Sucrase Is an Intramolecular Chaperone Located at the C-terminal End of the Sucrase-Isomaltase Enzyme Complex*

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The sucrase-isomaltase enzyme complex (pro-SI) is a type II integral membrane glycoprotein of the intestinal brush border membrane. Its synthesis commences with the isomaltase (IM) subunit and ends with sucrase (SUC). Both domains reveal striking structural similarities, suggesting a pseudo-dimeric assembly of a correctly folded and an enzymatically active pro-SI. The impact of each domain on the folding and function of pro-SI has been analyzed by individual expression and coexpression of the individual subunits. SUC acquires correct folding, enzymatic activity and transport competence and is secreted into the external milieu independent of the presence of IM. By contrast, IM persists as a mannose-rich polypeptide that interacts with the endoplasmic reticulum resident molecular chaperone calnexin. This interaction is disrupted when SUC is co-expressed with IM, indicating that SUC competes with calnexin for binding of IM. The interaction between SUC and the membrane-anchored IM leads to maturation of IM and blocks the secretion of SUC into the external milieu. We conclude that SUC plays a role as an intramolecular chaperone in the context of the pro-SI protein. To our knowledge all intramolecular chaperones so far identified are located at the N-terminal end. SUC is therefore the first C-terminally located intramolecular chaperone in mammalian cells.

Membrane and secretory proteins are subject to a complex array of cotranslational and post-translational processes that precede sorting to the organelle in which they exert their biological functions. The most crucial events take place in the ER and include folding, subunit assembly, and in many cases oligomerization (for review see Ref. 1). These steps, individually or altogether, lead ultimately to the acquisition of the protein to a transport-competent conformation that enables its egress from the ER. A number of resident proteins of the ER, such as the molecular chaperones BiP and calnexin and protein disulfide isomerase, play primordial roles in these processes that vary from binding of unfolded proteins to the formation of disulfide bonds between individual subunits or within a single polypeptide chain (2–4). A large number of proteins assemble into homodimers, oligomers, or heterodimers preceding protein exit from the ER (5, 6). Correct protein folding of the individual monomers or subunits is required for optimal completion of these events. Many proteins are known not to go into dimers or heterodimers, such as the brush border proteins sucrase-isomaltase (SI) (7), angiotensin-converting enzyme (8), and maltase-glucoamylase (9). An interesting and common feature of these proteins is their composition of an even number of homologous domains. SI, for example, is an enzyme complex that is made of two subunits that share ≥ 35% of amino acid sequence similarity and 41% of conserved sequences (10). Although no three-dimensional structure of this large protein is so far available, algorithmic predictions suggest that the two large domains are folded similarly and that the native folded state of the SI enzyme complex can be referred to as a pseudo-dimer (10). The striking similarity between the two subunits has led to the concept that SI has emerged from one cycle of duplication of a single gene (10). An important structural aspect of SI is that the two subunits reside on the same polypeptide. The enzyme is synthesized as a single chain polypeptide that is cleaved extracellularly in the intestinal lumen by pancreatic trypsin, generating the two subunits that remain closely associated with each other by strong non-covalent ionic interactions (7, 11). The subunits are therefore generated very late in the biosynthesis after the single chain form of SI, pro-SI, has acquired its complete transport efficiency and has reached its final destination in the brush border membrane. The question that arises in this respect is that of a possible role of each of the domains in the folding of the other and of the significance of either subdomain in the context of ensuring a correct protein folding and transport-competent configuration of the whole enzyme complex. The structural requirements and the mechanisms of assembly of heterodimeric complexes have been analyzed for several proteins such as fibrinogen (12), acetylcholine receptor (13), B cell antigen receptor (14), insulin receptor (15), major histocompatibility complexes (16), T-cell receptor (17), and hepatitis C virus glycoproteins E1 and E2 (18). In many of these examples the individual components of the heterodimers fold independently, and in some cases are also transported and secreted into the extracellular milieu. Nevertheless, for the generation of functional units or efficiently transported heterodimeric complexes along the secretory pathway each subunit must associate or interact with other members of the complex.

Not as well explored are the roles and mechanisms of folding of homologous subdomains within one single polypeptide chain. By virtue of its structural features SI is an exquisite model protein for analyzing molecular aspects of protein-protein interactions and their consequence on intracellular trafficking and function.

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The abbreviations used are: ER, endoplasmic reticulum; IM, isomaltase; SUC, sucrase; SI, sucrase-isomaltase; mAB, monoclonal antibody; endo H, endo-β-N-acetylglucosaminidase H; endo F, endo-β-N-acetylglucosaminidase F; glycopeptidase F; LPH, lactase-phlorizin hydrolase; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein.

