drosophila AD3 mutation, impairs Ca\(^{2+}\)-dependent self-oligomerization activity, and this activity is inhibited by inositol 1,3,4,5-tetrakisphosphate, a powerful blocker of neurotransmitter release, in vitro (29, 30). How self-oligomerization of Syt I regulates synaptic vesicle fusion, however, remains unclear.

Our previous study showed that Syt VII, which is involved in Ca\(^{2+}\)-dependent lysosomal exocytosis in fibroblasts (31) and insulin secretion in pancreatic \(\beta\)-cells (32), has the strongest Ca\(^{2+}\)-dependent self-oligomerization capacity in the Syt family (26, 27). The Syt VII Ca\(^{2+}\)-dependent self-oligomerization via the cytoplasmic domain occurs without tethering at the amino-terminal domain, whereas the Syt II Ca\(^{2+}\)-dependent self-oligomerization via the C2B domain occurs only when the two molecules are preassembled at the amino-terminal domain (26). Although the Ca\(^{2+}\)-dependent self-oligomerization of Syt I (or II) has been shown to be mediated by the C2B domain (22–24), no such information is available for other Syt isoforms, including Syt VII. In this study, we sought to determine the structural basis of Ca\(^{2+}\)-dependent oligomerization of Syt VII. Unlike Syt I, both the C2A and C2B domains of Syt VII function as a Ca\(^{2+}\)-dependent oligomerization site (i.e. Syt VII has “two hands”), and this explains its robust Ca\(^{2+}\)-dependent oli-
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N915-cto, (N328A)-cto, (N328L)-cto, (N328D)-cto, (N329K)-cto, or (N329P)-cto) (36, 37).

**Cellulotransfection of pEF-T7-Syts and pEF-FLAG-Syts into COS-7 cells was carried out (5 $\times$ 10$^5$ cells the day before transfection/10-cm dish) by the DEAE-dextran method, as described previously (33). Proteins were solubilized with a buffer containing 1% Triton X-100, 250 mM NaCl, 50 mM HEPEs-KOH, pH 7.2, 0.1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g leupeptin, and 10 $\mu$g pepstatin A at 4°C for 2 h. T7-Syts were precipitated by anti-T7 tag antibody-conjugated agarose in the presence of various concentrations of Ca$^{2+}$, or 2 mM EGTA, as described previously (26). SDS-polyacrylamide gel electrophoresis and immunoblot analyses were also performed, as described previously (33). The blots shown in this paper are representative of at least three independent experiments. Immunoreactive bands on x-ray film (X-Omat AR, Kodak) were captured by Gel Print 2000i/VGA (Bio Image) and analyzed with Basic Quantifier Software (version 1.0) (Bio Image). Multiple sequence alignment of the mouse synaptotagmin family was performed by using the ClustalW program, as described previously (38).

**RESULTS**

Mapping of the Domain Responsible for Ca$^{2+}$-Dependent Self-oligomerization of Synaptotagmin VII—In our previous study we showed that the Syt VII cytoplasmic domain is sufficient for Ca$^{2+}$-dependent self-oligomerization activity (26, 27). To identify the domain responsible for the Ca$^{2+}$-dependent self-oligomerization of Syt VII, we produced various Syt VII deletion mutants and tagged them with T7 (see Fig. 1A). Each T7-tagged mutant was coexpressed with the FLAG-Syt VII cytoplasmic domain (Syt VII-cyto) in COS-7 cells, and the associations between the T7- and FLAG-tag proteins were evaluated by immunoprecipitation, as described previously (33). Briefly, T7-Syt proteins were immunoprecipitated by anti-T7 tag antibody-conjugated agarose in the presence and absence of 500 $\mu$M Ca$^{2+}$. The co-immunoprecipitated FLAG-Syt-cto proteins were first detected by anti-FLAG rabbit polyclonal antibody (Fig. 1B, upper panel), and the same blot was then stripped and reprobed with HRP-conjugated anti-T7 tag antibodies (Fig. 1B, lower panel). Surprisingly, with the exception of T7-Syt VII-cyto$\Delta$C2AB (spacer domain alone), all the deletion mutants interacted with FLAG-Syt VII-cyto only in the presence of Ca$^{2+}$, indicating that each of the C2 domains functions as an acceptor for the Ca$^{2+}$-dependent self-oligimerization. By contrast, Syt I Ca$^{2+}$-dependent self-oligimerization has been shown to be mediated by the C2B domain alone and not by the C2A domain (22, 28).

