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Association between the 15-kDa Selenoprotein and UDP-glucose:Glycoprotein Glucosyltransferase in the Endoplasmic Reticulum of Mammalian Cells*

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Mammalian selenocysteine-containing proteins characterized with respect to function are involved in redox processes and exhibit distinct expression patterns and cellular locations. A recently identified 15-kDa selenoprotein (Sep15) has no homology to previously characterized proteins, and its function is not known. Here we report the intracellular localization and identification of a binding partner for this selenoprotein which implicate Sep15 in the regulation of protein folding. The native Sep15 isolated from rat prostate and mouse liver occurred in a complex with a 150-kDa protein. The latter protein was identified as UDP-glucose:glycoprotein glucosyltransferase (UGTR), the endoplasmic reticulum (ER)-resident protein, which was previously shown to be involved in the quality control of protein folding. UGTR functions by glucosylating misfolded proteins, retaining them in the ER until they are correctly folded or transferring them to degradation pathways. To determine the intracellular localization of Sep15, we expressed a green fluorescent protein-Sep15 fusion protein in CV-1 cells, and this protein was localized to the ER and possibly other perinuclear compartments. We determined that Sep15 contained the N-terminal signal peptide that was essential for translocation and that it was cleaved in the mature protein. However, C-terminal sequences of Sep15 were not involved in trafficking and retention of Sep15. The data suggest that the association between Sep15 and UGTR is responsible for maintaining the selenoprotein in the ER. This report provides the first example of the ER-resident selenoprotein and suggests a possible role of the trace element selenium in the quality control of protein folding.

Selenium is an essential trace element in the diet of many organisms, including humans. It is present in the form of a selenocysteine (Sec)[1] residue in several naturally occurring enzymes and proteins (1, 2). In selenoenzymes with established function, such as glutathione peroxidases, thyroid hormone deiodinases, and thioredoxin reductases in mammals, and hydrogenases and formate dehydrogenases in bacteria and archaea, Sec is present at the enzyme active center and participates in various redox reactions (2).

Functions of many other mammalian selenoproteins, including the 15-kDa selenoprotein (Sep15), selenoprotein P, selenoprotein W, selenoprotein R (also named selenoprotein X), selenoprotein T, and selenoprotein N, have not been established. However, most of these proteins have clearly identifiable Sec-containing redox motifs, such as the Cys-Xaa-Xaa-Sec motif in selenoprotein W and selenoprotein T, suggesting their possible involvement in redox processes (3).

Sep15 was recently identified in human T-cells (4). The gene for this protein is expressed in various human tissues with highest expression levels in the prostate and thyroid. In addition to humans, genes encoding Sep15 were detected in mice and rats. Sep15 exhibits no homology to previously characterized proteins, which precluded its functional characterization. However, Sep15 has a highly conserved motif, Cys-Gly-Sec-Lys, suggesting that this center could constitute an active center, in which Sec and Cys form a reversible seleno-sulfide bond. Besides this putative redox center, a previously noted feature in the Sep15 sequence was the lack of N-terminal sequences in the isolated human T-cell selenoprotein, which suggested the possibility of post-translational processing of the protein. In addition, Sep15 migrated as the 15-kDa protein on SDS-PAGE gels, whereas the migration properties of the native protein were consistent with a protein of ~160–240 kDa. The low abundance of the 15-kDa selenoprotein in human T-cells and its lability during isolation did not permit isolation of the native protein to homogeneity to test whether the 160-kDa complex was composed of multiple selenoprotein subunits or if other protein components were involved in the complex (4).

The finding that the protein was expressed in the prostate at elevated levels compared with other tissues (4, 5) provided an opportunity to determine the oligomeric composition of Sep15 by isolating the selenoprotein from this organ. In this report, we describe isolation of Sep15 from rat prostate and mouse liver. In both preparations, the native selenoprotein occurred as a complex with UDP-glucose:glycoprotein glucosyltransferase (UGTR), an enzyme involved in the quality control of protein folding (6). Further characterization revealed that Sep15 was located in perinuclear cellular compartments, consistent with the finding that UGTR is located in the endoplasmic reticulum (ER). The observation that Sep15 was found only in a complex with UGTR suggests that it may be involved in the regulation of protein folding.

EXPERIMENTAL PROCEDURES

Materials—Rat prostate and mouse liver were purchased from Pel-Freez. [^{75}Se]Selenious acid (specific activity 1,000 Ci/mmol) was from the University of Missouri Research Reactor Facility (Columbia, MO).

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[1] The abbreviations used are: Sec, selenocysteine; Sep 15, 15-kDa selenoprotein; PAGE, polyacrylamide gel electrophoresis; UGTR, UDP-glucose:glycoprotein glucosyltransferase; ER, endoplasmic reticulum; HPLC, high performance liquid chromatography; GFP, green flourescent protein; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride; DTT, dithiothreitol.

Phenyl-TSK and DEAE-TSK HPLC columns were from TosoHaas. The phenyl-Superose FPLC column, Q-Sepharose, and ConA-Sepharose were from Amersham Pharmacia Biotech. LipofectAMINE was from Life Technologies, Inc. Polyclonal anti-green fluorescent protein (GFP) antibodies were from Invitrogen. *Escherichia coli* strain NovaBlue was from Novagen. The Maxi kit for plasmid isolation was from Qiagen, BODIPY®TR ceramide was from Molecular Probes, native and SDS-PAGE gels and immunoblot membranes were from Novex, and immunoblot detection systems ECL-Plus and SuperSignal were from Amersham Pharmacia Biotech and Pierce, respectively. Other reagents were of the highest quality available.

