Molecular Chaperones Stimulate the Functional Expression of the Cocaine-sensitive Serotonin Transporter*

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The serotonin transporter (SERT) is an N-glycosylated integral membrane protein that is predicted to contain 12 transmembrane regions. SERT is the major binding site in the brain for antidepressant drugs, and it also binds amphetamines and cocaine. The ability of various molecular chaperones to interact with a tagged version of SERT (Myc-SERT) was investigated using the baculovirus expression system. Overexpression of Myc-SERT using the baculovirus system led to substantial quantities of inactive transporter, together with small amounts of fully active and, therefore, correctly folded molecules. The high levels of inactive Myc-SERT probably arose because folding was rate-limiting due, perhaps, to insufficient molecular chaperones. Therefore, Myc-SERT was co-expressed with the endoplasmic reticulum (ER) molecular chaperones calnexin, calreticulin and immunoglobulin heavy chain binding protein (BiP), and the foldase, ERP57. The expression of functional Myc-SERT, as determined by an inhibitor binding assay, was enhanced nearly 3-fold by co-expressing calnexin, and to a lesser degree on co-expression of calreticulin and BiP. Co-expression of ERP57 did not increase the functional expression of Myc-SERT. A physical interaction between Myc-SERT-calnexin and Myc-SERT-calreticulin was demonstrated by co-immunoprecipitation. These associations were inhibited in vivo by deoxyo-jirimycin, an inhibitor of N-glycan precursor trimming that is known to prevent the calnexin/calreticulin-N-glycan interaction. Functional expression of the unglycosylated SERT mutant, SERT-QQ, was also increased on co-expression of calnexin, suggesting that the interaction between calnexin and SERT is not entirely dictated by the N-glycan. SERT is the first member of the neurotransmitter transporter family whose folding has been shown to be assisted by the molecular chaperones calnexin, calreticulin, and BiP.

The serotonin transporter (SERT) is a member of a small family of integral membrane proteins that transport neurotransmitters and osmolytes into cells by coupling uptake to the influx of Na+ and Cl− ions down their concentration gradients (reviewed in Refs 1–3). SERT is of particular pharmacological interest, because it is the primary binding site in the brain for antidepressant drugs, and it also interacts with cocaine and amphetamines (4, 5). All members of this family share two structural characteristics, namely 12 putative transmembrane domains and a large extracellular loop between transmembrane domain 3 and transmembrane domain 4. This particular loop is glycosylated in all members of the family, although the number of potential N-glycans varies between 1 and 4, and the sites of glycosylation are not absolutely conserved. SERT has two N-glycans in this region, and their importance for its activity has been tested by altering the consensus N-glycosylation sequence from Asn-X-Ser/Thr to Gln-X-Ser/Thr; deletion of both N-glycosylation sites in SERT neither changed the Kᵢ₅₅ for binding of the inhibitor RTI55 (6). However, the number of functional molecules of SERT/cell decreased to 5% that of the levels of fully glycosylated SERT, suggesting that N-glycosylation plays an important role in either the initial folding of SERT or for its stability in the membrane (6). Other members of the neurotransmitter transporter family were later shown to share this characteristic with SERT (7–9).

In addition to N-glycosylation, the large extracellular loop between transmembrane domains 3 and 4 may also contain a disulfide bond between two Cys residues 8 amino acids apart. Alteration of either Cys residue severely reduced cell surface expression of functional SERT or the dopamine transporter, but replacement of both Cys residues led to only a slight reduction in activity (10, 11). The two Cys residues proposed to form the disulfide bond are absolutely conserved throughout this neurotransmitter transporter family, suggesting that the disulfide bond may be another essential structural feature for efficient, stable, cell surface expression.

