Evolutionary Gain of Function for the ER Membrane Protein Sec62 from Yeast to Humans

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Because of similarity to their yeast orthologues, the two membrane proteins of the human endoplasmic reticulum (ER) Sec62 and Sec63 are expected to play a role in protein biogenesis in the ER. We characterized interactions between these two proteins as well as the putative interaction of Sec62 with ribosomes. These data provide further evidence for evolutionary conservation of Sec62/Sec63 interaction. In addition, they indicate that in the course of evolution Sec62 of vertebrates has gained an additional function, the ability to interact with the ribosomal tunnel exit and, therefore, to support cotranslational mechanisms such as protein transport into the ER. This view is supported by the observation that Sec62 is associated with ribosomes in human cells. Thus, the human Sec62/Sec63 complex and the human ER membrane protein ERJ1 are similar in providing binding sites for BiP in the ER-lumen and binding sites for ribosomes in the cytosol. We propose that these two systems provide similar chaperone functions with respect to different precursor proteins.

INTRODUCTION

In eukaryotic cells, protein secretion begins with the translocation of presecretory proteins across the membrane of the rough endoplasmic reticulum (ER). Translocation is mediated by a protein transloca- (also termed translocon), that resides in the ER membrane, and occurs co- or posttranslational. The posttranslational mechanism is abundant in the yeast Saccharomyces cerevisiae and the human parasite Trypanosoma brucei (Goldshmidt et al., 2008), whereas the cotranslational mechanism dominates in mammalian cells. In uni- as well as multicellular organisms and for both modes of operation, the protein transloca- contains the heterotrimeric Sec61 complex as a central pore-forming component. In yeast ER, the cotranslationally operating protein transloca comprises Sec63p (a membrane-integrated heat shock protein [Hsp] 40), Sec71p, Sec72p, and Kar2p (a lumenal Hsp70) as additional components. The posttranslational mechanism is abundant in the yeast Saccharomyces cerevisiae and the human parasite Trypanosoma brucei (Goldshmidt et al., 2008), whereas the cotranslational mechanism dominates in mammalian cells. In uni- as well as multicellular organisms and for both modes of operation, the protein transloca- contains the heterotrimeric Sec61 complex as a central pore-forming component. In yeast ER, the cotranslationally operating protein transloca comprises Sec63p (a membrane-integrated heat shock protein [Hsp] 40), Sec71p, Sec72p, and Kar2p (a lumenal Hsp70) as additional components. The posttranslational mechanism is abundant in the yeast Saccharomyces cerevisiae and the human parasite Trypanosoma brucei (Goldshmidt et al., 2008), whereas the cotranslational mechanism dominates in mammalian cells. In uni- as well as multicellular organisms and for both modes of operation, the protein transloca- contains the heterotrimeric Sec61 complex as a central pore-forming component.

Sec62 is associated with ribosomes in human cells. Thus, the human Sec62/Sec63 complex and the human ER membrane protein ERJ1 are similar in providing binding sites for BiP in the ER-lumen and binding sites for ribosomes in the cytosol. We propose that these two systems provide similar chaperone functions with respect to different precursor proteins.

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Abbreviations used: BiP, immunoglobulin heavy-chain binding protein; ER, endoplasmic reticulum; GST, glutathione S-transferase; Hsp, heat shock protein; NAC, nascent polypeptide-associated complex; PDI, protein disulfide isomerase; SPR, surface plasmon resonance; SRP, signal recognition particle.
2005) and that mammalian Sec62 also may be able to bind to ribosomes. We note that similar basic oligopeptide motifs are absent from yeast Sec62p (see Figure 1A).

There also is considerable medical interest in human Sec62 and Sec63. Genetic work linked mutations in the SEC63 gene to polycystic liver disease (Davila et al., 2004). According to the two-hit hypothesis for this disease, mutations in the second allele in liver cells of heterozygous carriers of a SEC63 mutation do not result in cell death, but rather lead to cell proliferation and the progressive development of liver cysts. Furthermore, mutations in the SEC63 gene were described for HNPC-associated small-bowel cancer (Schulmann et al., 2005) and microsatellite-unstable gastric and colorectal cancers (Mori et al., 2002). In addition, over-expression of the SEC62 gene was found to be associated with sporadic colorectal cancer (Escrich et al., 2005) and prostate cancer (Jung et al., 2006).

Here, we provide direct evidence for conservation of Sec62/Sec63 interaction from yeast to humans and show that in the course of evolution Sec62 of vertebrates has gained a function, i.e., the ability to interact with the ribosomal tunnel exit. Two basic oligopeptide motifs are responsible for this interaction and are absent from yeast Sec62p as well as from Sec62 in invertebrates or plants. Thus, the human Sec62/Sec63 complex and human ERj1 are similar in providing a binding site for ribosomes on the cytosolic face and a binding site for BiP on the luminal face of the ER membrane, i.e., may both be involved in cotranslational transport of polypeptides into the ER. This is consistent with the observation that Sec62 is protected against externally added antibodies by ribosomes in permeabilized human cells.

MATERIALS AND METHODS

Materials

Aurintricarboxylic acid (ATA) and CHAPS were from Calbiochem (La Jolla, CA). 35S-methionine and enhanced chemiluminescence (ECL) were obtained from GE Healthcare (Waukesha, WI). Sulfo-SMCC was purchased from Pierce (Rockford, IL). Antibodies against calnexin and protein disulfide isomerase (PDI) were from Stressgen (San Diego, CA); peroxidase-conjugated anti-penta-histidine antibodies were from Qiagen (Hilden, Germany); peroxidase conjugate against rabbit IgG goat antibodies were from Sigma (St. Louis, MO). Antibodies against yeast Sec62 (CNKKAINEAQEN) and human ribosomal proteins L4 (CEKPPTEEKKPAAP) and S3 (CGKPEPPAMPQPV) were raised against the indicated C-terminal oligopeptide.

Peptides were synthesized on a 433A peptide synthesizer (Applied Biosystems, Foster City, CA), cleaved, and deprotected as described previously (Diersk et al., 1996). Oligopeptides 62-11mer 1 and 2 corresponded to amino acid residues 2 through 12 and 157 through 167 of human Sec62, respectively. Peptide ivD2-11mer corresponded to amino acid residues 2 through 12 of Sec62 isoform A of Dracopilus melunogaster (SEKRRKRRK).

