ERp44 Mediates a Thiol-independent Retention of Formylglycine-generating Enzyme in the Endoplasmic Reticulum*

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Inside the endoplasmic reticulum (ER) formylglycine-generating enzyme (FGE) catalyzes in newly synthesized sulfatases the post-translational oxidation of a specific cysteine. Thereby formylglycine is generated, which is essential for sulfatase activity. Here we show that ERp44 interacts with FGE forming heterodimeric and, to a lesser extent, also heterotetrameric and octameric complexes, which are stabilized through disulfide bonding between cysteine 29 of ERp44 and cysteines 50 and 52 in the N-terminal region of FGE. ERp44 mediates FGE retrieval to the ER via its C-terminal RDEL signal. Increasing ERp44 levels by overexpression enhances and decreasing ERp44 levels by siRNA, small interfering RNA; MSD, multiple sulfatase deficiency; STS, steroid sulfatase; PBS, phosphate-buffered saline; NEM, i-formylglycine-generating enzyme (FGE) complex formation nor interferes with ERp44-mediated activation of sulfatases in vivo. This in vivo activity is affected neither by overexpression nor by silencing of ERp44, indicating that a further ER component interacting with the N-terminal extension of FGE is critical for sulfatase activation.

The biogenesis of sulfatases involves as an essential step the generation of a unique amino acid, Cα-formylglycine (FGly)4 (1, 2). FGly is the catalytic residue in the active site of nearly all eukaryotic and prokaryotic sulfatases (1–5), 17 of which are encoded in the human genome and 10 of which so far have been shown to fulfill highly specific functions (6–9). The FGly participates as an aldehyde hydrate in the hydrolysis of sulfate esters according to a novel transsulfation/elimination mechanism (10–12). In eukaryotes newly synthesized sulfatase polypeptides undergo FGly modification in the lumen of the endoplasmic reticulum (ER) by late co- or early post-translational oxidation of a critical cysteine residue that is part of a highly conserved consensus motif (C(T/S/C/A)PSR for human sulfatases) (13, 14). The oxidation of this cysteine is catalyzed by the recently discovered FGly-generating enzyme (FGE) (15, 16) through a novel mixed-functional oxygenase mechanism (17, 18).5 Failure to generate FGly, as found in patients with mutations in the FGE-encoding SLUMF1 gene, leads to multiple sulfatase deficiency (MSD), a rare but fatal inherited disorder that is characterized by synthesis of catalytically inactive sulfatases (15–17, 19–21).

FGE is an N-glycosylated single-domain protein with a novel fold (17, 18). Although it has little secondary structure, the catalytic core of FGE is a compact monomeric molecule that is stabilized by two intramolecular disulfide bridges and two Ca2+ ions. On its surface FGE harbors a binding groove for the sulfatase CXP5R substrate peptide with Cys336 and Cys341 of FGE being involved in FGly formation (17, 18). From this compact globular structure (residues 73–374), an N-terminal extension of 40 residues, the structure of which is unknown, obviously sticks out and is trimmed off in case of FGE secretion (22) by a furin-like protease.6 Recently we could show that residues 34–88 (residues 1–33 are cleaved off by signal peptidase) fulfill two functions. On the one hand they confer ER retention to FGE, and on the other hand they are required for in vivo sulfatase activation through FGly generation.7 Moreover, we could show that retaining FGE in the ER and ensuring the in vivo activity of FGE are two separate functional properties mediated by MSD patients; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PDI, protein-disulfide isomerase.

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4. The abbreviations used are: FGly, Cα-formylglycine; ASA, arylsulfatase A; ER, endoplasmic reticulum; FGE, FGly-generating enzyme; pFGE, paralog of FGE; MSD, multiple sulfatase deficiency; STS, steroid sulfatase; PBS, phosphate-buffered saline; NEM, N-ethylmaleimide; RNAi, RNA interference; siRNA, small interfering RNA; MSDi cells, immortalized fibroblasts from MSD patients; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PDI, protein-disulfide isomerase.

