Evolutionarily Conserved Multiple C₂ Domain Proteins with Two Transmembrane Regions (MCTPs) and Unusual Ca²⁺ Binding Properties

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C₂ domains are primarily found in signal transduction proteins such as protein kinase C, which generally contain a single C₂ domain, and in membrane trafficking proteins such as synaptotagmins, which generally contain multiple C₂ domains. In both classes of proteins, C₂ domains usually regulate the respective protein’s function by forming Ca²⁺-dependent or Ca²⁺-independent phospholipid complexes. We now describe MCTPs (multiple C₂ domain and transmembrane region proteins), a novel family of evolutionarily conserved C₂ domain proteins with unusual Ca²⁺-dependent properties. MCTPs are composed of a variable N-terminal sequence, three C₂ domains, two transmembrane regions, and a short C-terminal sequence. The invertebrate organisms Caenorhabditis elegans and Drosophila melanogaster express a single MCTP gene, whereas vertebrates express two MCTP genes (MCTP1 and MCTP2) whose primary transcripts are extensively alternatively spliced. Most of the MCTP sequences, in particular the C₂ domains, are highly conserved. All MCTP C₂ domains except for the second C₂ domain of MCTP2 include a perfect Ca²⁺/phospholipid-binding consensus sequence. To determine whether the C₂ domains of MCTPs actually function as Ca²⁺/phospholipid-binding modules, we analyzed their Ca²⁺- and phospholipid-binding properties. Surprisingly, we found that none of the three MCTP1 C₂ domains interacted with negatively charged or neutral phospholipids in the presence or absence of Ca²⁺. However, Ca²⁺ titrations monitored via intrinsic tryptophan fluorescence revealed that all three C₂ domains bound Ca²⁺ in the absence of phospholipids with a high apparent affinity (EC₅₀ of ~1.3–2.3 μM). Our data thus reveal that MCTPs are evolutionarily conserved C₂ domain proteins that are unusual in that the C₂ domains are anchored in the membrane by two closely spaced transmembrane regions and represent Ca²⁺-binding but not phospholipid-binding modules.

The C₂ domain is defined as a sequence motif in a comparison of the primary structures of different protein kinase C isoforms and named in an unbiased fashion as the “second constant sequence” of protein kinase C isoforms (1). Later studies revealed that a large number of proteins include C₂ domains, with >200 such proteins in the human genome alone (2). We observed that in synaptotagmin 1, a synaptic vesicle protein that binds Ca²⁺ and contains two C₂ domains (3), C₂ domains are autonomously folded Ca²⁺-binding modules (4, 5). Subsequently, most C₂ domain proteins were found to form Ca²⁺-dependent phospholipid complexes, although some appear to bind to phospholipids in the absence of Ca²⁺ (e.g. PTEN) (6) and others constitute protein-interaction domains instead of binding to either Ca²⁺ or phospholipids (e.g. the N-terminal C₂ domain of Munc13-1 (7) or the C-terminal RIM1 and C₂ domains (8)). Although the Ca²⁺-binding properties of many C₂ domain proteins remain to be examined, the large number of C₂ domain proteins in the vertebrate genome makes it likely that this domain represents the second most common Ca²⁺ binding motif after the EF-hand motif.

Most C₂ domain proteins are either signal transduction enzymes, such as protein kinase C, or membrane trafficking proteins, such as synaptotagmin 1. At least some isoforms of all major signal transduction enzymes, from ubiquitin ligases to kinases to various phospholipases, contain a C₂ domain. Without exception, these proteins are soluble cytosolic enzymes that include a single C₂ domain. In contrast, membrane trafficking proteins generally include at least two C₂ domains, although a few proteins such as the γ-RIM isoforms (9) and some splice variants of piccolo/aczonin and intersectin (10–12) contain only a single C₂ domain. In membrane trafficking proteins the different C₂ domains often feature conserved sequence differences, indicating that the C₂ domains are functionally specialized. For example, although in synaptotagmin 1 the C₂A and C₂B domains both bind Ca²⁺ and phospholipids with similar affinities (13), the C₂B domain contains an additional ”bottom” α-helix that is absent from the C₂A domain but is conserved in all of the C₂B domains of synaptotagmins (14). Membrane trafficking proteins with multiple C₂ domains either have no TMR1 or a single TMR either at the N terminal (e.g. synaptotagmins; see Fig. 1) (15) or the C terminal (e.g. ferlins) (16).

