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Production of Functional Human Selenocysteine-Containing KDRF/Thioredoxin Reductase in *E. coli*

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In a previous study, we reported the isolation of a cDNA encoding KDRF (KM-102-derived reductase like factor) from the human bone marrow-derived stromal cell line KM-102. Analysis of the sequence of this cDNA revealed it to be the previously reported human thioredoxin reductase cDNA. Human thioredoxin reductase, which was recently isolated from human lung adenocarcinoma NCI-H441 cells as a selenocysteine-containing selenoprotein, and its substrate thioredoxin are thought to be essential for protecting cells from the damage caused by reactive oxygen species. To obtain the selenocysteine-containing recombinant KDRF/thioredoxin reductase, we introduced a secondary structure, which is identical to the selenocysteine insertion signal of *Escherichia coli* formate dehydrogenase H mRNA, downstream of the TGA in the KDRF/thioredoxin reductase cDNA and expressed it in *E. coli*. As a result, a significant amount of selenocysteine was incorporated into the C-terminus of the KDRF/thioredoxin reductase protein. The selenocysteine-containing KDRF/thioredoxin reductase showed reducing activities toward human and *E. coli* thioredoxin, whereas non-selenocysteine-containing KDRF/thioredoxin reductase showed no enzyme activity. Our results suggest that this strategy will be applicable to the production of other mammalian selenocysteine-containing selenoproteins in *E. coli*.

Key words: KDRF, LC/ESI MS, selenium, selenocysteine, thioredoxin reductase.

Selenium (Se) is an essential trace element in mammals and birds (*1*). In many Se-dependent enzymes, Se occurs as an active center amino acid residue, selenocysteine (Sec) (*2*, *3*). The incorporation of Sec into a selenoprotein is directed by an in-frame UGA codon in its mRNA (*4*). The use of a UGA codon as a Sec codon occurs in both prokaryotes and eukaryotes, but the recognition mechanism of the UGA codon as a Sec differs between them. In the *Escherichia coli* selenoprotein formate dehydrogenase (FDH) isoenzymes FDH_H and FDH_N, a specific stem-loop immediately 3′ to the UGA codon is required for translation of this UGA as a Sec (*5–7*). On the other hand, a 3′-untranslated region of the mRNA for eukaryotic selenoprotein is required for recognition of UGA as a Sec codon (*8*, *9*). Therefore mammalian selenoproteins can not be expressed directly in their active forms in *E. coli* (*10*).

Recently, we reported the isolation from the human bone marrow-derived stromal cell line KM-102 of a cDNA encoding KDRF (KM-102-derived reductase like factor) that possesses reducing activities on 2,6-dichlorophenol-indophenol (DCIP) and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), but later we found that the nucleotide sequence of the KDRF cDNA was identical to that of a reported human thioredoxin reductase (hTR) cDNA (*11–13*). TR was originally identified in *E. coli* as part of the ribonucleotide reductase system, in which this enzyme catalyzes the reduction of the disulfide bridge in the thioredoxin (Trx) by NADPH (*14*). In mammals, TR and Trx are essential for protection against damage caused by reactive oxygen species (ROS) (*15–17*). ROS have been thought of as toxic by-products of cellular oxygen metabolism, and excessive production of ROS or an insufficiency of antioxidant defenses has been implicated in apoptosis, aging, and cancer (*18*). Since the inappropriate production of ROS can have severe pathological consequences in disease (*19*), the inhibition of radical generation has become a pharmaceutical target (*20*). Thus, we attempted to produce recombinant human KDRF/TR protein in *E. coli* to evaluate the its ability to protect human cells against ROS.

Recently, hTR was isolated from human lung adenocarcinoma NCI-H441 cells and Jurkat T cells as a selenoprotein (*21*, *22*). Sec was found to be incorporated at the position corresponding to TGA in the cDNA, previously thought to be the stop codon, and is followed by Gly-551 (*11*) [Gly-499 (*12*)], the actual C-terminal amino acid of hTR (*22*). Sec-containing hTR is not expressed as an active form in *E. coli* (*12*) because the incorporation mechanism of Sec into the UGA codon differs between human and *E. coli*, as previously described.

