Glucose Transporter Glut3 Is Targeted to Secretory Vesicles in Neurons and PC12 Cells*

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In rat brain and cultured neuroendocrine PC12 cells, Glut3 is localized at the cell surface and, also, in a distinct population of homogenous synaptic-like vesicles. Glut3-containing vesicles co-purify with “classical” synaptic vesicles, but can be separated from the latter by sucrose gradient centrifugation. Unlike classical synaptic vesicles, Glut3-containing vesicles possess a high level of aminopeptidase activity, which has been identified as aminopeptidase B. This enzyme has recently been shown to be a marker of the secretory pathway in PC12 cells (Balogh, A., Cadel, S., Foulon, T., Picart, R., Der Garabedian, A., Rousselet, A., Tougaard, C., and Cohen, P. (1998) J. Cell Sci. 111, 161–169). We, therefore, conclude that Glut3 is targeted to secretory vesicles in both neurons and PC12 cells.

Glucose transporters represent a family of proteins that are responsible for the cellular uptake of glucose and are ubiquitously expressed in mammalian cells in a tissue-specific fashion (1). Normally, these proteins function at the cell surface and recycle between the plasma membrane and intracellular compartment(s) at a rate that depends on the type of cell as well as the isoform of the transporter protein. The distribution of glucose transporters between the plasma and intracellular membranes is strictly regulated, because this distribution has a major impact on glucose uptake and, hence, cellular energy metabolism as well as on maintaining glucose homeostasis in the blood. This is certainly true for the fat and muscle-specific transporter isoform, Glut4, and, to some extent, for the “ubiquitous” transporter isoform, Glut1, both of which traffic in competent cells under tight insulin control (reviewed in Refs. 1 and 2). Glut3, which is expressed to some degree in cultured L6 muscle cells, has also been shown to redistribute to the plasma membrane after insulin or insulin growth factor-1 treatment (3). In addition, Glut3 is expressed in platelets in which it is translocated from α-granules to the cell surface in response to thrombin, a phenomenon that may reflect increased energy requirements of platelets upon activation (4, 5). The major Glut3-expressing tissue, however, is brain (1, 6), and it is unknown whether Glut3 function in brain may be regulated by compartmentalization.

We, therefore, studied the intracellular localization of Glut3 in rat brain as well as in cultured rat pheochromocytoma PC12 cells. Although the major part of the Glut3 moiety in both brain and PC12 cells is localized in the plasma membrane, a fraction of the transporter has been found inside the cell. Intracellular Glut3 is present in a biochemically homogenous population of membrane vesicles, which are co-purified with classical synaptic vesicles but can be separated from the latter in equilibrium density and velocity sucrose gradients. A unique feature of Glut3-containing vesicles that allows one to easily discriminate them from classical synaptic vesicles, which otherwise have a very similar protein composition (7), is the presence of aminopeptidase activity. We have determined that this activity belongs to aminopeptidase B, which has recently been shown to represent a marker of the regulated secretory pathway in PC12 cells (8). Thus, we suggest that Glut3-containing vesicles represent a novel secretory compartment in neurons and neuroendocrine cells. We have also found Glut3 in clathrin-coated vesicles, suggesting that Glut3 may recycle between the plasma membrane and intracellular membranes via a clathrin-mediated pathway.

MATERIALS AND METHODS

Antibodies—Affinity purified anti-Glut3 antibody was from Charles River Pharmaservices. Antibody to clathrin heavy chain was from ICN. Antibody to synaptophysin was from Roche Molecular Biochemicals. Antibodies to SV2 and synaptotagmin were kind gifts from Dr. K. Buckley (Harvard Medical School). Antibody to synaptobrevin was a kind gift from Dr. R. Jahn (Max Plank Institute, Gottingen).

Cell Culture—The pheochromocytoma cell line PC12 was grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin in 5% CO2 at 37 °C.

Isolation and Fractionation of Synaptic Vesicles from PC12 Cells—Confluent 15-cm plates were rinsed twice at 4 °C with buffer A (150 mM NaCl, 10 mM Hepes, pH 7.4, 1 mM EGTA, 0.1 mM MgCl2). Cells were scraped from dishes into 1–2 ml of buffer per plate, and homogenized with 10 strokes in a Dounce homogenizer followed by passage through a 25 gauge needle 10 times. The homogenate was centrifuged at 100,000 × g for 5 min, followed by centrifugation of the resulting supernatant (S1) at 27,000 × g for 35 min to yield a pellet and supernatant (S2) (9). Total intracellular membranes were pelleted by centrifugation of supernatant S2 at 180,000 × g for 2 h and centrifuged on a 10–30% (w/w) sucrose velocity gradient for 50 min at 48,000 rpm in a SW50.1 rotor. Fractions were collected and analyzed for synaptic vesicle proteins.