Work over the last few decades established Ca²⁺ as the major intracellular second messenger in eukaryotic cells, with specificity achieved by the spatial segregation of Ca²⁺ signals. In vertebrate genomes, proteins containing EF-hand Ca²⁺-binding sites are more common than C₂ domain proteins (2). However, Ca²⁺ binding in EF-hand proteins often does not serve a direct regulatory function but instead acts in Ca²⁺ buffering (e.g. parvalbumin and calbindin) or subserves a structural role (reviewed in Refs. 17 and 18). Among the EF-hand proteins with a regulatory Ca²⁺-binding site, one particular

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1 The abbreviations used are: TMR, transmembrane region; EYFP, enhanced yellow fluorescent protein; GST, glutathione S-transferase; MCTP, multiple C₂ domain and transmembrane region protein.
protein, calmodulin, appears to mediate more Ca$^{2+}$-dependent regulatory actions than all other EF-hand proteins combined and is in fact expressed as an identical sequence from multiple independent genes (reviewed in Refs. 19 and 20).

This situation seems to be completely different for C2 domain proteins. All functionally characterized proteins containing a C2 domain that binds Ca$^{2+}$ also act as Ca$^{2+}$ sensors, and no single C2 domain protein dominates the Ca$^{2+}$-dependent regulation of a cell. Thus, to fully understand the targets of Ca$^{2+}$-signaling in cells it is essential to characterize all principal C2 domain proteins. Only a complete overview of the Ca$^{2+}$-dependent properties of different C2 domain proteins will provide insight into how Ca$^{2+}$ signaling works. In view of these considerations, we have searched for conserved C2 domain proteins that might function as widely distributed Ca$^{2+}$ sensors and have focused on membrane-anchored C2 domain proteins because these are most likely involved in membrane traffic.

**MATERIALS AND METHODS**

**Sequence Analyses and Data Bank Searches**—Various data banks of the National Center for Biotechnology Information (NCBI) and Genomics were searched for multiple C2 domain proteins, expressed sequence tags, splice variants in reported full-length sequences, and the genes of MCTPs using programs available on the NCBI web site with default settings. The MCTP cDNA sequences were submitted to GenBank™ (accession numbers AY566715, AY566716, and AY566717).

**Expression and Purification of Recombinant Proteins**—The DNAs encoding the human MCTP1L-CA (residues 243–391), MCTP1L-CB (residues 451–598), MCTP1L-C-C (residue 608–755), and MCTP2-C-C (residues 491–638) domains were PCR amplified, subcloned into pGEX-KG vector, and verified by DNA sequencing. All recombinant proteins were produced as bacterial GST fusion proteins, purified essentially as described (21), additionally treated with Benzonase, and with 20 mM CaCl$_2$ and high salt buffers to remove the contaminating bacterial GST fusion proteins. After the incubations of C2 domains with liposomes, liposomes with bound C2 domains were re-isolated by centrifugation essentially as described (14, 23), and bound proteins were precipitated, resuspended in 30 μl of buffer B (40 mM Tris-HCl, pH 8.0, 1 mM NaCl, and 0.5 mM sodium EGTA) and centrifuged at 10,000 × g for 10 min to precipitate unbroken cells and nuclei. The supernatant was additionally centrifuged at 100,000 × g for 1 h, and total membrane and cytosol fractions were separated. All fractions were adjusted to the same volume, and Western blotting using a polyclonal antibody to human MCTPs was performed.

**Characterization of MCTP Sequences**—In searching for protein sequences containing C2 domains and transmembrane regions, we identified four classes of evolutionarily conserved proteins as follows: (i) synaptotagmins, which are expressed in at least 15 isoforms and are defined by the presence of a single N-terminal TMR and two C-terminal domains with N- or C-terminal TM sequences (reviewed in Ref. 15); (ii) forlins, which contain 3–6 C2 domains and a C-terminal TM (reviewed in Ref. 16); and (iii) two novel protein families that comprise 3–6 C2 domains as E-Syts (for “extended synaptotagmins”), because the topology of these proteins resembles that of synaptotagmins. One member of the E-Syt family was described previously as an unnamed plasma membrane protein of adipocytes (26); these proteins will be
are highly variable; furthermore, both human MCTP1 and C. elegans genes, the precise colinearity of the MCTPs, we aligned the human C. elegans sequences with the same overall architecture and a high degree of sequence identity, whereas invertebrate animals (C. elegans and Drosophila melanogaster) contain a single MCTP gene. For comparison of MCTPs, we aligned the human MCTP1 and MCTP2 and the Drosophila and C. elegans MCTP sequences (Fig. 2A). The Drosophila sequences were assembled from two separate predicted transcription units in the data bank (CG33148 and CG33146). Although Drosophila MCTP is predicted from the genome sequence to represent two separate genes, the precise colinearity of the Drosophila sequence with the human and C. elegans sequences (Fig. 2A) and the corresponding Anopheles gambiae sequence (data not shown) indicates that this represents a single transcription unit.