To produce the active form KDRF/TR in *E. coli*, we introduced a secondary structure identical to the Sec insertion signal (SECIS) of the *fdhF* (encoding the 80 kDa subunit of FDH_H) mRNA downstream of the TGA in the KDRF/TR

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Abbreviations: FDH, formate dehydrogenase; KDRF, KM-102-derived reductase like factor; TR, thioredoxin reductase; Trx, thioredoxin.

cDNA and expressed it in *E. coli*. Using this strategy, we succeeded in incorporating Sec into human selenoprotein in an *E. coli*–expression system. The recombinant KDRF/TR obtained here was found to have a Sec residue in its C-terminus and to show enzymic activity.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids—a) pNIa31-7K: An expression plasmid, pNIa31-7K, which starts with Lys^{49} in KDRF/TR (*11*), was constructed in the Clover Yellow Vein Virus (ClYVV) nuclear inclusion-a (NIa) protease system (*23*) using the polymerase chain reaction (PCR) (Fig. 1).

b) pNIa31-7Kfdh-9: To introduce a secondary structure that closely resembles that of the *fdhF* mRNA downstream of TGA in pNIa31-7K, we synthesized four oligonucleotides (oligo i to iv), including two pairs of complementary oligos: i, 5′-GCTCTGGGGCAAGCATCCTCCAGGCTGGCTGCTG-AGGTTAACCTCGAGTCTAGA-3′; ii, 5′-TCTAGACTCGA-GGTTAACCTCAGCAGCCAGCCTGGAGGATGCTTGCCC-CAGAGC-3′; iii, 5′-GGTTAACATCGGTTGCAGGTCTGCA-CCAATCTTAACCTAATGGCGCCTCGAGTC-3′; iv, 5′-GA-CTCGAGGCGCCATTAGGTTAAGATTGGTGCAGACCTG-CAACCGATGTTAACC-3′. Firstly, 7 μg each of oligos i and ii was annealed in annealing buffer (7 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 20 mM NaCl, and 7 mM $MgCl_2$) at 70°C for 5 min and cooled to room temperature, followed by kination using T4 polynucleotide kinase (Toyobo, Osaka). pUCKM31-7 DNA (*11*) was digested with *Aor*51HI (all restriction enzymes in this study were purchased from Takara Shuzo, Tokyo), dephosphorylated, and ligated with the above mentioned annealing fragment (pUCKM31-7fdh1-2-1). Next, 7 μg each of oligos iii and iv was annealed under the same conditions and digested with *Xho*I and *Hpa*I. pUCKM31-7fdh1-2-1 DNA was digested with *Xho*I and *Hpa*I, dephosphorylated, and ligated with the *Xho*I/*Hpa*I digested annealing fragment (pUCKM31-7fdh1-2-1-31). Then, the pUCKM31-7fdh1-2-1-31 DNA was digested with *Xba*I and *Sma*I, and polyacrylamide gel electrophoresis (PAGE) was performed in 8% polyacrylamide gels to yield a 978 bp *Xba*I/*Sma*I-digested fragment that was purified from the gel. The pNIa31-7K DNA was digested with *Xba*I and *Sma*I, dephosphorylated, and ligated with the 978 bp *Xba*I/*Sma*I fragment (pNIa31-7Kfdh-9) (Fig. 2a). The nucleotide sequence was analyzed to confirm that no abnormalities existed in the portion in which the fragment was inserted.

Purification of Recombinant Proteins and Amino Acid Sequence Analysis—a) pNIa31-7K product: pNIa31-7K DNA–transformed *E. coli* (JM109) were grown aerobically in LB medium (*24*) containing 50 μg/ml ampicillin with or without 10 μM Na_2SeO_3 (Sigma) at 37°C, and 1 mM isopropyl-thio-β-D-galactoside (IPTG) (Wako Pure Chemical, Osaka) was added when an absorbance of 0.9 at 600 nm was reached. After the addition of IPTG, the medium was further cultured for 48 h at 28°C. The cells were then collected and washed with 10 mM Tris-HCl, pH 7.26 (buffer A), and disrupted by sonication. The supernatant was collected after centrifugation and applied to 10 ml of DEAE-Sepharose Fast Flow (Pharmacia Biotech) contained in an XK16/20 column (φ2.0 × 20 cm, Pharmacia Biotech). Proteins were eluted with a stepwise gradient of 20, 40, and 60 mM NaCl (each 30 ml), 80 and 100 mM NaCl (each 60 ml) and 200, 300, and 400 mM NaCl (each 30 ml) in buffer A. The fractions were subjected to SDS-PAGE (*25*) under reducing conditions, and the band (M_r 55,000) was detected by silver staining. The collected fractions were combined and dialyzed against 10 mM Tris-HCl, pH 7.26, 1.5 mM EDTA and 2 mM DTT (buffer B). Next, affinity chromatography was performed using 7 ml of 2′,5′-ADP Sepharose 4B (Pharmacia Biotech) contained in an XK16/20 column. The protein was eluted with a stepwise gradient of 200 mM NaCl (21 ml), 300 mM NaCl (21 ml), and 1 M NaCl (28 ml) in buffer B. The fractions containing the pNIa31-7K product [Ala-KDRF/TR (Lys^{49})] identified by silver staining were collected and combined. We subjected the combined sample to SDS-PAGE under reducing conditions and transferred the resulting bands electrophoretically to a polyvinylidine difluoride film (Pro Blot, Perkin-Elmer). The area corresponding to the band (M_r 55,000) was cut out for protein microsequencing. Amino-terminal sequence determination was carried out with a gas-phase protein sequencer (PPSQ-10, Shimadzu, Kyoto).

