Formylglycine-generating enzyme (FGE) catalyzes the oxidation of a specific cysteine residue in nascent sulfatase polypeptides to formylglycine (FGly). This FGly is part of the active site of all sulfatases and is required for their catalytic activity. Here we demonstrate that residues 34–68 constitute an N-terminal extension of the FGE catalytic core that is dispensable for in vitro enzymatic activity of FGE but is required for its in vivo activity in the endoplasmic reticulum (ER), i.e. for generation of FGly residues in nascent sulfatases. In addition, this extension is needed for the retention of FGE in the ER. Fusing a KDEL retention signal to the C terminus of FGE is sufficient to mediate retention of an N-terminally truncated FGE but not sufficient to restore its biological activity. Fusion of FGE residues 1–88 to secretory proteins resulted in ER retention of the fusion protein. Moreover, when fused to the N-terminal extension of pFGE, where it functions as an ER retention signal (17). Hence a C-terminal PGEL tetrapeptide is found only in pFGE, where it functions as an ER retention signal (17). The N-terminal 55 residues of mature FGE are the N-terminal extension cysteine 52 is critical for the biological activity. We postulate that this N-terminal region of FGE mediates the interaction with an ER component to be identified and that this interaction is required for both the generation of FGly residues in nascent sulfatase polypeptides and for retention of FGE in the ER.

Sulfatases carry in their active site a unique amino acid, Cα-formylglycine (FGly), which is crucial for their catalytic activity (1, 2). In most eukaryotic and prokaryotic sulfatases, FGly is generated from a cysteine residue (3–5). This posttranslational protein modification in eukaryotes occurs in the endoplasmic reticulum (ER) during or shortly after translocation of sulfatase polypeptides into the lumen of the ER and before their folding (1, 3). The oxidation of cysteine to FGly is catalyzed by the FGly-generating enzyme (FGE) (6, 7), which uses molecular oxygen as the terminal electron acceptor. The critical role of FGE for the synthesis of sulfatases is highlighted by multiple sulfatase deficiency (MSD), a rare inherited disorder caused by mutations in the gene encoding FGE and characterized by the synthesis of catalytically inactive sulfatases (6–10).

FGE is localized in the ER and generates the FGly residue by oxidation of the cysteine in the C(T/S/C/A)PSR pentapeptide conserved in all human sulfatases. The N-terminal 33 residues of human FGE represent a cleavable signal peptide. The mature form of FGE is composed of a compact N-glycosylated monomeric core with a novel fold and little secondary structure that is stabilized by two Ca2+ ions and two intramolecular disulfide bonds (11–13). The N-terminal 55 residues of mature FGE form a domain of unknown structure, which obviously extends from the compact fold of the core protein, as indicated by very high protease sensitivity (11, 12). In the active site two crucial cysteines have been implicated in binding and oxidation of the substrate (sulfatase) cysteine according to a novel oxygenase mechanism (12, 13).

A paralog of FGE (pFGE) is found in most species from mammals down to unicellular eukaryotes (6, 7). It shares with FGE a high sequence and structural homology but lacks FGly-generating activity (14, 15). Major structural differences between FGE and pFGE are the N-terminal extension of 55 residues (residue 34–88 in human FGE) and a loop of a 22-residue length (FGE residues 304–325), both found only in FGE (12, 16); furthermore a C-terminal PGEL tetrapeptide is found only in pFGE, where it functions as an ER retention signal (17). Hetero-complex formation of FGE, pFGE, and sulfatases has been observed and speculated to confer ER retention also to FGE (17, 18). However, FGE trafficking out of the cell and re-uptake by other cells have also been described (19). Interestingly, the majority of FGE that escapes retention in the ER is secreted as an N-terminally truncated form starting with glutamate 73. A peptide inhibitor for furin-type proteinases prevents this cleavage, suggesting that a Golgi-localized furin-like proteinase initiates the proteolytic processing of FGE during secretion. In

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2 The abbreviations used are: FGly, Cα-formylglycine; ER, endoplasmic reticulum; FGE, formylglycine-generating enzyme; pFGE, paralog of FGE; MSD, multiple sulfatase deficiency; STS, steroid sulfatase; HA, hemaggulitin.
3 J. Peng, T. Dierks, A. Preusser-Kunze, M. Mariappan, K. von Figura, and B. Schmidt, unpublished results.
4 M. Mariappan, unpublished results.