^{75}Se Labeling of the 15-kDa Protein—Rat cell line RLE was grown on RPMI 1640 medium for 48 h in the presence of 0.5 mCi of sodium [^{75}Se]selenite/100 ml of cell culture medium. Cells were washed four times with phosphate-buffered saline, collected by trypsinization, and stored at −80 °C prior to use. ^{75}Se-Labeled mouse liver was obtained as described (7). Briefly, 0.5 mCi of freshly neutralized [^{75}Se]selenious acid was injected intraperitoneally, the mouse was sacrificed 48 h later, and ^{75}Se-labeled tissues were collected and stored at −80 °C prior to use.

Isolation of Sep15 from Rat Prostate—133 g of rat prostate was mixed with ^{75}Se-labeled rat RLE cells and homogenized at 4 °C in 400 ml of 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM AEBSF, 1 mM DTT, 5 μg/ml leupeptin, and 5 μg/ml aprotinin, and the homogenate was further treated by sonication. Insoluble material was removed by centrifugation, and the supernatant was applied to a 200-ml Q-Sepharose column equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 1 mM DTT (buffer A). After washing the column with 2 volumes of buffer A, the bound proteins were eluted by application of a linear gradient from buffer A to 1 M NaCl in buffer A. ^{75}Se was determined with a γ-counter, and column fractions containing peaks of radioactivity were analyzed by SDS-PAGE followed by detection of ^{75}Se on gels with a PhosphorImager. Fractions containing the ^{75}Se-labeled Sep15 were identified based on migration properties of this protein on SDS-PAGE, combined, concentrated, made in 1 M NaCl, filtered, and applied to a phenyl-HPLC column equilibrated in 1 M NaCl in buffer A. The protein was eluted by application of a linear gradient from 1 M NaCl in buffer A to buffer A, and the column was washed further with a short gradient from buffer A to water, which eluted Sep15. Fractions containing ^{75}Se were analyzed by SDS-PAGE followed by the PhosphorImager analysis as described above. Fractions containing Sep15 were then applied directly to a DEAE-HPLC column equilibrated in buffer A. Sep15 was eluted with a gradient from buffer A to 0.5 M NaCl in buffer A. Radioactive fractions were concentrated and stored at −80 °C until ready for use.

Gel Electrophoretic Analyses of the Rat Prostate 15-kDa Selenoprotein—Fractions containing Sep15 were analyzed on native and SDS-PAGE gels. To determine if the ~160-kDa protein band, which appeared on native gels and was labeled with ^{75}Se, contained Sep15 and UGTR, a native PAGE gel was briefly stained with Coomassie Blue, the band of interest cut from the gel, minced, incubated overnight in SDS-PAGE sample buffer containing 10 mM DTT, and the liquid fraction analyzed by SDS-PAGE. The 150-kDa protein band, which appeared on an SDS-PAGE gel after staining with Ponceau S, was used for the subsequent sequence analysis.

Tryptic Peptide Analysis of the 150-kDa Protein Band—A gel slice containing a Ponceau S-stained 150-kDa protein band was sent to Harvard Microchem (Boston) where the protein was digested with trypsin, and the resulting peptides were separated by reverse phase chromatography and their masses determined by electrospray mass spectrometry. Four tryptic peptides were sequenced by Edman degradation comprising a total of 88 amino acid residues.

Isolation of the 15-kDa Selenoprotein from Mouse Liver—Initial fractionations of mouse liver Sep15 and UGTR were carried out as described for rat prostate. Subsequent isolations took advantage of the fact that UGTR could be isolated efficiently by affinity chromatography on a ConA-Sepharose resin. 150 g of mouse liver was mixed with 3 g of ^{75}Se-labeled mouse liver and homogenized at 4 °C in 3 volumes of buffer A containing 0.5 mM sodium orthovanadate and protease inhibitors used in the rat prostate fractionation. The homogenate was sonicated, clarified by centrifugation, and the clear supernatant was applied to a Q-Sepharose column equilibrated with buffer A. The bound proteins were eluted using a linear 0–1.5 M gradient of NaCl in buffer A. Fractions were analyzed for the presence of ^{75}Se-labeled proteins as described for rat prostate. The fractions containing labeled Sep15 were combined, concentrated, and applied to a ConA-Sepharose column that was equilibrated with 20 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl (buffer B). The column was washed with buffer B, and proteins were eluted by the application of a step gradient of 10, 50, 100, 200, and 300 mM methyl α-D-mannopyranoside in buffer B. Fractions containing ^{75}Se-labeled Sep15 were pooled, concentrated, and applied to a phenyl-HPLC column as described for rat prostate. The protein was eluted in two overlapping peaks, the first peak containing both UGTR and Sep15, and the second peak containing only UGTR. Fractions containing Sep15 were pooled and subsequently applied onto a DEAE-HPLC column as described above. Proteins eluted from the DEAE column were analyzed by native and SDS-PAGE and by immunoblot assays with antibodies specific for Sep15 or UGTR.

Constructs with GFP—N-Sep15-C-GFP denotes the following: N is the 28-residue N-terminal signal peptide of Sep15; Sep15 is the 15-kDa selenoprotein without its signal peptide and 4 C-terminal residues; C is the C-terminal tetrapeptide of Sep15; and GFP is the 239-residue GFP. N-Sep15-C-GFP and Sep15-C-GFP constructs were made using the pEGFP-N1 expression vector. Human Sep15 cDNA (U93C), in which the Sec codon, TGA, was mutated to a cysteine codon, TGC, was amplified with primers T7 and XhoI-15-1h (5′-CCACTCGAGGCGTTC-CAACTTTTCACT-3′) and designated N-Sep15-C-GFP. U93C amplified with primers Sal-15-1h (5′-CGCAACGTCGACATGTCTGCTTTT-GGGGCAGAG-3′) and XhoI-15-1h was designated Sep15-C-GFP. The resulting polymerase chain reaction products were cloned into the *Xho*I site of pEGFP-N1. The GFP-Sep15-C construct was made using the pEGFP-C3 expression vector. Human Sep15 mutant cDNA, U93C, was amplified with primers Sal-15-1h and T3 and cloned into the *Xho*I/*Bsp*120I sites of pEGFP-C3. The fragment encoding N-terminal sequences of the 15-kDa protein was obtained by amplification of the U93C cDNA with primers T7-*Nhe*I, 5′-CGATGCTAGCTAATACGACT-CACTATAGGG-3′, and *Age*I-15-1h, 5′-CACGACCGGTGCCTCCGAT-GAAAACTCTGCC-3′. N-GFP-Sep15-C and N-GFP constructs were made by cloning this fragment into the *Nhe*I/*Age*I sites of GFP-Sep15-C and pEGFP-N1, respectively. The N-GFP-Sep15 construct was obtained by mutagenesis of N-GFP-Sep15-C with primers 15-1h-159stopF, 5′-CCTGAGTGAAAAGTAGGAACGCATATAAATCTTGC-3′ and 15-1h-159stopR, 5′-GCAAGATTTATATGCGTTCCTACTTTTCACTCAGG-3′. All constructs were transformed into the *E. coli* strain NovaBlue, and the plasmids were isolated using a Maxi kit.

