Insulin-responsive tissues contain the core complex protein SNAP-25 (synaptosomal-associated protein 25) A and B isoforms in addition to syntaxin 4 and synaptobrevins 1 and 2

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SNAP-25 (synaptosomal-associated protein 25), syntaxin and synaptobrevin are the three SNARE [soluble NSF attachment protein receptor (where NSF = *N*-ethylmaleimide-sensitive fusion protein)] proteins that form the core complex involved in synaptic vesicle docking and subsequent fusion with the target membrane. The present study is aimed at understanding the mechanisms of fusion of vesicles carrying glucose transporter proteins with the plasma membrane in human insulin-responsive tissues. It describes the isolation and characterization of cDNA molecules encoding SNAP-25 A and B isoforms, syntaxin 4 and synaptobrevins (also known as vehicle-associated membrane proteins) from two major human insulin-responsive tissues, skeletal muscle and fat. The DNA and deduced amino acid sequences of SNAP-25 revealed perfect identity with the previously reported human neural SNAP-25 A and B isoforms. Our results indicate the presence of both isoforms both in insulin-responsive tissues and in *in vitro* cultured 3T3-L1 cells, but suggest a differential pattern of gene expression: isoform A is the major species in adipose tissue, and isoform B is the major species in skeletal muscle. The presence of SNAP-25 protein in 3T3-L1 cells was demonstrated by immunofluorescence microscopy using an anti-SNAP-25 monoclonal antibody. Immunoprecipitation experiments using the same monoclonal antibody also revealed the presence of SNAP-25 protein in plasma membrane fractions from rat epididymal fat pads. The syntaxin 4-encoding region from skeletal muscle contains five nucleotide differences from the previously reported placental cDNA sequence, two of which result in amino acid changes: Asp-174 to Glu and Val-269 to Ala. The synaptobrevin 1 cDNA from skeletal muscle contains two nucleotide differences when compared with the corresponding clone from neural tissues, one of which is silent and the other resulting in the amino acid change Thr-102 to Ala. The cDNA sequence of the protein from fat is identical with that of human synaptobrevin 1 from neural tissues. Furthermore, we have confirmed the presence of syntaxin 4 in fat and of synaptobrevin 2 in skeletal muscle by PCR amplification and Southern hybridization analysis. Using the yeast two-hybrid system, an interaction was observed between the full-length cytoplasmic domains of syntaxin 4 and synaptobrevin 2, a vesicle membrane SNARE previously shown by others to be associated with vesicles carrying the GLUT4 glucose transporter protein, but no interaction was seen with synaptobrevin 1. Flow cytometry of low-density microsomes isolated from fat cells was used to demonstrate the binding of syntaxin 4 to a subset of vesicles carrying GLUT4 protein; whereas SNAP-25 on its own bound poorly to these vesicles, the syntaxin 4–SNAP-25 complex gave a strong interaction.

INTRODUCTION

The SNARE hypothesis [1] proposes the existence of unique forms of vesicle membrane (v-) and target membrane (t-) SNAREs [soluble NSF attachment protein receptors (where NSF = *N*-ethylmaleimide-sensitive fusion protein)] to ensure docking and fusion of vesicles to the correct target membrane. Syntaxins, SNAP-25 (synaptosomal-associated protein 25) and synaptobrevins [also known as vehicle-associated membrane proteins (VAMPs)] are the three synaptic proteins that form the core complex around which the subsequent cascade of protein–protein interactions that control membrane fusion and exocytosis occurs [1,2]. During the docking phase the t-SNAREs syntaxin and SNAP-25 bind tightly to each other at the target membrane to form a high-affinity binding site for the v-SNARE, synaptobrevin/VAMP [2].

Multiple isoforms of each of these SNAREs have now been reported. Three isoforms of synaptobrevin have been identified in mammalian neural tissues that are involved in either regulated (VAMP-1, VAMP-2) or constitutive (cellubrevin) vesicular trafficking pathways [3]. Several isoforms of syntaxins have been analysed from the rat [4,5] and have been classified into five groups (syntaxins 1–5) with broad but differing tissue distributions [5]. The different syntaxin isoforms have been proposed to function in a highly specific manner at various stages of the secretory pathway within the cell and to be used in different tissues at particular stages of the pathway [5]. Only one SNAP-25 gene has been identified to date, and it is highly conserved in *Drosophila*, *Torpedo*, chicken, mouse and human. Unlike the synaptobrevins and syntaxins, SNAP-25 lacks a distinct transmembrane domain and is believed to be membrane-associated through post-translational palmitoylation of a quartet of cysteine

Abbreviations used: $DiIC_{18}(5)$, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; GST, glutathione S-transferase; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP-25, synaptosomal-associated protein 25; SNARE, soluble NSF attachment protein receptor; t-SNARE, target membrane SNARE; v-SNARE, vesicle membrane SNARE; VAMP, vesicle-associated membrane protein.

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residues [6,7]. There are two isoforms of SNAP-25, A and B, that are generated by alternative splicing of exons 5a and 5b and which differ at nine amino acids, including a slight variation in the arrangement of the cysteine quartet [8]. In SNAP-25 A, the cysteine residues occur at positions 84, 85, 90 and 92, while in SNAP-25 B the positions are 85, 88, 90 and 92. The two isoforms are differentially expressed in neural cells and are suggested to have distinct roles in the process of axonal outgrowth and exocytosis of neurotransmitters [9].

Functional homologues of different members of the SNARE family of proteins have been found in yeast, *Drosophila*, plants and different types of tissues in higher eukaryotes, suggesting conservation of common mechanisms for secretion [5,8,10–18].

In insulin-responsive tissues such as adipocytes and skeletal muscle, a major response to insulin is the translocation of vesicles containing the glucose transporter protein GLUT4 from the cytoplasm to the plasma membrane [19–24]. The first evidence to suggest that homologues of the neural synaptic proteins may be involved in insulin-mediated regulation of glucose transport was the immunological detection of synaptobrevins in GLUT4-enriched vesicles from rat adipocytes [11]. Since then, the presence of synaptobrevin in insulin-responsive tissues has been confirmed immunologically in the mouse adipocyte 3T3-L1 cell line [21], rat skeletal muscle and the rat L6 muscle cell line [22,23]; by PCR analysis of mRNA from rat adipose tissue and rat skeletal muscle [22]; and by Northern blot analysis in rat skeletal muscle and the rat L6 muscle cell line [23].

Northern analysis has indicated that syntaxin 4 is the predominant isoform in rat skeletal muscle [5,24], although syntaxins 2 and 5 are also present [5]. Syntaxin 4 mRNA has also been identified in the rat L6 muscle cell line by Northern analysis, confirming that it is a genuine muscle cell transcript and is not derived from muscle-associated neural or endothelial tissue [24]. In addition, syntaxin 4 protein has been detected in membrane preparations of rat skeletal muscle and L6 muscle cells using an affinity-purified antibody raised against the cytoplasmic region (residues 1–274) of rat syntaxin 4 [24].

The only report of SNAP-25 in non-neural tissues of mammals is the detection of SNAP-25 mRNA and protein in isolated pancreatic islets of Langerhans [15], suggesting that exocytosis of insulin-containing granules also involves similar processes. SNAP-25 protein was undetectable in membrane preparations from rat skeletal muscle and L6 myotubes [23].