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fact, consensus sequences for furin-type proteinases are found at arginine residues 69 and 72 of FGE. It has been demonstrated that a truncated FGE, lacking residues 34–68, is catalytically active (7, 11). This suggested that the N-terminal extension of FGE, harboring a sequence motif that is fully conserved in all eukaryotic FGEs from unicellular eukaryotes to mammals (see “Discussion”), contributes a functional property that is independent of its Fgly-generating activity.

Here we show that the N-terminal extension of FGE is required for the activation of sulfatases in vivo. Furthermore, the N-terminal extension is required for FGE retention in the ER and carries a transferable ER-retention signal. Retention of N-terminally truncated FGE in the ER, however, is not sufficient to restore the activation of sulfatases. Surprisingly, co-expression of a fusion protein carrying the N-terminal extension of FGE partially restores the in vivo activation of sulfatases by N-terminally truncated FGE. We conclude from these data that the N-terminal extension of FGE mediates the interaction with an ER component and thereby ensures both the retention of FGE in the ER and the generation of Fgly residues in nascent sulfatase polypeptides.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Plasmids**—The plasmids used to express galactose-6-sulfatase, steroid sulfatase (STS), and differ-ent forms of His₆- or HA-tagged FGE and pFGE have been described previously (11, 15, 17). For co-expression of FGE-HA, Δ34–68FGE-HA, or Δ34–68FGE-HA-KDEL with steroid sulfatase, the pBI vector (BD Biosciences) was used, which allows expression of two cDNAs from a bidirectional tetracycline/doxycycline-responsive promoter (15). In the first step of cloning, FGE-HA, Δ34–68FGE-HA, or Δ34–68FGE-HA-KDEL-encoding PCR products were equipped with a 5' EcoRV or BamHI site and a 3' HA tag sequence followed by a stop codon (for Δ34–68FGE-HA-KDEL, extended by KDEL and stop codon) and a NotI site by add-on PCR using pBI-FGE-HA (15) as template. The following primers were used: GGATATCGGG-GACCAACATGGCTGC (EcoRV-FGE); CCGGATCCCGCA- CATTGGACGCAAGGTTGG (pBI-BamHI); ATAGTTTGGGGCGGCGTATCGTAGCGGACATCATACGG- ATATGCTCATGTTGGCGACGC (FGE-HA-NotI); TAGTGTAGCGGGGCTTATGCGTAGTCAGGCACATCATACGG-GACCACTAGATGCTGC (FGE-C50A), GGGTTCTTGGCGGCGGCTGGGACACCTAGATGCTGC (FGE-52A), and TGGGGATCCGCCACGCGGCGC (FGE-52A). All PCR reactions were performed with Pfu-Ultra polymerase (Stratagene). The resulting constructs were analyzed by full-length sequencing of coding regions to preclude any PCR-derived errors.