Immunoblot Analyses—Immunoblot assays with rabbit polyclonal antibodies raised against UGTR isolated from rat liver (8) and against the keyhole limpet hemocyanin-conjugated synthetic peptide corresponding to the C-terminal portion of Sep15 (4) were performed using the ECL-Plus detection system. Rabbit polyclonal antibodies specific for GFP were used for the detection of GFP-Sep15 fusion proteins with an ECL system.

Cell Growth, Transfection, and Dual Fluorescence Imaging Confocal Microscopy—Growth of monkey CV-1 cells and transfection were carried out as described (9). 5 μg of plasmid DNA and 30 μl of LipofectAMINE were used for transfection of each 60-mm plate. CV-1 cells transfected with the appropriate constructs were incubated for 12 h in a CO_2 incubator. We used a fluorescent BODIPY®TR ceramide as a reference marker for perinuclear structures. This reagent has been shown to be accumulated in the ER and Golgi and has been used to study protein trafficking (10, 11). The transfected cells were rinsed with serum-free Dulbecco's modified Eagle's medium containing 10 mM HEPES (DMEM-HEPES) and then incubated for 25 min at room temperature in the same medium containing 2 μM BODIPY®TR ceramide. The cells were washed twice in serum-free DMEM-HEPES and were used immediately for image collection. Double-labeled images of live cells were collected with a water immersion lens using a dual excitation/emission and dual channel mode on a Bio-Rad MRC1024ES laser-scanning microscope.

RESULTS

Native Rat Prostate Sep15 Is Composed of Multiple Polypeptides—Sep15 was previously isolated from a human T-cell line, but small amounts of isolated proteins and protein lability precluded molecular characterization of native Sep15 (4). Subsequent studies found that the protein is expressed at higher levels in the prostate (4, 5). In the present study, a procedure for isolation of Sep15 from rat prostate was developed which included fractionation of protein extracts on conventional Q-Sepharose and phenyl-HPLC columns followed by an HPLC procedure on a DEAE column. To allow efficient detection of Sep15 in chromatographic fractions by tracing γ-radioactivity, rat prostate homogenates were mixed with the ^{75}Se-labeled extracts obtained from a rat cell line, and the combined extract

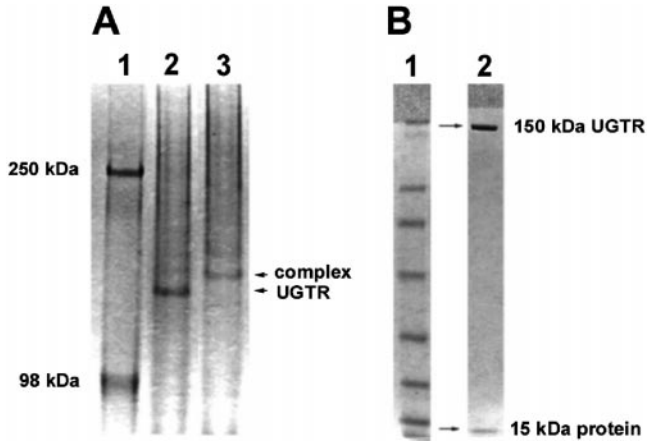

FIG. 1. **Gel electrophoretic analysis of the UGTR·Sep15 complex.** Rat prostate extracts were fractionated sequentially using Q-Sepharose, phenyl-HPLC, and DEAE-HPLC columns. Isolation of Sep15 was followed by immunoblot assays. In *A*, Coomassie Blue staining of a native gradient PAGE gel is shown, and the lanes contain: *1*, protein standards; *2*, 150-kDa band (UGTR); and *3*, 165-kDa band (the UGTR·Sep15 complex). In *B*, Coomassie Blue staining of an SDS-PAGE gel is shown, and the lanes contain: *1*, protein standards (6–180-kDa range); and *2*, Sep15 preparation. The material for *lane 2* was obtained by excising a protein band similar to that shown in *panel A*, *lane 3*, from the gel, soaking it overnight in SDS sample buffer in the presence of 10 mM DTT, and analyzing the extracted proteins by SDS-PAGE.