Ribosomes were purified from canine pancreas by gradient centrifugation and washed with puromycin (0.5 mM) and high salt (500 mM KOAc). The A260/A280 of the purified ribosomes was 1.98. Ribosomal subunits were prepared by incubation of washed ribosomes in high salt (1 M KC1) and subsequent sucrose gradient centrifugation according to published procedures (Sedding, 1990). The integrity of 80S ribosomes and ribosomal subunits was confirmed by analysis of molecular mass and hydrodynamic radius by a combination of asymmetric-flow field-flow fractionation and light-scattering analysis as 35 kDa (Supplemental Figure 2), which is consistent with a monomer (calculated mass of monomer: 23 kDa).

Pulldown Assay

Purified GST or related hybrid proteins (10 μg) were immobilized on GSH-Sepharose. Then, buffer (20 mM HEPES-KOH, pH 7.5, 150 mM KC1, 2 mM MgCl2, 0.65% CHAPS) or purified hexa-histidine fusion protein (12 μg) in the same buffer was applied to the resin. Where indicated oligopeptides (final concentration: 300 μg/ml) were added simultaneously. After washing, the bound material was eluted and analyzed by SDS-PAGE as followed by either protein staining with Coomassie Brilliant Blue or Western blotting plus immunodetection with anti-penta-histidine antibodies or antibodies against ribosomal proteins plus peroxidase conjugate of anti-rabbit IgG goat antibodies. The antibodies were visualized by incubation of the blots in ECL and subsequent luminescence imaging (Lumi-Imager F1 with LumiAnalyst software 3.1, Roche Diagnostics, Mannheim, Germany).

Ribosome-Binding Assay

Recombinant proteins (150 pmol) were diluted into 20 mM HEPES-KOH, pH 7.5, 200 mM KC1, 1.5 mM MgCl2, 1 mM EDTA, and 0.65% CHAPS and incubated in the simultaneous absence or presence of ribosomes (50 pmol) for 15 min at 30°C (Dudek et al., 2005). Where indicated oligopeptides (50 nmol) were added. Subsequently, the mixture was layered onto a low-salt cushion (0.5 M sucrose in 40 mM HEPES-KOH, pH 7.5, 150 mM KOAc, 5 mM MgOAc, 2 mM DTT) and subjected to centrifugation for 90 min at 356,000 × g and 2°C (Beckman table top ultracentrifuge Optima MAX-E, Beckmann rotor TL-A-120.2, Fullerton, CA). The supernatants were subjected to protein precipitation with trichloroacetic acid. The precipitates and the pellets, respectively, were subjected to SDS-PAGE and subsequent protein staining or Western blotting plus immunodetection with peroxidase-conjugated anti-penta-histidine antibodies or antibodies against ribosomal proteins plus peroxidase conjugate of anti-rabbit IgG goat antibodies. The antibodies were visualized by incubation of the blots in ECL and subsequent luminescence imaging.

Alternatively, ribosomal complexes were subjected to sucrose gradient centrifugation (linear sucrose gradient between 10 and 60%, wt/vol) in low-salt buffer adjusted to 33 μg/ml BSA (for 60 or 90% at 280,000 × g and 2°C (Beckman ultracentrifuge Optima L-80, Beckmann rotor SW 55 Ti; Dudek et al., 2002). After fractionation of the gradients, proteins were precipitated and subjected to SDS-PAGE and subsequent protein staining or Western blotting plus immunodetection as described above.

Surface Plasmon Resonance Spectroscopy

Surface plasmon resonance (SPR) spectroscopy was performed in a BIAAlte upgrade system (Biacore, Freiburg, Germany). Sensor chip NTA was activated with N3+ according to the manufacturer’s protocol (Biacore). Hexa-histidine-tagged proteins were immobilized on the chip surface at a flow rate of 10 μl/min in 10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 50 μM EDTA, and 0.005% surfactant P20. For interaction analysis, the chip was equilibrated with running buffer (10 mM HEPES-KOH, pH 7.6, 150 mM NaCl, 0.125% digitonin) at a flow rate of 30 μl/min. Solutions containing increasing concentrations of analyte were passed over the chip surface. Each analyte application was followed by application of running buffer and eventually with running buffer that was supplemented with high salt. The analysis was carried out by employing BIA evaluation software version 3.1 (Biacore).

In Vitro Translation

Synthesis of firefly luciferase, bovine preprolactin (ppl), or nascent preprolactin (ppl)(s) was carried out in rabbit reticulocyte lysate in the presence of 35S-methionine (translation kit type I or II, Roche Diagnostics; Dudek et al., 2005). The translation reactions contained either buffer (20 mM HEPES-KOH, pH 7.5, 500 mM KC1, 2 mM MgCl2, 2 mM DTT, 0.65% CHAPS), recombinant protein (final concentration: 1 μM) in buffer, DMSO (final concentration: 2%), or oligopeptide (final concentration: 150 μM) in DMSO. After incubation for various times at 30°C, the translation reactions were subjected to SDS-PAGE and phosphorimaging (Molecular Dynamics, Sunnyvale, CA; model SF with Image Quant software).

Chemical Cross-Linking

ppl(s) was synthesized in reticulocyte lysate in the presence of 35S-methionine for 20 min (Dudek et al., 2005). After adjusting the translation reaction to 500 mM KOAc for 5 min at 0°C, the ribosomes were pelleted by centrifugation through a high-salt cushion. The ribosomes were resuspended in XL-buffer (50 mM HEPES-KOH, pH 7.5, 200 mM KOAc, 2 mM MgOAc, 0.2 M sucrose, 0.65% (wt/vol) CHAPS), divided into various aliquots, and supplemented with buffer or recombinant protein (2 μM). After incubation for 15 min at 30°C, ribosomes were resolated by centrifugation through a low-salt cushion and incubated with Sulfo-SMCC (0.25 mM) for 20 min at 0°C. All samples were subjected to SDS-PAGE and phosphorimaging.
Yeast Manipulations

Yeast SEC62 ts mutant strain RDM50-94C (leu2-3 leu2-112 his4 ura3-52 sec62-1 MAT/H9251) was kindly provided by R. Schekman (Berkeley, CA). The CEN-multicopy vector YEp with URA3 as a selection marker was provided by G. Schlenstedt (Homburg). Cells were transformed with a derivative of YEp that contained the human SEC62 cDNA under control of the GAL1 promoter and were grown on SD medium without uracil at 24 or 37°C in the presence of glucose (2%) or galactose (2%).

