Expression of vesicle-associated membrane protein 2 (VAMP-2)/synaptobrevin II and cellubrevin in rat skeletal muscle and in a muscle cell line

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Molecular studies have identified a family of synaptic vesicle-associated membrane proteins (VAMPs, also known as synaptobrevins) which have been implicated in synaptic vesicle docking and/or fusion with plasma membrane proteins. Here we demonstrate the expression of two members of this family, VAMP-2/synaptobrevin II and cellubrevin, in skeletal muscle, a tissue with both constitutive and regulated membrane traffic. The 18 kDa VAMP-2 polypeptide was detected in purified membrane fractions from adult skeletal muscle and from L6 myotubes in culture, demonstrating that the presence of this protein in the isolated muscle membrane fractions is not the result of contamination by ancillary tissues such as peripheral nerve. Furthermore, skeletal muscle and the muscle cell line also expressed cellubrevin, a VAMP-2 homologue of 17 kDa, which is much less abundant in brain cells. Both VAMP-2 and cellubrevin were preferentially isolated in membrane fractions rich in plasma membranes, and were less concentrated in light microsomes and

other internal membrane fractions of mature muscle or muscle cells in culture. Interestingly, both VAMP-2 and cellubrevin were much more abundant in the differentiated L6 myotubes than in their precursor myoblasts, suggesting that they are required for functions of differentiated muscle cells. The identity of both polypeptides was further confirmed by their susceptibility to proteolysis by *Clostridium tetanus* toxin. Expression of these products was further established by the presence of mRNA transcripts of VAMP-2 and cellubrevin, but not of VAMP-1, in both skeletal muscle and L6 myotubes. In contrast, other synaptic vesicle and docking/fusion components were undetectable, such as VAMP-1, SNAP25 and syntaxin 1A/1B, as were synaptophysin and synapsin Ia/Ib, proteins which are believed to be involved in sensing the signal for neuronal exocytosis. It is concluded that VAMP-2 and cellubrevin are expressed in skeletal muscle cells and may each participate in specific processes of intracellular membrane traffic.

INTRODUCTION

Intracellular membrane traffic is an essential process of all mammalian cells. Constitutive membrane traffic is involved in the movement of most membrane proteins to particular compartments and organelles, and in steady-state secretion. Regulated membrane traffic is involved in the specific and often acute changes in cellular localization of membrane proteins and the secretion of vesicle contents in response to hormones and other stimuli.

One of the basic steps in intracellular membrane movement is the docking and fusion of a vesicle with a target membrane. Membrane-bound proteins participating in these processes have recently been identified in neuronal tissues and within the Golgi apparatus [1–3]. These elements can be isolated as a high-molecular-mass (20 S) complex comprising membrane proteins of both the vesicle and the target membranes [2–4]. Additional components of the complex are ubiquitous cytosolic proteins known to participate in intra-Golgi transport, which include the *N*-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment factors (SNAPs) [2,3]. The membrane proteins on the donor vesicle and the acceptor membrane that constitute the 20 S complex (docking/fusion complex) have been termed SNAP receptors or SNAREs [2]. SNAREs isolated from detergent extracts of mammalian brain include the synaptic vesicle VAMP-

2 (vesicle-associated membrane protein 2)/synaptobrevin II, while plasma membrane SNAREs include syntaxins 1A, 1B and SNAP25 [2,4]. A direct interaction between VAMP-2 and syntaxin 1A was recently demonstrated *in vitro* [5], indicating that these proteins may play a role in the docking and/or the fusion of the vesicle to the membrane.

Recently, VAMP-like molecules were detected in two non-neuronal cells, i.e. rat adipocytes [6] and pancreatic acinar cells [7]. In addition, another member of the VAMP family was identified and named cellubrevin. mRNA transcripts of cellubrevin were detected in testis, lung, kidney, intestine, brain, cerebellum, spinal cord and liver, and the corresponding polypeptide was also detected in parotid, thyroid and adrenal glands [8]. It was thus hypothesized that cellubrevin may play a role in endocytic/exocytic mechanisms. To date, expression of VAMP has been found to be restricted to secretory tissues, whereas cellubrevin is found in both secretory and non-secretory cells. The participation of VAMP in synaptic secretion has been documented, but it is not known if either VAMP or cellubrevin are involved in non-secretory intracellular membrane transit. Skeletal muscle is a tissue which displays both constitutive and regulated membrane traffic. In particular, membrane structures containing glucose transporters and $\alpha 2$ and $\beta 1$ subunits of the Na^+/K^+ -ATPase are recruited to the plasma membrane by insulin [9–13], and cell-surface insulin receptors are increased by adrena-