**Cell Culture and Transfections**—The immortalized fibroblasts from an MSD patient, which lack endogenous FGE activity due to a severe homozygous mutation (p.S155P) in the SLMF1 gene (8), were kindly provided by Dr. Andrea Ballabio (Telethon Institute of Genetics and Medicine, Naples, Italy). These “MSDi cells” and HT1080 cells were cultured at 37°C under 5% CO₂ in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum. Stable Tet-On cell lines were established by transfection with pUHrT62 (kindly provided by Dr. Nadja Jung) encoding the reverse tetracycline-controlled transactivator (20) and neomycin resistance vector pBS4.7pA (11) or blasticidin resistance vector (Invitrogen) in a 10:1 ratio into HT1080 and MSDi cells, respectively. Transfectants were selected in medium supplemented with neomycin (Invitrogen), increasing from 0.2 to 0.8 mg/ml, or blasticidin (Invitrogen), increasing from 1 to 5 µg/ml. The stable clones were screened first for doxycycline-dependent fluorescence after transient transfection with a pBI-EGFP plasmid (BD Biosciences). The best clones were then rescreened through Western blotting for doxycycline-dependent pFGE production after transient transfection with pBI-pFGE (17). The stable transfection of FGE-His or Δ34–68FGE-His alone and with galactose-6-sulfatase was performed as described earlier (15). Transient transfections were performed with Lipofectamine 2000 as recommended by Invitrogen. For single or co-expression experiments, the HT1080 Tet-On or MSDi Tet-On cells were transfected with 2 µg of pBI vector carrying one or two cDNAs from a bidirectional tet-responsive promoter. For triple expression experiments (and the double expression controls, see Figs. 6A and 7B), 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 containing one or two different cDNAs, as indicated in the figure. Unless otherwise stated, the medium was replenished 6 h after transfection with medium containing 2 µg/ml doxycycline (BD Biosciences), and the cells were cultured for 48 h before further experiments.
and media were collected 30 h after induction for further analysis.

**Indirect Immunofluorescence**—For recombinant expression of pBl-Δ34−68FGE-HA, pBl-Δ34−68FGE-HA-KDEL, pBl-pFGE, pBl-pFGEΔPGE, and pBl-pFGE1−88-pFGEΔPGE, the MSDi Tet-On or HT1080 Tet-On cells were grown on coverslips for 1 day and transiently transfected as described above. After 30 h of transfection, the cells were analyzed by indirect immunofluorescence as described previously (15). For co-localization studies, rabbit antisera raised against FGE (11) or pFGE (15) and monoclonal antibodies against marker proteins protamine sulfatases a cysteine into the active site FGly residue that is indispensable for sulfatases to become catalytically active. FGE is synthesized as a precursor of 374 amino acids with a cleavable signal peptide of 39 residues at its N terminus (11). A fraction of mature FGE escapes retention in the ER and is secreted. The majority of secreted FGE lacks the N-terminal 39 amino acids of mature FGE (residues 34−72) but retains catalytic activity. For a recombinant form of FGE lacking N-terminal residues 34−68 (Δ34−68FGE), we could unequivocally demonstrate catalytic activity in vitro when using a synthetic peptide as a substrate that contains the C(T/S/C/A)PSR consensus motif of human sulfatases (11). To examine whether truncated FGE retains biological activity in intact cells, we co-expressed cDNAs encoding full-length FGE or Δ34−68FGE together with cDNAs encoding sulfatases in two different experimental systems and determined the catalytic activity of the sulfatases. The activity of the sulfatases depends on FGE-mediated generation of the FGly residue and, therefore, can serve as an indirect indicator for the modification of the newly synthesized sulfatases by FGE.

In the first system full-length FGE was stably expressed in human fibrosarcoma HT1080 cells that stably co-expressed galactose-6-sulfatase as a reporter sulfatase. We observed an up to a 340-fold increase in galactose-6-sulfatase activity due to FGE co-expression. The increase was proportional, although not linear, to the expression level of FGE (Fig. 1A). Expression of truncated FGE at levels comparable with that of full-length FGE did not increase galactose-6-sulfatase activity significantly above the basal level (Fig. 1A), indicating that the N terminus of FGE is required for the activation of sulfatases.

In a second system we utilized for expression immortalized fibroblasts from a patient with multiple sulfatase deficiency (MSDi Tet-On cells, see “Experimental Procedures”), which almost completely lack endogenous FGE activity due to a severe homozygous mutation (p.S155P) in the SUMF1 gene (8). Transient co-expression of steroid sulfatase with full-length FGE increased the activity of steroid sulfatase 95-fold compared with cells expressing steroid sulfatase only. Co-expression of truncated FGE with steroid sulfatase failed to increase the activity of the sulfatase also in this expression system (Fig. 1B).

