Co-chaperone Specificity in Gating of the Polypeptide Conducting Channel in the Membrane of the Human Endoplasmic Reticulum*


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Capsule
Background: The molecular chaperone immunoglobulin heavy-chain-binding protein (BiP) modulates gating of the polypeptide-conducting and calcium-permeable channel (Sec61 complex) in the membrane of the endoplasmic reticulum (ER).
Results: Two co-chaperones, ERj3 and ERj6, support BiP in preventing ER calcium leakage via Sec61 complex.
Conclusions: ERj3 and ERj6 facilitate Sec61 channel closing.
Significance: Different co-chaperones assist BiP in Sec61 channel gating.

In mammalian cells, signal peptide-dependent protein transport into the endoplasmic reticulum (ER) is mediated by a dynamic polypeptide-conducting channel, the heterotrimeric Sec61 complex. Previous work has characterized Sec61 complex as a potential ER Ca^{2+} leak channel in HeLa cells and identified ER luminal molecular chaperone immunoglobulin heavy-chain-binding protein (BiP) as limiting Ca^{2+} leakage via the open Sec61 channel by facilitating channel closing. This BiP activity involves binding of BiP to the ER luminal loop 7 of Sec61α in the vicinity of tyrosine 344. Of note, the Y344H mutation destroys the BiP binding site and causes pancreatic β-cell apoptosis and diabetes in mice. Here, we systematically depleted HeLa cells of the BiP co-chaperones by siRNA-mediated gene silencing and used live cell Ca^{2+} imaging to monitor the effects on ER Ca^{2+} leakage. Depletion of either one of the ER luminal BiP co-chaperones, ERj3 and ERj6, but not the ER membrane resident co-chaperones (such as Sec63 protein, which assists BiP in Sec61 channel opening) led to increased Ca^{2+} leakage via Sec6 complex, thereby phenocopying the effect of BiP depletion. Thus, BiP facilitates Sec61 channel closure, that is limits ER Ca^{2+} leakage via the Sec61 channel with the help of ERj3 and ERj6. Interestingly, deletion of ERj6 causes pancreatic β-cell failure and diabetes in mice and humans. We suggest that co-chaperone controlled gating of the Sec61 channel by BiP is particularly important for cells, which are highly active in protein secretion, and that break down of this regulatory mechanism can cause apoptosis and disease.
Therefore, gating of the Sec61 channel has to be tightly regulated by its allosteric effectors, ER luminal immunoglobulin heavy-chain-binding protein (BiP) and cytosolic Ca\(^{2+}\)-calmodulin (CaM). In addition, the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) counteracts Ca\(^{2+}\) leakage from the ER (15).

The Hsp70-type molecular chaperone BiP plays a role in Sec61 channel closure (8, 9, 16, 17) (Fig. 1). By combining siRNA mediated gene silencing and live cell Ca\(^{2+}\) imaging, it was observed in HeLa cells that reduced levels of BiP lead to increased Ca\(^{2+}\) leakage from the ER via the Sec61 complex. This increased leakage was seen in imaging of both cytosolic as well as ER luminal Ca\(^{2+}\) when cells were challenged with thapsigargin the presence of extracellular EGTA (16). Furthermore, we found that BiP binds to loop 7 of Sec61\(\alpha\) in order to facilitate Sec61 channel closure. BiP’s action was highly specific, i.e. it could not be compensated by other abundant ER luminal chaperones (PDI, Grp94, or calreticulin) or even its yeast ortholog, Kar2p (16). Strikingly, mutant variant BiPR197E, which is deficient in its ability to cooperate with Hsp40-type co-chaperones, was also unable to mediate Sec61 channel closure, pointing towards the involvement of a Hsp40-type co-chaperone in Sec61 channel closure (16). Furthermore, we showed with a similar experimental approach that Ca\(^{2+}\)-CaM can bind to an IQ motif present in the cytosolic amino terminus of the \(\alpha\)-subunit of the heterotrimeric Sec61 complex and, with the help of ER membrane protein Sec62, limit Ca\(^{2+}\) leakage from the ER after Ca\(^{2+}\) has started to leak out (13, 18).

Of note, a mouse model for diabetes has indicated that a single amino acid exchange in the ER luminal loop 7 of murine Sec61\(\alpha\) leads to a partially deficient Sec61 complex and to β-cell death as well as diabetes (19). When wildtype Sec61\(\alpha\) was replaced with the corresponding mutant Sec61\(\alpha\)Y344H in human cells, Ca\(^{2+}\) leakage from the ER was increased and was no longer affected by manipulation of the BiP concentration (16). Therefore, it was suggested that failure of BiP to facilitate Sec61 channel closure in the homozygous \(SEC61A1Y344H\) mouse contributes to apoptosis of cells with high secretory activity, such as pancreatic β-cells. It is interesting to mention that various other mutations and knock-outs of resident ER proteins can cause diabetes in mice, such as deletion of the Hsp40-type co-chaperones of BiP ERj4 and ERj6 (20, 21), or of the BiP-interacting protein PKR-like kinase (PERK) (22). However, diabetes can also be caused in man and mice by mutations in genes coding for non-ER proteins, such as the insulin gene.

We asked if the Hsp40-type co-chaperones of BiP that are involved in Sec61 channel closure can be identified and whether the mechanism of Sec61 channel closure by BiP can be further elucidated. To the latter, we addressed the questions if artificial ligands of Sec61\(\alpha\) loop 7, i.e. specific antibodies or Fab fragments thereof, can substitute for BiP in mediating Sec61 channel closure in single channel measurements. We identified ERj3 (also termed DnaJB11) and ERj6 (also termed DnaJC3, p58\(^{IPK}\)) as the BiP co-chaperones that are involved in Sec61 channel closure in HeLa cells. Furthermore, we observed that Fabs, directed against Sec61\(\alpha\) loop 7, can indeed trigger Sec61 channel closing in single channel recordings. In various tumor cells, inefficient Sec61 channel closure after \(SEC62\) gene silencing or CaM inhibition is associated with reduced migratory potential (18, 23). In our current study, we observed that ERj3 and ERj6 silencing causes the similar phenomenon, confirming their significance in the regulation of the Sec61 channel.

**EXPERIMENTAL PROCEDURES**

*Materials*-Enhanced chemiluminescence (ECL\(^\text{TM}\)), ECL\(^\text{TM}\) Plex goat-anti-rabbit IgG-Cy5 and ECL\(^\text{TM}\) Plex goat-anti-mouse IgG-Cy3 conjugate were purchased from GE Healthcare. Thapsigargin, ionomycin, and Fura-2 AM were from Invitrogen/Molecular probes. The nucleic acid gel stain GelRED was from GeneON. Antibodies against ERj5 were from Abnova, against ERj6 from Cell Signaling, and against β-actin from Sigma. Antibodies against ERj4 were a kind gift from Linda Hendershot (Memphis, USA). Antibodies against BiP and GFP for immunoprecipitation were from Santa Cruz and against hexahistidine from Cell Signaling.
Rabbit antibodies were raised against the carboxy-terminal peptide (14-mer) or loop 7 (12-mer) of human Sec61α, the carboxy-terminal peptides of ERj3 (14-mer), Sec62 (11-mer), or Sec63 (13-mer), and the amino-terminal peptides of human BiP (12-mer) or Grp170 (11-mer) plus an amino- or carboxy-terminal cysteine, and against ERj1C-ΔN21 (24), respectively. Where indicated, antibodies were affinity purified on Sulfolink-immobilized peptides (Thermo/Fisher Scientific). Fabs were produced from affinity purified antibodies with the Pierce Fab Preparation Kit (Thermo/Fisher Scientific) according to the manufacturer’s protocol. GST-tagged ERj proteins and His-tagged BiP from hamster were purified as previously described (25-28). Fabs were analyzed by reductive or non-reductive (+/-β-mercapto-ethanol in the sample buffer) SDS-PAGE.

