The Role of ERp44 in Maturation of Serotonin Transporter Protein*

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Background: ERp44 favors maturation of disulfide-linked oligomeric proteins. Results: ERp44 bound to SERT but preferentially to Cys mutants; in ERp44-silenced cells, 5-HT uptake was down-regulated; MTSEA-biotin labeled SERT with a higher affinity, indicating more free Cys on SERT in silenced cells. Conclusion: A disulfide link between Cys-200 and Cys-209 is a prerequisite for SERT oligomerization. Significance: This is the first study showing the involvement of ERp44 in maturation of SERT.

This article has been withdrawn by Samuel Freyaldenhoven, Yicong Li, Arif M. Kocabas, Grover P. Miller, and Fusun Kilic. Despite attempts, Enrit Ziu, Serra Ucer, and Raman Ramanagoudr-Bhojappa could not be reached for conference on this decision. Dr. Kilic contacted the editorial office to report errors in Fig. 3A and Fig 8 of their article. The Journal requested the original data for Figs. 1A, 1D, 3A, 6A, 8A, 8B, and 9A. Due to the dated material, she could not provide all of them. The data that were provided to the Journal was not 300 ppi. The investigation by the Journal determined the following. Lanes 2 and 3 of the total SERT immunoblot in Fig. 1A were duplicated in lanes 5 and 6. The actin immunoblot from Fig. 1A was reused in Figs. 8B and 9A as actin. The first two lanes of the SERT (PM) immunoblot in Fig. 3A were duplicated. Additionally, the first lane of the Total SERT immunoblot in Fig. 3A was reused in lane 4. In Fig. 6A, lanes 2 and 5 of the actin immunoblot were duplicated.

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2 The abbreviations used are: SERT, serotonin transporter; 5-HT, serotonin; MSH, β-mercaptoethanol; ER, endoplasmic reticulum; Ero1-L, ER oxidase 1-α; Ab, antibody; WB, Western blot; IP, immunoprecipitation; QQ, two glycosylation sites mutated to glutamine; MTSEA-biotin; N-biotinylaminomethylmethanethiosulfonate; PM, plasma membrane.
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importance of a disulfide bond for SERT folding and surface expression suggest a thiol-dependent quality control mechanism in SERT maturation (11, 22). Moreover, the modification of exposed thiols with β-mercaptoethanol (MSH) altered the density of SERT molecules on the plasma membrane and the 5-HT uptake rates (10). Pretreatment of the cells with MSH led to much lower 5-HT uptake rates when compared with control cells, although MSH treatment released more SERT molecules to the plasma membrane. The association between SERT molecules was also obstructed by MSH treatment (10). Altogether, these findings indicate the involvement of SERT in a thiol-mediated retention mechanism. However, neither the mediators aiding in disulfide bond formation nor the role of disulfide bonds in SERT maturation has been vigorously studied yet.

To explore factors involved in SERT maturation, we examined whether the SERT maturation pathway utilizes ERp44 and ER oxidase 1-α (Ero1-Lα) because of their roles in the maturation and quality control of disulfide-containing oligomeric proteins (15, 19, 23–29). An exposed thiol for a protein localized to the ER favors formation of mixed disulfide bonds with thioredoxin family members, notably ERp44 (16, 23–27). During this process, ERp44 preferentially associates with unassembled subunits of disulfide-containing oligomeric proteins (15, 26, 27). In this study, we employed biochemical and molecular biological techniques using endogenous and heterologous expression systems to assess an association between ERp44 and SERT in the maturation process, and the importance of a disulfide bond for SERT localization and hence structure and function were shown to preferentially associate with ERp44. The present studies suggest that (i) disulfide bond formation represents a critical step in SERT folding to a fully active form, and (ii) SERT utilizes ERp44 as well as its partner Ero1-Lα, an oxidoreductase, in the disulfide bond formation process.