Quantitative Fluorescence Microscopy

Cells were grown on eight-chambered Lab-Tek glass coverslips (Naperville, IL) in the respective standard media to 40–70% confluence. Cells were fixed with fresh 3.7% formaldehyde in PBS for 15 min at room temperature, permeabilized with PBS with 0.1% Triton X-100, and—where indicated—treated with 50 μg/ml RNase A during the block step in 10% fetal bovine serum/PBS. Subsequently, the cells were labeled with affinity-purified primary antibodies, followed by Alexa 555–conjugated anti-rabbit IgG secondary antibodies. Cells were imaged using fluorescence microscopy with a widefield microscope (Axiovert 200; Carl Zeiss Microimaging, Thornwood, NY; 63× oil 1.4 NA objective, 450–490 excitation/500–550 emission bandpass filter) and a Retiga 2000R camera. Image analysis was performed with ImageJ 1.39 (http://rsb.info.nih.gov/ij/). Figures were prepared using Microsoft Excel 2004 (Redmond, WA) and Adobe Photoshop CS2 and Adobe Illustrator 11.0 (San Jose, CA).

RESULTS

Human Sec62 and Sec63 Interact with Each Other in a Manner Similar to their Yeast Orthologues

In yeast, the negatively charged carboxyterminus of Sec63p interacts with the overall positively charged aminoterminal (N-terminal) domain of Sec62p (Wittke et al., 2000; Figure 1A). Thus, we asked if this mode of interaction is conserved in the two human proteins. Purified GST hybrid proteins that comprised the complete carboxyterminus (C-terminal) domain of human Sec62 and synthetic basic oligopeptides that are used throughout this study.

Figure 1. Structural and functional features of human Sec62 and Sec63. (A) Domain organization of Sec62, Sec63, and ERj1. Sec63 and ERj1 are membrane resident Hsp40s with ER lumenal J-domains and cochaperones for ER-lumenal Hsp70s (BIP and Kar2p). Sil1, Grp170, and Lhs1p act as nucleotide exchange factors for these Hsp70s. We note that loss of Sil1 function is linked to the human neurodegenerative disease Marinesco-Sjögren syndrome and may be nonlethal due to functional redundancy of Sil1 with Grp170 (Zimmermann et al., 2006). (B) Basic oligopeptides that are present in Sec62 and established ribosomal ligands in Homo sapiens. (C) Recombinant derivatives of human Sec62 and synthetic basic oligopeptides that are used throughout this study.
Indicated oligopeptide (225 residues) were immobilized on GSH-Sepharose. Buffer, Sec62N, or Sec62N-GST or the two GST hybrid proteins, GST-Sec63C or GST-Sec63C26, served as a negative control. Human Sec62N interacts with human Sec63C. (A and B) SPR analysis of the Sec62/Sec63 interaction. Human Sec62N was immobilized in the measuring cell of a NTA sensor chip via its terminal domains of mammalian and yeast NAC orthologues. Both mammalian proteins behaved like their yeast orthologues. To further characterize the Sec62/Sec63 interaction, SPR experiments were carried out. Human Sec62N was immobilized in the measuring cell of a NTA sensor chip via its hexa-histidine tag. Human Sec62C served as a negative control and was immobilized in the reference cell. Then increasing concentrations of human Sec63C were passed over the chip and were followed by buffer. Association of the analyte and its dissociation were recorded and analyzed (Figure 2D). We could fit the kinetics with a 1:1 binding model and determined an apparent affinity (Kd) of Sec63C for Sec62N of 4.78 nM. This Kd was consistent with the fact that native Sec62 was communoprecipitated with Sec63 from a microsomal detergent extract (Tyedmers et al., 2000).

**Human Sec62 Interacts with 80S Ribosomes**

The N-terminal domain of mammalian Sec62 (Sec62N) contains two basic oligopeptide motifs that are reminiscent of similar peptides in established ribosomal tunnel exit ligands (such as NAC and ERj1; Ferbitz et al., 2004; Blau et al., 2005; Figure 1B). Deletion of the basic oligopeptides in the N-terminal domains of mammalian and yeast NACβ (Grallath et al., 2006; Wegrzyn et al., 2006) as well as the cytosolic domain of ERj1 (Dudek et al., 2005) led to loss of ribosome-binding ability. Therefore, Sec62N was analyzed with respect to its ribosome-binding ability by a number of different experimental approaches, and the role of the two basic oligopeptide motifs was characterized. The same experimental strategies were previously used for the characterization of the ribosome interaction of ERj1 (Dudek et al., 2002, 2005).

