Sortilin Is a Major Protein Component of Glut4-containing Vesicles*

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In fat and skeletal muscle cells, glucose transporter isoform 4 (Glut4) is translocated to the cell surface in response to insulin via a system of specialized recycling vesicles. Besides Glut4, these vesicles include the novel insulin-regulated aminopeptidase, receptors for insulin-like growth factor-II/Mann-6-phosphate and transferrin, and a glycoprotein with the molecular mass of 110 kDa. We report here by the criteria of the partial protein sequencing and subsequent cDNA cloning that this glycoprotein, 110, the last unidentified major protein component of Glut4-containing vesicles, is sortilin, a novel type 1 receptor-like protein recently cloned from human brain (Petersen, C. M., Nielsen, M. S., Nykjar, A., Jacobsen, L., Tommerup, N., Rasmussen, H. H., Roiggaard, H., Gliemann, J., Madsen, P., and Moestrup, S. K. (1997) J. Biol. Chem. 272, 3399–3605). This protein is highly expressed in fat, brain, and lung and is dramatically up-regulated during differentiation of adipocytes in vitro.

The regulation of blood glucose levels by insulin in mammals is achieved by the hormone-dependent movement of the fat and muscle-specific glucose transporter, Glut4, from an intracellular storage vesicle to the cell surface (1–4). As an approach to understand the mechanisms underlying this process, we and others have isolated these vesicles using anti-Glut4 antibodies and have analyzed their protein content by several techniques. Thus, immunosolation of Glut4-containing vesicles following cell surface biotinylation in the presence of insulin revealed three major component proteins in these vesicles (gp230, gp160, and gp110)1 that corresponded to major silver staining vesicular proteins present in the basal state (no insulin) (5). These proteins bind to wheat germ agglutinin-agarose and can be detected in an overlay assay with labeled wheat germ lectin, and therefore they are glycoproteins (5). Recently, we were able to detect an additional protein, gp180, which follows the same trafficking pathway as the former proteins but represents a relatively minor component of Glut4 vesicles. We and others have studied these proteins and have identified gp160 as a novel insulin-regulated aminopeptidase, or IRAP (6, 7), gp230 as the IGF-II/Mann-6-P receptor (8), and gp180 as the transferrin receptor.2 Here, we report the identification of gp110, apparently the last major component protein of Glut4-containing vesicles, as the recently described putative sorting protein/receptor, sortilin (9).

MATERIALS AND METHODS

Adipocyte Fractionation and Isolation of Glut4-containing Vesicles—Adipocytes were isolated from the epididymal fat pads of male Sprague-Dawley rats (150–175 g) by collagenase digestion and fractionated into subcellular fractions by differential centrifugation according to Simpson et al. (10). Light microsomes were immunosolated on monoclonal anti-Glut4 antibody, 1F8 (11), and covalently immobilized on Reacti Gel GF 2000 (Pierce), and the bound material was eluted with 1% Triton X-100 in phosphate-buffered saline. Nonspecific adsorption was controlled by passage of microsomes over total mouse IgG (Sigma) immobilized on the same beads.

Isolation of Rat Sortilin cDNA Clone—A peptide excised from purified p110 (peptide 2 in Fig. 1) was found to be 93.7% identical to the translated peptide of Homo sapiens cDNA clone 249708 (accession number X85743) derived from a normalized human expressed sequence tag (EST) cDNA library. This clone was purchased from Genome Sequencing Center at Washington University School of Medicine, and the whole insert of the clone (1380 base pairs) was sequenced. The sequenced insert was found 99.5% identical to Human sortilin (9) (accession number X98248). This sequence was used as a probe to screen rat skeletal muscle cDNA library. All probes were labeled by random priming using the Klenow fragment of DNA polymerase (Promega) and [α-32P]dCTP (NEN Life Science Products). The DNA insert of Homo sapiens cDNA clone 249708 was excised with Xhol/Nofl digestion, labeled with [α-32P]dCTP by random priming, and used to screen 5' stretch cDNA libraries from skeletal muscle (primed with oligo(dT) + random primers, respectively) of adult male Sprague-Dawley rats (CLONTECH). Seventy-150 mm agar plates each containing 50,000 phage plaques were transferred to nylon filter disks (NEN Life Science Products) and hybridized to probe as described in manufacturer's instruction. Positive clones were picked and rescreened until single clones were obtained. Lambda DNA clones were purified by Nucleobond AX (The Nest Group, Inc.) and directly sequenced using gt11 primers and synthetic oligonucleotide primers corresponding to the gene-specific sequence.

