The Glucose Transporter (GLUT-4) and Vesicle-associated Membrane Protein-2 (VAMP-2) Are Segregated from Recycling Endosomes in Insulin-sensitive Cells

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Abstract. Insulin stimulates glucose transport in adipocytes by translocation of the glucose transporter (GLUT-4) from an intracellular site to the cell surface. We have characterized different synaptobrevin/vesicle-associated membrane protein (VAMP) homologues in adipocytes and studied their intracellular distribution with respect to GLUT-4. VAMP-1, VAMP-2, and cellubrevin cDNAs were isolated from a 3T3-L1 adipocyte expression library. VAMP-2 and cellubrevin were: (a) the most abundant isoforms in adipocytes, (b) detectable in all insulin responsive tissues, (c) translocated to the cell surface in response to insulin, and (d) found in immunoadsorbed GLUT-4 vesicles. To further define their intracellular distribution, 3T3-L1 adipocytes were incubated with a transferrin/HRP conjugate (Tf/HRP) and endosomes ablated following addition of DAB and H_2O_2. While this resulted in ablation of >90% of the transferrin receptor (TfR) and cellubrevin found in in-

tracellular membranes, 60% of GLUT-4 and 90% of VAMP-2 was not ablated. Immuno-EM on intracellular vesicles from adipocytes revealed that VAMP-2 was colocalized with GLUT-4, whereas only partial colocalization was observed between GLUT-4 and cellubrevin. These studies show that two different v-SNAREs, cellubrevin and VAMP-2, are partially segregated in different intracellular compartments in adipocytes, implying that they may define separate classes of secretory vesicles in these cells. We conclude that a proportion of GLUT-4 is found in recycling endosomes in nonstimulated adipocytes together with cellubrevin and the transferrin receptor. In addition, GLUT-4 and VAMP-2 are selectively enriched in a postendocytic compartment. Further study is required to elucidate the function of this latter compartment in insulin-responsive cells.

I̲NSULIN stimulates glucose transport in muscle and adipose tissue by provoking the translocation of glucose transporters, specifically glucose transporter-4 (GLUT-4),[1] from an intracellular vesicular pool to the cell surface (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). Despite considerable progress in our understanding of this process, a number of major questions remain. What is the nature of the intracellular GLUT-4 compartment

and is it present in all cells? Is this compartment of endosomal origin or is it analogous to a secretory compartment? What other proteins are located in this compartment and how does GLUT-4 move from this site to the cell surface? The soluble *N*-ethyl maleimide-sensitive factor attachment protein receptor (SNARE) hypothesis provides an avenue for addressing such questions. Vesicles interact with their target membranes via the binding of v- and t-SNAREs (Sollner et al., 1993). The specificity that is required to regulate the myriad of intracellular transport reactions is seemingly provided by different v- and t-SNARE isoforms. For instance, v- and t-SNAREs that regulate endoplasmic reticulum to Golgi transport (Bennett and Scheller, 1993), intra-Golgi transport (Banfield et al., 1995), and Golgi to plasma membrane traffic (Bennett and Scheller, 1993) have all been identified in yeast. Vesicle-associated membrane protein (VAMP)/synaptobrevin is a mammalian homologue of the yeast v-SNAREs. VAMP was first identified in brain (Trimble et al., 1988;

1. *Abbreviations used in this paper:* CD-MPR, cation-dependent mannose 6-phosphate receptor; ceb, cellubrevin; GLUT, glucose transporter; HDM and LDM, high- and low-density microsomes; PM, plasma membrane; SNARE, soluble *N*-ethyl maleimide-sensitive factor attachment protein receptor; SSV, small synaptic vesicle; Tf/HRP, transferrin/HRP conjugate; TfR, transferrin receptor; TGR, *trans*-Golgi reticulum; VAMP, vesicle-associated membrane protein.

Baumert et al., 1989) where it has a specialized function in the fusion of small synaptic vesicles (SSVs) with the presynaptic plasma membrane (Schiavo et al., 1992; Südhof et al., 1993). More recently, VAMP has also been identified in adipocytes (Cain et al., 1992). This raises the possibility that the presence of VAMP could define a specialized class of intracellular GLUT-4 vesicles that are regulated in a manner similar to SSVs.

To date, the nature of the intracellular GLUT-4 compartment is poorly understood. GLUT-4 has been localized to endosomes and the *trans*-Golgi reticulum (TGR) and to associated tubulo-vesicular elements using immuno-EM (Slot et al., 1991a,b). However, it is not clear if the tubulo-vesicular elements are subdomains of the TGR/endosomal system or a discrete specialized secretory system like SSVs. To make this distinction, it will be necessary to undertake a high resolution analysis of the protein composition of the intracellular GLUT-4 vesicles. In the present study, we have identified three different synaptobrevin homologues in 3T3-L1 adipocytes (VAMP-1, VAMP-2, and cellubrevin). Using endosomal ablation and immuno-EM on intracellular vesicles isolated from adipocytes, we have compared the intracellular distribution of VAMP-2 and cellubrevin in adipocytes with GLUT-4. These data provide evidence for the intracellular segregation of these different synaptobrevins. We have identified at least four separate intracellular compartments using these markers containing either: *a*) cellubrevin alone, *b*) cellubrevin and GLUT-4, *c*) GLUT-4 and VAMP-2, or *d*) GLUT-4 alone.

Materials and methods

Materials

Restriction enzymes were obtained from New England Biolabs, Inc. (Beverly, MA). Radioactive nucleotides, nylon membranes (Hybond-N$^+$), HRP-conjugated goat anti–rabbit IgG, and enhanced chemi-luminescence (ECL) detection kits were from Amersham (Aylesbury, UK). All tissue culture reagents were from GIBCO BRL (Gaithersburg, MD), with the exception of fetal calf serum, which was from Commonwealth Serum Laboratory (Parkville, Vic, Australia). Poly(vinylidene difluoride) blotting membranes were from Millipore (Bedford, MA). Bicinchoninic acid (BCA) protein assay kits were from Pierce (Rockford, IL). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer (model 394; Foster City, CA). All other chemicals were of high quality commercial grades.

Antibodies

Antibodies specific for GLUT-4 were raised against a peptide corresponding to the COOH-terminal 12 amino acids of the rat adipocyte isoform (James et al., 1987) for use in immunoblotting, or against the COOH-terminal 17 amino acids for use in immuno-EM. Antibodies specific for GLUT-1 were raised against a peptide corresponding to the COOH-terminal 12 amino acids (Piper et al., 1991). An antibody specific for the human cation-dependent mannose 6-phosphate receptor (CD-MPR) cytosolic tail was a gift from Dr. Annette Hille (Universität Göttinggen, Göttingen, Germany), and is described in Schulze-Garge et al. (1993). Affinity purified antibodies raised against a synthetic peptide corresponding to the unique NH$_2$-terminal 20 amino acids of rat brain VAMP-2 with an additional NH$_2$-terminal cysteine (sequence SATAATVPPAAPAGQGG-PPA(C)) were a gift from Dr. Masami Takahashi (Mitsubishi Kasei Institute of Medical Research, Tokyo). Affinity-purified antibody raised against a synthetic peptide corresponding to the NH$_2$-terminal 12 amino acids of cellubrevin was provided by Dr. Thomas Südhof (Southwestern Medical School, Dallas, TX), and an antibody raised against a synthetic peptide corresponding to the NH$_2$-terminus of VAMP-1 was a gift from Dr. Pietro De Camilli (Yale University, New Haven, CT). All synaptobre-

vin antibodies were specific for their respective isoforms. An antibody specific for the α subunit of the Na-K/ATPase was kindly provided by Dr. R. Mercer (Washington University, St. Louis, MO).