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Not only is the protein structure of SI interesting, but also pathophysiological cases in which naturally occurring mutant phenotypes of SI provide a suitable model to gain insights into the molecular mechanisms underlying intracellular protein transport and polarized sorting (19–21). One of the SI mutant phenotypes, phenotype V, revealed an unusual intracellular cleavage after complex glycosylation in the Golgi apparatus. After this cleavage the sucrase (SUC) domain was degraded, and the isomaltase (IM) domain was correctly transported and sorted to the apical membrane (19). These data have proposed that the complete polarized sorting information of pro-SI is located in the IM region. The role of the IM or SUC domains in the events preceding sorting, e.g., ER to Golgi transport, remained unanswered. If SUC is not needed for sorting, then what would be its role in the context of transport and folding of the whole pro-SI species? A possible role of SUC in attenuating the folding of the pro-SI polypeptide is suggested upon consideration of another phenotype of sucrase-isomaltase deficiency. In phenotype II a Q1098P mutation in the SUC domain leads to a transport block of pro-SI (22). In this paper we have addressed questions related to the possible role of SUC and IM in cotransfected COS-1 cells expressing for 901 amino acids of IM were deleted in-frame to generate SUC, which is not transport-competent. This panel of constructs should provide insights into the events preceding sorting, e.g., ER to Golgi transport, remained unanswered. If SUC is not needed for sorting, then what would be its role in the context of transport and folding of the whole pro-SI species? A possible role of SUC in attenuating the folding of the pro-SI polypeptide is suggested upon consideration of another phenotype of sucrase-isomaltase deficiency. In phenotype II a Q1098P mutation in the SUC domain leads to a transport block of pro-SI (22). In this paper we have addressed questions related to the possible role of SUC and IM in cotransfected COS-1 cells expressing pro-SI (pSG8-SUCMA). The resulting cDNA of SUC MA was verified by sequencing, and the plasmids obtained were denoted pSG8-SUCMA, pcDNA3-SUC MA, pIM-YFP, and pJB20-LPH.

**MATERIALS AND METHODS**

**Construction of cDNA Clones—Oligonucleotide-directed mutagenesis** with the QuikChange kit in vitro mutagenesis system from Stratagene was applied to introduce a stop codon at nucleotides 2878–2881. This exact mutation was the last amino acid of IM. The template consisted of a full-length cDNA encoding pro-SI cloned into pSG8-vector (22), pSG8-SIAM (23), or pSI-YFP (24). The following oligonucleotides were used in this context: IsoStop upstream, 5'-AAC ACT GCA AAT GGC TGA ATA AAG GTA CCA TCT G-3' and IsoStop downstream, 5'-CAG ATG GTA CCT TTA TTC AGG CAT TTG CAG TAT T C-3'. The mutations were confirmed by sequencing, and the plasmids obtained were denoted pSG8-IM, pSG8-SUC SOL, or pIM-YFP.

**SUC SOL** was cloned in two steps. At first the cDNA encoding the signal sequence of pro-LPH, LPH signal, was amplified by PCR as described before (25). The resulting cDNA comprising the last 20 nucleotides of the LPH signal sequence (nucleotides 5253–5274) was synthesized by PCR of the pro-SI cDNA template and the oligonucleotides LPHsig (5'-TTT CCT CAT GCT GGG GGT CAA TAA AGC TAT CCT GCA GGA GGG GGT CAA TAA AGC TAT CCT GCA GGA GGG GGT CAA TAA AGC TAT CCT GCA GGA GGG GGT CAA T) and ISUCSOL (5'-ACC CCC ATG GTC GAG GGT CTC GAG GGT CTC GAG GGT CTC GAG GGT CTC GAG GGT CTC GAG G). Both DNA fragments were then fused by assembly PCR and cloned into the unique EcoRI/XhoI sites of the pSG8 expression vector. The resulting construct, denoted pSG8-SUC SOL, was sequenced and found to contain the signal sequence of pro-LPH fused in-frame to the SUC cDNA beginning with nucleotide 2882.

**Transient Transfection of COS-1 Cells—**pSG8-IM, pSG8-IM SOL, pSG8-SUC SOL, pDNA-SUC SOL, pMYF-P, and pJB20-LPH (an expression vector for the LPH signal sequence of intestinal lactase-phlorizin hydrolase) were transfected into COS-1 cells using Lipofectamine (26). The transient expression of pSG8-SUC SOL was verified by sequencing.