As part of a study aimed at increasing our understanding of the mechanism of insulin-induced GLUT4 vesicle docking and fusion, the present paper describes the identification, cloning and characterization of cDNA molecules encoding SNAP-25 A and B isoforms isolated from human fat and skeletal muscle cDNA and from the mouse 3T3-L1 adipose cell line. The presence of syntaxin 4 mRNA in skeletal muscle and of synaptobrevin 1 in skeletal muscle and fat tissues has been confirmed by PCR, cDNA cloning and sequence analysis, while mRNAs for syntaxin 4 in fat and synaptobrevin 2 in skeletal muscle have been amplified by PCR and confirmed by Southern hybridization. The interaction of syntaxin 4 with synaptobrevin 1 and 2 proteins *in vivo* in yeast and the interaction of recombinant syntaxin 4 and SNAP-25 proteins (both singly and in combination) with low-density microsomes consisting of vesicles carrying GLUT4 protein have also been investigated.

MATERIALS AND METHODS

Cell culture

3T3-L1 fibroblasts obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) were maintained and passaged as preconfluent cultures in Dulbecco's modified Eagle's medium (DMEM) (Sigma) with 10 % (v/v) newborn calf serum (CSL Ltd.). Cells for differentiation were maintained at confluence for 48 h, then induced to differentiate by the addition of DMEM containing 5 % (v/v) foetal calf serum (FCS) (CSL Ltd.), 4 μg/ml insulin, 0.25 mM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine. After 72 h, induction medium was replaced with fresh FCS/DMEM containing 4 μg/ml insulin. After differentiation (during which > 90 % of fibroblasts differentiated into mature adipocytes), the cells were washed with PBS, pH 7.4, before preparation of RNA by standard techniques. PC12 cells were maintained and passaged in DMEM containing 10 % horse serum and 5 % FCS. They were stimulated to form neurite outgrowths by culture in GMEM containing Monomed (CSL Ltd.) and 10 ng/ml nerve growth factor 2.5 S (Sigma).

Preparation of template DNA

Poly(A)$^+$ RNA from human skeletal muscle (Clontech) was used as the template to make a cDNA pool using reverse transcriptase. DNA from a human fat cell λgt11 library (Clontech; human fat cell 5′-stretch plus library; HL3016b) was isolated by using standard procedures [26] to produce template DNA for PCR analysis. For some experiments, the total λgt11 library inserts were amplified by PCR from purified DNA using specific forward and reverse primers. To produce cDNA template from 3T3-L1 adipocytes, total RNA (600 μg) was isolated from approx. 0.7 ml of packed, differentiated 3T3-L1 cells using guanidinium thiocyanate/phenol (Trisol reagent; Gibco BRL). Traces of genomic DNA were removed by treatment with RNase-free DNase (Promega). DNA was synthesized from 3T3-L1 RNA using oligo(dT) as the primer and reagents from the Strata Script kit (Stratagene).

Isolation and characterization of cDNA clones

DNA sequences of oligonucleotides used in PCR and Southern hybridization experiments are shown in Figure 1. The PCR primer combinations used for cloning SNAP-25 were: (i) oligonucleotides N2903 (forward; nt 1–24) and N2905 (reverse; nt 594–621) to identify and clone the full-length SNAP-25-encoding region; (ii) oligonucleotides N3187 (forward; nt 171–189) and N3188 (forward; nt 171–189), each in combination with the reverse primer N2905, to distinguish isoforms A and B respectively; (iii) oligonucleotide N3063 (forward; nt 28–49), which starts from the 28th base downstream of the initiation codon, with just one base change between human and mouse SNAP-25 sequences, was used in combination with N2905 (reverse) to identify the presence of SNAP-25 in 3T3-L1 cells, and PCR amplifications using N3187 and N2905 or N3188 and N2905 were carried out for identification of the A and B isoforms respectively.

The PCR primers used for cloning syntaxin 4 were N2872 (forward; nt 1–27) and N2873 (reverse; nt 867–894), based on the syntaxin 4 gene sequence from human placenta [25]. The PCR primers used to amplify synaptobrevin 1 from the muscle cDNA were N2804 (forward; nt 2–23) and N2853 (reverse; nt 307–331). The PCR primers used for synaptobrevin 1 amplification from the fat library insert DNA were N2869 (forward; nt 1–23) and N2870 (reverse; nt 307–354). The PCR primers used to amplify the total cytoplasmic domain of synaptobrevin 2 from human muscle cDNA were N2963 (forward; nt 1–21) and N2961 (reverse; nt 324–348), and those used to amplify exons II and III were N2963 (forward; nt 1–21) and N2806 (reverse; nt 266–282).

The conditions for PCR amplification of SNAP-25 and syntaxin 4 using *Pfu*I enzyme were typically as follows: 1 cycle at

SNAP-25	
N2903/FWD	5′ATGGCCGAAGACGCAGACATGCGC3′
N2904/FWD	5′TCAGGACGGAGTGGTGGCCAGCCA3′
N2905/REV	5′TTAACCACTTCCCAGCATCTTTGTTGC3′
N3061/FWD	5′GCACCCGTCGTATGCTGCAACT3′
N3062/FWD	5′TGAGGAAGGGATGGACCAAATC3′
N3063/FWD	5′GAGCTGGAGGAGATGCAGCGAA3′
N3187/FWD	5′CGATCGTGTCGAAGAAGGC3′
N3188/FWD	5′GGAACGCATTGAGGAAGGG3′
SYNTAXIN 4	
N2872/FWD	5'ATGCGCGACAGGACCCACGAGCTG3′
N2873/REV	5'TTATCCAACCACTGTGACGCCAATGAT3'
N2875/INT	5'CAGCATGGGGTCCTGTCCCAGCAA3'
N3122/INT	5'GGGCTCCCCAGCCGTGCCGT3'
SYNAPTOBREVIN 1	
N2800/REV	5′AGTAAAAAAGTAGATTACAATAAC3′
N2804/FWD	5′GTCTGCTCCAGCTCAGCCACC3′
N2806/REV	5′CTTGCAGTTTTTCCACC-3′
N2819/REV	5′CCAGGACCTTGTCCACGTTC3′
N2853/REV	5′TACCACGATGATGGCACAGATGGC3′
N2869/FWD	5′CGTAGGATCCATGTCTGCTCCAGCTCAGCCACC3′
N2870/REV	5′AGCCTAGAATTCAGTAAAAAAGTAGATTACAATAAC TACCACGATGATGGCACAGATGGC3′
N2973/REV	5′ATGCTGAATTCTCATTACTTCAGGTTTTTCCACCAATACTTCC3′
N3110/FWD	5′ GCATTCATATGTCTGCTCCAGCTCAGCC3′
N3112/REV	5′ TTATGCATATGTCATTACTTGCAGTTTTTCCACC3′
N2328/INT	5′GTGAACGTGGACAAGGTCCT3′
SYNAPTOBREVIN 2	
N2806/REV	5′CTTGCAGTTTTTCCACC-3′
N2961/REV	5′AGAGCTGAAGTAAACTATGATGATG-3′
N2963/FWD	5′ATGTCTGCTACCGCTGCCACG-3′
N3351/FWD	5′ACATTCATATGTCGGCTACCGCTGCCACC 3′
N3245/REV	5′TTGTGCATATGTCATTACTTGAGGTTTTTCCACCAG 3′

Figure 1 Oligonucleotide sequences

FWD, forward; REV, reverse.