Ca$^{2+}$-Dependent Homo- and Hetero-oligimerization Properties of the C2 Domains of Synaptotagmin VII—To investigate whether a single C2 domain alone is sufficient for the Ca$^{2+}$-dependent self-oligomerization capacity of Syt VII, Ca$^{2+}$-dependent associations between T7- and FLAG-Syt VII-C2A (or C2B) was investigated as described above. As shown in Fig. 2A, both C2 domains showed Ca$^{2+}$-dependent self-oligomerization activity, although some fractions (about 40%) of the C2A domain self-oligomerized even in the absence of Ca$^{2+}$. Because the entire cytoplasmic domain of Syt VII did not show Ca$^{2+}$-inde-
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Because the cytoplasmic domain of Syt VII showed Ca\(^{2+}\)-dependent self-oligomerization activity with an EC\(_{50}\) value of about 150 \(\mu\)M Ca\(^{2+}\), we investigated the Ca\(^{2+}\)-dependence of the self-oligomerization activity of each of the C2 domains of Syt VII (Fig. 3A). The EC\(_{50}\) values for self-oligomerization by the Syt VII C2A and C2B domains were \(-150\) \(\mu\)M Ca\(^{2+}\) and 250 \(\mu\)M Ca\(^{2+}\), respectively (Fig. 3B; Syt VII-C2A, closed squares; Syt VII-C2B, open squares). Thus, the EC\(_{50}\) values for self-oligomerization with the isolated C2 domains and the full cytoplasmic domain are essentially the same.

Identification of the Amino Acid Residues Responsible for the Ca\(^{2+}\)-dependent Self-oligomerization of Synaptotagmin VII—In the next set of experiments we sought to identify the amino acid residues responsible for the robust Ca\(^{2+}\)-dependent self-oligomerization of Syt VII. First, we made sequence comparisons among mouse Syts I–XI C2 domains in a search for specific amino acid residues found only in Syt VII and not in other Syt isoforms. We found one Asn residue that was only present in the C2 domains of Syt VII (Asn-195 in the C2A domain and Asn-328 in the C2B domain of Syt VII), with the corresponding positions of Syts I–VI and VIII–XI being occupied by Thr (Fig. 4A, asterisk). These Asn residues of Syt VII were located between the \(\beta4\) and \(\beta5\) strands of the eight-stranded \(\beta\)-sandwich structure of the C2 domain and are in close proximity to the C2B effector domain responsible for inositol polyphosphate, AP-2 binding, and self-oligomerization of Syt I (two Lys residues in Fig. 4A, number signs (#)) (24, 29, 35, 39). To examine the involvement of these Asn residues in the self-oligomerization of Syt VII, we performed site-directed mutagenesis, and obtained Syt VII-C2A(N195T) and -C2B(N328T) mutants.

In our previous study, we showed that the Syt VII cytoplasmic domain can interact with various Syt isoforms in a Ca\(^{2+}\)-dependent manner (26, 27). Syt VII preferentially interacts with Syts V, VI, and X, weakly with Syts I, II, and IX, but not essentially with Syts III, IV, VIII, and XI (26, 27). To determine which C2 domains of Syt VII are involved in hetero-oligomerization with other Syt isoforms, FLAG-Syt VII-C2A (or -C2B) was coexpressed with T7-Syts II, IV, VI, and VIII cytoplasmic domains in COS-7 cells, and their associations were evaluated as described above. Surprisingly, Syt II-Cyto weakly interacted with both C2 domains of Syt VII in the presence of Ca\(^{2+}\), whereas Syt-VI-Cyto interacted only with the Syt VII C2A domain in the presence of Ca\(^{2+}\) (Fig. 2B). By contrast, Syt IV-Cyto and Syt VIII-Cyto did not interact with either C2 domain of Syt VII, consistent with the findings in a previous report (27). To further specify the domains of Syt II (or Syt VI) that act as an acceptor for Ca\(^{2+}\)-dependent hetero-oligomerization with Syt VII, each C2 domain of Syt II (or Syt VI) with a T7-tag was similarly coexpressed with FLAG-Syt VII-Cyto in COS-7 cells. As shown in Fig. 2C, the Syt II-C2B and Syt VI-C2A domains were found to be essential for Ca\(^{2+}\)-dependent hetero-oligomerization with Syt VII. Thus, the contribution of the C2 domains to Syt Ca\(^{2+}\)-dependent oligomerization differs among isoforms.
mutants. As expected, the C2B(N328T) mutant had completely lost Ca\(^{2+}\)-dependent oligomerization activity (Fig. 4B, right panel), although some fractions of C2B(N328T) mutant proteins showed Ca\(^{2+}\)-independent oligomerization activity with the wild-type C2B domain. By contrast, the N198T mutation had no effect on Ca\(^{2+}\)-dependent oligomerization by the C2A domain (Fig. 4B, left panel). Similar experiments were performed by using the full cytoplasmic domain carrying the N195T or N328T mutation (Fig. 4C), and interestingly, the N328T mutation completely abolished the Ca\(^{2+}\)-dependent self-oligomerization activity (i.e. Ca\(^{2+}\)-independent self-oligomerization), even when the C2A domain was intact.