b) pNIa31-7Kfdh-9 product: pNIa31-7Kfdh-9 DNA–transformed *E. coli* (JM109) were grown under the same conditions as a). Cells were collected and washed with 10 mM Tris-HCl, pH 7.26, and 1 mM DTT (buffer C) and disrupted by sonication. The supernatant was applied to 10 ml of DEAE-Sepharose Fast Flow, and the protein was eluted with increasing concentrations of NaCl. Fractions were combined, dialyzed against buffer C, and applied to 10 ml of 2′,5′-ADP Sepharose 4B. The protein was eluted with a stepwise gradient of 200 mM NaCl (100 ml), 300, 500, and 1,000 mM NaCl (each 50 ml) in buffer C. The fractions were subjected to SDS-PAGE under reducing conditions and the band (M_r 55,000) was detected as in a). The collected fractions were combined and dialyzed against buffer C. The protein solution from 2′,5′-ADP Sepharose 4B was applied to 5 ml of DEAE-Sepharose Fast Flow (in XK16/20) for concentration, and the protein was eluted with 200 mM NaCl (18 ml) in buffer C. The collected fractions were applied to Superdex 200 (2.6 × 60 cm, Pharmacia Biotech), and eluted with phosphate-buffered saline (5 ml × 50). The protein was monitored by SDS-PAGE and silver staining. Fractions containing the protein were pooled, 50 ml of buffer C was added, and this protein solution was again applied to 5 ml of DEAE-Sepharose Fast Flow. The protein was eluted with 200 mM NaCl (24 ml) in buffer C. This preparation was used as the purified protein [Ala-KDRF/TR (Lys^{49}-fdh)]. The amino-terminal sequence of this protein was determined by the same method as described in a). Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad) in which bovine serum albumin (Sigma) was used as a standard.

Se Content Determination—The Se contents of Ala-KDRF/TR (Lys^{49}-fdh) and Ala-KDRF/TR (Lys^{49}) were determined by inductively coupled plasma (ICP) mass spectrometry (SPQ6500, Seiko, Chiba).

Characterization of C-Terminal Peptide by Liquid Chromatography Mass Spectrometry—Seventeen micrograms of the purified Ala-KDRF/TR (Lys^{49}-fdh) was further purified by SDS-PAGE under reducing conditions. The gel was stained with Coomassie Brilliant Blue and the visualized protein band (M_r 55,000) was excised. The excised gel piece was prewashed three times with 0.2 M NH_4HCO_3 in 50% acetonitrile according to Hellman *et al.* (*26*). The protein in

the gel piece was reduced, carbamoylmethylated, and then digested with endoproteinase Lys-C (Boehringer Mannheim) according to Shevchenko *et al.* (*27*) except that ^{18}O-enriched (50 atom%) water (Isotec, Miamisburg, OH) was used. After overnight digestion, the peptides were extracted from the gel by one change of 20 mM NH_4HCO_3 and three changes of 5% formic acid in 50% acetonitrile at room temperature. The combined solution was concentrated to 100 μl, and a 10 μl aliquot was analyzed directly by liquid chromatography electrospray ionization mass spectrometry (LC/ESI MS): column, Vydac (Hesperia, CA) C_{18} 1.0 × 150 mm (218TP5115); solvent delivery, Perkin-Elmer 140C dual syringe pumps; solvent A, 0.09% trifluoroacetic acid (TFA) in water; solvent B, 0.075% TFA in acetonitrile; flow rate, 50 μl/min; initial mobile phase composition, 5% solvent B; linear gradient elution, started 5 min after injection and 5 to 60% solvent B over 55 min; mass spectrometer, Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK, tuned to unit mass resolution) with an electrospray ion source.

TR Assay—The purified proteins and *E. coli* TR (American Diagnostica) were pretreated with 1 mM DTT at 4°C for 2 h. The assays were monitored for the change in absorbance at 340 nm due to the oxidation of NADPH (Boehringer Mannheim) in the presence of human Trx (American Diagnostica) or *E. coli* Trx (Promega) and bovine insulin (Sigma) (*28*). The assay mixture contained 55 mM potassium phosphate, pH 7.0, 22 mM EDTA, 97 μM insulin, 133 μM NADPH, with human or *E. coli* Trx and enzyme to a final volume of 1.8 ml. All assays were done at room temperature using a Shimadzu UV-180 spectrophotometer. Assay mixtures lacking Trx served as controls. No reduction of insulin disulfides was obtained without TR (purified proteins or *E. coli* TR).