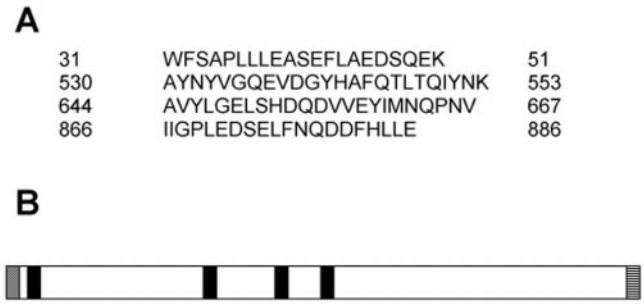

FIG. 2. **Internal peptide sequences of rat prostate UGTR.** In *A*, amino acid sequences of four peptides obtained by tryptic digest of isolated rat liver UGTR are shown. Residue *numbers* correspond to the rat liver UGTR sequence. In *B*, the relative location of the four peptides (*filled boxes*) within the putative UGTR from rat liver (*long*, *rectangular box*) is shown. Other features shown within UGTR are the N-terminal signal peptide (*diagonal pattern*) and the C-terminal ER retention signal (*horizontal pattern*).

was used for further isolation. Immunoblot and PhosphorImager detection of Sep15 on gradient nondenaturing and SDS-denaturing PAGE gels in fractions from the first two columns revealed that the native protein migrated as a large, ~165-kDa, species. However, these same fractions migrated as the 15-kDa species on SDS-PAGE gels (data not shown). Although native gradient PAGE does not accurately determine the molecular weight of a protein, an 11-fold difference in masses between denaturing and nondenaturing conditions suggested that the native Sep15 was bound to another protein or proteins in the rat prostate or was composed of multiple identical selenoprotein subunits. These data were also consistent with the previous electrophoretic and gel filtration experiments of partially purified human T-cell Sep15 (4).

Native Rat Prostate Sep15 Is Associated with the 150-kDa Protein—Electrophoresis of the protein preparation isolated by the three purification steps described above is shown in Fig. 1. Native gel electrophoresis revealed a Coomassie Blue-stained band of ~165 kDa (Fig. 1*A*, *lane 3*). This band contained Sep15 as it was labeled with ^{75}Se and was immunoreactive with anti-Sep15 antibodies (data not shown). SDS-PAGE analysis of the 165-kDa band showed that it consisted of a 150-kDa protein and Sep15 (Fig. 1*B*, *lane 2*). It was noted that the 150-kDa protein was also present in a selenoprotein-free form (Fig. 1*A*, *lane 2*) which eluted later than the 150-kDa protein-Sep15 complex from a phenyl-HPLC column. In contrast, we were not able to detect Sep15 that was free of the 150-kDa protein in rat prostate fractions.

The 150-kDa Protein Is UGTR—The 150-kDa protein was digested with trypsin, and the sequence of four peptides consisting of a total of 88 amino acid residues was determined (Fig. 2). Two of these peptides were not homologous to any known sequences, but the other two showed partial sequence homology with *Drosophila melanogaster* UGTR (GenBank accession number U20554) (12). During these initial studies, no mammalian UGTR sequences were available in GenBank. However, all four 150-kDa protein peptides were identical with internal peptides of the rat liver UGTR sequence that was deduced from the corresponding cDNA sequence (kindly provided by Dr. A. Parodi). In addition, several mammalian UGTR sequences subsequently became available in GenBank, including rat UGTR (accession number AF200359) (13) and two human UGTRs (accession numbers AF227905 and AF227906) (14). Sequences of internal peptides obtained from the 150-kDa protein were identical with the internal sequences of the rat protein and were highly homologous with the human sequences. This further confirmed that the 150-kDa protein is UGTR. The relative locations of the four peptide sequences within rat liver UGTR are shown in Fig. 2*B*. UGTR has been shown to be responsible for the quality control of protein folding in yeast and vertebrates (6). This protein is located in the ER and functions by reglucosylating misfolded proteins in the ER lumen, thus allowing them to interact with calnexin/calreticulin chaperones (15, 16).

UGTR and Sep15 were also detected by Western analysis in fractions from a phenyl-HPLC column (Fig. 3). UGTR eluted in two overlapping peaks, the first of which contained Sep15 (Fig. 1*A*, *lane 3*; Fig. 3, *lanes 1*, *3*, *5*, and *7*), whereas the second peak did not contain the selenoprotein (Fig. 1*A*, *lane 2*; Fig. 3, *lanes 2*, *4*, *6*, and *8*). The proteins shown in Fig. 3 were separated by SDS-PAGE in the presence (Fig. 3, *lanes 1* and *2*, and *5* and *6*) and absence (Fig. 3, *lanes 2–4*, and *7* and *8*) of a reducing agent DTT, which did not influence mobility of UGTR and Sep15 on SDS-PAGE. This suggests that the isolated UGTR·Sep15 complex did not contain a significant amount of interprotein disulfide bonds.

Association between the 15-kDa Protein and UGTR in Mouse Liver—To determine whether the association between Sep15 and UGTR is a general phenomenon rather than a cell type-specific event, we fractionated mouse liver extracts and tested elution patterns of these two proteins (Figs. 4 and 5). Initially, ^{75}Se-labeled liver homogenates were fractionated on DEAE and phenyl columns, and protein fractions were analyzed for the presence of UGTR by immunoblot assays and for the presence of Sep15 by detection of ^{75}Se with a PhosphorImager. These assays revealed that UGTR and Sep15 coeluted from these columns (Fig. 4). We further attempted isolation of UGTR to near homogeneity and tested whether isolated UGTR contained Sep15. For this purpose, we utilized a ConA column, which is an affinity column used previously in the purification of UGTR (8) as an additional intermediate step. The apparently homogeneous preparation of UGTR was then analyzed by immunoblot assays for the presence of Sep15. Analyses of five subsequent fractions from the phenyl column in the procedure that employed the ConA column are shown in Fig. 5. Detection of proteins on native (Fig. 5*A*) and SDS-PAGE (Fig. 5*B*) gels with antibodies specific for rat liver UGTR, as well as the use of antibodies specific for Sep15 on native (Fig. 5*C*) and SDS-