94 °C for 5 min; denaturation at 94 °C for 1 min; annealing at 60 °C for 1 min; and extension at 72 °C for 1 min (40 cycles), followed by continuation of incubation for 3 min at 72 °C (one cycle). For cloning, multiple PCRs were performed in 10 separate tubes and the final reaction tubes were mixed and cloned into pCR-Script(SK+) or pBluescript(SK+) phagemids (Stratagene). The conditions for PCR amplification of synaptobrevins 1 and 2 were 45 s each at 95 °C, 50 °C and 72 °C (30 cycles). Plasmid DNA was isolated according to standard procedures [26] and sequenced [27] using the PCR primers, vector primers and primers internal to each gene.

Restriction enzyme analysis, [γ-^{32}P]ATP end-labelling of PCR fragments and oligonucleotides, and Southern hybridization were carried out according to standard procedures [26]. Radiolabelled oligonucleotides N2904 (nt 324–347), N3122 (nt 91–110) and N2328 (nt 1143–1162) [28], specific to the internal regions of SNAP-25, syntaxin 4 and synaptobrevins 1/2 respectively, were used as probes in separate Southern hybridization experiments. Sequence alignment was carried out using the software package of the Genetics Computer Group.

Cloning and expression of syntaxin 4 and SNAP-25 in *Escherichia coli*

The PCR fragments encoding the cytoplasmic domain of syntaxin 4 (from a skeletal muscle cDNA pool), and full-length SNAP-25 (from a fat cDNA library), were engineered to contain *Bam*HI and *Eco*RI at their 5′ and 3′ ends respectively, and were cloned into the *Bam*HI–*Eco*RI sites of the expression vector pGEX2T. *E. coli* strains XL1Blue (Stratagene), DH5α or BL21(DE3) were transformed and clones were selected on ampicillin-containing agar plates. Recombinant GST fusion protein was purified from transformants by glutathione–Sepharose beads using standard procedures [29].

Analysis of syntaxin 4 and GLUT4 vesicle interactions by flow cytometry

Fat cells were isolated from rat epididymal fat pads by collagenase digestion [30] in modified Krebs–Ringer bicarbonate/Hepes buffer, pH 7.4 (containing 1.15 mM Ca^{2+}, 3% BSA and 5 mM glucose). The cells were homogenized, and low-density microsomes were prepared by differential centrifugation as described in [31–33]. For immunostaining and protein interaction studies, 30 μl of microsomal suspension containing approx. 7.2 μg of protein was placed in a total volume of 150 μl of HES buffer (20 mM Hepes, 1 mM EDTA, 250 mM sucrose, pH 7.4) with 1% BSA. Proteins (4 μg) biotinylated by the method described in [34] were added with or without purified anti-GLUT4 monoclonal antibody 1F8 (1.4 μg of protein) [35]. The suspension was incubated at 4 °C overnight and then centrifuged at 100000 ***g*** for 60 min in a Beckman TL100 ultracentrifuge. The pellets were washed in 150 μl of HES buffer and then resuspended in 150 μl of HES/BSA. To detect 1F8 binding, 2 μl of fluorescein isothiocyanate (FITC)-labelled anti-mouse immunoglobulin (Silenus, Melbourne, Australia) was added, and to detect the binding of biotinylated protein, 2 μl of streptavidin–phycoerythrin conjugate (Dako, Carpinteria, CA, U.S.A.) was added. Tubes were incubated overnight at 4 °C, centrifuged as before and the pellet resuspended in 180 μl of HES buffer. Resuspension of the microsomes was ensured by a 1 s sonication with a Microson sonicator (Heat Systems, Farmingdale, NY, U.S.A.) using a 3/16 inch (4.7 mm) probe at the minimum power setting. A membrane-selective dye, $DiIC_{18}(5)$ (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate; Molecular Probes, Eugene, OR, U.S.A.), was added to 5 μM and the microsomes were analysed by flow cytometry on a Coulter EPICS cytometer with excitation at 488 nm for fluorescein and phycoerythrin, and at 633 nm for $DiIC_{18}$. Fluorescein emission was measured at 525 nm, phycoerythrin emission was measured at 575 nm and $DiIC_{18}(5)$ fluorescence was measured at 675 nm. Microsomes were identified as distinct from instrument and solvent noise by a combination of light scatter and $DiIC_{18}$ fluorescence, and all data were gated on the microsomal population as indicated by these parameters.

Analysis of syntaxin 4 and synaptobrevin protein interactions using the yeast two-hybrid system

Experiments to test for protein interactions were carried out using the MATCHMAKER Two-Hybrid System kit (Clontech).

Briefly, the DNA fragments encoding the cytoplasmic domains of synaptobrevins 1 and 2 and syntaxin 4 were cloned into yeast two-hybrid vectors as follows. The clone for the human synaptobrevin 1 cytoplasmic domain was modified using primers N3110 (forward) and N3112 (reverse) to contain an *Nde*I site; the product was cleaved with *Nde*I and then cloned into the *Nde*I site of vector pAS2 (Clontech). The synaptobrevin 2 cytoplasmic domain (from rat brain; a gift from Dr. R. Scheller and Dr. D. E. James) was generated using primers N3351 (forward) and N3245 (reverse) and cloned into pAS2 by the same procedure. Constructs were sequenced to verify the integrity of the clones. The DNA fragment encoding the cytoplasmic domain of syntaxin 4 from human skeletal muscle was subcloned as a *Bam*HI to *Sal*I fragment from pBluescript II SK(+) phagemid into the GAL4 activation domain fusion vector pGAD424. Yeast (*Saccharomyces cerevisiae*) strain CG1945 was transformed with combinations of either synaptobrevin 1 or 2 cytoplasmic domains and either pGAD424/syntaxin 4 cytoplasmic domain or vector pGAD424 as a control, and interactions were analysed by measuring the activation of the β-galactosidase reporter gene [36,37].

Metabolic labelling and immunoprecipitation

[^{35}S]-Trans label (ICN Biomedicals) ([^{35}S]methionine/[^{35}S]-cysteine 70:15, mol/mol) was added to cells in cysteine- and methionine-free DMEM containing 0.5% FCS and incubated overnight (12–14 h). Cells were washed three times in PBS, then lysed by the addition of 800 μl per dish of 50 mM Hepes buffer, pH 7.2, containing 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 200 units/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin and 2 mM PMSF. Dishes were incubated for 5 min on ice, and the lysate was collected and incubated for a further 5 min on ice, then clarified by centrifugation at 12500 ***g*** for 5 min. The supernatants were then immunoprecipitated by dilution (1:3, v/v) in 20 mM Hepes buffer, pH 7.2, containing 150 mM NaCl, 10% glycerol and 0.1% Triton X-100 (HNTG buffer), and incubation with 4 μl of anti-SNAP-25 (SMI 81; Sternberger Monoclonals Inc.) or 4 μl of rabbit serum (control) and 20 μl of Protein A–Sepharose (Sigma) slurry overnight at 4 °C. The Sepharose beads were pelleted by centrifugation, washed three times in HNTG buffer and analysed following PAGE by autoradiography of gels incubated with Amplify (Amersham). In experiments where fat cell membrane lysates were examined, 1.4 mg of plasma membranes purified from rat epididymal fat pads was solubilized in HNTG buffer containing 1% Triton X-100 for 10 min and the supernatant was clarified by centrifugation at 10000 ***g*** for 5 min at 4 °C, prior to immunoprecipitation as detailed above. Immunoprecipitates were analysed, after PAGE and transfer of proteins to nitrocellulose, by blotting with anti-SNAP-25 antibody (SMI 81) and ECL (Amersham) detection.