Sec62N was incubated in the presence or absence of non-translating 80S ribosomes. An aliquot of ribosomes was incubated in the absence of Sec62N and served as reference (Figure 3A). The samples were analyzed by gradient centrifugation and subsequent SDS-PAGE and protein staining. In the absence of ribosomes, Sec62N stayed at the top of the gradient (Figure 3B). After incubation with ribosomes, Sec62N comigrated with ribosomes (Figure 3C). Thus the observed comigration of Sec62N with ribosomes was not due to aggregation, but rather reflected an interaction between the two molecules.
Figure 3. Ribosome binding of human Sec62N and inhibition by basic oligopeptides. (A–C) Sec62N was adjusted to KCl concentration of 100 mM and incubated without (B) or with ribosomes (C). A third mixture contained ribosomes but was free of Sec62N (A). Subsequently the samples were subjected to sucrose gradient centrifugation. After fractionation of the gradients, aliquots of the fractions and the pellets (p) were subjected to SDS-PAGE and protein staining. (D–F) Recombinant proteins were incubated in the presence (D and E) or absence (F) of nontranslating ribosomes. Subsequently, the mixtures were layered onto a sucrose cushion and subjected to ultracentrifugation. The pellets (p) and supernatants (s) were analyzed by SDS-PAGE and subsequent protein staining (D) and Western blotting plus immunodetection with anti-penta-histidine antibodies (E and F). (G) Sec62N (150 pmol) or Sec62N in combination with the indicated oligopeptide (50 nmol) were incubated in the presence of nontranslating ribosomes. Subsequently, the mixtures were layered onto a sucrose cushion and subjected to ultracentrifugation. The pellets and supernatants were analyzed by SDS-PAGE and subsequent protein staining. (H–J) Recombinant proteins were incubated in the presence (H and I) or absence (J) of nontranslating ribosomes. Subsequently, the mixtures were analyzed as in D–F. We note that the recombinant proteins did not pellet in the absence of ribosomes (F and J). yeast, yeast Sec62N; h-yeast, humanized yeast Sec62N.
Next, Sec62N and three truncated derivatives (Figure 1C) were each incubated in the presence or absence of nontranslating ribosomes in order to address the question of which part of Sec62N was involved in this interaction. Yeast Sec62N that lacks similar basic oligopeptide motifs as compared with human Sec62N (Figure 1A) served as negative control. Subsequently, the ribosomes were reisolated by centrifugation and the relative amount of ribosome associated Sec62N was determined by SDS-PAGE and protein staining (Figure 3D) or Western blotting plus immunodetection with anti-penta-histidine antibodies (Figure 3, E and F). All proteins stayed in the supernatant in the absence of ribosomes (Figure 3F). However, Sec62N and constructs that contained at least a single positively charged oligopeptide were pelleted together with ribosomes, i.e., were active in ribosome binding (although to a varying degree), whereas the construct with the double deletion (termed Sec62N-DΔN10-ΔC40) was almost completely inactive in ribosome binding (Figure 3, D and E, lanes 7 and 8). Furthermore, yeast Sec62N that lacks similarly charged oligopeptides was unable to bind ribosomes (Figure 3, D and E, lanes 9 and 10). Although both basic oligopeptides within Sec62N contributed to ribosome binding, the aminoterminal peptide seemed to be more important.

To further substantiate the role of the two highly charged oligopeptides for ribosome binding, synthetic oligopeptides were used as potential competitors of Sec62N in ribosome binding (peptides 62-11mer1 and 2; Figure 1C). The aminoterminal oligopeptide from an invertebrate Sec62 served as negative control (peptide iv62-11mer). Only the aminoterminal peptide from human Sec62 competed with Sec62N for ribosome binding (Figure 3G, lanes 3 and 4).

Having observed an interaction of human Sec62N with ribosomes, i.e., a gain of function of the human protein compared with the yeast protein, we asked if yeast Sec62N can be turned into a ribosome-binding protein by the addition of the aminoterminal dodecapeptide from human Sec62 and if human SEC62 is able to rescue the thermosensitive growth phenotype of a translocation-deficient yeast SEC62 mutant. Yeast Sec62N was extended at its aminoterminus by the aminoterminal dodecapeptide from human Sec62 (termed humanized Sec62N) and incubated in the presence or absence of nontranslating ribosomes. Subsequently, the ribosomes were reisolated by centrifugation and the relative amount of ribosome associated humanized Sec62N was determined by SDS-PAGE and protein staining or Western blotting plus immunodetection with anti-penta-histidine antibodies. In contrast to wild-type yeast Sec62N, humanized Sec62N bound to ribosomes (Figure 3, H–J). Thus the aminoterminal peptide that is present in human Sec62 is sufficient for ribosome binding. Because of a protein translocation defect at the nonpermissive temperature, the yeast mutant strain RDM50-94C hardly grows at 37°C (Deshaies and Schekman, 1990). However, when the human SEC62 gene was expressed in this strain after the addition of galactose, the cells grew at 37°C (Figure 4). Thus, the additional positively charged oligopeptides that are present in human Sec62 compared with yeast Sec62p do not interfere with the posttranslational function of yeast Sec62p.

To quantitatively characterize the ribosome interaction of mammalian Sec62, SPR experiments were carried out. Human Sec62N was immobilized in the measuring cell of a NTA sensor chip via its hexa-histidine tag. Based on the experiments that are depicted in Figure 3, D and E, yeast Sec62N served as a negative control and was immobilized in the reference cell. Then increasing concentrations of mammalian ribosomes were passed over the chip and were followed by buffer. Association of the analyte to and its dissociation from the ligand were recorded and analyzed (Figure 5A). We determined an apparent affinity (K_d) of ribosomes for Sec62N of 0.13 nM.

Ribosome Association of Sec62 Is Salt- and RNase-sensitive

The interaction of ERj1 with ribosomes was shown to be salt-sensitive and to involve rRNA (Dudek et al., 2002 and 2005). Here, we asked if this is also true for Sec62N. Thus, ribosome binding assays and SPR experiments were carried out above the standard 100–150 mM salt concentration and by substituting ribosomes by RNase-treated ribosomes, respectively. Human Sec62N was immobilized in the measuring cell of a NTA sensor chip via its hexa-histidine tag, and yeast Sec62N was immobilized in the reference cell. After association of native ribosomes, the dissociation was carried out at 200 and 500 mM KOAc concentration (Figure 5B). According to the sensorgram, the interaction of Sec62N with ribosomes was salt-sensitive. When RNase treated ribosomes were used as analyte the sensorgram suggested that the interaction of Sec62N with ribosomes was RNase-sensitive (Figure 5C). Similar observations were made in the ribosome-binding assays (Figure 5, D and E vs. F). Thus binding of Sec62N to ribosomes most likely involves electrostatic interactions with rRNA. We note that the observed salt sensitivity of ribosome binding of Sec62N may explain why Sec62 was not characterized as ribosome-associated membrane protein after solubilization of microsomes in de-
tergent (salt concentration: 400 mM KCl; Meyer et al., 2000; Tyedmers et al., 2000).

**Sec62 Interacts with Ribosomes near or at the Tunnel Exit**

Next, we used three independent experimental approaches to assess whether or not the ribosomal exit site of nascent polypeptides is also the binding site for Sec62N, because this site has previously been observed to provide a docking site for several proteins that are involved in protein biogenesis at the ER (SRP, NAC, Sec61 complex, ERJ1; Beckmann et al., 2001; Halic et al., 2004; Ferbitz et al., 2004; Blau et al., 2005).