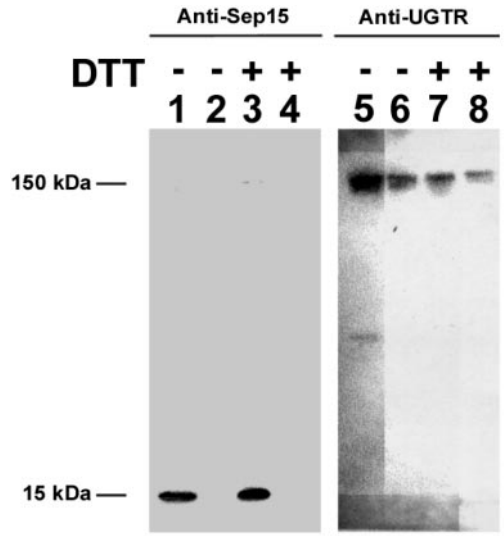

FIG. 3. **Immunoblot analyses of rat prostate Sep15 and UGTR.** Fractions containing hydrophobic proteins that eluted from a phenyl-HPLC column were examined for the presence of Sep15 and UGTR by Western blotting. Two fractions in which Sep15 and UGTR signals peaked are shown in the figure. The first eluted fraction is shown in *lanes 1*, *3*, *5*, and *7*; and the second fraction is in *lanes 2*, *4*, *6*, and *8*. Immunoblot analysis with antibodies specific for Sep15 is shown in *lanes 1–4* and with antibodies specific for UGTR, in *lanes 5–8*. *Lanes 1*, *2*, *5*, and *6* correspond to reducing SDS-PAGE (in the presence of 10 mM DTT), and *lanes 3*, *4*, *7*, and *8* correspond to nonreducing SDS-PAGE.

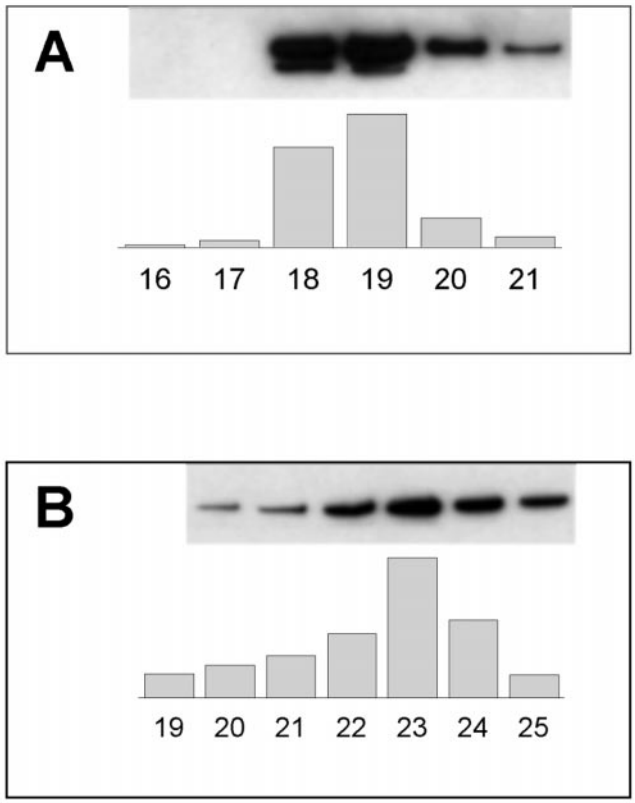

FIG. 4. **Copurification of Sep15 and UGTR in mouse liver fractions.** Elution of Sep15 and UGTR from a DEAE-HPLC column (*A*) and from a phenyl-Superose column (*B*) is shown. *Bar*s represent relative ^{75}Se radioactivity of the 15-kDa protein determined with a PhosphorImager, and bands represent immunoblot detection of UGTR with anti-rat liver UGTR antibodies in the corresponding fractions. The phenyl-Superose column did not resolve UGTR and the UGTR·Sep15 complex.

PAGE (Fig. 5*D*) gels, suggested coelution of these proteins. Overall, these data suggested that mouse liver preparations contained Sep15 bound to UGTR. However, further studies are required to address the quantitative relationship between the UGTR·Sep15 complex and the remaining cellular UGTR.

Intracellular Localization of the 15-kDa Protein—Because UGTR exhibits specific intracellular localization (ER resident protein) (17), we determined the cellular location of Sep15. For this study, we employed a set of constructs that encoded various fusion proteins between Sep15 and GFP (Fig. 6). These constructs lacked a 3′-untranslated region of Sep15 gene which contains the Sec insertion sequence element (5). All known eukaryotic selenoprotein genes, including Sep15, contain Sec insertion sequence elements, which are stem-loop structures located in 3′-untranslated regions and are necessary to dictate Sec insertion and prevent termination of protein synthesis at in-frame UGA codons (1, 18). Accordingly, to express a full-size polypeptide in the absence of the Sec insertion sequence element, TGA that encodes Sec at position 93 in Sep15 was replaced in these constructs with TGC that encodes cysteine. These mutations were made to increase translation efficiency because efficiency of selenoprotein synthesis from transfected constructs in mammalian cells is low (19). The constructs were transiently transfected into monkey CV-1 cells, and confocal microscopy was used to localize fusion proteins by detecting the GFP green fluorescence (Fig. 7). To determine the location of the transiently expressed proteins, we used a cell-permeable fluorescent ceramide conjugate, which is known to label the ER and Golgi (11, 20). In addition, expression of fusion proteins and their experimental molecular masses was obtained by assaying transfected cellular extracts by immunoblot assays with anti-GFP antibodies (Fig. 8). Sep15 was located in the membranous reticular structures in the perinuclear region, when it was transiently expressed in the form fused through its C-terminal region to GFP (Fig. 7).

The N-terminal Signal Peptide of Sep15 Is Necessary for ER Localization—Known ER resident proteins contain N-terminal signal peptides that are required for translocation of proteins into the ER. The signal peptide may be subsequently cleaved from translocated proteins. As expected, Sep15 contained a highly hydrophobic N-terminal signal peptide. We observed previously that Sep15 isolated from a human T-cell line lacked the N-terminal sequence (4), which is consistent with it being an ER luminal protein.