**Biosynthetic Labeling and Immunoprecipitation—**Transfected COS-1 cells were cultivated in Dulbecco's modified Eagle's medium, 10% fetal calf serum and labeled with 50 µCi of [35S]methionine as described previously (26). Biosynthetically labeled cells were solubilized for 1 h at 4 °C in lysis buffer (25 mM Tris-Cl, pH 8.0, 50 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, and a mixture of protease inhibitors) containing 1 µg/ml pepstatin, 5 µg/ml leupeptin, 17.4 µg/ml benzamidine, and 1 µg/ml aprotinin. Usually 1 ml of ice-old lysis buffer was used for each 100-mm culture dish (~2–4 × 10^6 cells). The medium and cell lysates were immunoprecipitated with epitope-specific mAbs directed against IM, SUC, or SI. These products were the following hybridomas: HBB 1/691, HBB 2/219, and HBB 7/705 (27). LPH was immunoprecipitated with a mAb that was a product of hybridoma HBB 1/909/34/74 (27). The immunoprecipitation procedures were performed essentially according to Naai et al. (27). In some experiments the culture media after the biosynthetic labelings were collected, protease inhibitors were added and centrifuged at 10,000 × g for 30 min, and the supernatant was immunoprecipitated with anti-IM or anti-SUC. A possible interaction of SUC and IM in cotransfected COS-1 cells was assessed using mAbs that recognize either IM (HBB 3/705) or SUC (HBB 2/219) (27). The immunoprecipitates were analyzed by SDS-PAGE according to Laemmli (27). After electrophoresis the gels were fixed and analyzed by a phosphorimaging device (Bio-Rad).

**Confocal Fluorescence Microscopy—**Transfected COS-1 cells grown on coverslips were fixed 2 days post-transfection with 3% paraformaldehyde and analyzed using a Leica TCS SP2 microscope with a ×63 water planapochromat lens (Leica) (24). Immunolabeling of SUC in cotransfected cells was performed using HBB 2/219 as primary antibody and rhodamine-conjugated goat anti-mouse IgG (Sigma). Sequential scans with a 643-nm HeNe laser or the 458- and 514-nm excitation lines of an argon laser and the optimal emission wavelength spectra for rhodamine, CF, and YFP were employed for dual color CF and YFP imaging.

**RESULTS AND DISCUSSION**

The impact of SUC and IM domains on the generation of a transport-competent configuration of pro-SI was addressed in conjunction with the question of whether either domain can fold independently. For this, we made several cDNA constructs encoding each individual domain, expressed these in COS-1 cells, and assessed the folding patterns and transport kinetics of the recombinant proteins. One construct included the entire IM sequence containing its own transmembrane segment, the non-cleavable signal sequence, and the heavily O-glycosylated stalk region (denoted IM). To generate a potentially soluble form of IM, the sequences encoding the entire transmembrane domain were deleted. This signal sequence for ER translocation contained in the transmembrane of IM was replaced by the cleavable signal sequence of intestinal lactase-phlorizin hydrolase containing IM SOL. Analogous to IM, a SUC construct was made containing the cleavable LPH signal sequence (SUC SOL). An additional construct was made in which the membrane-anchoring domain of LPH was fused to the C-terminal end of SUC (SUC SOL). This panel of constructs should provide information regarding the folding of each domain and the influence of membrane anchoring in this context.
Expression of a Full-length IM—The cDNA construct encoding IM was transfected into COS-1 cells, which were biosynthetically labeled with [35S]methionine and immunoprecipitated with a mixture of mAb anti-SI. Fig. 1A shows that IM was revealed exclusively as a mannose-rich glycosylated polypeptide as determined by its complete sensitivity to treatment with endo H. Longer labeling periods did not result in a different band pattern. The IM domain of pro-SI contains a Ser/Thr-rich domain, the site of heavy O-glycosylation within the pro-SI species. This type of glycosylation commences in the cis-Golgi and is terminated in the trans-Golgi network (31). Because IM is not O-glycosylated and persists exclusively as an endo H-sensitive polypeptide the data demonstrate that this form did not traverse the ER to the Golgi apparatus to be processed to a complex glycosylated protein. To delineate the basis for this transport block, we analyzed the folding pattern of IM in protease sensitivity studies. In these experiments trypsin was used because this protease cleaves wild type pro-SI to IM and SUC (7), which are trypsin-resistant. An alteration in the sensitivity of these species toward trypsin is therefore reminiscent of an altered folding pattern. Fig. 1B shows that IM was entirely degraded upon trypsin treatment. By contrast, native and correctly folded IM generated from wild type pro-SI by trypsin was resistant to further treatment with protease during similar periods. Altogether, the biosynthetic labeling data and the trypsin assay demonstrate that individual expression of IM in COS-1 cells results in a malfolded and transport-incompetent glycoprotein. Furthermore, these findings shed light on a potentially critical role of SUC in facilitating the folding of IM.