The sequence alignments show that the N termini of MCTPs are highly variable; furthermore, both human MCTP1 and C. elegans MCTP are expressed with two alternative 5′-sequences (referred to as MCTP1L and MCTP1S and as MCTP1L and MCTP1S, respectively), possibly because the genes contain two independent promoters. Motif searches demonstrated that all MCTPs contain three C2 domains (referred to as C2A, C2B, and C2C domains; shown in red in Fig. 2A) followed by two transmembrane regions (shown in gray). Only short linkers separate the three C2 domains, whereas a longer region connects the C2 domains to the TMRs (presumptive cysteoplastic non-C2 domain sequences are shown in yellow in Fig. 2A). The sequence that connects the two TMRs is short and highly charged. All of the MCTP sequences except for the N-terminal region are highly conserved, with the highest degree of conservation of MCTPs being in the C2C domain, the second TMR, and the short C-terminal cysteoplastic sequence. The latter includes a 12-residue stretch that is identical in all species and isoforms (NNELLDFLSRV; Fig. 2A).

MCTPs Contain Two Functional TMRs—The proposed transmembrane topography for MCTPs (Fig. 1) is based on the absence of an N-terminal signal peptide in the MCTP sequences and the fact that all known C2 domains are cysteoplastic. With this topography, MCTPs are the only C2 domain proteins that include more than one TMR. Interestingly, analysis of the MCTP sequences from expressed sequence tags reveals evidence of at least two sites of alternative splicing. First, the linker that separates the C2A and C2B domain varies in size dependent on alternative splicing. Second, we observed in multiple independent clones alternative splicing of the first TMR (Fig. 2B). As a result of this alternative splicing, some mRNAs for both MCTP1 and MCTP2 only encode the second TMR. If correct, this alternative splicing would convert the conserved C-terminal cysteoplastic sequence of MCTPs into a non-cysteoplastic sequence, i.e. turn the topography of the C terminus around (Fig. 1).

Although the predicted TMRs of MCTPs have all the features of classical TMRs, including a high degree of hydrophobicity, prediction of TMRs is not always accurate. To test whether each TMR is individually capable of anchoring MCTPs into a membrane, we generated constructs that encode EYFP fusion proteins of MCTP2 (Fig. 3). In these proteins, EYFP is fused to the C-terminal part of MCTP2 containing either both TMRs or only the first or second TMR. Of these proteins, the one carrying a deletion of the first TMR corresponded precisely to the sequence of the expressed sequence tag clones containing alternatively spliced MCTP variants that lack the first TMR (see Fig. 2B).

We first transfected the EYFP-MCTP2 fusion constructs into COS cells and examined whether the presence of either TMR would render the protein particulate as expected for a TMR protein (Fig. 3). Indeed, we found that EYFP itself was soluble, but when fused to MCTP2 fragments containing either one of the two TMRs it became particulate. We next examined the localization of the transfected EYFP-MCTP2 fusion proteins in transfected HEK293 cells. All three proteins were localized to intracellular vesicular structures, suggesting that each TMR by itself is competent to anchor the protein to membranes and indicating that MCTP2 may normally be a vesicular protein (Fig. 4).

Structure of the MCTP Genes—Using public and the Celera Genomics databases, we determined the organization and chromosomal localizations of the human MCTP genes. MCTP1 is encoded by a large gene (~600 kb) on chromosome 5q15, whereas MCTP2 is encoded by a smaller gene (~200 kb) on chromosome 15q26 (Fig. 5). The C-terminal half of the MCTPs, which exhibits the most sequence similarity, contains the same exon-intron organization (Fig. 2A). Small differences in the number and placement of introns interrupting homologous se-
FIG. 2. Structure of MCTPs. A, primary amino acid sequences of human MCTP1 and MCTP2 (hMCTP1 and hMCTP2), Drosophila MCTP (DmMCTP), and C. elegans MCTP (CeMCTP). Residues that are present at the same position in at least two of the four sequences are highlighted with a color code as follows: yellow letters on a red background, cytoplasmic C2 domain sequences; black letters on a yellow background, cytoplasmic non-C2 domain sequences; white letters on a gray background, TMRs; yellow letters on a blue background, extracytoplasmic loop between the TMRs; and yellow letters on a purple background, C-terminal sequence. Sequences are numbered on the right; note that the C. elegans sequence includes a 66-residue insertion at amino acid number 535 that is missing from the other species. Alternatively spliced sequences in the linker between the C2A and C2B domains are underlined. B, alternative splicing of the C-terminal TMRs of MCTPs. The full-length variant and two variants for MCTP1 (GenBank™ accession number AK057694 and AK058012) and one variant for MCTP2 (GenBank™ accession number R98750) are aligned. Note that the splice variants truncate TMR1 but leave TMR2 intact, thereby causing an inversion of the topology of the C terminus (see Fig. 1).
quences are observed in the N-terminal half of the proteins (Tables I and II). The different N-terminal sequences observed in MCTP1L and MCTP1S are encoded by distinct exons (referred to as 1a and 1b) that are separated by 200 kb in the genome (Fig. 5). Most introns disrupt the coding sequence of the MCTPs at the same position, most often the “0” position that lies precisely between individual codons. The gene organization explains the alternative splicing that we observed. The alternative splicing of sequences separating the C2A and C2B domains is due to the presence or absence of exon 7 in MCTP1 and MCTP2, and that of the first TMR is due to the presence or absence of exon 18 in MCTP1 and exon 17 in MCTP2.