Cell Culture

3T3-L1 fibroblasts (American Type Culture Collection, Rockville, MD) were grown in DME supplemented with 10% newborn calf serum, 2 mM L-glutamine, 100 U/l penicillin, and 100 mg/l streptomycin at 37°C in an atmosphere of 5% CO$_2$. Cells were passaged at subconfluency. Confluent cells were differentiated into adipocytes as described (Frost and Lane, 1985). Cells were used at day 8 after differentiation. Adipocyte primary cultures were isolated from rat epididymal fat by collagenase digestion as described previously (Simpson et al., 1983). To establish basal conditions before use, 3T3-L1 cells were incubated for 2 h in serum-free DME, and primary cultures of rat adipocytes were incubated for 30 min in modified KRP (12.5 mM Hepes, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO$_4$, 1 mM CaCl$_2$, 1 mM sodium phosphate, 2 mM sodium pyruvate, 2% BSA, pH 7.4). When used, insulin was included in the incubation media at 4 μg/ml for 30 min at 37°C.

Cloning and Sequence Analysis of VAMP-s in 3T3-L1 Adipocytes

Two oligonucleotide primers (CGCGGATCCAGCAAACCCAGGCAC-AAGTGGA; sense primer and CGCGGATCCTTCCACCAATACTT-CCTTTT; antisense primer) corresponding to the conserved central domain of rat brain VAMP-1 cDNA (Elferink et al., 1989) were synthesized and used to obtain a 193-bp DNA fragment by RT-PCR from rat brain RNA. Rat brain RNA was isolated by the method of Chirgwin et al. (1979). The PCR cycling profile was 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, repeated for 35 cycles. The authenticity of the amplified DNA fragment was verified by DNA sequencing. The PCR product was isolated from a 1% agarose gel, radiolabeled with random hexamer primers (Promega, Madison, WI), and used to probe a randomly primed 3T3-L1 adipocyte cDNA library constructed in λ ZAP II, kindly provided by Dr. F. Fiedorek (University of North Carolina, Chapel Hill, NC). A total of 250,000 plaques were screened. From 14 positives, isolated after sequential purifications, two distinct cDNAs, VAMP-1 and cellubrevin, were identified. The 3T3-L1 adipocyte cDNA library was also screened with a labeled rat brain VAMP-2 cDNA, kindly provided by Dr. Masami Takahashi. A total of 250,000 plaques were screened, and from 36 positives isolated, four were sequenced and found to be homologous to rat brain VAMP-2. Positive clones were sub-cloned into pBluescript II SK$^-$ (Stratagene, La Jolla, CA) and sequenced manually using Sequenase (version 2.0; U.S. Biochemical Corp., Cleveland, OH) or by automated DNA sequencing (model 373A; Applied Biosystems Inc.). All cDNAs were sequenced in both strands.

Preparation of Membranes

Preparation of Membranes for Immunoblotting. All steps were performed at 4°C or on ice. 3T3-L1 cells were grown in 100-mm culture dishes, scraped into 5 ml of HES Buffer (20 mM Hepes, 250 mM sucrose, 1 mM EDTA, pH 7.4) containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 250 μM PMSF, and homogenized by 10 passes through a 22-gauge needle. The homogenate was centrifuged at 60,000 rpm (model TLA100.3; Beckman Instruments, Fullerton, CA) for 60 min. The membrane pellets were resuspended and solubilized in PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 8.0 mM Na$_2$HPO$_4$, pH 7.4)/0.1% SDS for 1 h. Insoluble material was removed by centrifugation in a microfuge for 10 min. The supernatants were assayed for protein using the BCA assay and used to prepare Laemmli gel samples.

Sub-cellular Fractionation of 3T3-L1 Adipocytes. Cells were homogenized in HES buffer containing protease inhibitors by 10 passes through a homogenizer (Balch et al., 1984) using a 0.0011" clearance ball bearing. Subcellular fractionation was performed as described previously (Simpson et al., 1983; Piper et al., 1991). This fractionation procedure, which separates membrane fractions on the basis of differential centrifugation, generates four membrane fractions. The M/N fraction is enriched in mitochondria and nuclei and contains little GLUT-4 or other endosomal markers (Simpson et al., 1983; James and Pilch, 1988; Piper et al., 1991). The plasma membrane (PM) fraction is highly enriched in plasma membrane markers such as the α subunit of the Na-K/ATPase (see Fig. 2). The HDM is enriched in ER markers (Simpson et al., 1983) and fluid phase

markers, but not markers of the recycling endosomal system (James and Pilch, 1988). The LDM contains Golgi markers, recycling endosomes, and the majority of the insulin-regulatable GLUT-4 compartment (Simpson et al., 1983; James and Pilch, 1988; Piper et al., 1991).

Preparation of Vesicles for Immuno-EM. When vesicles were prepared for immuno-EM, cells were resuspended in 2× vol. HES buffer containing protease inhibitors and homogenized using a 22-gauge needle. The homogenate was centrifuged at 13,000 rpm for 20 min (model SS-34; Sorvall Instruments, Wilmington, DE) to remove cell debris, mitochondria, nuclei, and plasma membranes. The supernatant was layered onto a 1.5 M sucrose cushion (1 ml) containing Hepes (pH 7.4, 20 mM) and EDTA (1 mM) and centrifuged at 30,000 rpm for 60 min using a Beckman rotor (model SW-41). The intracellular membrane vesicles above the 1.5 M sucrose cushion were harvested and immediately fixed in a final concentration of 2% paraformaldehyde/PBS or incubated with 1 M KCl for 10 min before fixation. Fixed membranes were stored at 4°C.

Endosome Ablation

The transferrin/HRP-conjugate (Tf/HRP) was prepared using the carbodiimide method of Kishida et al. (1975). HRP (10 mg) was dissolved in 0.1 M NaCl and 10 mM sodium phosphate, pH 7.2 (1 ml), at 4°C and dialyzed overnight against the same buffer. Disodium succinate (200 mg) and succinic anhydride (70 mg) were added to the protein solution, and this was stirred for 30 min at 0°C and for 30 min at room temperature, and then passed over an 8-ml G-50 Sephadex column. The solution was concentrated to 0.5 ml using a centriprep concentrator (Amicon, Beverly, MA). *N*-hydroxysuccinimide (25 mg) and *N*-ethyl-n-(3-dimethylaminopropyl) carbodiimide hydrochloride (40 mg) were added and the solution stirred for 3 h at 0°C. This was again passed over an 8-ml G-50 Sephadex column in 0.1 M NaCl/1 mM sodium phosphate, pH 7.2, and the eluate was collected. This activated HRP was immediately added to 1 ml phosphate buffer containing transferrin (10 mg/ml) and ^{125}I-transferrin (10^7 cpm) and stirred for 2 d at 2°C. The conjugation reaction was quenched by addition of excess glycine and a sample of the reaction run on a 10% polyacrylamide gel to check, by autoradiography, for an increase in molecular weight of a proportion of the iodinated transferrin. Samples from three reactions were pooled and chromatographed on a 75 × 2-cm Sephacryl S-300 column in order to separate unconjugated transferrin from the Tf/HRP conjugate. Conjugate was aliquotted and stored at −80°C until use. The conjugate was iron loaded as described by West et al. (1994).

After 2 h in serum-free DME, 3T3-L1 adipocytes were incubated with 20 μg/ml Tf/HRP for the times indicated. Cells were chilled by washing in ice-cold isotonic citrate buffer and kept on ice to prevent further vesicle trafficking during the DAB cytochemistry reactions. Cell surface–attached Tf/HRP was removed by acid washing for 10 min in ice-cold isotonic citrate buffer (150 mM NaCl and 20 mM sodium citrate, pH 5.0) with three changes of buffer and then the monolayer was washed once in ice-cold PBS, pH 7.4. Cells were incubated with DAB (100 μg/ml) in the absence or presence of H_2O_2 (0.02%, vol/vol) for 60 min at 4°C in the dark. Cells were washed once with PBS containing 5 mg/ml BSA and three times with ice-cold HES buffer before subcellular fractionation. GLUT-4–containing vesicles were immunoabsorbed from the LDM fraction as previously described (Martin et al., 1994).