Analysis of the function of a protein is a useful tool in providing information on its folding status. Thus, we measured the enzymatic activity of IM and compared it with that of pro-SI. There was no detectable activity of IM supporting the view that IM is not correctly folded.

Expression of a Soluble Form of SUC—Having examined the folding and biosynthesis of IM the next step was to analyze the biosynthetic and structural features of individually expressed SUC as an essential step toward the delineation of the influence of either domain, IM or SUC, on the folding and transport behavior of the other.

SUC does not possess a signal sequence for translocation into the ER, and the signal sequence of the full-length pro-SI molecule is located in the transmembrane domain of the IM subunit (10). A construct was therefore generated that contained the entire SUC sequence to which the cleavable signal sequence of intestinal lactase-phlorizin hydrolase has been fused at the N-terminal end (SUC_{SOL}). Transfection of this fusion protein into COS-1 cells revealed a mannose-rich endo H-sensitive polypeptide in the cell lysates of the biosynthetically labeled cells. Because SUC_{SOL} does not possess a membrane-anchoring domain, transport competence of this form would be compatible with its secretion into the external milieu. As shown in Fig. 2A a diffuse band larger than the mannose-rich cellular SUC_{SOL} species was immunoprecipitated with mAb anti-SUC antibodies from the culture medium. This species was also endo H-resistant, reminiscent of a complex glycosylation and maturation in the Golgi apparatus. These results demonstrate that SUC_{SOL} is a hydrophilic protein that, unlike IM, has egressed the ER and has been processed in the Golgi apparatus and secreted from the cell. On the other hand, the proportion of secreted mature SUC relative to its mannose-rich polypeptide (55 versus 45%) is reminiscent of a slow transport and processing rate of this form as compared with complete pro-SI. Here, almost 80% of mannose-rich pro-SI was converted to a complex glycosylated species during a similar labeling period. We asked whether an altered folding pattern of SUC_{SOL} upon individual expression may account for the delay in transport and processing kinetics. Therefore, we probed the folding of SUC_{SOL} using the trypsin sensitivity assay previously employed for IM. Fig. 2B demonstrates that SUC was resistant to trypsin. Treatment of wild type pro-SI with trypsin generated the SUC and IM subunits that were similarly trypsin-resistant. These results demonstrate that no additional trypsin cleavage sites were exposed in the individually expressed SUC, strongly suggesting that the folding pattern of SUC and SUC within the pro-SI species is similar. To further corroborate these data we measured the enzymatic activity of SUC_{SOL}. Here individually expressed SUC revealed an enzymatic activity comparable with that within the pro-SI enzyme complex, supporting the view that SUC_{SOL} is a correctly folded protein. Together, the similar reactivity toward trypsin of individually expressed SUC and the enzymatic activity of SUC_{SOL} suggest that gross
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structural alterations or novel folding determinants are unlikely to have been elicited in individually expressed SUCsol.