Cell Culture—3T3-L1 preadipocytes were maintained in growth medium consisting of Dulbecco’s modified Eagle’s medium with 10% fetal calf serum (Intergen, Co.) and were induced to differentiation as described previously (12). Adipogenesis was induced by feeding with fresh medium containing 10% fetal bovine serum (FBS), 0.5 mM methyisobutylxanthine, 1 µM dexamethasone, and 5 µg of insulin/ml for 48 h. The cells were subsequently maintained in medium containing 10% FBS and 2.5 µg of insulin/ml for an additional 48 h and were refeed every 2 days with 10% FBS medium.

RNA Isolation and Analysis—Total RNA was isolated from rat tissues or 3T3-L1 cells as described by Chomczynski and Sacchi (13). Tissues were homogenized in solution D (4 µg guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% Sarkosyl, 1 mM 2-mercaptoethanol). Cells were washed twice with ice-cold phosphate-buffered saline and lysed with solution D. The lysate was extracted with acidic phenol-chloroform and then subjected to an isopropanol precipitation at −20 °C. Poly(A)+ RNA was selected using a oligo(dT) cellose (Type 3, Collaborative Biomedical Products) according to the manufacturer’s instructions. For Northern blot analysis, 20 µg of total RNA or 5 µg of poly(A)+ were separated on formaldehyde agarose gels and transferred to Gene Screen Plus Plus by capillary transfer (NEN Life Science Products).
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RESULTS

As discussed in the Introduction, the polypeptide composition of Glut4-containing vesicles as revealed by the silver staining and/or biotinylation of the material specifically bound to 1F8-beads consists of three major glycoproteins that we have called gp230, gp160, and gp110 (5, 6, 16). The first two are the IGF-II/Man-6-phosphate receptor (8) and IRAP, respectively (6, 17). To identify gp110, we isolated adipocytes from 60 rats, one of the sequenced peptides, namely the 16-mer NECSL-HHASISISQK (peptide 2 in Fig. 1), was synthesized in vitro with several lines of differentiating myoblasts (not shown).

FIG. 1. Identification of gp110 as rat sortilin. The figure shows the alignment of the amino acid sequences of human sortilin (top) and rat gp110. Three fragments obtained by protein sequencing of purified gp110 are in bold type. Peptide 2, used as an antigen for immunization, is underlined. Hyphens indicate the amino acid residues that are identical to those found in rat gene, and gaps are added for optimal alignment.

After ultraviolet+ cross-linking, filters were prehybridized, hybridized, and subjected to analysis as described previously (14). For rehybridization, the probe was stripped from membrane by washing the membrane in boiled 0.1% SDS, 2x SSC, 0.1% SDS twice, each for 10 min. The cDNA utilized in these studies were: sortilin (rat) and Glut4.

Gel Electrophoresis and Immunoblotting—Protein samples were separated in SDS-polyacrylamide gels according to Laemmli (15) and transferred to Immobilon-P membrane (Millipore) in 25 mM Tris, 192 mM glycine, and subjected to analysis as described previously (14). For rehybridization, the probe was stripped from membrane by washing the membrane in boiled 0.1% SDS, 2x SSC, 0.1% SDS twice, each for 10 min. The cDNA utilized in these studies were: sortilin (rat) and Glut4.

FIG. 2. Northern blot analysis of sortilin's expression. A, poly(A)+ RNA from male Sprague-Dawley rat tissues was isolated as described under "Materials and Methods." After electrophoresis in 1% formaldehyde agarose gel and transfer to nylon membrane, RNA was hybridized with specific probes. Each lane contains 5 μg of total RNA. B, confluent 3T3-L1 preadipocytes were induced to differentiate as described under "Materials and Methods." Lane 1 corresponds to preadipocytes; lanes 2 and 3 correspond to 2 and 7 days after induction of differentiation, respectively. Total RNA was isolated from the 3T3-L1 cells (bottom panel), and 20 μg of total RNA was analyzed by Northern blot with specific probes (top panels).

Chem laboratory (Fig. 1). The sequence of peptide 2, a 16-mer, was found in the EST data base with one amino acid difference. The corresponding human clone was purchased, sequenced, and used as a probe to screen a rat skeletal muscle cDNA library as described under "Materials and Methods." Nine independent clones were isolated, and sequencing of the largest one yielded 1872 base pairs in the coding region of the rat cDNA with a predicted protein sequence having 93% identity to the EST sequence and subsequently to the recently published human sortilin clone (Fig. 1) (9).