MATERIALS AND METHODS

JAR cells were provided by the American Type Culture Collection (Manassas, VA). Protein A-Sepharose beads and non-immune rabbit serum were purchased from Zymed Laboratories Inc. (South San Francisco, CA). 3H-Labeled 5-HT was purchased from PerkinElmer Life Sciences. HA-tagged and untagged forms of ERp44 and the Ero1-Lα construct were a generous gift from Dr. Sitia Roberto (Salute San Raffaele, Milan, Italy). Lentiviral small hairpin RNA (shRNA) plasmid, anti-ERp44, and Ero1-Lα antibodies were generous gifts from Dr. Scherer at the University of Texas Southwestern and used by Wang et al. (30). The second-generation packaging plasmid, pS-PAX2, and VSV-G were purchased from Addgene Inc. (Cambridge, MA). Expression vectors, cell culture materials, Lipofectin, and Lipofectamine 2000 were purchased from Invitrogen. ERp44 and Ero1-Lα antibody (Ab) were purchased from Cell Signaling Technology (Beverly, MA). NHS-SS-biotin, the Micro BCA protein assay reagent kit, and Pico-West Supersignal ECL substrate were purchased from Pierce. Scintillation mixture was purchased from Fisher. A monoclonal SERT Ab recognizing amino acid residues 51–66 on the N terminus was purchased from Mab Technologies (Stone Mountain, GA).

Plasmids, Constructs, and Cell Line Expression Systems—JAR cells were cultured in RPMI 1640 medium with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, referred to as “full RPMI.” Cells (2 × 10^5 cells/assay) were used in biotinylation, Western blot (WB), membrane preparation, transport assay, and immunoprecipitation (IP) assays 48 h postseeding.

Transporters with both glycosylation sites mutated to glutamine, QQ (N208Q and N17Q), were constructed utilizing a Stratagene QuikChange XL site-directed mutagenesis kit as described previously (7, 10).

The three Cys residue (C109A, C200S, and C209S) mutants were introduced by site-directed mutagenesis using oligonucleotides 5′-CTT GCG CAT AGC TTA CCA GAA TGG AG-3′, 5′-CGA ACC ACC AGC TCC AAG AAC TCC TGG AG-3′, and 5′-CGT GGC ACA CTG GCA ACT GCT CAG TGG AG-3′, respectively, on SERT cDNA expressed in JAR cells by using the vac

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Sequin, and hence structure and function were shown to preferen-

[372x503]-CTG CCC TGG ACC AGC TCC AAG AAC

[312x420]Sequencing Facility. In addition, mutants with Cys-200

[312x479]ACT CCA CCA ATT ACT TCT CCG AG-3'

[312x491]-CCT GGA ACA CTG GCA

[312x514]-CTT CCC CTA CAT AGC TTA CCA GAA

[312x479]/H11032

[312x491]TCC TGG AAC AC-3

[312x479], and 5 [312x479]

[312x503]-CTG CCC TGG ACC AGC TCC AAG AAC

[312x456]same primers, the double mutant was generated.

SERT and the FLAG- and Myc-tagged forms of SERT. Using the

[312x456]ACT CCA CCA ATT ACT TCT CCG AG-3'

[312x479]/H11032

[312x491]TCC TGG AAC AC-3

[312x479], and 5 [312x479]

[312x503]-CTG CCC TGG ACC AGC TCC AAG AAC

[312x456]same primers, the double mutant was generated.
the transporter binds 5-HT in a 1:1 stoichiometry. When these conditions are not satisfied, the kinetic profile may deviate from a simple hyperbola, and thus we also fit data to the Hill equation describing cooperative effects of 5-HT concentrations on the uptake rate. Equation 1 depicts the Hill equation such that \( v \) is the observed uptake rate, \( V_{\text{max}} \) is the maximal uptake rate, \( K_H \) is the Hill coefficient or measure of cooperativity. When the Hill coefficient is 1.0, the equation reduces down to the one used for fitting the data to the traditional transport model. The fits of the data to these kinetic models were compared, and the most probable one was identified by the Akaike Information Criterion using GraphPad Prism software (San Diego, CA).