Expression of a Membrane-bound Form of SUC—The striking structural homologies between SUC and IM have suggested that the evolution of pro-SI implicates one cycle of gene duplication whereby IM is coded by the initial gene and SUC by the later emerging gene (10). The high proportion of identical amino acids found in both proteins that are organized in numerous stretches over the pro-SI protein as well as the high number of conserved amino acids has led to the idea that SUC and IM are similarly folded and therefore form pseudo-dimers (10). Along this concept and in a fashion similar to its homologous counterpart SUC, a correctly folded and transport-competent configuration of individually expressed IM would be anticipated. Although this was not the case, as the experiments presented above clearly demonstrated, two important differences in the structural features between IM and SUC should be considered, which may ultimately play a substantial role in influencing the folding pattern and transport competence of IM. First, IM is associated with the membrane through its non-cleavable signal sequence, and second, it contains immediately upstream the membrane-anchoring domain a Ser/Thr-rich stalk domain, the site of heavy O-glycosylation of the pro-SI in the Golgi. One attractive hypothesis would attribute the ability of SUC or the failure of IM to adopt a correct folding to the absence or presence of the transmembrane domain and the stalk region. The corollary to this would be that the transmembrane domain in the IM constitutes a constraint that hinders an efficient and flexible formation of secondary structures of the luminal part of IM. To test this hypothesis we fused the transmembrane domain of IM containing the signal sequence into the N-terminal end of SUC and analyzed the biosynthetic and structural features of this hybrid protein (denoted SUCMA). This hybrid protein was sensitive to treatment with endo H, reminiscent of a mannose-rich type of glycosylation and ER localization (Fig. 3A). We further examined whether this SUCMA is indeed membrane-bound, and for this purpose we performed phase separation using Triton X-114. As shown in Fig. 3B SUCMA was entirely recovered in the detergent phase compatible with its association with the membrane. Therefore, in contrast to the soluble form of SUC (SUCsol) SUCMA did not acquire correct folding in the ER and as a consequence failed to egress the organelle. This was further corroborated by the complete degradation of SUCMA by trypsin and the lack of enzymatic activity (data are not shown). Altogether, the data demonstrate that fusing the membrane region of IM to the soluble form of SUC results in a protein that did not acquire correct folding and is ultimately blocked in the ER as a mannose-rich glycosylated protein. The reverse experiment was also performed. Here, a soluble IM protein was generated in which the membrane-anchoring and stalk domains of IM were truncated and replaced by the cleavable signal sequence of lactase-phlorizin hydrolase. Expression of this form, IMsol, revealed an exclusively mannose-rich glycosylated polypeptide (Fig. 3C) that was not secreted into the external milieu. It is obvious that the conversion of IM to a soluble protein is not associated with the acquisition of transport competence. It is likely, therefore, that the folding pattern of IM is not entirely similar to that of SUC. Despite the strong similarities between SUC and IM some subtle differences may exist that affect the overall patterns of these two domains. One such difference is the presence of a trefoil domain in the IM subunit immediately upstream of the stalk domain (10). A mutation in the immediate vicinity of this domain generates a folding intermediate in pro-SI that affects substantially the sorting fidelity of pro-SI to the apical membrane leading to a random distribution of the protein on both membranes (32). Given that neither the membrane-anchoring domain of pro-SI, nor its cytoplasmic tail expresses a retention signal for the ER, the conversion of a transport-competent soluble form of SUC to an ER-localized membrane-bound SUCMA can be explained only by a direct effect of the membrane anchor of IM on the luminal part of SUC, for instance, by limiting the flexibility of SUC to acquire its correct folding.

Implication of SUC in the Folding of IM—The observations made so far support the notion that SUC adopts a native conformation similar to SUC within the pro-SI molecule independent of the presence of IM, whereas IM does not. A growing family of membrane and secretory proteins contain pro-domains at the N-terminal end that undergo post-translational proteolytic cleavage in the late secretory pathway after the protein has acquired transport competence (26, 33–36). These domains, called intramolecular chaperones, modulate the folding of this class of proteins directly through interaction with other regions of the protein or by substituting for ER resident chaperones. We asked the question whether a correct folding of GI requires the presence of SUC that would function as an intramolecular chaperone in the context of the entire pro-SI protein. SUC and IM were coexpressed, and the folding and processing of IM were examined. First we wanted to determine whether both proteins interact with each other. For this the cell lysates containing cotransfected SUC and IM were immunoprecipitated with antibodies that recognize either SUC (mAb 219) or IM (mAb 705) but not both (37). Fig. 4A depicts the results obtained with the individual antibodies in cells trans-
fected with the individual genes or with both. Within 6 h of labeling SUC was precipitated from the cell lysates as a mannose-rich polypeptide as well as from the culture medium as a complex glycosylated protein. IM was found in the cell lysates as a mannose-rich polypeptide. In cells cotransfected with both genes, SUC and IM are associated with each other because they are immunoprecipitated with mAb anti-SUC (HBB 219), which recognizes only the IM subunit. Moreover, the SUC subunit was no longer secreted into the extracellular milieu, supporting the view that it has exhibited a strong interaction with the membrane-anchored protein IM in a fashion similar to the in vivo situation in the brush border membrane (7).

The influence of SUC on the trafficking of IM was also examined in transfected cells using confocal laser microscopy. First, the intracellular transport of IM was monitored in COS-1 cells transfected with a cDNA encoding IM and fused to the yellow variant of the green fluorescent protein (pIM-YFP). Confocal analysis of these cells revealed a uniform pattern of distribution of IM-YFP in the ER (Fig. 4B), supporting the biochemical data. In the presence of SUC in cotransfected cells YFP-tagged IM was localized in the Golgi apparatus and at the cell surface in addition to the ER. SUC was visualized in fixed cells using rhodamine-conjugated anti-IgG antibodies and localized in the ER and to a lesser extent in the Golgi (Fig. 4B). The reason for this is that SUC is not membrane-bound and exits the Golgi rapidly into the extracellular milieu. Altogether, the data support the notion that SUC is implicated in the folding events of IM and its acquisition of transport competence from the ER.