5. J. Peng, T. Dierks, A. Pruesser-Kunze, M. Mariappan, K. von Figura, and B. Schmidt, unpublished observations.

6. M. Mariappan, unpublished observations.

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by the N-terminal extension of FGE, as fusion with a C-terminal KDEL signal led to effective ER retention of FGE but not to biological activity.7 The latter is surprising, because in vitro the N-terminal extension is not needed for FGly modification of synthetic sulfatase peptide substrates (15, 22). It was even more surprising to find that the N-terminal extension of FGE in living cells at least partially can complement in trans an N-terminally truncated, and therefore inactive, FGE.7 This complementation, however, was only observed when FGE1–88 was expressed as an N-terminal fusion with the paralog of FGE (pFGE), which itself is catalytically inactive because of the absence of the two catalytic cysteines in the substrate-binding groove (24, 25). pFGE lacks an N-terminal extension but is otherwise structurally very similar to FGE and shares with it localization in the ER lumen (24, 25). Obviously the structural similarity of pFGE to FGE enables the FGE1–88-pFGE fusion described above to present the N-terminal extension to its catalytic partner and thereby to complement in vivo functionality, i.e. FGly generation in nascent sulfatase polypeptides inside the ER.7 We could further show that a Cys-Gly-Cys motif in this N-terminal extension of FGE, which is fully conserved in all known eukaryotic FGE sequences, is critical for activation of sulfatases, whereas it is dispensable for ER retention of FGE.7 This led us to postulate that the N-terminal extension of FGE mediates the interaction with ER components, which are required for the generation of FGly residues and the retention of FGE in the ER. In this study we describe the identification of an ER component interacting with the N-terminal extension of FGE, the biochemical basis of this interaction, and its direct function relevance for ER retention in the ER.

EXPERIMENTAL PROCEDURES

Fishing of FGE-interacting Proteins and Identification of ERp44—HT1080 cells stably expressing His6-tagged FGE (22) were incubated for 5 min with PBS, pH 7.4, containing 150 mM NaCl and 20 mM NEM (buffer I) prior to harvesting by trypsinization and lysis by sonication (3 × 20 s on ice) in buffer II, containing 20 mM Hepes, pH 7.6, 100 mM NaCl, 10 mM CaCl2,5 mM MgCl2,20 mM NEM, and protease inhibitor mixture (Sigma). The lysate was cleared by centrifugation at 100,000 × g for 1 h. The supernatant was incubated with Affi-Gel-10 matrix. After spinning, the supernatant was incubated with either Affi-Gel-10 matrix derivatized with the ASA scrambled peptide (PVSLPTRSCAAATLGTR) or Affi-Gel-10 matrix derivatized with the ASA-Asp69 peptide (PVSLSTPSRAALLTGR) (22) for 2 h at 4 °C. The beads were extensively washed with lysis buffer and eluted with 400 mM ASA-Asp69 peptide. The eluted material was subjected to precipitation with 10% trichloroacetic acid and boiled in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer with or without 100 mM dithiothreitol. The samples were resolved by SDS-PAGE (10% acrylamide), visualized by silver staining, and identified as indicated.

Expression Plasmids—The ERp44 cDNA was synthesized from total RNA, isolated from HeLa cells, by reverse transcription using the Omniscript RT kit (Qiagen) and an oligo(dT) primer. The first strand cDNA was amplified by PCR with primers ERp44-Nhe-fwd and ERp44-EcoRV-rev (see below). The PCR fragment was digested with NheI and EcoRV and cloned into MCS I of the pBI vector (BD Biosciences). Full-length sequencing verified the correctness of the cDNA insert. A c-Myc tag was inserted after the signal peptide cleavage site by sequential PCR-based reactions, in a similar way as reported for human ERp44 cloning (27). Briefly, ERp44-Nhe-fwd/Myc-ERp44-1R were used for first amplification reaction and Myc-ERp44-2f/ERp44-EcoRV-rev were used for the second. The resulting PCR products were used as templates in an overlapping extension PCR with primers ERp44-Nhe-fwd/ERp44-EcoRV-rev to generate a product coding for Myc-tagged ERp44. This product was digested with NheI and EcoRV for cloning into MCS I of pBI vector. The following primers were used: ERp44-Nhe-fwd, 5′-CTAGCTAGCATGATCCCTGCCGTCTTATCCATCC-3′; Myc-ERp44-1R, 5′-CAGCTCTCTCTCGAGATCGCTTTCTGCCATCAGTTGTTACAGGAG-TAAAAAC-3′; Myc-ERp44-2f, 5′-GAAGAGACAGAAGCTGTCTCAAGGAGACCTGCACTGACCTGCTGTTTGACATG-3′. For coexpression of Myc-ERp44 with FGE or its mutants, Myc-ERp44 cDNA was cloned into the MCS I of pBI vector that already had a cDNA encoding wild-type FGE (or its mutants) in the MCS II. These pBI-FGE constructs have been described earlier (24).