Abbreviations used: VAMPs, vesicle-associated membrane proteins; NSF, *N*-ethylmaleimide-sensitive factor; SNAPs, soluble NSF attachment factors; SNAREs, SNAP receptors; PMSF, phenylmethanesulphonyl fluoride; PVDF, poly(vinylidene difluoride).

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line [14]. The identification of proteins implicated in intracellular traffic at all levels may lead to a better understanding of regulated membrane flux in skeletal muscle.

The objective of this study was to determine whether members of the synaptic vesicle docking/fusion apparatus are expressed in mature muscle and in muscle cells in culture. Protein products and mRNA transcripts of the VAMP-2 and cellubrevin genes were detected in these cells and the subcellular distribution of the polypeptides determined. Their identity was confirmed by their susceptibility to proteolysis by tetanus toxin, a clostridial neurotoxin recently shown to specifically cleave cellubrevin and VAMP-2.

EXPERIMENTAL

Membrane and tissue fractions

Subcellular fractionation of rat skeletal muscle

This was carried out as described previously [9,15–17] and the membranes were characterized on the basis of their content of immunodetectable marker proteins and enzymic activities [9,11,15–17].

Brain microsomes

These were prepared according to the procedure of Nagamatsu et al. [18].

Sciatic nerve extract

The visible portion of the nerve of 250 g rats was excised, cut into small fragments and incubated in 4% SDS, at pH 7.0, with the protease inhibitors 1 mM phenylmethanesulphonyl fluoride (PMSF), 5 μ M leupeptin, 5 μ M pepstatin A, 50 μ M E-64, and 5 mM EDTA, for 30 min at room temperature. The extract was homogenized with a Wheaton Teflon–glass homogenizer and centrifuged in a microfuge at maximum speed to sediment insoluble material. The supernatant, containing all membrane and cytosolic components, was frozen until used.

Growth, differentiation and subcellular fractionation of L6 muscle cells

L6 muscle cells were grown to the stages of myoblasts and myotubes [19–23]. Membrane fractions of cells at both stages of differentiation were prepared as described previously [19,20].

Immunoblots

Samples were solubilized in Laemmli's sample buffer [24], resolved by SDS/PAGE and transferred on to poly(vinylidene difluoride) (PVDF) filters as described [23]. Filters were incubated with primary antibody solution overnight at 4 °C (antibody dilutions: anti-VAMP-2, 1:200; anti-cellubrevin, 1:1000; anti-SNAP25, 1:1000; anti- α 1 Na⁺/K⁺-ATPase, 1:50; anti-synaptophysin, 1:20; anti-syntaxin 1A/1B, 1:500; anti-synapsin Ia/Ib, 1:5000). For detection of bound primary antibodies, filters were washed twice for 15 min and then twice for 10 min each wash with buffer A [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.05% Tween 20/0.04% Nonidet P40] and incubated with 0.1 μ Ci/ml

of ¹²⁵I-Protein A (for polyclonal primary antibodies) or ¹²⁵I-labelled sheep anti-(mouse IgG) antibody (for monoclonal primary antibodies) for 1 h at room temperature. The filters were then washed four times (15 min each wash) with buffer A at room temperature, dried and exposed to either Kodak XAR-5 film or Dupont Autoradiography film for various lengths of time at –70 °C. When the enhanced chemiluminescence detection system was used the blots were washed as indicated above, then incubated with horseradish peroxidase-labelled donkey anti-(rabbit IgG) secondary antibody for 1 h at room temperature, washed again three times (15 min in buffer A) and developed according to the manufacturer's instructions (Amersham). For alkaline phosphatase detection, the filters were stained with 5-bromo-4-chloro-3-indoyl phosphate-*p*-toluidine salt and *p*-Nitroblue tetrazolium chloride as described [25]. Scanning of X-ray films was done using a PDI model DNA35 scanner with the version 1.3 of the Discovery Series one-dimensional-gel-analysis software.