SDS-PAGE and Western Blotting

SDS-PAGE was performed by the method of Laemmli (1970) using 10 or 15% acrylamide resolving gels. Proteins resolved by SDS-PAGE were electrophoretically transferred to poly(vinylidene difluoride) by the method of Towbin et al. (1979). Membranes were incubated with rabbit polyclonal antibodies specific for either VAMP-1 (1:250), VAMP-2 (10 μg/ml), cellubrevin (1:1,000), GLUT-1 (5 μg/ml), GLUT-4 (1:500), or the α subunit of the Na-K/ATPase (1:500). Immunoreactive proteins were detected using HRP-conjugated goat anti–rabbit IgG (1:10,000) and ECL.

Electron Microscopy

Paraformaldehyde-fixed intracellular membrane vesicles were randomly adsorbed to form var-coated copper grids. Dry grids were layered on 10-μl drops of vesicle suspensions and floated on them for 10 min. The nonbound vesicles were washed away during the sequential incubations described below. All subsequent incubations of grids were performed on ~500-μl drops at room temperature unless stated otherwise. Grids were incubated sequentially on 2% gelatin for 10 min, 0.02 M glycine/PBS (4 × 1 min), and 0.1% BSA/PBS for 1 min. Grids were then incubated with 10-μl

drops of primary antibodies diluted in 1% BSA/PBS as follows: GLUT-4 serum (1:400), VAMP-1 IgG (1:100), VAMP-2 IgG (10 μg/ml), cellubrevin IgG (1:500), and CD-MPR serum (1:100). After a 30-min incubation, grids were washed four times with 0.1% BSA/PBS and then incubated with 20 μl gold-conjugated protein A in 1% BSA/PBS for >30 min. Grids were then washed with 0.1% BSA/PBS for 5 min and then four times with PBS for 5 min each. The reaction was stabilized by a 5-min incubation in 1% glutaraldehyde/PBS. When double labeling was performed, grids were washed twice for 5 min with PBS and five times for 2 min each with 0.02 M glycine/PBS, after which the above procedure was repeated using a different primary antibody and different-sized protein A–gold conjugate. All grids were then washed twice for 5 min with PBS, five times for 2 min with ultrapure water, and stained with uranyl acetate/methyl cellulose (1:9; 4% uranylacetate in 0.15 M oxalic acid, pH 7–8: 2% methylcellulose) for 5 min. Grids were dried and viewed using a transmission electron microscope (model 1010; JEOL U.S.A., Inc., Peabody, MA).

Results

Cloning and Sequencing of 3T3-L1 Adipocyte Synaptobrevins

To identify synaptobrevin isoforms expressed in adipocytes, a 3T3-L1 adipocyte cDNA expression library was screened with rat brain VAMP-1 or VAMP-2 cDNA probes. Full length clones encoding VAMP-1, VAMP-2, and cellubrevin were isolated and characterized. Both 3T3-L1 adipocyte VAMP-2, and cellubrevin were 100% identical to their respective rat brain homologues at the amino acid level. VAMP-1, the least abundant VAMP homologue detected in adipocytes, was 96% identical to the rat brain sequence (Elferink et al., 1989) at the amino acid level. There were substitutions (rat brain → 3T3-L1) at amino acid residues 28 (T → M), 48 (I →M), 78 (V → Q), and 116 (I → F). One of these changes, V78 in rat to Q78 in mouse, occurred within the putative tetanus toxin cleavage site and may render murine VAMP-1 tetanus toxin sensitive. In this respect, murine VAMP-1 more closely resembles the human (Archer et al., 1990) and drosophila (Südhof et al., 1989) brain VAMP-1 homologues than the rat brain homologue. Similarly, the amino acid substitution I48 to M48 changes the distal recognition site for botulinum D toxin to that found in VAMP-2 (Yamasaki et al., 1994) suggesting that murine VAMP-1 also has increased sensitivity to this toxin.

Fig. 1 shows a representative immunoblot of the tissue distribution of cellubrevin, VAMP-1, and VAMP-2. Cellubrevin is ubiquitously expressed, which is consistent with previous studies (McMahon et al., 1993). VAMP-2 is expressed at high levels in brain, but it was also detected in all tissues that exhibit insulin-regulated glucose transport. All three synaptobrevin isoforms were detected in 3T3-L1 fibroblasts. After differentiation of these cells into adipocytes, the expression of VAMP-2 increased 2.4-fold, and on average there was also a slight (1.2-fold) increase in the expression of cellubrevin (Fig. 1). An immunoreactive species corresponding to VAMP-1 could not be detected in 3T3-L1 adipocytes.

Subcellular Distribution of Synaptobrevins in Adipocytes

To identify the cellular location of synaptobrevins, adipocytes were incubated in the presence or absence of insulin and then subjected to subcellular fractionation followed
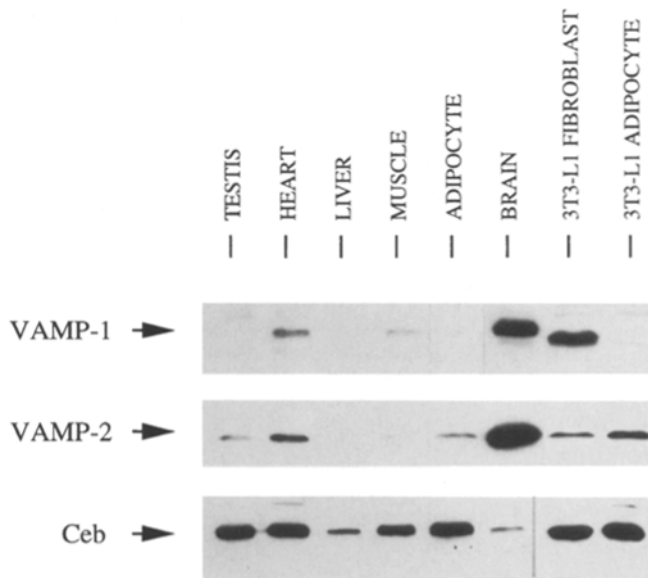
Figure 1. Distribution of VAMP-1, VAMP-2, and cellubrevin in rat tissues and 3T3-L1 cells. Total cell membranes were prepared from rat tissues and from 3T3-L1 fibroblasts and adipocytes. The distribution of synaptobrevins were then determined by immunoblotting. Equal amounts (50 μg) of cell membranes were separated by SDS-PAGE and analyzed by immunoblotting using antisera specific for VAMP-1, VAMP-2, or cellubrevin (Ceb).

Figure 2. Subcellular distribution of VAMP-2 and cellubrevin in basal and insulin-stimulated 3T3-L1 adipocytes. Basal (−) or insulin-stimulated (+) 3T3-L1 adipocytes were subjected to differential centrifugation. Samples containing either plasma membranes (PM), low density microsomes (LDM), high density microsomes (HDM), or mitochondria/nuclei (M/N), each containing 50 μg protein, were separated by SDS-PAGE and analyzed by immunoblotting using antisera specific for Na-K/ATPase, GLUT-4, VAMP-2, or cellubrevin (Ceb).

by immunoblotting (Fig. 2). This fractionation procedure has been used extensively to study the subcellular distribution of glucose transporters and other markers in adipocytes (Cushman and Wardzala, 1980; Simpson et al., 1983; James et al., 1987; James and Pilch, 1988; Piper et al., 1991; Cain et al., 1992; Hanpeter and James, 1995). Relevant to the present studies, endosomal markers such as internalized transferrin (Livingstone et al., 1996), internalized insulin (James and Pilch, 1988), and the transferrin receptor (Hanpeter and James, 1995) are enriched predominantly in the LDM fraction and to a lesser extent in the HDM fraction. In agreement with previous studies, the α subunit of the Na-K/ATPase, a cell surface marker, is highly enriched in the PM fraction (Fig. 2) and its distribution is unaffected by insulin. In the absence of insulin, GLUT-4 was found both in the LDM (~60%) and the HDM (~30%). In response to insulin, there was a four- to fivefold increase in the level of GLUT-4 in the PM, a twofold decrease in the LDM, and a slight decrease in the HDM. These data support previous studies showing that insulin stimulates the movement of GLUT-4 from intracellular membranes (LDM) to the cell surface (PM).