Furthermore, we estimated the proportion of the cells containing both proteins. Almost 25% of all transfected COS-1 cells expressed both polypeptides, thus providing an explanation for the reduced intensity of the SUC band co-precipitated with IM in Fig. 4A. The specific role of SUC in influencing the folding of IM was examined in a control experiment in which LPHβ, an irrelevant intestinal protein, was fused to CFP (LPHβ-CFP) (26) and cotransfected with IM-YFP into COS-1 cells. Fig. 4C demonstrates that LPHβ-CFP had no effect on the intracellular transport of IM-YFP because the latter was not transported beyond the ER in the presence of LPHβ-CFP. This finding supports the view that the observed change in the transport behavior of IM-YFP in the presence of SUC is due to a specific interaction of both proteins.

**Interaction of IM and Calnexin**—Individual expression of IM in COS-1 cells followed by immunoprecipitation with mAb anti-IM (HBB 705) resulted in the identification of IM associated with a band of an apparent molecular mass of 97 kDa (Fig. 5A). This band corresponded to the ER molecular chaperone calnexin as assessed by immunoblotting with anti-calnexin antibody (Fig. 5A). On the other hand, intracellular SUC found in the cell lysates, i.e., as a mannose-rich polypeptide, did not react with calnexin (Fig. 5A). Therefore, we wanted to explore a possible role of this chaperone in regulating the interaction between SUC and IM. Fig. 5B shows that during early phases of biosynthesis SUC, IM, and calnexin co-precipitated with mAb anti-IM (HBB 705). 3 h into the chase, at the time SUC has acquired correct folding and mature conformation, the calnexin band disappeared. This result was obtained with either anti-IM or -SUC antibodies. It is known that anti-IM (HBB 705) recognizes only a transient immature form of IM and pro-SI (37). It was therefore difficult to determine whether coexpression of SUC and IM had resulted in a maturation of IM. Nevertheless, detection of an enzymatically active IM in the cellular lysates strongly suggested that this subunit had acquired a correctly folded pattern in the presence of SUC. Furthermore, immunoprecipitation with anti-SUC (HBB 219) revealed SUC, IM, and a diffuse band above the SUC protein that has an apparent molecular mass similar to the native brush border IM form. Finally, IM was no longer efficiently recognized by mAb anti-IM (HBB 705) antibody after 3 h of chase, supporting the view that this IM form has an altered folding pattern when it is associated with SUC. The specificity of SUC as an interaction partner with IM was further assessed by cotransfection of IM with the unrelated intestinal glycoprotein LPHβ (Fig. 5C). Here, calnexin co-precipitated with IM was not displaced by LPHβ after 3 h of chase, in sharp contrast to the situation when SUC was used in the cotransfection experiments. Altogether, the results demonstrate that SUC
competes with calnexin for binding to IM in the ER followed by a strong association of mature SUC and IM.

**Concluding Remarks**—The SUC subunit of the pro-SI enzyme complex folds independently of other domains of this enzyme complex. Most notably, it does not require the presence of its strikingly homologous counterpart, the IM subunit. SUC is a transport-competent protein, and because it is not membrane-anchored it is secreted into the external milieu. Interestingly, however, conversion of the hydrophilic SUC form to a membrane-bound protein results in its complete retention in the ER. Examples with a variety of membrane-bound proteins have assigned membrane-anchoring domains a decisive role in the formation of correctly folded oligomeric protein structures that acquire transport competence and exit the ER (38–40). The data obtained in this paper with a membrane-anchored form of SUC provide an entirely opposite view to this concept and show that a membrane anchor can also act negatively by distorting the folding of an otherwise correctly folded molecule.