Molecular chaperones are a diverse collection of proteins that interact transiently with unfolded and misfolded proteins, thus preventing improper protein-protein interactions until the protein folds correctly (reviewed in Refs 12 and 13). When the protein reaches its final correct three-dimensional structure, the molecular chaperone is released. If the protein never folds correctly, then the interaction between the molecular chaperone and the misfolded protein may be more stable (12, 13). A few membrane proteins, including some ion channels (14, 15), transporters (16–19), and G protein-coupled receptors (20–22) have been shown to interact in vivo with various molecular chaperones. Most molecular chaperones, including immunoglobulin heavy chain binding protein (BiP), interact directly

iodophenyl)tropane; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline-Tween 20; p.i., post-infection.
with the polypeptide chain of the unfolded protein (23), but two molecular chaperones (calnexin and calreticulin) have been shown to interact primarily with a precursor N-glycan attached to the proteins (24–27). Calnexin contains a single transmembrane domain that anchors it to the ER, where it is thought to play a crucial role in retention of misfolded proteins (24, 28). Despite being related at the sequence level, calreticulin and calnexin often show different binding characteristics to an unfolded protein (27, 29–31). The protein disulfide isomerase ERp57 also acts only on N-glycosylated proteins (32, 33), but this is because of its recruitment by either calnexin or calreticulin (34). The requirement of the neurotransporter transporters for N-glycosylation for efficient functional expression suggests that N-glycan-specific molecular chaperones and foldases may be involved in their synthesis. In this paper we test this hypothesis by the co-expression of calnexin, calreticulin, and ERp57 with SERT in insect cells using the baculovirus expression system.

The baculovirus expression system is currently used to over-express many glycosylated integral membrane proteins in sufficient quantities for purification and structural studies (35, 36). Expression of SERT in insect cells using recombinant baculoviruses normally results in about 500,000 copies/cell of functional SERT (determined by a binding assay using the cocaine analogue 125I-RTI55) and substantially greater amounts of inactive transporter (6). The amount of inactive, unglycosylated, transporter increases dramatically after day 2 post-infection when there is no further increase in the amount of functional SERT expressed. One explanation for this is that the folding of SERT, presumably co-translational (37, 38), has become uncoupled from membrane insertion and protein translocation. If folding has become the rate-limiting step during the overexpression of SERT, then increasing the amounts of specific molecular chaperones or foldases that help SERT to fold could increase the amount of functional SERT expressed. Consequently, a selection of different folding assistance factors, including the N-glycan binding molecular chaperones (calnexin, calreticulin), an Hsp70 family member (BiP), and the foldase ERp57 were co-expressed with SERT in insect cells to examine their roles in the generation of active SERT. The specific folding factors required by SERT in vivo are not yet known, so any molecular chaperones or foldases that improve the overexpression of functional SERT in insect cells can be regarded as potential candidates for efficiently folding the SERT in native tissues.

MATERIALS AND METHODS

Construction of Recombinant Baculoviruses—A cDNA encoding Myc-SERT was constructed from SERT-TAG (6) by ligating an oligonucleotide encoding a calmodulin binding domain in the unique NcoI site around the initiating Met codon, and an oligonucleotide encoding a His10 tag was ligated into the NotI/XhoI sites before the termination codon. All molecular biology techniques were as described in Ausubel et al. (39). The predicted amino acid sequence of Myc-SERT at the N and C termini is as follows (SERT amino acid residues are underlined, and the cMyc tag is in bold): "...METT.... NAVAAAHHH-...". All oligonucleotides ligated into the SERT-TAG cDNA were checked by DNA sequencing. The Myc-SERT cDNA was ligated into plasmid pVL1392 (Pharmingen) and a recombinant baculovirus was constructed using Baculogold (Pharmingen) exactly as described by the manufacturer. Recombinant viruses were isolated after one round of plaque purification and amplified using standard techniques (40, 41). A recombinant baculovirus expressing ERp57 was constructed from the cDNA ligated in pBlueBacIII (BaculoGold) using the MluI cloning site (Invitrogen). A baculovirus-expressing calreticulin was constructed from the human calreticulin cDNA (43) (a kind gift from D. H. Llewellyn) by ligating it into pVL1392 and using the BacVector-3000 transfection kit (Novagen) to construct the recombinant baculovirus. Construction of the murine chaperone BiP baculovirus has been described previously (44). The virus containing the canine calnexin gene (45) was constructed using the BacPAK baculovirus system and techniques (CLONTECH).

