Glycosyl Modification Facilitates Homo- and Hetero-oligomerization of the Serotonin Transporter

A SPECIFIC ROLE FOR SIALIC ACID RESIDUES*

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The serotonin transporter (SERT) is an oligomeric glycoprotein with two sialic acid residues on each of two complex oligosaccharide molecules. In this study, we investigated the contribution of N-glycosyl modification to the structure and function of SERT. We report that N-glycosylation of SERT strongly facilitates its oligomerization into homo- and hetero-oligomers in a cell-dependent and -independent manner. Sialylation of Asn-208 (to Gln) and Asn-217 (to Gln) of SERT, QQ, expressed in parental Chinese hamster ovary (CHO) cells and endogenous (placental choriocarcinoma JAR cells) cells required sialylation of Asn-208 and Asn-217 to the structure and function of SERT in two model systems, SERT overexpressed in CHO cells and SERT in JAR cells. The QQ mutant form (after site-directed mutagenesis of Asn-208 to Gln and Asn-217 to Gln) of SERT, QQ, expressed in parental CHO cells and JAR cells had a lower degree of glycosylation than the wild-type SERT, and the QQ mutant form of SERT could not provide all of the original immunoblots, but they state that the immunoblots were not reused.

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* The abbreviations used are: SERT, serotonin transporter; rSERT, rat SERT; NET, noradrenaline transporter; DAT, dopamine transporter; CHO, Chinese hamster ovary; NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate; PNGase F, peptide N-glycosidase F; MTSEA, (2-aminoethyl)methanethiosulfonate; IP, immunoprecipitation; NEM, N-ethylmaleimide.

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WITHDRAWN
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required for normal cell-surface expression; and (iii) sialylated glycanes are also required for normal cytoskeletal associations of SERT with myosin IIA. Based on these findings, we propose a structural role for sialylated N-glycans in conferring an optimum conformation to SERT that facilitates homo-oligomerization upon biosynthesis and, in turn, exposes the domain(s) for myosin cytoskeletal associations. To explore if the impaired oligomeric properties of SERT were due to the lack of myosin interaction or branching, we also used Lec4 cells and found the same result with those using Lec4 cells. Utilizing the Lec mutant cells in this study was a novel approach to elucidate the significance of sialic acid residues in SERT function.

EXPERIMENTAL PROCEDURES

Materials—The mutant form of rat SERT (rSERT, Asn206 and Asn177 of the N-linked glycosylation consensus sequences replaced with glutamine residues by site-directed mutagenesis), the QQ construct in plasmid pCGT148 (31), was a generous gift from Dr. C. G. Tate (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK). Lec2 was purchased from American Type Culture Collection (ATCC, CRL 1736) (Manassas, VA). Lec4 cells were a generous gift from Dr. P. Stanley (Albert Einstein College of Medicine, Bronx, NY). Myosin IIA cDNA was kindly provided by Dr. R. Adelstein (NHBLI, NIH, Bethesda, MD). Endonucleases and ligases were from New England Biolabs Inc. (Beverly, MA). Expression vectors, cell culture materials, Lipofectin, and LipofectAMINE 2000 were from Invitrogen. The micro BCA protein assay kit, the enhanced chemiluminescence (ECL) Western blotting system, streptavidin-agarose beads, sulfo-coumidimydil 2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin), and immunoreversal peroxidase-conjugated streptavidin were from Pierce. Mouse monoclonal anti-Myc antibody was from Chemicon International, Inc. (Temecula, CA). Mouse monoclonal anti-FLAG mouse and rabbit anti-FLAG reagents, thioglycolic acid, and rabbit anti-rabbit secondary antibodies were from Promega (Madison, WI). Proteins were recovered with streptavidin-agarose beads during overnight incubation. Biotinylated proteins were eluted and separated on SDS-polyacrylamide gels. The biotinylated proteins were treated with 100 mM glycine to remove biotin and normalized by ECL detection system. The signals were visualized using a VersaDoc 1000 gel visualization and analysis system (Bio-Rad). Immunoblot Analysis—Following transfection, cells were solubilized in phosphate-buffered saline containing 0.44% SDS, 1 mg/ml phenylmethylsulfonyl fluoride, and protease inhibitor mixture. The protease inhibitor mixture contained 5 mM EDTA, 1 mM Na3VO4, and 0.44% SDS.