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**Figure 5.** Characterization of the Sec62/ribosome interaction. (A) Human Sec62N was immobilized on an activated NTA sensor chip in the measuring cell and yeast Sec62N as a negative control in the reference cell. Increasing concentrations of nontranslating ribosomes (as indicated) were passed over the chip and were followed by running buffer. Association and dissociation kinetics were recorded. (B) Sensorgram after application of ribosomes, running buffer, and subsequent application of running buffer with 200 and 500 mM KOAc, respectively. (C) Sensorgram after application of ribosomes and the same concentration of ribosomes that had been pretreated with RNase A (240 μg/ml), respectively, for 30 min at 30°C. (D) Sec62N was incubated in the presence of nontranslating ribosomes at the indicated salt concentrations. Subsequently, the mixture was layered onto a sucrose cushion and subjected to ultracentrifugation. The pellets and supernatants were analyzed by SDS-PAGE and subsequent protein staining. (E) and (F) Sec62N was incubated in the presence of ribosomes that had been pretreated with buffer (E) or RNase A in buffer (80 μg/ml; F) for 30 min at 30°C. Subsequently the samples were subjected to sucrose gradient centrifugation. The fractions were analyzed by SDS-PAGE and subsequent Western blotting plus immunodetection with anti-penta-histidine (■), anti-L4 (○), and anti-S3 (○) antibodies. The Western blot signals were quantified by luminescence imaging and plotted against the fraction number.
First we assessed if there was a preference of Sec62N for the large ribosomal subunit. Sec62N was incubated in the presence or absence of 60S or 40S ribosomal subunits. The samples were analyzed by gradient centrifugation and subsequent SDS-PAGE as followed by Western blotting plus immunodetection with anti-penta-histidine, anti-L4, or anti-S3 antibodies. After incubation with 60S subunits, a fraction of the Sec62N comigrated with ribosomes (Figure 6B). In the absence of ribosomes and in the presence of 40S subunits, Sec62N stayed at the top of the gradient (Figure 6, A and C). Thus, Sec62 can only interact with large ribosomal subunits.

Next, we analyzed the possible overlap in binding sites between Sec62N and ERj1 using SPR experiments (Blau et al., 2005). Sec62N was immobilized in the measuring cell of a NTA sensor chip via its hexa-histidine tag and analyzed with respect to binding of native ribosomes that had been preincubated with ERj1C (Figure 6D). Native ribosomes that had been preincubated with Sec62N served as positive control, and native ribosomes that had been preincubated with BSA as negative control. The results demonstrate that Sec62N and ERj1 compete for an overlapping binding site on ribosomes, most likely at or near the ribosomal tunnel exit.

To directly address this point, Sec62N or its derivatives were allowed to form complexes with ribosomes that contained radiolabeled nascent polypeptide chains of defined length (i.e., peptidyl-tRNAs comprising 86 amino-terminal amino acid residues of preprolactin, ppl86mer). Subsequently, the ribosome/nascent chain/Sec62N complexes were reisolated and subjected to chemical cross-linking. ERj1 served as a positive control (Dudek et al., 2005; Figure 6E, lane 6). In the presence of Sec62N or a construct that contains a single positively charged oligopeptide, cross-linked products of the nascent polypeptide were detected (Figure 6E, lanes 2–4). These were absent when buffer, Sec62N/H9004N10/H9004C40 (Figure 6E, lane 1 and 5), yeast Sec62N (Figure 6F, lane 2), or human Sec62C were used (Figure 6F, lane 3). Thus, like ERj1, Sec62N binds to the ribosome near the tunnel exit. We note that different types of translation kit were used in the two experiments, which may account for the differences in cross-linking efficiencies.
amino-terminal oligopeptide has a more pronounced effect on binding of Sec62N compared with removal of the carboxy-terminal oligopeptide.

**Sec62N Does Not Simultaneously Interact with Sec63C and Ribosomes**

Having seen an interaction of Sec62N with ribosomes (Figure 3) and with Sec63C (Figure 2), we asked if Sec62N can recruit Sec63C to ribosomes. In ribosome binding assays, however, we failed to detect a trimeric complex (data not shown). Therefore, the immobilized complex between GST-Sec63C and Sec62N was used (Figure 7, left panels). In parallel, a complex was formed between GST-Sec63C and Sec62NΔN10-ΔC40, i.e., the truncated Sec62N that lacks the two positively charged oligopeptides (Figure 7, right panels). The resins were eluted with buffer or nontranslating ribosomes in the same buffer. Subsequently, the resins were eluted with SDS sample buffer, and all samples were analyzed as described above. On elution with buffer, Sec62N and Sec62NΔN10-ΔC40 remained bound to the immobilized Sec63C as expected (Figure 7, lanes 4 and 9). On elution with ribosomes, however, Sec62N eluted together with the ribosomes (lane 3 vs. 5). In contrast, Sec62NΔN10-ΔC40 that was unable to bind to ribosomes was not eluted with ribosomes (lane 8 vs. 10). Thus, interaction of Sec62N with Sec63C does not prevent binding of Sec62 to ribosomes, which is consistent with the observed K_d values for the interactions of Sec62N with Sec63C (4.78 nM) and ribosomes (0.13 nM).

**Mammalian Sec62 Inhibits Translation at the Level of Initiation**

ERj1 was shown to be able to bind to ribosomes as well as to inhibit protein synthesis at the level of initiation (Dudek et al., 2005). Therefore, Sec62N and its derivatives were tested for their ability to inhibit synthesis of firefly luciferase or bovine preprolactin in reticulocyte lysate (Figure 8, A and B). In this assay, Sec62N and constructs that contained at least a single positively charged oligopeptide were active in inhibiting protein synthesis (although to a varying degree), and the construct with the double deletion (termed Sec62NΔN10-ΔC40) was less active (Figure 8A). Furthermore, yeast Sec62N that lacks similarly charged oligopeptides was inactive (Figure 8A). We note that a similar inhibitory effect of human Sec62N on the synthesis of luciferase, and preprolactin was observed in the presence of canine pancreatic microsomes (Supplemental Figure 5). A control experiment demonstrated that the translational inhibition activity of Sec62N was specific: the inhibitory effect of Sec62N on translation correlated reciprocally with the ribosome content of the reticulocyte lysate (Figure 8C).