RESULTS

Expression of KDRF/TR in E. coli—In order to produce KDRF/TR protein in *E. coli*, we first tried to construct an expression plasmid starting with Lys^{49} in KDRF/TR using the ClYVV NIa protease system (*23*). This Lys^{49} corresponds to the first amino acid in the shortest of the three proteins that are secreted by COS-1 cells when the mammalian expression plasmid pSRα31-7 is introduced into them (*11*). The *E. coli* expression plasmid pNIa31-7K is shown in Fig. 1, and the purification method for the protein (M_r 55,000) is described in "MATERIALS AND METHODS." SDS-PAGE analysis of the protein is shown in Fig. 3 [lane 1, Ala-KDRF/TR (Lys^{49})]. The amino-terminal sequence of the protein is Ala-Lys-Leu-Leu-Lys-Met-Asn-Gly-Pro-Glu. This sequence corresponds to that of Lys^{49} to Glu^{57} in KDRF/TR (*11*). The KDRF/TR and ClYVV-NIa protease cDNAs are joined by a specific linker that codes for a proteolytic processing amino acid sequence (Asn-Cys-Ser-Phe-Gln-Ala). As the ClYVV-NIa protease cuts this fusion protein between the Gln and Ala residues, the Ala residue remains at the amino-terminus of the protein after processing.

Next, we investigated whether this protein [Ala-KDRF/TR (Lys^{49})] is able to reduce *E. coli* Trx in an insulin reduction assay (*28*). It has been reported that hTR reduces both *E. coli* Trx and rat Trx in insulin reduction assay and that this broad specificity is typical of mammalian TRs (*21*). Even when Ala-KDRF/TR (Lys^{49}) was purified from pNIa31-7K–transformed *E. coli* cultured in LB medium containing Na_2SeO_3, it displayed no TR activity (data not shown). One possible explanation for this observation is Sec misincorporation into the Ala-KDRF/TR (Lys^{49}) protein. As Berry *et al.* (*8*) have reported that the Sec insertion mechanism in selenoprotein translation differs between prokaryotes and eukaryotes, we tried to introduce a secondary structure identical to the SECIS of bacterial selenoenzyme *fdhF* mRNA downstream of the TGA in the pNIa31-7K.

Expression of pNIa31-7Kfdh-9 in E. coli—In a previous study, Zinoni *et al.* demonstrated that the insertion of Sec into the *E. coli fdhF* selenopolypeptide requires the presence of a 40-base stretch on the 3′ side of the UGA_{140} codon in *fdhF* mRNA (*5*). To achieve Sec incorporation into KDRF/TR, we introduced a synthetic oligonucleotide identical to the SECIS of *E. coli fdhF* mRNA into the expression plasmid pNIa31-7K (Fig. 2a) and obtained expression plasmid pNIa31-7Kfdh-9. The predicted mRNA secondary structure downstream of the UGA in pNIa31-7Kfdh-9 is shown in Fig. 2b. The plasmid pNIa31-7Kfdh-9 was introduced into *E. coli* and the transformant was cultured in the presence of Na_2SeO_3. From this culture, we purified the protein with a molecular weight of 55,000, and the results of SDS-PAGE analysis of the protein are shown in Fig. 3 [lane 2, Ala-KDRF/TR (Lys^{49}-fdh)]. The subunits of *E. coli* TR (M_r 35,000) were not detected in the SDS-PAGE analy-

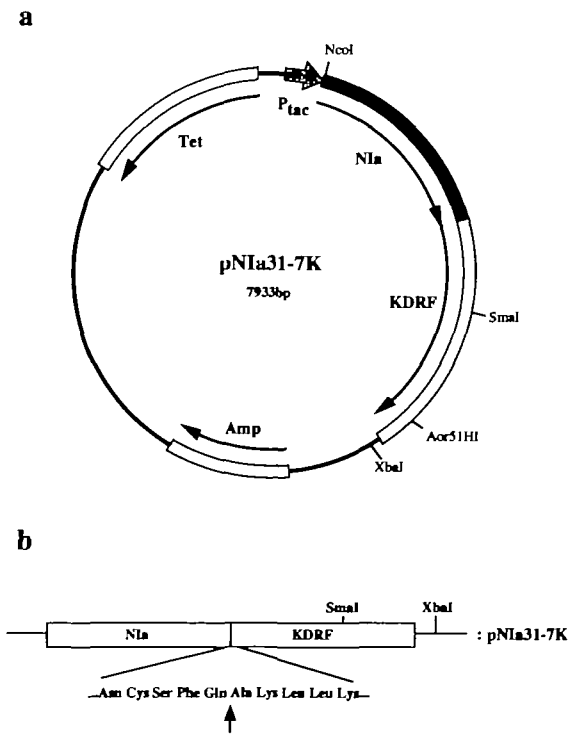

Fig. 1. **Schematic structure of pNIa31-7K.** a: pNIa31-7K plasmid DNA was constructed by PCR. Expression of the NIa and KDRF/TR-fused gene is controlled by the *tac* promoter. The remaining segment was derived from pKK388-1. Open bars represent other functional genes in the plasmid DNA; the directions of transcription are indicated by arrows. b: The amino acid sequence in the joint region of the NIa-KDRF fusion protein is indicated. NIa protease processes the fusion protein between Gln and Ala residues (arrow) in this region.

sis. In addition, amino-terminal analysis of this protein revealed its identity to Ala-KDRF/TR (Lys[49]). As shown in Fig. 4, the purified Ala-KDRF/TR (Lys[49]-fdh) has a visible absorption spectrum typical of flavoproteins (*29*), with two intensity maxima at 365 and 463 nm.