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Immunoblot Analysis—Following transfection, cells were solubilized in phosphate-buffered saline containing 0.44% SDS, 1 mg/ml phenylmethylsulfonyl fluoride, and protease inhibitor mixture. The protease inhibitor mixture contained 5 mM EDTA, 1 mM Na3VO4, and 0.44% SDS. Cells were treated with 100 mM glycine to remove biotin and normalized by ECL detection system. The signals were visualized using a VersaDoc 1000 gel visualization and analysis system (Bio-Rad). Immunoblot Analysis—Following transfection, cells were solubilized in phosphate-buffered saline containing 0.44% SDS, 1 mg/ml phenylmethylsulfonyl fluoride, and protease inhibitor mixture (18) containing 5 mM NEM. Cell lysates were first precleared by incubation with nonimmune rabbit serum and protein A for 1 h and then centrifuged. The precleared lysate was combined with an equal volume of a 1:1 slurry of rabbit anti-mouse A and rabbit anti-rabbit secondary antibodies. The antibodies were preincubated with 2% SDS, 0.1% bromphenol blue, 10% glycerol, and 1% β-mercaptoethanol. Samples were separated on a 15% SDS-polyacrylamide gel (55). After electrophoresis, gels were analyzed by immunoblotting with either biotinylated monoclonal anti-FLAG antibody (diluted 1:1500) or polyclonal anti-myc antibody following the manufacturer’s instructions (Amersham Biosciences). Briefly, the synthetic peptide corresponding to the last 26 amino acids of the C terminus of SERT was dissolved in coupling buffer (0.1 M NaHCO3, and 0.5 M NaCl (pH 8.0)) and bound to CNBr-activated Sepharose 4B beads (Amersham Biosciences) that were previously washed with 1 ml HCl buffered saline. The column was packed, and crude antibody was run through the column. After washing the beads with 10 column volumes of phosphate-buffered saline, the antibody was eluted sequentially with 0.1 M glycine (pH 3.0) to tubes containing 50 ml of 1 M Tris (pH 8.9). The protein concentrations of each fraction were obtained using the BCA protein assay kit, and protein-containing fractions were stored at –80 °C.

Immunoblot Analysis—Following transfection, cells were solubilized in phosphate-buffered saline containing 0.44% SDS, 1 mg/ml phenylmethylsulfonyl fluoride, and protease inhibitor mixture. The protease inhibitor mixture contained 5 mM EDTA, 1 mM Na3VO4, and 0.44% SDS. Immobilon analysis (Millipore) and ECL Western blot analysis were performed to detect rSERT. Cells were washed with 1 ml HCl buffered saline (pH 6.8) and a 10% SDS sample buffer was added to tubes containing 50 ml of 1 M Tris (pH 8.9). The protein concentrations of each fraction were obtained using the BCA protein assay kit, and protein-containing fractions were stored at –80 °C.
an aliquot of this mixture was run on the gel. The positions of molecular under

lanes 4 and lane 7) were resolved and immunoblotted with affinity-purified biotinylated anti-SERT antibody. The lysate immune complexes were recovered and then eluted in sample buffer supplemented with PNGase F. After a 3-h incubation at 37 °C (34, 35), the

were separated by SDS-PAGE, and immunoblot analysis was performed with biotinylated anti-SERT antibody as described under "Experimental Procedures." All lanes contain protein recovered from the same number of cells equivalent to 30% of one well from a confluent 24-well dish. Three wells for each condition were pooled, and an aliquot of this mixture was run on the gel. The positions of molecular mass standards run on the same gel are shown in kilodaltons.