Fractions in the gradient that were enriched in SV2, synaptophysin and synaptobrevin, were pooled, pelleted, and reapplied on a 10–50% (w/w) sucrose equilibrium density gradient and centrifuged for 18 h at 48,000 rpm in a SW50.1 rotor. Fractions were collected starting from the bottom of the gradient.

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bottom of the gradient and blotted for GLUT3 as well as for individual synaptic vesicle proteins.

Isolation and Fractionation of Synaptic Vesicles from Rat Brain—Synaptosomes were prepared from rat brains according to the procedure of Huttner et al. (10). The resulting synaptosomal pellet was diluted with 70 volumes of ice cold H₂O (hypotonic lysis of synaptosomes to release synaptic vesicles) and immediately homogenized with three strokes in a Dounce homogenizer. 1 mM HEPES, pH 7.4, was added to a final concentration of 7.5 mM HEPES, and the homogenate was centrifuged for 20 min at 25,500 × g to remove the synaptosomal membrane. The supernatant was centrifuged for 2 h at 48,000 rpm, in a Ti60 rotor, and the resulting pellet was resuspended in 1 ml of 30 mM sucrose, 4 mM HEPES, pH 7.4, and homogenized by passage through a 25 gauge needle 5 times back and forth. This material was loaded on a continuous gradient of 50–800 mM sucrose, 4 mM HEPES, pH 7.4, and the gradient was centrifuged for 5 h at 22,000 rpm in an AH627 rotor. Fractions containing synaptic vesicle marker proteins were pooled, pelleted by centrifugation at 48,000 rpm for 2 h, resuspended in buffer A and then loaded onto an equilibrium density sucrose gradient (10–50% w/v) in buffer A and centrifuged at 48,000 rpm for 18 h in a SW50.1 rotor. For velocity centrifugation, material from the equilibrium density gradient was further fractionated in 10–30% (w/v) gradients in a SW50.1 rotor at 48,000 rpm for 55 min. Following each centrifugation, fractions were collected starting from the bottom of the gradient and analyzed for total protein content, presence of specific proteins by Western blotting, and aminopeptidase activity.

Purification of Clathrin-coated Vesicles from Rat Brain—Clathrin-coated vesicles were purified from rat brain in MES buffer (0.1 M MES, 1 mM EGTA, 0.5 mM MgCl₂, pH 6.5) by the procedure of Maycox et al. (11). The final pellet containing purified coated vesicles was resuspended in 0.2 ml of MES buffer, and coated vesicles were then centrifuged in an equilibrium density sucrose gradient (20–65% w/v) in MES buffer, pH 6.5) at 48,000 rpm for 18 h in a SW50.1 rotor. Fractions were collected from the bottom of the gradient and analyzed for total protein and for specific proteins by Western blotting. Fractions that were positive for clathrin as well as for the synaptic vesicle proteins SV2, synaptophysin, and synaptobrevin were further purified by recentrifugation in an additional equilibrium sucrose gradient (20–65% w/v) for 18 h.

Anion Exchange Chromatography—To separate proteins by anion exchange chromatography, membrane samples were solubilized in 1% Triton X-100 for at least 2 h at 4 °C, centrifuged, and applied to a 1-ml Pharmacia Mono-Q column equilibrated with buffer A with 50 mM NaCl and 0.1% Triton X-100 and spun for 16 h at 33,000 rpm in a SW50.1 rotor. Fractions were reapplied on a 10–50% (w/v) sucrose equilibrium density gradient, and centrifuged for 18 h at 48,000 rpm in a SW50.1 rotor. Fractions (10 ml drops of a flow rate of 0.5 ml/min) were collected starting from the bottom of the gradient and blotted for GLUT3, SV2, synaptophysin, and synaptobrevin. Autoradiograms were quantitated in a computing densitometer (Molecular Dynamics). The figure shows a representative result of six independent experiments.

Isolation and Fractionation of Synaptic Vesicles from PC12 cells—Synaptic vesicles were partially purified from PC12 cells as described under “Materials and Methods,” reappplied on a 10–50% (w/v) sucrose equilibrium density gradient, and centrifuged for 18 h at 48,000 rpm in an SW50.1 rotor. Fractions were collected starting from the bottom of the gradient and blotted for GLUT3, SV2, synaptophysin, and synaptobrevin. Autoradiograms were quantitated in a computing densitometer (Molecular Dynamics). The figure shows a representative result of six independent experiments.

Identification and Isolation of the Intracellular Glut3-containing Vesicles—Fig. 1 shows localization of Glut3 in the plasma membrane and perinuclear region of PC12 cell. Glut3-containing Vesicles in Brain

FIG. 1. Localization of Glut3 in PC12 cells. The plane of focus at an intermediate section through the cells shows localization of Glut3 in the plasma membrane and perinuclear region of PC12 cell.