Se Contents of Ala-KDRF/TR (Lys[49]-fdh) and Ala-KDRF/TR (Lys[49])—To confirm the incorporation of Sec into Ala-KDRF/TR (Lys[49]-fdh), we conducted ICP mass spectrometry analysis. The amount of Se in Ala-KDRF/TR (Lys[49]-fdh) was determined to be 0.086 Se per subunit of protein, whereas Se was not detected in Ala-KDRF/TR (Lys[49]). These results indicate that Se was incorporated into Ala-KDRF/TR (Lys[49]-fdh).

Verification of Selenocysteine Incorporation near the C-Terminus of Ala-KDRF/TR (Lys[49]-fdh)—As the codon coding for Sec was introduced at the position corresponding to TGA in the KDRF/TR cDNA, and the Sec is followed by Gly-551 (*11*) [Gly-499 (*12*)], which is the actual C-terminal amino acid (*22*), we analyzed Lys-C peptides of Ala-KDRF/TR (Lys[49]-fdh) by LC/ESI MS and searched for the peptide containing the original C-terminus of the protein to confirm the incorporation of a Sec residue. To facilitate identification of the C-terminal peptide, Ala-KDRF/TR (Lys[49]-fdh) was digested in 50 atom% ^{18}O-containing buffer. Endoproteinase digestion in 50 atom% ^{18}O-containing buffer generates C-terminally ^{18}O-labeled peptides, which give characteristic doublet or triplet molecular-related ion signals with a spacing of 2 daltons (Da) (*30*, *31*). However, the C-terminal carboxyl group of the protein is not involved in proteolysis and the C-terminal peptide can be easily recognized in an ESI mass spectrum (*31*). In the present study, we observed molecular-related ions for only two peptides that do not show the characteristic 2 Da spacing (*i.e.*, m/z 560.4 observed at retention time (*tR*) 22.0 min and m/z 692.9 at *tR* 22.3 min) in an LC/ESI MS experiment. The first ion, m/z 560, was assigned to the doubly protonated Arg[539]-Ser-