Cell culture-HeLa cells (ATCC no. CCL-2) were cultivated at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) with 10% foetal bovine serum (FBS) (Biochrom) and 1% penicillin/streptomycin (PAA) in a humidified environment with 5% CO₂. For live cell calcium imaging, cells were grown on 25-mm glass cover slips pre-treated with poly-L-lysine (1 mg/ml) for 1 h. Cell growth was monitored using the Countess® Automated Cell Counter (Invitrogen) according to the manufacturer’s instructions.

A HeLa cell line that stably expresses the FRET-based calcium sensor D1ER (HeLa-D1ER) was generated to measure the luminal calcium concentration in ER. D1ER was kindly provided by R. Y. Tsien (29). HeLa-D1ER cells were maintained in culture under selection with G418 (Minimal Essential Medium, MEM (Gibco), 10% FBS, 0.5 mg/ml G418) and transferred to poly-L-lysine coated glass cover slips previous to silencing and live cell calcium imaging experiments, as described below for naïve HeLa cells.

Silencing of gene expression by siRNA- For gene silencing, 5.2 x 10⁵ HeLa cells were seeded per 6 cm culture plate in normal culture. The cells were transfected with targeting siRNA (Table 1) or control siRNA (AllStars Negative Control siRNA, Qiagen) using HiPerFect Reagent (Qiagen) according to the manufacturer’s instructions (typical final concentration of siRNAs: 20 nM). After 24 h, the medium was changed and the cells were transfected a second time. SEC61A1 silencing was carried out as described previously (30). Silencing efficiencies were evaluated by western blot analysis using the respective antibodies and an anti-β-actin-antibody from mouse. The primary antibodies were visualized using ECL™ PLEX goat-anti-rabbit IgG-Cy5- or ECL™ PLEX goat-anti-mouse IgG-Cy3 conjugate and the Typhoon-Trio imaging system in combination with the Image Quant TL software 7.0 (GE Healthcare). Alternatively, signals were detected using a secondary peroxidase (POD)-coupled anti-rabbit antibody (Sigma) and ECL™, visualized with a Fusion SL (peqlab) luminescence imaging system.

Complementation analysis-To rescue the phenotype of ERJ3 silencing, the ERJ3 cDNA was inserted into the multi-cloning sites of a pCDNA3-IREs-GFP-vector. Cells were treated with ERJ3-UTR siRNA as described above for 48 h. Subsequently, control siRNA and ERJ3 siRNA treated cells were transfected with either vector or ERJ3 expression plasmid using Fugene HD (Promega) according to the manufacturer’s instructions.

Real-time cell proliferation analysis-The xCELLigence SP system (Roche) was used for the real-time analysis of cell proliferation. In this system, 5.0 x 10³ HeLa cells treated with control siRNA or siRNAs against ERJ3 and ERJ6, respectively, for 48 h were seeded into a 96-well e-plate (Roche) according to the manufacturer’s instructions. Cell proliferation was monitored for 48 h and the data evaluated with RTCA 2.0 software (Roche).

Quantitative real-time PCR analysis-Cells were harvested and total RNA was isolated by using the RNA Blood Kit (Qiagen). Reverse transcription was performed with Superscript II RT (Invitrogen) and oligo-dT primers (Eurofins Genomics) and the cDNA was purified using the PCR Purification Kit (Qiagen). TaqMan® Gene Expression Assays (Applied Biosystems) were used for quantitative real-time PCR of SEC61A1 (Hs00273698_m1), ERJ3 (Hs00212527_m1), ERJ4 (Hs00202448_m1), ERJ6 (Hs00534483_m1), CHOP (Hs99999174_m1) and BIP (Hs99999174_m1) in StepOne Plus 96-well system (Applied Biosystems). Δct-values
were calculated using *ACTB* (Hs00357333_m1) as a standard and the values were then normalized to control siRNA treated cells.

Alternatively, *XBP1* was amplified in the same cDNA with *Pfu* polymerase (Thermo/Fisher Scientific) and established primers (CACCTGAGCCCGAGGAG and TTAGTTCAATGACTCCAGC) (31). The PCR products were subjected to gel electrophoresis in the presence of GelRED and visualized with GelDoc XR imaging system (Biorad).

3D structured illumination microscopy-Cells were seeded on glass cover slips and treated as indicated. After 96 h the glass slides were removed and washed twice with cold PBS. Cells were fixed with 4% Paraformaldehyde for 20 min at 4 °C. Fixed cells were permeabilized and blocked with PSS (PBS + 0.1% Saponin + 10% FCS) for 1 h at room temperature. To improve the antigen accessibility RNase A (Roche) was added to a final concentration of 50 µg/ml. After washing with PSS, indirect immunofluorescence staining with an affinity purified rabbit antipeptide antibody directed against the COOH terminal undecapeptide of human Sec62 protein (plus an aminoterminal cysteine) and Alexa Fluor594-coupled secondary antibody from goat (Invitrogen) was performed. We note that the anti-Sec62 antibody is specific for Sec62 under denaturing as well as native conditions (i.e. Western blot and fluorescence microscopy-signals were quenched after silencing of the *SEC62* gene) (22). Cells were analyzed by microscopy on an Elyra SIM PS1 (Carl Zeiss-MicroImaging). The microscope was equipped with a Plan-Apochromat Oil DIC lense with 63x magnification and 1.4 numerical aperture (Carl Zeiss) and an iXonEM+885 EMCCD camera (Andor Technology). Mounting medium was Roti®-Mount FluorCare DAPI (Carl Roth), the oil was Immersol 518F (Carl Zeiss).

**Protein transport into semi-permeabilized cells**-Precursor polypeptides were synthetized in reticulocyte lysate in the presence of [35S]methionine for 16 min at 30 °C. After 5 min of incubation with RNase A (final concentration: 80 µg/ml) and cycloheximide (final concentration: 100 µg/ml) at 30 °C, buffer or semi-permeabilized cells, resulting in a final concentration of 12,800 cell equivalents/µl, were added and the incubation was continued for 30 min (post-translational transport experiment). The cells had previously been treated with targeting or control siRNA for 96 h. Semi-permeabilized-cells were prepared by digitonin treatment from identical cell numbers according to published procedure (32, 33). Their concentrations were adjusted according to OD280 in 2% SDS and, eventually, confirmed by SDS-PAGE and protein staining. All samples were analyzed by SDS-PAGE and phosphorimaging (Typhoon-Trio imaging system). Image Quant TL software 7.0 was used for quantifications. Silencing efficiency was evaluated by western blot.

**Live cell calcium imaging**-Live cell calcium imaging for cytosolic Ca2+ was carried out as previously described (14, 30, 34, 35). HeLa cells were loaded with 4 µM Fura-2 AM in DMEM for 45 min at 25 °C. Cells were washed twice and incubated in a calcium-free buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 0.5 mM EGTA, 10 mM glucose in 10 mM HEPES-KOH, pH 7.35) at room temperature. During the experiment, cells were treated with thapsigargin (1 µM) and ratiometric measurements were carried out for 7.5 or 12.5 min. Where indicated, HeLa cells were treated with siRNA for 96 h prior to calcium imaging. Data were collected on an iMIC microscope and the polychromator V (Till Photonics) by alternate excitation at 340 nm and 380 nm and measurement of the emitted fluorescence at 510 nm. The microscope was equipped with a Fluor M27 lense with 20x magnification and 0.75 numerical aperture (Carl Zeiss) and an iXonEM+ camera (Andor Technology). Images containing 50-55 cells/frame were sampled every 3 s using TILLvisION software (Till Photonics). Fura-2 signals were recorded as the ratios F340/F380.
where F340 and F380 correspond to the background-subtracted fluorescence intensity at 340 and 380 nm, respectively. Cytosolic $[\text{Ca}^{2+}]$ was estimated from ratio measurements using an established calibration method (36). Data were analyzed using Excel 2007. $P$ values were determined by unpaired $t$ tests. Alternatively, HeLa cells were loaded with 4 µM Fura-2 AM and washed and incubated in the calcium-free buffer. After ratiometric measurements were carried out for 1 min, ionomycin (5 µM) was added and the measurements continued. Data were collected on the iMIC microscope.