FIG. 2. Fractionation of synaptic vesicles from PC12 cells in an equilibrium density gradient. Synaptic vesicles were partially purified from PC12 cells as described under “Materials and Methods,” reappplied on a 10–50% (w/v) sucrose equilibrium density gradient, and centrifuged for 18 h at 48,000 rpm in an SW50.1 rotor. Fractions were collected starting from the bottom of the gradient and blotted for GLUT3, SV2, synaptophysin, and synaptobrevin. Autoradiograms were quantitated in a computing densitometer (Molecular Dynamics). The figure shows a representative result of six independent experiments.

slides. Staining was examined by confocal laser scanning microscopy (Bio-Rad, Microscope Division; MRC/600).

Electron Microscopy—Glut3-containing vesicles purified by sucrose gradient centrifugation were pelleted, resuspended in PBS to a final concentration of 0.1–0.5 mg/ml, and fixed with 2% paraformaldehyde in PBS. Formvar-carbon coated nickel grids were layered on 25-μl drops of vesicle suspension for 1–5 min. Grids were then stained with 1% uranyl acetate for 30 s, dried, and viewed through a Phillips transmission electron microscope. Micrographs were taken at magnification ×10,000–48,000.

Gel Electrophoresis and Immunoblotting—Proteins were separated by SDS-polyacrylamide gel electrophoresis according to Laemmli (12) and transferred to an Immobilon-P membrane in 25 mM Tris, 192 mM glycine. Following transfer, the membrane was blocked with 10% non-fat dry milk in PBS for 1 h at 37 °C and probed with specific antibodies. Autoradiograms were quantitated in a computing densitometer (Molecular Dynamics).

Protein Determination—Protein content was determined with the BCA kit (Pierce) according to the manufacturer’s instructions.

RESULTS

Identification and Isolation of the Intracellular Glut3-containing Vesicles—Fig. 1 shows localization of Glut3 in the neuroendocrine PC12 cell line by immunofluorescent staining. In this figure, it is evident that in addition to the plasma membrane, Glut3 is present in an intracellular perinuclear compartment.
To understand the nature of this intracellular Glut3-containing compartment, we fractionated PC12 cells as described previously (9). After cell homogenization, heavy subcellular structures and the plasma membrane were removed by pelleting, and the resulting intracellular membranes were fractionated in a linear equilibrium density gradient as described under “Materials and Methods.” Under these conditions, Glut3-containing intracellular membranes have a very narrow density distribution that substantially, although not completely, overlaps with the position of the synaptic vesicle marker proteins SV2 and synaptophysin, with Glut3-containing vesicles having a buoyant density less than synaptic vesicles (Fig. 2).

The limited amount of the material available from cultured cells did not allow us to purify intracellular Glut3-containing vesicles from PC12 cells in the preparative amounts that were required for detailed biochemical analysis of this compartment. Therefore, further characterization of Glut3-containing vesicles was performed using rat brain as a source of biological material.

In the following experiments, we took advantage of a well established procedure for the isolation and subcellular fractionation of synaptosomes (10). Briefly, we isolated synaptosomes from rat brain, lysed them with osmotic shock, removed synaptic membranes, and purified intra-synaptosomal membranes by preparative sucrose gradient centrifugation (10). This preparation was fractionated further by equilibrium density sucrose gradient centrifugation as shown in Fig. 3 and as described previously (7). This procedure allows the separation of the intra-synaptosomal membrane material into two major peaks according to their buoyant densities (7), one of which is likely to represent classical synaptic vesicles (7). The nature of the second less dense population of vesicles remains unknown. Fig. 3 shows that Glut3 is specifically enriched in this second population of vesicles. Based on these results, we conclude that the buoyant density distribution of Glut3 vesicles and synaptic vesicles in PC12 cells (Fig. 2) and rat brain (Fig. 3) is very similar if not identical.

As we have shown earlier (7) and reconfirm here, this second population of “synaptic-like” vesicles from rat brain, unlike classical synaptic vesicles, possesses a high level of aminopeptidase activity (Fig. 3), which serves as a convenient and reliable marker of this compartment and allows one to readily distinguish the two populations of vesicles.

The peak 2 fractions, which contained both Glut3 and aminopeptidase, were pooled and, following pelleting, recentrifuged in a sucrose velocity gradient (Fig. 4). Under these conditions, the Glut3-containing material sediments as homo-
geneous vesicles whose distribution completely overlaps with the total protein profile and also with synaptic vesicle marker proteins and aminopeptidase activity.