To further substantiate the role of the two highly charged oligopeptides for translational modulation by Sec62N, the synthetic oligopeptides were used in translation (peptides 62-11mer1 and 2; Figure 1C). Again, the amino-terminal oligopeptide from an invertebrate Sec62 served as negative control (peptide iv62-11mer). Indeed, the amino-terminal peptide from human Sec62N inhibited synthesis of preprolactin as well as luciferase (Figure 8, D and E).

In addition, Sec62N was tested for its ability to inhibit translation after inhibition of initiation (Figure 8F). Sec62N did not affect protein synthesis under these conditions. Thus, Sec62N inhibits synthesis of presecretory as well as nonsecretory proteins at the level of initiation.

**Mammalian Sec62 Is Protected from Antibody Access by Ribosomes in Permeabilized Cells**

Snapp et al. (2004) established a microscopic method to address the organization of protein translocon in the ER of mammalian cells. The experimental strategy was cell fixation, cell permeabilization, optional ribosome destruction by RNase treatment, and incubation with specific primary and fluorescently labeled secondary antibodies (Figure 9A). In the subsequent fluorescence microscopy and image analysis, the quantitative data from the RNase-treated cells were compared with the minus RNase control. Positive differential effects in fluorescence intensity were taken as an indication of association of the respective protein with ribosomes. Here, two different cell types (Madin-Darby canine kidney [MDCK] and HeLa) were analyzed with respect to Sec62 (Figure 9B). The Sec61α and Sec61β subunits of the translocon served as positive controls, the ER-membrane protein calnexin and the ER-luminal PDI served as negative controls (Snapp et al., 2004). Although the extents of the differential effects varied between the two cell types, we detected a significant increase in fluorescence intensity after RNase treatment for Sec61α, Sec61β, and Sec62, but not for calnexin and PDI. Similar results were obtained for Cos-7 and HepG2 cells (data not shown). Thus, Sec62 is in the vicinity of ribosomes and, by extrapolation, of Sec61 complexes in the intact ER. This is consistent with our previous findings that Sec61 subunits can be communoprecipitated from microsomal detergent extracts with antibodies against Sec62 and vice versa (Tyedmers et al., 2000).

To rule out the possibility that the observed epitope protection is due to the ribonucleoprotein particles SRP rather than ribosomes, the α-subunit of the SRP receptor (SRα) was analyzed under identical conditions (Figure 9C). There was no increase in fluorescence intensity after RNase treatment for SRα in either MDCK or HeLa cells. Thus, the observed epitope protection in the case of Sec61α, Sec61β, and Sec62, was indeed due to ribosomes.

Next we analyzed if the effect of RNase treatment can be mimicked by puromycin treatment of MDCK and HeLa cells, i.e., under conditions where the nascent polypeptides are released from translating ribosomes (Figure 10). There was no intensity increase detected for any of the proteins.
after addition of puromycin. This supports the notion that many ribosomes or 60S ribosomal subunits do not leave the ER surface after termination of protein synthesis (Potter et al., 2001). Furthermore, these data suggested that the RNase experiments visualized translating as well as nontranslating ribosomes.

**DISCUSSION**

**Similarities between Human and Yeast Sec62**

Here we demonstrated that the human ER membrane proteins Sec62 and Sec63 interact in the same manner as compared with their yeast orthologues, i.e., involving the conserved oppositely charged regions at the central region of Sec62N and the carboxyterminus of Sec63C (Wittke et al., 2000; Figure 1A). This is consistent with the observations that in *T. brucei* there is no Sec62 ortholog and that the trypanosomal Sec63 lacks the negatively charged carboxy-terminal region (Goldshmidt et al., 2008; Supplemental Figures 6 and 7). Taken together with the fact that the two proteins are associated with each other and with the Sec61 complex in detergent extracts from both yeast (Jermy et al., 2006) and canine pancreatic microsomes (Meyer et al., 2000; Tyedmers et al., 2000), it is very likely that the two mammalian proteins play a similar role in protein biogenesis compared with their yeast orthologues, i.e., are involved in protein transport into the ER. This view is supported by our immunofluorescence analysis (see below).

**Differences between Human and Yeast Sec62**

Furthermore, we have shown that in the course of evolution Sec62 of vertebrates has gained a function, i.e., the ability to interact with the ribosomal tunnel exit. This is consistent with our observation that human Sec62 is associated with ribosomes at the ER of mammalian cells and with the fact that the majority of transport into the mammalian ER is cotranslational. In the case of Sec62, two basic oligopeptide motifs may be responsible that are absent from yeast Sec62p.
as well as from Sec62 orthologues in invertebrates (such as *Caenorhabditis elegans* or *Drosophila melanogaster*) or plants (such as *Arabidopsis thaliana* or *Oryza sativa*; Supplemental Figures 6 and 7). Interestingly, this apparent gain of function did not interfere with the original function of the yeast protein because human Sec62 was able to complement the respective yeast *ts* mutant strain, as had previously been shown for the insect protein (Noel and Cartwright, 1994). Thus Sec62 showed most of the characteristics that were previously observed for ERJ1 (Figure 1): 1) high salt– and RNase-sensitive ribosome binding of the cytosolic domain, 2) essential basic oligopeptide(s) in the cytosolic domain, 3) binding near the ribosomal tunnel exit, and 4) modulatory effect on initiation of protein synthesis (Dudek et al., 2002, 2005; Blau et al., 2005). Because Sec63 shares with ERJ1 the ability to bind to BiP via its ER-luminal domain (J-domain), the human Sec62/Sec63 complex may have overlapping functions with ERJ1. This view is supported by the observations that human ERJ1 can complement a yeast Sec63p knockout (Krocynska et al., 2004) and that loss of Sec63 function in human polycystic liver disease is not lethal (Davila et al., 2004; Zimmermann et al., 2006). We note that in the case of ERJ1 the basic oligopeptide motif is also found only in vertebrate orthologues (Supplemental Figure 8).

