The Glucose Transporter GluT4 and Secretory Carrier Membrane Proteins (SCAMPS) Colocalize in Rat Adipocytes and Partially Segregate during Insulin Stimulation*

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SECRETORY CARRIER MEMBRANE PROTEINS (SCAMPS) mark the recycling system for the insulin-responsive glucose transporter, GluT4, in rat adipocytes. Anti-GluT4 and anti-SCAMP antibodies each immunoadsorb vesicles containing both antigens from a low density microsomal fraction that is enriched in both antigens. The immunoadsorbed vesicles also contain VAMPs (synaptobrevins), synaptic vesicle membrane proteins. All three antigens were colocalized in low density microsomal vesicles from both basal and insulin-stimulated adipocytes. The SCAMPS have the same electrophoretic mobility as a major polypeptide detected in GluT4 vesicles. During insulin stimulation, 40% each of GluT4 and VAMPs redistribute from low density microsomes to the plasma membrane fraction; however, <10% of the SCAMPS redistribute. Immunocytochemical staining of adipose tissue shows almost complete coincidence of SCAMPS and GluT4 in the basal state and extensive redistribution of both antigens to the cell periphery during insulin stimulation. Segregation of antigens during stimulation is not as distinct as observed by fractionation, although there are regions at the cell border where the SCAMPS appear more concentrated than GluT4. These data suggest that during insulin stimulation, in contrast to the behavior of GluT4, SCAMPS remain tightly associated with the recycling system.

The maintenance of glucose homeostasis involves the uptake of glucose into fat and muscle in response to circulating insulin. In these tissues, in the basal state, the glucose transporter GluT4 resides in intracellular vesicles that are concentrated in the region of the trans-Golgi network and also more peripherally. Upon insulin stimulation, these vesicles translocate to and fuse with the plasma membrane, redistributing ~40% of the GluT4 to the cell surface and increasing glucose transport into the cells 20–30-fold (Holman et al., 1990; Slot et al., 1991a, 1991b; Smith et al., 1991; Rodnick et al., 1992). The GluT4-containing vesicles have been identified using immunoelectron microscopy (Slot et al., 1991a, 1991b; Rodnick et al., 1992) and purified from low density microsomal fractions using vesicle immunoadsorption with antibodies against the cytoplasmic tail of the GluT4 molecule (Zorzano et al., 1989; Calderhead et al., 1990; Cain et al., 1992; Rodnick et al., 1992).

Translocation of GluT4 to the cell surface in response to insulin resembles regulated exocytosis in specialized secretory cells. Guided by this functional analogy, Cain et al. (1992) have shown recently that the synaptic vesicle protein VAMP(s) (vesicle-associated membrane protein, synaptobrevin) is enriched in the GluT4 vesicles isolated from adipocytes. Brand et al. (1991) have identified a family of secretory carrier membrane proteins (SCAMPS; M, 35,000–40,000) that are found in all regulated secretory carrier membranes. More extensive examination of the SCAMPS has shown that they are present in a wide variety of cells regardless of their specialization for regulated secretion. The SCAMPS are localized in general to membranes (secretory and endocytic vesicles) that share the function of transport to and from the cell surface and thus constitute markers of a general recycling system.

We now show that GluT4 and VAMPs colocalize with the SCAMPS in adipocytes. Using biochemical and immunocytochemical approaches, the coincidence is practically complete in unstimulated cells, and the SCAMPS appear to be major polypeptides of GluT4 vesicles. Interestingly, immunocytochemical observations indicate that both SCAMPS and GluT4 relocate from the perinuclear region to the cell periphery during insulin stimulation. Although colocalization remains extensive upon reorganization of recycling membranes, we detect a partial segregation of GluT4 and SCAMPS that is especially clear using cell fractionation; only GluT4 redistributes appreciably to the plasma membrane fraction. Thus as GluT4 cycles to the cell surface in response to insulin, it may separate in part from the SCAMPS with which it is associated in vesicles.