**N-terminally Truncated FGE Is Rapidly Secreted**—A possible explanation for the inability of Δ34−68FGE to support the formation of catalytically active sulfatases could be that the N-terminal fragment is required for the retention of FGE in the ER where it catalyzes the generation of FGly residues in nascent sulfatase polypeptides. To examine the role of the N-terminal residues 34−68 for the retention of FGE in the ER, HT1080 cells expressing full-length or Δ34−68FGE were metabolically labeled, and the secretion of the newly synthesized FGE was monitored during a subsequent chase for up to 8 h (Fig. 2). The data clearly show that Δ34−68FGE is rapidly secreted with less than 10% being retained intracellularly after chasing for 8 h, whereas after the same chase time half of the full-length FGE is still recovered intracellularly. The half-life of intracellular FGE was estimated to be 7.3 h for full-length FGE and 2.5 h for Δ34−68FGE.

**Retention of Truncated FGE in the ER Does Not Restore Its Biological Activity**—To prevent secretion of Δ34−68FGE, we fused to its C terminus the KDEL tetrapeptide, an ER retrieval signal widely used by soluble ER proteins. The addition of the KDEL tetrapeptide resulted in the effective retention of N-terminally truncated FGE in MSDi Tet-On cells.
Residues 34–88 of FGE Contain a Transferable ER Retention Signal—The previous experiments had shown that the N-terminal residues 34–68 of FGE are required for the biological activity and the retention of FGE in the ER. To determine whether the N terminus of FGE contains a transferable ER retention signal, we fused it to secretory proteins. As a passenger protein we used a secretory form of pFGE. pFGE is a paralog protein we used a secretory form of pFGE. pFGE is a paralog of FGE that lacks FGE activity. Both share a unique fold, high sequence identity (Fig. 4A), which is lacking in FGE (Fig. 4B). The secretion of N-terminally truncated FGE is biologically inactive. A, wild-type (wt) FGE (open circles) or Δ34–68FGE (filled circles), both equipped with a C-terminal His6 tag, were stably transfected into galactose-6-sulfatase-expressing HT1080 cells. The concentrations of FGE and galactose-6-sulfatase were quantified by Western blotting and determination of cell protein. The concentration of galactose-6-sulfatase was comparable in all cell lysates (not shown). The specific activity of galactose-6-sulfatase (Gal6S) is expressed as relative specific activity by referring to that in cells expressing Gal6S only (normalized to 1 relative value, see “Experimental Procedures”). B, STS, wild-type FGE, Δ34–68FGE, and Δ34–68FGE-KDEL were transiently co-expressed in MSDi Tet-On cells in the combinations indicated above the lanes. The FGE constructs carried a C-terminal HA tag, which was extended by a KDEL tetrapeptide or not as indicated. In the cell extracts the activity of STS was determined, and the amounts of STS and FGE were quantified by Western blotting. The two bands for Δ34–68FGE and Δ34–68FGE-KDEL represent differentially N-glycosylated forms. The band marked by an asterisk represents N-terminally truncated FGE resulting from proteolytic cleavage at arginine 72 (11). The specific activity of STS was referred to that in cells expressing STS only.

(FA) The secretion of Δ34–68FGE-KDEL was greatly reduced and even lower than that of wild-type FGE. Immunofluorescence of Δ34–68FGE-KDEL showed a typical ER-staining pattern and co-localization with the ER marker protein disulfide isomerase (Fig. 3B). Co-expression of the ER-retained Δ34–68FGE-KDEL with steroid sulfatase only slightly increased steroid sulfatase activity but was still nearly 50-fold less efficient than observed for wild-type FGE (Fig. 1B, second and fourth lanes). These data clearly show that ER retention is not sufficient to restore the biological activity of Δ34–68FGE.
FGE in Vivo Function and ER Retention

A

| Residues | FGE | pFGE |
|----------|-----|------|
| 34–68FGE | . . . | RQL AHS KMP1 PAGVFT MGT. | . . . |
| 34–88FGE | . . . | NTPDS ASHLNL GFCRAA DR PTMD |
| 27 | AT5MQLQGGRFLMGTTT | NTPDS ASHLNL GFCRAADGP PPE |