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\nu = \frac{V_{\text{max}} \times [5\text{-HT}]^n}{(K_H^n + [5\text{-HT}]^n)} \quad \text{(Eq. 1)}
\]

Preparation of shRNA Lentiviral Particles—Lentiviral shRNA expression constructs were created utilizing a second-generation packaging plasmid, psPAX2, obtained from Addgene. HEK-293FT cells were cultured for 48 h and transiently transfected with the respective shRNA construct graciously provided by Dr. Scherer (University of Texas Southwestern), psPAX2, and VSV-G in a 1:2 ratio of Lipofectamine 2000 to Opti-MEM medium. The medium was replaced 12 h later with full DMEM. After 48 h post-transfection, the medium was selected, filtered with a 0.45-μm PDVF filter, and precipitated in PEG 8000 Na+/Cl− solution for 20 h. The lentiviral particles were collected with centrifugation at 4 °C and stored at −80 °C in PBS. Viral particles were stored at −80 °C. Viral particles were titrated with a standard immunofluorescence assay using a 2-color fluorescent antibody labeling.

Transfection with Lentiviral Expression Constructs—JAR cells were grown to 60% confluence in 100-mm dishes. Cells were transfected with lentiviral expression constructs in full medium for further experiments. These cells (2 × 10^5 cells/assay) were solubilized in PBS containing 0.44% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture, which contained 5 mg/ml pepstatin, 5 mg/ml leupeptin, and 5 mg/ml aprotinin. In lysis buffer, the alkyllating agent N-ethylmaleimide at a final concentration of 5 mM was added to prevent oxidation and formation of nonspecific disulfide bonds during lysis and to retain the native monomeric structures in the gel (7). Samples were analyzed by 10% SDS-PAGE and transferred to nitrocellulose membrane. WB analysis was performed with anti-ERp44 (diluted 1:1000), anti-SERT (diluted 1:500), or anti-Ero1-1α (diluted 1:1000) Ab and then with horseradish peroxidase-conjugated anti-rabbit secondary Ab (diluted 1:7500), respectively. The signals were visualized using the ECL detection system. WB analysis of densitometric scans was done using a two-sided \( t \) test.

Cell Surface Biotinylation—The surface expression of the SERT and the mutant forms were monitored by biotinylation as described (5). In brief, cells (2 × 10^5 cells/assay) were treated with the membrane-impermeant biotinylating reagent NHS-SS-biotin (Pierce) or 1 mM MTSEA-biotin (Toronto Research Chemicals). These reagents selectively modify only the external lysine residues on membrane proteins. The unbound reagent was washed away and quenched with glycin. The cells were lysed in Tris-buffered saline containing 1% SDS, 1% Triton X-100, and protease inhibitor mixture/PMSF. The biotinylated proteins were recovered with streptavidin-agarose beads (Pierce). Quenching unreacted MTSEA-biotin was not required because of its rapid hydrolysis in aqueous buffers. The labeled proteins were resolved by 8% SDS-PAGE, were transferred to nitrocellulose, and were detected with streptavidin-agarose beads (Pierce). SEM analysis of SDS-PAGE gels was done to selectively modify lysine amino groups exposed on the cell surface. SEM analysis was done to selectively modify lysine amino groups exposed on the cell surface.