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* The abbreviations used are: GluT4, insulin-responsive glucose transporter; GluT4 adipose tissue; ECL, enhanced chemiluminescence; HDM, high density microsomal fraction; HSTBS, TBS containing 16% heat-inactivated horse serum; LDM, low density microsomal fraction; M6PR, mannose-6-phosphate receptor; PBS, phosphate-buffered sodium chloride; PM, plasma membrane fraction; SCAMP, secretory carrier membrane protein; TBS, Tris-buffered sodium chloride; TR, transferrin receptor; VAMP, synaptic vesicle-associated membrane protein.

** SCAMPS were originally stated to have apparent M, in the range 31,000–35,000 (Brand et al., 1991). According to more recent studies including adipose cloning (S. H. Brand and J. D. Castle, manuscript in preparation), the estimated range should be revised to 35,000–40,000.

§ Brand, S. H., and Castle, J. D. (1993) EMBO J., in press.
MATERIALS AND METHODS
Antibodies—The affinity-purified rabbit antibodies against the carboxyl-terminal peptide of the rat GluT4 (amino acid residues 491–509) have been previously described (Calderhead et al., 1990). The monoclonal antibody PC9/36 (Brand et al., 1991) was purified from hybridomas culture medium by concentration with a Centricron device, M, cut-off 30,000 (Amicon, Beverly, MA), or purified from ascites on a Protein A/G column (Pierce Chemical Co.). The anti-VAMP antibody was an affinity-purified rabbit antibody prepared against a peptide of rat VAMP 1–2 (Cain et al., 1992). A mouse monoclonal antibody (C8) against rat GluT4 was purchased from Genzyme (Cambridge, MA). A monoclonal antibody against the human transferrin receptor was a gift from Dr. I. Trowbridge (Salk Institute, San Diego, CA), and two polyclonal antibodies against the mannose-6-phosphate/GF-11 receptor were gifts from Drs. D. Measner and S. Koerbling (Washington University, St. Louis, MO) and Drs. C. Scott and R. Baxter (Royal Prince Alfred Hospital, Camperdown, Australia).

Subcellular Fractionation—Adipocytes were prepared from the epididymal fat pads of 6 or 12 male Sprague-Dawley rats (200–225 g) using collagenase digestion (Simpson et al., 1983). In preparations from 12 rats, one half of the adipocytes were treated with 20 ng insulin for 30 min at 37 °C. Cells were washed prior to homogenization in 250 mM sucrose, 20 mM Tris (or 20 mM Hepes), 1 mM EDTA, pH 7.4, and fractionated into four membrane fractions: low density microsomes (LDM), high density microsomes (HDM), plasma membrane (PM), and mitochondria/nuclei as described (Simpson et al., 1983). In some experiments, samples (750 μg of protein) of the LDM were reconstituted in 50 mM Tris-HCl buffer, pH 7.4, and centrifuged at 60,000 g for 90 min in a SW 41 rotor. Samples were homogenized with a 23-gauge needle, and subjected to velocity sedimentation (2 h, 37,000 rpm, SW 41 rotor (Beckman Instruments, Sunnyvale, CA)) on 12 ml, 5–25% glycerol gradients containing 150 mM NaCl, 10 mM Hepes, 1 mM EDTA, and 0.1 mM MgCl₂, pH 7.4 (Chir-O'Grady et al., 1990). Fractions (1 ml) were collected and analyzed by immunoblotting.

Electrophoresis and Immunoblotting—Samples were reduced with 100 mM dithiothreitol and electrophoresed on 12%, 10%, or 7.5% polyacrylamide gels (Laemmli, 1970) and electrotransferred to 0.45-μm nitrocellulose. Parallel lanes were used for parallel samples with primary antibody omitted (again with negligible vesicle binding). The membranes were stained with Ponceau S before they were immersed in Coomassie brilliant blue R-250 for 2 h at room temperature and then incubated for 2–3 h at room temperature. Following washing, by goat anti-rabbit IgG conjugated to 10 nm gold (EY Laboratories, San Mateo, CA) used at a 1:25 dilution (Brand et al., 1991).