Immunofluorescence labelling

For immunofluorescence labelling, cells were grown on glass coverslips, washed in PBS, fixed in 2% paraformaldehyde, quenched with 100 mM glycine/PBS, permeabilized in 1% Triton X-100, incubated with primary antibody overnight [anti-SNAP-25 (SMI 81) or anti-GLUT4 (R1159 [33]; a gift from Dr. J. Proietto, University of Melbourne, Australia) at a 1:100 dilution] and detected with FITC-conjugated second antibody (1:1000 dilution; Silenus, Hawthorn, Victoria, Australia). Cells were washed in PBS and fixed in 25 mg/ml 1,4-diazabicyclo[2.2.2]octane (Sigma) in 90% glycerol. Analysis was performed by confocal microscopy using a Leitz Ortholux II microscope (40 × objective) and fluorescence was observed with a Bio-Rad Lasersharp confocal imaging system.

RESULTS

Cloning and characterization of SNAP-25

Using one set of oligonucleotides [N2903 (nt 1–24) and N2905 (nt 594–621)] as forward and reverse primers respectively, and cDNA pools from human skeletal muscle and fat tissues as templates, the PCR generated very low amounts of a 0.62 kb product from both reactions. However, using nested forward primers at the 5′ end [N3063 (nt 28–49), N3061 (nt 83–104), N3062 (nt 180–201) and N2904 (nt 324–347)] and the same reverse primer, N2905, PCR products of the expected sizes (594, 539, 443 and 298 bp respectively) were generated. In Southern hybridization analysis, N2904, a primer specific to the internal region of human SNAP-25, hybridized to all fragments (Figures 2a and 2b). Longer exposure of the blots clearly showed the presence of the full-length (621 bp) SNAP-25 DNA fragment. PCR amplifications were carried out in multiple tubes using the two extreme-end primers (N2903 and N2905) to produce large amounts of the full-length 621 bp fragment from both fat and skeletal muscle. The DNA fragments were separately cloned into pCR-Script SK(+) phagemid and subjected to DNA sequence analysis. Sequencing of two clones each indicated that both clones from fat cells were isoform A, while both clones sequenced from skeletal muscle were isoform B (results not shown). The

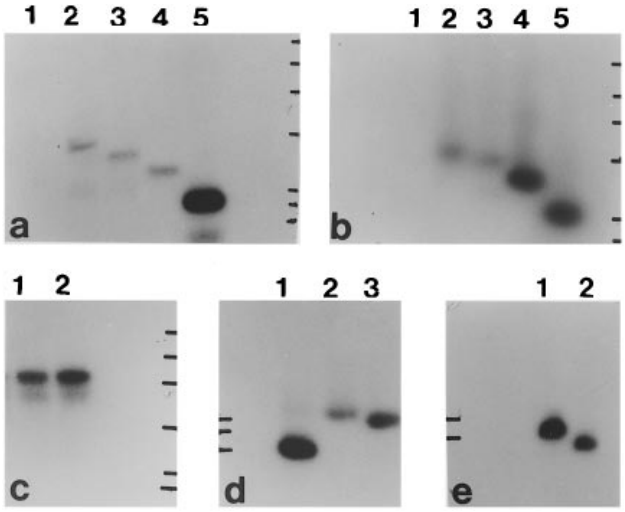

Figure 2 Southern hybridization analysis

Nitrocellulose blots carrying DNA fragments generated by PCR were probed with radiolabelled primers: (**a**) and (**b**) N2904 (SNAP-25); (**c**) N3122 (syntaxin 4); (**d**) and (**e**) N2328 (a common region for both synaptobrevins 1 and 2). In (**a**) (fat) and (**b**) (skeletal muscle), lanes 1–5 represent products generated from PCR amplifications using nested 5′ forward primers (N2903, N3063, N3061, N3062 and N2904 respectively) and a common reverse primer, N2905. In (**c**), lanes 1 and 2 represent syntaxin 4 PCR products using fat and skeletal muscle cDNA templates respectively. In (**d**) (skeletal muscle), lanes 1, 2 and 3 represent synaptobrevin 1 PCR products obtained using the common forward primer N2804 and reverse primers N2819, N2853 and N2806 respectively. In (**e**) (skeletal muscle), lanes 1 and 2 represent synaptobrevin 2 PCR products obtained using the common forward primer N2963 in combination with the reverse primers N2961 and N2806 respectively. The positions of the ØX174 DNA *Hae*III size standards are indicated on the right margins of (**a**)–(**c**) or on the left margins of (**d**) and (**e**). From top: (**a**) 1.35, 1.07, 0.87, 0.60, 0.31, 0.28/0.27, 0.23 kb; (**b**, **c**) 1.35, 1.07, 0.87, 0.60, 0.31, 0.28/0.27 kb; (**d**) 0.31, 0.28/0.27, 0.194 kb; (**e**) 0.31, 0.28/0.27 kb.

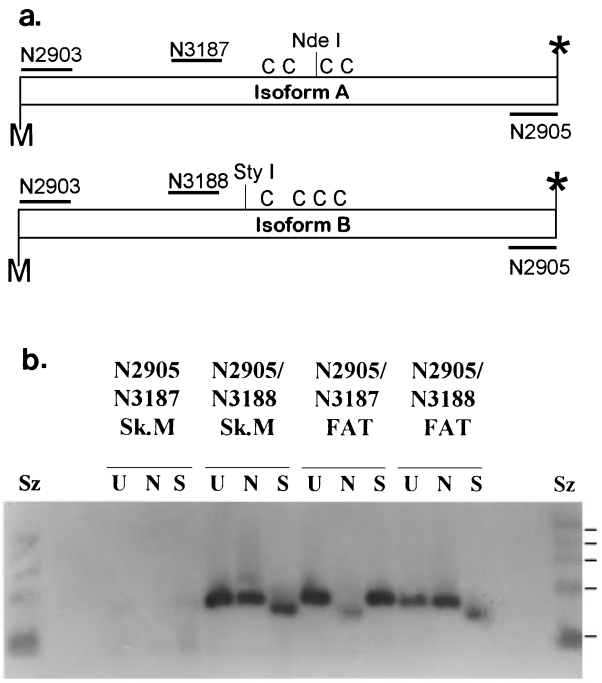

Figure 3 Analysis of SNAP-25

(**a**) Schematic drawing of SNAP-25 sequences showing the location of the cysteine quartet regions of the A and B isoforms, to indicate unique restriction enzyme sites and regions corresponding to the forward primers N2903, N3187 and N3188 and the reverse primer N2905. (**b**) Autoradiogram showing differences in mobility of radiolabelled DNA fragments generated from isoform A- and isoform B-specific PCR, digested with the restriction enzymes *Nde*I and *Sty*I. Lanes U, N and S correspond to uncut, *Nde*I-cut and *Sty*I-cut DNA fragments respectively; Sz refers to ØX174 DNA *Hae*III size standards (from top: 1.35, 1.07, 0.87, 0.60, 0.30/0.28/0.27 kb).

sequences were identical to the previously reported SNAP-25 isoforms from human neural tissues [16,38].

