Sortilin Is the Major 110-kDa Protein in GLUT4 Vesicles from Adipocytes

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Vesicles containing the glucose transporter GLUT4 from rat adipocytes contain a major protein of 110 kDa. We have isolated this protein, obtained the sequences of peptides, and cloned a large portion of its cDNA. This revealed that the protein is sortilin, a novel membrane protein that was cloned in another context from a human source while this work was in progress. Subcellular fractionation of rat and 3T3-L1 adipocytes, together with GLUT4 vesicle isolation, showed that sortilin was primarily located in the low density microsomes in vesicles containing GLUT4. Insulin caused a 1.7-fold increase in the amount of sortilin at the plasma membranes of 3T3-L1 adipocytes, as assessed by cell surface biotinylation. The expression of sortilin in 3T3-L1 cells occurred only upon differentiation. Previous characterization of sortilin has led to the suggestion that it functions to sort luminal proteins from the trans Golgi. The significance of its insulin-stimulated increase at the cell surface and of its expression upon differentiation will require definitive delineation of its function.

Insulin stimulates the transport of glucose in fat and muscle cells by causing the translocation of the glucose transporter GLUT4 from an intracellular location to the plasma membrane (1). The basis for this effect is largely an enhancement of the rate constant for externalization of GLUT4 by insulin (2, 3). Intracellular GLUT4 is most probably located in both endosomes and specialized insulin-regulatable secretory vesicles (4, 5). The enhanced rate constant for externalization is likely to reflect in part or entirely the enhanced fusion of these insulin-regulatable vesicles with the plasma membrane (6, 7).

To search for other proteins that undergo translocation in response to insulin, we and others have undertaken the characterization of other proteins in the intracellular vesicles containing GLUT4 (8). This approach led to the discovery of a novel membrane aminopeptidase, now referred to as the insulin-regulated aminopeptidase (IRAP) (9, 10). IRAP is entirely colocalized with GLUT4 in adipocytes and, like GLUT4, undergoes marked translocation in response to insulin (5, 11, 12).

In the present study we have partially cloned and then characterized another major novel protein of 110 kDa present in the GLUT4-containing intracellular vesicles from rat adipocytes. While this work was in progress, the homologous human protein was cloned in another context and named sortilin (13). The function of sortilin has not been established. However, the fact that it has a cytoplasmic carboxyl-terminal sequence highly homologous to that of the cation-independent mannose 6-phosphate receptor (CIM6PR) and a luminal domain homologous to the yeast vacuolar sorting protein 10, together with the finding that it appears to be colocalized with the CIM6PR in COS cells, suggest that sortilin is a receptor involved in sorting luminal proteins from the trans-Golgi to late endosomes (13). Here we show that in adipocytes sortilin is largely located in vesicles containing GLUT4 and that in response to insulin sortilin translocates to the plasma membrane, although not as markedly as does GLUT4 or IRAP.

EXPERIMENTAL PROCEDURES

Antibodies—A rabbit antiserum was raised against the entire luminal domain of human sortilin expressed as a recombinant protein, and the immunoglobulin fraction was isolated from the serum. The details of this preparation will be described elsewhere. A rabbit antiserum was generated against the carboxy-terminal peptide of rat sortilin (terminal 14 amino acids with Cys at the amino terminus of the peptide) conjugated via the Cys to keyhole limpet hemocyanin. The anti-peptide antibodies were affinity-purified on the peptide conjugated to SulfoLink (Pierce) as described in Ref. 14. The antibodies against the sortilin luminal domain were used throughout for immunoblotting, with the exception of the immunoblot in Fig. 6, which was performed with these antibodies (not shown) as well as the ones against carboxy-terminal peptide (shown). The antibodies against GLUT4 and IRAP were the affinity-purified rabbit antibodies against the carboxy-terminal 19 amino acids of GLUT4 and the entire cytoplasmic domain of IRAP, respectively, described previously (10, 15).

Adipocytes—Rat adipocytes were obtained by collagenase digestion of epididymal fat pads, as described in Ref. 16. 3T3-L1 fibroblasts were cultured and differentiated into adipocytes as described in Ref. 17.

Purification and Peptide Analysis of Sortilin—The procedures used here have been described in detail previously (18). GLUT4 vesicles were isolated from the adipocytes of 96 rats. The vesicle proteins were separated by SDS gel electrophoresis, transferred to nitrocellulose, and stained with Amido Black. The strips containing the 110-kDa vesicle protein were subjected to peptide analysis. This consisted of digestion with the protease LysC, separation of the peptides by microbore high performance liquid chromatography, and microsequencing of selected peptides, as described in Ref. 19.