Immunochemistry—Immunocytochemical localization of SCAMPS and GluT4 in brown adipose tissue was carried out according to Slot et al. (1986). Rat brown adipose tissue was perfused between 20 and 30 min post-injection, they were perfused initially (for 45 min) with saline solution containing 0.5% formaldehyde and 0.5% glutaraldehyde and then with saline containing 3% sucrose, 100 mM potassium phosphate, pH 7.4, and centrifuged for 6 min at 11,000 × g in a microcentrifuge prior to S. aureus addition. Following incubation, vesicle-S. aureus complexes were washed twice with homogenization buffer with added salt, and all bound proteins were eluted in 133 μl of SDS sample buffer containing 2.5% SDS, 125 mM Tris-HCl, pH 6.8, 10% glycerol, and 8 μl urea. Low density microsomes remaining in the 48,000 × g supernatant after immunoadsorption were recovered by centrifugation at 150,000 × g for 1 h. These membrane pellets were resuspended in 133 μl of SDS sample buffer. Equal volumes of samples were used for immunoblotting.

To titrate immunoadsorption of vesicles from the LDM at limiting concentrations of antibody, 10 μl of 8. aureus cells were coated with 11 μg of rabbit anti-mouse IgG and decreasing amounts of anti-SCAMP antibody (as indicated in Fig. 4) prior to combining with samples of the 48,000 × g supernatant and processing as above. The vesicles remaining in the 48,000 × g supernatant after immunoadsorption were concentrated as described above, and their content of GluT4 and SCAMP was assessed using immunoblotting and the ECL method. A series of antigen-containing standards and duplicate samples were included on all blots.

To examine the polyepitope profile of the GluT4 vesicles, the samples were immunoprecipitated exactly as described (Cain et al., 1992) and used with the nonionic detergent C8E₅ (Calbiochem). Vesicle proteins were stained with colloidal gold (Bio-Rad) after separation by electrophoresis and transfer to nitrocellulose. Parallel lanes were used for immunoblotting with anti-SCAMP.

Vesicle Immunoisolation with Magnetic Beads—Electron microscopy was performed on samples of vesicles (from the 48,000 × g supernatant) that had been adsorbed from supernatant to 300 μg of bead-coated magnetic beads. Bound antigens were eluted with SDS sample containing 2.5% SDS, 125 mM Tris-HCl, pH 6.8, 10% glycerol, and 8 μl urea. Low density microsomes remaining in the 48,000 × g supernatant after immunoadsorption were recovered by centrifugation at 150,000 × g for 1 h. These membrane pellets were resuspended in 133 μl of SDS sample buffer. Equal volumes of samples were used for immunoblotting.

For electron microscopy, pellets were resuspended in SDS sample buffer containing 2.5% SDS, 125 mM Tris-HCl, pH 6.8, 10% glycerol, and 8 μl urea. Low density microsomes remaining in the 48,000 × g supernatant after immunoadsorption were recovered by centrifugation at 150,000 × g for 1 h. These membrane pellets were resuspended in 133 μl of SDS sample buffer. Equal volumes of samples were used for immunoblotting.

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antibody with the wrong secondary antibody. In all controls, no staining above background was observed.

Immunofluorescent images were collected using a Laser Sharp MRC-600 Bio-Rad confocal box adapted to a Nikon Diaphot microscope equipped with a 100× UV apochromatic objective (N.A. 1.3) and a microcomputer outfitted with COMOS software (Bio-Rad). Illumination was provided by an argon/krypton laser. For detection of Cy3, excitation and emission filters were 568 DF10 and 585 EFLP, respectively, and for Cy5, excitation and emission filters were 647 DF10 and 680 EF32, respectively. Most images were recorded with the confocal detector aperture set near its maximum for the low intensity signals. The contrast of the recordings was enhanced using a standard adaptive histogram procedure. Illustrations were prepared using ANALYZE software 5.0.1 (Mayo Foundation, Rochester, MN) using a Sun Sparc2 workstation.