Expression of SERT and Molecular Chaperones in Insect Cells—Methods for culturing and infecting insect cells (40, 41) were exactly as described in (6, 46). Two different conditions were used for expression experiments. For comparing the effects of molecular chaperones on the expression of SERT, 100-μl aliquots containing 1× 10⁶ cells were infected with recombinant baculoviruses at a multiplicity of infection of 5. Cultures were grown at 27 °C in stirred flasks (Techne, Cambridge, UK) that were stirred at 60 rpm. Aliquots were removed at specific time points, and membranes were prepared (see below). For experiments involving 1-deoxynojirimycin (Sigma), 2 × 10⁶ cells were grown in 25-cm² flasks containing 4 ml of medium, all the cells were harvested after incubation at 27 °C for a specified time. The medium used in all these experiments was TNM-FH insect medium (Sigma) supplemented with 10% bovine calf serum (PAA Laboratories, Austria) and 0.1% Pluronic F68 (Sigma).

Membrane Preparation and Western Blotting—Membranes (1 ml) were prepared from 1 × 10⁶ cells as described by Tate (40) by shearing the cells through a 28-gauge needle in 0.1× phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and 2.5 μg/ml leupeptin. Western blotting and SDS-PAGE were performed using standard techniques (39). Western blots were prepared using ECL-nitrocellulose (Amersham Pharmacia Biotech) and developed using the enhanced chemiluminescent system (ECL, Amersham Pharmacia Biotech). Antibodies against N-glycosylated calnexin (C terminus), BiP (Grp78), and ERp57 were purchased from Stressgen (Victoria, Canada). A culture supernatant of anti-c-Myc antibody was prepared from the hybridoma cell line 9E10 (47) and was used without further purification. An anti-SERT antibody (C72A) that recognizes the C terminus of SERT was a kind gift from R. Blakely. All the antibodies used in Western blots were incubated for 1 h at room temperature at a dilution of 1:500 in 5% nonfat milk in TBST (20 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20).

Inhibitor Binding Assays for SERT—Binding assays were performed as described (6, 46). Briefly, crude membranes were incubated with 2 nM 125I-RTI55 in the presence or absence of 200 μM serotonin to determine specific binding. Membranes were pelleted by centrifugation (10,000 g, 5 min), the supernatant was removed, and the radioactivity in the membranes was determined using a gamma counter. Assays were performed in triplicate. Total binding represented 10% or less of the total available 125I-RTI55. Typically 1 μl of membranes containing Myc-SERT (as prepared above) was used per binding assay, but 10 μl of membranes containing SERT-QQ were used, because it is expressed at about 20-fold lower levels than glycosylated SERT.

Immunoprecipitation and Western Blotting—Membrane samples were treated with 1% digitonin (Sigma-Aldrich) buffer containing 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) to solubilize membrane proteins. Crude membrane samples were clarified at 16,000 g for 15 min to separate aggregates from soluble fractions. Protein A-Sepharose beads (50 μl/sample) were washed twice with cold phosphate-buffered saline and incubated with 5 μl of anti-c-Myc antibody (9E10) or 1% anti-SERT antibody in 50 μl of phosphate-buffered saline for 1 h at 4 °C on a rotator. After incubation, 100 μl of clarified sample was added to each tube and immunoprecipitated overnight on a rotator at 4 °C. Samples were then centrifuged and washed four times with cold phosphate-buffered saline. Immunoprecipitates were analyzed by SDS-PAGE under reducing and denaturing conditions. The gel was then transferred onto a nitrocellulose membrane (Life Technologies, Inc.) and incubated overnight in 10% skim milk (ICN) in TBST on an orbital shaker. Subsequent to blocking, nitrocellulose membranes were washed three times for 15 min in TBST and then incubated for 1 h at room temperature in primary antibody solution (2% skim milk (ICN), 20% fetal bovine serum (Life Technologies, Inc.), 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), 0.02% Thimerosal (Sigma-Aldrich)) containing primary antibody (either anti-calnexin C terminus (Stressgen) at 1:6000 or anti-calreticulin (Stressgen) at 1:8000). After 3-15 min washes in TBST, the blots were incubated for 1 h in 1:15,000 dilution of peroxidase-conjugated goat anti-rabbit IgG (Pierce) in 5% skim milk in TBST. After 3 more washes in TBST for 15 min each, blots were developed with the Super Signal chemiluminescence system (Pierce).

