Definition of Munc13-homology-domains and characterization of a novel ubiquitously expressed Munc13 isoform

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Munc13 proteins constitute a family of three highly homologous molecules (Munc13-1, Munc13-2 and Munc13-3). With the exception of a ubiquitously expressed Munc13-2 splice variant, Munc13 proteins are brain-specific. Munc13-1 has a central priming function in synaptic vesicle exocytosis from glutamatergic synapses. In order to identify Munc13-like proteins that may regulate secretory processes in non-glutamatergic neurons or non-neuronal cells, we developed protein profiles for two Munc13-homology-domains (MHDs). MHDs are present in a wide variety of proteins, some of which have previously been implicated in membrane trafficking reactions. Taking advantage of partial sequences in the human expressed sequence tag (EST) database, we characterized a novel, ubiquitously expressed, rat

protein (Munc13-4) that belongs to a subfamily of Munc13-like molecules, in which the typical Munc13-like domain structure is conserved. Munc13-4 is predominantly expressed in lung where it is localized to goblet cells of the bronchial epithelium and to alveolar type II cells, both of which are cell types with secretory function. In the present study we identify a group of novel proteins, some of which may function in a Munc13-like manner to regulate membrane trafficking. The MHD profiles described in the present study are useful tools for the identification of Munc13-like proteins, that would otherwise have remained undetected.

Key words: lung, protein profile, secretion.

INTRODUCTION

Synaptic vesicles are the key organelles in neurotransmitter release at synapses. In the presynaptic terminal of a nerve cell synaptic vesicles pass through a complex cycle of membrane fusion and fission reactions that govern the release process. Vesicles are probably generated by budding from early endosomes. They are loaded with transmitter, translocate to the plasma membrane and dock at the active zone, an electron dense presynaptic membrane region where the release process is restricted to. Docked vesicles have to be primed, that is mature to a fusion competent state, before a rise in the intracellular calcium concentration can trigger fusion and secretion of the transmitter. After fusion, vesicular membrane and protein components are retrieved by clathrin-mediated endocytosis and recycled directly or via early endosomes [1].

Munc13 proteins constitute a family of three molecules (Munc13-1, Munc13-2 and Munc13-3) with homology to *Caenorhabditis elegans* unc-13p. With the exception of a ubiquitously expressed Munc13-2 splice variant, Munc13 proteins are specifically expressed in brain, where Munc13-1 has a central role in synaptic vesicle priming. Munc13 proteins contain a phorbol ester-binding C₁-domain and two C₂-domains, which are Ca²⁺/phospholipid binding domains [2,3]. The most abundant isoform in rat brain, Munc13-1, is specifically targeted to presynaptic active zones [4]. It binds to syntaxin, a component of the synaptic vesicle fusion apparatus [5], as well as to DOC2 ('double C₂') [6], and acts as a phorbol ester-dependent enhancer of neurotransmitter secretion when overexpressed in *Xenopus* neuromuscular junctions [4]. Loss of Munc13-1 in deletion mutant

mice leads to an arrest of the synaptic vesicle cycle of hippocampal neurons at the synaptic vesicle priming step, resulting in a functional shutdown of synapses. These contain normal numbers of docked vesicles which are not primed and therefore unable to fuse upon physiological stimulation [7], demonstrating that Munc13-1 is an essential synaptic vesicle priming protein in neurons. Interestingly, this Munc13-1 mutant phenotype is restricted to excitatory/glutamatergic neurons while inhibitory/ γ -aminobutyric acid (GABA)ergic nerve cells are unaffected by the Munc13-1 deficiency [7]. Analysis of deletion mutations in the *C. elegans* and *Drosophila unc-13* genes yielded results that are very similar to those obtained in Munc13-1 mutant mice, demonstrating that the functional role of Unc-13 homologues is evolutionary conserved [8,9].