ERp44 mutants were created by the QuikChange site-directed mutagenesis method (Stratagene) using pBI-MycERp44 as template and complementary mutagenesis primers. The coding sequences of these primers were: TTTTTATGCTGACTGCGGCT-CGTTTCAGTGACATG (ERp44-C29A) and GTTTCAGTGACATGCTCCTGCTGTTTGACATG-3′. For triple expression, the MSDi Tet-On or HT1080 Tet-On cells were transfected with 2 g of pBI vector containing steroid sulfatase alone and 2 g of pBI vector carrying one or two cDNAs driven by a bidirectional tetracycline responsive promoter. For triple expression, the MSDi Tet-On cells were transfected with a mixture of two plasmids: 2 μg of pBI vector containing steroid sulfatase alone and 2 μg of pBI vector carrying two different cDNAs. 6 h after transfection, the medium was replenished with medium containing 2 μg/ml doxycycline (BD Biosciences), and after 30 h, the cells and medium were collected for further analysis.

An HT1080 Tet-On cell line stably expressing c-Myc-ERp44 under control of a doxycycline-responsive promoter was established by cotransfecting HT1080 Tet-On cells (neomycin-resis-
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Identification of ERp44 as an in Vivo Interaction Partner of FGE—To identify interacting partners of FGE, we aimed at pulling out complexes of FGE with its partners from cells over-expressing FGE. We took advantage of an affinity column with an immobilized ASA peptide that binds with high affinity to the active site of FGE (15). To control for specificity, we used in parallel an affinity column carrying an immobilized peptide of identical amino acid composition but having the three key residues of the FGlY modification motif of sulfatases in scrambled order (see Ref. 15).

HT1080 cells stably overexpressing FGE were treated with NEM prior to lysis to stabilize disulfide bonds that had formed in vivo. Using the original FGE purification protocol (15), we observed binding of FGE only to the sulfatase peptide column (Fig. 1, lanes 3 and 4). In the eluate, obtained with free ASA peptide, two additional bands in the range of 70–75 kDa were detected (Fig. 1, lane 3), which contained no FGE, as evidenced by Western blotting (not shown). Tryptic digestion and MALDI-TOF mass spectrometry identified them as the two Hsp70 chaperone isoforms Hsp70-5 and Hsp70-9. These chaperones were also detectable in the eluate from the control affin-
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EGF and ERp44 Form Hetero- and Homodimeric Complexes in Vivo

FIGURE 2. FGE and ERp44 form hetero- and homodimeric complexes. A, NEM lysates of HT1080 cells coexpressing FGE-HA and c-Myc-ERp44 were incubated with either rabbit preimmune serum (Mock) or rabbit ant-FGE antibodies (α-FGE) and precipitated with Pansorbin. The pellet was boiled in sample buffer (without β-mercaptoethanol). Equal parts of the input (Load) and pellet fractions were analyzed by SDS-PAGE under nonreducing conditions and Western blotting (WB) using either anti-c-Myc or anti-HA antibodies, as indicated. The asterisks indicate background bands. B, the same lysates (see A) were subjected to immunoprecipitation with either control α-His IgG (Mock) or mouse anti-c-Myc antibodies. 10% of the input (Load) and pellet fractions were analyzed as above, using, however, rabbit anti-FGE antibodies (α-FGE) on the Western blot, as indicated. The positions of FGE mono- and homodimers (FGE and FGE$_{ox}$) of FGE/ERp44 heterodimers, tetramers, and octamers (FGE/ERp44$_{2}$, FGE/ERp44$_{4}$, and FGE/ERp44$_{8}$) are indicated.