RESULTS

In order to evaluate ER thiol-mediated SERT retention at the plasma membrane and its role in SERT function, human placental JAR cells were pretreated with MSH at different concentrations, as it was previously studied for IgG (26). The
surface expression of SERT in MSH-pretreated JAR cells increased in parallel with MSH concentrations (Fig. 1A), whereas their 5-HT uptake rates were significantly lower than the control cells (Fig. 1B). The results indicate the involvement of the disulfide bond formation in the maturation process of SERT in ER.
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**ERp44 and Ero1-Lα Interact with Endogenous SERT—**JAR cells endogenously express SERT and have proven to be very useful in studies relating to the regulatory aspects of the placental transporter. We took advantage of this system in monitoring the processing of SERT. The JAR cell line in the transient transfection system offers an *in situ* environment in processing of the newly synthesized SERT. As reported previously (10, 31), transient transfection produced an at least 50-fold higher level of protein than the endogenous system. Therefore, using JAR cells only mimics the endogenous condition for the transiently transfected plasmids. The endogenous expression of SERT, ERp44, and Ero1-Lα were confirmed in JAR cells through WB assays with the corresponding Abs (Fig. 1C).

Associations between SERT and these chaperones were then demonstrated by IP assays. Cellular proteins pulled down by monoclonal SERT-Ab were analyzed with WB assays using anti-ERp44 or Ero1-Lα polyclonal Abs (Fig. 1D). Densitometric quantification of the levels of ERp44 and Ero1-Lα proteins on SERT-Ab showed that a significant level of chaperone proteins were bound to SERT-Ab compared with the bands when the primary Ab was omitted (Fig. 1E, gray filled bars). Although these results suggest the presence of an association between these ER proteins and SERT in the endogenous expression system, we further examined the specificity of our IP assay conditions by analyzing the levels of ERp44, Ero1-Lα, and SERT in SERT-Ab-depleted cell lysate (Fig. 1D). In SERT-Ab-depleted cell lysate, the levels of these three proteins are significantly less than their expression levels in the original lysate (SERT-Ab-excluded IP). The intensities of the bands were quantified as representative of the amount of bands (black filled bars). These data showed that 45.8% of Ero1-Lα of the ERp44 levels (gray bars versus black bars) were pulled down by SERT-Ab coating protein A beads. SERT Ab-bound cellular proteins were sorted for positive GFP expression on a FACS Aria at the University of Arkansas for Medical Sciences Cytomtery Facility 72 h post-transfection, equal numbers of cells were lysed, detergent-soluble cell lysate was pooled, and then WB analysis was performed using a Versa Doc 1000 gel documentation system (Bio-Rad). Data show that in ERp44-shRNA-expressing JAR cells, there was an unexpected greater than 50% reduction in 5-HT uptake rates (Fig. 3B). SERT-ERp44 Protein Associations in Ero1-Lα shRNA-expressing JAR Cells—SERT-ERp44 and SERT-Ero1-Lα associations were tested with IP assays in Ero1-Lα- and ERp44-shRNA-expressing JAR cells, respectively (Fig. 3A). The quantification of the levels of SERT on the plasma membrane of these cells determined a 75 and 100% increase in SERT expression. Despite the increase in the cell surface of SERT expression in ERp44- or Ero1-Lα-silenced JAR cells, there was an unexpected greater than 50% reduction in 5-HT uptake rates (Fig. 3B).

**SERT Expression and Transport Activity in shRNA-expressing Cells**—In order to evaluate the role of interactions between SERT and two ER chaperones, ERp44 and Ero1-Lα we measured the plasma membrane and the whole cell expression of SERT in ERp44 or Ero1-Lα shRNA-expressing JAR cells first and then compared the 5-HT uptake abilities of these cells. WB analysis of ERp44- and Ero1-Lα-silenced JAR cell lysates showed no difference in the total cellular expression of SERT compared with the control (scrambled plasmid) or JAR cells (Fig. 3A).

