Expression of the Synaptic Vesicle Proteins VAMPs/Synaptobrevins 1 and 2 in Non-neural Tissues*

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The VAMPs/synaptobrevins (Vp/Sybs) are small integral membrane proteins. Two isoforms, Vp/Syb 1 and Vp/Syb 2, are considered to be specific to neural tissue. They are associated with synaptic vesicles and are believed to play an important role in neurotransmitter release. A third isoform, cellubrevin, has recently been found in non-neural tissues. We now report that the distribution of Vp/Syb 1 and Vp/Syb 2 is wider than previously thought. RNA transcripts for both Vp/Syb 1 and Vp/Syb 2 were found in rat skeletal muscle and in several other rat non-neural tissues, and antibodies specific for Vp/Syb 2 detected a protein in the endoplasmic reticulum-Golgi area of skeletal muscle. Thus Vp/Syb 1 and Vp/Syb 2 are not restricted to the nervous system but appear to be co-expressed with cellubrevin in many different tissues. This redundancy of Vp/Sybs in a single cell may be required to control the specificity of vesicle-target interaction in the several pathways of intracellular vesicle traffic that are operative within each cell.

A recent exciting development in biological chemistry has been the discovery that intracellular trafficking of vesicles, from yeast to neurons, apparently utilizes a common mechanism (1, 2). Several lines of research converged in this discovery. Neurobiologists had identified many proteins of nerve terminals (reviewed in Ref. 3) that are possibly involved in neurotransmitter release. Among these are synaptotagmin/p65 (4), synapsin (5), synaptophysin (6), and the VAMPs/synaptobrevins (7,8), all in synaptic vesicles, and SNAP-25 (9) and the syntaxins (10, 11) in the nerve terminal membrane. Independently, cell biologists identified NSF, the N-ethylmaleimide-sensitive fusion protein (12) and the SNAPs (small NSF attachment proteins) (13), which are cytoplasmic proteins required for the vectorial movement of vesicles from the ER to the Golgi complex and from the Golgi complex to the plasma membrane. Docking and fusion of vesicles was shown to involve the formation of a complex in which NSF and SNAPs bring together a vesicle protein and a target membrane protein. An assay looking for membrane components of this complex in brain (2) identified the VAMPs/synaptobrevins, syntaxins, and SNAP-25, thereby linking two different forms of trafficking: calcium-induced synaptic vesicle release and constitutive secretion.

VAMP/synaptobrevins are a family of small vesicle-associated proteins, independently discovered in Torpedo cholinergic vesicles (VAMP-1, Ref. 7) and in rat brain (synaptobrevin 1, Ref. 8). Molecular cloning (14) revealed that the two proteins were the product of homologous genes. Subsequently, a second gene from the same family (15, 16) was isolated and characterized. The importance of the Vp/Sybs in synaptic vesicle fusion has been emphasized by the discovery that they were the target of tetanus toxins (17), which block neurotransmitter release. Recent work (18) has suggested that specificity in vesicle-target docking is provided by protein-protein interactions between Vp/Sybs and syntaxins. Differential expression and subcellular localization of the different VAMPs and syntaxins may then be one of the most important aspects of intracellular traffic regulation, both at the synapse and in non-neural cells. The Vp/Sybs have a three-domain organization. A variable proline-rich N-terminal sequence of 28 amino acids is followed by a highly conserved central hydrophobic core of 69 amino acids and a hydrophobic sequence of 23 amino acids, presumed to be the membrane anchor. Vp/Sybs 1 and 2 were reported to be expressed only in nerve cells (8, 15, 19). A new member of the Vp/Syb family, cellubrevin, has been cloned recently (20) and appears ubiquitous in non-neural cells. Since Vp/Syb 1 and Vp/Syb 2 have different cellular distributions in the brain (15), it appeared that each cell type would express a single specific form of Vp/Syb.

As part of our ongoing work on the characterization of insulin and exercise-mediated translocation of GLUT4 in skeletal muscle, we are interested in the potential role of the Vp/Sybs. This report describes the detection of transcripts for Vp/Sybs 1 and 2, as well as cellubrevin, in skeletal muscle and several other tissues in the rat. Expression in muscle was confirmed by immunoblotting and immunofluorescence. Thus, in contrast to the current opinion, we show that the tissue distribution of Vp/Sybs 1 and 2 is wider than previously recognized and that several Vp/Sybs may be expressed in a single cell.