Ero1, unpolymerized immunoglobulin chains, and adiponectin are readily reduced by free thiols. Only through blocking free thiol groups prior to lysis by alkylation reagents such as NEM, can the complexes be recovered (Fig. 3, lanes 3 and 4). In vivo, the complexes are highly labile, because no complexes could be recovered from cell extracts when the NEM treatment of the cells prior to lysis was omitted. Thus, the majority of FGE is engaged in different covalent complexes either with itself or with ERp44.

ERp44 and FGE Form Noncovalent and Disulfide Bridge-Stabilized Complexes—Under the given expression levels in the cells (FGE exceeding ERp44), approximately half of FGE is recovered as a complex with ERp44 (Fig. 2A, lane 6), whereas ERp44 was quantitatively present in the complexes with FGE. In most experiments monomeric ERp44 barely was detectable (Fig. 2, lanes 1 and 3, Fig. 3, lanes 3 and 4). In vivo, the complexes are highly labile, because no complexes could be recovered from cell extracts when the NEM treatment of the cells prior to lysis was omitted (Fig. 3, lanes 1 and 2). The complexes are readily split into their monomeric constituents by β-mercaptoethanol (Fig. 3, lanes 7 and 8) or other thiols. This suggests that upon cell lysis the disulfide bridges stabilizing the complexes are readily reduced by free thiols. Only through blocking free thiol groups prior to lysis by alkylation reagents such as NEM, can the complexes be recovered (Fig. 3, lanes 3 and 4).
It should be noted that in the cells used for this experiment (stably expressing ERp44 and transiently expressing wild-type FGE or Δ34–68FGE), large amounts of monomeric ERp44 were detectable (Fig. 4, lanes 1 and 4). In these cells traces of monomeric ERp44 also were detected in the immunoprecipitates obtained with anti-FGE antibodies (Fig. 4, lanes 2 and 5). This indicates that ERp44 also forms noncovalent complexes with both full-length and truncated FGE, which after SDS-PAGE are disassembled into their monomeric constituents. They are detectable, however, only under conditions of ERp44 excess over FGE.

Cys\(^{50}\) and Cys\(^{52}\) of FGE Mediate Disulfide Bonding with Cysteine 29 of ERp44 and with One Another—To identify the cysteines engaged through mixed disulfide linkage in FGE and ERp44, the following alanine mutants were generated: FGE-C50A, FGE-C52A, FGE-C50A/C52A, ERp44-C29A, and ERp44-C63A. Expression of FGE-C50A or FGE-C52A led to efficient recruitment of coexpressed wild-type ERp44 into heterodimeric covalent complexes, i.e. with similar efficiency as observed for wild-type FGE (Fig. 5). As observed earlier (22) FGE homodimer formation requires Cys\(^{50}\) and Cys\(^{52}\). Notably, the heterotetrameric and heteroctameric complexes were not found with each of the cysteine mutants. With the double mutant FGE-C50A/C52A no covalent complex with ERp44 was formed at all. Thus, the two cysteines Cys\(^{50}\) and Cys\(^{52}\) are equally well capable of forming mixed disulfide bonds with ERp44, but both cysteines are required for formation of the higher heterooligomeric complexes. It should be noted that in the FGE-C50A/C52A-coexpressing cells ERp44 is forming higher oligomeric complexes with itself (Fig. 5, top of lane 10). This obviously is the consequence of lacking disulfide stabilization of complexes with FGE, at least under the applied in vitro conditions.
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conditions (cell lysis and immunoprecipitation). The observation, on the other hand, of significant amounts of ERp44 that were immunoprecipitated by the anti-FGE antibody even from FGE-C50A/C52A coexpressing cells again demonstrates that noncovalent heteromeric complexes between FGE and ERp44 are formed, which, however, are disrupted during SDS-PAGE (Fig. 5, lane 11).

Cys\textsuperscript{29} of ERp44 has been shown to form disulfide-bonded complexes with other proteins. Also Cys\textsuperscript{63} of ERp44 was proposed to have a surface-exposed localization and found to be accessible for NEM (30). In cells coexpressing FGE and ERp44-C63A, heteromeric complex formation between FGE and ERp44 was as efficient as in cells coexpressing wild-type ERp44 (Fig. 6, lanes 5, 6, 11, and 12), whereas in cells coexpressing FGE and ERp44-C29A, none of the heteromeric complexes were detectable (Fig. 6, lanes 3, 4, 9, and 10). Thus, disulfide bonding occurs between Cys\textsuperscript{29} of ERp44 and either Cys\textsuperscript{50} or Cys\textsuperscript{52} of FGE.