The luminal calcium concentration in ER ([Ca$^{2+}$]$_{\text{ER}}$) was analysed using the FRET-based calcium sensor D1ER, which comprises two fluorescent proteins, CFP and citrine, and two sensing proteins, calmodulin and a calmodulin-binding peptide derived from skeletal muscle myosin light chain kinase (29). D1ER was stably expressed in the HeLa-D1ER cell line (see Cell culture). The experiments were carried out with the iMIC microscope, the polychromator V and the Live Acquisition Software (Till Photonics). HeLa-D1ER cells were exposed to 433 nm and the emitted fluorescence was split at 469/23 nm and 536/27 nm to obtain the CFP and citrine components, respectively. The cell fluorescence was additionally passed through a dichrotome and projected on the chip of the microscope camera to obtain simultaneous CFP and citrine images. Image pairs containing 10-15 cells/frame were obtained at 20x magnification every 10 sec. The FRET ratios were calculated from background-subtracted CFP and citrine image pairs as $F536/F469$, where $F536$ and $F469$ represent the citrine and CFP fluorescence intensities, respectively (29). In order to measure $[\text{Ca}^{2+}]_{\text{ER}}$ and $[\text{Ca}^{2+}]_{\text{cytosol}}$ simultaneously in the same cell, HeLa-D1ER cells were loaded with Fura-2 AM and incubated in a calcium-free buffer as described for naïve HeLa cells. Fura-2 signals were recorded and analysed as described for naïve HeLa cells. To allow the recording of D1ER and Fura-2 signals in the same cells, the filtersets were automatically exchanged in a way that two pairs of images were obtained every 10 s. The ratios $F536/F469$ and $F340/F380$, which reflect $[\text{Ca}^{2+}]_{\text{ER}}$ and $[\text{Ca}^{2+}]_{\text{cytosol}}$, respectively, were measured in each image pair.

Surface plasmon resonance spectroscopy-Surface plasmon resonance (SPR) spectroscopy was performed in a BIAlite upgrade system (BIACORE) according to our established procedure (25-28). Monoclonal goat anti-GST antibodies were immobilized on a sensor chip CM5 (BIACORE). GST hybrid proteins were bound to the immobilized antibodies in the measuring cell; GST was bound to the antibodies in the reference cell. For interaction analysis, the chip was equilibrated with running buffer (150 mM NaCl, 3 mM KCl, 1 mM MgCl$_2$, 0.1% Tween 20, 1 mM ATP in sodium phosphate pH 7.4) at a flow rate of 5 µl/min. Subsequently, solutions containing increasing concentrations of BiP were passed over the chip surface. Each 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 2.2.4 (BIACORE).

Peptides corresponding to the peptides used for generation of the loop 7 (339-353) antibodies were synthesized with biotinylated dipeptide KG at the carboxy-terminus and immobilized in the measuring cell of Biacore avidin sensor chip SA. The reference cell was loaded with carboxy-terminally biotinylated Sec61α peptide (325-339 with biotinylated dipeptide KG). The running buffer was 10 mM HEPES/KOH (pH 7.4), 150 mM NaCl, 6.4 mM KCl, 2 mM MgCl$_2$, 0.005% Surfactant P2. Antibodies were injected at a flow rate of 10 µl/min at 1 µM IgG or 500 nM Fab in runnig buffer, respectively. The bound antibodies were removed by injecting a pulse of 100 mM glycine/HCl (pH 2.8). The analysis was carried out on the BIAlite upgrade system, employing the BIA evaluation software version 3.1 (BIACORE).

Immunoprecipitation-For co-immunoprecipitation, HeLa cells were transfected with an expression plasmid (pCAGGS2-IRES-GFP) coding for BIP-His6 using Lipofectamine® (Invitrogen) according to the manufacturer's instructions. Two days after transfection, cells were washed twice with PBS (Gibco) and lysed in ice-cold CellLytic-M lysis buffer with protease inhibitor cocktail (Sigma) (37). After centrifugation at 16,000 x g and 4 °C
for 10 min, supernatant was collected and precleared for 1 h with protein G beads (GE Healthcare). Subsequently, aliquots were incubated with two negative control antibodies (non-immune murine immunoglobulins and anti-GFP) or anti-hexahistidine- and anti-BiP antibodies, at 4 °C overnight, respectively. Precipitation of immune-complexes was performed by addition of protein G beads for 8 h at 4 °C. The beads were washed three times with NP-40 buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 1% Nonidet-P40) and subjected to SDS-PAGE and western blotting. For detection, Sec61α and BiP rabbit polyclonal antibodies were used. The primary antibodies were visualized using secondary peroxidase (POD)-coupled anti-rabbit antibody (Sigma) and ECL.

For immunoprecipitation, canine pancreatic microsomes were stripped of ribosomes with puromycin and high salt and solubilized in 50 mM HEPES/KOH pH 7.8, 500 mM KAc, 15% glycerol, 2.5% digitonin for 10 min on ice as described (25). Supernatant was collected after centrifugation at 16,000 x g and 4 °C for 10 min. Antibodies were coupled according to the manufacturers protocol to Dynabeads-Protein A (DYNAL BIOTECH) and the loaded beads incubated with the solubilized PKRM overnight at 4 °C with slow shaking. After removing the supernatant, the beads were washed three times with 1 ml of NP-40 buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 1% Nonidet-P40) and, subsequently, incubated with 100 mM glycine/HCl (pH 2.8) for 30 min at room temperature. Supernatant was collected, neutralized, SDS-sample buffer was added and the sample was incubated for 10 min at 56°C. The samples were analyzed by SDS-PAGE and western blotting. For Sec61-detection, the Sec61α-subunit specific rabbit polyclonal antibody was used as well as a secondary peroxidase (POD)-coupled anti-rabbit antibody (Sigma) and ECL™, visualized with a Fusion SL (peqlab) luminescence imaging system.

Single channel recordings-Vesicles for planar bilayer experiments were prepared by mixing (3:2, v/v) the Sec61-containing vesicles with preformed liposomes (egg L-α phosphatidylcholine, 10 mg/ml) in 50 mM KCl and 10 mM Mops/Tris (pH 7.0) (9). Mega-9 (nonanoyl-N-methylglucamide) was added to a final concentration of 80 mM. After mixing, the sample was dialysed for 4 h at room temperature and then overnight at 4°C against a buffer containing 50 mM KCl and 10 mM Mops/Tris (pH 7.0). Aliquots (10 µl, typically 10 mg/ml protein, lipid/protein 2:1 [w/w]) of the proteoliposomes derived from RM vesicles were incubated with 200 µM puromycin and 250-500 mM KCl for 15-30 min on ice. Planar lipid bilayers were produced by the painting technique described previously (38). An osmotic gradient was used for vesicle fusion. Membrane potentials refer to the trans compartment. Data recording and analysis was performed as previously described (9). Voltage ramps were conducted at a rate of 6.6 mV/s.

Cell migration analysis-Migration was tested with the BD Falcon FluoroBlok system (Becton Dickinson) in 24-well inserts (22). A total of 2.5 x 10⁴ cells treated with control siRNA or siRNAs against ERJ3 and ERj6, respectively, for 48 h were loaded into this system in DMEM-medium containing 1.0% FBS. The inserts were placed in DMEM-medium with 10% FBS as an attractant. After 48 h the cells were fixed with methanol and stained with DAPI and migrating cells were analyzed at room temperature on the backside of the membrane by fluorescence microscopy on a TE2000-S inverted microscope (Nikon). The microscope was equipped with a Plan Fluor lens with 10x magnification and 0.3 numerical aperture (Nikon) and a Digital Sight DS-5Mc camera (Nikon). Migrated cells of at least three individual images were automatically counted using NIS-Elements AR Software (Nikon).