PNGase F Treatment—CHO and Lec cells transiently transfected with transporters were first lysed in IP buffer containing NEM at a final concentration of 5 mm. The cell lysate was then treated with rabbit anti-mouse protein A-Sepharose beads coated with biotinylated anti-SERT antibody. The lysate immune complexes were recovered and then eluted in sample buffer supplemented with PNGase F. After a 3-h incubation at 37 °C (34, 35), the

was separated by SDS-PAGE, and immunoblot analysis was performed with biotinylated streptavidin and the ECL blotting system.

Transport Assay—CHO and Lec cells infected with recombinant vaccinia virus (Fig. 1, lane 1) and total cell extracts. Analysis of membrane proteins with anti-SERT antibody (Fig. 3A, lane 3) demonstrated that, on the plasma membrane, the level of SERT transporters appeared at the 90-kD species. In CHO cells, all three of these SERT species were more abundant. However, all three of these SERT species were reduced to 50 kDa in the presence of PNGase F; therefore, we believe that SERT proteins are either differen-

tially or partially glycosylated during their biosynthesis process.

Data Analysis—Nonlinear regression fits of experimental and calculated data were performed with Origin (MicroCal Software, Northampton, MA), which uses the Marquardt-Levenberg nonlinear least-squares curve-fitting algorithm. Each figure shows a representative experiment that was performed at least twice. The statistical analysis given under "Results" is from multiple experiments. Data with error bars represent means ± S.D. of triplicate samples.

RESULTS

Glycosylation Pattern of SERT—We first evaluated expression of SERT proteins and their delivery to the cell surface in both model systems. After transfection, expression of wild-type SERT in sialic acid-defective Lec4 cells and the QQ mutant form in CHO cells was demonstrated by immunoblot analysis of cell-surface proteins labeled with extracellular NHS-SS-biotin and total cell extracts. Analysis of membrane proteins with anti-SERT antibody produced three major bands at 90, 65, and 60 kDa in the SERT construct-transfected CHO cells (Fig. 1, lane 1) and Lec4 cells (lane 5). Analysis of total cell extracts with biotinylated anti-SERT antibody also gave rise to the same resolution pattern for SERT in CHO cells (lane 2). The major observable difference between Lec4 and CHO cells is that the 60-kD species was more abundant in Lec4 cells than in CHO cells, whereas the reverse was true for the 65-kDa species. Under our experimental conditions, sialic acid moieties do not contribute to the mobility of SERT upon SDS-PAGE. Control experiments in which either no biotinylation reagent was added or mock-transfected cells were used did not reveal the bands visible on these immunoblots (data not shown).

Removal of potential glycosylation sites during migration of transporter proteins was observed in two different approaches: (i) surface expression of QQ in CHO cells (Fig. 1, lane 4) and Lec4 cells (lane 7) and (ii) PNGase F treatment of SERT. PNGase F removed N-linked glycosylation sites from expressed proteins regardless of oligosaccharide residues.

PNGase treatment eliminated the bands of the 90-, 65-, and 60-kDa species of SERT and revealed a faster migrating single band at ~50 kDa (Fig. 1, lane 3), similar to the QQ transporter (lane 4). Migration of transporters from PNGase F-treated CHO-SERT (lane 3) and CHO-QQ (lane 4) confirmed that the 50-kDa species represents the non-glycosylated form of the SERT polypeptide. However, the 50-kDa species did not appear in the blotting of CHO-SERT cells. Consistent with previous studies (31–33), a defect in the glycosylation processing of SERT did not impair its membrane trafficking or assembly.

Comparison by Western blot analysis of the protein pool labeled with external NHS-SS-biotin (Fig. 1, lane 1) and total cell extracts (lane 2) showed that, on the plasma membrane, the level of SERT transporters appeared at the 90-kD species. In CHO cells, all three of these SERT species were more abundant. However, all three of these SERT species were reduced to 50 kDa in the presence of PNGase F; therefore, we believe that SERT proteins are either differen-

tially or partially glycosylated during their biosynthesis process.

To explore whether the location or number of sialylated N-glycans affects the serotonin uptake ability of SERT, mutant transporters with a single glycosylation site (Q1 (N208Q) or Q2 (N217Q)) were prepared by site-directed mutagenesis and characterized in CHO cells for their serotonin uptake capacit-

ties. Interestingly, as the data in Fig. 2 show, a single deletion of either asparagine did not change the transport function.