RESULTS

Co-expression of Molecular Chaperones Affects the Amount of Functional SERT Expressed—The serotonin transporter and the molecular chaperones calnexin, calreticulin and BiP, and the foldase ERp57 were incorporated into separate baculovirus vectors for expression in insect cells. The serotonin transporter...
used in this study was altered to contain a cMyc tag at the N terminus (Myc-SERT) to allow easy identification of SERT using an anti-cMyc antibody (9E10) (47). Myc-SERT also contained a calmodulin binding domain at the N terminus and a His10 tag at the C terminus; both tags are used for the purification of Myc-SERT. Placing tags at the N and C termini of SERT does not affect either the $K_d$ for RTI55 binding or the $K_m$ for serotonin uptake into insect cells (6, 46). The amino acid sequences of the molecular chaperones were unaltered. All the molecular chaperones and Myc-SERT were expressed under the control of the polyhedrin promoter. Sf9 insect cells were infected with these recombinant baculoviruses at a multiplicity of infection of 5. Total cellular membranes were prepared from aliquots of cells up to 4 days post-infection (p.i.) and assayed for the amount of functional Myc-SERT using the inhibitor $^{125}$I-RTI55. It was assumed that if $^{125}$I-RTI55 bound to Myc-SERT in a serotonin-protectable manner, then Myc-SERT was functional i.e. if it was present at the plasma membrane, then it could transport serotonin. It was not possible to quantitate Myc-SERT levels by measuring $[^3]$Hserotonin uptake, because the cells become leaky on Myc-SERT expression, preventing any serotonin transport (6).

To examine the role of molecular chaperones and foldases in the folding of the serotonin transporter, Myc-SERT was initially expressed in the presence of either calnexin, calreticulin, ERp57, BiP or the control virus, by-pVL. The control virus (by-pVL (6)) does not express any protein from the polyhedrin promoter and was used to maintain a constant multiplicity of infection per experiment, because increasing the amount of virus infecting the cells can decrease levels of Myc-SERT expression. Western blots of insect cells expressing mammalian molecular chaperones were probed with antibodies to each molecular chaperone and confirmed that they were expressed (results not shown). The amount of functional Myc-SERT expressed, as determined by inhibitor binding, increased over time, with a maximum functional expression 2–3 days p.i. depending on which molecular chaperone was present (Fig. 1a). Calnexin consistently increased the amount of functional Myc-

FIG. 1. Expression levels of functional Myc-SERT co-expressed with molecular chaperones. Insect cultures (100 ml) co-infected with baculoviruses expressing Myc-SERT and various molecular chaperones were grown at 27 °C. Membranes were prepared from 10-ml aliquots of cells at various time intervals and assayed for functional Myc-SERT using the inhibitor $^{125}$I-RTI55. a, time course of Myc-SERT expression. Myc-SERT was co-expressed with baculoviruses expressing molecular chaperones calnexin (black squares), calreticulin (open squares), ERp57 (open circles), BiP (open triangles), control virus by-pVL (black circles). b, comparison of functional Myc-SERT expression on day 3 post-infection. Myc-SERT was co-expressed with the molecular chaperones as indicated, and the control was Myc-SERT co-infected with by-pVL. Error bars represent the S.E. with $n = 6$ for CXN, $n = 4$ for BiP, and $n = 2$ for CRT and ERp57. c, comparison of functional Myc-SERT expression on day 3 post-infection when co-expressed with calnexin and additional molecular chaperones as indicated. Error bars represent the S.E. ($n = 2$). d, comparison of functional SERT expression on day 3 post-infection when co-expressed with the molecular chaperones as indicated. Error bars represent the S.E. ($n = 3$).