Alternative splicing of the SNAP-25 gene generates cDNA molecules which differ within the region encoding residues 58 and 89, with unique restriction enzyme sites for the two isoforms (Figure 3a). Plasmid DNA from transformed *E. coli* cells was isolated and tested for the presence of the unique restriction enzyme site specific for each isoform. All 16 clones from fat tissue were SNAP-25 isoform A (cut once with *Nde*I, a unique site in the whole construct) and 43 clones from skeletal muscle were SNAP-25 isoform B (uncut with *Nde*I), suggesting the prevalence of one isoform in each tissue. Analysis of the PCR-amplified products generated by using the same set of primers (N2903/N2905) common to both isoforms indicated the prevalence of isoform A in fat and isoform B in skeletal muscle.

To further investigate the apparent presence of just one isoform in each tissue, PCR reactions involving the forward primers N3187 (isoform A-specific) and N3188 (isoform B-specific, with seven out of 19 nucleotides being different from N3187) (Figures 1 and 3a), and the common reverse primer (N2905), were performed on cDNA pools from both tissues (four reactions). The PCR was extended to 50 cycles to increase the probability of detecting the second isoform in each tissue. Analysis of the PCR products on an agarose gel (results not shown) indicated that there was a product of the expected size (approx. 456 bp) in all four reactions, suggesting the presence of both isoforms in both tissues. However, with the fat cDNA as template, the intensity of the band resulting from the isoform A primer was greater than that of the band resulting from the isoform B primer (results not shown). Similarly, the results from PCR using the cDNA pool from skeletal muscle as template indicated a strong bias towards the B isoform.

To examine whether the isoform-specific PCR products were generated by heterologous annealing of the forward primer used, the 456 bp DNA fragment was purified from agarose gels, end-

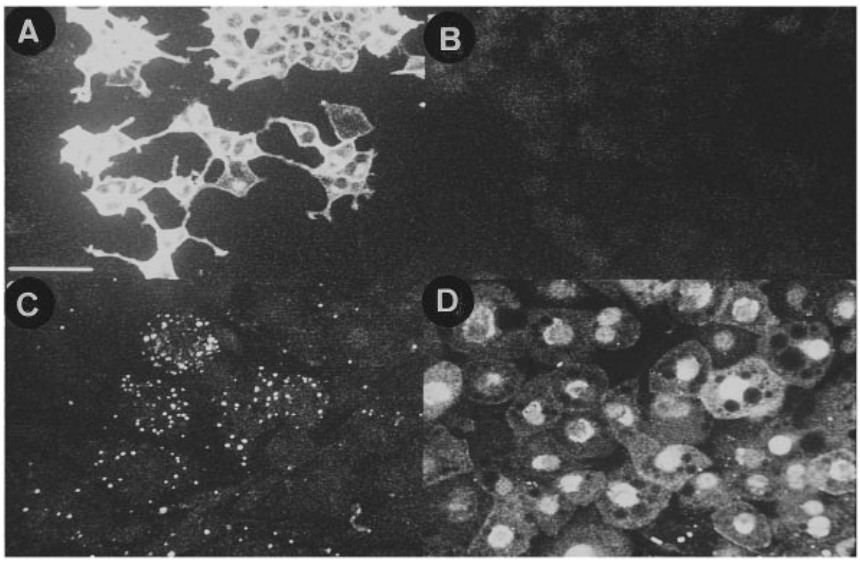

Figure 4 Immunolocalization of SNAP-25 and GLUT4 in 3T3-L1 and PC12 cells

PC12 cells (**A** and **B**) or 3T3-L1 cells (**C** and **D**) were fixed, permeabilized and labelled with antibody specific for SNAP-25 (SMI 81) (**A** and **C**) or the C-terminus of GLUT4 (R1159) (**B** and **D**) each at a 1:100 dilution, followed by FITC-conjugated sheep anti-rabbit or anti-mouse second antibody, as appropriate, at a 1:1000 dilution. Representative fields of cells were analysed by confocal microscopy. The bar represents 50 μm.

labelled with radioactive ATP and digested with *Nde*I or *Sty*I, which are unique to isoforms A and B respectively. The fragments were separated on an agarose gel and autoradiographed. The results (Figure 3b) indicate a lack of heterologous priming. For example, using fat cDNA as template and isoform A-specific N3187 as the forward primer and N2905 as the reverse primer, the PCR generated a 456 bp product that was cut only with *Nde*I and not with *Sty*I (Figure 3b), indicating that the PCR product was an isoform A-specific fragment of SNAP-25. Similarly, using the same DNA template and the reverse primer, but the isoform B-specific primer N3188, the PCR generated a 456 bp fragment that was cut with *Sty*I but not *Nde*I (Figure 3b), indicating that the PCR product was an isoform B-specific fragment of SNAP-25. Similar results were obtained using skeletal muscle cDNA as template, confirming the presence of both isoforms in both tissues. It remains to be investigated if the differential pattern indicated by the amount of transcripts determined by the earlier PCR experiments and subsequent analysis of the PCR products is reflected at the protein level. Antibodies specific to each of the two isoforms would be essential to distinguish between the two and to obtain their respective distributions in fat and skeletal muscle.

Detection of cDNAs for the two SNAP-25 isoforms in cultured 3T3-L1 adipocytes

To demonstrate that the origin of SNAP-25 transcripts identified by PCR was not due to contamination from neural or endothelial cells, cultured murine 3T3-L1 adipocytes were used to detect the presence of SNAP-25. Since there are some sequence differences between the human and murine SNAP-25 encoding regions, particularly at the 5′ end, the forward primer N3063 (nt 28–49), with just one base change between human and mouse sequences, was used with the same reverse primer (N2905). The PCR generated a fragment of the expected size (594 bp) which hybridized to a radiolabelled internal primer, indicating the expression of SNAP-25 in 3T3-L1 adipocytes cultured *in vitro*. The PCR reactions using the isoform-specific forward primers (N3187 and N3188) and the same reverse primer (N2905) revealed that the A isoform of SNAP-25 was also predominantly expressed in 3T3-L1 adipocytes, consistent with the previous results with the fat cDNA library. Partial sequencing of cloned DNA fragments from both reactions confirmed the identity of the two isoforms.

Detection of SNAP-25 protein in 3T3-L1 adipocytes

The presence of SNAP-25 protein in 3T3-L1 cells was investigated using a monoclonal antibody to SNAP-25, SMI 81 (Sternberger Monoclonals). Differentiated PC12 cells, which display a neural phenotype, were used as a positive control. Two approaches were taken: direct labelling of fixed cells and immunoprecipitation of SNAP-25 from cell lysates (see Figures 4 and 5). Labelling of fixed PC12 cells with the SNAP-25 monoclonal and FITC-labelled second antibody was marked (Figure 4A), especially at the cell margins, consistent with a predominant plasma membrane location. In contrast, only very faint fluorescence was seen in 3T3-L1 cells (Figure 4C). This pattern was diffuse within the cells and not uniform throughout the cell population. Cells were also labelled with a polyclonal antibody to GLUT4, R1159 [33] (Figures 4B and 4D). In this case, PC12 cells were not labelled at all (Figure 4B), whereas 3T3-L1 cells showed marked labelling around the nucleus (consistent with a perinuclear location) and at the cell margins in insulin-stimulated cells, consistent with a plasma membrane localization (Figure 4D).