Antibodies

An antibody specific for the VAMP-2 isoform (named V2R) was generated in rabbits by injecting a synthetic peptide corresponding to the 16 N-terminal amino acids of this protein (MSATAATAPPAAPAGE) coupled to BSA through an N-terminal cysteine residue. The peptide was coupled to maleimide-activated BSA (Pierce) according to manufacturer's instructions. The specificity of the rabbit polyclonal anti-VAMP-2 serum was confirmed by its ability to recognize VAMP-2 expressed as a recombinant fusion protein on Western blots. No cross-reactivity to other members of the VAMP family was detected, or to fusion proteins comprising common regions of the VAMPs including amino acids 33–116. The antibody to cellubrevin was a kind gift from Dr. Thomas C. Sudhof (Howard Hughes Medical Institute, Dallas, TX, U.S.A.) and was raised to the N-terminus segment as reported [8]. Monoclonal antibodies to synaptophysin (clone SY38) and SNAP25 (clone SMI81) were obtained from Boehringer–Mannheim and Sternberger Monoclonals Inc. respectively. A monoclonal antibody (number SPM-1) recognizing syntaxins 1A and 1B [4] was the kind gift of Dr. T. Abe (University of Niigata, Niigata, Japan). A monoclonal antibody recognizing synapsin I (mouse monoclonal hybridoma cell line 97.8B2.4) was the kind gift of Dr. T. Ueda (University of Michigan, Ann Arbor, MI, U.S.A.). A monoclonal antibody directed to the α 1 isoform of the Na⁺/K⁺-ATPase was the kind gift of Dr. K. Sweadner (Harvard University, Boston, MA, U.S.A.) [26].

Treatment with tetanus toxin

Membrane fractions derived from L6 myotubes and rat skeletal muscle respectively were dried down in a speed-vac and resuspended in 20 μ l of potassium glutamate buffer (138 mM potassium glutamate, 20 mM Hepes, 8 mM MgCl₂, 0.285 mM CaCl₂, 1 mM EGTA, 1 mM dithiothreitol, pH 7.15) with or without the indicated concentrations of tetanus toxin light chain prepared as in ref. [27]. The samples were incubated at 37 °C in a water bath under constant agitation for 1 h; a brief vortex was given at the 30 min interval. The samples were then boiled for 3 min in 20 μ l of double-strength Laemmli sample buffer and immediately resolved by SDS/PAGE. Proteins were then electrotransferred and immunoblotted with VAMP-2 or cellubrevin antibodies.

Isolation of RNA from rat skeletal muscle and L6 muscle cells and Northern-blot analysis

Total RNA was extracted from rat brain, soleus muscle and L6 muscle cells, using the single-step RNA isolation with acid guanidinium thiocyanate-phenol-chloroform [28], quantified by its 260/280 nm u.v. absorbance spectrum and electrophoresed under denaturing conditions in 1.2% (w/v) agarose gels containing 8% (v/v) formaldehyde. RNA was then transferred on to nylon membranes and baked for 90 min at 80 °C in a vacuum oven. Equal loading of all samples containing 20 µg of RNA (muscle and L6 myotubes) was confirmed by the ethidium bromide fluorescence incorporated into nucleic acids. The nylon membranes were prehybridized overnight at 42 °C with 200 µg/ml salmon sperm DNA in 6 × SSPE (1 × SSPE: 0.15 M NaCl/10 mM NaH₂PO₄/1 mM EDTA, pH 7.4)/10 × Denhardt's solution/0.5% SDS. Hybridization was carried out in 50% formamide/6 × SSPE/0.5% SDS/5% dextran sulphate/100 µg/ml salmon sperm DNA for 16 h at 37 °C by adding [α -³²P]dCTP-labelled probes for VAMP-1 (6.8 × 10⁶ c.p.m./ml; 5.5 × 10⁸ c.p.m./µg of DNA), VAMP-2 (1.14 × 10⁷ c.p.m./ml; 5.7 × 10⁸ c.p.m./µg of DNA) or cellubrevin (9.22 × 10⁶ c.p.m./ml; 4.6 × 10⁸ c.p.m./µg of DNA), labelled by the random primer method. The VAMP probes corresponded to nucleotides 1–1470 of rat VAMP-1 and nucleotides 1–432 of rat VAMP-2 [29]. The cellubrevin probe was a 0.68 kb *Eco*R1–*Bgl*II fragment from plasmid vector PCMV 18-5 (kind gift of Dr. Thomas C. Sudhof, Howard Hughes Medical Institute, Dallas, TX, U.S.A.). After hybridization, nylon membranes were washed three times (5 min each) with 1 × SSC (0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS at room temperature and twice, for 1 h, with 0.1 × SSC/0.5% SDS at 65 °C before exposure to Kodak XAR-5 film at –70 °C.