FIGURE 2. ERp44 and Ero1-Lα expressions in cell lines expressing ERp44 or Ero1-Lα shRNA. JAR cells were transfected with the shRNA for ERp44 or Ero1-Lα were sorted for positive GFP expression on a FACS Aria at the University of Arkansas for Medical Sciences Cytomtery Facility 72 h post-transfection, equal numbers of cells were lysed, detergent-soluble cell lysate was pooled, and then WB analysis was performed using a Versa Doc 1000 gel documentation system (Bio-Rad). Data show that in ERp44-shRNA-expressing JAR cells, there was an unexpected greater than 50% reduction in 5-HT uptake rates (Fig. 3B).
Each Cys residue in the 5-HT uptake rates to 3-fold the rate in JAR cells. Compared with the wild-type transporter, the relative 5-HT uptake rates for C200S, C209S, and the double mutant (C200S/C209S) were 18.76, 3.38, and 9.66%, respectively.

Glycosylation is an important prerequisite for the self-association of transporter protein (10), and the SERT monomers cannot be fully functional because they are in the oligomeric form (5). In relating these findings together with the kinetic characteristics of SERT in ERp44-silenced cells, next the role of Cys residues in the self-association ability of transporter protein was studied.

Self-association Abilities of C200S, C209S, and C109A and Role of ERp44 in This Process—We tested the self-association ability of SERT mutants as a measure of the possible role of the disulfide-bridge in SERT oligomerization.

JAR cells transfected with a 1:1 mixture of Myc- or FLAG-tagged mutant plasmids and either FLAG- or Myc-tagged proteins were precipitated from the mixture by using protein A beads coated with polyclonal FLAG or monoclonal Myc Ab. FLAG-SERT or Myc-SERT and any associated proteins eluted from the beads were analyzed in WB assays. The FLAG-SERT-associated proteins were probed with monoclonal Myc Ab; Myc-SERT and associated proteins were blotted with polyclonal FLAG Ab.
The self-association abilities of SERT molecules were shown under both IP assay settings, with either FLAG or Myc Ab-coated protein A beads (Fig. 8, A or B, respectively); Myc-SERT was found in association with FLAG-SERT, indicating that although the two forms of Myc-SERT/FLAG-SERT and FLAG-C109A/Myc-SERT remained associated after detergent disruption of the cells, the two other Cys mutants, C200S and C209S, associated neither by themselves nor with wild-type SERT.

Surface Labeling of Cells with MTSEA-Biotin—When SERT is present at the cell surface, there are three exposed Cys residues that are susceptible to modification and thus can be interrogated to assess the presence of disulfide bonds. MTSEA-biotin in the external medium reacts with free Cys residues on SERT and the mutants containing exposed Cys residues (1, 2, 15). JAR and ERp44-silenced JAR cells were transfected with SERT or one of the Cys mutant forms (C109A, C200S, and C209S). The next day, cells were treated with MTSEA-biotin as described previously (5); after removing unreacted reagent, the cells were lysed in detergent, and Streptavidin beads were used to precipitate biotinylated surface proteins. Fig. 9A shows that JAR-C109A cells were not labeled with MTSEA-biotin at all, indicating the absence of free Cys residue on the external loop of the C109A form; however, JAR-SERT cells were labeled at a level 50% less than the labeling of JAR-C200S or JAR-C209S cells. However, MTSEA-biotin labeling of ERp44-silenced JAR cells expressing SERT or one of the Cys mutant forms of SERT showed a different pattern. The level of MTSEA-biotin labeling of the JAR cells expressing C109A, C200S, and C209S was almost 50% of the labeling level of SERT in JAR cells. These data show that in JAR cells, SERT must have only one Cys that can interact with MTSEA-biotin, but in ERp44-silenced cells, it has more free Cys residues to be labeled by MTSEA-biotin. In a similar way, in JAR cells, C109A should not have free Cys residues, but in ERp44-silenced JAR cells, C109A must have two free Cys residues.

Next, we wanted to verify these findings in an endogenous expression system. JAR, ERp44 or Ero1-L/H9251-silenced JAR cells were treated with MTSEA-biotin. Biotinylated proteins were pulled down and analyzed using anti-SERT-Ab. The level of biotin labeling of JAR cells was much less than that in ERp44- or Ero1-L/H9251-silenced JAR cells (Fig. 9B).