Next, we attempted to determine whether the lower activities of transporters are due to lower protein expression levels. Surface and whole cell expression of mutant transporters (QQ, Q1, and Q2 in CHO cells and wild-type SERT in Lec4 cells) was measured and compared with that of wild-type SERT in CHO cells using the standard curve (Fig. 3A). Standard curves were generated with Myc-SERT-FLAG (wild-type SERT tagged with Myc and FLAG epitopes at the N and C termini, respectively). Different amounts (in a broad range of 1–25 μg of protein) of detergent-soluble total cell extracts from CHO or Lec4 cells expressing Myc-SERT-FLAG were separated by SDS-PAGE, and immunoblot analysis was performed with biotinylated anti-SERT antibody (Fig. 3A, inset). The integrated density
value for each band was converted to an equivalent amount of Myc-SERT-FLAG for anti-SERT antibody using the VersaDoc 1000 analysis system. Using this standard curve, the whole cell and plasma membrane expression of the transporters was then determined by quantitative Western blotting as the amount of Myc-SERT-FLAG proteins (Table I).

In other cell contexts (9, 31, 33) and for other transporters such as human NET (34–36) and DAT (47), the transfected cells are demonstrated that more than one band is detected when expressed at the surface and the intensity will increase at the surface similarly to that determined previously. Because of that, the SERT protein is expressed as the combination of glycosylated forms in CHO and Lec4 cells. Therefore, we used co-IP to demonstrate whether self-association occurs upon co-IP, appeared only as 90-kDa bands. Because of that, the SERT protein still gave rise to three species (data not shown), but upon excluding 5 mM NEM in the lysis buffer. Upon resolving the proteins associated with Myc-SERT under nonreducing conditions, a FLAG band with a very low intensity was detected in CHO cells (lane 4) or anti-FLAG antibody (Fig. 3, lane 2). These results suggest that the 90-kDa species is the one that associated to form homo-oligomeric transporters (lane 2). Under similar nonreducing conditions, resolution of the SERT protein still gave rise to three species (data not shown), but upon co-IP, appeared only as 90-kDa bands.

To determine the expression of sialylated N-glycans on the physical association of SERT monomers, different epitope tags (Myc-SERT, SERT-FLAG, Myc-QQ, and QQ-FLAG) were prepared and characterized for their serotonin uptake functions and whole cell and surface expression in CHO and Lec4 cells to explore whether epitope tag alters expression or membrane assembly of transporters. Standard curves were generated with 10 mM NEM for 30 min. The prominent FLAG band was seen upon IP of CHO cells (Fig. 4, lane 1). These results indicate that the two forms of SERT associate in CHO cells; hatched bars represent Lec4 cells (Origin plotting program, MicroCal Software). Mutation of both potential glycosylation sites caused a 70% decrease in the serotonin uptake function in CHO cells, but only 37% decrease in Lec4 cells. No difference was observed with QQ or Q2.

FIG. 2. Serotonin uptake function of CHO-QQ and Lec4-SERT cells. The uptake of serotonin by CHO-QQ cells or Lec4-SERT cells was greatly reduced compared with wild-type SERT in CHO cells. [3H]Serotonin uptake was measured in intact cells transiently expressing the transporters as described under "Experimental Procedures." Background accumulation of [3H]serotonin was measured in the same experiment using mock-transfected cells and subtracted from each experimental value. Maximum background accumulation was 0.01 pmol/mg of protein/min. White bars represent CHO cells; hatched bars represent Lec4 cells (Fig. 2, inset). The uptake of serotonin by CHO-QQ cells or Lec4-SERT cells (Fig. 2, inset) or anti-FLAG antibody (Fig. 3, inset) was determined by quantitative Western blotting as the integrated density value for each band was converted to an equivalent amount of Myc-SERT-FLAG for anti-Myc and anti-FLAG antibodies using the VersaDoc 1000 analysis system. Using this standard curve, the integrated density value for each band was converted to an equivalent amount of Myc-SERT-FLAG for anti-Myc and anti-FLAG antibodies using the VersaDoc 1000 analysis system. Table I shows that neither the epitope tag nor mutation changed the expression efficiency or membrane trafficking of transporter proteins and that their expression level was similar to that observed for unmodified wild-type SERT.