Munc13-1 is expressed in all neurons of the rat central nervous system [3]. In contrast, Munc13-2 and Munc13-3 exhibit strikingly differential expression patterns. Munc13-2 is only present in rostral brain regions, including cerebral cortex and 'cornu ammonis' regions of the hippocampus, while Munc13-3 expression is mostly restricted to the cerebellum [3]. Thus neurons in the rat brain co-express Munc13-1 with either Munc13-2 or Munc13-3, depending on the brain region under examination. On the basis of these findings and preliminary observations in deletion mutant mice [7], it was concluded that Munc13-2/Munc13-3 and Munc13-1 have partially redundant roles in neurotransmitter release. Neurons utilize transmitter-specific synaptic vesicle priming mechanisms where excitatory/glutamatergic cells are dependent on Munc13-proteins, while inhibitory/GABAergic cells are not [7].

In view of this striking differential dependence of neurons, in

Abbreviations used: BAI1, brain-specific angiogenesis inhibitor 1; BAP3, BAI1-associated protein 3; CAPS, Ca²⁺-dependent activator protein for secretion; CIRL, Ca²⁺-independent receptor for α -latrotoxin; CPD2, cerebellum postnatal development associated protein 2; EST, expressed sequence tag; GABA, γ -aminobutyric acid; GST, glutathione S-transferase; MHDs, Munc13-homology-domains; ORFs, open reading frames.

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The Munc13-4 nucleotide sequence data reported here appears in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AF159356.

Figure 1 Proteins containing MHDs

Domain structure of proteins identified with protein profiles for two Munc13-consensus sequences. Proteins are identified on the right and GenBank accession numbers are also given (ce-BAP3 was assembled manually from the genome; domain structure of the MR form of *C. elegans* unc-13 from [8]). MHDs are present in a diverse set of proteins, many of which are involved in the regulation of secretory processes. Note that *C. elegans* unc-13 is expressed in two variants (LR and MR). The L region is conserved in rat Munc13-1 and rat ubMunc13-2, while all other unc-13 homologues have unrelated N-termini. at, *A. thaliana*; C1, C₁-domain; C2, C₂-domain; ce, *C. elegans*; dr, *D. melanogaster*; h, human; m, mouse; PH, pleckstrin-homology-domain; r, rat; sc, *S. cerevisiae*; sp, *S. pombe*.

the central nervous system, on vesicle priming by the three known Munc13 isoforms, we initiated a search for novel Munc13-like proteins. Based on the assumptions that (1) synaptic vesicle priming is a general feature of neurotransmitter release from all neurons, and that (2) similar priming processes may be involved in non-neuronal cells, the aim of this search was the identification of novel vesicle priming proteins with central roles in secretory processes. We used distantly related Munc13-like protein sequences from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* to develop protein profiles for two Munc13-homology-domains (MHDs). We found that MHDs are present in a wide variety of proteins from *Arabidopsis thaliana*, *C. elegans*, *Drosophila melanogaster*, mouse, rat and human. Taking advantage of partial sequences in the human expressed sequence tag (EST) database we identified, cloned and characterized a novel, ubiquitously expressed rat protein (Munc13-4) that belongs to a subfamily of Munc13-like molecules in which the typical Munc13-like domain structure, with two C₂-domains separated by a sequence containing the two MHDs, is conserved. Munc13-4 is predominantly expressed in lung where it is localized to goblet cells of the bronchial epithelium and to alveolar type II cells, both of which are cell types with secretory function. In the present study we identify a group of novel proteins, some of which may function in a Munc13-like manner to promote vesicle priming during secretion from non-glutamatergic neurons and cells outside of the central nervous system.

MATERIALS AND METHODS

Sequence analysis and identification of Munc13-like proteins

Initial database searches for Munc13-like proteins were performed using the BLAST server at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). Multiple sequence alignments were calculated with either DNASTAR (Madison, WI, U.S.A.) or the PILEUP program of the GCG program suite and improved manually [10]. Profiles for MHD consensus sequences were constructed from aligned sequences [11] using the BLOSUM45 amino acid similarity matrix [12] with a gap penalty of 2.1 and a gap-extension penalty of 0.2. These profiles were used to search the current protein and DNA database releases [13].