To further investigate the importance of Asn-328 of the C2B domain in Ca\(^{2+}\)-dependent self-oligomerization, we mutated this residue to other amino acids with different side-chain structures (N328A, N328L, N328D, N328K, N328P). As shown in Fig. 4D, except for the N328P mutant, self-oligomerization of these mutants was also stimulated by Ca\(^{2+}\) (to an extent similar to that of the wild-type protein), but the Ca\(^{2+}\)-independent self-oligomerization activity was significantly increased. By contrast, the N328P mutant resulted in the loss of Ca\(^{2+}\)-dependent oligomerization, the same as the N328T mutation. These results indicate that Asn-328 in the C2B domain is associated with efficient Ca\(^{2+}\)-dependent self-oligomerization.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Ca\(^{2+}\)-dependent homo- and hetero-oligomerization properties of the C2 domains of synaptotagmin VII. pEF-T7-Syts and pEF-FLAG-Syt VII-cyto (or -C2A, -C2B) were cotransfected into COS-7 cells. The proteins expressed were solubilized with 1% Triton X-100 and immunoprecipitated by anti-T7 tag antibody-conjugated agarose, as described previously (33). Co-immunoprecipitated FLAG-Syts were first detected with anti-FLAG rabbit antibody (5 \(\mu\)g/ml, upper panels in A–C). The same blots were stripped and reprobed with HRP-conjugated anti-T7 tag antibody to ensure loading of the same amounts of T7-Syt proteins (lower panels in A–C). A, Ca\(^{2+}\)-dependent self-oligomerization properties of the Syt VII-C2A and -C2B domain. B, Ca\(^{2+}\)-dependent hetero-oligomerization between FLAG-Syt VII-C2A (or -C2B) and T7-Syt II, IV, VI, VII, and VIII-cyto. C, Ca\(^{2+}\)-dependent hetero-oligomerization between FLAG-Syt VII-cyto and T7-Syts II-, VI-C2A, or -C2B. The positions of the molecular weight markers (\(\times 10^{-3}\)) are shown on the left. D, schematic representation of the homo- and hetero-oligomerization of Syts II, VI, and VII. The transmembrane domain (TM) and two C2 domains are shown by open and hatched boxes, respectively. The shaded region around the transmembrane domain of Syt II indicates Ca\(^{2+}\)-dependent self-oligomerization. -S-S- represents disulfide bonding (26, 27, 33).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Ca\(^{2+}\) dependence of synaptotagmin VII C2A (or C2B) domain self-oligomerization. A, pEF-T7-Syt VII-C2A (or -C2B) and -FLAG-Syt VII-C2A (or -C2B) were cotransfected into COS-7 cells. The proteins expressed were solubilized with 1% Triton X-100 and immunoprecipitated by anti-T7 tag antibody-conjugated agarose in the presence of various concentrations of Ca\(^{2+}\), as described previously (26). Co-immunoprecipitated FLAG-Syts were first detected with anti-FLAG rabbit antibody (5 \(\mu\)g/ml, upper panels). The same blot was then stripped and reprobed with HRP-conjugated anti-T7 tag antibody to ensure loading of the same amounts of T7-Syt proteins (lower panels). B, the EC\(_{50}\) values of the Syt VII C2A and C2B domain self-oligomerization were about 150 \(\mu\)M Ca\(^{2+}\) and 250 \(\mu\)M Ca\(^{2+}\), respectively. The bars indicate the S.D. of three independent experiments.}
\end{figure}
dependent Syt clustering may regulate opening and/or expansion of a “fusional pore.” Nevertheless, it remains unknown how many Syt molecules cluster in response to Ca\(^{2+}\), even though such information is quite important in evaluating whether Syt molecules can regulate the fusion pore. To address this, we used gel filtration to estimate the molecular weight of each Syt VII C2 domain in the presence and absence of 1 mM Ca\(^{2+}\) (see “Experimental Procedures” for details). In the absence of Ca\(^{2+}\), the Syt VII-C2B proteins were eluted as a single peak (Fig. 5, top panel, fractions 75–80), and their apparent molecular mass was estimated to be ~10 kDa. Because the calculated molecular weight of T7-Syt VII-C2B is 17,451 and Syt VII-C2B did not form oligomers in the immunoprecipitation assay (Fig. 2A), we concluded that Syt VII-C2B is present as a monomer in the absence of Ca\(^{2+}\) (Fig. 5, top panel). By contrast, in the presence of 1 mM Ca\(^{2+}\), Syt VII-C2B proteins were eluted as a single peak with a very high molecular weight (Fig. 5, second panel, fractions 31–36) that was identical to the elution profile of blue dextran \((M_r, 2,000,000)\), an indicator of the void volume of the column (Fig. 5, bottom). Thus, we were unable to determine the exact number of the C2B domain clustered by Ca\(^{2+}\). The molecular weight shift induced by Ca\(^{2+}\) probably reflects direct clustering of the Syt VII C2B domain itself, and it is most unlikely to be mediated by certain adapter proteins (i.e., Ca\(^{2+}\)-dependent binding partner of Syts, such as syntaxin I) (40), because after Coomassie Brilliant Blue R-250 staining of the gel we were unable to observe any bands other than Syt molecules and the anti-T7-tag antibody used for immunoprecipitation, and syntaxin I is not expressed in COS-7 cells (data not shown). We therefore concluded that the Syt VII-C2B domain forms a multimer in the presence of Ca\(^{2+}\). We also evaluated the apparent molecular weight of Syt VII-C2B(N328T) mutant protein by using the same gel filtration column. Interestingly, Syt VII-C2B(N328T) proteins form multimers irrespective of the presence of Ca\(^{2+}\) (Fig. 5, fifth and sixth panels).