We were unable to detect a significant difference in the subcellular distribution of VAMP-2 and cellubrevin using this procedure (Fig. 2). Both synaptobrevin isoforms underwent insulin-dependent translocation from the LDM fraction to the cell surface. However, the magnitude of the insulin-dependent increase at the cell surface was much less for VAMP-2 and cellubrevin (two- to threefold, n = 4) compared to GLUT-4 (four- to fivefold, n = 4). This difference mainly reflected the very low levels of GLUT-4 in the plasma membrane of non-stimulated cells, whereas this was not the case for each of the synaptobrevins (Fig. 2).
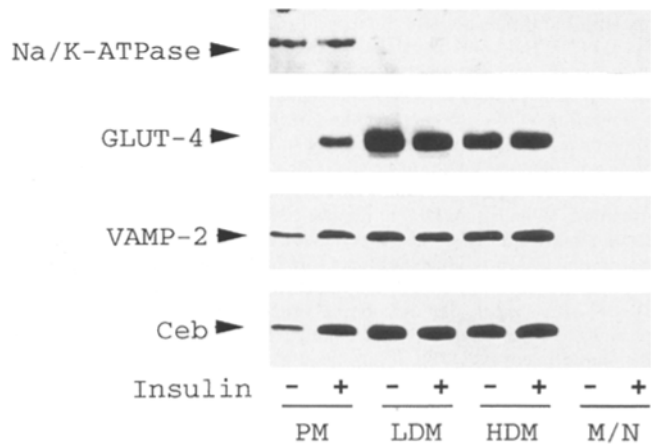
Similar results comparing the distribution of GLUT-4, VAMP-2, and cellubrevin were also obtained in primary cultured rat adipocytes (data not shown).

Co-localization of VAMP-2, Cellubrevin, and GLUT-4 with the Transferrin Receptor

A technique has been previously developed to investigate the recycling of proteins through endosomal compartments utilizing HRP-catalyzed complexing of DAB with neighboring lumenal and integral membrane proteins in the presence of hydrogen peroxide (Stoorvogel et al., 1988, 1989, 1991; West et al., 1994). By allowing cells to take up Tf/HRP before DAB/peroxide treatment, the compartments containing the TfR, specifically early endosomal compartments, can be selectively segregated from other compartments. In view of the high number of transferrin receptors expressed in 3T3-L1 adipocytes (Tanner and Lienhard, 1987, 1989), it is possible to use this technique to study the composition of the endosomal system in these cells. In combination with the subcellular fractionation program described here (Fig. 2), it is possible to determine the relationship between the endosomal system and intracellular compartments containing GLUT-4 and other markers in adipocytes. The LDM fraction was used for all of these studies because it is highly enriched in the insulin-regulatable GLUT-4 compartment (Fig. 2). We have used this technique to show that GLUT-4, GLUT-1, and the cation-independent MPR/insulin-like growth factor II receptor partially co-localize with the TfR in the endosomal system of 3T3-L1 adipocytes (Livingstone et al., 1996), which is consistent with previous studies using vesicle immunoadsorption (Piper et al., 1991; Hanpeter and James, 1995). TGN38, a membrane protein that is predominantly found in the TGR at steady state, was not ablated following treatment with Tf/HRP (Livingstone et al., 1996), indicating that this technique is reasonably specific for recycling

endosomes. The ablation of a protein may occur either through a change in vesicle density (density shift) or due to chemical cross-linking of proteins that are located within the lumen of the same compartment as the Tf/HRP conjugate. We have observed both types of modification in 3T3-L1 adipocytes treated with Tf/HRP (Livingstone et al., 1996). Irrespective of the mechanism of ablation, this method provides a powerful approach for identifying the composition of the recycling/endosomal system.

3T3-L1 adipocytes were incubated with the Tf/HRP conjugate for either 1 or 3 h at 37°C. DAB cytochemistry was then performed in the presence or absence of peroxide and the amount of GLUT-4, VAMP-2, and cellubrevin in the LDM was determined by immunoblotting (Fig. 3). Quantitative ablation of the TfR (Livingstone et al., 1996) and cellubrevin (Table I) was achieved following either a 1 or 3 h incubation with the conjugate. In contrast, only about 40% of the GLUT-4 and 10% of VAMP-2 found in the LDM fraction was removed under similar conditions. Longer incubation of the cells (3 h at 37°C) with the Tf/HRP conjugate confirmed that this result was not due to a difference in time required for the TfR to enter the VAMP-2-containing intracellular site. As endocytotic and exocytotic recycling of proteins is temperature-dependent, incubating the cells for 3 h at 4°C confirmed that the observed results were due to membrane recycling, as at this temperature neither VAMP-2 nor cellubrevin were removed.

To confirm the segregation of VAMP-2 and cellubrevin in the intracellular LDM fraction isolated from 3T3-L1 adipocytes, further experiments using vesicle immunoadsorption were performed. Adipocytes were incubated with Tf/HRP and some were treated with peroxide. Intracellular membranes (LDM) were isolated and incubated with
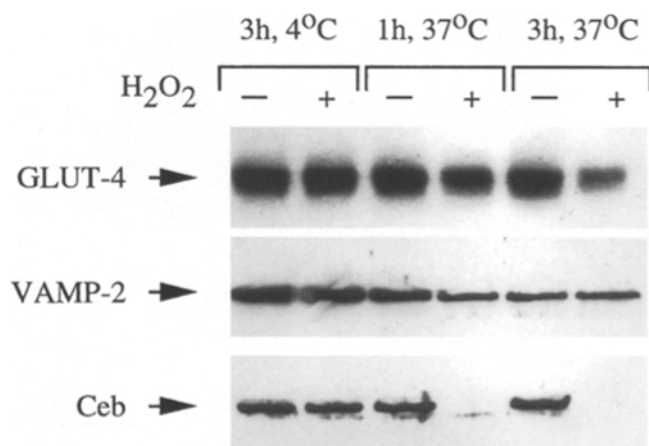
Figure 3. Effect of ablation of the TfR endocytotic pathway on VAMP-2 and cellubrevin in the LDM fraction of 3T3-L1 adipocytes. Nonstimulated 3T3-L1 adipocytes were incubated with Tf/HRP for either 1 or 3 h at 37°C, or for 3 h at 4°C. DAB cytochemistry was then performed either in the presence (+) or absence (−) of hydrogen peroxide. Cells were homogenized and intracellular membranes harvested following differential centrifugation. Intracellular membrane fractions, corresponding to 10% of that isolated from a single 10-cm plate of cells, were subjected to SDS-PAGE followed by immunoblotting with antisera specific for VAMP-2, cellubrevin (Ceb), or GLUT-4.

Table I. Effects of Tf/HRP Ablation on Intracellular GLUT-4 and Synaptobrevin Levels

| | | 4°C, 1 h | | 37°C, 1 h | | 37°C, 3 h | |
	n^*	−	+	−	+	−	+
GLUT-4	6	100	105 ± 8	102 ± 6	63 ± 6	103 ± 7	61 ± 2
VAMP-2	3	100	103 ± 14	101 ± 3	90 ± 11	104 ± 8	88 ± 9
Ceb	3	100	98 ± 6	107 ± 11	10 ± 4	101 ± 14	17 ± 6

(header spanning: Incubation conditions)

3T3-L1 adipocytes were treated with Tf/HRP for either 1 h or 3 h at 37°C or for 3 h at 4°C, after which the DAB cytochemistry reaction was performed in the presence (+) or the absence (−) of hydrogen peroxide. After subcellular fractionation, the LDMs were immunoblotted for the proteins shown. The data is expressed as a percentage ± SEM of the amount of protein present in control samples (control samples were treated with Tf/HRP for 3 h at 4°C, after which the DAB cytochemistry was performed in the absence of hydrogen peroxide). The GLUT-4 signal was detected using an iodinated anti–rabbit IgG and the radioactive bands quantified by gamma counting. The VAMP-2 and cellubrevin signals were detected by ECL and quantified using densitometry.
*Number of blots quantified (where $n = 3$, this is from three separate ablation experiments).

an antibody specific for the cytoplasmic carboxyl terminus of GLUT-4 to immunoadsorb GLUT-4–containing vesicles (Fig. 4). GLUT-4–containing vesicles isolated from nonablated cells contained both VAMP-2 and cellubrevin, indicating that these three proteins colocalize. Vesicles isolated from ablated cells, however, contained only VAMP-2 (Fig. 4). These data confirm the above findings (Fig. 3) and suggest that there are at least two distinct populations of GLUT-4–containing vesicles in adipocytes: one that is enriched in cellubrevin and another that is enriched in VAMP-2. Equivalent control vesicles isolated using nonspecific IgG contained neither cellubrevin nor VAMP-2.