It is likely that membrane-anchoring of SUC exerts constraints on particular protein domains at the N-terminal end in an immediate vicinity of the fused anchor, preventing thus an autonomous and flexible formation of these folding determinants. Obviously this protein does not require any of its neighboring protein domains within the enzyme complex for correct folding. By contrast, alteration of the native constellation of IM by its individual expression in the absence of SUC results in a protein that does not exit the ER. This form is readily degradable by trypsin, pointing to a malfolded conformation. However, correct folding of IM is restored by the addition of SUC through direct interaction between both subunits. The acquisition of IM to correct folding is accompanied by a strong interaction of both subunits, leading to an inhibition of the secretion of SUC into the external milieu. This resembles the situation in *vivo*, which occurs after the uncleaved mature pro-SI has been inserted into the membrane and cleaved thereafter by luminal trypsin. Here both subunits, the membrane-associated IM and
the hydrophilic SUC, remain strongly associated with each other via non-covalent strong ionic interactions. We propose that SUC therefore functions as an intramolecular chaperone implicated in the folding of IM. This chaperone function competes with that of the ER resident molecular chaperone, calnexin (4). Although SUC does not bind calnexin and acquires correct folding independently, IM binds strongly and persistently to calnexin. Only when SUC is coexpressed with IM is the interaction of the latter with calnexin disrupted, indicating a direct competition between SUC and calnexin. This competition provides another support for a chaperone function of the subunit and at the same time explains why individually expressed SUC does not bind calnexin. Based on the striking homologies between SUC and IM, although only IM binds calnexin, one may conclude that the binding sites for calnexin on IM are located in the non-homologous regions of SUC and IM. Scanning of these domains would provide important clues regarding the characterization of the mechanism implicating proteinaceous motifs in the binding of calnexin to IM, which also would correspond to the contact sites of IM with SUC. These sites can not be sugar-dependent because modification of carbohydrate processing has no influence on the trafficking and hitherto domain interaction of SUC and IM within the pro-SI protein (41). In view of this it seems unlikely that IM binding to calnexin would involve several cycles of deglucosylation/reglucosylation of the outmost end of the core N-linked glycans units (42). This is also supported by the fact that SUC and IM possess a comparable number of N-glycans that are distributed similarly over the individual protein domains (7) although only IM binds calnexin. The function of SUC as an intramolecular chaperone in the folding of IM is thus to prevent IM from binding calnexin.

**Fig. 5. IM interaction with calnexin.** A, IM interacts with calnexin. COS-1 cells were transiently transfected with pSG8-IM or pSG8-SUCsol and labeled 48 h after transfection with [35S]methionine for 1 h. IM or SUC were immunoprecipitated from the cellular lysates with mAb anti-IM (HBB 705) or mAb anti-SUC (HBB 219), respectively, and subjected to SDS-PAGE and phosphorimaging. The identification of calnexin was performed by Western blotting using a polyclonal anti-calnexin antibody. IP, immunoprecipitation; WB, Western blotting; CNX, calnexin. B, interaction of IM with calnexin is disrupted by SUC. COS-1 cells were cotransfected with pSG8-IM and pSG8-SUCsol. The cells were labeled for 1 h with [35S]methionine or labeled for 1 h and chased for 3 h. Cellular lysates were immunoprecipitated with either mAb anti-IM (HBB 705) or mAb anti-SUC (HBB 219), and the isolated proteins were analyzed by SDS-PAGE and phosphorimaging. C, calnexin is not displaced from IM by the irrelevant glycoprotein LPHβ. Cotransfection of COS-1 cells was performed with pSG8-IM and pJB20-LPHβ. The cells were labeled and chased as indicated in B, followed by immunoprecipitation either with mAb anti-IM (HBB 705) or together with mAb anti-LPH. The immunoprecipitates were analyzed by SDS-PAGE and phosphorimaging.
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erone in the context of pro-SI is the first of its kind for a domain that is located at the C-terminal end of a protein. All intramolecular chaperones so far identified in mammalian cells are located immediately at the N-terminal end (33, 34, 43), presumably to facilitate an early implication in the folding processes of the downstream domains (44). Only two known examples of C-terminally located intramolecular chaperones have been described in bacteria (45) and fungus (43). The scenario of the function of SUC in the context of the type II membrane protein pro-SI proposes that in the early phase after synthesis the IM domain binds calnexin. After acquisition of a correct folding in SUC this mature form binds tightly to IM, disrupting its interaction with calnexin. The consequence is that IM acquires correct folding and SUC is no longer secreted into the external milieu.