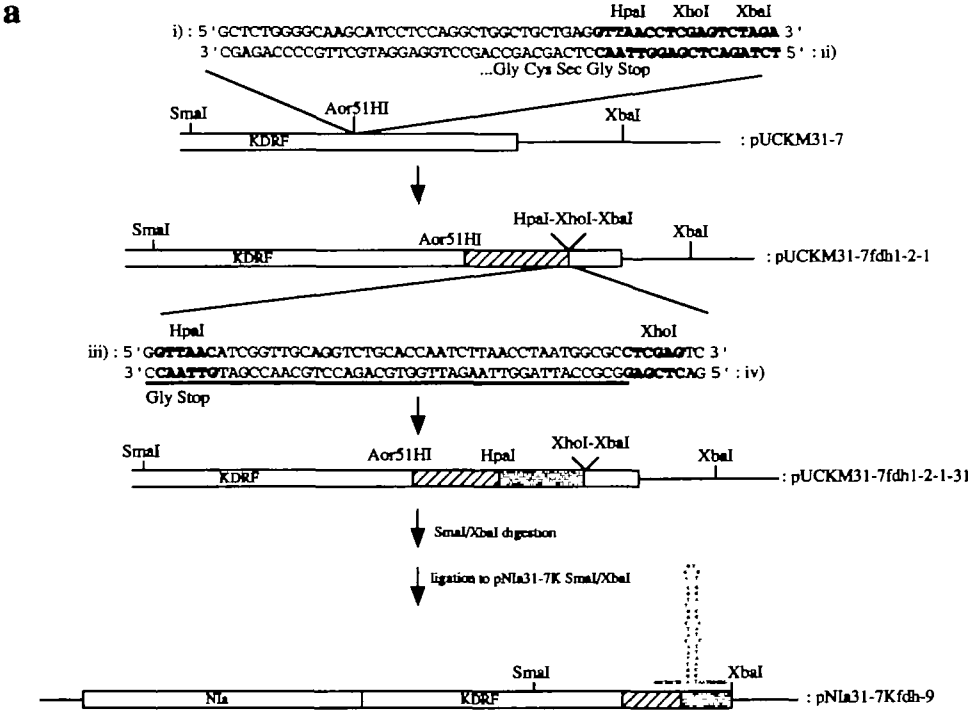

Fig. 2. **Construction of pNIa31-7Kfdh-9.** a, construction of pNIa31-7Kfdh-9 plasmid DNA containing a Sec insertion signal of *E. coli fdhF* mRNA. The open bars represent the cDNA sequence of the KDRF open reading frame. The hatched bars represent an annealing fragment (oligo i and ii) introduced into the *Aor*51HI site in pUCKM31-7 plasmid DNA. The dotted bars represent an *HpaI*/*Xho*I digested annealing fragment (oligo iii and iv) introduced into the *HpaI*/*Xho*I sites in pUCKM31-7fdh1-2-1 plasmid DNA. The synthetic DNA fragment identical to the Sec insertion signal of *E. coli fdhF* mRNA (underlined) was introduced downstream of the TGA codon in the pNIa31-7K plasmid DNA as indicated. Restriction enzyme recognition sequences are indicated by bold characters. b, predicted mRNA secondary structure downstream of the UGA codon in the pNIa31-7Kfdh-9. Ala-KDRF/TR (Lys[49]) and Ala-KDRF/TR (Lys[49]-fdh) are products of pNIa31-7K and pNIa31-7Kfdh-9, respectively. The UGA codon in pNIa31-7K is recognized as a stop codon in *E. coli* (-Gly-Cys-Stop), while it is recognized as a Sec codon in mammals (-Gly-Cys-Sec-Gly-Stop). Surrounding region represents the C-terminal portion of Ala-KDRF/TR (Lys[49]-fdh).

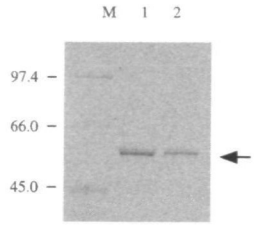

Fig. 3. **SDS-PAGE analysis of KDRF/TR.** Coomassie Brilliant Blue–staining of an 8% SDS-PAGE gel is shown. Lane 1, 10 μg Ala-KDRF/TR (Lys[49]); lane 2, 10 μg of Ala-KDRF/TR (Lys[49]-fdh) (indicated by arrow). JM109 transformed with plasmid pNIa31-7K or pNIa31-7Kfdh-9 was grown aerobically in the presence or absence of Na_2SeO_3. Lane 1 represents Ala-KDRF/TR (Lys[49]) purified from *E. coli* cultured without Na_2SeO_3 and lane 2 represents Ala-KDRF/TR (Lys[49]-fdh) purified from *E. coli* cultured in the Na_2SeO_3-containing medium. Molecular weight markers are shown on the left (in thousands).

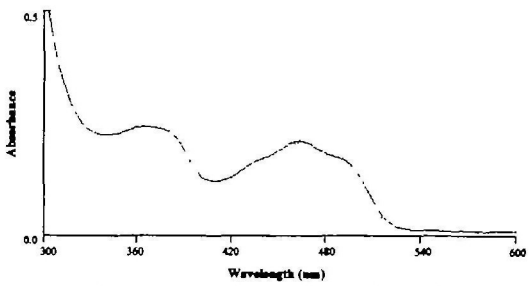

Fig. 4. **Absorption spectrum of purified Ala-KDRF/TR (Lys[49]-fdh).** The absorption spectrum was recorded in 10 mM Tris-HCl (pH 7.26) with 1.2 μg/μl of purified protein. The two peaks in the visible part of the spectrum show absorption maxima at 365 nm (absorbance = 0.238) and 463 nm (absorbance = 0.205).

Gly-Ala-Ser-Ile-Leu-Gln-Ala-Gly-Cys[549] (calcd. m/z for $[M+2H]^{2+}$: 560.6, as *S*-carbamoylmethyl derivative using average mass), the C-terminal peptide without Sec incorporation. Just after the elution of this peptide, a second ion, m/z 692.9, was observed with m/z 560.4 (Fig. 5). The ion at m/z 692.9 was assigned to the doubly protonated Arg[539]-Ser-Gly-Ala-Ser-Ile-Leu-Gln-Ala-Gly-Cys-Sec-Gly[551] on the basis of the m/z value (calcd. m/z for $[M+2H]^{2+}$: 692.7, as *S,Se*-bis-carbamoylmethyl derivative using average mass), and the characteristic peak shape was attributable not to ^{18}O incorporation but to Se isotopes (see inset of Fig. 5). The C-terminal of Ala-KDRF/TR (Lys[49]) was also analyzed by mass spectrometry in the same way. In contrast to Ala-KDRF/TR (Lys[49]-fdh), the only C-terminal peptide detected from the Ala-KDRF/TR (Lys[49]) digest was Arg[539]-Ser-Gly-Ala-Ser-Ile-Leu-Gln-Ala-Gly-Cys[549] observed at m/z 560 (data not shown). From these results, we concluded that Sec was incorporated into Ala-KDRF/TR (Lys[49]-fdh) as the 550th residue. However, the relative abundance of the protein with and without Sec incorporation could not be determined from the signal intensities at *tR* 22.0 min (61,600 counts at m/z 560.4) and *tR* 22.3 min (1,150 counts at m/z 692.9), because the C-terminal peptides with and without Sec incorporation should have different recoveries and ionizing efficiencies.