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SERT expressed nearly 3-fold (Fig. 1b), with calreticulin and BiP giving only a slight increase in functional SERT expression (1.3-fold and 1.4-fold, respectively). Co-expression of ERp57 did not enhance the amount of functional SERT expressed.

Calnexin and calreticulin can recruit ERp57 to promote the folding of proteins containing disulfide bonds (34), so co-expression experiments were conducted using combinations of Myc-SERT, calnexin, calreticulin, and ERp57. No further increase in functional Myc-SERT expression above that observed in the presence of calnexin was observed (Fig. 1c). Similar experiments co-expressing Myc-SERT, BiP, and calnexin showed that the individual effects of BiP and calnexin on improving functional Myc-SERT expression were not additive and actually resulted in a decrease in functional Myc-SERT expression (Fig. 1c). However, this result may be linked to the simultaneous expression of three different recombinant proteins from the polyhedrin promoter, leading to an overload of the protein synthesis capacity of the insect cells and a reduction in total SERT produced.

All the experiments above were performed on Myc-SERT, which differs from the native SERT by having tags at the N and C termini (see “Materials and Methods”). The effect of co-expression of molecular chaperones with untagged SERT was also studied (Fig. 1d). Calnexin clearly increases the level of functional SERT, but the co-expression of BiP and CRT was not helpful. In addition, calnexin did not improve SERT activity as much as was observed for Myc-SERT, suggesting that the effects of BiP and CRT on Myc-SERT activity would likely be too small to be detected on untagged SERT.

The amount of total Myc-SERT expressed in the presence of the molecular chaperones was assessed by Western blotting using an anti-cMyc antibody (Fig. 2). Quantitation was performed using the program Geltrak (49, 50) by simple integration of the area under the peaks. Both glycosylated and unglycosylated Myc-SERT were always expressed regardless of the type or presence of other co-expressed proteins. However, the amount of glycosylated Myc-SERT expressed on day 2 p.i. decreased slightly when BiP and calnexin were co-expressed (62 and 91% of the control, respectively). This contrasts with the increase in functional Myc-SERT expressed (the binding assays for these samples are shown in Fig. 1a) i.e. 110 and 180%, respectively. This phenomenon is most apparent in the experiment co-expressing Myc-SERT, calnexin, and BiP on day 1 p.i. compared with the control (Fig. 2b). On day 1 p.i. there were identical amounts of functional SERT (Fig. 2b), yet the Western blotting signal for glycosylated Myc-SERT co-expressed with calnexin and BiP was considerably weaker than the control. Quantitation of these bands by scanning densitometry showed that glycosylated and unglycosylated Myc-SERT represented 8 and 3%, respectively, of the levels observed in the absence of co-expressed chaperones, despite equal amounts of functional SERT present in both cases. In this instance, co-expression of calnexin and BiP with Myc-SERT resulted in a 18-fold increase in the ratio of active to inactive transporter, assuming that all the Myc-SERT expressed in the presence of calnexin and BiP is active.

Another effect of co-expressing Myc-SERT with calnexin is observed when SERT is solubilized from membranes with the detergent digitonin. The total and soluble Myc-SERT levels with and without co-infection are shown in Fig. 3a. As noted previously, co-infection with multiple baculoviruses reduces the total SERT expression level. However, the soluble Myc-SERT levels in Fig. 3a actually increase following co-infection with pVL, although not to the level observed with CXN co-expression. As has been observed previously with insect cells and Escherichia coli, reducing expression levels can often increase the solubility of heterologous proteins (51, 52). Comparing the two infections with pVL and CXN indicated comparable total SERT levels according to densitometric analysis, but a 2-fold enhancement in the soluble SERT levels was detected following CXN co-expression. This increase is comparable with the 2-fold increase observed on day 2 p.i. in the activity measurements following CXN co-expression (Fig. 1a).