RESULTS

SCAMPs Are Components of Vesicles Containing GluT4 and VAMPS—The SCAMPs are widely distributed among recycling cell surface carriers (Brand et al., 1991). Thus, we investigated whether the SCAMPs are components of the vesicles involved in the translocation of GluT4 to the plasma membrane. The LDM from rat adipocytes, which contains most of the insulin-responsive GluT4 (Cain et al., 1992), also contains the SCAMPs detected immunochemically in the LDM as bands of apparent Mr, 36,000–39,000. As the LDM is a heterogeneous fraction (Zorzano et al., 1989), we assessed the extent to which both SCAMPs and GluT4 (and VAMPs) are present in the same membranes by immunoblotting vesicle fractions that were immunoadsorbed from the LDM. As shown in Fig. 1A, immunoadsorption with anti-GluT4 results in coadsorption of SCAMPs and VAMPs, and immunoadsorption with anti-SCAMP results in the coadsorption of GluT4 and the VAMPs. There is no detectable immunoadsorption of any of these antigens when control nonspecific rabbit or goat anti-mouse IgG is substituted in the procedure. Under conditions in which GluT4 is completely depleted from the LDM by immunoadsorption with anti-GluT4 (Fig. 1A, Super), the SCAMPs and VAMPs are also completely depleted from the LDM. In addition, immunoadsorption of all the SCAMPs using anti-SCAMP completely depletes the LDM of all GluT4 and the VAMPS. These results show that the distributions of SCAMPs, GluT4, and VAMPs in the LDM overlap. There are no sizable pools of SCAMPs or VAMPS in vesicles that do not contain enough GluT4 to allow immunoadsorption, and the converse applies to GluT4 and VAMPs with regard to immunoadsorption by the SCAMPs.

During insulin stimulation, GluT4 is redistributed to the cell surface yet continues to recycle through the endosomal system (Slot et al., 1991a; Jhun et al., 1992). We repeated the immunoadsorption experiments on the LDM prepared from insulin-treated adipocytes (Fig. 1B). Immunoadsorption of GluT4 still resulted in the complete depletion of the SCAMPs and VAMPS present in the LDM (Fig. 1B, Super). Similarly, immunoadsorption with anti-SCAMP still completely depleted the LDM of GluT4 and VAMPS. These data indicate that the SCAMPs, GluT4, and VAMPS that remain in the LDM during insulin stimulation are colocalized.

Distribution of Antigens among Vesicles in the LDM—Immunoadsorption of vesicles is an efficient and sensitive procedure and may result from the interaction of only a few antigen and antibody molecules. It is possible that adsorbed vesicles are heterogeneous, containing subpopulations that are highly enriched in SCAMPs as compared to GluT4 and vice versa. Three different experiments were performed to check for subpopulations of SCAMP-rich, GluT4-poor vesicles in the LDM: vesicle immunoadsorption from the LDM using limiting amounts of antibody to bind the potential SCAMP-rich vesicles selectively, velocity gradient sedimentation of the LDM to compare antigen distributions among different-sized vesicles, and immunocytochemical labeling of immunoadsorbed vesicles.
with an excess of goat anti-mouse vesicles remaining in the 48,000 of the LDM from one rat) were incubated with 10 μl of S. aureus coated with an excess of goat anti-mouse IgG and 0–2 μg of anti-SCAMP. The vesicles remaining in the 48,000 x g supernatant after immunoprecipitation were recovered by centrifugation at 100,000 x g and assayed for the SCAMPs and GluT4 by Western blotting. Vesicle proteins were resolved by electrophoresis on 10% (GluT4) or 13% (SCAMP) gels, transferred to nitrocellulose, and visualized using the ECL method. Duplicate samples and antigen standard curves (0 μg of antibody) were run on each gel; three or four different exposures of each gel were quantitated. The averages and standard deviations of the different exposures of duplicate samples from a single gel are shown. The results presented are representative of three separate experiments.

The finding that there was little or no effect of insulin on the relative distributions of GluT4 and the VAMPS between the more slowly and more rapidly sedimenting vesicles of the LDM, they are SCAMP-richeGluT4- and VAMP-poor compartments in the LDM, they are quite as was the case for GluT4 and VAMP antigens (which are not visible in the protein staining pattern).