Similar results were obtained with the Syt VII-C2A domain (Fig. 5, third and fourth panels), although in the absence of Ca\(^{2+}\) the Syt VII-C2A domain eluted as a broad peak. Because the calculated molecular weight of T7-Syt VII-C2A is 16,667 and Syt VII-C2A formed Ca\(^{2+}\)-independent oligomers in the immunoprecipitation assay (Fig. 2A), this peak probably consists of the Syt VII-C2A dimer and monomer.

**DISCUSSION**

In the present study we analyzed the Ca\(^{2+}\)-dependent multimerization properties of the C2 domains of Syt VII to determine why Syt VII is the only member of the Syt family to exhibit robust Ca\(^{2+}\)-dependent oligomerization activity (26), why Syt VII is able to interact with various Syt molecules (27), and how many Syt VII molecules cluster in response to Ca\(^{2+}\).

The results presented in this paper indicate that both C2 domains of Syt VII contribute to Ca\(^{2+}\)-dependent homo- and/or hetero-multimerization, with slightly different affinities for Ca\(^{2+}\) (EC\(_{50}\) value for C2A domain self-multimerization of about 150 \(\mu\)M, and for the C2B domain self-multimerization, of about 250 \(\mu\)M). This finding is in considerable contrast to the results obtained for Syt I or II, because only the C2B domain of Syts I and II is involved in Ca\(^{2+}\)-dependent self-oligomerization (22–24). The Syt VII C2A domain efficiently interacts with itself and the Syt VI C2A domain, and weakly with the C2B domain of Syt II, but cannot interact with its C2B domain. Similarly, the Syt VII C2B domain interacts with itself and weakly with the C2B domain of Syt II, but not with its C2A domain. The Ca\(^{2+}\)-dependent homo- and hetero-oligomerization properties of Syt VII are summarized in Fig. 2D. Because of this two handed structure of Syt VII, it can oligomerize more efficiently than other Syt isoforms and can interact with various Syt isoforms without being tethered at the amino-terminal domain (26, 27).