To ensure that the majority of the GLUT-4–containing vesicles were being adsorbed from the LDMs, the membranes remaining in the supernatant after immunoadsorption were also blotted for VAMP-2, cellubrevin, and GLUT-4. The amount of GLUT-4 remaining after immunoadsorption was found to be less than 5% of the level present in the LDMs absorbed with nonspecific IgG, confirming that most of the GLUT-4–containing vesicles had been immunoadsorbed (results not shown). In control cells, immunoadsorption of GLUT-4–containing vesicles resulted in the removal of most VAMP-2 and cellubrevin (Fig. 4), compared to the levels remaining after control immunoadsorption with nonspecific IgG. In treated cells, GLUT-4 removal also resulted in VAMP-2 removal compared to control immunoadsorption. However, cellubrevin could not be detected regardless of whether specific anti-GLUT-4 IgG or nonspecific IgG was used. As cellubrevin is also not present in the equivalent vesicle preparation, this confirmed its removal from the LDM. These data indicate that VAMP-2 and a substantial proportion of GLUT-4 must be located in an intracellular compartment(s) distinct from the endosomal system that contains the TfR and cellubrevin.

To determine if the nonablated component of VAMP-2 and GLUT-4 could arise from the biosynthetic pathway, similar studies to those described above were performed in 3T3-L1 adipocytes following incubation with cycloheximide (20 μg/ml or 50 μg/ml) for either 45 min or 2 h at 37°C, conditions that have previously been shown to inhibit protein synthesis (Piper et al., 1992). However, there
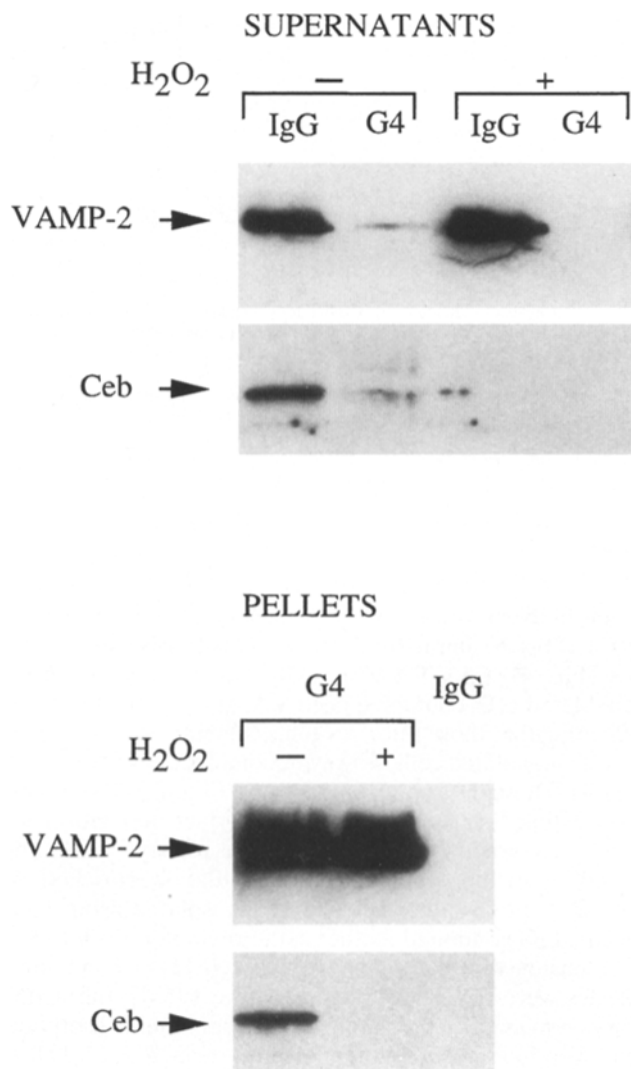
SUPERNATANTS

H_2O_2

VAMP-2 →

Ceb →

PELLETS

H_2O_2

VAMP-2 →

Ceb →

Figure 4. Effect of TfR pathway ablation on the immunoadsorption of GLUT-4–containing vesicles from 3T3-L1 adipocytes. 3T3-L1 adipocytes were loaded with Tf/HRP for 1 h at 37°C. After DAB cytochemistry in the presence (+) or absence (−) of hydrogen peroxide, cells were subjected to subcellular fractionation. GLUT-4–containing vesicles were then immunoadsorbed as described previously (Martin et al., 1994). Formaldehyde-fixed *Staphylococcus aureus* cells were incubated with GLUT-4 antibodies (*G4*) or nonspecific IgG (*IgG*). This complex was then incubated with an intracellular LDM fraction isolated from the 3T3-L1 adipocytes. The pellets containing the immunoadsorbed complex and the supernatant, containing the nonadsorbed membranes, were subjected to SDS-PAGE followed by immunoblotting using antisera specific for VAMP-2 or cellubrevin (*Ceb*).

was no significant change in the ablation efficiency following incubation with cycloheximide (data not shown), indicating that the nonablated component of GLUT-4 and VAMP-2 does not correspond to newly synthesized protein.

Immuno-electron Microscopy on Adipocyte Vesicles

DAB/peroxide complexation of the 3T3-L1 adipocyte-recycling endosomal pathway and immunoadsorption of GLUT-4 vesicles from intracellular membranes indicated that VAMP-2 colocalizes with GLUT-4 outside of the gen-

eral recycling pathway. This does not correspond to newly synthesised GLUT-4 or VAMP-2, as this compartment persisted in cells following incubation with cycloheximide. To further investigate the nature of this compartment, immuno-EM was performed on adipocyte intracellular vesicles. This technique provides several advantages not available using other methods, such as subcellular fractionation or immunocytochemistry in tissue or cell sections. First, it is possible to analyze the protein composition of a uniform population of vesicles. Thus, in order to examine the entire intracellular GLUT-4 compartment in these experiments, we isolated a membrane fraction that represented a combined HDM/LDM fraction. Whereas the majority of membranes in this fraction comprised small vesicles of an average diameter 80 nm, larger structures were occasionally observed and approximately 5% of these were labeled to varying degrees for GLUT-4, VAMP-2, and cellubrevin (see Fig. 5). These structures are presumably derived from early endosomal vacuoles or interconnected structures derived from the TGR. In view of their size, these are likely to be the major component of the HDM fraction. In addition, these strucutures are not observed in the LDM fraction (data not shown). Second, it is possible to detect variations in protein content across a population of vesicles. Third, the labeling obtained using this technique is typically quite high because of the mild fixation conditions and because labeling of whole vesicles can be achieved. For ex-
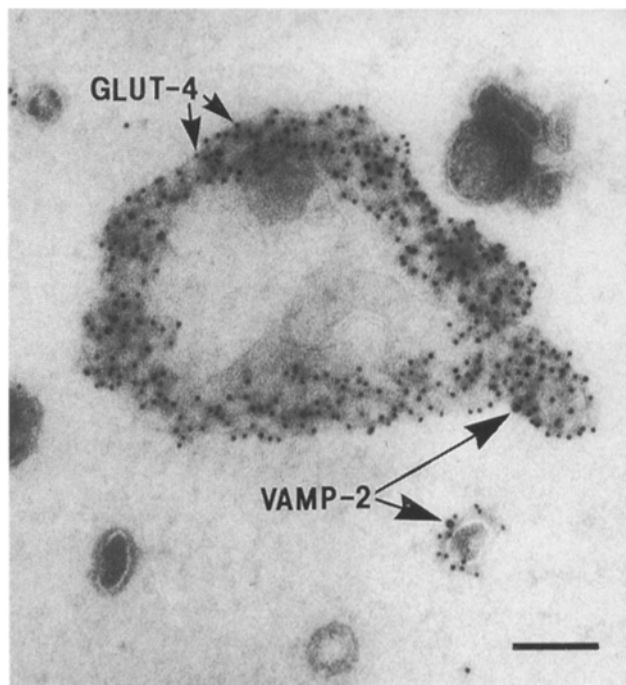

Figure 5. Immunoelectron microscopy labeling of larger, complicated structures in intracellular membranes from rat adipocytes. An intracellular membrane fraction was prepared from rat adipocytes and fixed in 2% paraformaldehyde. After adsorption to EM grids, these membranes were immunolabeled for GLUT-4 and VAMP-2 and specific labeling detected using 5- and 10-nm gold, respectively. Occasional labeling of large, complicated membrane structures was observed, as well as the labeling of small (50–140 nm) vesicles. Shown is one of the heavily labeled large structures together with both labeled and unlabeled vesicles. Bar, 100 nm.

ample, in the case of GLUT-4 some of the labeled vesicles contained >30 gold particles per vesicle. Finally, it is possible to colocalize different antigens in the same field of vesicles using secondary antibodies conjugated with different-sized gold particles.