**Subcellular Distribution of GluT4 and SCAMPs**—In view of the well established redistribution of both GluT4 and the VAMPS in response to insulin (Cain et al., 1992), we were quite...
interested by the observation that insulin did not appear to alter the levels of the SCAMPs in the LDM (Fig. 5). Consequently, we compared the distribution of the SCAMPs among all adipocyte fractions in the absence and presence of insulin stimulation. Examination of the same proportion of each fraction by immunoblotting showed that, as expected, the SCAMPs are mostly located in the LDM and are found at low levels in the HDM and PM fractions while being negligible in the mitochondrial/nuclear and cytosol fractions (Fig. 6a). Notably, neither the distribution nor the total amount of detectable antigen appear to change significantly with insulin stimulation.

SCAMP and GluT4 levels in the LDM and PM of control and insulin-stimulated samples are compared in Fig. 6 (b and c) on immunoblots of equal protein loads. The distributions of both antigens in these two fractions were quantitated for six different parallel fractionations of basal and insulin-treated adipocytes. The SCAMP distribution was affected only slightly by insulin stimulation as compared to the GluT4 distribution. The corresponding gold stained bands represent major polypeptides in the GluT4 vesicle profile. Molecular weights (×10^3) are indicated at left.

**Fig. 4.** Immunoadsorption and immunocytochemical labeling of vesicles on antibody-coated magnetic beads. Magnetic beads coated with anti-SCAMP (a and c-f; identified by SC on the bead) or an anti-GluT4 antibody (anti-GluT4 for b; monoclonal antibody 1F8 for g and h, both identified by G4 on the bead) were incubated with LDM-containing 48,000 × g supernatant from basal or insulin-stimulated (+I adipocytes. Proteins were eluted from S. aureus (-) or insulin-stimulated (+) adipocytes. Proteins were eluted from S. aureus with CaCl2, electrophoresed on a 10% gel, and transferred to nitrocellulose. Gold stained samples and immunoblotted samples represent the HDM and PM fractions while being negligible in the LDM (mitochondrial/nuclear) and cytosol (CYTO) from the adipocytes of a single rat were loaded. Protein loads were 38 µg (WCH), 13.5 µg (CYTO), 10 µg (LDM), 10 µg (PM), 12.4 µg (HDM), and 28 µg (MITO). Bands were visualized using the ECL method. B and C, immunoblots of LDM and PM (equal protein loads) comparing the content of GluT4 (B) and SCAMPS (C) before and after insulin stimulation. Bands were visualized using 125I-secondary antibody and autoradiography.

**Fig. 5.** Polypeptide composition of GluT4 vesicles. Comparative colloidal gold staining (left panel) and immunoblotting with anti-SCAMP (right panel) of GluT4-containing vesicles (G) isolated by immunoadsorption from the 48,000 × g supernatant from basal or insulin-stimulated (+) adipocytes. Proteins were eluted from S. aureus with CaCl2, electrophoresed on a 10% gel, and transferred to nitrocellulose. Gold stained samples and immunoblotted samples represent 75 and 11% respectively, of the immunoadsorbed fractions of the LDM from one rat. For comparison, protein staining is shown for total vesicle proteins (LDM; 2.5% of the sample) and proteins immunoadsorbed with nonspecific rabbit IgG (C). SCAMPS are identified by arrowheads, and the corresponding gold stained bands represent major polypeptides in the GluT4 vesicle profile. Molecular weights (×10^3) are indicated at left.

**Fig. 6.** Subcellular distribution of the SCAMPS and GluT4 in basal and insulin-stimulated rat adipocytes. Immunoblot showing the distribution of SCAMPS in subcellular fractions from basal or insulin-stimulated (+) adipocytes. A, proportional distribution of SCAMPS in which 7% of the total protein in each membrane fraction (LDM, PM, HDM, MITO) is represented and 1.4% of the whole cell homogenate (WCH) and cytosol (CYTO) from the adipocytes of a single rat were loaded. Protein loads were 38 µg (WCH), 13.5 µg (CYTO), 10 µg (LDM), 10 µg (PM), 12.4 µg (HDM), and 28 µg (MITO). Bands were visualized using the ECL method. B and C, immunoblots of LDM and PM (equal protein loads) comparing the content of GluT4 (B) and SCAMPS (C) before and after insulin stimulation. Bands were visualized using 125I-secondary antibody and autoradiography.
redistribution of the SCAMPs in response to insulin as assayed by subcellular fractionation is significantly less.