Sequence alignment of the Syt family and site-directed mu-
tagenesis enabled us to identify Asn-328 in the C2B domain as a crucial residue for Ca\(^{2+}\)-dependent self-multimerization by Syt VII. This Asn residue is found to be conserved in human, rat, and mouse Syt VII, but the corresponding position in other mouse Syt isoforms and Syts from other species is occupied by Thr (data not shown). Thus, the strong Ca\(^{2+}\)-dependent multimerization properties are probably retained, at least in mammals.

Recent crystallographic analysis of C2 domains of Syt I, phospholipase C\(\gamma\)1 (PLC\(\gamma\)1), and cytosolic phospholipase A\(\gamma\)1 (cPLA2) showed the C2 domains to be composed of a common eight-stranded anti-parallel \(\beta\)-sandwich consisting of four-stranded \(\beta\)-sheets, although their structures have been classified into two groups based on their topology (e.g. synaptotagmin I C2A domain with type I topology and PLC\(\gamma\)1 C2 domain with type II topology) (41–45). Three flexible loops (named loops 1–3) protrude from the tip of the \(\beta\)-sandwich structure, and two of them (loops 1 and 3) are involved in Ca\(^{2+}\)-binding in the Syt I-C2A domain, all three of which are loops in the PLC\(\gamma\)1- and cPLA2-C2A domains (41–45). Interestingly, Asn-677 (PLC\(\gamma\)1) and Asn-65 (cPLA2) in loop 2 bind Ca\(^{2+}\), and these residues correspond closely to the Syt VII Asn-328 residue, suggesting that the Asn-328 residue is crucial for direct Ca\(^{2+}\) stabilizing Ca\(^{2+}\)-binding (loops 1–3) may change electrostatic charges or induce structural changes, which would allow the putative Ca\(^{2+}\) effector domain of Syt VII (middle of the \(\beta\)-sandwich structure) to participate in self-multimerization. Further three-dimensional studies are required to elucidate how Asn-328 of Syt VII regulates Ca\(^{2+}\)-dependent self-oligomerization of C2 domains.

In summary, we showed that both the C2A and C2B domains of Syt VII contribute to Ca\(^{2+}\)-dependent multimerization. Because of this two handed structure, Syt VII can cluster heterogeneous Syt isoforms (Syts I, II, V, VI, VII, IX, and X) in response to Ca\(^{2+}\), and this Ca\(^{2+}\)-dependent Syt multimer may regulate vesicular exocytosis.

Acknowledgments—We thank Dr. Shigekazu Nagata for the expression vector and Eiko Kanno for technical assistance.

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FIG. 5. Determination of the apparent molecular weight of Syt VII-C2A and -C2B domains by gel filtration. Cell extracts containing T7-Syt VII-C2A (or -C2B) proteins were loaded on a gel filtration column of Sepharose CL-6B (1 × 120 cm) that had been equilibrated with buffer 1 containing 0.2% Triton X-100, 1 mM MgCl\(_2\), and 2 mM EGTA (or 1 mM CaCl\(_2\)). The fractions were collected in 0.75 mL, and each fraction was analyzed by 12.5% SDS-polyacrylamide gel electrophoresis and immunoblotting with HRP-conjugated anti-T7 tag antibody, as described previously (33). The apparent molecular weight of Syt VII-C2A (or -C2B) in the presence and absence of 1 mM Ca\(^{2+}\) was estimated by loading gel filtration molecular weight markers (blue dextran = 2000 kDa; bovine serum albumin (BSA) = 66 kDa; carbonic anhydrase (CA) = 29 kDa; and cytochrome c (Cyt C) = 12.4 kDa) on the same Sepharose CL-6B column. Blue dextran, an indicator of the void volume of the Sepharose CL-6B column, was monitored at an absorbance at 595 nm (A\(_{595}\), bottom trace) with an Hitachi U-2001 spectrophotometer. Note that, in the presence of Ca\(^{2+}\), the Syt VII C2 domains eluted with the same profile as blue dextran, indicating that they form multimers with very high molecular weight values.
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J. Biol. Chem. 2001, 276:27670-27676.
doi: 10.1074/jbc.M100851200 originally published online May 23, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M100851200

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