To test a possible role of the N-terminal peptide, we developed a construct that encoded Sep15 located downstream of GFP (the GFP-Sep15-C construct). This fusion protein, transiently expressed in CV-1 cells, was distributed in a non-ER-specific manner, including localization to the cytosol (Fig. 7). Similar patterns were also observed in cells expressing a fusion protein lacking the N-terminal peptide (Sep15-C-GFP) (Fig. 7). Thus, removal of N-terminal sequences from the selenoprotein resulted in a failure to direct Sep15 to the ER/Golgi domain, suggesting an essential role of the N-terminal peptide in selenoprotein translocation.

Sep15 Lacks an ER Retention Signal—ER resident proteins generally contain a common C-terminal tetrapeptide, KDEL, or very similar analogs. This tetrapeptide is necessary to prevent ER resident proteins from exiting the ER cellular compartment. However, the Sep15 sequence lacked such a signal and instead terminated with LERI. To determine if the last four residues of Sep15 constitute a novel ER retention signal or if this selenoprotein is retained in the ER by another mechanism, we developed a construct in which the GFP gene was inserted between the N-terminal signal peptide and the rest of Sep15 (the N-GFP-Sep15-C construct). Such a design allowed the GFP-Sep15 fusion protein to terminate on the natural C-terminal sequence of Sep15 while containing the N-terminal sig-

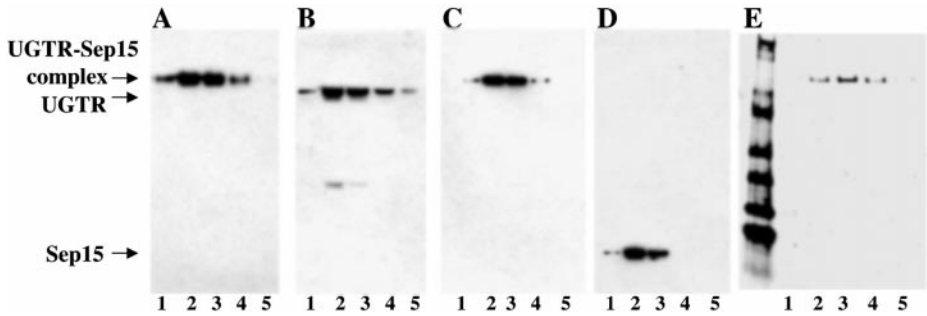

FIG. 5. **Immunoblot analyses of UGTR and Sep15 on native and SDS-PAGE gels.** Mouse liver extracts were fractionated on Q-Sepharose, ConA-Sepharose, phenyl-HPLC, and DEAE-HPLC columns as described under "Experimental Procedures." Sep15 was detected during isolation by the PhosphorImager and immunoblot assays and UGTR, by immunoblot assays. Late eluting fractions from a phenyl column were electrophoresed, transblotted, and blots were probed with antibodies specific for Sep15 or UGTR. *A*, analysis with antibodies specific for UGTR on a nondenaturing (native) PAGE gel. *B*, analysis with antibodies specific for UGTR on a SDS-PAGE gel. *C*, analysis with antibodies specific for Sep15 on a native PAGE gel. *D*, analysis with antibodies specific for Sep15 on a SDS-PAGE gel. *E*, Coomassie Blue staining of the native gel.

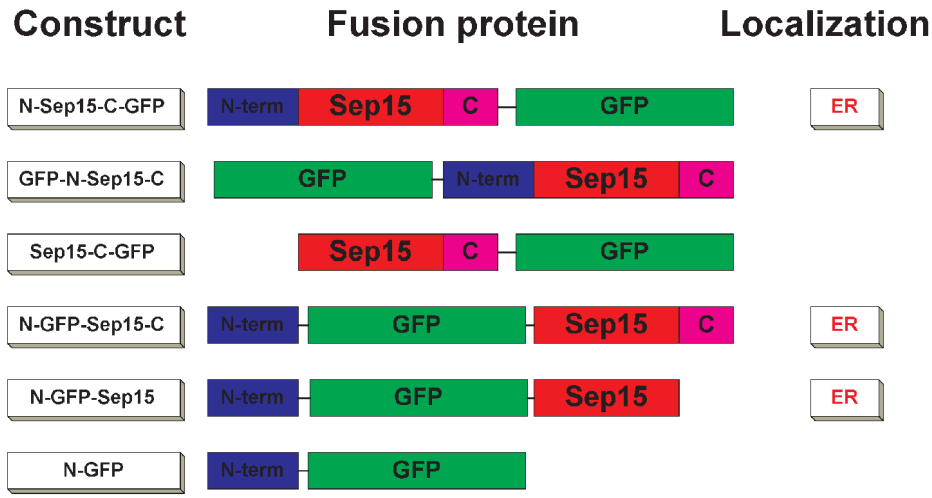

FIG. 6. **Schematic representation of selenoprotein-GFP fusion constructs.** Sep15-GFP fusion constructs were developed to determine the intracellular localization of the 15-kDa protein. *Construct*, *Fusion protein*, and *Localization* in the figure show (i) the specific construct, (ii) the organization of the fused protein, where *N* designates the 28-residue N-terminal signal peptide of Sep15; *Sep15*, the 15-kDa selenoprotein without its signal peptide and its four C-terminal residues; *C*, the 4 C-terminal residues of Sep15; and *GFP*, a 239-residue GFP; and (iii) polypeptides localized in the ER, respectively. For details, see "Results."

nal peptide for ER translocation. In addition, a construct was made which differed from the N-GFP-Sep15-C construct in that it lacked the last four residues (N-GFP-Sep15; Fig. 6). Upon transient expression in CV-1 cells, no differences were observed between fusion proteins with and without the C-terminal peptide, and both proteins were found residing in the ER/Golgi structures (Fig. 7). Thus, the C-terminal sequence was not important for intracellular trafficking and retention of Sep15.