Tissue Distribution of MCTPs—Data bank analysis revealed that the expressed sequence tags encoding MCTP1 and 2 were isolated from a large number of tissues such as brain, bone marrow, pancreas, spleen, thymus, placenta, blood vessel, and kidney. To test if the protein encoded by the MCTP mRNAs is actually synthesized and determine which tissues contain the highest steady-state levels of these proteins, we produced two antibodies against recombinant proteins derived from MCTP1 and MCTP2 (Fig. 6A). Immunoblotting of various rat tissues revealed that MCTP1 is highly expressed in skeletal muscle and to a lesser degree in heart muscle, whereas MCTP2 was primarily detectable in heart muscle and testis (Fig. 6B). The immunoblotting results for MCTP1 are likely to be an accurate reflection of the protein levels because two different antibodies, raised to non-overlapping MCTP1 epitopes, gave the same results, whereas only one of the two MCTP2 antibodies was usable. MCTP1 in heart muscle appears to be slightly smaller than in skeletal muscle, possibly because of alternative splicing. Because of the limited sensitivity of the antibodies, the...
results do not exclude the possibility of a low amount of expression of MCTP1 in non-muscle tissues. Nevertheless, the results clearly demonstrate that by far the highest levels of MCTPs are present in excitable muscle cells and that MCTPs are not enriched in brain, another tissue with a large number of excitable cells.

Structure of the MCTP C2 Domains—Because the three closely spaced C2 domains are the major feature of the cytoplasmic sequences of MCTPs, these domains presumably determine the function of these proteins. RNA interference experiments in *C. elegans* revealed that the MCTP homolog (1H206) is an essential gene and that its ablation leads to early...
embryonic lethality (27). Most, but not all, C2 domains function as Ca\(^{2+}\)-binding modules (reviewed in Ref. 28). As a first approach toward understanding the function of MCTPs, we therefore studied its C2 domains. Specifically, we investigated the two major activities associated with C2 domains, namely Ca\(^{2+}\)-binding and interaction with phospholipid membranes.

Alignment of the C2 domain sequences of MCTPs reveals that all three C2 domains belong to class 2 C2 domains. Class 1 and 2 C2 domains have similar structures composed of an eight-stranded \(\beta\)-sandwich but differ in strand topology. In class 2 C2 domains, the \(\beta\)-strand corresponding to the first \(\beta\)-strand of class 1 C2 domains is transplanted to the end of the C2 domain, making this the last \(\beta\)-strand (referred to as \(\beta1\); see Fig. 5). As a result, the topology of class 2 C2 domains represents a circular permutation of the topology of class 1 C2 domains (28), with the N- and C termini being on the top of the domain in class 1 C2 domains and at the bottom of the domain in class 2 C2 domains. Although all MCTP C2 domains belong to class 2, they are otherwise not very similar (Fig. 7). The C2A, C2B, and C2C domains are well conserved between various MCTP isoforms, but the degree of identity between C2 domains is low.

Most C2 domains bind Ca\(^{2+}\) with a low intrinsic binding affinity in the absence of phospholipid membranes but with a high apparent affinity in the presence of phospholipid membranes. This behavior probably results from the fact that the coordination spheres for bound Ca\(^{2+}\) ions are incomplete in these C2 domains in the absence of phospholipids but are completed by the phospholipid head groups. The Ca\(^{2+}\)-binding mode of C2 domains has been best characterized for the C2A domain from protein kinase C (29, 30), the C2A and C2B domains of synaptotagmin 1 (5, 14, 22), and the C2 domain from phospholipase C\(\delta\) (31). In all of the C2 domains studied, Ca\(^{2+}\) is bound exclusively by the loops emerging from the top of the \(\beta\)-sandwich. Ca\(^{2+}\) is coordinated by five aspartate, glutamate, glutamine, and valine-containing residues in the loop emerging from the top of the \(\beta\)-sandwich. Ca\(^{2+}\) is coordinated by five aspartate, glutamate, glutamine, and valine-containing residues in the loop emerging from the top of the \(\beta\)-sandwich.