Peptide-spot assay for antibody characterization-Peptides (12 amino acid residues with a overlap of 10 residues) covering the whole lenght of Sec61α were synthesized on acid hardened cellulose membranes, derivatized with a polyethylene glycol spacer, as described (39). Membranes were equilibrated in 150 mM NaCl, 50 mM Tris/HCl (pH 7.5) for 30 min at room temperature. The diluted antibody (1:1000) in PBS (5% milk powder) was added and the membrane incubated at 4°C overnight. Thereafter, the membrane was washed twice with PBS buffer for 10 min, incubated with peroxidase (POD)-coupled anti-rabbit antibody (Sigma), washed twice with PBS, incubated with
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ECL and analyzed using a LumiImager F1 (Roche). Stripping of the bound antibodies was performed by incubating the membranes with 100 mM glycine/HCl (pH 2.8) for 30 min at room temperature and repeated washing with PBS.

RESULTS

Silencing of ERJ3 and ERJ6 stimulate ER calcium leakage in intact cells - Based on the previous in vitro and cellular level experiments, we expected Hsp40-type co-chaperones of BiP to contribute to the limiting of Sec61-mediated Ca\textsuperscript{2+} efflux from the ER (16, 17). Seven such co-chaperones are currently known in the mammalian ER and were termed ERdj- or ERj-proteins 1 through 7 (40, 41) (Fig. 1, Table 2). Since the ER membrane protein Sec63 acts as BiP co-chaperone in facilitating protein transport into the mammalian ER (33), we first investigated whether silencing the SEC63 gene in HeLa cells with an established siRNA enhanced Ca\textsuperscript{2+} efflux from the ER. The same siRNA was previously shown to result in precursor polypeptide-specific inhibition of protein transport into the ER of HeLa cells (33). Using the Ca\textsuperscript{2+} dye Fura-2 in the absence of extracellular Ca\textsuperscript{2+} allows visualization of Ca\textsuperscript{2+} leakage from the ER indicated by the increased cytosolic calcium concentration in intact cells in response to the irreversible SERCA inhibitor thapsigargin. In Ca\textsuperscript{2+} imaging experiments, HeLa cells were treated with SEC63 siRNA for 96 h and, subsequently, Ca\textsuperscript{2+} leakage was unmasked by application of thapsigargin in the presence of extracellular EGTA. Cells treated with a non-targeting siRNA were subjected to the same procedure and served as negative control. In comparison to the control siRNA, the SEC63 siRNA had no effect at all on the thapsigargin-induced Ca\textsuperscript{2+} efflux, with a silencing rate of about 80% (data not shown). This suggests that Sec63 does not contribute to the limiting of Ca\textsuperscript{2+} leakage from the ER in human cells. Since the Sec63-related ER membrane resident Hsp40, ERJ1, was previously found to be unable to support BiP in Sec61 channel closure (14), we asked in the next experiment whether simultaneous depletion of all three known ER membrane resident Hsp40s with a luminal J-domain (ERJ1, ERJ2/Sec63, ERJ7) enhanced Ca\textsuperscript{2+} efflux from the ER (25, 28, 42, 43). Still no effect was observed, although the silencing rate was above 75% (data not shown). Thus, the membrane resident BiP co-chaperones do not apparently contribute to the limiting of Ca\textsuperscript{2+} leakage from the ER in human cells.

Next, we investigated whether knock down of the ER lumenal Hsp40s (ERJ3, ERJ4, ERJ5, ERJ6) enhances Ca\textsuperscript{2+} efflux from the ER in HeLa cells (44-49). Since ERJ3 and ERJ6 were found to associate with the Sec61 complex (50, 51), we first treated HeLa cells with two different ERJ3 or ERJ6 siRNAs and carried out Ca\textsuperscript{2+} imaging as described above. In contrast to the control siRNA, the ERJ3 and ERJ6 siRNAs significantly enhanced the thapsigargin-induced Ca\textsuperscript{2+} efflux (Fig. 2, A-D). In these experiments, the silencing rate was above 80% (Fig. 2E). Thus, the ER lumenal BiP co-chaperones, ERJ3 and ERJ6, contribute to the limiting of Ca\textsuperscript{2+} leakage from the ER in human cells. We suggest that in the case of ERJ3 knock down with two different siRNAs, which resulted in similar silencing efficiencies but quantitatively different effects in Ca\textsuperscript{2+} efflux from the ER, the differences may have been due to different silencing rates.

Control experiments, using ionomycin instead of thapsigargin, confirmed that the enhanced ER Ca\textsuperscript{2+} leakage in ERJ3 or ERJ6 depleted cells was not due to elevated cellular Ca\textsuperscript{2+} concentration in these cells (Fig. 2F) and gross alterations in cell- or ER-morphology (3D SIM-data not shown). Additional control experiments demonstrated the integrity of the depleted cells in the course of the experiments: ERJ3 and ERJ6 depletion i) hardly affected cell growth (Fig. 3A and legend to Fig. 2), ii) did not affect ER import of three model precursor polypeptides (preprolactin, proproapelin, prestatherin; data not shown), iii) did not lead to activation of the unfolded protein response, it is BIP, ERJ4, and CHOP mRNAs were not overproduced and XBP1 mRNA was not spliced (Figs 3B and 3C), and iv) did not result in global effects on the ER proteome (Figs 3, D and E). However, ERJ6 silencing reproducibly led to an increased ERJ3 protein content (Figs 2E and 3E). This raised the question why increased ERJ3 levels did not compensate for the decreased ERJ6 levels in limiting Ca\textsuperscript{2+} leakage from the
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ER. We propose that the two co-chaperones may act in a complex and will pursue this idea in future experiments.

We wondered whether simultaneous depletion of ERj3 and ERj6 has a more pronounced effect on Ca\(^{2+}\) efflux from the ER, as compared to the separate knock downs, and found this to be the case (Fig. 4, A-C). We did not only analyze Ca\(^{2+}\) efflux from the ER in response to ERj3 plus ERj6 knock down indirectly, that is as increased Ca\(^{2+}\) concentration in the cytosol, but also did so directly by simultaneous monitoring ER luminal Ca\(^{2+}\) concentration. HeLa cells were stably transfected with a plasmid, which codes for the ER targeted Ca\(^{2+}\) sensor protein D1ER. ERj3 and ERj6 were simultaneously depleted for 96 h and the cells were loaded with Fura-2 as before. Subsequently, Ca\(^{2+}\) efflux was unmasked by thapsigargin and the Ca\(^{2+}\) concentrations in cytosol and ER lumen were monitored by Fura-2- and FRET-imaging, respectively (Fig. 5). In contrast to the control siRNA, the combination of ERJ3 and ERJ6 siRNAs simultaneously enhanced the thapsigargin-induced Ca\(^{2+}\) decrease in the ER lumen and increase in the cytosol. Thus there was a correlation between increasing Ca\(^{2+}\) concentration in the cytosol and decreasing Ca\(^{2+}\) concentration in the ER lumen in response to ERj3 plus ERj6 depletion, that is Ca\(^{2+}\) is indeed leaking from the ER into the cytosol after ERj3 and ERj6 depletion. A similar additive effect as after simultaneous depletion of ERj3 and ERj6 was observed when ERJ3 or ERJ6 siRNA was combined with BIP siRNA (Fig. 4, D-F). We note that in these latter experiment silencing was carried out for only 48 h, which explains why the separate Hsp40 knock downs had hardly any effects. These results were consistent with the notion that both ERj3 and ERj6 contribute to BiP mediated Sec61 channel closure in human cells.