The anti-SNAP-25 monoclonal antibody was also used to immunoprecipitate SNAP-25 from [^{35}S]methionine metabolically labelled PC12 and 3T3-L1 cells (Figure 5A). A protein of approx. 25 kDa, consistent with the reported molecular mass of SNAP-25, was seen in PC12 extracts. This protein was not detected in extracts from 3T3-L1 cells. Since immunofluorescence (Figure 4) indicated the presence of a low level of SNAP-25 in 3T3-L1 cells, an alternative approach was used to investigate the presence of SNAP-25 in fat cells. Purified plasma membranes from large-scale rat fat cell fractionation (1.4 mg in total) were solubilized in 1% Triton X-100. The solubilized extract was then immunoprecipitated with anti-SNAP-25 antibody, separated by PAGE, transferred to a nitrocellulose membrane and analysed by ECL (Amersham) using the same anti-SNAP-25 antibody (Figure 5B). The Triton X-100-insoluble pellet was also analysed. A band of molecular mass approx. 25 kDa was seen in the solubilized extract, clearly indicating the presence of SNAP-25 in the plasma membrane of rat adipocytes. These results are consistent with a very low level of expression in fat cells.

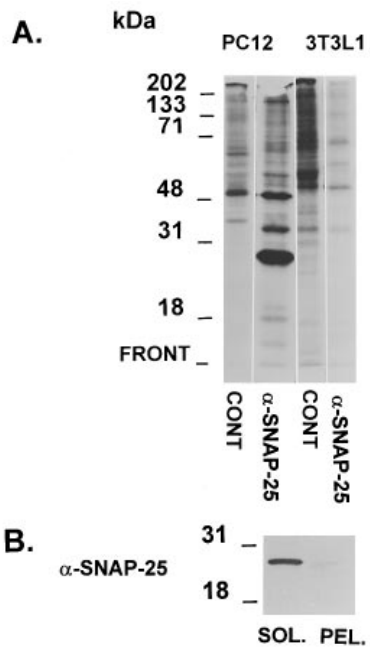

Figure 5 Immunoprecipitation of SNAP-25 from PC12 and adipose cells

Two approaches were used to identify SNAP-25 in PC12 and fat cells. In the first (**A**), three 90 mm dishes each of 3T3-L1 and PC12 cells were metabolically labelled with [^{35}S]methionine/[^{35}S] cysteine as described in the Materials and methods section, and immunoprecipitated with 4 μl of anti-SNAP-25 antibody (SMI 81) and 20 μl of Protein A–Sepharose. Immunoprecipitates were solubilized in reducing Laemmli/SDS loading buffer and analysed on 10% PAGE. Gels were incubated with Amplify (Amersham) prior to autoradiography. In the second approach (**B**), plasma membranes (1.4 mg), purified from rat epididymal adipocytes as described in the Materials and methods section, were solubilized in 1% Triton X-100, cleared by centrifugation at 15000 ***g*** (5 min at 4 °C), and SNAP-25 in the supernatant was analysed after immunoprecipitation with 4 μl of anti-SNAP-25 antibody (SMI 81) or with control serum (CONT) and 20 μl of Protein A–Sepharose (SOL.). The insoluble membrane pellet (PEL.) was also analysed after SDS/PAGE under non-reducing conditions. Samples were immunoblotted with SMI 81 and visualized with ECL (Amersham) detection. Similar results were obtained in two separate experiments.

Cloning and characterization of syntaxin 4 from human skeletal muscle

The PCR experiment using oligonucleotide N2872 (nt 1–27) as forward primer and N2873 (nt 867–894) as reverse primer generated a single product of size 894 bp from both human