MATERIALS AND METHODS

RT-PCR and 5' RACE PCR—Total RNA from rat brown adipose cells was generously provided by Dr. Omatsu-Kanbe (National Institutes of Health). Total RNA was extracted from various rat tissues and white adipose cells (male Wistar rats, 300 g body weight) using a monophase solution of phenol and guanidine isothiocyanate (RNA STAT-60, Tel Test "B"). RNA (1 μg) was reverse-transcribed in a reaction mixture containing 8 units of RNAsin (Promega, Madison, WI), 20 μmol of dNTPs (Pharmacia Biotech), 100 μmol of DTT, 200 units of mouse Moloney leukemia virus reverse transcriptase (Life Technologies, Inc.), 100 pmol of random hexamers (Pharmacia), and the buffer provided with the enzyme, in a final volume of 10 μl at 37 °C for 1 h. The sample was then amplified with 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer) in a total volume of 100 μl with 50 μmol of dNTPs and 100 pmol of sense and anti-sense primers in 10 mM Tris-HCl, pH 8.5, 50 mM KCl, 2.5 mM MgCl2, and 200 μg/ml gelatin. The oligonucleotides used for PCR are described in Fig. 1. The cycling profile was 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, repeated for 30 cycles. The 5' RACE protocol (21) was performed with a kit (Life Technologies, Inc.), according to the manufacturer's instructions. Reverse transcription of 1 μg of RNA was primed with oligo(dT)12-18, and the

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1 VAMP, vesicle-associated membrane protein; NSF, N-ethylmaleimide-sensitive fusion protein; SNAP, small NSF attachment proteins; ER, endoplasmic reticulum; Vp/Syb, VAMP/synaptobrevin; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR, RACE, rapid amplification of cDNA ends.
cDNA was 5′-tailed with dCTP. Two successive rounds of PCR were then performed, each with 35 cycles at 94 °C for 1 min, 42 °C for 1 min, and 72 °C for 1 min. The first round used a commercial anchor primer that recognizes the 5′ tail and Vp/Syb-specific oligonucleotide 110. The second round, performed on 3 μl of the first amplification mix, used the anchor primer and oligonucleotide 122.

PCR products were ligated into the pT7-Blue T-vector (pT7T) (Novagen, Madison, WI) and transformed into Escherichia coli DH5α (Life Technologies, Inc.). Plasmid DNA was purified with the Magic (Wizard) Mini-prep system (Promega) and analyzed by DNA agarose gel electrophoresis following restriction digestion with XbaI and BamHI. Clones with an insert larger than 100 base pairs were retained for sequencing. DNA sequences were obtained by manual sequencing using Sequenase version 2.0 (U. Biochemical Corp.) or by automatic sequencing at the NINDS DNA Sequencing Facility.

Antibodies—Two rabbit sera raised against rat brain Vp/Syb 2 were generously provided by Dr. Trimbble (University of Toronto). The first one was prepared against a peptide common to all Vp/Sybs (see Fig. 1) and will be referred to as anti-Vp/Sybs. For immunofluorescence, we used an affinity-purified fraction, and for immunoblots, the crude serum. The second serum was raised against the N-terminal peptide of Vp/Syb 2 (see Fig. 1) and is specific for this isoform; it will be referred to as anti-Vp/Syb 2. The monoclonal mouse anti-rat transferrin receptor antibody (3F4) was purchased from American Type Culture Collection (Rockville, CA). GLUT4 was detected with an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to the C-terminal part of the transporter (22). Secondary antibodies were purchased from Cappel (Organon Teknika, Durham, NC).

Cell Extracts and Immunochemistry—Rat brain and soleus muscles were homogenized in buffer at pH 7.4 containing sucrose (210 mM), EDTA (5 mM), NaCl (40 mM), HEPES (30 mM), and phenylmethylsulfonyl fluoride (1.5 mM). Total crude membranes were obtained by centrifugation for 90 min at 50,000 rpm in a Beckman 70.1 Ti rotor. An enriched muscle membrane fraction was prepared as described (23). 8 μg of protein was electrophoretically separated on a 14% SDS-polyacrylamide mini-gel (Novex, San Diego, CA), electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and processed for immunoblotting. Enhanced chemiluminescence was used to visualize antigen-antibody complexes (Amersham Corp.).

Preparation and Staining of Single Muscle Fibers—Hindquarters of male Wistar rats (300 g) were fixed by perfusion with 2% depolymerized paraformaldehyde in phosphate buffer as described (24). Soleus fibers were blocked for 1 h in phosphate-buffered saline, rinsed with phosphate-buffered saline, and stained with a drop of Vectashield (Vector, Burlingame, CA). They were observed and photographed with a Zeiss Axioskop microscope.