To confirm these conclusions, we attempted complementation by the ERJ3 cDNA lacking the ERJ3-UTR in the presence of the ERJ3-UTR siRNA and observed rescue of the ERJ3 silencing phenotype in form of restoration of the basal ER Ca\(^{2+}\) efflux seen in control cells (Fig. 6, A-C). Of note, transfection with a negative-control plasmid had no effect.

To address the question if the observed effects of ERJ3 and ERJ6 on cellular Ca\(^{2+}\) homeostasis is specific or a general phenomenon of ER luminal BiP co-chaperones, we investigated whether silencing the ERJ5 gene in HeLa cells with validated siRNAs enhanced Ca\(^{2+}\) efflux from the ER. In contrast to the ERJ3 and ERJ6 depletion, the depletion of ERJ5 did not have a stimulatory effect on Ca\(^{2+}\) efflux (Fig. 6, D-F), under conditions of an average silencing rate of 80%. Similar results were obtained with two established siRNAs against ERJ5 (data not shown), which caused an ER protein export defect (52). Thus, the observed co-chaperoning effect is specific to ERJ3 and ERJ6. We note that ERJ4 was omitted from this analysis since it is typically detectable only after induction of the unfolded protein response (40, 41, 45).

Next, we asked whether the effects of ERJ3 and ERJ6 on ER Ca\(^{2+}\) leakage could be linked to the Ca\(^{2+}\) permeable Sec61 complex. To address this question, we treated HeLa cells for 96 h with siRNA directed against ERJ3 or ERJ6 plus either SEC61A1 siRNA or a negative-control siRNA. Ca\(^{2+}\) imaging experiments showed that additional silencing of SEC61A1, but not the control silencing, in the presence of ERJ3 or ERJ6 siRNA prevented the ERJ silencing-induced Ca\(^{2+}\) efflux (Fig. 7, A-D). According to western blot analysis, the silencing efficiency of all three siRNAs was above 80% (Fig. 7E). Thus, ERJ3 and ERJ6 contribute to reducing Ca\(^{2+}\) leakage from the ER at the level of the Sec61 complex. We note, that SEC61A1 silencing led to depletion of ERJ3 and ERJ6 in these experiments, which must be due to their Sec61 dependent ER import.

In the next experiments we characterized the interaction between BiP and its Hsp40-type co-chaperones by surface plasmon resonance spectroscopy. GST fusion proteins of ER resident Hsp40s, ERj3 and ERj6, were purified and functionally characterized by surface plasmon resonance spectroscopy. As expected, both Hsp40s were able to productively interact with BiP, albeit with different affinities (Fig. 8, Table 2). The affinity of ERJ3 for hamster BiP was calculated, as based on Fig. 8, as 0.17 µM (Table 2). The affinity of ERJ6 had not previously been determined and was calculated as 5 µM (Table 2).
BiP and antibodies directed against the BiP binding site can bind to the Sec61α subunit that is present in the native Sec61 complex—Our previous analysis demonstrated the interaction of BiP with loop 7 of Sec61α at the peptide level and in silico (16). Here, we addressed the question if loop 7 of Sec61α is available to BiP binding in the native Sec61 complex, using various experimental strategies. First, we asked if Sec61α can be co-immunoprecipitated together with BiP from HeLa cell extract under established immunoprecipitation conditions (37). HeLa cells were transfected with a plasmid, coding for BiP with a hexahistidine tag. Two days after transfection, the cells were lysed and, after a pre-clearing step with protein G beads, aliquots were incubated with two negative control antibodies (non-immune murine immunoglobulins or anti-GFP) and anti-hexahistidine- and anti-BiP antibodies, respectively. Subsequently the immunocomplexes were harvested with protein G beads and, after three washing steps, subjected to SDS-PAGE and western blotting. The chemiluminescent signals were detected (Fig. 9A). Both anti-hexahistidine- and anti-BiP antibodies precipitated BiP and co-immunoprecipitated Sec61α, while the negative control antibodies failed to do so. Thus, BiP and Sec61 complex are associated with each other under normal growth conditions, as had been observed previously (53).

Next we asked if antibodies and Fab fragments directed against the di-tyrosine containing motif within loop 7, which was characterized as BiP interaction site (16), can access their epitope within native Sec61 complex. An antipeptide antibody was raised against the oligopeptide GGLCYYLSPPESC, corresponding to amino acid residues 339 through 350 of human Sec61α plus a carboxy-terminal cysteine residue, and affinity purified. The purified antibodies were characterized as monospecific and of high affinity by western blot, peptide library- and surface plasmon resonance experiments (Fig. 10A, B, and D). These antibodies were able to immunoprecipitate native Sec61 complex from canine pancreatic microsomal extracts (Fig. 9B), albeit less efficiently as compared to Sec61β-antibodies (25). Thus, the tyrosine 344 containing motif within loop 7 of Sec61α is available to antibody binding in the native Sec61 complex.

As we previously have observed Sec61 channel activity and its inhibition by BiP in single channel recordings from reconstituted canine pancreatic microsomes (9), we also addressed the question if anti-loop 7-Fabs can access their epitope in the membrane integrated Sec61 complex and whether or not their binding affects channel gating. Fab fragments were purified and characterized by surface plasmon resonance spectroscopy (Fig. 10, C and D). In contrast to an affinity purified anti-Sec61β antibody, binding of the anti-loop 7-Fabs to the Sec61 complex induced channel closure (Fig. 9, C-F): Specifically, the slopes of the current voltage relations in C and D are nearly identical, (C) slope=1.29 nS and (D) slope= 1.22 nS. The two single current voltage sweep measurements were from the same bilayer containing two channels, which did not change their open state during the voltage sweep between $V_m=-50$ to $V_m=+50$ mV. Closure of Sec61 channels was mainly but not exclusively observed at $|V_m|>50$ mV. The conductance histograms in Figs E and F are averages from three different single channel recordings during a 1 min voltage gate of $V_m=40$ mV. The data indicate, that the anti-Loop7 Fab shifted the population of the Sec61 open channel states towards the closed state. Thus, these results confirmed that loop 7 is accessible to soluble proteins in native Sec61 complexes and suggested that binding of even an artificial ligand to loop 7 is sufficient to induce Sec61 gating. We note that Fabs against the cytosolic ribosome binding site in Thermus thermophilus SecYE were able to induce partial channel opening in this ortholog of the Sec61 complex (54).

ERJ3 and ERJ6 silencing phenocopies the effects of SEC62 silencing and calmodulin inactivation in inhibition of migration of human cells—We sought to independently, albeit indirectly, confirm the notion that depletion of ERJ3 or ERJ6 from human cells leads to Ca$^{2+}$ leakage from the ER and, therefore, a rise in the cytosolic Ca$^{2+}$ concentration. In previous experiments we observed that Ca$^{2+}$ leakage from
the ER can be induced in various human tumor cells by depletion of the ER membrane protein Sec62 as well as by inhibition of CaM by CaM-antagonists, such as Ophiobolin A and Trifluoperazin (TFP) (18, 23). Both treatments resulted in a rise in the cytosolic Ca\(^{2+}\) concentration and a reduction of the cell's ability to migrate in an established transwell migration assay (18, 23, 55). Therefore, we tested if silencing of the ERJ3 or ERJ6 gene has a similar effect and found this to be the case, it is migration was down to 22 ± 4%, 12 ± 3%, 5 ± 1%, and 14 ± 2% after knock down of ERJ3 and ERJ6, respectively, with two siRNAs each as compared to control siRNA treated cells (data not shown). We note that silencing of both genes hardly affected cell growth (Fig. 3A) and that silencing of the ERJ1 or SEC63 gene did not have any effect on cell migration (23). Thus, the independent approach confirmed our interpretation that ERJ3 and ERJ6 are involved in controlling Ca\(^{2+}\) leakage from the ER.