**ERp44 Is a Retention Factor for FGE**—A fraction of FGE is constitutively secreted. This fraction increases upon overexpression of FGE (22). During secretion the majority of FGE (42 kDa) is processed by a furin-like activity to a 37-kDa form (Δ34–72FGE). We established HT1080 Tet-On cells that allowed to coexpress FGE and ERp44 from a bidirectional doxycycline-responsive promoter. After induction for 24 h, the cells and medium were analyzed by Western blotting. 15–20% of total FGE are found intracellularly, whereas the rest is secreted largely as Δ34–72FGE (Fig. 7, sample 1). Coexpression of ERp44 led to an up to 4-fold increase of intracellular FGE (Fig. 7, sample 2). ERp44 itself was fully retained intracellularly. ERp44 lacking the C-terminal RDEL retention signal was largely secreted and unable to increase FGE retention (Fig. 7, sample 3), indicating that the increase of ER retention by ERp44 relies on retrieval of the FGE-ERp44 complexes through KDEL receptors.

To determine whether disulfide bonding between ERp44 and FGE is required for mediating ER retention, we examined the retention when the cysteine mutants C50A and C52A of FGE were coexpressed with wild-type ERp44 and also wild-type FGE with the C29A mutant of ERp44. The results clearly show that ERp44-mediated retention of FGE is independent of disulfide bonding between ERp44 and FGE (Fig. 7, samples 4–7).

Recently we could show that the N-terminal extension (residues 34–68) of FGE is required for the retention of FGE in the ER\textsuperscript{7} After induction for 24 h, ∼10% of the N-terminally truncated FGE (Δ34–68FGE) were found intracellularly. Coexpression with ERp44 increased the retention ∼2-fold (Fig. 7, samples 8 and 9), indicating that the N-terminal extension of FGE is one but not the only part of FGE that contributes to ERp44-mediated retention.

Overexpression of ERp44 clearly increased the intracellular retention of FGE. To determine whether
ERp44 is necessary for ER retention of FGE, we attempted to reduce ERp44 levels by silencing. By expressing different RNAi constructs (si-ERp44-3U and si-ERp44-ORF) for ERp44, the levels of ERp44 in HT1080 cells were reduced to less than 5 and 20% of control, respectively (Fig. 8, shown for si-ERp44-3U). Reducing the ERp44 level to 4% of control decreased the fraction of FGE recovered intracellularly 12 h after induction from 36 to 8% (Fig. 8). Likewise silencing of ERp44 in cells coexpressing FGE and ERp44 from the same inducible promoter diminished the retention of FGE from 57 to 19%, which agrees with the less complete ERp44 silencing in these cells (data not shown). Please note the shorter induction time (12 h) as compared with Fig. 7 (24 h), which was chosen to start at relatively higher intracellular FGE levels (18% retention), because less FGE could accumulate in the medium.

FGE-mediated Activation of Sulfatases Is Independent of ERp44—The N-terminal extension of FGE is required for sulfatase activation. Mutation of Cys<sup>52</sup> to alanine abrogates the FGE-mediated activation of newly synthesized sulfatases, and mutation of Cys<sup>50</sup> to alanine reduced the activation to approximately half. To examine whether ERp44 is involved in the activation of sulfatases, we overexpressed steroidsulfatase (STS) in immortalized MSD Tet-On cells (MSDi cells), which lack endogenous FGE. Cotransferring these cells with bidirectional promoter-driven cDNAs coding for FGE and ERp44 and an STS encoding plasmid allowed for coexpression of all three proteins under the control of doxycycline (Fig. 10). Expression of STS alone led to the synthesis of STS polypeptides that were barely active. Coexpression of FGE increased the activity of STS to more than 50–100-fold. This effect is solely due to the activation of the STS polypeptides and not to an increase of STS synthesis. Coexpression of ERp44 with FGE did not further increase the activation of STS (Fig. 10). If at all, it decreased the activity of STS slightly (by 5–20% in three independent experiments). Coexpression of FGE with ERp44-C29A or ERp44-C63A, which potentially are dominant negative mutants of ERp44, did not affect STS activity (Fig. 10). A comparison of the expression levels of endogenous and recombinant ERp44 forms revealed that the level of endogenous ERp44 exceeded that of the recombinant forms at least 4-fold. The experiment was repeated with HT1080 Tet-On cells. In these cells the recombinant forms of ERp44 reach levels that are 2-fold higher than that of endogenous ERp44. However, in these cells, which express endogenous FGE, the overexpression of FGE increases the activity of STS only 2–3-fold. Also in this system coexpression of ERp44 with FGE did not affect STS activity (not shown). These data indicate that ERp44 is not a limiting factor for activation of STS by FGE when the latter two are overexpressed.