The rat tissues expressing sortilin were determined by Northern blot using human (not shown) and rat sortilin cDNA (Fig. 2A) giving identical results. Sortilin is highly expressed in lung, fat, and brain and to a lesser extent in muscle and heart and is practically absent from liver. During 3T3-L1 adipocyte differentiation, sortilin is not expressed in the preadipocytes but is dramatically induced during differentiation of these cells, along with Glut4 (Fig. 2B). Similar results were obtained with several lines of differentiating myoblasts (not shown).

One of the sequenced peptides, namely the 16-mer NECSL-HHASISISQK (peptide 2 in Fig. 1), was synthesized in vitro, coupled to keyhole limpet hemocyanin, and used as an antigen for immunization of two rabbits. As shown in Fig. 3, these antisera recognize a protein in Glut4-immunoadsorbed vesicles of M, 110,000. As a function of time of insulin exposure, this protein is depleted from Glut4-containing vesicles to the same extent as Glut4 (Fig. 3). This result is consistent with our earlier data showing insulin-dependent translocation of gp110/sortilin from Glut4 vesicles to the plasma membrane obtained using the independent technique of cell surface biotinylation (5). On the other hand, we were unable to directly confirm sortilin translocation by blotting plasma membrane and light microscopic fractions from insulin-treated and untreated adipo-
Sortilin is a major component of Glut4-containing vesicles. Freshly isolated rat adipocytes were incubated with or without 10 nM insulin for the indicated times and fractionated as described under “Materials and Methods.” Light microsomes (120 μg) were immunoadsorbed with 1F8 and nonspecific IgG beads, and the eluted material was analyzed by Western blot with anti-sortilin serum and 1F8 antibody.

During the course of our efforts to purify and clone gp110 from rat Glut4-containing vesicles, Petersen et al. (9) described the purification from human brain and the cDNA cloning of what they called sortilin. As shown in Fig. 1, gp110 is rat sortilin. What then is sortilin, and what is it doing as a companion of Glut4? Sortilin is a novel type I receptor-like protein that has a significant homology in its extracellular/lumenal portion with the vacuolar sorting receptor from yeast Vps10p, hence the name, sortilin. Interestingly, human sortilin has nine amino acids in its cytoplasmic tail identical to analogous regions of the cation-dependent Man-6-P receptor and the cation-independent IGF-II/Man-6-P receptors (9). This sequence contains well known targeting motifs, and therefore it is likely to be responsible for the co-localization of sortilin and the IGF-II/Man-6-P receptor in the same membrane compartments, including Glut4-containing vesicles (8). Because sortilin’s extracellular domain suggests it may be a receptor of some kind, it is possible that it serves an analogous function to that of the mannose-6-phosphate receptor in fat cells. The latter protein may function in Glut4-containing vesicles to clear IGF-II from the circulation, and sortilin may serve to clear as yet unknown circulating protein(s)/ligands.

A second possibility is that the presence of sortilin may be essential for the regulated translocation of Glut4-containing vesicles via its phosphorylation. Petersen et al. (9) found a potential phosphorylation site in the cytoplasmic C-terminal portion of sortilin in the region of high homology with the cation-dependent and cation-independent Man-6-P receptors. We have recently shown that gp110 in Glut4-containing vesicles may indeed be phosphorylated by an unidentified vesicle-associated protein kinase in an insulin-dependent manner. Although the biological effect of this phosphorylation is not yet known, it may mediate interaction of sortilin with adaptor complex, as is the case with Man-6-P receptors.

A third possibility is that sortilin may be involved in the biogenesis of vesicles in which it resides. Sortilin was purified from brain via its interaction with receptor-associated protein (RAP). RAP is a luminal protein of the endoplasmic reticulum and Golgi apparatus with chaperone-like functions, and it interacts with a variety of receptors (20, 21). The ability of sortilin to interact with RAP (9) suggests that RAP may play a role in the formation and functioning of Glut4-containing vesicles in insulin-sensitive fat and skeletal muscle cells. This remains to be determined, and RAP has never been studied in these cell types or with this perspective. If RAP has some particular role in fat cells, it is more likely in biogenesis of Glut4-containing vesicles rather than in regulation of their translocation.

Obviously, a more defined role in biology for sortilin awaits its more detailed study, particularly with regard to the identification of a putative ligand. Because it is very abundant in the brain, it will be intriguing to explore localization of this protein in this tissue and, more specifically, its potential presence in synaptic vesicles. Our preliminary data (not shown) suggest that this indeed may be the case. If so, this will provide another interesting parallel between the molecular composition and functioning of Glut4-containing and synaptic vesicles and may shed light on the biological role of sortilin.

REFERENCES


3 K. V. Kandror and P. F. Pilch, unpublished data.