B

| Chase (h) | wt-FGE | pFGE - PGEL | FGE1–88-pFGE - PGEL |
|-----------|--------|-------------|---------------------|
| 0         | C      | C           | C                   |
| 3         | C      | M           | M                   |
| 6         | M      | M           | M                   |
| 3         | C      | C           | C                   |
| 6         | M      | M           | M                   |
| 0         | C      | C           | C                   |
| 3         | M      | M           | M                   |
| 6         | M      | M           | M                   |

Intracellular pFGE (% of total)

- FIGURE 4. The N terminus of FGE mediates retention of pFGE - PGEL. A, alignment of the N and C termini of FGE and pFGE. The N- and C-terminal amino acid sequences of human FGE (residues 34–103 and 352–374) and pFGE (residues 27–42 and 277–301) are shown. Residues 1–33 of FGE and 1–26 of pFGE represent cleavable signal peptides. Sequence homology between FGE and pFGE is highest between residues 101 and 368 of FGE, which correspond to residues 40–293 of pFGE showing a sequence identity of 47.2%; only the N- and C-terminal borders of this homologous region are shown. For FGE the two N-terminal cysteines (Cys-50 and 52) and glutamate 73, which represents the N terminus after furin cleavage, are indicated. The C-terminal ER retention signal of pFGE is underlined. B, wild-type (wt) pFGE, pFGE - PGEL, and FGE1–88-pFGE - PGEL were transiently expressed in HT1080 Tet-On cells. 6 h after transfection the cells were induced with 10 ng/ml doxycycline. 12 h after induction with doxycycline, cells were metabolically labeled for 90 min. After a chase for 0, 3, and 6 h, cells and media were collected. pFGE was immunoprecipitated from cell extracts (C) and media (M) and analyzed by SDS-PAGE and phosphorimaging. Filled arrows indicate the full-length pFGE forms, and the open arrow indicates the N-terminally truncated form of the FGE1–88-pFGE - PGEL fusion protein resulting from cleavage in the secretory pathway (11). In the secretions up to three differentially N-glycosylated forms of pFGE are observed. The intracellularly retained pFGE is given as a percentage of total pFGE in cells plus medium.

(17) A major structural difference between the two paralogous proteins is the N-terminal region of FGE (residues 1–88, with residues 1–33 being cleaved off by signal peptidase), which is lacking in pFGE (Fig. 4A). We reasoned that transplanting residues 1–88 of FGE to the N terminus of mature pFGE - PGEL, i.e. to alanine-27 (Fig. 4A), would help to preserve possible functions of the fused FGE residues 34–88 due to the structural similarity of the pFGE carrier with FGE. The retention was determined after metabolic labeling of HT1080 Tet-On cells transiently expressing pFGE, pFGE - PGEL, or the FGE1–88-pFGE - PGEL fusion protein by following the distribution of newly synthesized pFGE forms during a chase for up to 6 h (Fig. 4B). After chasing for 6 h, 65% of wild-type pFGE and only 12% of pFGE - PGEL were recovered intracellularly, confirming the secretory nature of the latter. Fusing FGE residues 1–88 to pFGE - PGEL increased the retention to 34%, indicating that residues 34–88 of FGE harbor a transferable ER retention signal. Immunofluorescence showed that the preferential co-localization of pFGE - PGEL with the Golgi marker GM130 by fusing FGE residues 1–88 to its N terminus was shifted into a reticular pattern (Fig. 5A), then colocalizing, like wild-type pFGE, with the ER marker protein disulfide isomerase (Fig. 5B). The presence of a transferable ER retention signal within FGE residues 34–88 was further confirmed by fusing FGE residues 1–88 to lysozyme, a secretory protein. The fraction of intracellularly retained lysozyme increased from 1% for wild-type lysozyme to 77% for lysozyme carrying the N terminus of FGE (data not shown). An FGE-pFGE Fusion Protein Carrying Residues 34–88 of FGE Restores in Trans the Biological Activity of N-terminally truncated FGE—Next we examined whether the pFGE - PGEL-carrying residues 1–88 of FGE at its N terminus could restore the biological activity of N-terminally truncated FGE. Co-expression of steroid sulfatase with FGE, but not with truncated FGE, leads to the synthesis of active steroid sulfatase (see Fig. 1B and Fig. 6A, lanes 1–3). Co-expression of steroid sulfatase with the N-terminal fragment 1–88 of FGE fused to the secretory form of pFGE (FGE1–88-pFGE - PGEL) had no effect on steroid sulfatase activity (Fig. 6A, lane 4). However, when co-expressing FGE1–88-pFGE - PGEL and, in addition, truncated FGE, activation of steroid sulfatase was partially restored, as was observed in three independent experiments (Fig. 6A, lane 5). Co-expression of wild-type pFGE with truncated FGE did not restore the activation of steroid sulfatase (Fig. 6A, lane 6), demonstrating that the restoration of FGE activity by the FGE-pFGE fusion protein is due to its FGE1–88 part. Co-expression of the FGE1–88-pFGE - PGEL fusion protein with wild-type FGE showed about the same activation of steroid sulfatase as observed for wild-type FGE alone (Fig. 6A, compare lanes 2 and 7). Fusing FGE residues 1–88 to other carrier proteins such as lysozyme or enhanced cyan fluorescent protein and co-expressing the fusion proteins with N-terminally truncated FGE and steroid sulfatase did not lead to an activation of steroid sulfatase (data not shown). Restoration of the biological activity of Δ34–68FGE apparently requires the presentation of the N-terminal residues 34–88 by a carrier sharing structural similarity with FGE.