Cloning of Munc13-4

Initial database searches led to the identification of two yeast open reading frames (ORFs) with similarity to Munc13 proteins; yor296w from *S. cerevisiae*, and c11e3 from *S. pombe* (Figures 1 and 2). Databank searches with MHD profiles, developed on the basis of the two yeast sequences and known Munc13-sequences, identified, among other sequences (Figures 1 and 2), three ESTs with similarity to Munc13 proteins (Clone ID/GenBank accession numbers: 231903/H92853; 700912/AA287381;

Figure 2 Conservation of MHDs

Alignments and corresponding dendrograms of MHD1 (top panels) and MHD2 (bottom panels) from the indicated proteins. Proteins and residue numbers are identified on the left. Sequences are shown in single letter amino acid code and are aligned for maximal homology. Conserved residues are shown on black background and residues that are similar are shown on shaded background [similarity groups: (F,Y,W); (I,L,V,M); (H,R,K); (D,E); (G,A); (T,S); (N,Q)]. Dots indicate gaps. at, *A. thaliana*; ce, *C. elegans*; dr, *D. melanogaster*; h, human; m, mouse; r, rat; sc, *S. cerevisiae*; sp, *S. pombe*.

1035452/AA621650). The respective clones were then obtained (Research Genetics, Huntsville, AL, U.S.A.), and their inserts were used to screen a rat forebrain cDNA library in λ ZAPII (J. Boulter, University of California, Los Angeles, CA, U.S.A.) as well as a rat whole-brain library in λ ZAPII (Stratagene, Heidelberg, Germany) at high stringency. From 1×10^6 plaques, three positive clones were isolated. Rescued plasmids were sequenced using the dideoxy chain termination method with dye terminators on an Applied Biosystems 373 DNA sequencer (Applied Biosystems, Weiterstadt, Germany). All three clones overlapped with the EST clones used in the screen. Inserts of positive clones were then used in a screen of a rat lung cDNA library in λ gt11 (Clontech, Heidelberg, Germany) at low stringency. From 1×10^6 plaques, 71 clones were isolated, subcloned

into pBluescript (Stratagene, Heidelberg, Germany) and sequenced as described above. Of the sequenced clones, 45 contained parts of the appropriate cDNA, while the rest represented unrelated artefacts. A full-length sequence of the novel cDNA, designated Munc13-4, was assembled from multiple overlapping cDNA fragments and subcloned into pcDNA3 (pcDNA3-Munc13-4; Invitrogen, San Diego, CA, U.S.A.). A human embryonic kidney cell line (HEK-293) was transfected with this vector, using calcium phosphate [14].

Generation of antibodies to Munc13-4

Polyclonal antibodies directed against Munc13-4 were generated with a glutathione-S-transferase (GST) fusion protein as the

Figure 3 Primary structure of Munc13-4

The amino acid sequence of rat Munc13-4 was deduced from the nucleotide sequence assembled from multiple overlapping cDNA clones (GenBank accession number AF159356). Sequences are shown in single letter amino acid code and are aligned for maximal homology with human BAP3 (GenBank accession number AB017111). Conserved residues are shown on black background and residues that are similar are boxed [similarity groups: (F,Y,W); (L,L,V,M); (H,R,K); (D,E); (G,A); (T,S); (N,Q)]. Dots indicate gaps. C₂-domains are indicated by hatched bars and MHDs are indicated by shaded bars above the respective sequence stretch. Munc13-4 and BAP3 are 34% identical and form a new subfamily of Munc13-like proteins. h, human; r, rat.

antigen. Recombinant GST–Munc13-4 fusion protein was generated by using the expression plasmid pGEX-Munc13-4, which represents residues 889–1088 of Munc13-4 in the pGEX-KG vector [15]. Antisera were affinity-purified on fusion proteins that was either coupled to CNBr-activated Sepharose (Pharmacia, Freiburg, Germany) [16] or blotted to nitrocellulose [17].