Cloning of Rat Sortilin cDNA—BLAST searches (20) revealed that a human expressed sequence tag (AA017125) encoded a peptide similar to...
one of the peptides (amino acids 1–16, see Fig. 2) and a peptide identical to a second peptide (amino acids 65–73). Two additional human expressed sequence tags (H85459 and NT1981) contained overlapping sequences. This information was used to design two sense primers based on the first peptide (CGGCGAGGACGARG (a), where N = A, T, C, or G and R = A or G, and CGGACTTCGTCGCGGAAA), and an antisense primer based on the second peptide (TTCCCATATCCTC- CACTTGATA (c)). In addition an antisense primer was designed on the basis on another peptide (amino acids 170–176, see Fig. 2) (CGGG- GATCTCCTRTGDDTTCYTCCCA (d), where R = A or G; D = A, T, or G; and Y = C or T). Marathon-ready rat adipocyte cDNA (CLONTech) was initially amplified with PCR with primers a and d and AmpliTaq (Perkin-Elmer). The product of the reaction was purified with QiAquick PCR purification kit (Qiagen) and amplified with primers b and c. The product from this amplification was reamplified with primers b and c. This yielded the expected 200-base pair fragment, which was isolated from the gel piece with QiAquick gel extraction kit (Qiagen) and sequenced with primer c. With this sequence information, two sense (nucleotides 17–39 and 50–73, see Fig. 2) (CGGGGATCTCCTRTGDDTTCYTCCCA) and two antisense primers were designed and used in the 5' and 3' RACE procedures, respectively, with the Marathon-ready rat adipocyte cDNA according to the manufacturer’s instructions. 3' RACE yielded products of 900 base pairs and 2 kb, which were identical at the 5' end over the 900 base pairs. 5' RACE did not yield sortilin cDNA fragments. The PCR products were isolated and sequenced on both strands. Sequencing was performed on the Applied Biosystems 373 DNA Sequencing System with the Perkin-Elmer DNA Sequencing Kit FS.

**Subcellular Fractionation**—Rat adipocytes were either left in the basal state or treated with 10 mM insulin for 15 min. The cells were then fractionated into plasma membranes, LDM, high density microsomes, mitochondria/nuclei, and cytosol by differential centrifugation, as described in Ref. 21. 3T3-L1 adipocytes were either left in the basal state or treated with 170 nM insulin for 15 min and then fractionated into the same fractions by a very similar method (22).

**Isolation of GLUT4 Vesicles**—The method for the isolation of GLUT4 vesicles and their subsequent solubilization is described in detail in Ref. 23. According to this method, vesicles were adsorbed from the LDM/ cytosol fraction of rat or 3T3-L1 adipocytes with antibodies against the carboxy-terminal peptide of GLUT4 bound to protein A on the surface of Staph A cells. In the case of rat adipocytes, approximately 1.5 ml of this fraction (b), which was derived from the cytosol (a) and a cell lysate (c), was adsorbed with 7.5 µg of antibodies on 2.5 µl of Staph A cells. In the case of the 3T3-L1 adipocytes, 1 ml of this fraction, which was derived from one 10-cm plate (about 107 cells), was adsorbed with 11 µg of antibodies on 4 µl of Staph A cells. Vesicles bound to the adsorbent were solubilized with 0.5% nonyl ethylene glycol dodecyl ether.

**Cell Surface Biotinylation**—Plates (10 cm) of 3T3-L1 adipocytes in serum-free medium were left in the basal state or treated with 10 nM insulin for 15 min. Cell surface biotinylation, lysis, and immunoprecipitation of IRAP and sortilin were then carried out as described in Ref. 11. In this method aliquots of the cleared lysates (0.5 ml from 10% of a plate) were immunoprecipitated with antibodies against the carboxy terminus of sortilin (10 µg), antibodies against the cytoplasmic domain of IRAP (2 µg), or irrelevant rabbit immunoglobulin G. Under these conditions, the immunoprecipitations of sortilin and IRAP were approximately 75 and 90% complete, respectively. SDS samples of the immunoprecipitates were separated by gel electrophoresis and blotted for biotin with streptavidin-horseradish peroxidase (11). The relative loads of sortilin or IRAP in the lanes were determined by stripping the Immobilon P membranes according to the manufacturer’s (Millipore) instructions and then immunoblotting for sortilin or IRAP.