RESULTS

When total RNA from rat soleus muscle was reverse-transcribed and amplified with the oligonucleotides 107–109, 108–110, and 107–110 that cover the conserved central sequence of the Vp/Sybs (see Fig. 1 for the sequence of the oligonucleotides), single bands were obtained that were identical in size to those obtained from rat brain RNA (Fig. 2a). Since the gene sequence corresponding to Vp/Syb 2 is in a different exon than that corresponding to the Vp/Sybs 108–109 and 110 (16), the observation of bands of the expected size rules out amplification of contaminating genomic DNA. In addition, no bands were observed if one of the oligonucleotides was omitted or in the absence of reverse transcription (not shown). PCR products were cloned into the vector pT7T, and single clones were sequenced. The sequence obtained was identical to that of Vp/Syb 1.

Next, we amplified rat soleus RNA with oligonucleotides 121–124 covering the less conserved 3′-terminal sequence of Vp/Syb 2. Again, a single band was obtained (Fig. 2b). Its sequence, obtained after cloning into pT7T, was identical to that of Vp/Syb 2.

Amplification of muscle or brain RNA with oligonucleotides corresponding to the very 5′-terminal sequence of Vp/Syb 2 (oligonucleotide 120) were unsuccessful. We therefore resorted to a 5′RACE protocol (21) in order to find the 5′ end sequences expressed in muscle (see “Materials and Methods”). No PCR product could be detected after the first round of amplification (Fig. 2c, lane 1), but there was one PCR product of approximately 320 base pairs with two minor bands (Fig. 2c, lane 2) after the second round. Southern blotting using labeled oligonucleotide 107 as probe confirmed that the PCR product resulted from amplification of Vp/Syb cDNA (not shown). PCR products were subcloned and sequenced. Of the 29 sequences that were read, 15 corresponded to cellubrevin, 4 to Vp/Syb 1, and 2 to Vp/Syb 2; all were identical to published sequences, both in the coding and in the 5′-untranslated regions. The remaining 8 sequences could not be identified. Thus it appears that mRNAs for SbVp1 and Sb/Vp2 are expressed in skeletal muscle.

In order to verify that our observation was not limited to one specific muscle fiber type, we probed total RNA from a different muscle, the red gastrocnemius, and from heart, white adipose
cells, brown adipose cells, liver, and kidney. RT-PCR experiments were performed with oligonucleotides 107 and 110 as primers. A single band was observed for each tissue tested (Fig. 3, a and b). The PCR product obtained after amplification of RNA from white adipose cells with oligonucleotides 107-110 was cloned and sequenced. A sequence identical to that of Vp/Syb 1 for the corresponding fragment was obtained. We did not obtain PCR products from other tissues.

To examine the expression of the Vp/Sybs in non-neural tissues at the protein level, we took advantage of the availability of antibodies with differential specificity. Extracts from rat brain and soleus, as well as membrane-enriched mixed muscle fractions (see "Materials and Methods") were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-Vp/Sybs (Fig. 4, lanes 1-3), or anti-Vp/Syb 2 (Fig. 4, lanes 4-6). In brain extracts (lanes 1 and 4), both antibodies gave a band of 18 kDa, corresponding to the molecular mass of Vp/Sybs 1 and 2. In crude muscle extracts (lanes 2 and 5), no reactivity was detectable with the film exposure shown here. In contrast, bands were present in the lanes containing an enriched muscle membrane fraction (lanes 3 and 6). Anti-Vp/Sybs gave two bands (lane 3); the weaker one migrated like brain Vp/Sybs, whereas the stronger one (16-17 kDa) had a mobility corresponding to cellubrevin (20). Anti-Vp/Syb 2 (lane 6) gave a single band that migrated like brain Vp/Sybs. We conclude that also at the protein level, both Vp/Syb 2 and cellubrevin are expressed in muscle. Since VAMP-1 specific antibodies were not available, we could not determine if that protein is expressed as well.

Intracellular localization of the epitopes recognized by the antibodies in muscle was obtained by immunofluorescence. Teased soleus fibers were stained with the two anti-Vp/Syb antibodies (Fig. 5, a and b) and, in parallel, with antibodies to the transferrin receptor and to the glucose transporter GLUT4 (Fig. 5, c and d). All four antibodies gave a basically similar pattern. In mature muscle fibers, which are large single multinucleated cells, the core of the fibers contains the myofibrils responsible for muscle contraction. Nuclei and most of the apparatus responsible for protein synthesis, ER and Golgi complex, are concentrated in a thin band of cytoplasm at the periphery of the fiber, just below the plasmalemma (25). The staining obtained with all four antibodies was mostly concentrated in that peripheral cytoplasm. There was a ring of staining around the nuclei (arrowheads on the figure), as well as punctate staining between the nuclei. There were differences, however, between the TfR and GLUT4 staining (Fig. 5, c and d), and the Vp/Syb staining (Fig. 5, a and b). The former antibodies showed a strong interrupted perinuclear staining and coarse dots between the nuclei, resembling a Golgi complex pattern, whereas the latter antibodies gave a smoother perinuclear staining and finer dots between nuclei, closer to an ER pattern (26). The perinuclear staining was weak for anti-Vp/Syb 2 (Fig. 5b). Although Vp/Sybs may partially overlap with TfR and GLUT4, they demonstrate a distinct distribution in skeletal muscle.