**DISCUSSION**

**BiP co-chaperones ERJ3 and ERJ6 in BiP-mediated gating of the Sec61 channel**

Previous work from a number of laboratories characterized the ER membrane resident Sec61 complex, which facilitates transport of presecretory proteins into the ER and insertion of plasma membrane proteins into the ER membrane (10, 11), as a major ER Ca\(^{2+}\) leak channel (12-16, 56-59). Furthermore, it was observed in vitro as well as in intact cells that the ER lumenal Hsp70-type molecular chaperone, BiP, is involved in limiting ER Ca\(^{2+}\) leakage at the level of the Sec61 complex (8, 9, 16, 17, 53, 60) (Fig. 1). Taking advantage of mutant BiP variants, such as BiPR197E or BiPR197H, which cannot productively interact with Hsp40-type co-chaperones, it was concluded that the action of BiP in Sec61 channel closure involves a Hsp40-type co-chaperone (16, 17, 61). Here, we observed that the ER luminal Hsp40s, ERJ3 (44, 62, 63) and ERJ6 (48, 49), act as BiP co-chaperones in Sec61 channel closure and that the ER membrane resident Hsp40s, ERJ1, ERJ2/SEC63, and ERJ7, do not play a role. This finding is consistent with the previous reports that ERJ3 and ERJ6 are in association with the Sec61 complex (50, 51) and that loss of ERJ6 function in mice and humans causes pancreatic β-cell failure and diabetes (20, 64), which was also observed with the murine Sec61αY344H mutation that destroys the BiP binding site (16, 19). We note that ERJ2/Sec63 was previously shown to be involved in protein transport in a substrate-specific manner and that lack of this function can cause polycystic liver disease in man and mouse (31, 65, 66).

In the case of Sec63, physical association with Sec62 and Sec61 allows the co-chaperone Sec63 to recruit BiP to the Sec61 complex for protein translocation (Fig. 1), while for ERJ3 and ERJ6 the mechanism should be different. We suggest that the substrate binding domains of the two co-chaperones, which are the carboxy-terminal domain and tetratricopeptide repeat, respectively, may facilitate recruitment of BiP to the ER luminal loop 7 of Sec61α for channel closure (67). Because of the spatial constraints it appears to be highly unlikely that the co-chaperones also bind to loop 7.

**BiP/Sec61 channel interaction via ER luminal loop 7 of Sec61α-Our previous work demonstrated the interaction of BiP with loop 7 of Sec61α at the level of synthetic peptides and in silico (16). Therefore, we suggested that BiP mediates Sec61 channel gating via loop 7 (Fig. 1). This view was supported by the observations that i) replacement of tyrosine 344 by histidine in loop 7 leads to reduced BiP binding (16), ii) BiP and Sec61 complex co-immunoprecipitate (53), iii) replacement of Sec61α by Sec61αY344H results in increased Ca\(^{2+}\) leakage from the ER and the inability of BiP to suppress this in HeLa cells (16), and iv) the homozygous Sec61αY344H mutation causes pancreatic β-cell death and diabetes in mice (19). Here, we reproduced the co-immunoprecipitation data (Fig. 9A) and demonstrated that antibodies against a tyrosine 344 containing peptide have access to their epitope in detergent-solubilized Sec61 complexes (Fig. 9B) and that Fabs thereof not only have access to their epitope in membrane resident Sec61 complex but also mediate channel closure (Fig. 9E and F). Therefore, the concept that BiP mediates Sec61 channel gating via loop 7 interaction was further substantiated and the idea that the energy of BiP
binding to loop 7 is sufficient to mediate channel gating was experimentally confirmed.

Implications for the link between ER protein mis-folding, Ca\(^{2+}\) homeostasis, and apoptosis-As we have previously described, depletion of available BiP in cells by either BIP gene silencing or protein misfolding in the ER leads to Ca\(^{2+}\) leakage from the ER (16). It has been known for some time that protein misfolding in the ER initiates the unfolded protein response and, when the latter is unable to turn the tide, apoptosis, of which the underlying mechanisms are not fully understood (68, 69). Based on the observations that the SEC61A1Y344H mutation causes the unfolded protein response and apoptosis in a diabetes mouse model and that the same mutation destroys the BiP binding site and leads to increased ER Ca\(^{2+}\) leakage, we proposed that the role of BiP in limiting Ca\(^{2+}\) leakage from the ER at the level of the Sec61 complex contributes to the connection between ER protein misfolding and apoptosis: misfolding polypeptides sequester BiP - in the absence of BiP Sec61 complexes become leaky for Ca\(^{2+}\) - and Ca\(^{2+}\) transmission to mitochondria triggers apoptosis. This view was already partially confirmed (53). Furthermore, this notion is consistent with the observations that apoptosis can also be induced in HeLa cells by an inhibitor of CaM (70) and that BIP overexpression can protect cells from ER stress associated cell death (71). This concept was further strengthened by the observations that the BiP co-chaperones ERj3 and ERj6 support BiP in Sec61 channel closure (Figs 2 and 4) and that loss of ERj6 function in mice and humans also causes pancreatic β-cell apoptosis and diabetes (20, 64). The observation that loss of ERj4 function also leads to pancreatic β-cell apoptosis and diabetes may indicate that under stress conditions ERj4 may be an additional co-chaperone for BiP in Sec61 channel closure (21).

Sec61 channel function that is due to either subunits of the Sec61 complex or direct or indirect effectors thereof, such as BiP and its co-chaperones. Examples of such diseases are certain forms of diabetes in mice and humans, polycystic liver disease in man and mouse, hemolytic uremic syndrome in man (Table 2). We note that there is also a human hereditary disease that is caused by apoptosis and relates to the level of available BiP, the neurodegenerative Marinesco-Sjögren syndrome (72). In this case, the mutated gene codes for the BiP-nucleotide exchange factor, termed Sil1, and the phenotype is most evident in Purkinje cells of the cerebellum. Of further note, too much suppression of ER Ca\(^{2+}\) leakage can also cause disease, such as cancers of the prostate and the lung, which are linked to overexpression of the SEC62 gene (18). The corresponding protein is involved in CaM mediated Sec61 channel closure (Fig. 1).

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions
SS, MCK, and IG carried out Ca\(^{2+}\) imaging experiments under supervision by AC. AM purified proteins and performed Biacore experiments under supervision by MJ. QW and NS performed co-ip experiments under supervision by XZC. BH and RW contributed the single channel measurements. SS did 3D-SIM and performed qPCR experiments with help from FB and MG. PL, SU, and MG studied cell proliferation in real-time and cell migration. SH carried out protein transport experiments. JD supervised all cloning work and silencing...
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experiments. MJ purified, characterized, and used the anti-loop 7 antibodies in pull-down experiments. AC and RZ planned and supervised the project and wrote the manuscript together with SS. All authors reviewed the results and approved the final version of the manuscript.

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1To whom correspondence should be addressed: R. Zimmermann, Tel.: +49 6841 1626510; Fax: +49 6841 1626288; E-mail: richard.zimmermann@uks.eu
2The abbreviations used are: BiP, immunoglobulin heavy-chain-binding protein; CaM, calmodulin; ER, endoplasmic reticulum; Hsp, heat shock protein; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; SIM, structured illumination microscopy.

LEGENDS TO THE FIGURES

FIGURE 1. Topology and domain organisation of Sec61 complex, Sec62, BiP and its Hsp40-type co-chaperones and nucleotide exchange factors in the ER of human cells. Protein names, synonyms, and systematic chaperone names (in parentheses) are indicated. C, carboxy-terminal or substrate binding domain; CaM, calmodulin; Cys, cysteine-repeat or zinc finger-like domain; EF, EF hand motif; GF, glycine-phenylalanine rich domain; IQ, IQ motif; NBD, nucleotide binding domain; SBD, substrate binding domain; TPR, tetratricopeptide repeat; TRX, thioredoxin domain.