RESULTS AND DISCUSSION

VAMP-2 polypeptide in membrane fractions of rat muscle and muscle cell cultures

VAMP-2 is a polypeptide of 18 kDa containing a C-terminal transmembrane anchor which exposes the remainder of its mass,

Figure 1 Presence of VAMP-2 polypeptide in brain microsomes, sciatic nerve extract, and purified membranes from rat skeletal muscle

Membrane fractions were isolated and proteins resolved by SDS/PAGE on 13% polyacrylamide gels prior to immunoblotting with anti-(VAMP-2)-specific antibody V2R followed by ¹²⁵I-labelled Protein A as outlined in the Experimental section. The figure shows a representative immunoblot with samples of rat brain microsomes (Br, 10 µg of protein), sciatic nerve extract (Ne, 40 µg of protein), and the purified membrane fractions from skeletal muscle (50 µg of protein each) which band on top of 25%, 30% and 35% sucrose layers (labelled 25, 30, 35) (see text). The molecular mass of the immunoreactive VAMP-2 band is indicated. The distribution of the plasma membrane marker $\alpha 1$ Na⁺/K⁺-ATPase is shown to confirm the purity and origin of the muscle membrane fractions.

including the N-terminus, to the cytoplasm [29]. The presence of this polypeptide was assessed using an antibody directed to the N-terminal 16 amino acids (see the Experimental section). Figure 1 illustrates the detection of VAMP-2 protein in purified membrane fractions from rat skeletal muscle. Analysis of the relative concentration of this polypeptide in the various membrane fractions isolated revealed a preferential distribution in two fractions (denoted 25 and 30) which were rich in plasma membrane markers. Fractions 25 and 30 are isolated above 25% and 30% sucrose layers of a discontinuous sucrose gradient respectively. Their complete biochemical characterization has been published previously [9,11,15–17]. Both are rich in membrane markers such as the $\alpha 1$ Na⁺/K⁺-ATPase subunit, GLUT1 glucose transporters, alkaline phosphatase and 5'-nucleotidase. The presence of the plasma membrane marker $\alpha 1$ Na⁺/K⁺-ATPase is presented in the figure. The abundance of VAMP-2 in these fractions suggests that they may contain membranes with the ability to dock and fuse; these could be of plasma membrane origin or may include an endosomal population which may not be separated out by the fractionation procedure. This suggests that the VAMP-2 polypeptide could be involved in either exocytic or endocytic processes involving the membrane constituents isolated in these muscle fractions.

The membranes recovered over the 35% sucrose layer (sample 35), which contain the insulin-sensitive pool of glucose transporter and $\alpha 2$ Na⁺/K⁺-ATPase molecules, contained the lowest, albeit clearly detectable, level of VAMP-2 when compared with the 25% and 30% sucrose fractions per milligram of protein (Figure 1). By quantitative scanning of three individual experiments the relative distribution of VAMP-2 was 5:8:1 in fractions 25, 30 and 35 respectively. The results in Figure 1 also demonstrate that the VAMP-2 polypeptide is considerably more abundant in brain than in muscle. Unfractionated brain microsomes gave an approximately 30-fold higher signal of VAMP-2/mg of protein than did fraction 30, the purified skeletal muscle membranes richest in VAMP-2.