Next, the self-association property of Myc-SERT with SERT-FLAG was analyzed in co-IP assays in CHO and Lec4 cells by cotransfecting the cells with a 1:1 ratio of Myc-SERT and SERT-FLAG constructs. After transfection, cells were treated with 10 mM NEM for 30 min (21). A prominent FLAG band was seen upon IP of CHO cells (Fig. 4, lane 1). These results indicate that the two forms of SERT associate in CHO and Lec4 cells (Fig. 4, lane 1). The difference was created by excluding β-mercaptoethanol from the lysis buffer and including 5 mM NEM in the lysis buffer. Upon resolving the proteins associated with Myc-SERT under nonreducing conditions, one major FLAG band appeared at ~90 kDa (Fig. 4, lane 3). These results suggest that the 90-kDa species is the one that associates to form homo-oligomeric transporters (lane 2). Under similar nonreducing conditions, resolution of the SERT protein still gave rise to three species (data not shown), but upon co-IP, appeared only as 90-kDa bands.
our previous techniques (18, 21), that were originally used by MacKinnon (57) with potassium channel. Sensitive (wild-type) and toxin-insensitive mutant forms were mixed, and the toxin sensitivity of the mixture was analyzed to determine the subunit stoichiometry. In a similar fashion, the functional consequences of homo- and hetero-oligomerization between SERT and the mutants was determined in total cell lysate and in the cell-surface pool isolated using streptavidin-agarose (Table I), and the expression levels for SERT forms could be compared even though they were detected with different antibodies. Immunoblots were quantitated using a VersaDoc 1000 system.

**Table I**

| Transport function and expression characteristics of SERT and mutants in CHO and lec4 cells |
| Uptake rate | Total SERT expression | Surface expression | Uptake rate | Total SERT expression | Surface expression |
|------------|----------------------|-------------------|------------|----------------------|-------------------|
|            | pmol/min/mg          | µg                | µg         | pmol/min/mg          | µg                |
|**CHO cells** |                      |                   |            | **lec4 cells**       |                   |
| SERT       | 0.356 ± 0.037        | 23.6              | 3.50       | 0.148 ± 0.025        | 22.4              | 3.75              |
| QQ         | 0.101 ± 0.002        | 22.1              | 4.33       | 0.093 ± 0.005        | 21.8              | 4.66              |
| Myc-SERT   | 0.295 ± 0.008        | 22.5              | 4.90       | 0.171 ± 0.011        | 23.0              | 4.10              |
| SERT-FLAG  | 0.325 ± 0.043        | 22.1              | 3.75       | 0.181 ± 0.020        | 22.5              | 3.70              |
| Myc-QQ     | 0.098 ± 0.004        | 22.2              | 3.90       | 0.109 ± 0.009        | 22.7              | 3.90              |
| QQ-FLAG    | 0.100 ± 0.004        | 23.0              | 3.70       | 0.100 ± 0.025        | 22.0              | 4.01              |
| Q1         | 0.308 ± 0.007        | 22.8              | 3.85       | 0.108 ± 0.021        | 21.6              | 3.80              |
| Q2         | 0.310 ± 0.010        | 22.0              | 3.90       | 0.100 ± 0.018        | 22.6              | 4.30              |

**Fig. 3.** Standard curves for quantitation of SERT mutant expression. The indicated amounts of detergent-solubilized cell lysate from Myc-SERT-FLAG-expressing CHO and lec4 cells were separated by SDS-PAGE and visualized by Western blot analysis. The integrated density value for each band was converted to the equivalent amount of Myc-SERT-FLAG for antibodies (A) against peptide-purified and biotinylated SERT (A), Myc (B), and FLAG (C). In this way, the relative amount of SERT constructs was determined in both total cell lysate and the cell-surface pool isolated using streptavidin-agarose (Table I), and the expression levels for SERT forms could be compared even though they were detected with different antibodies. Immunoblots were quantitated using the VersaDoc 1000 system.
(18) and NET (21) proteins were demonstrated.