To test further if the N-terminal signal peptide of Sep15 was alone responsible for ER localization of the selenoprotein, we expressed a GFP form containing N-terminal signal peptide of Sep15 (N-GFP). This protein was detected in the ER and other cellular compartments. Thus, the N-terminal signal was not sufficient for exclusive ER localization of the protein. These data suggested that the internal (without the N-terminal peptide and the C-terminal tetrapeptide) selenoprotein sequence was responsible for retention of the protein in the ER. It is likely that the ER-translocated Sep15 is kept in this cellular compartment through its tight interaction with UGTR, which does have the C-terminal ER retention signal and the N-terminal signal peptide.

To test whether the N-terminal single peptide was cleaved from the fusion proteins upon translocation into the ER, we calculated molecular masses for seven fusion products and for their predicted forms obtained by cleavage of the N-terminal signal peptide (Table I). These values were compared with experimental masses obtained from immunoblot assays (Fig. 8). This experiment revealed that the signal peptide of Sep15 was cleaved in every case in which it was present as an N-terminal sequence in a fusion protein, *i.e.* upstream of either Sep15 or GFP. Indeed, immunoblot assays indicated similar mobility of N-Sep15-GFP-C (Fig. 8, *lanes 2* and *4*) and Sep15-GFP-C fusion proteins (Fig. 8, *lane 3*) on SDS-PAGE gels, as well as similar mobility of GFP (Fig. 8, *lane 1*) and N-GFP (Fig. 8, *lane 8*). It should be noted that the proteins being compared should have differed by 2.7 kDa if the N-terminal signal peptide was retained. The difference of 2.7 kDa should be sufficient to be resolved by our SDS-PAGE analysis because a difference of 1.8 kDa was clearly seen when Sep15-C-GFP and GFP-Sep15-C fusion proteins were compared (see Fig. 8, *lanes 3* and *5*, respectively).

DISCUSSION

In this report, we described the association between Sep15 and UGTR in the ER of mammalian cells. The data show that Sep15 is tightly bound to UGTR, which suggests that this selenoprotein may be linked to the quality control of protein folding.

Sep15 had previously been isolated only from a human T-cell line and only under denaturing conditions (4). Thus, a possibility remained that Sep15 was a component of a multiprotein complex or a homomultimer. Isolation of Sep15 from mammalian tissues and cell lines was proven to be difficult because of its extreme lability and low abundance. However, taking advantage of the finding that Sep15 exhibits high expression levels in prostate (4), we isolated the protein from rat prostate. A procedure for isolation of Sep15 was developed which combined conventional chromatography and HPLC. This procedure allowed rapid isolation of the protein and minimized losses through denaturation.

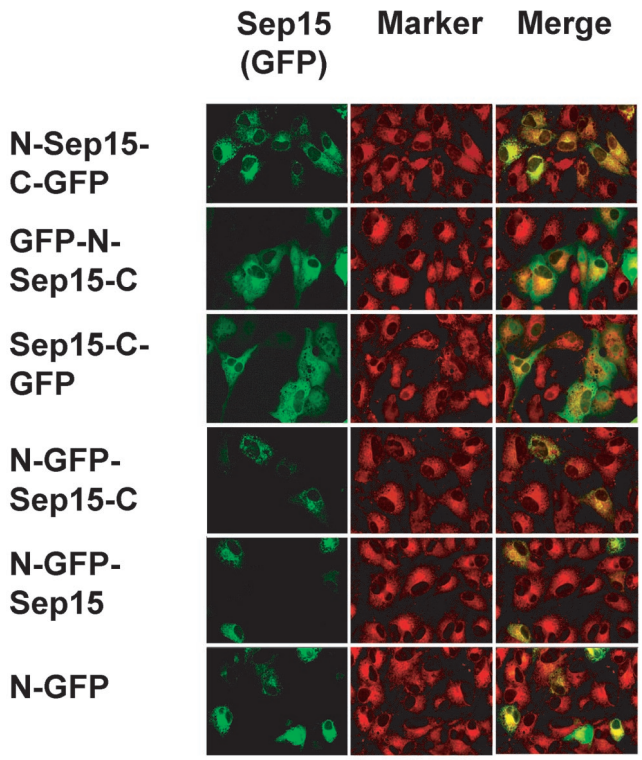

FIG. 7. **Confocal microscopy.** Confocal images of CV-1 cells expressing various GFP-tagged Sep15 and control proteins are shown. A set of three images is shown for each construct. *Left panels* show green fluorescence corresponding to transiently expressed fusion proteins. *Center panels* show fluorescence of the ER/Golgi marker. *Right panels* show images obtained by merging *left* and *center panels*. The *scale bar* is 100 μm. The GFP fusion constructs used in this experiment are shown on the *left*.

TABLE I
Characteristics of Sep15-GFP constructs
The design of the constructs is described under "Experimental Procedures."

Construct[a] and composition[b]	Predicted molecular mass[c]	Predicted molecular mass (N terminus is cleaved)[d]
	kDa	*kDa*
N-Sep15-C-GFP		
161-aa Sep15	47.0	44.3
22-aa linker		
239-aa GFP		
Sep15-C-GFP		
136-aa Sep15	44.6	44.6
22-aa linker		
239-aa GFP		
GFP-Sep15-C		
239-aa GFP	42.8	42.8
6-aa linker		
136-aa Sep15		
N-GFP-Sep15-C		
37-aa N-terminal	46.9	44.1
4-aa linker		
239-aa GFP		
6-aa linker		
136-aa Sep15		
N-GFP-Sep15		
37-aa N-terminal	46.3	43.6
4-aa linker		
239-aa GFP		
6-aa linker		
132-aa Sep15		
N-GFP		
37-aa N-terminal	30.9	28.2
4-aa linker		
239-aa GFP		
GFP		
239-aa GFP	26.9	26.9

[a] Sep15 is a protein without its 28 N-terminal and its 4 C-terminal residues; N is the N-terminal signal peptide of Sep15; and C represents four C-terminal residues of Sep15.