In an attempt to characterize further the interaction of FGE1–88-pFGE - PGEL and N-terminally truncated FGE, underlying the ability of the former to transactivate the latter, we analyzed whether FGE1–88-pFGE - PGEL is able to improve ER retention of Δ34–68FGE in the absence of a C-terminal KDEL signal. When expressed alone, indirect immunofluorescence analysis detected FGE1–88-pFGE - PGEL in the ER (see above, Fig. 5, A and B), whereas the steady-state localization of intracellular Δ34–68FGE is mainly the Golgi (Fig. 5C, upper panels). This localization did not change when co-expressing the two components together (Fig. 5C, lower panels),
indicating that their interaction is not strong enough to impose ER co-localization. It should be noted, however, that FGE1–88-pFGE/H9004PGEL itself is only moderately retained in the ER, i.e. clearly less efficient than wild-type pFGE (Fig. 4B). These considerations prompted us to do the experiment the other way round, namely to efficiently retain /H900434–68FGE in the ER by adding a KDEL signal and to quantify its effect on retention of FGE1–88-pFGE/H9004PGEL. The results obtained (Fig. 6B) indicate

with 10 ng/ml doxycycline. pFGE forms were detected with pFGE rabbit antiserum (red) and the Golgi marker GM130 (in A) or the ER marker protein disulfide isomerase (PDI) (in B) with a mouse monoclonal antibody (green). The merge reveals a co-localization of the pFGE forms with GM130 in yellow. C, /H900434–68FGE was transiently expressed (at 1 μg/ml doxycycline) alone or together with FGE1–88-pFGE/H9004PGEL as indicated and detected by a polyclonal FGE antibody (red). Golgi, ER markers, or FGE1–88-pFGE/H9004PGEL (green) were detected as in A and B.

FIGURE 5. Subcellular localization of FGE1–88-pFGEΔPGEL and Δ34–68FGE in HT1080 cells. A and B, wild-type pFGE, pFGEΔPGEL, and FGE1–88-pFGEΔPGEL were transiently expressed in HT1080 Tet-On cells and induced with 10 ng/ml doxycycline. pFGE forms were detected with pFGE rabbit antiserum (red) and the Golgi marker GM130 (in A) or the ER marker protein disulfide isomerase (PDI) (in B) with a mouse monoclonal antibody (green). The merge reveals a co-localization of the pFGE forms with GM130 in yellow. C, /H900434–68FGE was transiently expressed (at 1 μg/ml doxycycline) alone or together with FGE1–88-pFGE/H9004PGEL as indicated and detected by a polyclonal FGE antibody (red). Golgi, ER markers, or FGE1–88-pFGE/H9004PGEL (green) were detected as in A and B.