DISCUSSION

Despite a wealth of knowledge on key amino acid residues needed for SERT activity, there are limited data on the protein
mediators and quality control checkpoints in SERT maturation. Studies have shown that an exposed thiol at Cys-200 or Cys-209 on EL2 is sufficient for the intracellular retention of SERT, but SERT mutants without Cys residues on the second extracellular loop are able to reach the PM despite the lack of a disulfide bond (11). These studies suggest a quality control mechanism involved in SERT maturation, which recognizes exposed Cys in SERT molecules and retains them intracellularly. The ability of Cys mutants of SERT to reach the PM further implies that the quality control mechanism does not recognize non-native structure, such as hydrophobic patches or immature glycans, but rather, the retention of Cys mutants of SERT is entirely thiol-dependent.

The retention of proteins through reversible disulfide bonds has become the primary feature of thiol-mediated retention involving two ER resident proteins, ERP44 and Ero1-Lα (15, 26, 27, 30). ERP44 and Ero1-Lα have been described as a quality control mechanism in the maturation of disulfide-containing oligomeric proteins (16, 26, 27, 30). Given the importance of disulfide bond formation in the maturation of SERT and the nature of thiol-mediated retention in ER, herein, we tested a novel hypothesis of ERP44-mediated disulfide bond formation and the role of disulfide in oligomerization of SERT monomers.

In initial experiments, we were able to demonstrate that endogenous SERT interacts with ERP44 and Ero1-Lα (15, 26, 30). Given the importance of ERP44 and Ero1-Lα in the regulation of disulfide bond formation and the role of disulfide in oligomerization of SERT monomers, we hypothesized that ERP44 might mediate disulfide bond formation in SERT. To test this hypothesis, we performed experiments to determine if ERP44 interacts with SERT and if the thiol group of Cys residues in SERT is involved in the interaction.

We transiently transfected JAR cells with a plasmid expressing wild-type SERT and a plasmid expressing a Cys mutant of SERT (C200S or C209S) and co-immunoprecipitated the proteins with anti-SERT and anti-ERP44 antibodies. As shown in Figure 6, the interaction between SERT and ERP44 is significantly enhanced in the presence of a Cys mutant of SERT, indicating that the thiol group of Cys residues in SERT is involved in the interaction with ERP44.

In addition to determining the interaction between SERT and ERP44, we measured the 5-HT uptake rate of wild-type SERT and Cys mutants of SERT in JAR cells and in ERp44-silenced JAR cells. As shown in Figure 7, the 5-HT uptake rate of wild-type SERT is significantly lower than that of Cys mutants of SERT, indicating that the thiol group of Cys residues in SERT is involved in the function of SERT.

In summary, our results provide evidence for a novel hypothesis that ERP44 mediates disulfide bond formation in the maturation of SERT and the role of disulfide in oligomerization of SERT monomers. Additionally, our findings suggest that thiol-mediated retention in ER is a critical mechanism for regulating disulfide bond formation and the function of SERT.
sufficient for the retention of SERT (Fig. 6A), and furthermore, ERp44 may be involved in the quality control of disulfide bond formation.

ERp44 also preferentially interacts with unassembled subunits of oligomeric proteins (27). This association enables ERp44 to selectively retain cargo proteins, like SERT, in an environment suitable for their maturation and concentrate subunits in a local environment to promote their polymerization (15, 27, 30). SERT mutants (e.g. QQ) that cannot undergo glycosylation demonstrate compromised oligomerizations and are unable to properly transport 5-HT (10); here, this mutant form of SERT was included in our studies to investigate if SERT undergoes disulfide bond-mediated oligomerization process.

Data showed that if SERT molecules cannot associate in an oligomeric form, then this facilitates the association between ERp44 and SERT (Fig. 6).