Proteins immunologically related to VAMP-1 and VAMP-2 have been detected in non-neuronal cells [6,7] but the isotype expressed in these cells was not determined given that the antibodies used to visualize them do not differentiate among the members of the VAMP family. Because the VAMP-2 isotype had previously been shown to be expressed only in neuronal cells and neuroendocrine cells, we explored the possibility that some, or all, of the VAMP-2 present in the muscle fractions might originate from ancillary tissues. The membrane fractions isolated from skeletal muscle were previously shown to be devoid of neuronal markers, based on lack of the $\alpha 3$ subunit of the Na⁺/K⁺-ATPase (Y. Mitumoto, H. Hundal and A. Klip, unpublished work) and of endothelial markers [17]. We further explored whether sciatic nerve contains VAMP-2 which might contaminate the muscle-derived fractions. Figure 1 (lane Ne) shows that total extracts of sciatic nerve do not contain VAMP-2 protein. This suggests that the VAMP-2 polypeptide present in the muscle membranes is likely to be of myocyte origin, and demonstrates that VAMP-2 is not present in motor nerve axons.

The above results, however, do not exclude formally the possibility that the VAMP-2 polypeptide detected in muscle-derived membrane fractions could originate from the presynaptic membrane of the neuromuscular endplate. Therefore, to investigate directly the expression of VAMP-2 in cells of muscle origin devoid of exogenous tissues we explored the presence of VAMP-2 in L6 muscle cells in culture. We have previously established that these clonal cells express a variety of muscle-specific proteins at the myotube stage, including myogenin, the sarcoplasmic reticulum Ca²⁺-ATPase, the GLUT4 glucose trans-

Figure 2 Assessment of the presence of VAMP-2 polypeptide in membranes from L6 myotubes and myoblasts

Purified light microsome (LM) and plasma membrane (PM) fractions from L6 myotubes (Mt) and myoblasts (Mb) were probed for $\alpha 1$ Na^+/K^+ -ATPase (a plasma membrane marker) and VAMP-2. Samples (20 μg of protein) of each fraction were resolved by SDS/PAGE on 13% polyacrylamide gels and transferred to PVDF filters. Guided by the migration of prestained molecular-mass markers (Bio-Rad), the filters were cut just below the expected molecular mass of the $\alpha 1$ - Na^+/K^+ -ATPase. The top piece was probed with anti-($\alpha 1$ - Na^+/K^+ -ATPase) monoclonal antibody McK1 and the bottom piece was probed with anti-(VAMP-2) antibody V2R. Brain microsomes (10 μg of protein) were run as a control for the 18 kDa VAMP-2 polypeptide. ^{125}I -labelled sheep anti-(mouse IgG) and ^{125}I -labelled Protein A were used to detect the anti-($\alpha 1$ Na^+/K^+ -ATPase) and anti-(VAMP-2) primary antibodies respectively.

Figure 3 Presence of cellubrevin polypeptide in brain microsomes, sciatic nerve extract and membranes from rat skeletal muscle and L6 muscle cells

The following membrane fractions were separated by SDS/PAGE using 14% polyacrylamide gels: brain microsomes, 40 μg of protein (Br); sciatic nerve extract, 40 μg of protein (Ne); skeletal muscle membrane fractions banding on top of 25%, 30% and 35% sucrose, 50 μg of protein each (lanes 25, 30, 35); L6 muscle cell plasma membranes (PM) and light microsomes (LM), 30 μg of protein each, from L6 myoblasts (Mb) or myotubes (Mt) (Notice that Mb samples precede Mt samples in this figure, while the reverse applies to Figure 2). After electrotransfer, PVDF filters were cut as described in Figure 2 and immunoblotted for $\alpha 1$ Na^+/K^+ -ATPase (top portion) or cellubrevin (bottom portion). Immunoreactive bands were detected by ^{125}I -labelled sheep anti-(mouse IgG) antibodies (top) or the enhanced chemiluminescence method (bottom) as outlined in the Experimental section.