To study the effect of sialylated N-glycans on the functional association of SERT proteins, we used the previously reported SERT monomers: Res-FLAG (resistant to MTSEA inactivation and with a FLAG epitope tag at the C terminus) and Sens-Myc (sensitive to MTSEA inactivation and with a Myc epitope tag at the N terminus). MTSEA inactivates the serotonin transport function of Sens-Myc, but not of Res-FLAG.

If the physical association between these two forms of SERT has no effect on transport function, then the amount of inactivation in a 1:1 mixture should be equal to the amount of activity contributed by Sens-Myc, if the two forms are independent, as expected from the prediction for a two-hit inactivation process in which both subunits need to be modified for inactivation to occur; but in Lec4 cells, SERT monomers function independently.

In Lec4 cells, in addition to SERT, also other sialic acid-modified glycoproteins are defective. This fact limits our interpretation of the direct effect of sialic acid residues on the functional association of SERT monomers. To address this issue, we prepared mutant forms of the transporter (Res-FLAG-QQ and Sens-Myc-QQ) and monitored them in CHO cells. In this way, we could compare the effect of glycosylation modification in a system where it is known that the only difference is the glycosylation of SERT. Res-FLAG-QQ and Sens-Myc-QQ were expressed in the presence of 0.25 mM MTSEA and in CHO cells, the serotonin uptake by the mixed transporters was then measured in the presence of 0.25 mM MTSEA (Fig. 5C). In the absence of sialylated N-glycans, the experimental points did not functionally interact. The function of Res-FLAG-QQ and Sens-Myc-QQ coexpressed in CHO cells was found to be dependent on the presence of 0.25 mM MTSEA. Both proteins showed no inhibition or inactivation.

Fig. 4. Self-association ability of SERT proteins under different glycosylation patterns. CHO cells (lanes 1–4 and 6) and Lec4 cells (lane 5) were cotransfected with SERT-FLAG and Myc-SERT constructs at a 1:1 ratio where indicated, solubilized, and treated with mouse anti-Myc antibody (Ab)-coated rabbit anti-mouse protein A-Sepharose beads. The immunoprecipitates were separated and blotted with biotinylated anti-Myc antibody as described under "Experimental Procedures." Control experiments in which only SERT-FLAG- or SERT-transfected CHO cells were tested did not reveal a visible band (lane 6) on the immunoblot. All lanes contain protein recovered from the same number of cells equivalent to 30% of one well from a confluent 6-well dish. The positions of molecular mass standards run on the same gel are shown in kilodaltons. MSH, β-mercaptoethanol.

In Fig. 5, three possible results are plotted according to predictions of the amount of activity remaining after MTSEA treatment. If no functional association occurs between Res-FLAG and Sens-Myc, the amount of inactivation should be equal to the amount of activity contributed by Sens-Myc, i.e., 50% inactivation by MTSEA should be seen in a 1:1 mixture of Sens-Myc and Res-FLAG plasmids, and the resulting activities should fall on the straight dotted lines in Fig. 5. However, if SERT proteins functionally associate in a dimeric form, then a 75% inhibition would be expected from the same 1:1 mixture. The dashed lines assume random association of Res-FLAG and Sens-Myc into dimers and that all activity of all dimers containing Sens-Myc is sensitive to MTSEA. The solid lines assume random dimeric formation and that only dimers containing two Sens-Myc subunits would be sensitive to MTSEA.