**Possible Function(s) of Human Sec62/Sec63 and ERJ1**

What are the specific functions of Sec62/Sec63 and ERJ1 in the mammalian ER? We are convinced that the properties of
the two proteins/complexes suggest a cotranslational function, most likely in protein transport into the ER. Both could serve in recruiting BiP to Sec61 complexes and/or incoming polypeptides (Dierks et al., 1996; Tyedmers et al., 2003; Alder et al., 2005). Furthermore, both proteins/complexes could be involved in the transport of those nascent polypeptides that are synthesized by ribosomes or 60S ribosomal subunits that stay permanently associated with the ER surface (Potter et al., 2001), i.e., do not depend on SRP and its receptor for targeting to the ER (Figure 10). In this case it would make perfect sense to allow initiation of protein synthesis only if BiP is available, i.e., is bound to an ER-lumenal J-domain. Indeed, we observed for ERj1 that binding of BiP to the J-domain still allows binding of the ERj1/BiP complex to the ribosome and that this heterodimeric complex does not inhibit translation (Dudek et al., 2005). We suggest that a similar mechanism may be in operation for the Sec62/Sec63-complex. Here the model is that binding of BiP to the ER-lumenal J-domain of Sec63 induces a Sec63/Sec62-interaction that allows binding of the aminoterminal domain of Sec62 to ribosomes in a way that is compatible with translation.

Why would there be two similar systems? There could be functional specialization: the one system could recruit BiP to ribosomes and the Sec61 complex for sealing of the Sec61 channel (Alder et al., 2005), whereas the other system could recruit BiP as a molecular ratchet for incoming polypeptide chains (Tyedmers et al., 2003). Alternatively, the two systems may have different substrate specificities. A first indication for a potential substrate specificity of ERj1 was reported by S. Y. Blond and coworkers (Kroczynska et al., 2005, 2004, 2005). In a two-hybrid screening these authors identified two interaction partners of the SANT-2 domain that is present in the cytosolic domain of human ERj1, the secretory proteins α1-antichymotrypsin (ACT, residues 140-400) and inter-α-trypsin inhibitor heavy chain 4 (ITIH4, residues 588-930). The two proteins have in common that they are secreted in the human liver, are serum protease inhibitors, and are acute-phase proteins. In both cases the signal peptides were not present, indicating that the mature proteins interacted with the SANT-2 domain. This may suggest that ACT and ITIH4 are substrates of ERj1 in the human liver. Therefore, we used the aminoterminal domain of human Sec62 as bait in a two-hybrid screening of a human liver cDNA library (data not shown). We identified four putative interaction partners, two uncharacterized proteins and the precursors of two secretory proteins, haptoglobin (residues 1-296) and complement factor B (residues 1-237). The two secretory proteins have in common that they are secreted in the human liver, are serum proteins with a serine protease domain, and contain complement control protein (CCP)-domains or SUSHI repeats in their aminoterminal regions (two in residues 1-296 of haptoglobin and three in residues 1-237 of complement factor B). In both cases the signal peptides were present, possibly indicating that the signal peptides interacted with Sec62N. A signal peptide receptor function of human Sec62 would not be unexpected because the yeast Sec62p was shown to be part of the signal peptide receptor that is involved in posttranslational transport. Thus, these observations may indicate that haptoglobin and complement factor B may be substrates of Sec62 in the human liver. In future work, this interpretation of the two two-hybrid screenings has to be evaluated after siRNA-mediated silencing of the ERj1 and the SEC62 gene, respectively.

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REFERENCES

Alder, N. N., Shen, Y., Brodsky, J. L., Hendershot, L. M., and Johnson, A. E. (2005). The molecular mechanism underlying BiP-mediated gating of the Sec61 translocon of the endoplasmic reticulum. J. Cell Biol. 168, 389–399.

Beckmann, R., Spahn, C.M.T., Esswar, N., Helmers, J., Penczek, P., Sal, A., Frank, J., and Bölö, G. (2001). Architecture of the protein-conducting channel associated with the translating 80S ribosome. Cell 107, 361–372.

Blau, M., Mallapudi, S., Becker, T., Dudek, J., Zimmermann, R., Penczek, P., and Beckmann, R. (2005). ERj1p uses a universal ribosomal adapter site to coordinate the 80S ribosome at the membrane. Nat. Struct. Mol. Biol. 12, 1015–1016.

Davila, S., et al. (2004). Mutations in SEC63 cause autosomal dominant poly-cystic liver disease. Nat. Genet. 36, 575–577.

Deshaies, R. J., and Schekman, R. (1990). Structural and functional dissection of Sec62p, a membrane-bound component of the yeast endoplasmic reticulum protein import machinery. Mol. Cell. Biol. 10, 6624–6635.

Dierks, T., Volkmer, J., Schlenstedt, G., Jung, C., Sandholzer, U., Zachmann, K., Schlottenhose, P., Neiher, K., Schmidt, B., and Zimmermann, R. (1996). A microsomal ATP-binding protein involved in efficient protein transport into the mammalian endoplasmic reticulum. EMBO J. 15, 6931–6942.

Dudek, J., et al. (2002). A novel type of co-chaperone mediates transmembrane recruitment of DnaK-like chaperones to ribosomes. EMBO J. 21, 2958–2967.

Dudek, J., Greiner, M., Müller, A., Hendershot, L. M., Kopsch, K., Nastanczyk, W., and Zimmermann, R. (2005). ERj1p has a basic role in protein biogenesis at the endoplasmic reticulum. Nat. Struct. Mol. Biol. 12, 1038–1041.

Elschirch, S., et al. (2005). Molecular staging or survival prediction of colorectal cancer patients. J. Clin. Oncol. 23, 3526–3535.

Ferblitz, L., Maier, T., Patzelt, H., Bukau, B., Desauerl, E., and Ban, N. (2004). Structure of the trigger factor chaperone complex with the ribosome defines the molecular environment of the emerging nascent protein chain. Nature 431, 590–596.

Frank, R. (1992). Spot synthesis: an easy technology for positionally addressable parallel chemical synthesis on a membrane support. Tetrahedron 48, 9217–9232.