porter and the voltage-sensitive Ca^{2+} channel [21–23]. Two clearly identifiable membrane fractions were analysed: one, banding on top of 32% sucrose, which is enriched in plasma membranes [19,20] but may also contain endosomes (PM fraction), and a second, light microsomal (LM) fraction, which has been shown to contain the donor pool of GLUT4 glucose transporters [19–21]. The fractions were further characterized in Figure 2 by the presence of the $\alpha 1$ subunit of the Na^+/K^+ -ATPase, which segregates with the PM fraction and whose content does not change during maturation from myoblasts to myotubes. Figure 2 demonstrates that whereas the VAMP-2 polypeptide was not visualized in either of the two membrane fractions derived from L6 myoblasts, it was clearly detectable in the membranes from

differentiated L6 myotubes. This suggests that the VAMP-2 polypeptide may fulfill some role essential for membrane traffic in myotubes but not in myoblasts. In this context, it is interesting to note that the insulin-sensitive intracellular membrane pool containing glucose transporters forms only at the myotube stage [21]. The VAMP-2 protein of L6 myotubes was largely concentrated in the PM fraction, with only a small amount present in the LM fraction when compared per milligram of protein. The relative proportions of VAMP-2 in the two fractions was found to be 0.24:1.0 (LM:PM) in four independent experiments. Interestingly, when the amount of VAMP-2 in mature skeletal muscle and in the muscle cells in culture was compared by analysing similar amounts of the corresponding plasma membrane fractions in the same gel, L6 myotubes were found to be a richer source of this polypeptide.

Muscle cellubrevin: subcellular distribution and expression during myogenesis

Cellubrevin is a 17 kDa polypeptide closely related to VAMP-2 but with wider tissue distribution. Using a cellubrevin-specific antibody which does not cross-react with VAMP-1 or VAMP-2, we demonstrate in Figure 3 cellubrevin immunoreactivity in isolated membranes of rat skeletal muscle, but interestingly not in crude brain microsomes. This is in contrast with VAMP-2, which was shown above to be more abundant in brain microsomes than in the muscle membranes. Given the rather impure nature of the brain microsomes compared with the purified muscle membranes, it cannot be ruled out that cellubrevin may be expressed in brain cells albeit at much reduced levels. Indeed, cellubrevin mRNA has been found in brain RNA [8]. In tissues where the cellubrevin polypeptide has been detected, this required extraction of the samples with Triton X-114 [8]. In contrast, the muscle membrane fractions used in the present study are of sufficient purity to allow detection without the need for detergent extraction. Results from three independent experiments showed that the relative distribution of cellubrevin in fractions 25:30:35 was 1.0:1.8:0.22. This distribution of the 17 kDa cellubrevin in skeletal muscle closely parallels that of the 18 kDa VAMP-2.

Figure 3 also shows that cellubrevin is expressed in membranes of L6 muscle cells in culture. As is the case for VAMP-2, cellubrevin was found to be highly concentrated in the PM fraction, and largely depleted from the LM fraction (the relative proportion in three separate experiments averaged 0.13:1.0 for LM:PM). However, in contrast with VAMP-2, cellubrevin was clearly detectable in L6 cells even at the myoblast stage (PM fraction), and its content further increased in the myotubes, where LM localization also became apparent. In addition, cellubrevin was found to be more abundant in membranes from L6 myotubes than in the cognate fraction of mature rat muscle (notice the difference in intensity of the bands in Figure 3, using 30 μg and 50 μg of protein of L6 and rat muscle membranes respectively).

The relative roles of VAMP-2 and cellubrevin within muscle cells remain to be determined, but it may be hypothesized that, as suggested by recent studies [2,8], each participates in specific membrane docking/fusion processes. Interestingly, although cellubrevin is present in hepatic endosomes, it does not appear to mediate endosome–endosome fusion assayed *in vitro* [30].

Susceptibility of VAMP-2 and cellubrevin to proteolysis by tetanus toxin

Both VAMP-2 and cellubrevin have been found to be specifically cleaved by the endopeptidase activity of the catalytic chain of

Figure 4 Effect of purified tetanus toxin light-chain on VAMP-2 and cellubrevin of rat skeletal muscle and L6 myotubes