**Immunofluorescent Localization of SCAMPs and GluT4**

*Fig. 7. Distribution of SCAMPs (C-SC) and GluT4 (C-G4) in unstimulated brown adipose tissue by double-label indirect immunofluorescence. Colocalization of SCAMPs, detected with Cy3, and GluT4, detected with Cy5, at intracellular (often perinuclear) sites. Arrows identify a clear exception to colocalization where GluT4 is absent from a perinuclear region of concentrated SCAMPs. Nuclei (n); bar, 5 μm.*

*Fig. 8. Comparative distribution of SCAMPs (SC) and GluT4 (G4) as viewed by indirect immunofluorescence in unstimulated and insulin-stimulated brown adipose tissue. C-SC and C-G4, unstimulated (control) cells showing extensive concentration and colocalization of antigens at perinuclear sites that are removed from the capillary lumen (I; I-SC and I-G4, insulin-stimulated sample showing extensive redistribution of both antigens to the cell periphery. Brackets identify sites at or near the adipocyte surface where SCAMP staining appears more focally concentrated than that of GluT4. Nuclei (n); bar in lower right panel corresponds to 5 μm.*

The distributions of SCAMPs and GluT4 were compared by immunocytochemistry in brown adipose tissue. Brown adipose tissue exhibits the same insulin-stimulated translocation of GluT4 to the plasma membrane as white adipose tissue (Slot et al., 1991a) and can be experimentally depleted of stored triglyceride, enabling examination of antigen distribution by indirect immunofluorescence. As shown in Figs. 7 and 8, the distributions of the two antigens in unstimulated tissue are nearly identical. Both are focally concentrated at intracellular, often perinuclear, sites having a reticular appearance. These sites are mostly located at a visible distance from cell borders. Notably, occasional examples have been found where SCAMPs but not GluT4 are highly concentrated (Fig. 7), attesting to the specificity of antibody staining. The differences may stem from either the broader distribution of SCAMPs among different cell types (Slot et al., 1990; Brand et al., 1993) or possibly the presence of occasional adipocytes with very attenuated expression of GluT4.

In insulin-stimulated adipocytes, both GluT4 and SCAMPs undergo a striking and parallel redistribution to the cell periphery such that much of the staining is concentrated at or near surfaces that frequently border the capillary endothelium (Fig. 8). This dramatic redistribution of GluT4 has been reported previously in brown fat (Slot et al., 1991a). In all specimens examined, the staining of SCAMPs and GluT4 showed the same overall pattern, although in certain cell profiles, SCAMP staining at the cell border was more focally concentrated than that of GluT4. This local difference may be significant; the more diffuse GluT4 staining may be indicative of a broader cell surface distribution. This may be the basis for the partial segregation of GluT4 and the SCAMPs that is observed by fractionation of insulin-treated cells (Figs. 5 and 6; see “Discussion”).

**DISCUSSION**

**Implications from Colocalization of the SCAMPs and GluT4**

Our findings regarding the presence of SCAMPs (and
VAMPs) in adipocytes and their codistribution with GluT4 provide clear support for the idea that the insulin-regulated carriers of GluT4 share some of the same functional machinery as secretory vesicles in regulated secretory cells. Not only does this relationship extend to synaptic vesicles as recognized previously (Cain et al., 1992), it also now extends to protein storage granules of exocrine and endocrine cells. Moreover, the similarity to the latter organelles is increased by the observation of at least two sizes of SCAMPs in GluT4 vesicles rather than the single-sized M, 37,000 form(s) observed in synaptic vesicles (Brand et al., 1991).