Due to the relatively low expression of VAMP-2 in 3T3-L1 adipocytes, it was not possible to detect immunogold labeling on vesicles isolated from these cells (data not shown). However, VAMP-2 is expressed at higher levels in rat adipocytes and intracellular vesicles isolated from these cells yielded consistent specific VAMP-2 labeling (Table II). Therefore, an extensive analysis at the immuno-EM level was performed on vesicles isolated from these cells. No specific VAMP-1 labeling was detected in adipocyte vesicles, probably because of its very low expression in these cells (data not shown). We observed significant variation in labeling for different antigens (see Table II). This may be due to epitope masking, different levels of protein expression, or differential sensitivity to the fixative. However, this caveat only becomes problematic, at least in the present studies, if the low labeling efficiency results in an underestimation of the total number of vesicles containing a particular antigen. However, as described below this does not appear to be the case.

The overall labeling for GLUT-4 was much higher, ranging from 3–>30 gold particles per vesicle, than cellubrevin (2–6 particles per vesicle) or VAMP-2 (1–3 particles per vesicle). We attempted to determine if the low VAMP-2 labeling was related to epitope masking or to the particular antibody used. Vesicles were incubated with either 1 M KCl, glycine buffer, pH 2.4, or bicarbonate buffer, pH 11.9, before fixation. However, none of these treatments had a significant effect on VAMP-2 labeling. In addition, we obtained essentially identical results with two separate VAMP-2 antibodies. Thus, we found no evidence for selective protection of the VAMP-2 epitope. In addition to variations in antigen labeling per vesicle, there were also differences in the percentage of total vesicles labeled for each marker (Table II). However, there was no correlation between these two parameters. For instance, the percent of total vesicles containing cellubrevin and GLUT-4 was similar (31 and 36%, respectively), but the

overall labeling per vesicle was much higher for GLUT-4 (Table II). These data suggest that the labeling obtained using these different antibodies provides a reasonably accurate representation of the distribution of the majority of these different proteins. The diameter of the vesicles labeled varied over the range 50–140 nm, with an average vesicle diameter of 80 nm. There was no correlation between vesicle size and specific protein content.

Once the single-labeling profiles had been determined for each of the antibodies, double labeling was used to determine the extent of overlap between different antigens. Characteristic double labeling of vesicles is shown in Fig. 6 and quantitation of the overlap between different markers is shown in Table II. In interpreting these data, it is important to recognize the variation in the pool size for the different antigens. For example, the total number of vesicles containing GLUT-4 (36%) is much larger than the total number of VAMP-2 vesicles (12%). Thus, despite the very high degree of overlap between these two markers, only 26% of vesicles that contain GLUT-4 also contain VAMP-2.

Almost half of the GLUT-4 (+ve) vesicles contained cellubrevin and nearly a quarter contained VAMP-2. Conversely, almost all of the VAMP-2 (+ve) vesicles contained GLUT-4, whereas in the case of cellubrevin (+ve) vesicles, there was a significant population of these that were GLUT-4 (−ve). In conjunction with the Tf/HRP ablation data, where about half of the GLUT-4 was ablated under conditions that ablate almost all of the cellubrevin, these data suggest that those GLUT-4 (+ve)/cellubrevin (+ve) vesicles observed by immuno-EM colocalizing with cellubrevin must partly derive from the recycling endosomal pool. However, only a relatively small proportion of VAMP-2 was ablated following incubation with Tf/HRP (Table I), yet 63% of the VAMP-2 (+ve) vesicles contain cellubrevin (Table II). Because the VAMP-2 pool size is much smaller than the cellubrevin pool (12 versus 31%, Table II), we would have predicted that the VAMP-2 pool would only contain 18% of the total amount of cellubrevin. This value is very close to the measured amount of nonablated cellubrevin following incubation with Tf/HRP (Table I). Thus, we assume that the majority of VAMP-2 (90%, Table I) sorts into a separate compartment from the recycling endosomes together with a large proportion (>60%, Table I) of the recycling GLUT-4. Other recycling proteins, such as cellubrevin and MPR, also sort into this compartment but with a much lower fidelity. So, the majority of these proteins remain in the recycling compartment together with the TfR and thus are subject to efficient ablation (Table I).

Although it was not possible to visualize VAMP-2 labeling of vesicles isolated from 3T3-L1 adipocytes, it was possible to detect GLUT-4 and cellubrevin labeling of 3T3-L1 adipocyte vesicles. In these cells, the segregation of cellubrevin and GLUT-4 was consistent with the results obtained in rat adipocytes. A significant proportion of the GLUT-4 (50%) was localized to vesicles that did not contain cellubrevin, and 60% of the cellubrevin containing vesicles were GLUT-4 negative (data not shown).

The TfR and cellubrevin are likely to be predominantly located in early endosomes, and so one possible explanation for the results obtained in this study is that a large proportion of GLUT-4 may reside in a late endosomal com-

Table II. Analysis of the Protein Composition of Adipocyte Vesicles Determined Using Immuno-EM

| Vesicles containing* | Analysis of labeling | | Percentage overlap with [†] | | | |
	Percentage total	Range	GLUT-4	VAMP-2	Ceb	MPR
GLUT-4	36 ± 7	3–25	100	26 ± 6	46 ± 1	48 ± 7
VAMP-2	12 ± 1	1–3	87 ± 5	100	63 ± 3	36 ± 1
Ceb	31 ± 2	2–6	66 ± 4	24 ± 5	100	54 ± 4
MPR	23 ± 7	1–11	76 ± 2	45 ± 5	63 ± 6	100