Data presented in Fig. 4 suggest that Glut3-containing vesicles at this purification step are close to homogeneity. This was confirmed by electron microscopy (Fig. 5), which demonstrated that this preparation consists of homogenous round-shaped vesicles with an average diameter of 47 ± 6 nm. Using electron microscopy, we could not find any significant differences between Glut3-containing synaptic-like vesicles and classical synaptic vesicles (not shown).

Identification of the Aminopeptidase Activity Associated with Glut3-containing Vesicles as Aminopeptidase B—To identify aminopeptidase activity associated with Glut3-vesicles, we used an antibody against aminopeptidase B described by Cadel et al. (13). For these experiments, we solubilized Glut3-containing vesicles in 1% Triton X-100 and centrifuged this material in a 5–20% sucrose gradient (Fig. 6).

In the next experiment, we fractionated Triton-solubilized Glut3-containing vesicles on a Mono-Q column (Fig. 7). Unlike sucrose gradient centrifugation, fast protein liquid chromatography allows the separation of aminopeptidase activity into two peaks, the major of which co-elutes with aminopeptidase B. The nature of the minor peak remains unknown, although it may represent a degradation product of the major peak or may result from binding of aminopeptidase B to other proteins. Thus, we conclude that aminopeptidase B is the only or, at least, the major aminopeptidase present in Glut3-containing vesicles. Because aminopeptidase B has recently been shown to be targeted to the regulated secretory pathway in PC12 cells (8), we believe that Glut3-containing vesicles, which we describe here, represent a secretory compartment in both PC12 cells and neurons.

Glut3 in Clathrin-coated Vesicles—Glucose transporter proteins, Glut1, and Glut4, in particular, are known to recycle...
between the plasma membrane and an intracellular storage compartment. To determine whether this may also be the case for Glut3, we isolated clathrin-coated vesicles from rat brain according to the procedure of Maycox et al. (11) and fractionated them in an equilibrium density sucrose gradient (Fig. 8). Glut3 is clearly present in clathrin-coated vesicles suggesting that this isoform of glucose transporter recycles in neurons, although the quantitative parameters of this recycling have yet to be determined.

**DISCUSSION**

In this study, we examine the intracellular localization of Glut3, the predominant glucose transporter isoform in brain. We show that Glut3 is present in a distinct homogenous population of synaptic-like vesicles that we have recently identified in rat brain (7) where these vesicles are as abundant as classical synaptic vesicles and can be separated from the latter by equilibrium density gradient centrifugations. The overall protein composition of these synaptic-like vesicles is very close to classical synaptic vesicles, with SV2, synaptotagmin, synaptophysin, and synaptoprevin being the major components in both pools (7). Several important differences have nevertheless been found in protein content in the two vesicular populations. First, synaptic-like vesicles have a much lower content of V-type H⁺-ATPase (proton pump) (7); second, they specifically compartmentalize Glut3 (this study); finally, they have very high aminopeptidase activity that has been identified as aminopeptidase B (this study). Because this aminopeptidase is known to be targeted to the secretory pathway (8), we believe that these synaptic-like vesicles represent a secretory compartment in neurons and PC12 cells. This is consistent with the fact that Glut3 is targeted to the secretory granules in platelets, another cell type with high Glut3 content (4, 5). Because Glut3 is also a predominant component of clathrin-coated vesicles in rat brain, we suggest that this protein may recycle between its intracellular compartment and the plasma membrane via an as yet uncharacterized pathway involving clathrin-coated vesicles.

There are interesting parallels between Glut3-containing vesicles in brain and Glut4-containing vesicles in insulin-sensitive fat and skeletal muscle tissues. Both types of vesicles have similar size, sedimentation coefficient, and buoyant density in sucrose solutions (this study and Ref. 14). Moreover, both incorporate aminopeptidases that, in addition to glucose transporters, serve as marker proteins for these vesicular compartments. The specific vesicle-associated aminopeptidases, however, are quite different; insulin-sensitive aminopeptidase, or IRAP, was found in Glut4-containing vesicles (15, 16) and aminopeptidase B (8, 17) in Glut3-containing vesicles. An even more significant difference is that Glut4-containing vesicles represent a specialized endosomal compartment in adipocytes (18), whereas Glut3-containing vesicles in brain are likely to be a regulated secretory compartment. In this regard, it will be interesting to determine whether Glut3- and aminopeptidase B-containing synaptic-like vesicles are relevant to the vesicular compartment identified by transfection of Glut4 into PC12 cells (19). This may reveal interesting aspects of protein targeting into different specialized intracellular membrane compartments. Another important question has to do with the regulation of Glut3 translocation from intracellular secretory vesicles to the cell surface and its possible return to an intracellular pool, which presumably takes place via a clathrin-mediated mechanism. This problem is currently under investigation in our laboratory.

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