### Table II

**Genomic organization of the human MCTP2 gene (15q26)**

Data are based on the analysis of human genome sequences in the Celera and GenBank databases. Nucleotide numbers correspond to those of the assembled Celera genome. UTR, untranslated region. Initiator and stop codons are boldfaced and underlined.

| Exon no. | Nucleotide no. | Size (bp) | Intron/exon junction sequences |
|----------|----------------|-----------|-------------------------------|
| 1        | 9807022–9807486 | 465       | 5' UTR ATG, GAT, TAG, GAT, — GAA, GAG, CCA, GAG, gtgagataggg |
| 2        | 9823038–9823100 | 63        | ttcttgcag AAG, CTA, TGT, GGA, — GAA, GAG, CCA, GAG, gtgtgtgtgttta |
| 3        | 9824285–9824393 | 143       | atctgtgcag GTA, CGG, GGG, GAA, — CGA, GAT, GGG, GGA, gtaaagctggg |
| 4        | 9848046–9848188 | 77        | cttcttaag GC, ACA, AGT, GAT, — CTA, CTT, GTC, AAG, gtaatacagata |
| 5        | 9848955–9849031 | 112       | etcttttag GTA, TAT, GAT, CA, — GAG, CTT, AAC, AGtcagattta |
| 6        | 9854969–9854980 | 98        | tttggctttag TCC, TCT, TTG, ATC, — ATT, ATA, CAG, GGA, gtaaagttatgg |
| 7        | 9853885–9853920 | 36        | ttgttttag CCT, TCT, TTG, ATA, — TAG, GCC, AGC, AAG, gtaaatatactt |
| 8        | 9864893–9865057 | 165       | ttgcttttag TCC, TCT, TTG, ATA, — TAG, AAA, AGA, AAG, gtaatacttata |
| 9        | 9876298–9876368 | 131       | ttttttag GAT, TAT, GAT, AAG, — GAA, CTT, GCC, AGC, AAG, gtaatacagata |
| 10       | 9876361–9876547 | 187       | accttttag ACG, GTG, AGT, — AAA, CTT, ACA, ATG, gtaatacagata |
| 11       | 9878843–9878936 | 94        | caacccctcag GTG, TTA, CAG, AAC, — GCA, GAT, TTC, CCA, Gttgaggtttta |
| 12       | 9892778–9892880 | 103       | atctttcag GG, AAG, AGT, GAC, — AAA, GTT, TTT, ACA, TTtgtaagcttt |
| 13       | 9894179–9894281 | 103       | ttgtaacat TCC, ATT, AAA, GAT, — CCC, CCT, GTC, TTC, gtaatgcttt |
| 14       | 9909717–9909718 | 123       | ttgtaacat GT, AAG, GAT, GGA, — ATA, TAT, AAT, CCG, gtaaagttatgg |
| 15       | 9909677–9909675 | 75        | tttttag CTG, AAA, GCA, AGT, — GAT, TAT, GTT, CTA, gtaaatatactt |
| 16       | 9910656–9910775 | 120       | ttaaaatcag TTC, TTA, AGA, — ATA, GCA, TTC, GGC, gtaaatatactt |
| 17       | 9948902–9949054 | 123       | tttttag CTG, AAA, GCA, AGT, — GAT, TAT, GTT, CTA, gtaaatatactt |
| 18       | 9951675–9951716 | 42        | tttttag GAG, ACC, AAG, — GAA, GAT, GAC, gtcgcttgcct |
| 19       | 9966939–9967002 | 110       | tttttag TAG, TGT, GGA, — GAA, AGG, ATT, AAG, gtaaagttatgg |
| 20       | 9975961–9976103 | 110       | tttttag GAA, TCT, GAG, AAA, — GAA, AGG, ATT, AAG, gtaaagttatgg |
| 21       | 9985452–9985549 | 98        | tttttag CTG, AAA, GCA, AGT, — GAT, GTT, CAA, AAG, gtaaatatactt |
| 22       | 9987722–9987790 | 66        | tttttag CTG, AAA, GCA, AGT, — GAT, GTT, CAA, AAG, gtaaatatactt |