Immunocytochemical detection of Munc13-4

Adult rats were anaesthetized with tribromoethanol and fixed by transcardial perfusion with 4% (w/v) paraformaldehyde/0.1 M phosphate buffer (pH 7.4). Lungs were immediately dissected, post-fixed for 1 h and cryoprotected with 30% (w/v) sucrose. On a frozen microtome, 14 µm thick sections were cut and mounted onto microscope slides for light microscopic immunocytochemistry. Endogenous peroxidase activity was blocked by incubation for 20 min at room temperature with 0.5% (v/v) H₂O₂ in methanol. Sections were probed with affinity-purified antibodies to Munc13-4 and labelled according to published procedures with the streptavidin/biotin/horseradish peroxidase ('ABC') method (Dako, Glostrup, Denmark), followed by a histochemical nickel-intensified horseradish peroxidase reaction with 3,3'-diaminobenzidine [4]. Sections were counterstained with Kernechtrot (Merck, Darmstadt, Germany).

Miscellaneous

Multiple rat tissue mRNA blots were purchased from ClonTech (Heidelberg, Germany) and hybridized at high stringency with an 0.7 kb *SacI*–*ApaI*-fragment (bp 2851–3554) from the Munc13-4 cDNA. SDS/PAGE and immunoblotting were performed using standard procedures [18,19].

RESULTS

Identification of proteins containing MHDs

In initial cDNA database searches we identified two yeast ORFs that exhibited similarities with Munc13 proteins outside the conserved C₂-domains; *S. cerevisiae* yor296w, presumably involved in bud formation, and *S. pombe* c1le3 (Figures 1 and 2). Two profiles for MHD consensus sequences, designated MHD1 and MHD2 for MHDs 1 and 2, were constructed from alignments between Munc13-sequences and the novel yeast proteins. Interestingly, MHD1 and MHD2 enclose a central C₂-domain in the two yeast ORFs, while they are arranged between two flanking C₂-domains in Munc13 proteins (Figure 1). Using these profiles multiple proteins containing MHDs were detected in the current databases (Figures 1 and 2). Apart from the rat, human, *C. elegans* and *D. melanogaster* Munc13 proteins and the yeast ORFs described above, these included the following proteins: (1)

Figure 4 Tissue distribution of Munc13-4 mRNA

Blots containing polyadenylated-enriched RNA from the indicated rat tissues were hybridized at high stringency with a uniformly labelled probe from the coding region of Munc13-4 and exposed to film for 24 h. The same blots were rehybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase to ensure uniform loading (not shown). Apart from skeletal muscle, Munc13-4 mRNA was expressed in all tissues examined. Strongest expression was observed in lung, spleen and testis.

C. elegans and human brain-specific angiogenesis inhibitor 1 (BAI1)-associated protein 3 (BAP3) which exhibit the typical Munc13-like domain structure with two C_2 -domains flanking the

two MHDs, but which lack the long N-terminus with the phorbol ester-binding C_1 -domain (human BAP3 binds to BAI1, a member of the secretin-receptor family [20]); (2) rat Ca^{2+} -dependent activator protein for secretion (CAPS), a regulator of large dense-core vesicle secretion [21–23], and its *C. elegans* homologue unc-31p, both of which contain only MHD1 and are otherwise unrelated to Munc13 proteins; (3) human cerebellum postnatal development associated protein 2 (CPD2) (partial sequence which contains only MHD1), a protein with a putative role in cerebellum development; (4) four *A. thaliana* proteins of unknown function.

Cloning of Munc13-4

In addition to the above sequences our database searches resulted in the identification of several human ESTs containing MHDs. Three of these encoded parts of a protein with weak homology to human BAP3 (Clone ID/GenBank accession numbers: 231903/H92853; 700912/AA287381; 1035452/AA621650), demonstrating the existence of a family of mammalian BAP3-like proteins. Since the domain structure of BAP3 resembles that of the known Munc13 proteins, indicating that BAP3 and its homologues may also be functionally related to Munc13 proteins, we cloned the full-length sequence of the novel EST-encoded protein from rat lung cDNA. The resulting cDNA contained an ORF that encoded a protein of 1088 amino acid residues with a calculated molecular mass of 125 kDa (Figure 3). The primary structure of the novel protein was 34% identical to that of BAP3. In addition, the domain structures of the two proteins were identical, with two C_2 -domains at the N- and C-termini flanking the central MHDs (Figures 1 and 3). Due to the highly conserved Munc13-like domain arrangement, and because its two C_2 -domains were most closely related to the C_2 -domains of Munc13 proteins (see [20] for a comparison of C_2 -domains in BAP3 and Munc13), the novel protein was named Munc13-4.