To investigate the role of glycosylation in the functional interaction of SERT monomers, Res-FLAG and Sens-Myc were coexpressed first in CHO (Fig. 5A) and then in Lec4 cells (Fig. 5B) by mixing them in different ratios. The activity profile of the mixed transporters was then characterized in the presence of MTSEA. In Fig. 5A, the squares represent the amount of serotonin transport measured in the absence of MTSEA reagent. After treatment with MTSEA, less of the total activity (circles) was inactivated than expected based on the content of Sens-Myc, which means that the experimental points deviated noticeably from the dotted and dashed lines, an indication that the two forms of SERT functionally interact in CHO cells (Fig. 5A). However, in Lec4 cells, the experimental points fell on the dotted line, showing a linear relationship between these two forms of SERT and that they function independently (Fig. 5B). Our data from three independent experiments fit best with the solid line in CHO cells, but with the dotted line in Lec4 cells. Therefore, in CHO cells, the experimental points coincide with the prediction for a two-hit inactivation process in which both subunits need to be modified for inactivation to occur; but in Lec4 cells, SERT monomers function independently.

To understand the involvement of glycosylation in myosin IIA-SERT association, co-IP assays were performed with Lec4 cells expressing SERT (Fig. 6A, lane 3) or with CHO cells coexpressing Myc-QQ and myosin IIA (lane 2). Although transiently transfected myosin IIA protein was detected in Lec4 cells (lane 4), it was not detected in the co-immunoprecipitate with Myc-SERT (lane 3). However, studying protein-protein interactions in heterologous expression systems suffers from drawbacks such as possible overexpression problems. To further explore the possible obstacles of a heterologous expression system, we tested myosin IIA-SERT interaction in placental choriocarcinoma JAR cells, which constitutively express both proteins (Fig. 6B, lanes 1 and 2). Immunoblot analysis of JAR cell lysate with biotin
ylated anti-SERT antibody produced three major bands at 90, 65, and 60 kDa, similar to those observed for the transiently transfected SERT-expressing CHO or Lec4 cells (Fig. 1, lanes 1 and 5). After confirming the endogenous expression of myosin IIA and SERT in JAR cells, a co-IP assay with anti-myosin IIA antibody was performed to test their association in JAR cells in the presence of 10 mM MgATP. Probing the myosin IIA-bound proteins with biotinylated anti-SERT antibody resulted in a SERT protein stain at 65 kDa, indicating an endogenous association between myosin and SERT proteins (Fig. 6B, lane 4). Similarly, under reduced electrophoretic conditions, the 90- and 60-kDa species appeared to be associated with myosin IIA (lane 7). We found that even though the 90-kDa species was predominantly stained by biotinylated anti-SERT antibody, the 60-kDa species also associated with myosin IIA.

Although transiently transfected Myc-QQ protein was easily detected in JAR cells using anti-Myc antibody (Fig. 6B, lane 3), it was not detected in the co-immunoprecipitate with myosin IIA (lane 5), an observation that strengthened our confidence in the results of glycosyl modification in myosin IIA-SERT co-precipitation. Our data strongly indicate glycosylation-dependent association of myosin IIA and SERT. To control for nonspecific precipitation of myosin IIA, the same analysis was done without anti-mycin IIA antibody (lane 6), and no protein band was labeled, demonstrating that myosin IIA did not bind nonspecifically to the protein A beads.

**Glycosylation and Regulation of rSERT by cGMP**—The functional consequence of the involvement of N-glycosyl modification on myosin IIA-SERT interaction was tested by measuring the serotonin uptake function of transporters upon stimulation with a cGMP donor, 8-bromo-cGMP, in both our model systems. CHO and Lec4 cells were transiently transfected with SERT or QQ constructs. The following day, they were preincubated in serum-free medium containing 1 mM 8-bromo-cGMP for 1 h at 37 °C (60) and then assayed for serotonin uptake function in the dark in the presence of 8-bromo-cGMP. Fig. 7 shows that SERT expressed in CHO cells was also activated in the cGMP pathway. Addition of 8-bromo-cGMP increased the activity of heterologously expressed SERT by 66% of the control value in the absence of the cGMP donor. In contrast, neither the QQ mutant in CHO cells nor SERT in Lec4 cells was significantly affected by 8-bromo-cGMP.