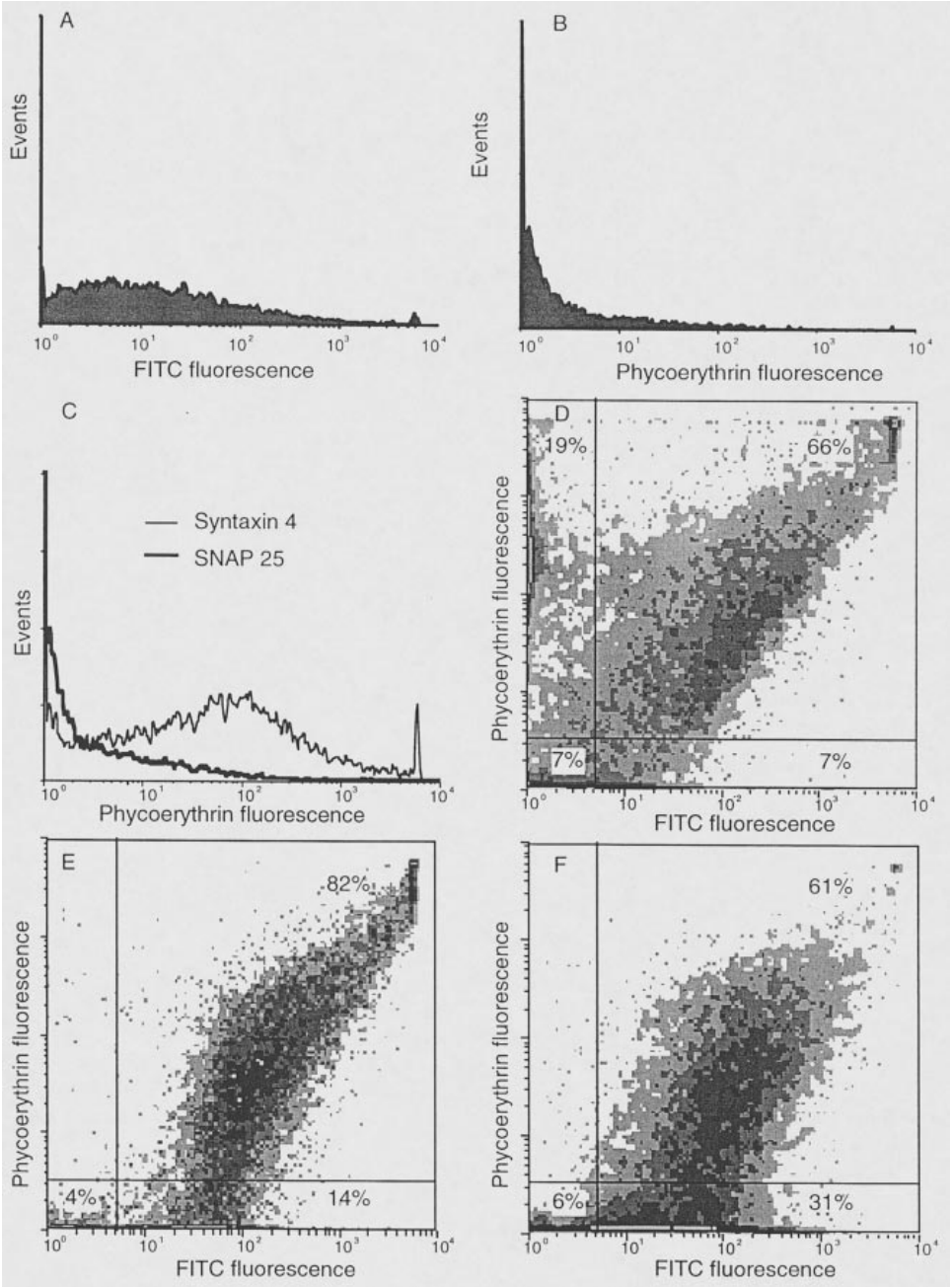

Figure 6 Interaction of syntaxin 4 and SNAP-25 with GLUT4 vesicles

Flow cytometry of low-density microsomes labelled with 1F8 (anti-GLUT4) antibody and FITC-labelled anti-mouse immunoglobulin (**B**, **D**–**F**) and/or biotinylated proteins and phycoerythrin/streptavidin (**C**–**F**). Microsomes were prepared, treated with antibody, syntaxin 4 and SNAP-25, and analysed as described in the Materials and methods section. (**A**) Control antibody, IgG1 isotype; (**B**) GST–biotin (control); (**C**) biotinylated syntaxin 4 or SNAP-25; (**D**) antibody 1F8 and syntaxin 4–biotin; (**E**) antibody 1F8, syntaxin 4–biotin and SNAP-25; (**F**) antibody 1F8, syntaxin 4 and SNAP-25–biotin. The two-dimensional plots have event numbers coded as grey scale densities. The quadrant positions define populations considered to be positive for phycoerythrin (upper left), fluorescein (lower left) and both (upper right). The percentages of data points in each quadrant are indicated, except where they are insignificant.

skeletal muscle and adipose tissue cDNA templates (Figure 2c). The PCR product generated from skeletal muscle cDNA was cloned and sequenced. The sequence data revealed that the cloned fragment corresponded to the entire coding region of syntaxin 4a (297 amino acids), beginning at the initiation codon and ending at the stop codon. Only five sequence differences were found when compared with the published sequence for syntaxin 4a from human placenta [25]. Two of these, T to G at nucleotide 588 (numbering according to the sequence of Li et al. [25]) and T to C at nucleotide 873, resulted in the amino acid sequence changes Asp-174 to Glu and Val-269 to Ala respectively. The other three were silent changes at the third position in codons 49 (T to G at nucleotide 213), 166 (C to G at nucleotide 564) and 190 (T to G at nucleotide 636). A PCR product of size 894 bp was also obtained using primers N2872 and N2873 with the adipose tissue cDNA template. It hybridized to the syntaxin 4

internal oligonucleotide N3122, similar to the fragment from skeletal muscle (Figure 2c).

Cloning and characterization of synaptobrevins from human skeletal muscle and adipose tissue

PCRs using the forward primer N2804 (nt 3–23) and the reverse primer N2800 (spanning nt 1131–1140 and nt 1167–1180) failed to amplify synaptobrevin 1 using the skeletal muscle cDNA as template. Since the reverse primer N2800 covers the transmembrane region and is AT-rich, a shorter reverse primer, N2853 (nt 307–331), beginning just upstream of the AT-rich region, was employed in conjunction with N2804. This resulted in the generation of a 330 bp fragment from skeletal muscle cDNA, which hybridized to N2328 (nt 921–940), an oligonucleotide specific to the internal region of synaptobrevins. The primers N2804 and N2870 (nt 307–354) were used to amplify a 330 bp fragment from the human fat cDNA library. The amplified fragments isolated from muscle and fat were subcloned and sequenced. The deduced amino acid sequence obtained for fat synaptobrevin 1 was identical to the published deduced human genomic sequence [28], while the muscle synaptobrevin 1 clone varied at nucleotides 251 (silent) and 306 resulting in a Thr-102 to Ala amino acid substitution. The PCR reactions for synaptobrevin 2 using the forward primer N2963 (nt 1–21) and the reverse primers N2806 (nt 266–282) and N2961 (nt 324–348) generated 280 and 340 bp fragments respectively from the human skeletal muscle cDNA template, and these fragments hybridized to the oligonucleotide N2328 [28] (Figure 2e).

Interaction of syntaxin 4 and SNAP-25 with GLUT4 vesicles

Biotin-labelled human syntaxin 4 as a GST fusion protein bound to low-density microsomes. Binding was detected with phycoerythrin-labelled streptavidin and was indicated by vesicles exhibiting phycoerythrin fluorescence that was significantly greater than that observed when the vesicles were treated with either GST–biotin or GST–SNAP-25–biotin (Figures 6B and 6C). Double labelling with the FITC-labelled anti-GLUT4 antibody, 1F8, allowed the identification of microsomes that exhibited both fluorescent labels and therefore also contained GLUT4 (data points in upper right quadrant, Figure 6D). The GST–SNAP-25 fusion protein did not show any significant binding to the low-density microsomes (Figure 6C). However, incubation of the microsomes with preformed complexes containing biotin-labelled GST–syntaxin 4 and unlabelled GST–SNAP-25 resulted in greater binding of GST–syntaxin 4 (increased percentage of points in upper right quadrant, Figure 6E), while complexes of unlabelled GST–syntaxin 4 and biotin-labelled GST–SNAP-25 resulted in the binding of GST–SNAP-25 to the microsomes (upper right quadrant, Figure 6F). Again, double labelling with 1F8 demonstrated that a high proportion of the microsomes binding the syntaxin–SNAP 25 complexes were also GLUT4-positive. In all diagrams, those microsomes that were GLUT4-positive but did not bind biotin-labelled proteins are located in the lower right quadrant, while those binding biotin-labelled protein but which were GLUT4-negative are located in the upper left quadrant. The lower left quadrants contain events that were negative for both labels.

Interaction of syntaxin 4 with synaptobrevins 1 and 2 in the yeast two-hybrid system

The yeast two-hybrid system [36] was used to characterize the interactions between syntaxin 4, cloned from human skeletal muscle, and synaptobrevin 1 from human skeletal muscle or synaptobrevin 2 from rat brain. The sequences encoding the cytoplasmic domain of synaptobrevin 1 and synaptobrevin 2 were cloned separately into the bait vector pAS2. Syntaxin 4 cDNA was cloned into the prey vector pGAD424. *S. cerevisiae* strain CG1945 was transformed with the bait vector containing either synaptobrevin 1 or 2 in combination with the prey vector with or without syntaxin 4. Interaction between the SNAREs transcriptionally activates both a nutritional reporter gene (*HIS 3*) as well as the synthesis of β-galactosidase, permitting colorimetric quantification of interaction strengths. β-Galactosidase specific activity (units) was determined by a colorimetric assay [37] of extracts of cells grown in liquid medium. The results (Table 1) indicated a clear interaction between the cytoplasmic domains of syntaxin 4 and synaptobrevin 2, but not between syntaxin 4 and synaptobrevin 1.

Table 1 Interactions between synaptobrevins and syntaxin 4 in the yeast two-hybrid system

The specific β-galactosidase activity generated by the interactions is given, where 1 unit is 1 nmol/min per mg of protein.