Characterization of Munc13-4 mRNA and protein

RNA blots loaded with polyadenylated-enriched RNA from different tissues were hybridized at high stringency with a probe

Figure 5 Tissue distribution of Munc13-4 protein

Left panel: homogenates from untransfected HEK-293 cells (Mock) and from HEK-293 cells that had been transfected with a eukaryotic expression vector encoding full-length Munc13-4 (pcDNA-Munc13-4) (20 μ g/lane) were analysed by SDS/PAGE and immunoblotting using affinity-purified antibodies to Munc13-4. Full-length Munc13-4 (arrow) migrates as a 115 kDa polypeptide. Additional bands represent unidentified cross-reactive proteins. Right panel: homogenates from the indicated tissues (20 μ g/lane) were analysed by SDS/PAGE and immunoblotting using affinity-purified antibodies to Munc13-4. Like Munc13-4 mRNA, Munc13-4 protein (arrow) was expressed most strongly in rat spleen and lung. Additional bands represent unidentified cross-reactive proteins.

Figure 6 Munc13-4 localization in lung

Section through the bronchial epithelium of rat demonstrating the presence of Munc13-4 in goblet cells (examples are indicated by arrowheads).

specific for Munc13-4 (0.7 kb *SacI*-*ApaI* fragment from the Munc13-4 cDNA). A 4.5 kb Munc13-4 mRNA transcript was detected in lung, spleen and testis (Figure 4). In heart muscle, kidney, liver, brain and skeletal muscle a very weak signal of similar size was detected. This demonstrates that in contrast to the brain-specific BAP3, Munc13-4 is expressed in a more ubiquitous manner.

For the basic protein chemical characterization of Munc13-4, we generated a polyclonal antibody to the C-terminus of Munc13-4 (residues 889–1088). The resulting rabbit antiserum was affinity-purified on immobilized antigen. Using the affinity-purified antibodies to Munc13-4 in Western blots of HEK-293 cells, a 115 kDa band was detected in cells transfected with the full-length eukaryotic Munc13-4 expression construct pcDNA-Munc13-4, but not in untransfected cells (Figure 5, left panel). A protein of the same size was detected by Western blot analysis in lung and spleen, confirming the non-neuronal expression pattern of Munc13-4 as determined by mRNA blotting (Figure 5, right panel). Protein levels in other tissues expressing Munc13-4 mRNA were below the detection limit of the antibody.

Munc13-4 protein was most strongly expressed in lung, and our affinity purified antibodies to Munc13-4 were essentially mono-specific for Munc13-4 in Western blots of lung homogenates (Figure 5, right panel). Therefore the distribution of Munc13-4 was examined in lung by light microscopic immunocytochemistry, employing affinity-purified antibodies to Munc13-4. We found that Munc13-4 was mainly present in goblet cells of the bronchial epithelium (Figure 6). Weaker staining was also observed in alveolar type II cells (results not shown).

DISCUSSION

With the exception of a ubiquitously expressed Munc13-2 splice variant, rat Munc13 proteins are expressed in a brain-specific manner [2,3]. Munc13-1, the most abundant isoform, is selectively targeted to presynaptic active zones where it plays an essential role in synaptic vesicle priming [4,7]. Since vesicle priming by Munc13 proteins appears to be specific for glutamatergic neurons [7], alternative pathways for (synaptic) vesicle priming have to be postulated for cells in which priming of secretory vesicles occurs (e.g. non-glutamatergic neurons or neuroendocrine cells). It is likely that some of these priming reactions, and particularly those that are taking place in neurons, are mediated by Munc13-like proteins. In addition, unrelated proteins may participate in vesicle priming reactions as exemplified by the roles of phosphatidylinositol transfer protein, phosphatidylinositol 4-kinase, and phosphatidylinositol-4-phosphate 5-kinase in the priming of chromaffin granules [24].