Isolated membrane fractions from rat skeletal muscle (40 μ g of protein of fraction 25; 50 μ g of fraction 30) and from L6 myotubes (20 μ g of protein of fraction PM) were dried under vacuum and incubated with or without tetanus toxin light chain (TeTx-LC) as described in the Experimental section. The L6 myotube fractions were tested in the absence or presence of 0.5% Triton X-100 (tr). Immediately after the *in vitro* incubation, samples were boiled for 3 min in double-strength Laemmli sample buffer and analysed for the presence of VAMP-2 (top panel) or cellubrevin (lower panel) by SDS/PAGE in 13% and 14% polyacrylamide gels respectively, and immunoblotting with the corresponding antibodies. (a) Effect of 50 nM tetanus toxin on VAMP-2 of skeletal muscle and L6 myotube membranes. (b) Effect of 90 nM tetanus toxin on L6 myotube membranes. (c) Effect of 90 nM tetanus toxin on cellubrevin of skeletal muscle and L6 myotube membranes.

tetanus toxin [8,31]. To confirm that the polypeptides identified in our study by the VAMP-2-specific and cellubrevin-specific antibodies were indeed the corresponding antigens, we assessed the integrity of the immunoreactive bands following *in vitro* treatment of isolated membranes with tetanus toxin light chain. Figure 4 shows clear reductions in the amount of immunoreactivity with both the anti-VAMP-2 antibody and the anti-cellubrevin antibody in both skeletal muscle and L6 myotube membranes. Triton X-100 was used to facilitate hydrolysis of VAMP-2 and cellubrevin by the toxin. The detergent was previously used in studies *in vitro* of *Clostridium* toxin action [32] and may increase accessibility of the substrate to the enzyme. The Coomassie Blue staining pattern of other polypeptides of the membrane fractions in Figure 4 was not altered by treatment with the toxin (results not shown), indicating that there was no random hydrolysis of proteins by the light-chain enzyme. Figure 4(a) shows complete cleavage of the 18 kDa band recognized by the anti-VAMP-2 antibody in skeletal muscle by 50 nM tetanus toxin. Although cleavage was substantial in L6 myotubes, the remnant immunoreactive band could suggest the presence of an unknown, toxin-resistant isoform of VAMP. Figure 4(b) shows, however, that all immunoreactivity disappeared upon *in vitro* treatment of L6 myotube membranes with a higher concentration of the clostridial toxin (90 nM), demonstrating that all the immunoreactivity was toxin-sensitive and thus most likely to be VAMP-2. This concentration of the toxin also fully cleaved cellubrevin in both skeletal muscle and L6 myotubes (Figure 4c).

Test of expression of synaptophysin, SNAP25, syntaxin 1A/1B and synapsin Ia/Ib in skeletal muscle and L6 myotubes

To investigate whether muscle cells express other proteins thought to be required for synaptic vesicle fusion in nerve cells, we examined the presence in muscle and L6 myotube membranes of two other proteins involved in vesicle docking/fusion, i.e. SNAP25 and syntaxin 1A/1B, which are known to be membrane proteins of the presynaptic membrane. In addition, the presence of two synaptic vesicle proteins, synapsin I and synaptophysin, was also explored in the muscle membranes. Synapsin I is a synaptic vesicle-associated protein which becomes phosphorylated in response to agonist stimulation of neuronal cells [33], and has been proposed to mediate synaptic vesicle sequestration [33] or to inhibit excess neurotransmitter release by residual Ca^{2+} [34].

Figure 5 Examination of the presence of synapsin Ia/Ib, synaptophysin, syntaxin 1A/1B and SNAP25 in brain microsomes and purified membrane fractions from L6 myotubes and skeletal muscle

Samples of brain microsomes (5 μ g of protein) (Br) or of membranes (20 μ g of protein) from L6 myotubes (LM and PM fractions as indicated) and from rat skeletal muscle (fractions 25, 30 and 35) were subjected to SDS/PAGE on 13% polyacrylamide minigels, electrotransferred and subjected to immunoblotting as described in the Experimental section, using the primary antibodies indicated on the right-hand side. Synapsin Ia/Ib and syntaxin 1A/1B were detected with alkaline phosphatase-conjugated goat anti-(mouse IgG) antibodies. Synaptophysin and SNAP25 were detected with ^{125}I -labelled sheep anti-(mouse IgG) antibodies. The molecular mass of each protein is indicated on the left-hand side. In the immunoblot with anti-(synapsin Ia/Ib) monoclonal antibody, a non-specific band of approx. 75 kDa was also noted in the membrane fractions derived from rat skeletal muscle; this band was visualized even when the primary antibody was omitted (result not shown).