		β-Galactosidase activity (units)	
Bait vector	Prey vector ...	GAD	GAD/syntaxin4
Synaptobrevin 1		5.7 ± 0.7	5.3 ± 1.1
Synaptobrevin 2		10.0 ± 1.3	23.0 ± 2.7

DISCUSSION

Intracellular protein trafficking involves budding of vesicles from a donor membrane, vesicle translocation, docking with the correct target membrane, priming, membrane fusion and exocytosis, endocytosis and subsequent fusion with early endosomes [2]. Several proteins responsible for docking and fusion of vesicles in a constitutive or a regulated fashion have been identified and characterized, revealing a high degree of evolutionary conservation of this process from yeast to mammalian neurons [1,2,14]. This has led to the expectation that isoforms of these neural proteins will be involved in insulin-regulated glucose transport responses. Cells of the major insulin-responsive tissues, i.e. skeletal muscle and adipose tissue, have been shown to actively transport vesicles carrying the glucose transporter GLUT4 and to a lesser extent GLUT1 in response to insulin stimulus [19–21,39].

In the present study, the expression of SNAP-25 isoforms A and B in adipose and skeletal muscle tissues has been demonstrated by PCR cloning and sequence analysis. Furthermore, the presence of SNAP-25 cDNA has been shown in cultured mouse 3T3-L1 adipocytes, cells known to display insulin-mediated GLUT4 vesicle translocation [20]. Our results suggest that the SNAP-25 A isoform is the major species present in adipose tissue, while the B isoform, which is implicated in fast-release neurotransmitter responses [6], is the major species in skeletal muscle. The expression of SNAP-25 had been considered to be neural-cell-specific, with a significant functional role for each isoform in synaptogenesis and neurotransmission [6,9]. The only other reports of SNAP-25 expression in non-neural tissues are the localization of SNAP-25 in islets of Langerhans by immunofluorescence and Western blot analysis [15,17], and the presence of the functional homologue Sec9 in yeast [13]. Further investigations are necessary to test the predominance of one specific SNAP-25 isoform over the other at the protein level in skeletal

muscle and adipose tissues and to establish its significance at the functional level.

The presence of syntaxin 4 in human adipose tissue and skeletal muscle has been shown by PCR amplification and DNA sequence determination of the skeletal muscle clone. This DNA was highly similar to the syntaxin 4a DNA cloned from human placenta [25], with only five nucleotide differences, two of which result in amino acid sequence changes, Asp-174 to Glu and Val-269 to Ala. The presence of Glu-174 and Ala-269 in skeletal muscle syntaxin 4 is consistent with the corresponding positions in other syntaxins [40–42]. The Val to Ala change is in the region required for binding synaptobrevin [43,44]. Syntaxin 4 mRNA has been detected in rat skeletal muscle and L6 myoblasts and myotubes by Northern blotting, and the protein was detected in muscle membrane fractions with an affinity-purified specific antibody [24]. In support of our results indicating the presence of syntaxin 4 in fat tissue, a partial cDNA clone (length 417 bp) from a 3T3-L1 cDNA library has been isolated and sequenced, showing 98 % identity to the corresponding region of rat syntaxin 4 (J. Tellam and D. E. James, personal communication).

Using the yeast two-hybrid system, a clear interaction was shown between the full-length cytoplasmic domain of syntaxin 4 and synaptobrevin 2, but not synaptobrevin 1. These findings are in contrast with previous studies which reported that synaptobrevin 2 cannot interact with syntaxin 4 in a yeast two-hybrid system [45] and that synaptobrevin 1 can interact with GST–syntaxin 4 fusion protein *in vitro* [46]. The reasons for these differences in findings are unclear.

In the present study, flow cytometry has been shown to be a useful tool for analysing the binding of proteins to isolated microsomes. Direct binding of syntaxin 4 to microsomes could be observed, while the binding of SNAP-25 to the same population of microsomes was dependent on formation of a complex with syntaxin 4. A high proportion of these microsomes was also found to be positive for GLUT4. It is not known whether the high proportion of microsomes binding the anti-GLUT4 antibody 1F8 indicates the true proportion of GLUT4 in the microsomes isolated, or whether characteristics of data collection on the cytometer favoured the subpopulation of vesicles which carry GLUT4. This flow cytometry approach to vesicle binding is novel and provides a powerful method to screen peptides or protein domains for their capacity to bind or interfere with complex formation. The present findings with synaptobrevin 2, syntaxin 4 and SNAP-25 mirror those of Hayashi et al. [43], who showed that synaptobrevin 2 bound weakly to both SNAP-25 and syntaxin 1a, that SNAP-25 bound tightly to syntaxin 1a, and that the presence of all three components led to a dramatic increase in the interaction strength of the synaptobrevin 2–syntaxin 1a–SNAP-25 complex, which was resistant to denaturation by SDS.

Expression of synaptobrevin 2 and cellubrevin has been previously detected in rat skeletal muscle, rat L6 myotubes, rat adipose tissue or mouse 3T3-L1 adipocytes by PCR cloning and partial sequencing [22], Northern blotting [23] and immunoblotting [11,21–23]. Our results indicate the expression of synaptobrevin 2 in human skeletal muscle. Both synaptobrevin 2 and cellubrevin have been shown to be more abundant in differentiated L6 myotubes than in precursor myoblasts [23]. Like GLUT4, both cellubrevin and synaptobrevin 2 have been shown to be translocated to the plasma membrane from the low-density microsomal fraction of rat adipocytes [11] and mouse 3T3-L1 adipocytes [21] in response to insulin, but were located on distinct subsets of GLUT4-containing vesicles [21].

The situation with synaptobrevin 1 is less clear. The present study has demonstrated that synaptobrevin 1 transcripts can be detected in human skeletal muscle and adipose tissue, as found for rat skeletal muscle and adipose tissue [22]. However, synaptobrevin 1 transcripts were not detected in rat skeletal muscle or L6 myotubes by Northern blotting [23,47], suggesting that the mRNA levels are low compared with those of synaptobrevin 2 [23]. In addition, in the absence of a synaptobrevin 1-specific antibody, tetanus toxin treatment of isolated membrane fractions indicated that the levels of the toxin-resistant synaptobrevin 1 protein were minimal in these rat tissues [23]. The present data are in agreement with this conclusion. In addition, the observation that the pre-complex of syntaxin 4 and SNAP-25 binds to GLUT4 vesicles *in vitro* implies that synaptobrevin 2 is the dominant isoform present in GLUT4 vesicles, since only synaptobrevin 2, but not synaptobrevin 1, was capable of interacting with syntaxin 4 in the yeast two-hybrid system.

The data presented here add further support to the theory that glucose transporter translocation in insulin-responsive tissues such as skeletal muscle and fat may follow the general principles of intracellular protein traffic. The subcellular localization of synaptobrevin 2 before and after insulin treatment in adipocytes [11,21] or muscle [22], the increased expression of synaptobrevin 2 [23] and syntaxin 4 [24] during differentiation of L6 myoblasts into myotubes, and the localization of syntaxin 4 to plasma membrane-enriched fractions are all consistent with this hypothesis. The current report of the presence of SNAP-25 in insulin-responsive tissues completes the identification of the three proteins that form the core complex of SNAREs (synaptobrevin, syntaxin and SNAP-25). This, coupled with the detection of non-neural forms of synaptotagmin [48], Munc18 [49], Rabs [50,51] and Rab GDP dissociation inhibitor [52] in adipose tissue, indicates that the machinery for regulated vesicle transport appears to be present. Further investigations are required to demonstrate the functional link between the other t- and v-SNAREs described in these tissues and the transport, docking and fusion of glucose transporter vesicles.

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