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**Fig. 6. Antibodies to MCTPs and the tissue distribution of MCTP1 and MCTP2 expression.** A. Location of antibody epitopes in the domain structure of MCTPs. Two different epitopes were used for raising antibodies to each MCTP as indicated. B, immunoblots of rat tissues with the two independent MCTP1 antibodies (left), one of the two MCTP2 antibodies (top right), and vasoconstrictor protein (VCP) antibodies (bottom right); used as a loading control). Positions of molecular mass markers are shown on the left. Note that the two independent MCTP1 antibodies result in exactly the same pattern of reactivity on the multi-tissue immunoblots despite being raised with distinct epitopes, suggesting that MCTP1 is primarily expressed in skeletal muscle and, to a lesser extent, in heart. The second MCTP2 antibody was not successful (not shown).
or asparagine residues that are present in the top loops. All of these residues are conserved in the MCTP C2 domains, including the Drosophila and C. elegans MCTP C2 domains, except for the C2B domain of MCTP2, which lacks two of the five Ca\(^{2+}\)-binding residues (Fig. 7). In addition, the other conserved residues of the Ca\(^{2+}\)-binding loops of C2 domains are also retained in the MCTP domains (e.g. the typical GAXSD sequence of the first top loop that is also present in all synaptotagmins) (28), indicating that these domains have the expected features of Ca\(^{2+}\)/phospholipid binding domains. However, recent results (32) revealed that the Ca\(^{2+}\)-binding properties of C2 domains cannot be predicted from sequence analyses because the C2B domain of synaptotagmin 4, despite a perfect Ca\(^{2+}\)-binding consensus sequence, exhibits no intrinsic or phospholipid-dependent Ca\(^{2+}\)-binding. This raises the question of whether MCTPs indeed function as Ca\(^{2+}\)-binding molecules.

Phospholipid Binding Properties of the MCTP C2 Domains—We first tested whether the MCTP C2 domains bind to phospholipids similarly as other C2 domains. We produced all three C2 domains of MCTP1 and the C2C domain of MCTP2 as recombinant GST fusion proteins, we then tested the binding of these domains to liposomes with five different phospholipid compositions that included neutral phospholipids, phosphatidylserine, and negatively charged phospholipids (Fig. 8). Binding was examined in the presence of EGTA, Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) using a sensitive centrifugation assay that detects Ca\(^{2+}\)-dependent phospholipid binding by measuring the amount of C2 domain protein that can be isolated with liposomes in the presence of Ca\(^{2+}\) but not EGTA (23). This binding assay is important in assessing the ability, or lack thereof, of C2 domains to interact with phospholipids, because the pull-down assay that we originally developed (4) and has been widely adopted does not detect weaker interactions such as the binding of the synaptotagmin C2B domain (14). Surprisingly, using the centrifugation assay we detected no significant Ca\(^{2+}\)-dependent binding of any MCTP C2 domain to any of the phospholipid membranes (Fig. 8). Some weak binding was observed, especially for the C2C domain of MCTP1, but this binding never reached the level observed with typical phospholipid-binding C2 domains. This result indicates that MCTPs are not phospholipid-binding molecules for either charged or neutral phospholipids, raising the question of whether they are at all involved in Ca\(^{2+}\)-binding (32).

**Intrinsic Ca\(^{2+}\)-Binding to MCTP C2 Domains**—To monitor Ca\(^{2+}\) binding to MCTP C2 domains, we purified the recombinant C2 domains as GST fusion proteins, cleaved them off the GST moiety with thrombin, and examined their intrinsic tryptophan fluorescence as a function of Ca\(^{2+}\) (Fig. 9). This experiment was prompted by the observation that all MCTP C2 domains contain a tryptophan in the middle of \(\beta\)-strand 5 (see Fig. 7) and that the C2B domains additionally contain a tryptophan in top loop 3 and in the bottom N-terminal sequence.

The tryptophan fluorescence spectra of the C2 domains exhibited characteristic differences in the number of fluorescence maxima and their emission wavelength. In every C2 domain, however, the addition of a saturating concentration of Ca\(^{2+}\) increased the intrinsic tryptophan fluorescence in a manner that was fully reversible upon the addition of excess EGTA (Fig. 9). The Ca\(^{2+}\)-induced fluorescence increase was quite large (>10%) for the C2A and the C2B domains and smaller for the MCTP1 C2C domain. For this reason we also studied the C2C domain from MCTP2, because the C2C domain of this isoform contains an extra tryptophan (Fig. 7), and we included this domain in all other assays as well. Indeed, the MCTP2 C2C domain does exhibit a significantly larger Ca\(^{2+}\)-dependent fluorescence increase (Fig. 9). Ca\(^{2+}\) induced no shift in emission maxima, and Mg\(^{2+}\) had no effect on any tryptophan fluorescence property of the C2 domains (data not shown).