**Immunofluorescence**—3T3-L1 adipocytes in the basal and insulin- treated state (170 nM for 15 min) were fixed and examined separately for sortilin or GLUT4 immunofluorescence, as described in Ref. 11. Sortilin was labeled with the antibodies against the lumenal domain at 10 µg/ml, and GLUT4 was labeled with antibodies against the carboxy-terminal peptide at 5 µg/ml.

**Immunoblotting**—Immunoblotting of GLUT4 and IRAP was performed as described in Ref. 11. The same procedure was used for blotting sortilin, with either antibodies against the lumenal domain at 10 µg/ml or those against the carboxy-terminal peptide at 5 µg/ml. The relative intensities of the bands on each blot were quantitated by computer enhanced video densitometry using the program NIH Image, and the relative amounts of each protein were calculated from the standard curve given by a dilution series of the protein run on each blot.
FIG. 2. Partial cDNA and amino acid sequence of rat sortilin. The nucleotide sequences of the cDNA encoding most of rat sortilin and the corresponding amino acid sequence are presented. The putative membrane spanning domain is boxed (678–699), and the sequenced tryptic peptides are underlined (1–16, 65–73, 170–175, and 461–469). Beneath the amino acid sequence is the predicted amino acid sequence of the recently cloned human sortilin (GenBank yX98248; Ref. 13) is presented; dots represent identical amino acids. It should be noted that some rat cDNA fragments obtained by PCR did not contain AAG at nucleotides 563–565; this results in the replacement of Lys and Ala at 199 and 200 with Thr.
membranes increased by $6.2 \pm 1.2$- and $2.2 \pm 0.6$-fold, respectively, for the rat adipocytes and by $13 \pm 6.8$- and $5.1 \pm 2.8$-fold, respectively, for the 3T3-L1 adipocytes (Fig. 3, lane 6 versus lane 5 and lane 18 versus lane 17). Correspondingly, the amounts of GLUT4 and IRAP in the LDM decreased to $58 \pm 12$ and $69 \pm 22\%$ of the level in basal cells, respectively, for rat adipocytes and to 50 and 45\% of the level in basal cells, respectively, for 3T3-L1 adipocytes (Fig. 3, lane 4 versus lane 3 and lane 16 versus lane 15). In contrast, sortilin showed a small increase or no increase in the plasma membrane ($1.0 \pm 0.2$- and $1.7 \pm 0.5$-fold for rat and 3T3-L1 adipocytes, respectively) and a small decrease or no decrease in the LDM ($110 \pm 7$ and $90\%$ of the level in basal rat and 3T3-L1 adipocytes, respectively) (Fig. 3, lanes given above).

To determine what fraction of the sortilin in the LDM was located in vesicles containing GLUT4, the vesicles were isolated from the LDM with antibodies against the carboxyl terminus of GLUT4; then the original LDM, the depleted LDM, and the vesicles were analyzed for sortilin, as well as for GLUT4 and IRAP, by immunoblotting (Fig. 4). With the LDM from rat and 3T3-L1 adipocytes, $90 \pm 2$ and $87 \pm 6\%$ of the GLUT4 vesicles, respectively, were specifically adsorbed by the antibodies against GLUT4, as measured by the percentage of depletion of both GLUT4 and IRAP from the LDM$^3$ (Fig. 4, lane 6 versus 5 and lane 17 versus lane 16) and evidenced by the recovery of the IRAP in the vesicles (lanes 8 and 19). A substantial portion of the sortilin, $75 \pm 7$ and $70 \pm 0\%$ in rat and 3T3-L1 adipocytes, respectively, was located in the adsorbed GLUT4 vesicles, measured by its depletion from the LDM and shown by its recovery in the vesicle proteins (Fig. 4, lanes as given above).

**Translocation of Sortilin to the Plasma Membrane in Response to Insulin**—As described above, analysis by subcellular fractionation revealed little or no translocation of sortilin to the plasma membrane in response to insulin in either rat or 3T3-L1 adipocytes. However, this method can underestimate the degree of translocation, because some intracellular vesicles often contaminate the plasma membranes. In fact, on the basis of a previous report (24), we expected some translocation of sortilin in response to insulin. It had been found that GLUT4 vesicles contain a 110-kDa protein that could be biotinylated by treating rat adipocytes with a membrane-impermeant biotinylating reagent and that the biotinylation of this protein was greater when the adipocytes were in the insulin-treated state. This result was interpreted to mean that more of the 110-kDa protein was at the cell surface subsequent to insulin treatment. Most likely this 110-kDa protein was sortilin.