By drawing analogies between insulin-responsive GluT4 carriers and stimulus-regulated secretory vesicles, we are not implying that GluT4 in unstimulated adipocytes is entirely concentrated in discrete stimulus-regulated carriers in the same way that synaptic vesicle marker proteins are concentrated in synaptic vesicles in neurons. Instead, the almost completely coincident localization of GluT4 with the SCAMPs suggests a different situation. The SCAMPs have recently been identified as markers for a general cell surface recycling system as broadly defined by Cameron et al. (1991). This system encompasses endosomal pathways and also includes regulated secretory pathways as derivatives in specialized cells. Accordingly, if SCAMPs delineate this system in adipocytes, then GluT4 also delineates this system, and its distribution may include not only regulated transport compartments but also components of constitutive recycling pathways. This view is supported by the findings presented in Fig. 3 showing that GluT4 and the SCAMPs (and the VAMPs) coseident with the TR and M6PR in rapidly sedimenting vesicles in addition to sedimenting in a slower peak in vesicles that appear to lack (or are relatively depleted of) these constitutively recycling receptors. Interestingly, the distribution on glycerol gradients of GluT4 and the SCAMPs (and the VAMPs) relative to that of the TR (Fig. 3) is quite similar to the distribution of synaptophysin relative to that of the TR in PC12 cells (Cameron et al., 1991). Thus GluT4/SCAMP/VAMP-rich, TR-poor membranes in adipocytes may be analogous to synaptic-like microvesicles in neuroendocrine cells. Although regulated exocytosis of synaptic-like microvesicles has not been demonstrated as yet, insulin-stimulated export of GluT4 to the adipocyte cell surface may occur from such a compartment.

Constitutive export of TR and M6PR may involve a different route within the general recycling system that also would contain some SCAMPs and GluT4. Recently, evidence for constitutive recycling of GluT4 in adipocytes has been reported (Jhun et al., 1992). It should be noted that there is evidence that in adipocytes, insulin also increases the rate constant for exocytosis of constitutively recycling cell surface proteins (Tanner and Lienhard, 1987) and at least one constitutively secreted protein (Kitagawa et al., 1989). This effect may account for the smaller increases in cell surface TR, M6PR, and GluT1 (relative to GluT4) caused by insulin (Davis et al., 1986; Tanner and Lienhard, 1987, 1988; Appell et al., 1988; Zorzano et al., 1989) and suggests that regulated export extends beyond the GluT4 route.

An alternative, equally plausible proposal for the way in which GluT4 reaches the cell surface has been described by Robinson and James (1992). They suggest that in response to insulin, vesicles enriched in GluT4 (presumably corresponding to the less rapidly sedimenting ones lacking regulated secretory receptors; see Fig. 3) may fuse with the endosomal compartment rather than the plasma membrane. In this scenario, the GluT4 then proceeds to the plasma membrane from the endosomes via the constitutive recycling pathway, which, as noted above, is also stimulated by insulin.

Insulin-stimulated Trafficking of the SCAMPs and GluT4

—Given the codistribution of the SCAMPs and GluT4 in unstimulated adipocytes, their distinct distributions from one another following insulin stimulation (Fig. 6) are especially interesting. We believe that the different distributions arise by partial segregation of the SCAMPs from GluT4 when trafficking to the cell surface is increased. Several alternative explanations seem much less likely. First, segregation probably is not an artifactual reflecting underdetection of SCAMPs in the plasma membrane fraction during stimulation since the total SCAMP level in all fractions is unchanged upon stimulation (Fig. 6). Second, SCAMP relocation from plasma membranes via dissociation (as observed for the GTP binding protein rab 3a during exocytosis of synaptic vesicles; Fischer von Mollard et al. (1991)) is very unlikely because sequencing predicts that the SCAMPs are transmembrane proteins. Third, we have found no evidence for vesicle subpopulations that are separately highly enriched in GluT4 and SCAMP and could differ in their sensitivities to insulin, using either immunoprecipitation, velocity gradient sedimentation, EM immunogold labeling of isolated vesicles, or immunofluorescent staining of whole cells. These methods are unlikely to detect vesicles that only contain proportionately more SCAMPs than GluT4 as the result of partial segregation during insulin stimulation.