Next we examined whether reducing the expression of ERp44 by silencing would affect the activation of STS by FGE. Using the RNAi construct si-ERp44-3U, we succeeded in reducing the level of endogenous ERp44 in MSDi Tet-On cells by more than 90%. This, however, did not affect the relative
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FIGURE 10. Effect of ERp44 on FGE-mediated activation of steroid sulfatase. MSDi Tet-On cells were transiently transfected with the indicated combinations of cDNAs (see “Experimental Procedures”). The amounts of steroid sulfatase (STS), FGE and ERp44 were monitored in the cell extracts by Western blotting. The STS activity in MSDi cells expressing STS alone or STS together with FGE were 0.58 and 35 nmol/h/mg cell protein. The relative specific activity of STS given below the lanes was calculated from the STS activity (nmol/h/mg cell protein) divided by the Western blot signal of STS (arbitrary units/mg cell protein) and referred to that in cells expressing STS only.

stimulation of STS activity by FGE (not shown). In HT1080 cells, the two RNAi constructs (si-ERp44-3U and si-ERp44-ORF) reduced the ERp44 level to less than 5% (Fig. 8) and ~20%, respectively. The 2–3-fold stimulation of STS activity by coexpression of FGE was not affected by silencing ERp44 expression (not shown). These data indicate that either ERp44 is not involved in FGE-mediated activation of STS or that the residual amount of ERp44 left after silencing is sufficient to support FGE-mediated activation of STS.

DISCUSSION

In a recent study we found the N-terminal extension of FGE (residues 34–88) to be involved in two separable and essential properties of FGE. First, the retention of FGE in the ER is significantly enhanced by its N-terminal noncatalytic extension. This effect is independent of the conserved cysteine residues Cys⁵⁰ and Cys⁵² within the N-terminal extension. Second, the activation of sulfatases by FGE depends on the presence of the N-terminal extension, although the latter is dispensable for the generation of FGly residues under in vitro conditions. For the in vivo activation of sulfatases the cysteine residue Cys⁵² is critical, whereas mutating Cys⁵⁰ reduces FGE-mediated activation of sulfatases only to approximately half.

Here we show that the N-terminal extension of FGE significantly enhances the complex formation with the ER protein ERp44. These complexes are stabilized by disulfide bridges between Cys⁴⁹ of ERp44 and Cys⁵⁰ or Cys⁵² of FGE. The complex formation is shown to mediate the retention of FGE in the ER, whereas there is no evidence that activation of sulfatases by FGE depends on FGE-ERp44 complexes.

ERp44 Is a Thiol-independent Retention Factor for FGE—ERp44 has been thoroughly characterized as a thiol-dependent ER retention factor for Ero1, the major oxidase for PDI, and for other oxidoreductases of the ER (23, 27–30). ERp44-mediated retention relies on retrieval from post-ER compartments through KDEL receptors. The formation of mixed disulfides with Ero1 via Cys²⁹ of ERp44 was found to be essential for Ero1 retention (thiol-mediated retrieval, ref. 30). ERp44 also retains secretory proteins like immunoglobulin chains and adipoonectin through disulfide bond formation during biogenesis to assist their assembly and to retrieve nonpolymerized subunits back to the ER (thiol-mediated assembly and quality control). Both thiol-mediated processes localize ERp44 to the exit sites of the ER, to the ER-Golgi intermediate compartment and to the cis-Golgi (29). We observed that ERp44 localizes to both PDI- and GM130-positive compartments and that overexpression of FGE shifts the distribution of ERp44 to the ER (Fig. 9). This relocation suggested that ERp44 and FGE physically interact. In fact, FGE-ERp44 complexes could be detected by coimmunoprecipitation and coaffinity purification (Figs. 1–6).