Synaptophysin is an integral membrane glycoprotein of synaptic vesicles, of no known function [35]. Figure 5 presents the detection of the 86/80 kDa polypeptides synapsin Ia/Ib, the 38 kDa polypeptide synaptophysin, the 36 kDa polypeptide doublet corresponding to syntaxin 1A/1B, and the SNAP25 polypeptide at 30 kDa. The results clearly show that all four proteins were readily detectable in unfractionated brain microsomes, but were undetectable in 20 μ g of protein of pure membrane fractions derived from either rat skeletal muscle or L6 myotubes. The combined results of Figure 5 confirm the neuronal expression of all these proteins, and together with the evidence from Figures 1–5 establish that VAMP-2 is the only synaptic vesicle protein, among those tested, which is expressed in skeletal muscle and muscle cells in culture.

Figure 6 Presence of VAMP-2 and cellubrevin transcripts in total RNA extracts of brain, muscle and L6 myotubes

Total RNA was extracted from rat brain, soleus muscle or L6 myotubes, and used on Northern blots to analyse the presence of transcripts to VAMP-1 (a), VAMP-2 (b), or cellubrevin (c). A single transcript of the size indicated by the arrow was detected in each case. Hybridization was carried out with specific cDNA probes as indicated in the Experimental section. Samples (20 μ g) of total RNA from muscle or L6 myotubes were loaded in each instance; for brain total RNA, 20 μ g were loaded in the VAMP-1 gel; 5 μ g in the cellubrevin gel and 1 μ g in the VAMP-2 gel.

Detection of VAMP-2 mRNA in mature muscle and muscle cells in culture

The results from Figures 1–5 strongly suggest that VAMP-2 and cellubrevin are expressed at the protein level in skeletal muscle and muscle cells in culture. This is the first demonstration of expression of these two members of the VAMP family in non-neuronal tissues, given the fact that the isoform expressed in fat cells has not been identified [6] and the isoform expressed in acinar cells is thought to be VAMP-1 [7]. The conclusion of our study is based on the isoform-specificity of the antibodies used, and on the susceptibility of the rat VAMP-2 and cellubrevin, but not of VAMP-1, to proteolysis by tetanus toxin. However, final demonstration of the expression of these gene products in skeletal muscle cells requires detection of their mRNA transcripts. Indeed, the use of cDNA probes on Northern blots is a more stringent approach than the use of antibodies on immunoblots.

Figure 6(a) shows that transcripts of VAMP-1 were clearly detectable in brain RNA but not in samples containing 20 μ g of skeletal muscle or L6 myotube RNA. In contrast with VAMP-1, the 2.4 kb transcript corresponding to the VAMP-2 gene was clearly detectable in 20 μ g of muscle RNA (Figure 6b). Total RNA of L6 myotubes also showed detectable levels of VAMP-2 mRNA. These results confirm the known absence of VAMP-1 from skeletal muscle [36] and reveal the expression of the VAMP-2 gene product in muscle RNA extracts and in differentiated muscle cells. Figure 6(c) shows that the 1.8 kb mRNA of cellubrevin was clearly detectable in skeletal muscle RNA and in L6 myotubes.

In conclusion, the present study demonstrates that both VAMP-2 and cellubrevin are expressed in skeletal muscle and muscle cells in culture. Both proteins increase substantially upon muscle cell differentiation, suggesting that they may participate in membrane traffic processes particular to the muscle phenotype. Whereas VAMP-2 is not expressed in myoblasts, cellubrevin is already present at this stage in muscle development. The peak level of expression of both proteins occurred in the myotube stage, possibly suggesting an important developmental role for the protein. Furthermore, the absence of SNAP25, synaptophysin, syntaxin 1A/1B and synapsin Ia/Ib indicates that the

molecular mechanisms controlling regulated exocytosis in muscle cells are different from those of neurons. The role of VAMP-2 and cellubrevin in mediating intracellular traffic events, and in particular in regulated membrane fusion, can now be examined. Studies are currently underway to evaluate the sensitivity of insulin-mediated recruitment of glucose transporters to tetanus toxin, in order to assess the possible contributions of VAMP-2 and cellubrevin to the process of regulated membrane traffic in muscle cells.

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