Although presently we cannot rule out the possibility that partial segregation of the SCAMPs and GluT4 during insulin stimulation occurs intracellularly, our prediction is that it arises from sorting at the cell surface involving more efficient sequestration and reinternalization of the SCAMPs than of GluT4. As a consequence, we expect that proportionately more SCAMPs than GluT4 will be concentrated in coated pits, coated vesicles, and early endosomal elements near the cell surface. Since the latter fractionate as part of the LDM fraction, the extent of translocation of SCAMPs is less than that of GluT4. The co-redistribution of GluT4 and the SCAMPs to the cell border in insulin-treated brown fat, as assessed by immunofluorescence, strengthens our belief that both proteins cycle to the cell surface; however, the extensive colocalization appears in disagreement with the results from subcellular fractionation of white fat. Rather than attribute the difference to the type of fat used (because both brown and white adipocytes respond similarly to insulin) (Zorzano et al., 1989; Calderwood et al., 1990; Slot et al., 1991a; Smith et al., 1991; Cain et al., 1992), we suspect that the difference is only an apparent one arising from the substantial differences between the two methodologies (fixation and immunofluorescence versus subcellular fractionation) that limit the direct comparison. For immunofluorescence, fixation of cells acutely interrupts membrane trafficking during insulin stimulation and largely preserves spatial relationships in situ, but for two proteins that are thought to be recycling at different rates, local concentration differences indicative of partial segregation may not be detected readily. Specifically, GluT4 is likely to be partially concentrated in coated pits (Robinson et al., 1992), and as described above, we suspect that the extent of concentration will be even greater for the SCAMPs. The GluT4 concentrated in coated pits will give a stronger signal in immunofluorescence than the more diffuse GluT4 in the uncoated regions of the membrane. As a result, the co-localization of GluT4 and SCAMPs will appear greater than it really is. Additionally, our immunofluorescence method is not sufficiently resolving to distinguish clearly SCAMPs in coated vesicles and endosomal membranes close to the plasma membrane from SCAMPs actually in the plasma membrane. As noted with Fig. 8, SCAMP staining at the cell border sometimes appeared more concentrated than that of GluT4; this finding is consistent with the SCAMPs being proportionately more concentrated in vesicles.
bordering the plasma membrane (and thus partially segregated from GluT4). Although cell fractionation has enabled identification of partial segregation of SCAMP and GluT4, it can disrupt associations that exist in intact cells. Thus vesiculated microdomains of the plasma membrane containing co-concentrated SCAMPS and GluT4 may not fractionate with the PM fraction. Alternatively, such microdomains might bud from plasma membrane fragments during homogenization at ambient temperature (which is used to prevent congealing of triglyceride). Reduced relocation of antigens to the PM fraction would be the consequence in either case, and the effect would be greater on the SCAMPS if they are more efficiently concentrated within the cell surface. The finding that insulin-stimulated GluT4 translocation to the plasma membrane is higher when measured in intact cells than by cell fractionation (Zorzano et al., 1989; Calderhead et al., 1990; Holman et al., 1990; Cain et al., 1992) may be a relevant illustration of the limitation of the fractionation approach. We emphasize, however, that none of the procedural limitations we have discussed affects our basic observation that the SCAMPS and GluT4 become partially segregated from one another during insulin stimulation. When we have obtained antibodies against the SCAMPS that are suitable for EM immunocytochemistry, it will be possible to compare the distributions of SCAMPS and GluT4 at the cell border and to search for a direct morphological correlate of the partial segregation.

In conclusion, the partial segregation of the SCAMPS from GluT4 and the VAMPs/synaptobrevin upon exposure to insulin suggests that the SCAMPS are more rapidly internalized. Because of this property and because of the widespread distribution of SCAMPS among different cell types and their association with the general recycling system, it may be that the SCAMPS perform an important function in the trafficking process itself. A major challenge is to elucidate this function.

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