Direct Interaction of Myc-SERT with Calnexin and Calreticulin Shown by Co-immunoprecipitation—To examine the possible association of the molecular chaperones calnexin and calreticulin with SERT, co-immunoprecipitation experiments were performed. Insect cell membranes from day 2 p.i. were solubilized in digitonin, and the insoluble fraction was removed by centrifugation. The digitonin-soluble fraction was then used in the co-immunoprecipitations. Digitonin preferentially solubilizes the glycosylated form of SERT and does not solubilize any of the unglycosylated SERT (Fig. 3a). It is likely that all the unglycosylated SERT is inactive (6) and forms a digitonin-resistant aggregate in the ER, which can only be solubilized using harsher detergents.2 The soluble fraction also contained the molecular chaperones e.g. calnexin (Fig. 3b).

Digitonin extracts of membranes containing Myc-SERT and calnexin were immunoprecipitated with the anti-cMyc antibody, and the immunoprecipitate was separated by SDS-PAGE and Western blotted using an anti-calnexin antibody (Fig. 3c). Calnexin was immunoprecipitated with anti-cMyc antibody when Myc-SERT and calnexin were co-expressed, but calnexin was not precipitated when it was expressed alone (Fig. 3c). These data indicate that there is a specific interaction between Myc-SERT and calnexin. Similarly, co-immunoprecipitation of calreticulin and Myc-SERT with the anti-cMyc antibody showed that calreticulin also interacts with Myc-SERT (Fig. 4).
The protein observed at lower molecular weights in all the lanes of the immunoprecipitation blot in Fig. 4 is the immunoglobulin heavy chain from the anti-cMyc antibody.

The Interaction of Calnexin with SERT Is Predominantly via the N-Glycan—Previous studies have indicated that the interaction between calnexin and N-glycosylated proteins is mainly dependent on the presence of a monoglucosylated N-glycan precursor (GlcNac-Man_Glc). If glucose trimming by glucosidases I and II is inhibited by the presence of 1-deoxynojirimycin (dNJM), calnexin binding to the N-glycan can be prevented (24). Insect cells infected with baculoviruses expressing Myc-SERT and calnexin were, therefore, grown in the presence or absence of dNJM. Membranes from the cells were assayed for functional expression of Myc-SERT and the association between calnexin and Myc-SERT. In the absence of calnexin, dNJM has little effect on functional Myc-SERT expression (Fig. 5a). When Myc-SERT is co-expressed with calnexin, an increase in functional Myc-SERT expressed per cell is observed; in the presence of dNJM, functional Myc-SERT expressed is decreased to the level seen in the absence of calnexin (Fig. 5a). In these experiments, calnexin did not increase the amount of functional SERT as much as in the experiments depicted in Fig. 1. This is probably because the experiments using dNJM were conducted on cells adhered to tissue culture flasks containing 4 ml of medium, and the experiments described in Fig. 1 were grown in well aerated spinner cultures. An immunoprecipitation of Myc-SERT co-expressed in the presence of calnexin and dNJM showed minimal levels of calnexin associated with Myc-SERT compared with Myc-SERT and calnexin expressed in the absence of dNJM (Fig. 5b). The background levels of observed calnexin may be because of the inability to block all glucosidase-mediated N-glycan trimming in insect cells under the conditions employed, or perhaps there is a weak association between calnexin and Myc-SERT when the N-glycans are in the glucosylated form. Similar experiments showed no association between calreticulin and Myc-SERT in the presence of dNJM (Fig. 5c).

A second approach we used to look at the interaction between SERT and calnexin was to use an unglycosylated SERT mutant (SERT-QQ) that had both Asn residues (N-208 and N-217) normally glycosylated changed to Gln (Q) residues. SERT-QQ was expressed at 20-fold lower levels than glycosylated SERT but was still fully functional according to binding assays (6). Surprisingly, co-expression of SERT-QQ with calnexin led to a small but reproducible increase in the amount of SERT-QQ expressed (Fig. 6a), which was inhibitable by dNJM (Fig. 6b). Immunoprecipitations failed to show any stable interaction between SERT-QQ and calnexin (Fig. 6b).