We next exploited the tryptophan fluorescence changes to titrate Ca\(^{2+}\) binding (Fig. 10). Saturable Ca\(^{2+}\)-dependent increases in tryptophan fluorescence were observed for all C2 domains, with half-maximal changes between 1.3 and 2.3 \(\mu\)M free Ca\(^{2+}\). The signal-to-noise ratio was robust for the C2A and C2B domains, but the changes in the C2C domains were rather small (Fig. 10). However, multiple independent experiments provided similar results, indicating that the changes observed correspond to a reliable Ca\(^{2+}\) binding event.

**Effect of Purified MCTP C2 Domains on Ca\(^{2+}\)-triggered Secretion from Permeabilized PC12 Cells**—The properties of the MCTP C2 domains, high affinity intrinsic Ca\(^{2+}\) binding and no phospholipid binding, are unexpected. The apparent Ca\(^{2+}\) affinity of the C2 domains approximately corresponds to that of secretion from PC12 cells. In previous studies we have shown that C2 domains with an apparent high Ca\(^{2+}\) affinity in phospholipid complexes potently inhibit Ca\(^{2+}\)-induced exocytosis from permeabilized PC12 cells (23). However, for traditional C2
domains, altering phospholipid binding always leads to a change in apparent $Ca^{2+}$ affinity, because phospholipid binding and $Ca^{2+}$ binding are interdependent (33). Because the MCTP $C_2$ domains have the requisite $Ca^{2+}$ affinity to interfere with PC12 cell exocytosis but do not bind phospholipids, they may still function in $Ca^{2+}$-dependent exocytosis. To address this possibility, we compared the effect of purified MCTP $C_2$ domains with those of synaptotagmin 7 $C_2$ domains (which are potent inhibitors of exocytosis) on $Ca^{2+}$-induced secretion in permeabilized PC12 cells (Fig. 11). We observed a strong inhibitory effect of the synaptotagmin 7 $C_2$ domains on secretion but no effect by the MCTP $C_2$ domains. These results indicate that consistent with the tissue distribution of MCTPs (Fig. 6), MCTPs are not components of the secretory machinery. Furthermore, these results show that for inhibition in the permeabilized PC12 cell assay, a high $Ca^{2+}$ binding affinity is not sufficient for an effect.

**DISCUSSION**

In the present study we describe a novel family of $C_2$ domain proteins that exhibit unusual properties indicative of a role in


**Ca²⁺Binding by Novel Membrane-bound C₂ Domain Proteins**

**Fig. 10. Ca²⁺ titration of the tryptophan fluorescence of purified recombinant C domains from MCTPs.**

Intrinsic fluorescence of the indicated purified C domains from MCTPs (3 μM) was monitored as function of the free Ca²⁺ concentration (excitation, 282 nm; emission of the MCTP1 Cₐ domain, 332 nm; emission of the MCTP1 C₇ domain, 344 nm; emission of the MCTP1 C₉ domain, 328 nm; emission of the MCTP2 C₇ domain, 328 nm). Ca²⁺ concentrations were clamped with Ca²⁺-EGTA buffers (see "Materials and Methods"). Data shown are means ± S.D. (n = 3 for Cₐ and C₇ domains of MCTP1; n = 7 for C₉ domains from MCTP1 and MCTP2; each experiment was performed in triplicate). The numbers indicated display the Ca²⁺ concentration that caused a half-maximal fluorescence change, as calculated by curve fitting using GraphPad PRISM version 3.02 software.

**Fig. 11. Effect of MCTP C₉ domains on Ca²⁺-induced exocytosis from permeabilized PC12 cells.** PC12 cells were loaded with ³H-labeled norepinephrine, cracked, and preincubated with 6 μM purified C₉ domain GST fusion proteins. Exocytosis was measured as the amounts of norepinephrine released during a 30-min incubation period after the addition of a buffer containing no Ca²⁺ or 10 μM Ca²⁺. Data shown are means ± S.D. (n = 3; each experiment was performed in duplicate).

Ca²⁺ signaling. These properties, which differentiate MCTPs from other previously studied C₂ domain proteins, are described in the next two paragraphs.

First, the architecture of the MCTPs is unique in that they are composed of a large, presumptively cytoplasmic sequence primarily composed of three class 2 C₉ domains and two TMRs. With the MCTPs there are now four families of membrane-anchored multiple C₂ domain proteins (Fig. 1). MCTPs are the only membrane-bound C₂ domain proteins that contain two functional TMRs (Figs. 3 and 4).