To quantitate the translocation of sortilin, we employed a cell surface biotinylation method that we have previously developed to measure the translocation of IRAP in 3T3-L1 adipocytes (11). Basal and insulin-treated 3T3-L1 adipocytes were cooled to $4\,^\circ C$ to prevent membrane trafficking and then reactivated with a membrane-impermeant reagent that biotinylates amino groups. Subsequently, sortilin and, as a control, IRAP were isolated by immunoprecipitation with antibodies against their cytoplasmic domains, and the biotin content of each measured by blotting with streptavidin conjugated to horseradish peroxidase. The biotin content of the sortilin or IRAP from insulin-treated cells relative to that of the protein from basal cells measures the fold increase at the cell surface. Fig. 5 presents the results of a typical experiment. Insulin increased the amount of sortilin at the plasma membrane by $1.7 \pm 0.2$-fold (lane 2 versus lane 1). By contrast and in agreement with our previous results (11), the amount of IRAP at the cell surface increased by more than 8-fold (lane 9 versus lane 8).

The translocation of sortilin and GLUT4 in 3T3-L1 adipocytes was also examined by immunofluorescence (see “Experimental Procedures”). In basal cells the labeling pattern for both proteins consisted of a strong perinuclear patch and dots throughout the cell, as described previously for GLUT4 in 3T3-L1 adipocytes (11, 25) and sortilin in COS cells (13). Upon insulin treatment, a ring of GLUT4 fluorescence at the periphery of the cell, which reflects GLUT4 translocation to the plasma membrane, was evident (as in Ref. 25). However, no such ring was exhibited by sortilin (data not shown). This qualitative result thus agrees with the conclusion from subcellular fractionation and cell surface biotinylation that marked translocation of sortilin does not occur.

**Expression of Sortilin upon Differentiation of 3T3-L1 Adipocytes**—Fig. 6 shows the expression of sortilin as 3T3-L1 fibroblasts differentiate into adipocytes. There was no detectable expression in the fibroblasts; expression was first evident at day 4 of differentiation and maximal by day 8. This pattern is almost the same as that for GLUT4 expression (Fig. 6 and Ref. 11). In contrast, IRAP is expressed in fibroblasts, although at about one-sixth the amount per mg of protein as in adipocytes.
similar results. In support of this hypothesis, the rate constant for trafficking from the endosomes to the plasma membrane. This increase in the rate constant for externalization of the transferrin receptor in 3T3-L1 adipocytes is increased 2-fold by insulin (26), and kinetic analysis shows that this increase accounts for its redistribution in response to insulin (26). By contrast, GLUT4 and IRAP are located in both the endosomes and in the specialized insulin-regulatable vesicles, which are postulated to arise from the endosomes (6, 7). GLUT4 and IRAP are sequestered in these vesicles in the unstimulated adipocytes, and consequently the amounts of each on the cell surface are low in this state. Insulin causes a much increase in the rate constant for endocytosis of the specialized vesicles, and as a consequence the fold increases in GLUT4 and IRAP at the plasma membrane are much larger (6, 7).

The finding that a large fraction of the sortilin is isolated in the GLUT4 vesicles may at first seem inconsistent with the modest translocation of sortilin. The explanation probably lies in the nature of the GLUT4 vesicles. The GLUT4 vesicles are operationally defined as those vesicles that are isolated from the LDM fraction with antibodies against GLUT4. The LDM fraction contains not only the specialized insulin-regulatable vesicles, but also vesicles arising from the endosomes, Golgi, and endoplasmic reticulum, and there are also surely GLUT4 molecules in some of these latter vesicles. Because our procedure for the isolation of GLUT4 vesicles probably absorbs any vesicle with one copy of GLUT4, most of the sortilin may be in GLUT4 vesicles arising from endosomes and Golgi, whereas much more of the GLUT4 and IRAP may be in the specialized insulin-regulatable vesicles.

The function of sortilin remains to be determined. Although its known properties suggest that it may be a receptor for sorting luminal proteins from the Golgi to the late endosomes, similar to the CIM6PR (see the Introduction), it could also be a receptor for a specific extracellular ligand. For example, the CIM6PR is also the receptor for insulin-like growth factor II (30). If sortilin is a receptor for an extracellular ligand, then the insulin-elicited increase at the plasma membrane should lead to a corresponding increase in endocytosis of this ligand and consequently would be of physiological significance. The finding that fibroblasts have no detectable sortilin suggests that whatever its function, sortilin is not required for viability of all animal cell types.

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