FIGURE 6. Restoring the biological activity of truncated FGE in trans by co-expression of the FGE N terminus fused to pFGE. A, MSDi Tet-On cells were transiently transfected with the indicated combinations of cDNAs (see "Experimental Procedures"). The amounts of STS, FGE, and pFGE were monitored in the cell extracts by Western blotting. The specific activity of STS was referred to that in cells expressing STS only. Data shown are representative for three independent experiments; the statistics for the specific STS activity values were as follows: 47 ± 3.5 (lane 2), 3.6 ± 0.8 (lane 3), 1.6 ± 0.4 (lane 4), 15.1 ± 0.8 (lane 5), 1.8 ± 0.7 (lane 6). wt, wild type. B, coexpression of Δ34–68FGE-KDEL improves retention of FGE1–88-pFGEΔPGEL. HT1080 Tet-On cells were transiently transfected with the indicated cDNAs and expressed from a bidirectional promoter. Cells (C) and media (M) were collected after 12 h of induction with 2 μg/ml doxycycline. The amounts of FGE1–88-pFGEΔPGEL and Δ34–68FGE-KDEL in cells and medium were quantitated by Western blotting. Data shown are representative for three independent experiments; the KDEL on Δ34–68FGE improved intracellular retention of FGE1–88-pFGEΔPGEL by a factor of 1.44 ± 0.16 (rightmost sample/center sample).
FGE in Vivo Function and ER Retention

that Δ34–68FGE-KDEL slightly, but reproducibly, promotes intracellular retention of FGE1–88-pFGEΔPGEL (shifting from 17–20 to 28–30%, measured after 12 h of doxycycline-induced co-expression). Because this effect was a consequence of efficient ER retention of Δ34–68FGE imposed by the presence of KDEL (Fig. 6B, center sample/right-most sample), this finding indicates that both components do interact in the ER.

**Cysteine 52 Is Critical for the Biological Activity of FGE**—Previously we have shown that FGE residues 34–68 mediate homodimerization of FGE and that the homodimers are stabilized by disulfide bonding (11). The N-terminal extension of FGE contains a fully conserved Cys-Gly-Cys motif (see “Discussion”) with the two cysteines at positions 50 and 52. We mutated cysteine 50 and/or cysteine 52 to alanine and co-expressed the C50A, C52A, and C50A/C52A FGE mutants together with steroid sulfatase in MSDi Tet-On cells (Fig. 7A). Mutation of cysteine 52 clearly interfered with the activation of steroid sulfatase as did the mutation of both cysteine residues. Mutation of cysteine 50 decreased the biological activity of FGE, but activation of the sulfatase was still half as effective as by wild-type FGE. These data indicate that cysteine 52 is critical for the biological activity of FGE. Importantly, cysteine 52 is also critical for trans-activating Δ34–68FGE-KDEL by FGE1–88-pFGEΔPGEL, thereby confirming that this cysteine residue plays a crucial role in the activation of the FGE catalytic core

**DISCUSSION**

The presented data allow the assignment of two distinct functional properties to the N-terminal extension of FGE. On the one hand it confers retention of FGE in the ER; on the other hand it serves an auxiliary function in the process of sulfatase activation that obviously is essential in vivo.

The generation of active sulfatases depends on the oxidation of a specific cysteine in nascent sulfatase polypeptides to a FGly residue. Although the FGly-generating activity of FGE resides in its catalytic core domain comprising residues 89–374, this study shows that the in vivo generation of FGly residues in nascent sulfatase polypeptides requires not only the catalytic core domain of FGE but also the N-terminal extension of FGE. This extension is encoded in the first exon of eukaryotic FGE-encoding SLIMFI genes and comprises an orthologously placed Cys-Gly-Cys motif that can be found without exception from mammals, bird, lizard, amphibians, fish, tunicates, urchin, insects, cnidarian, and sponge to even unicellular eukaryotes (Emiliana huxleyi).5 We could show that FGE residues 34–68 are required for the in vivo function of FGE, whereas residues 34–88 are sufficient to restore the generation of active sulfatases by N-terminally truncated FGE. This functional complementation required the fusion of FGE residues 1–88 (with residues 1–33 being cleaved off by signal peptidase) to the structurally unrelated pFGE. Fusion of FGE residues 1–88 to structurally unrelated carriers such as lysozyme or enhanced cyan fluorescent protein or expression of the FGE1–88 alone did not restore the activation of sulfatases when co-expressed with the catalytic core domain of FGE (data not shown). We conclude from these observations that the N-terminal extension of mature FGE, i.e. residues 34–88, confers functional complementation and that fusion to a structurally related carrier is required to impose an active conformation to FGE residues 34–88. Surprisingly, this active conformation of a non-catalytic FGE–pFGE fusion is able to transmit enzymatic functionality onto the catalytic core residing in the truncated FGE partner. This trans effect obviously involves