**DISCUSSION**

Several proteins essential for the formation of a vesicle docking complex were initially discovered in synaptic vesicles. Then it was found that they were members of large families with relatives in non-neural mammalian cells, *Drosophila*, and yeast. The Vp/Sybs were originally found in cholinergic vesicles in *Torpedo* (7) and in synaptic vesicles in mammals (8), but new results showed that there were Vp/Syb isoforms outside the nervous system. Antibodies to a conserved sequence present in all known Vp/Sybs recognized a protein enriched in the vesicles that carry the glucose transporter GLUT4 in rat adipocytes (27). In addition, some yeast proteins involved in ER to Golgi and in Golgi to plasma membrane transport were shown to be homologs of the Vp/Sybs (reviewed in Ref. 2). Cellubrevin, a new member of the Vp/Syb family recently found in non-neural cells (20), appeared to be the non-neural counterpart of Vp/Syb 1 and 2. This report demonstrates that Vp/Sybs 1 and 2 are also expressed in non-neural tissues. Transcripts of both Vp/Syb 1 and 2 were amplified by RT-PCR from RNA extracted from skeletal muscle and several...
other tissues. Chromosomal DNA or plasmid contamination were ruled out by scanning exon/intron boundaries and by running standard PCR controls. Muscle contains very few nerve cell bodies, and amplification of muscle RNA with oligonucleotides corresponding to syntaxin 1a gave no detectable product (not shown), making it unlikely that the PCR bands might result from amplification of contaminating neuronal RNA. Our results thus argue for the presence of Vp/Syb transcripts that might have been missed by the less sensitive Northern technique (15).

Mature muscle fibers are very highly organized cells, with a central core essentially dedicated to contraction, whereas most constituents of the biosynthetic pathway are concentrated in a thin layer of peripheral cytoplasm. In a crude muscle extract, the contribution of this layer of cytoplasm is small compared to that of the densely packed contractile proteins. This probably explains why no bands were obtained from crude muscle extracts in immunoblots with anti-Vp/Syb antibodies (Fig. 4, lanes 2 and 5) and why others failed to detect Vp/Sybs 1 or 2 in skeletal muscle (8). In contrast, when a fraction enriched in intracellular membranes was blotted, Vp/Sybs were easily detected. The antibody against a peptide common to all known Vp/Sybs produced two bands, one of 16 kDa, the molecular mass of cellubrevin, and one of 18 kDa, the molecular mass of Vp/Sybs 1 and 2. Interestingly, Cain et al. (27) observed two bands of molecular mass 16 and 18 kDa when they immunoblotted low density microsomes and plasma membrane fractions from adipocytes with anti-Vp/Syb antibodies.

We compared the distribution of the Vp/Sybs to that of TIR and GLUT4. TIR is a marker of the endosome pathway and recycles through the trans-Golgi (28), whereas GLUT4 stains the trans-Golgi and vesicles located at or near the plasma membrane (24, 29, 33). The report that Vp/Sybs were enriched in vesicles containing GLUT4 in adipocytes (20) suggested that GLUT4 and Vp/Sybs might have the same distribution. Although the general pattern of staining was similar for all four antibodies, a detailed examination revealed differences which suggest that at least a fraction of muscle GLUT4 may be associated with the Vp/Sybs.

All pathways of intracellular trafficking, both constitutive and regulated, seem to be operative in muscle. Although generally not considered a secretory tissue, muscle can show a robust and sustained constitutive secretion (30, 31). Translocation of GLUT4 from an intracellular pool to the surface of the cell after stimulation by insulin (24) clearly represents a case of regulated traffic. Moreover, quantal calcium-mediated release of injected acetycholine from Xenopus myocytes (32) shows that muscle is capable of nerve-like calcium-induced regulated secretion. Thus the difference in trafficking modes between neural and non-neural cells may be quantitative rather than qualitative, with all possible pathways represented in each cell but to various degrees. Different forms of Vp/Sybs may be involved in different pathways where they may contribute to the specificity of vesicle-target interaction (18), in which case several isoforms would be required in each cell. This raises the possibility that other as yet undiscovered Vp/Sybs may coexist with the already known isoforms.

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