FIGURE 2. Effect of depletion of the ER luminal Hsp40s, ERj3 and ERj6, on \(\text{Ca}^{2+}\) leakage from the ER in HeLa cells. A,C. HeLa cells were treated with the indicated siRNAs for 96 h and loaded with the calcium indicator Fura-2 AM as described in Experimental procedures. Then live cell \(\text{Ca}^{2+}\) imaging was carried out according to our established procedure (13, 14, 16). \(\text{Ca}^{2+}\) release was unmasked by applying thapsigargin (1 \(\mu\)M) in the presence of external EGTA. Average values are given, and error bars represent standard errors of the mean (s.e.m.). B,D. Statistical analysis of the changes in the cytosolic \(\text{Ca}^{2+}\) concentration after the addition of thapsigargin in the experiments presented in A and C. Error bars represent s.e.m. \(P\) values < 0.001 were defined as significant by unpaired \(t\) tests and are indicated by three asterisks (**). The numbers of cells that were analyzed are indicated. Data were collected in six independent experiments with culture duplicates for each condition. In these experiments, the averaged numbers of silencing siRNA treated cells corresponded to 78 ± 9%, 83 ± 6%, 79 ± 12%, and 89 ± 10%, respectively, as compared to control siRNA treated cells. E. Silencing was evaluated by western blots. Averaged relative protein contents are given with s.e.m. in % of control siRNA treated cells and as normalized to \(\beta\)-actin (n is given in parenthesis). Only the areas of interest from single gels are shown. F. HeLa cells were treated and analyzed as described for A-D, except that ionomycin (5 \(\mu\)M) was used instead of thapsigargin in order to measure total cellular calcium according to our established procedure (16). Data were accumulated in a single experiment with culture duplicates for each condition. Since the
calibration did not cover the high concentrations in the cytosolic Ca\textsuperscript{2+} concentration after the addition of ionomycin, we refrained from converting these ratios to cytosolic Ca\textsuperscript{2+} concentrations.

**FIGURE 3. Effect of ERJ3 and ERJ6 gene silencing on cell proliferation and the content of selected mRNAs and proteins.** A, HeLa cells were treated with the indicated siRNAs for 48 h and seeded in e-plates. Proliferation was recorded in real-time for 48 h. Growth rates were measured in three independent experiments in triplicate and are given with s.e.m. as slope of the curves for the second 48 h. Silencing was evaluated by western blots (data not shown). B-E, HeLa cells were treated with the indicated siRNAs for 96 h as in Fig. 2 and their content of selected mRNAs and proteins, respectively, was evaluated by quantitative real-time PCR (B), agarose gel electrophoresis (C), or western blots (D,E). B,C. As positive control for UPR activation, control siRNA treated cells were treated with tunicamycin for 5 h at 2 µg/ml. B, Averaged relative mRNA contents from four independent experiments are given with s.e.m. in % of control siRNA treated cells and as normalized to ACTB. The 100% values are indicated by dashed line. C, XBP1 was amplified in the same cDNA as in B with appropriate primers and subjected to gel electrophoresis and imaging. Only the area of interest from a single 3% agarose gel is shown. Lanes 4 and 5 represent a longer exposure as compared to the other lanes. D,E. For western blots, averaged relative protein contents are given with s.e.m. in % of control siRNA treated cells and as normalized to b-actin.

**FIGURE 4. Effect of simultaneous depletion of ER luminal Hsp40s, ERJ3 and ERJ6, and BiP on Ca\textsuperscript{2+} leakage from the ER in HeLa cells.** A, HeLa cells were treated with the indicated combinations of siRNAs for 96 h and analyzed as described in the legend to Fig. 2. Average values are given, and error bars represent standard errors of the mean (s.e.m.). B,D,E, Statistical analysis of the changes in the cytosolic Ca\textsuperscript{2+} concentration after the addition of thapsigargin. Error bars represent s.e.m. P values < 0.001 were defined as significant by unpaired t tests and are indicated by three asterisks (** **). The numbers of cells that were analyzed are indicated. Data were collected in two (B) or three (D,E) independent experiments with culture duplicates for each condition. C,F, Silencing was evaluated by western blots. Only the areas of interest from single gels are shown.

**FIGURE 5. Effect of simultaneous depletion of ERJ3 and ERJ6 on ER luminal Ca\textsuperscript{2+} in HeLa cells.** The FRET-based sensor D1ER was stably expressed in HeLa-D1ER cell and used to image the ER luminal Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{ER}). The gene silencing was carried out with the indicated siRNAs for 96 h. Just before imaging, cells were loaded with Fura-2 AM to image the cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{cytosol}) and incubated in a Ca\textsuperscript{2+}-free solution containing EGTA. The Ca\textsuperscript{2+} leakage was unmasked with thapsigargin (1 µM). The time courses of [Ca\textsuperscript{2+}]\textsubscript{cytosol} (A) and [Ca\textsuperscript{2+}]\textsubscript{ER} (B) were obtained in the same cells. n: 38, Control siRNA; 22, ERJ3 + ERJ6 siRNA. To evaluate the effects gene silencing, we calculate the Δ[Ca\textsuperscript{2+}]\textsubscript{cytosol} as (R\textsubscript{peak}-R\textsubscript{0})/R\textsubscript{0}, where R represents the ratio F340/F380 of Fura-2 signals (A, inset). The time to 50% decay (τ1/2) of [Ca\textsuperscript{2+}]\textsubscript{ER} was measured in individual cells (B, inset).

**FIGURE 6. Effects of ERJ3 expression and ERJ5 depletion, respectively, on Ca\textsuperscript{2+} leakage from the ER in HeLa cells.** A, HeLa cells were treated with the indicated siRNAs and plasmids for 96 h and analyzed as described in the legend to Fig. 2. Average values are given, and error bars represent standard errors of the mean (s.e.m.). D, HeLa cells were treated with the indicated siRNAs (10 nM) for 72 h and analyzed as described in the legend to Fig. 2. Average values are given, and error bars represent standard errors of the mean (s.e.m.). B,E, Statistical analysis of the changes in the cytosolic Ca\textsuperscript{2+} concentration after the addition of thapsigargin in the experiments presented in A and D, respectively. Error bars represent s.e.m. P values < 0.001 were defined as significant by unpaired t tests and are indicated by three asterisks (** ** **). The numbers of cells that were analyzed are indicated. B, Data were collected in two independent experiments with culture triplicates for each condition. E, Data were collected in five independent experiments with culture duplicates for each condition. In these experiments, the averaged numbers of silencing siRNA treated cells corresponded to 94 ± 23% and 96 ± 15%, respectively, as compared to control siRNA treated cells. C,F, Expression and silencing, respectively, were evaluated by western blots.
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Averaged relative protein contents are given with s.e.m. in % of control siRNA treated cells and as normalized to β-actin (n is given in parenthesis). Only the areas of interest from single gels are shown.

FIGURE 7. Effect of simultaneous depletion of ER lumenal Hsp40s, ERj3 and ERj6, and Sec61 complex on Ca²⁺ leakage from the ER in HeLa cells. A,C, HeLa cells were treated with the indicated siRNAs for 96 h and loaded with the calcium indicator Fura-2 AM as described in Experimental procedures. Then live cell Ca²⁺ imaging was carried out. Ca²⁺ release was unmasked by applying thapsigargin in the absence of external Ca²⁺. Average values are given, and error bars represent standard errors of the mean (s.e.m.). B,D, Statistical analysis of the changes in the cytosolic Ca²⁺ concentration after the addition of thapsigargin in the experiments presented in A and C. Error bars represent s.e.m. P values < 0.001 were defined as significant by unpaired t tests and are indicated by three asterisks (**). The numbers of cells that were analyzed are indicated. Data were collected in two independent experiments with culture duplicates for each condition.

FIGURE 8. Interaction of ER resident Hsp40s with BiP. The GST hybrid proteins of ERj3 (A) and ERj6 (B), respectively, were immobilized in the measuring cell; GST was bound in the reference cell. BiP binding was analyzed in a BIAAlite upgrade system at the indicated analyte concentrations (in µM) in comparison to the reference cell, according to our established procedure (25-28).