Determination of TR Activity of Ala-KDRF/TR (Lys[49]-fdh)—Next, we used an insulin reduction assay to test

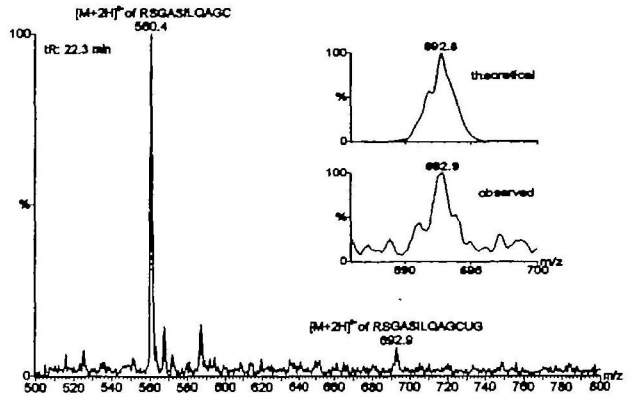

Fig. 5. **Mass spectrum at *tR* 22.3 min obtained in the LC/ESI MS experiment on the Lys-C digest of Ala-KDRF/TR (Lys[49]-fdh).** The inset shows an enlargement of the observed and theoretical peak shapes of a doubly protonated Sec-containing C-terminal peptide RSGASILQAGCUG (U: Sec). The theoretical peak shape was calculated for the predicted composition of the doubly protonated peptide, *i.e.*, $C_{51}H_{91}N_{19}O_{19}SSe$ (assuming as *S,Se*-bis-carbamoylmethyl derivative). A half-height peak width of 0.6 Da, the value typically observed in this experiment, was used for the calculation. The resolution is not sufficient to show the natural isotope distribution of Se (^{74}Se:^{76}Se:^{77}Se:^{78}Se:^{80}Se:^{82}Se = 0.9:9.0:7.6:23.5:49.6:9.4) but sufficient to reflect the characteristically dispersed peak shape of the Se-containing peptide ion.

whether Ala-KDRF/TR (Lys[49]-fdh) has Trx reduction activity. A human Trx–dependent reduction of insulin with an activity of 2.2 μmol NADPH oxidized/min/mg of protein was observed. The apparent K_m value for *E. coli* Trx was 26 μM and the relative specificity (k_{cat}/K_m) was 0.83. When cultured without Na_2SeO_3, Ala-KDRF/TR (Lys[49]-fdh) did not display human or *E. coli* Trx reduction activities. In the case of *E. coli* TR, *E. coli* Trx–dependent reduction with an activity of 12.4 μmol NADPH oxidized/min/mg of protein was observed, whereas human Trx–dependent reduction activity was not detected. This indicates that the measured enzyme activity of Ala-KDRF/TR (Lys[49]-fdh) was not due to contaminating *E. coli* TR. From these results, we conclude that Sec-containing Ala-KDRF/TR (Lys[49]-fdh) is the active form and that its reducing activity depends completely on Sec incorporation.

DISCUSSION

In a previous paper, we reported the isolation from the human bone marrow-derived stromal cell line KM-102 of a cDNA encoding KDRF that possesses reducing activities on DCIP and DTNB (*11*), but later found that the nucleotide sequence of the KDRF cDNA is identical to that of hTR cDNA (*12, 13*). Human TR was isolated as a selenoprotein by Tamura and Stadtman (*21*), and Gladyshev *et al.* (*22*) revealed that the Sec residue is incorporated into the hTR C-terminus (-Cys-Sec-Gly-stop).

Initially, we tried to produce the KDRF/TR protein [Ala-KDRF/TR (Lys[49])] in *E. coli*, but were unable to obtain it as an active form. The specific incorporation of Sec into distinct proteins is directed by in-frame TGA codons derived by all known selenoprotein genes (*4*). As TGA codons (UGA in the mRNA transcripts) are normally used as translation termination codons, a specific mechanism to discriminate

them from TGA codons directing Sec insertion exists. Indeed, use of TGA as a Sec codon occurs in both prokaryotes and eukaryotes, but their mechanisms of TGA-recognition as a Sec codon differ. In prokaryotes, previous studies have revealed that a specific stem-loop structure immediately downstream of the TGA codon is required for translation of the TGA as a Sec codon (*5–7*). On the other hand, in eukaryotes, the 3′-untranslated regions of the mRNAs are required for the incorporation of Sec (*8, 9*).