Because ERp44 associates with cargo proteins through exposed thiols, our findings suggest that QQ mutants may not be able to form a disulfide bond, and further, glycosylation of SERT may contribute to its disulfide modification. Although not previously reported for SERT, glycosylation has been shown to facilitate disulfide bond formation in other proteins, such as epidermal growth factors and human insulin receptor (33, 34). This result implies the requirement of disulfide bond formation in functional oligomerization of SERT monomers. Sequential modification would allow the stepwise maturation of SERT, and quality control checkpoints intimately associated with the folding process ensure that unmodified or misfolded proteins do not proceed to the next stage in maturation (15). In this manner, the ER folding machinery is able to couple the maturation and quality control of proteins within the secretory pathway (16, 23). However, additional studies are needed in order to confirm with certainty the interdependence among post-translational modifications of SERT.

Once associated with ERp44, previous data have shown that cargo molecules are displaced by Ero1-L/H9251 through binding with Cys-29 of ERp44 (36). Interactions with Cys-29 have also been shown to facilitate the association between ERp44 and other cargo proteins, such as adiponectin and IgM subunits (27, 30). However, using a mutant form of ERp44, C29S, we showed that SERT does not interact with ERp44 through Cys-29, but rather disruption of interactions through Cys-29 through mutations increases its association with the transporter. Considering the role of Ero1-Lα in displacement of other cargo proteins from ERp44, these findings suggest that Ero1-Lα is required to interact with ERp44 for efficient release of SERT, and the relative ratio of the two chaperones, ERp44 and Ero1-Lα, to each other as well as SERT is a crucial determinant for proper SERT maturation.

Our co-IP assays suggested a possible association between ERp44 and SERT; however, studying protein-protein interactions in co-IP experiments with detergent-disturbed cell lysate suffers from drawbacks, such as possible nonspecific interactions and variations in the efficiency of the IP. Therefore, we performed an analysis of these associations on 5-HT uptake function of SERT proteins. The kinetic studies along with the MTSEA-biotinylation assays strongly indicate that ERp44 and Ero1-Lα contribute to the maturation of SERT. Consequently,
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we examined the functional contribution of the interaction. We were able to create stable knockout cell lines of ERp44 and Ero1-Lα through the delivery of RNA interference (RNAi). Initial attempts at protein reduction with transient transfection of protein-specific shRNA to degrade targeted mRNA constructs were unsuccessful due to the long half-life of ERp44 and Ero1-Lα. As a solution, lentiviral particles containing shRNA targeted to ERp44 or Ero1-Lα were produced and transsected into target cell lines for long term RNAi. The expression levels of ERp44 and Ero1-Lα were successfully reduced to 60% of endogenous levels in JAR cells. Furthermore, as a primary ER localization mechanism of Ero1-Lα, we observed a 30% reduction in Ero1-Lα when ERp44 protein levels were significantly reduced. Our data confirm the previously published studies (26, 35) on the saturability of the ERp44-dependent retention mechanism. According to these studies, if the Ero1-Lα/ERp44 ratio exceeds its threshold for retention, a portion Ero1-Lα escapes from the ER (26, 35).

In our knockout expression system, SERT is endogenously expressed in JAR cells; thus, we are able to measure the functional consequence of reduced ERp44 or Ero1-Lα protein expression on SERT activity. However, these could be an indirect effect of the gene silencing on other proteins which are required for the SERT folding and maturation. Therefore, the kinetic and biochemical studies together with the data from MTSEA-biotinylation assays are more specific in demonstrating the direct action of ERp44 on SERT.

For untreated cells, the kinetic profile for 5-HT uptake was strongly sigmoidal, indicating that increasing concentrations led to positive cooperativity. The kinetic profile was improved by the absence of any change in cellular 5-HT concentration. Based on our observation of the biphasic relationship between extracellular 5-HT elevation, loss of surface SERT, and depletion of platelet 5-HT (31, 36, 37). The rise in concentration of oligomerized, active SERT improves 5-HT activity more than expected, as reflected in the sigmoidal kinetic profile.