Fixed vesicles from adipocytes were single or double labeled as described in Materials and Methods. The percentage of the total vesicles present in the membrane fraction used that were labeled with each antibody (*Percentage total*), and the range of gold labeling per vesicle (*Range*) was determined by single labeling. To determine colocalization, double labeling was performed. For each pool of the total vesicles labeled for a primary marker, (*) the degree of overlap with a second marker protein ([†]) was determined. The degree of overlap is expressed as a percentage only of the pool containing the first labelled protein (i.e. 36% of the total vesicles contain GLUT-4, and 26% of that 36% also contain VAMP-2). Between 200–300 individual vesicles were counted to determine percent overlap between each marker. Shown are the means ± SEM of three separate vesicle preparations.
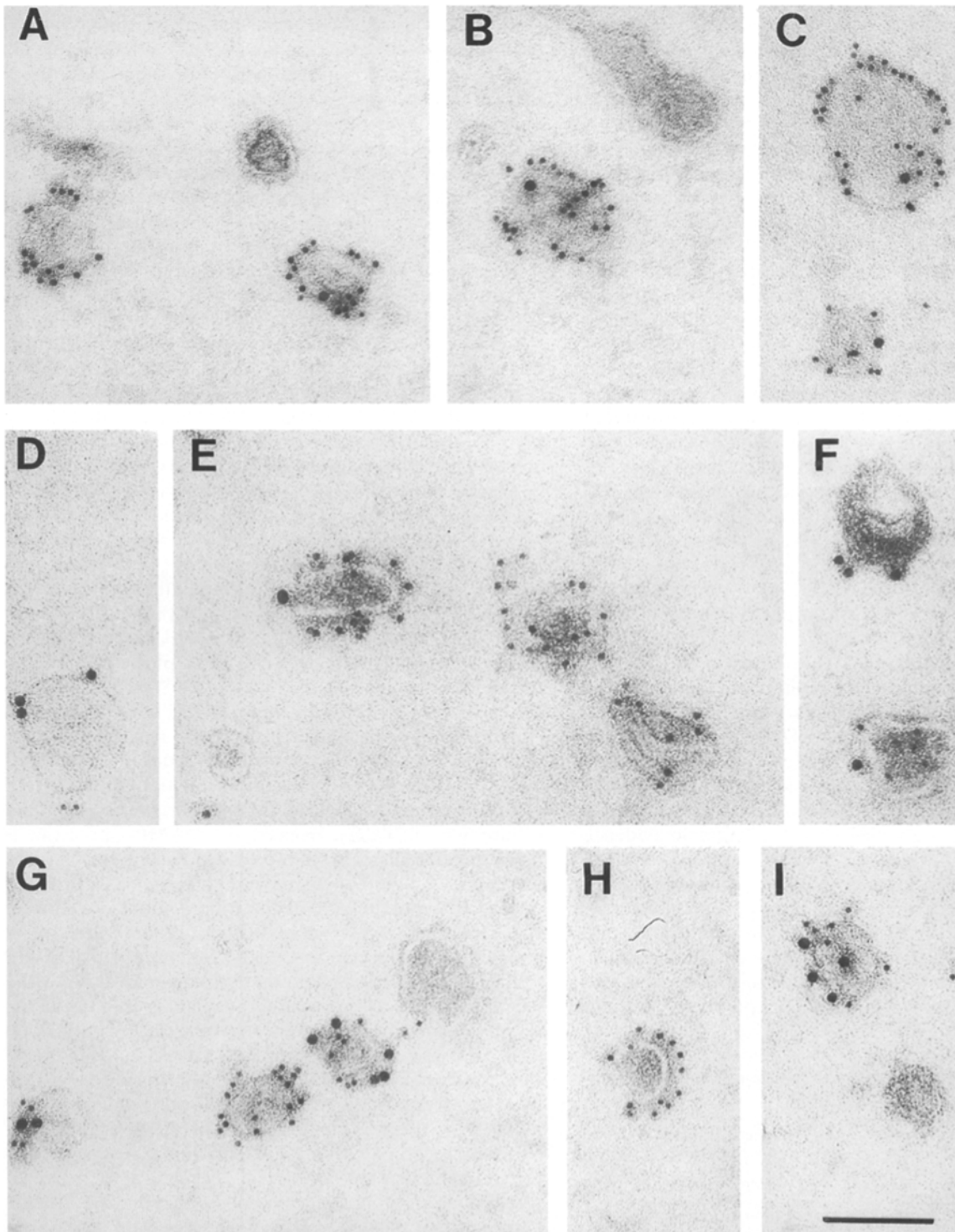
Figure 6. Immunoelectron microscopy of intracellular vesicles from rat adipocytes. An intracellular membrane fraction was prepared from rat adipocytes, fixed in 2% paraformaldehyde, and adsorbed to EM grids. The grids were then labeled with specific antibodies, which were detected using protein A conjugated to different sizes of gold. (*A–C*) Colocalization of GLUT-4 (5-nm gold) and VAMP-2 (10-nm gold). (*D–F*) Colocalization of GLUT-4 (5-nm gold) and cellubrevin (10-nm gold). (*G–H*) Colocalization of GLUT-4 (5-nm gold) and the CD-MPR (10-nm gold). Bar, 100 nm.

partment that is not accessible using the Tf/HRP conjugate. To test this possibility, we conducted localization studies using antibodies specific for the CD-MPR, as this receptor predominantly recycles between the Golgi, late endosomes, and cell surface (Duncan and Kornfeld, 1988).

Antibodies specific for the CD-MPR labeled a subpopulation (~23%) of vesicles isolated from rat adipocytes at an efficiency of 1–11 gold particles per vesicle. Double-labeling experiments revealed that the CD-MPR was partially colocalized with VAMP-2, GLUT-4, and cellubrevin (Table II).

It displayed a relatively high degree of co-localization with GLUT-4 in that 76% of the CD-MPR (+ve) vesicles also contained GLUT-4. However, this only represented 48% of the total GLUT-4 (+ve) vesicles. Only 36% of the VAMP-2 vesicles contained the CD-MPR; therefore, a large proportion of the MPR is excluded from the GLUT-4(+ve)/VAMP-2(+ve) vesicle pool.

Discussion

In this study we have shown that insulin-sensitive cell types express three different synaptobrevin homologues that have previously been shown to be involved in recycling to the cell surface (Schiavo et al., 1992; Südhof et al., 1993; Galli et al., 1994). The two most abundant isoforms, cellubrevin and VAMP-2, are located in intracellular vesicles together with the insulin regulatable glucose transporter GLUT-4 and undergo insulin-dependent movement to the cell surface in adipocytes. Using immuno-EM on intracellular vesicles isolated from adipocytes, we present evidence that GLUT-4 is segregated into distinct intracellular subcompartments as determined by the overlapping yet distinct colocalization with cellubrevin and VAMP-2. These findings were verified using independent biochemical techniques indicating that the segregation of these markers among different vesicles was not due to epitope masking or differential labeling efficiencies of the various antibodies. Incubation of 3T3-L1 adipocytes with Tf/HRP at 37°C for 1–3 h followed by incubation with DAB/H_2O_2 resulted in ablation of >90% of the TfR (Livingstone et al., 1996), >80% of cellubrevin, and ∼40% of GLUT-4 found in the intracellular LDM fraction (Table I). Most of the VAMP-2 (∼90%) and ∼60% of GLUT-4 was recovered in the nonablated fraction. These results suggest that v-SNAREs may be differentially localized within the recycling system in adipocytes and that a large proportion of intracellular GLUT-4, together with VAMP-2, is segregated in a postendocytic compartment.

Previous studies have identified synaptobrevins in insulin-sensitive cells (Cain et al., 1992). However, it was not possible to distinguish between the different synaptobrevin homologues because antibodies against the conserved central domain of VAMP-2 were used to immunoblot adipocyte membranes. We have identified different synaptobrevins in adipocytes by screening a 3T3-L1 adipocyte cDNA expression library with cDNA probes encoding rat brain synaptobrevin isoforms. We opted to use a cDNA library derived from 3T3-L1 adipocytes as opposed to either muscle or adipose tissue to avoid potential contamination with cDNAs of nonparenchymal origin, particularly nervous tissue. Full length cDNA clones encoding cellubrevin, VAMP-1, and VAMP-2 were identified. Isoform-specific antibodies against the extreme amino terminus of each synaptobrevin isoform were used to confirm the expression of different synaptobrevins in 3T3-L1 cells and rat tissues. Although we found no significant change in the levels of cellubrevin protein following differentiation of 3T3-L1 fibroblasts into adipocytes, there was a significant increase in VAMP-2. VAMP-2 expression was also detected in all rat tissues that exhibit insulin-regulated glucose transport, including white adipose tissue, cardiac, and skeletal muscle (Fig. 1). Although the expression of VAMP-2 was highest in brain, which is consistent with its role in SSV exocytosis, sig-nificant expression of the protein was also evident in most other tissues.