DISCUSSION

The serotonin transporter is a complex integral membrane protein composed of 12 transmembrane regions that requires N-glycosylation for efficient folding and stability (6) and the presence of a putative disulfide bond for activity (11). We have shown here that the production of Myc-SERT is enhanced by at least three molecular chaperones. The interactions between Myc-SERT and the molecular chaperones was investigated in insect cells using the baculovirus expression system. The presence of large quantities of inactive Myc-SERT expressed in
insect cells suggested that folding might be rate-limiting for the production of functional Myc-SERT using the baculovirus expression system. In this study, the role of molecular chaperones in generating active Myc-SERT was investigated, and we found that co-expression of three molecular chaperones increased the amount of functional SERT expressed. The role of these chaperones may be to retain Myc-SERT in a folding-competent conformation and prevent nonspecific aggregation. Calnexin was the most successful of the co-expressed molecular chaperones, improving functional Myc-SERT expression by nearly 3-fold. Smaller increases in functional Myc-SERT expression were also seen on co-expression of calreticulin and BiP. In addition, specific interactions between Myc-SERT and calnexin, and Myc-SERT and calreticulin, were confirmed in co-immunoprecipitation experiments. It was not possible to demonstrate an interaction between BiP and Myc-SERT in the same way because of nonspecific interactions in the immunoprecipitation. The N-glycan-specific protein disulfide isomerase, ERp57, did not increase functional Myc-SERT expression either in the presence or absence of other molecular chaperones.

Increased expression of functional Myc-SERT was a consequence of molecular chaperone activity and not because of increases in transcription or translation. This was apparent from Western blots of membranes prepared from cells coexpressing Myc-SERT and the molecular chaperones calnexin and BiP (Fig. 2). The levels of unglycosylated and glycosylated Myc-SERT did not increase in parallel with the increases in functional Myc-SERT determined from inhibitor binding, suggesting that the increases were because of better folding of Myc-SERT in the presence of molecular chaperones. In fact co-expression of calnexin and/or BiP actually decreased the amount of inactive Myc-SERT expressed. This was particularly apparent on co-expression of Myc-SERT, calnexin, and BiP, where on day 1 p.i., glycosylated and unglycosylated Myc-SERT represented 8 and 3%, respectively, of the control levels on a Western blot, despite containing equal amounts of functional SERT. A number of possible mechanisms could explain this...
decrease in nonfunctional Myc-SERT expression, including increased proteolysis of misfolded Myc-SERT or perhaps decreased rates of translation because of better coupling between folding and translation.

The simplest explanations for the large increase in functional Myc-SERT expression on co-expression of calnexin are that there is little or no calnexin expressed in insect cells or that insect calnexin does not interact with Myc-SERT. This was suggested from expressing Myc-SERT in the presence of dNJM. The generation of the monoglucosylated N-glycan precursor is prevented by dNJM; hence, dNJM is expected to prevent calnexin from interacting with Myc-SERT in vivo, as we have observed. However, in the absence of co-expressed calnexin, dNJM has little effect on functional Myc-SERT expression, suggesting that the folding of Myc-SERT in insect cells is not dependent on an insect calnexin. At this stage, it cannot be determined whether there is no endogenous insect calnexin in the cells or whether there is calnexin and that it does not interact with Myc-SERT like mammalian calnexin. Western blots of insect cell membranes using antibodies to mammalian calnexin showed weakly cross-reacting proteins, but it is not clear whether they represent calnexin homologues. SF9 cells may not be the only insect cells to lack calnexin activity, because this has also been reported for a Drosophila cell line (53).