This mechanism depends on ERp44 function to maintain the appropriate response of SERT to changing 5-HT levels and the resulting transport activity. Without ERp44 chaperone activity, relative levels of SERT at the cell surface were high prior to the addition of 5-HT. As 5-HT levels increased, the sigmoidicity of the kinetic profile was essentially abolished, which is probably due to the absence of any change in cellular SERT localization. Nevertheless, once SERT reached the cell surface, its activity was compromised based on a decreased maximal uptake rate. SERT maturation into an active transporter then requires ERp44 activity as well. Ero1-Lα is another contributor to these processes through its interactions with ERp44. Taken together, these data suggest that ERp44 plays a critical role in the maturation and membrane trafficking of SERT within cells.

To identify the cause of reduced SERT activity, we measured the SERT surface expression by biotinylation in shRNA-expressing cells. Despite decreased transport activity, we discovered a greater than 1- and 2-fold increase in SERT expression on the PM in cells expressing ERp44 or Ero1-Lα shRNA, respectively. A reduced transport activity can be attributed to an increase in nonfunctional SERT and/or a decrease in fully active SERT molecules at the cell surface, which was demonstrated by our biochemical studies in ERp44-silenced cells (Fig. 5). Therefore, significantly lowered ERp44 or Ero1-Lα is sufficient to allow improperly folded SERT molecules to reach the PM, and ERp44 and Ero1-Lα probably act as a quality control mechanism ensuring the fidelity of SERT maturation.

Previous data implicate ERp44 and Ero1-Lα in the quality control of cargo proteins, and a dynamic relationship exists between ERp44 and Ero1-Lα for the efficient retention and release cargo (27, 30, 32). It was previously shown that an interaction between Cys-29 of ERp44 and Ero1-Lα aids the release of the cargo protein from the ER (15, 26–28). We hypothesized that this mechanism is required for SERT release from ERp44. However, increased SERT surface expression in cells with knocked down ERp44 expression seemed contradictory; thus, we measured the association between ERp44 and Ero1-Lα. We discovered that ERp44 and Ero1-Lα expression levels are not able to adequately compensate for reduced ERp44 levels. This increases the cargo/chaperone ratio to increase, and as a result, misfolded SERT molecules are unable to be properly retained and bypass quality control.

SERT is a complex oligomeric glycoprotein, which needs significant post-translational modification to fold into its native structure (5, 9–11). The formation of such a large, multimeric complex is inherently error-prone and requires more time to fold. Whereas ERp44-mediated quality control recognizes improperly folded SERT molecules and prevents them from reaching the PM, ERp44-dependent ER retention may serve to increase the time SERT is in the secretory pathway and thus the opportunity for SERT to fold into its native structure. Furthermore, ERp44 is able to couple quality control and oxidative folding by directing non-native SERT to the ER machinery needed for disulfide bond formation.

In addition to a quality control mechanism, the regulation of ERp44 and Ero1-Lα may offer an additional layer of post-translational control of SERT activity. Increased expression of both chaperones, ERp44 and Ero1-Lα, has been shown to stimulate the functional oligomerization and expression of other cargo proteins involved in thiol-mediated retention (30). However, up-regulation of only ERp44 has been shown to favor the retention of cargo molecules, and Ero1-Lα overexpression stimulates their release (30). This is especially relevant due to the tissue- and sex-specific expression of ERp44 and Ero1-Lα. For example, there is a 50% reduction in ERp44 and Ero1-Lα expression in male mice as compared with female mice and a greater than 80% reduction in ERp44 and Ero1-Lα in ob/ob
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mice (30). Thus, our in vitro shRNA experiments are physiologically relevant and provide evidence that regulation of ERP44 and Ero1-Lα expression and activity could affect SERT in vivo.

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