Two other studies have recently demonstrated the presence of VAMP-2 (Ralston et al., 1994; Volchuk et al., 1994, 1995) and cellubrevin (Volchuk et al., 1994, 1995) in insulin-sensitive cell types. It has also been shown that insulin stimulates the movement of cellubrevin and VAMP-2 from intracellular vesicles to the plasma membrane in 3T3-L1 adipocytes (Volchuk et al., 1995), which is consistent with the present findings (see Fig. 2). In the study by Volchuk et al. (1995) and in a separate study using PC12 cells (Chilcote et al., 1995; Grote et al., 1995), it was suggested that cellubrevin and VAMP-2 are colocalized in the same intracellular vesicles, making it difficult to discern unique roles for these synaptobrevin homologues. However, in view of the similarity in physico–chemical characteristics of membranes derived from the post-Golgi secretory/endosomal system and because there is a constant flux of membrane through this system, it may be quite difficult to identify discrete subcompartments. It is noteworthy that we have been unable to resolve separate intracellular compartments containing VAMP-2 and cellubrevin using a variety of techniques, including vesicle immunoadsorption (see Fig. 4), velocity sedimentation in sucrose, or equilibrium density sedimentation in either sucrose or glycerol gradients (data not shown). Only by performing immuno-EM on intracellular vesicles isolated from rat adipocytes or by use of the Tf/HRP endosomal ablation technique have we been able to show that while there is overlap in the intracellular distribution of VAMP-2 and cellubrevin, there is also significant intracellular segregation of these two synaptobrevin isoforms. While this conclusion contradicts recent findings in PC12 cells (Chilcote et al., 1995; Grote et al., 1995), it is conceivable that the segregation of these two synaptobrevin isoforms is cell specific.

It has also been difficult to identify a unique intracellular GLUT-4 compartment in insulin-sensitive cells despite the fact that such a compartment has been postulated (Herman et al., 1994; Verhey et al., 1995). A major reason for this is because even in the absence of insulin, there is a substantial proportion of GLUT-4 in the endosomal system (Slot et al., 1991a,b), presumably because the sorting processes that give rise to these vesicles are somewhat inefficient. Similarly, as has been found in the present study (see below), low levels of endosomal recycling proteins are found in GLUT-4 vesicles, once again presumably due to inefficient sorting. These properties have made it difficult to segregate these compartments even using specific techniques such as immunoadsorption with specific antibodies. For example, we have previously found that the overall polypeptide composition of TfR-containing vesicles and GLUT-4–containing vesicles, isolated by immunoadsorption with antibodies specific for each protein, is not significantly different (Hanpeter and James, 1995). Thus, in order to separate GLUT-4 vesicles from recycling endosomes, we have employed two techniques: one which allows us to study the protein composition of individual vesicles and the other which enables us to remove the bulk of recycling endosomes using an endocytic marker. We believe that these studies provide reasonable evidence for the existence of an intracellular GLUT-4 compartment in adipocytes that communicates with the endocytic system.

The immuno-EM data on isolated vesicles and the Tf/HRP ablation data provide a cohesive view of the intracellular distribution of GLUT-4 and other markers in adipocytes. The partial but incomplete overlap between GLUT-4 and cellubrevin or MPR by immuno-EM was consistent with the partial ablation of GLUT-4 compared to these other proteins. Somewhat surprising was the efficiency with which the Tf/HRP conjugate resulted in ablation of the MPR (Livingstone et al., 1996) because these two proteins are thought to traverse distinct trafficking pathways (Van Weert et al., 1995). However, this is not the case in adipocytes as we have previously shown that immunoadsorption of vesicles using a TfR antibody results in efficient coadsorption of the MPR from the LDM fraction isolated from these cells (Hanpeter and James, 1995). Regardless, there is clearly a population of intracellular vesicles in adipocytes that contains as much as 60% of the total GLUT-4 and relatively little (<10%) of the total amount of MPR, cellubrevin, and TfR (Table II). The efficient coisolation of cellubrevin-containing vesicles with an antibody specific for GLUT-4 (see Fig. 4) was somewhat contradictory to this conclusion. This is probably not due to differences in cell type used for these two experiments because we also observed a significant proportion of GLUT-4 (50%) in vesicles that did not contain cellubrevin in 3T3-L1 adipocytes by immuno-EM (data not shown). We have observed similar discrepancies using the vesicle immunoadsorption technique to examine the overlap between GLUT-4 and GLUT-1 in adipocytes (Piper et al., 1991; Robinson and James, 1992). We suspect that these discrepancies arise because of the considerable heterogeneity in the concentration of different proteins among different vesicles. In direct support of this, the heterogeneity in GLUT-4 labeling among different vesicles was quite striking (Table II). Hence, we imagine that the cellubrevin (+ve)/GLUT-4 (−ve) vesicles detected by EM may contain very low levels of GLUT-4 that were sufficient to allow for efficient immunoadsorption but that are not detected by EM. It is highly unlikely that the heterogeneity in labeling per vesicle is an artifact of this technique because it was observed for almost all antigens tested including GLUT-4, MPR, cellubrevin (Table II), and GLUT-1 (data not shown). Hence, as suggested above, it is likely that this variability represents the nature of sorting for membrane proteins in the endosomal sytem. We have attempted to determine if there is a correlation between GLUT-4 density per vesicle and the presence of other markers. However, these analyses have not yet revealed the biological significance of this observation.

What is the nature of this separate intracellular compartment of GLUT-4 in adipocytes? It appears to be distinct from early and late endosomes as depicted by the absence of the TfR, cellubrevin, and MPR (Tables I and II). However, these recycling proteins may be present at low levels in this compartment, indicating that this separate GLUT-4 compartment is a derivative of the endosomal system. This is not surprising because kinetic experiments using reagents that label GLUT-4 at the cell surface have revealed that GLUT-4 constantly recycles both in the absence and presence of insulin (Yang and Holman, 1993). In doing so, it uses the same pathway as other recycling proteins (Robinson et al., 1992; Slot et al., 1991a,b). This postendocytic GLUT-4 compartment is probably not TGR-

derived because TGN38 is excluded from the GLUT-4 compartment (Martin et al., 1994) and relatively low levels of GLUT-4 are found in the TGR in adipocytes (Slot et al., 1991 a,b). In addition, this does not represent newly synthesized GLUT-4 because this compartment persists in cells treated with cycloheximide. This does not exclude the possibility of this being a post-TGR exocytic compartment. Interestingly, we have recently shown that a large proportion of GLUT-4 is targeted to secretory granules in atrial cardiomyocytes (Slot, J.W., S. Martin, and D.E. James, unpublished observation) and that GLUT-4 apparently joins this compartment at the TGR.

In VAMP-2, we have identified another protein constituent of this compartment which happens also to be a constituent of other regulated secretory compartments in a variety of other cell types: SSVs in neurons (Schiavo et al., 1992; Südhof et al., 1993); insulin granules in β cells (Jo et al., 1995; Liebenhoff and Rosenthal, 1995); water channel granules that respond to vasopressin (Regazzi et al., 1995); and zymogen granules in pancreas (Braun et al., 1994). Therefore, it may be predicted that VAMP-2 may serve a similar type of specialized function in adipocytes. Hence, cellubrevin may participate in regulating traffic through the constitutive recycling pathway, whereas VAMP-2 may regulate exocytosis of GLUT-4 from a separate storage compartment. However, in this case we would have predicted a very large insulin-dependent increase in VAMP-2 at the cell surface similar to GLUT-4, but this was not evident from the present studies (see Fig. 2). In fact, the effect of insulin on VAMP-2 movement was very similar to cellubrevin. Alternatively, VAMP-2 may play a role in facilitating the intracellular sequestration of GLUT-4 in the absence of insulin and not in the fusion of these vesicles with the cell surface. This may explain why many of the VAMP-2 (+ve) vesicles also contain cellubrevin. Future studies will be required to dissect the different roles that cellubrevin and VAMP-2 play in the regulated recycling of GLUT-4. Synaptobrevins are known to bind to t-SNAREs, specifically syntaxin (Bennett et al., 1993), and so another question is which syntaxins are these synaptobrevins binding to in adipocytes? We have only been able to identify a single post-Golgi syntaxin isoform in 3T3-L1 adipocytes, syntaxin 4, which is identical to the rat brain isoform (Tellam, J., and D.E. James, unpublished data). This is of considerable interest as VAMP-2 has been shown to bind specifically to syntaxin 1A and 4 but not to syntaxins 2 or 3 (Calakos et al., 1994).

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