The large increase in levels of functional Myc-SERT caused by co-expressing calnexin warranted a further investigation of the interaction between them. Therefore, an unglycosylated SERT mutant, SERT-QQ, was co-expressed with calnexin. Surprisingly, calnexin was capable of increasing the functional expression of SERT-QQ. Co-immunoprecipitations suggested that there was no measurable interaction between calnexin and SERT-QQ. However, this does not mean that there was never any transient association between SERT-QQ and calnexin, because any such interaction may well have been disrupted by digitonin solubilization before immunoprecipitation. This would be likely if calnexin interacted with SERT via its transmembrane region, as has been suggested for the association between calnexin and P-glycoprotein (54). The interaction between SERT-QQ and calnexin also seemed to be inhibited by dNJM. The most straightforward conclusion is that dNJM can interact directly with calnexin and prevent its function as a molecular chaperone; this may not be surprising given that glucosidase II, which is inhibited by dNJM, recognizes the identical monoglucosylated N-glycan precursor as calnexin. An alternative explanation is that a small proportion of SERT-QQ is N-glycosylated, but this seems unlikely as there are no other consensus N-glycosylation sites predicted to be in SERT facing the ER lumen, including the rarely used consensus Asn-X-Cys (see for example Ref. 55). These results could also be explained by an indirect effect of calnexin on SERT-QQ i.e. calnexin improved the folding of another unidentified molecular chaperone in insect cells that then helped SERT-QQ to fold.

The experiments described in Fig. 1, a–c all use a tagged version of SERT (Myc-SERT), with the tags at the N and C termini predicted to be in the cytoplasm. The molecular chaperones and foldases BiP, CRT, and ERp57 are all present in the ER lumen and, therefore, cannot interact directly with tags. The functional domain of calnexin is in the ER lumen, but it also has a single transmembrane domain and a short C-terminal domain that is in the cytoplasm. Thus the tags on SERT could only potentially interact directly with the C terminus of calnexin. However, although the C terminus of calnexin is essential for the proper localization of calnexin in the ER, it is not believed to be required for function of calnexin (34). Given the intracellular distribution of the molecular chaperones, it was surprising that the presence of the tags on SERT affected the ability of molecular chaperones to improve the expression of the transporter (Fig. 1). In addition, the amount of functional Myc-SERT per cell does not differ appreciably from the amount of functional untagged SERT per cell when they are expressed independently. Yet co-expression of calnexin with Myc-SERT led to a 3-fold improvement in functional activity as compared with a 1.4-fold improvement in expression of untagged SERT. It is possible that the tags on SERT have a subtle effect on either the folding pathway or kinetics of folding, which provide a greater opportunity for the co-expressed molecular chaperones to bind and have an effect. Presumably, in the absence of additional tags, SERT can be folded sufficiently using the molecular chaperones naturally found in insect cells, and additional molecular chaperones therefore have little effect.

We have shown that the folding of Myc-SERT in vivo can be assisted by the molecular chaperones calnexin, calreticulin, and BiP. In addition, it has recently been shown that the foldase NinaA, a membrane-bound peptidyl-prolyl cis-trans isomerase from Drosophila, can increase the cell surface expression of the dopamine transporter (56), a homologue of SERT. Elucidating the role of molecular chaperones and foldases in the folding of SERT will not only lead to an understanding of how complex membrane proteins fold, but it will also help us to overexpress proteins for subsequent purification and structural analysis. Many membrane proteins are expressed very poorly in heterologous expression systems (35, 36), and this may be a direct consequence of the lack of specific molecular chaperones in the cell lines used. Alternatively, there may be insufficient amounts of molecular chaperones to cope with the high level of protein translation induced in most heterologous expression systems. For example, recombinant IgG secretion from insect cells is enhanced by co-expressing BiP or protein disulfide isomerase (57, 58). Unfortunately, co-expressing multiple molecular chaperones simultaneously has not been successful, suggesting that their relative levels of expression may require careful modulation. The inclusion of several chaperones and foldases on one virus, and the use of vectors including lower strength promoters, such as IE1 or the basic protein promoter, may help to rectify this limitation.

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