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REFERENCES

1. Gething, M. J. (1997) Nature 388, 329–331
2. Freedman, R. B. (1989) Cell 57, 1069–1072
3. Munro, S., and Pelham, H. R. (1986) Cell 46, 291–300
4. Ou, W. J., Cameron, P. H., Thomas, D. Y., and Bergeron, J. J. (1993) Nature 364, 771–776
5. Kreis, T. E., and Lodish, H. F. (1986) Cell 46, 929–937
6. Tato, U., Hammond, C., and Helenius, A. (1995) EMBO J. 14, 1340–1348
7. Naim, H. Y., Sterchi, E. E., and Lentze, M. J. (1988) J. Biol. Chem. 263, 7242–7253
8. Naim, H. Y. (1992) Biochem. J. 286, 451–457
9. Nicholls, B. L., Elder, J., Avery, S., Hahn, D., Quaroni, A., and Sterchi, E. (1998) J. Biol. Chem. 273, 3076–3081
10. Hauri, W., Spier, M., Semenza, G., and Lodish, H. F. (1986) Cell 46, 227–234
11. Hauri, H. P., Wacker, H., Rickli, E. E., Bigler-Meier, B., Quaroni, A., and Semenza, G. (1982) J. Biol. Chem. 257, 4522–4528
12. Xu, W., Chung, D. W., and Davie, E. W. (1996) J. Biol. Chem. 271, 27348–27353
13. Wang, Z. Z., Hardy, S. F., and Hall, Z. W. (1996) J. Cell Biol. 135, 809–817
14. Luisiri, P., Lee, Y. J., Eifelder, B. J., and Clark, M. R. (1996) J. Biol. Chem. 271, 5158–5163
15. Fehlmann, M., Chvatchko, Y., Brandenburg, D., Van Obberghen, E., and Brossette, N. (1985) Biochimie (Paris) 67, 1155–1159
16. Gorga, J. C. (1992) Crit. Rev. Immunol. 11, 305–335
17. Meuer, S. C., Acuto, O., Hussey, R. E., Hodgdon, J. C., Fitzgerald, K. A., Schlossman, S. F., and Reinherz, E. L. (1983) Nature 303, 888–890
18. Deleersnyder, V., Pilze, A., Wyckowski, C., Blikt, K., Xu, J., Hahn, Y. S., Rice, C. M., and Dubuisson, J. (1997) J. Virol. 71, 677–704
19. Fransen, J. A., Hauri, H. P., Ginzel, L. A., and Naim, H. Y. (1991) J. Cell Biol. 115, 45–57
20. Hauri, H. P., Roth, J., Sterchi, E. E., and Lentze, M. J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4423–4427
21. Jacob, R., Zimmer, K. P., Schmitz, J., and Hauri, H. Y. (2000) J. Cell. Biol. 161, 281–287
22. Ouwendijk, J., Moolenaar, C. E., Peters, W. J., Hellenberg, C. P., Ginzel, L. A., Fransen, J. A., and Naim, H. Y. (1996) J. Clin. Invest. 97, 633–641
23. Jacob, R., Alfalah, M., Grunberg, J., Obendorf, M., and Hauri, H. Y. (2000) J. Biol. Chem. 275, 6566–6572
24. Jacob, R., and Naim, H. Y. (2001) Curr. Biol. 11, 1444–1450
25. Jacob, R., and Naim, H. Y. (2003) Curr. Opin. Cell Biol. 15, 451–457
26. Jacob, R., and Naim, H. Y. (2000) J. Biol. Chem. 275, 26933–26943
27. Laemmli, U. K. (1970) Nature 227, 680–685
28. Towbin, H., Staebelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
29. Dahlqvist, A. (1968) Anal. Biochem. 22, 99–107
30. Wilson, J. M., Whitney, J. A., and Neutra, M. R. (1987) J. Cell. Biol. 105, 691–703
31. Steiner, D. F., Docherty, K., and Carroll, R. (1984) J. Cell. Biochem. 24, 121–130
32. Wessberg, N., Jacob, R., Alfalah, M., Zimmer, K. P., and Naim, H. Y. (2001) J. Biol. Chem. 276, 23506–23510
33. Cao, J., Huang, H., and Oldfield, E. H. (2000) J. Biol. Chem. 275, 29646–29653
34. Cigic, B., Dahl, S. W., and Pain, R. H. (2000) Biochemistry 39, 12382–12390
35. Marie-Claire, C., Yabuta, Y., Suefui, K., Matsuzawa, H., and Shinde, U. (2011) J. Biol. Chem. 286, 151–165
36. Shinde, U., Li, Y., Chatterjee, S., and Inouye, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6924–6928
37. Hauri, H. P., Sterchi, E. E., Bienen, D., Fransen, J. A., and Marxer, A. (1985) J. Cell Biol. 101, 838–851
38. Doyle, C., Sambrook, J., and Gething, M. J. (1986) J. Cell Biol. 103, 1107–1204
39. Lazarro, J., Lin, S. P., Kitakis, N., Lee, M. S., Bird, C., and Roth, M. G. (1990) J. Biol. Chem. 265, 4760–4767
40. Naim, H. Y., and Naim, H. (1998) Eur. J. Cell Biol. 70, 198–208
41. Naim, H. Y., Joberty, G., Alfalah, M., and Jacob, R. (1999) J. Biol. Chem. 274, 17961–17967
42. Kim, P. S., and Arvan, P. (1995) J. Cell Biol. 128, 29–38
43. Conesa, A., Weelink, G., van den Hondel, C. A., and Punt, P. J. (2001) FEBS Lett. 503, 117–120
44. Zhu, M., Ohta, Y., Jordan, F., and Inouye, M. (1989) Nature 339, 483–484
45. Feller, G., D’Amico, S., Benostmane, A. M., Joly, P., van Beeumen, J., and Gerday, C. (1998) J. Biol. Chem. 273, 12109–12115