[b] Composition reflects sizes of Sep15 fragments, linkers, and GFP shown in numbers of amino acids (aa).

[c] Molecular masses were calculated for full-length proteins expressed from constructs.

[d] Molecular masses were calculated for proteins in which the N-terminal signal peptide of Sep15 was cleaved between Ala^{28} and Phe^{29}.

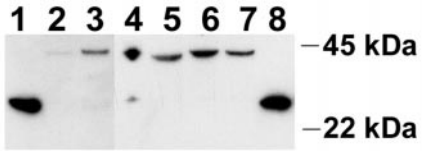

FIG. 8. **Immunoblot detection of the GFP-Sep15 fusion proteins.** CV-1 cells were transiently transfected with plasmids encoding GFP (*lane 1*), N-Sep15-C-GFP (*lanes 2* and *4*; two independent transfection experiments are shown), Sep15-C-GFP (*lane 3*), GFP-Sep15-C (*lane 5*), N-GFP-Sep15-C (*lane 6*), N-GFP-Sep15 (*lane 7*), and N-GFP (*lane 8*) as described under "Experimental Procedures." Samples were probed with antibodies specific for GFP.

The isolated selenoprotein was found to occur in a complex with a 150-kDa protein. Comparison of sequenced peptides from the 150-kDa protein with the deduced sequence of rat liver UGTR revealed 100% identity in four peptide sequences. Western blot analyses of the 150-kDa protein with the anti-UGTR antibodies, as well as analyses of fractionated mouse liver extracts, further supported the conclusion that Sep15 was purified in a complex with UGTR. This finding was unexpected for the following reasons. (i) UGTR is known for its role in the quality control of protein folding (6). This enzyme recognizes misfolded protein domains in the ER lumen of eukaryotic cells and specifically glucosylates these proteins, which retains misfolded proteins in the ER for the next cycle of folding by the calnexin/calreticulin glycoprotein folding system (15, 16, 21). Previously characterized eukaryotic selenoproteins were involved in redox processes (2), and redox function has been anticipated for Sep15, but the quality control of protein folding has not been linked to a redox process. (ii) UGTR is located in the ER (17), and no selenoprotein has yet been found to occur in this cellular compartment. In addition to Sep15, two other known mammalian selenoproteins, glutathione peroxidase 3 and selenoprotein P, contain N-terminal signal peptides. These proteins are secreted and are the major selenoproteins in the plasma of mammals (22). Most other known mammalian selenoproteins are cytosolic, nuclear, or mitochondrial proteins.

To determine the intracellular localization of Sep15, we made a series of constructs that encoded fusion proteins between Sep15 and GFP. In addition, we tested the relevance of the N-terminal portion of Sep15 for the ER translocation and of the C-terminal portion of the protein for ER retention. Expression patterns of the GFP fusion constructs containing Sep15 sequences in CV-1 cells were examined and compared with location of a marker by live-cell imaging confocal microscopy. Although the marker that was used in the present study is known to label both ER and Golgi, the fact that UGTR is the ER resident protein strongly suggests that Sep15 colocalized with UGTR in the ER. The data from imaging analyses in combination with other biochemical evidence demonstrated that the N-terminal signal peptide of Sep15 was necessary for ER localization. In contrast, the C-terminal tetrapeptide of the selenoprotein lacked a typical ER retention signal, and this sequence was not necessary to keep the protein in the ER. It appears that the selenoprotein sequence itself was responsible for retaining Sep15 in the ER and preventing its secretion. The data thus suggested that Sep15 was maintained in the ER because of its interaction with UGTR.

The binding between Sep15 and UGTR appeared to be very strong because these proteins copurified at each isolation step. Moreover, Sep15 was found exclusively in the UGTR-bound form. The lack of the UGTR-free selenoprotein may also be consistent with the idea that Sep15 and UGTR are subunits of a two-subunit protein. It is possible that the presence of the selenoprotein subunit in UGTR preparations was unnoticed previously because of the small size of Sep15, which made it difficult to visualize the selenoprotein by protein staining on SDS-PAGE gels. In addition, low percentage SDS-PAGE gels have been used previously for homogeneity assessment of isolated UGTR (8). In these gels, selenoprotein would migrate in the dye front.

In contrast to the exclusive binding of Sep15 to UGTR, the latter protein was detected in both selenoprotein-bound and selenoprotein-free forms. It remains to be determined if the selenoprotein-free form arose by the release of the denatured selenoprotein during protein isolation, if it was a natural UGTR form or if UGTR also occurred in a complex with other proteins and/or selenoproteins.

UGTR was shown previously, by immunoprecipitation, to associate with misfolded proteins, such as α_1-antitrypsin (23), and with other ER resident proteins, such as protein disulfide isomerases, carboxylesterase, and the glucose-regulated protein (24). However, these proteins do not copurify with UGTR, and only a small fraction of them was associated with this enzyme. Sep15, on the other hand, was found exclusively in the UGTR-bound form in rat prostate and mouse liver.

UGTR is the only known quality control protein that recognizes misfolded proteins in the ER, and its mechanism has been characterized in great detail. Interestingly, UGTR is able to glucosylate misfolded domains specifically while not reacting with properly folded domains within a protein composed of identical folded and misfolded domains (16).

The possible role of redox processes in the ER-based protein folding has received much attention recently. In particular, protein disulfide isomerase was found to remove electrons, through the disulfide bond formation, from folding proteins and to transfer reducing equivalents further to the ER membrane protein Ero1 (25, 26). The formation of disulfide bonds in nascent polypeptides is believed to be associated with folding by the calnexin/calreticulin chaperones. Although properly folded proteins may proceed further to secretory pathways, misfolded polypeptides including those containing disulfide bonds are glucosylated by UGTR to retain them for the next cycle of folding. Sensing or reduction of disulfides within misfolded proteins prior to folding appears to be required. Whether Sep15 is involved in such redox reactions is a direction for further research.

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