![Figure 8](image_url)

**FIGURE 8.** Intracellular retention of FGE is independent of cysteine residues 50 and 52. HT1080 Tet-On cells were transiently transfected with FGE and the FGE mutants C50A, C52A, and C50A/C52A. Cells (C) and media (M) were collected 30 h after induction with doxycycline (2 μg/ml) and analyzed for FGE by Western blotting. For FGE forms in the medium see Fig. 2. wt, wild type.

5 T. H. Pringle, personal communication.
heterodimer formation, as evidenced further by an increased intracellular retention of FGE1–88-pFGE∆PGEL due to co-expression of a KDEL-tagged and, therefore, ER-resident form of Δ34–68FGE.

FGE residues 34–68 are also required for the retention of FGE in the ER, and residues 34–88 are sufficient to mediate the retention of passenger proteins in the ER. Whether the shorter fragment such as residues 34–68 can act as a transferable ER retention signal needs to be tested. The mechanism of ER retention of FGE is not clear. The structure of the oligosaccharide of intracellular FGE indicated that FGE is only exposed to α-mannosidases, which can trim up to 5 of the 9 mannose residues of the Manα-GlcNac2 oligosaccharide linked to asparagine 141 (11) but not to one of the elongating glycosyltransferases of which N-acetylglucosaminyl transferase I, catalyzing the first of the elongation reactions in N-linked oligosaccharides, resides in the medial Golgi (24). This supports the notion that the N-terminal extension of FGE mediates either its retention in the ER or its retrieval to the ER through a recycling pathway involving compartments proximal to the medial Golgi.

It is interesting to note that the retention of N-terminally truncated FGE through KDEL-mediated recycling is not sufficient to ensure the generation of FGly residues in nascent sulfatase polypeptides. Based on the data of this study we hypothesize that the N-terminal extension of FGE mediates interaction with an ER-localized component, regulating the retention of FGE in the ER as well as the generation of FGly residues in nascent sulfatase polypeptides. The nature of this component in the ER needs to be identified. Its regulatory function on FGE biological activity may be related to the recruitment of nascent sulfatase substrates or to provide reducing cofactors (e.g. cysteine thiols) to the mixed-functional oxygenase reaction that in vitro are provided by dithiothreitol or other artificial thiols (7, 11).

As mentioned above, the N-terminal extension contains two fully conserved cysteine residues in positions 50 and 52 as part of a Cys-Gly-Cys motif. Disulfide bonds in CXC motifs are predicted to be labile (25) and expected to transfer readily to other dithiols. In Erv2p, a sulfhydryl oxidase generating disulfide isomerase using oxygen as a terminal electron acceptor. It is interesting to note that the retention of N-terminally truncated FGE through KDEL-mediated recycling is not sufficient to ensure the generation of FGly residues in nascent sulfatase polypeptides. Based on the data of this study we hypothesize that the N-terminal extension of FGE mediates interaction with an ER-localized component, regulating the retention of FGE in the ER as well as the generation of FGly residues in nascent sulfatase polypeptides. The nature of this component in the ER needs to be identified. Its regulatory function on FGE biological activity may be related to the recruitment of nascent sulfatase substrates or to provide reducing cofactors (e.g. cysteine thiols) to the mixed-functional oxygenase reaction that in vitro are provided by dithiothreitol or other artificial thiols (7, 11).

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