To obtain the active form of the KDRF/TR protein, we attempted to introduce a secondary structure downstream of the TGA codon, a structure that closely resembles that of *E. coli fdhF* mRNA, into the KDRF/TR cDNA (Fig. 2), and expressed this modified cDNA in *E. coli* in the presence of Na_2SeO_3. The *E. coli fdhF* gene product is a Sec-containing selenoprotein, and the mechanism for the incorporation of Sec into the protein has been studied in detail (*5, 7*).

To examine whether the protein incorporates a Sec residue into the C-terminus of the Ala-KDRF/TR (Lys^{49}-fdh), we measured the Se content and performed mass spectroscopic analysis. The results showed that the amount of Se in the Ala-KDRF/TR (Lys^{49}-fdh) was 0.086 Se per subunit of protein, whereas Ala-KDRF/TR (Lys^{49}) had no detectable Se. It was also demonstrated that the C-terminus of the Ala-KDRF/TR (Lys^{49}-fdh) contains the Sec residue (Fig. 5).

We next examined whether the Sec-containing Ala-KDRF/TR (Lys^{49}-fdh) catalyzes the NADPH-dependent reduction of human and *E. coli* Trx in an insulin reduction assay. Ala-KDRF/TR (Lys^{49}-fdh) did not exhibit reducing activity against human or *E. coli* Trx when the transformant *E. coli* were cultured without Na_2SeO_3. In addition, Ala-KDRF/TR (Lys^{49}) revealed no reducing activity even when it was purified from *E. coli* cultured in Na_2SeO_3-containing medium. On the other hand, Ala-KDRF/TR (Lys^{49}-fdh) purified from *E. coli* cultured in medium containing Na_2SeO_3 displayed both human and *E. coli* Trx reduction activities. It has been reported that purified hTR reduces both *E. coli* Trx and rat Trx in an insulin reduction assay, and that this broad specificity is typical of mammalian TRs (*21*). The measured enzyme activity of Ala-KDRF/TR (Lys^{49}-fdh) was not due to contamination by *E. coli* TR, since *E. coli* TR has no reducing activity against human Trx and *E. coli* TR subunits (M_r 35,000) were not detected in our preparation of Ala-KDRF/TR (Lys^{49}-fdh) by SDS-PAGE analysis (Fig. 3). However, further investigations are needed to compare the enzyme activity of Ala-KDRF/TR (Lys^{49}-fdh) to native hTR purified from human cells. In addition, it is necessary for us to examine whether or not the Ala residue remaining at the amino-terminus of the protein might perturb its enzyme activity.

From these results, we conclude that the Ala-KDRF/TR (Lys^{49}-fdh) is an active form and that the reducing activity of Ala-KDRF/TR (Lys^{49}-fdh) against human and *E. coli* Trx depends on Se. This result reveals that the incorporation of Sec at the C-terminus of the enzyme is essential for the reducing activity, a conclusion consistent with statements in recent literature (*32, 33*).

Mammalian TR has a broad substrate specificity and reacts not only with its homologous Trx but also with *E. coli* Trx, DTNB, GS-Se-GS, selenite, vitamin K, alloxan, lipid hydroperoxides, hydrogen peroxide, and organic hydroperoxides (*15*). In addition, hTR and Trx are secreted by cells (*11, 34*). Based on these findings, it is suggested that the mammalian TR-Trx system plays an important role in protecting cells against damage caused by ROS both inside and outside the cells. It is evident that the inappropriate production of ROS can have severe pathological consequences in, for example, rheumatoid arthritis, reperfusion injury, immune injury to lung and kidney, and cerebral trauma or ischemia (*19*). Thus, the inhibition of radical generation has become a major pharmaceutical target (*20*). Indeed, large numbers of inhibitors of superoxide generating oxidases have been developed, including standard and experimental anti-inflammatory and anti-rheumatic drugs, natural products, and so on (*20*). The TR-Trx system has also been reported to play an important role in preventing cell damage caused by UV-generated free radicals on the human skin. Schallreuter and Wood (*17*) have suggested a direct connection between radical reduction by hTR and the regulation of tyrosinase in melanin biosynthesis at the surface of cell membranes. Thus, we focused on the possibility that recombinant hTR can be applicable as an anti-oxidant drug. Although Arner *et al.* (*35*) reported success in incorporating Sec into rat TR in *E. coli*, rat TR may be antigenic in humans and so may not be appropriate for clinical use. Development of a system to produce KDRF/TR in *E. coli* would provide a useful tool not only for analyzing the biological role of KDRF/TR *in vitro*, but also for treatment of diseases caused by ROS.

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