Goldshmidt, H., Sheiner, L., Büttiker, P., Roditi, I., Ullei, S., Günzel, M., Engstler, M., and Michaël, S. (2008). Role of protein translocation pathways across the ER in Trypanosoma brucei. J. Biol. Chem. 283, 32085–32098.

Grallath, S., Schwarz, J. P., Böttcher, U.M.K., Bracher, A., Hartl, F. U., and Siegers, K. (2006). L25 functions as a conserved ribosomal docking site shared by nascent chain-associated complex and signal recognition particle. EMBO Rep. 7, 78–84.

Halic, M., Becker, T., Pool, M. R., Spahn, C.M.T., Grassucci, R. A., Frank, J., and Beckmann, R. (2004). Structure of the signal recognition particle interacting with the elongation-arrested ribosome. Nature 427, 808–814.

Hamman, B. D., Hendershot, L. M., and Johnson, A. E. (1998). BiP maintains the permeability barrier of the ER membrane by sealing the luminal end of the translocon pore before and early in translocation. Cell 92, 747–758.

Jermy, A. J., Willer, M., Davis, E., Wilkinson, B. M., and Stirling, C. J. (2006). The Bif domain of Sec63p is required for assembly of functional ER translocons. J. Biol. Chem. 281, 7899–7906.

Jung, V., Kamradt, J., Kindich, R., Jung, M., Mueller, M., Schulz, W. A., Engers, R., Stoeckle, M., Zimmermann, R., and Wullich, B. (2006). Genomic and expression analysis of the Sec63–Sec62 amplicon reveals BLOC1/SEC62 as a probable target gene in prostate cancer. Mol. Cancer Res. 4, 169–176.

Kalies, K.-U., Allan, S., Sergeyenko, T., Kröger, H., and Römsch, K. (2005). The protein translocation channel binds proteasomes to the endoplasmic reticulum membrane. EMBO J. 24, 2284–2293.
Kroczynska, B., Evangelista, C. M., Samant, S. S., Elguindi, E. C., and Blond, S. Y. (2004). The SANT2 domain of murine tumor cell DnaJ-like protein 1 human homologue interacts with α1-antichymotrypsin and kinetically interferes with its serpin inhibitory activity. J. Biol. Chem. 279, 11432–11443.

Kroczynska, B., King-Simmons, L., Alloza, L., Alava, M. A., Elguindi, E. C., and Blond, S. Y. (2005). BIP co-chaperone MTJ1/ERDJ1 interacts with inter-α-trypsin inhibitor heavy chain 4. Biochem. Biophys. Res. Comm. 338, 1467–1477.

Meyer, H.-A., Grau, H., Kraft, R., Kostka, S., Prehn, S., Kalies, K.-U., and Hartmann, E. (2000). Mammalian Sec61 is associated with Sec62 and Sec63. J. Biol. Chem. 275, 14550–14557.

Mori, Y., et al. (2002). Instabilotyping reveals unique mutational spectra in microsatellite-unstable gastric cancers. Cancer Res. 62, 3641–3645.

Noel, P. J., and Cartwright, I. L. (1994). A Sec62p-related component of the secretory protein translocon from Drosophila displays developmentally complex behavior. EMBO J. 13, 5253–5261.

Plemper, R. K., Bohmler, S., Bordallo, J., Sommer, T., and Wolf, D. H. (1997). Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. Nature 388, 891–895.

Potter, M. D., Seiser, R. M., and Nicchitta, C. V. (2001). Ribosome exchange revisited: a mechanism for translation-coupled ribosome detachment from the ER membrane. Trends Cell Biol. 11, 112–115.

Schulman, K., et al. (2005) HNPCC-associated small bowel cancer: clinical and molecular characteristics. Gastroenterology 128, 590–599.

Snapp, E. L., Reinhart, G. A., Bogert, B. A., Lippincott-Schwartz, J., and Hegde, R. S. (2004). The organization of engaged and quiescent translocons in the endoplasmic reticulum of mammalian cells. J. Cell Biol. 164, 997–1007.

Spedding, G. (1990). Isolation and analysis of ribosomes from prokaryotes, eukaryotes, and organelles. In: Ribosomes and Protein Synthesis: A Practical Approach, ed. G. Spedding, Oxford: IRL Press, 1–29.

Tyedmers, J., Lerner, M., Bies, C., Dudek, J., Skowronek, M. H., Haas, I. G., Heim, N., Nastainczyk, W., Volkmer, J., and Zimmermann, R. (2000). Homologs of the yeast Sec complex subunits Sec62p and Sec63p are abundant proteins in dog pancreas microsomes. Proc. Natl. Acad. Sci. USA 97, 7214–7219.

Tyedmers, J., Lerner, M., Wiedmann, M., Volkmer, J., and Zimmermann, R. (2003). Polypeptide-binding proteins mediate completion of cotranslational protein translocation into the mammalian endoplasmic reticulum. EMBO Rep. 4, 505–510.

Wegrzyn, R. D., Hofmann, D., Merz, F., Nikolay, R., Rauch, T., Graf, C., and Deuering, E. (2006). A conserved motif is prerequisite for the interaction of NAC with ribosomal protein L23 and nascent chains. J. Biol. Chem. 281, 2847–2857.

Willer, M., Jermy, A. J., Young, B. P., and Stirling, C. J. (2003). Identification of novel protein-protein interactions at the cytosolic surface of the Sec63-complex in the yeast ER membrane. Yeast 20, 133–148.

Wittke, S., Dunnwald, M., and Johnson, N. (2000). Sec62p, a component of the endoplasmic reticulum translocation machinery, contains multiple binding sites for the Sec-complex. Mol. Biol. Cell 11, 3859–3871.

Woolhead, C. A., McCormick, P. J., and Johnson, A. E. (2004). Nascent membrane and secretory proteins differ in FRET-detected folding far inside the ribosome and in their exposure to ribosomal proteins. Cell 116, 729–736.

Zimmermann, R., Muller, L., and Wullich, B. (2006). Protein transport into the endoplasmic reticulum: mechanisms and pathologies. Trends Mol. Med. 12, 567–573.

Zimmermann, R., and Blatch, G. L. (2009). A novel twist to protein secretion in eukaryotes. Trends Parasitol. 25, 147–150.