FIGURE 9. Interaction of Sec61 complex with BiP and anti-loop 7 antibodies, respectively. A, Co-immunoprecipitation of BiP and Sec61 complex from BiP-His6 containing HeLa cell extracts was carried out as described in Experimental procedures. The precipitates were analyzed for BiP and Sec61α, respectively. 40% of the HeLa cell extract that was used for the precipitation was analyzed in parallel (lane 1). Only the areas of interest from single gels are shown. B, Pull down of Sec61 complex from canine pancreatic microsomal extracts was carried out with anti-loop 7 antibodies as described in Experimental procedures. Affinity purified anti-Sec61ß antibodies served as positive control (lane 4). The precipitates were collected with Dynabeads. Dynabeads without antibody served as negative control (lane 2). Molecular mass marker (M) (lane 1) and rough canine pancreatic microsomes (RM) (lane 5) were subjected to gel electrophoresis in parallel. All samples were analyzed for Sec61α. Only the areas of interest from single gels are shown. C,D, Current voltage relationship from bilayers containing two active Sec61 channels in the absence or presence of affinity purified anti-Sec61ß at 2.5 µg/ml final concentration on both sides of the bilayer. PKRM, rough microsomes, which were treated with puromycin plus high salt, were used as the source of the Sec61 channels. E,F, Conductance histograms from single channel recordings obtained from bilayers with single active Sec61 channels, average of n=3 recordings at Vm=+40 mV in the absences and after 30 min. incubation with loop 7 Fabs (final concentration: 2 µg/ml).

FIGURE 10. Purification and characterization of anti-loop 7 antibodies and Fabs. A, Canine pancreatic rough microsomes (RM) were subjected to SDS-PAGE in parallel to molecular mass standards (M) and blotted to PVDF membranes. The loop 7 specific rabbit polyclonal antibody was used as well as a secondary peroxidase (POD)-coupled anti-rabbit antibody and ECL™ for visualization with a Fusion SL luminescence imaging system. B, Peptide spot array of Sec61α (corresponding to the complete amino acid sequence, in length of 12 amino acid residues with shift of two amino acids) was incubated with affinity purified anti-loop 7 IgGs and, thereafter, visualized by anti-rabbit-POD and ECL. Only the peptide epitope (339-353) showed signals for antibody binding. The peptide numbers are given as well as their corresponding sequences, the positive peptides are indicated by red writing of the amino acid residues, which were used for immunization. The BiP binding site is underlined, the minihelix given in italics (16). C, Fab production from affinity purified IgGs was evaluated by reductive and non-reductive SDS-PAGE. Coomassie staining of the resulting protein composition revealed the correct and efficient fragmentation of the IgGs by papain. D, IgG and Fab binding was evaluated in SPR experiments with immobilized peptide
from loop 7 (339-353) (measuring cell) and peptide 325-339 (control cell), respectively. Bound antibodies were released from peptides by application of 100 mM glycine/HCl (pH 2.8).
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TABLE 2. **Properties of BiP and its co-chaperones and nucleotide exchange factors.** The concentration of ERj6 was determined for a suspension of canine pancreatic microsomes as previously described for the listed proteins (27, 28). The binding constants of ERj4, ERj5 and ERj7 for hamster BiP were taken from previous publications (27, 28, 39). We note that our other previously published binding constants of ERj1, 2, and 3 as well as full length ERj5 referred to murine BiP and showed significant differences only with respect to ERj3 (K_D 3.6). Similar to human patients with MSS, mice homozygous with respect to the so called woozy mutation in the Sil1 gene develop ataxia due to Purkinje cell loss in the cerebellum, and myopathy (72). GST, glutathione-S-transferase; HUS, hemolytic uremic syndrome; MSS, Marinesco-Sjögren syndrome; PLD, polycystic liver disease.

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<td>GST-J1-domain (44-140)</td>
<td>4.2x10^4</td>
<td>1.9x10^-3</td>
<td>0.17</td>
<td>0.07</td>
<td>PLD</td>
<td></td>
</tr>
<tr>
<td>ERj2</td>
<td>-</td>
<td>GST-J2-domain (91-189)</td>
<td>1.2x10^3</td>
<td>3.4x10^-3</td>
<td>2.91</td>
<td>0.40</td>
<td>PLD</td>
<td></td>
</tr>
<tr>
<td>ERj3</td>
<td>+</td>
<td>GST-ERj3 (18-358)</td>
<td>4.1x10^3</td>
<td>2.2x10^-3</td>
<td>0.57</td>
<td>0.06</td>
<td>PLD</td>
<td></td>
</tr>
<tr>
<td>ERj4</td>
<td>+++</td>
<td>GST-ERj4 (24-222)</td>
<td>0.5x10^3</td>
<td>3.2x10^-3</td>
<td>6.07</td>
<td>not detectable</td>
<td>diabetes</td>
<td></td>
</tr>
<tr>
<td>ERj5</td>
<td>+</td>
<td>GST-J5-domain (26-123)</td>
<td>2.8x10^3</td>
<td>1.7x10^-3</td>
<td>0.59</td>
<td>0.40</td>
<td>diabetes</td>
<td></td>
</tr>
<tr>
<td>ERj6</td>
<td>+</td>
<td>GST-ERj6 (32-504)</td>
<td>3.9x10^2</td>
<td>1.9x10^-3</td>
<td>5.00</td>
<td>0.03</td>
<td>diabetes</td>
<td></td>
</tr>
<tr>
<td>ERj7</td>
<td>+</td>
<td>GST-J7-domain (39-149)</td>
<td>5.1x10^3</td>
<td>5.7x10^-3</td>
<td>1.13</td>
<td>0.46</td>
<td>diabetes</td>
<td></td>
</tr>
<tr>
<td>Grp170</td>
<td>+</td>
<td>---</td>
<td>not determined</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sill</td>
<td>-</td>
<td>GST-39-461</td>
<td>not detectable</td>
<td>0.001</td>
<td></td>
<td>MSS</td>
<td>„woozy“ phenotype</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2

(A) 1 µM TG

[Ca^{2+}]_{cytosol} (nM)

Time (s)

(B) 

Δ[Ca^{2+}]_{cytosol} (nM)

Control  ERJ3 #1  ERJ3 #2

837  921  977

D

Δ[Ca^{2+}]_{cytosol} (nM)

Control  ERJ6 #1  ERJ6 #2

837  802  912

(C) 1 µM TG

[Ca^{2+}]_{cytosol} (nM)

Time (s)

(D) 

Δ[Ca^{2+}]_{cytosol} (nM)

Control  ERJ6 #1  ERJ6 #2

837  921  977

(F)  

ΔRatio (340/380) x 1000

Control  ERJ3 #1  ERJ3 #2  ERJ6 #1  ERJ6 #2

144  140  138  120  142

(E) 

Western blot analysis of ERj3, ERj6, and β-Actin levels in control and siRNA-treated cells. 

Average ± SD for (8) samples.
Figure 3
Figure 4
Figure 5
Figure 6

A. 

B. 

C. 

D. 

E. 

F.
Figure 7
Figure 8
Figure 9

A. Western blot analysis showing BiP-His and Sec61α under different IP conditions.

B. Western blot analysis showing Sec61α under control, Loop 7, Sec61β, and RM conditions.

C. Graph showing current (pA) vs. voltage (mV) for PKRM.

D. Graph showing current (pA) vs. voltage (mV) for PKRM with anti-Sec61β.

E. Distribution of conductance (pS) for PKRM.

F. Distribution of conductance (pS) for PKRM with anti-Loop 7 Fab.
Co-chaperone Specificity in Gating of the Polypeptide Conducting Channel in the Membrane of the Human Endoplasmic Reticulum


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