Second, the functional properties of MCTPs are unique in that they bind Ca²⁺ but not phospholipids. We only showed this for MCTP1 and for the C₉-C domain of MCTP2, but the C₉-A domain of MCTP2 is highly homologous to that of MCTP1 and thus is likely to also bind Ca²⁺. The C₉-B domain of MCTP2, however, lacks the consensus sequence for C₂ domain Ca²⁺-binding sites (Fig. 7) and is unlikely to bind Ca²⁺. Invertebrate MCTPs from C. elegans and D. melanogaster also contain all Ca²⁺-binding site sequences (Fig. 2A), consistent with the notion that Ca²⁺ binding is evolutionarily conserved and that the C₉-B domain of MCTP2 is the only C₂ domain of an MCTP that does not bind Ca²⁺.

With these properties, MCTPs are veritable Ca²⁺-binding machines in which three Ca²⁺-binding C₂ domains are attached to the membrane. The high affinity of all three MCTP1 C₂ domains for Ca²⁺ without phospholipid binding was unexpected. We recently showed that the C₉-B domain of synaptotagmin 4 contains a perfect predicted Ca²⁺-binding site with a high degree of sequence identity to that of the C₉-B domain of synaptotagmin 1, but, nevertheless, it does not bind Ca²⁺ (32); thus, the presence of a predicted Ca²⁺-binding site is not sufficient to deduce the Ca²⁺ binding properties of a C₂ domain. In extension of this conclusion, the current data indicate that the presence of a consensus Ca²⁺-binding site in a C₂ domain also does not allow the prediction of the mode of Ca²⁺ binding. The standard mode for C₂ domains is to bind Ca²⁺ in a complex with phospholipids in which the phospholipid head groups are essential to complete the coordination spheres of the bound Ca²⁺ ions (33). The present data show that this mode of Ca²⁺ binding is not universally true in that the MCTP Ca²⁺-binding sites must have reasonably complete coordination spheres for Ca²⁺ in the absence of phospholipids. The sequences of the MCTP C₂ domains, however, provide no clue as to how this might work. It is possible that in the top loops of the C₂ domains, negatively charged residues, in addition to the canonical five aspartates, contact the Ca²⁺ ions. However, no conserved, additional negatively charged residues are present that could fulfill this role. Alternatively, it is possible that Ca²⁺ ions bind between two C₂ domains, with the coordination sphere formed by residues from the top loops of two different C₂ domains.

The Ca²⁺ binding properties and structures of MCTPs demonstrate that these proteins function in Ca²⁺ signaling at the membrane. Elucidating the nature of this function will require a genetic approach that builds on the observation that in C. elegans MCTP is an essential gene whose ablation causes embryonic lethality (27). The essential nature of MCTP is consistent with an important role in Ca²⁺ signaling that could, in
principle, consist of a Ca\(^{2+}\)-controlled regulatory function or an activity as a Ca\(^{2+}\) buffer. Although a Ca\(^{2+}\) buffer function cannot be excluded, a Ca\(^{2+}\) regulatory function appears more likely. With the generally fast Ca\(^{2+}\) binding, the relatively low Ca\(^{2+}\) affinities, and the obligatory formation of phospholipid complexes by C\(_2\) domains (reviewed in Ref. 28), C\(_2\) domain proteins are more suited than EF-hand proteins for some Ca\(^{2+}\) regulatory functions and less suited for Ca\(^{2+}\) buffering functions. In the case of MCTPs, however, this assumption may not likely. With the generally fast Ca\(^{2+}\), it cannot be excluded, a Ca\(^{2+}\) activity as a Ca\(^{2+}\) protein. Members of the third family, referred to here as extended synaptotagmins and E-Syts because they contain two TMRs, one of which (TMR1) may be spliced in and out in both isoforms (Fig. 2B). Alternative splicing that removes the first TMR would retain the membrane-bound nature of MCTPs, because the second TMR is sufficient to anchor the cytoplasmic C\(_2\) domains to the membrane (see Figs. 3 and 4) but would convert the normally cytoplasmic C-terminal sequence of MCTP into an extracellular sequence (see Fig. 1).

It is interesting that of the four families of proteins that contain both C\(_2\) domains and a TMR, the two families that have been functionally studied (synaptotagmins and ferlins) are involved in membrane fusion (reviewed in Refs. 15 and 16). Members of the third family, referred to here as extended synaptotagmins or E-Syts because of their structural similarity to synaptotagmins (Fig. 1), have not been studied beyond the cloning of one of their isoforms, and the fourth family consists of the MCTPs examined here. The fact that synaptotagmins and ferlins are involved in membrane traffic strongly supports the notion that MCTPs might also act in membrane traffic. Furthermore, the presence of two evolutionarily conserved TMRs, when one would have been sufficient to anchor the C\(_2\) domains to the membrane, and the presence of three C\(_2\) domains with distinct conserved sequences argue against a Ca\(^{2+}\) buffer function. Again, genetic experiments will have to address this important issue.

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