**DISCUSSION**

The oligosaccharide residues of glycoproteins in general are believed to be important for a variety of functions, including
correct protein folding, oligomerization, membrane trafficking, and targeting (37–42). SERT is a homo-oligomeric glycoprotein (18–21) with two sialic acid residues on each complex oligosaccharide residues. In two model systems, we demonstrated that defects in sialylated N-glycans do not alter whole cell or plasma membrane expression of SERT proteins, but do decrease serotonin uptake function. These findings suggest that glycosylation may contribute to the correct folding and oligomeric properties of SERT proteins, as reported for epidermal growth factors and insulin receptors (48, 49). Several studies using physical measurements such as gel filtration, cross-linking, and fluorescence resonance energy transfer techniques also reported, as we did (18), that SERT is a homo-oligomeric protein with functional interactions between subunits that occur in intracellular compartments (18–21, 61). We wondered if modifications with sialylated N-glycans directly participate in the serotonin uptake function or confer an optimum conformation to SERT proteins that facilitates their protein-protein interactions. Our data demonstrate that defects in glycosylation, specifically in sialic acid residues, impair homo-oligomerization of SERT proteins. The lower uptake rates of QQ in CHO cells and of SERT in Lec4 cells must be due to the transport capacity of the active SERT monomers.

To determine the impact of glycosyl modification with sialylated N-glycans, we studied the hetero-oligomeric interactions of SERT, specifically with myosin, under defective glycosylation conditions. Our rationale for choosing myosin is based on three related reasons. 1) Both glycosylation sites are located at the extracellular side of the plasma membrane; therefore, we sought a cytosolic partner for SERT. 2) Myosin is an abundant cytoskeletal protein and a strong candidate partner for SERT, as it is a protein kinase G-anchoring protein (50), and the
uptake function of SERT is regulated in the NO-dependent pathway via cGMP-dependent protein kinase G (15) through interactions with anchoring proteins (50). 3) The impact of glycosylation could be observed in the functional hetero-oligomerization of SERT. By measuring the serotonin uptake function in the presence of the cGMP donor 8-bromo-cGMP, the effect of glycosylation on the myosin-SERT interaction could be monitored.

The overall findings from both model systems suggest that modification with sialylated N-glycans is required for myosin-SERT hetero-oligomerization. Glycosyl modification of SERT should occur earlier than the myosin-SERT association. If glycosylation directly contributes to SERT function rather than having an impact on its conformation, then the cGMP donor should still be able to stimulate the uptake function of SERT under the defective glycosylation conditions, and the myosin-SERT association should not be impaired. If glycosylation has an impact on the conformation (positioning) of SERT proteins, then myosin-SERT interactions and regulation of SERT function in the NO-dependent pathway should be impaired under defective glycosylation conditions.

On the basis of our present data and previous reports about the sialic acid residues of SERT (27–30), we believe that modification with the sialylated N-glycans confers on SERT proteins an optimum position that exposes the homo-oligomeric domains on SERT for self-association. Defects in sialyl-N-glycan modification impair SERT-SERT association, and as a result, the myosin-SERT binding domain(s) are not accessible for hetero-oligomerization or regulation in the NO-dependent pathway. However, one other way to see the results is that other protein(s) that regulate SERT NO/cGMP-sensitive pathway may also have an impact on its conformation, then the cGMP donor should not function under defective glycosylation conditions. In the future, identification of these proteins an optimum position that exposes the homo-oligomeric domains on SERT for self-association, but could not function under defective glycosylation conditions.

The use of the Lee4 mutant cells in this study was a novel approach to elucidate the role of N-linked glycosylation in the structure and function of SERT and to demonstrate the significance of the sialic acid residues. Future studies using wild-type SERT in other Lee mutant cells should further advance our understanding of glycosyl modification of monoamine transporter structure, function, and regulation. Additionally, the application of fluorescence resonance energy transfer techniques in living cells will allow for a more direct observation of the role of sialylated N-glycans in oligomeric states of SERT proteins. Identification and characterization of the factors associated with the regulation of SERT uptake function will provide vital information that might lead to new treatments for patients with major neuropsychiatric disorders.

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WITHDRAWN
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