The complex formation between FGE and ERp44 serves to retrieve FGE back to the ER. Increasing ERp44 levels by overexpression (Fig. 7) improves and reducing ERp44 levels by silencing (Fig. 8) decreases the intracellular retention of FGE. The retention depends on the C-terminal RDEL retrieval signal of ERp44. Deletion of the latter fully abolished the retention of FGE. Interestingly, in case of ERp44ΔRDEL coexpression, FGE is mainly secreted without N-terminal truncation (Fig. 7, compare samples 1 and 3). Thus, ERp44ΔRDEL, which also is secreted, protects FGE from furin processing. This clearly indicates that FGE passes the furin-type protease in the secretory route as an FGE-ERp44ΔRDEL complex and corroborates the tight interaction of the two components.

It should be noted that ERp44 exerts its FGE retention function through a thiol-independent mechanism. FGE retrieval did not depend on the cysteine residues required to establish the disulfide bonds between ERp44 and FGE. The observation that FGE retention through coexpressed ERp44-C29A is fully functional in vivo (Fig. 7) clearly suggests that the noncovalent interaction between the two partners is strong enough to mediate retrieval to the ER. The partial retention of FGE lacking residues 34–68 by ERp44 (Fig. 7, sample 9) suggests that noncovalent FGE-ERp44 complexes are formed even if the N-terminal 35 residues of the N-terminal extension of FGE (residues 34–68) are lacking. It should be noted that a thiol-independent but ERp44-mediated retention of cargo proteins is not unique for FGE and has been observed for monomeric immunoglobulin K and J and mutant μ chains (27, 30).

Sulfatase Activation by FGE Does Not Depend on Complexes with ERp44—ERp44 has been shown to mediate the redox regulation of the inositol 1,4,5-trisphosphate receptor type 1, a prominent ion channel in the brain for the release of Ca²⁺ from the ER (26). The dependence of sulfatase activation on the N-terminal extension of FGE and requirement of the cysteine residues Cys⁵⁰ and Cys⁵² within this extension for the formation of covalent complexes with ERp44 prompted us to examine the involvement of such complexes in the FGE-mediated activation of sulfatases. Two experimental lines strongly suggest...
that complexes with ERp44 are not directly involved in FGE-mediated activation of sulfatases. First, overexpression of ERp44 augmented the FGE-mediated stimulation of STS neither in MSDi cells nor in HT 1080 cells. Second, reduction of the ERp44 level to less than 10% of control through silencing did not affect the FGE-mediated activation of STS. Although the result of the silencing cannot formally exclude a catalytic role of ERp44 in the activation of sulfatases, it clearly excludes a role for ERp44 as a coactivator required in stoichiometric amounts for FGE-mediated activation of sulfatases. ERp44, however, can affect FGE-mediated activation of sulfatases indirectly through increasing the retention of FGE in the ER.

Concluding Remarks—The N-terminal extension of FGE enhances its retention in the ER. Complex formation with the ER protein ERp44 is shown to mediate this retention. The complexes are stabilized by disulfide bridges involving Cys50 and Cys52 in the N-terminal extension of FGE and Cys29 in ERp44. The functional significance of the covalent linkages between FGE and ERp44, which in vivo are highly labile, remains unclear. They are not required for retention. The N-terminal extension is furthermore required for the biological function of FGE, the activation of sulfatases by post-translationally generating an FGly residue in the catalytic site of newly synthesized sulfatase polypeptides. This function apparently is not mediated by the complexes of FGE with ERp44. Silencing of ERp44 did not diminish FGE-mediated activation of sulfatases. The latter critically depends on Cys52 but not on Cys50 within the N-terminal extension of FGE. The requirement for complex formation of FGE with ERp44 did not distinguish between Cys50 and Cys52. Formation of noncovalent complexes was independent of either cysteine residue, whereas formation of covalent complexes was equally dependent on both cysteine residues. This indicates that for sulfatase activation the interaction of the N-terminal extension with a second, non-ERp44 component is required.

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