In the present study, we attempted to find novel Munc13-like proteins with putative roles in the regulation of membrane trafficking processes. For this purpose we developed protein

profiles for two Munc13 consensus sequences (designated MHD1 and MHD2) as tools for the detection of Munc13-like proteins in database releases. Using these profiles we identified several proteins whose similarity to Munc13 proteins was highly significant but had not been detected before (Figures 1 and 2). Apart from the yeast ORFs used in the initial development of MHD profiles, four *A. thaliana* proteins of unknown function, and a human protein with a putative role in cerebellar development, our analysis identified two extremely interesting protein families with similarity to Munc13 proteins. One of these included rat CAPS and its *C. elegans* homologue unc-31p, while the other one was represented by human and *C. elegans* BAP3 and the Munc13-4 protein cloned in the present study.

Both CAPS and unc-31p contain only MHD1 and are otherwise unrelated to Munc13 proteins (Figure 1). In PC12-cells CAPS is required for a post-priming Ca^{2+} -dependent step in neurosecretion [21]. In brain CAPS is specifically localized to large dense-core vesicles whose secretion it appears to regulate in a manner similar to that in PC12-cells [22,23]. The localization and function of CAPS is different from that of Munc13-1, which is localized to presynaptic active zones [4], not present on vesicles [3], and which mediates the vesicle priming step *per se* [7]. However, the involvement of CAPS and Munc13-1 in consecutive steps of vesicle secretion suggests that MHD1 has similar functions in the two proteins.

In contrast to CAPS, BAP3 and Munc13-4 share the same domain structure and exhibit significant similarity with Munc13 proteins throughout their sequence, indicating that BAP3, Munc13-4 and the three known Munc13 isoforms are evolutionary descendants of a common ancestor and have similar functional roles. Human BAP3 was originally identified as an interaction partner of BAI1, a member of the secretin-receptor family [20]. Interestingly, BAI1 is distantly related to the Ca^{2+} -independent receptor for α -latrotoxin (CIRL), which is involved in the regulation of synaptic neurotransmitter release [25,26]. This link to transmitter secretion and the brain-specific expression pattern of BAP3 raise the possibility that BAP3 mediates synaptic vesicle priming in neurons that are independent of priming by Munc13 isoforms (e.g. GABAergic neurons). Unlike BAP3, its homologue Munc13-4 is expressed in a ubiquitous fashion (Figures 4 and 5). Munc13-4 is strongly expressed in lung where it is mainly present in goblet cells of the bronchial epithelium (Figure 6) and alveolar type II cells (results not shown), suggesting a role in secretory processes. With the exception of an N-terminal splice variant of Munc13-2, which also appears to be expressed outside of the nervous system ([27] and N. Brose, unpublished work), this identifies Munc13-4 as the first bona fide ubiquitous Munc13 isoform.

Apart from the identification of novel Munc13-like proteins, an important objective of the present study was to use the newly identified molecules in order to gain insight into the molecular or enzymic function of Munc13 proteins and their relatives. Unfortunately, the function of the novel Munc13-like proteins identified here is either completely unknown (as in the case of the *A. thaliana* proteins) or has only been analysed 'phenomenologically' on the system or cellular level, but not in molecular terms. MHDs are present in organisms as phylogenetically distant as yeast and mammals, and some mammalian MHD-bearing proteins are expressed in a ubiquitous manner, suggesting that these domains take part in molecular processes common to all organisms and cells. Although Munc13 proteins (synaptic vesicle priming), CAPS/unc-31p (large dense-core vesicle exocytosis), BAP3 (CIRL-binding, neurosecretion), yor296w (bud formation) and Munc13-4 (present in secretory cells of the lung) are all linked to secretory processes of some

sort, this common molecular role of MHDs remains unclear. In most of these molecules MHDs do not coincide with functionally characterized domains. Only the syntaxin-binding region of the known Munc13 proteins [5] overlaps partially with MHD1, but Munc13-4 does not bind syntaxins (H. Koch and N. Brose, unpublished work).

The MHD profiles described in the present study are very useful tools for the identification of Munc13-like proteins, that would otherwise have remained undetected. As with other conserved protein sequence domains, it is probable that the molecular role of MHDs will become evident once the 'common theme' in the known MHD-bearing proteins is recognized.

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