Molecular cloning of thioredoxin reductase isozymes, *TrxR1* and *TrxR2*, from human lung adenocarcinoma cell line, NCI-H441

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Summary

The genes encoding mammalian thioredoxin reductase (*TrxR*) isozymes, *TrxR1* and *TrxR2*, were cloned from a human lung adenocarcinoma cell line, NCI-H441. *TrxR1* gene was amplified by conventional thermal cycle program to give an intensive signal on agarose gel electrophoresis. mRNA from normal lung cells also produced the amplified *TrxR1* gene by the same RT-PCR procedure. In contrast, the amplification of *TrxR2* gene from the NCI-H441 cells required the touch-down PCR program in which the annealing temperature was decreased from 75 to 65 degree by 0.4 degree in every cycle. The normal lung mRNA sample failed to yield the *TrxR2* gene, suggesting even smaller expression in the normal cells. The amplified genes were cloned on TOPO TA vector and sequenced to identify the sequences.

Mammalian thioredoxin reductases (*TrxR*s) are NADPH-dependent, FAD-containing disulfide reductases that catalyze the reduction of thioredoxin by NADPH. Unlike the well-characterized homologues from yeast and prokaryotes, the larger mammalian enzymes of 55- to 58-kDa subunits contain selenocysteine residue in the sequence -Cys-Secys-Gly(end) at the C-terminus[1-5)]. In this paper, genes of *TrxR* isozymes have been cloned and sequenced from cDNA library of human lung adenocarcinoma cells NCI-H441 and of normal lung cells for the comparison. Lung cancer cells appeared to have higher expression of *TrxR2* gene than normal lung cells, and this may be necessary for the malignant cells that are growing rapidly under such high oxygen pressure in the lungs. A point mutation Met101Thr was found in the adenocarcinoma *TrxR1*, which might be effective in preventing undesired oxidative modification of residues near the redox acive site in the selenoenzymes.

Materials and Methods

Cell growth and cDNA synthesis　Human lung adenocarcinoma NCI-H441 cells were grown and maintained by standard tissue culture techniques using RPMI 1640 medium with 10% (v/v) fetal bovine serum. The mRNA extraction and cDNA preparation were carried out using Poly (A) Pure mRNA Purification Kit (Ambion) and cDNA Synthesis System (GIBCO BRL) according to the manufacturer's specification. Total human lung RNA was purchased from Clontech.

Amplification of genes of TrxR isozymes　*TrxR1* cDNA was amplified by PCR using 5'-ATG AAC GGC CCT GAA GAT CT-3' as the forward primer and 5'-TTA ACC TCA GCA GCC AGC CT-3' as the reverse primer. *TrxR2* cDNA was amplified by PCR using 5'-ATG GCG GTG GCG CTG CGG GG-3' as the forward primer and 5'-TTA CCC TCA GCA GCC TGT CA-3' as the reverse primer. The PCR products were separated on a 1% agarose gel, and the ampli-

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fied 1.5-kilo base molecule was eluted from the gel with a Ultrafree-DA (Millipore). After ligation of the eluted DNA into pCR 2.1-TOPO vector (Novagen) and transformation of *E. coli* DH5α, positive clones were identified by treatment of the recombinant plasmids with restriction enzymes and DNA sequencing. DNA sequences were analyzed on ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with BigDye Terminator cycle sequencing kit with the manufacturer's specification.

Ternary Structure Prediction by SWISS-MODEL The ternary structure of *TrxR1* has been predicted on SWISS-MODEL using the ternary structures of mammalian glutathione reductase (1GETA, 1GETB, 1GERB, and 1GERA) as the template. The amino acid sequence of the lung adenocarcinoma cell has been submitted through the web page, http://www.expasy.org/swissmod/SWISS-MODEL.html, along with those template ternary structures. Since the SWISS-MODEL server will return all results via E-mail, the results options were set as "Send the results as plain ASCII mail instead of email attachment" for convenience on Macintosh computers.

Results

Molecular Cloning of TrxR1 and TrxR2 ---- The genes encoding *TrxR1* and *TrxR2* were amplified from cDNA which has been prepared from mRNA freshly extracted from the lung cancer cells. The yield of the PCR products on the agarose electrophoresis gel indicated that *TrxR1* appears to be the dominant form and *TrxR2* was detected in much smaller amount (Fig 1A). When cDNA was prepared from commercially available total RNA, which was prepared from normal lungs of 40-year old male caucasian adult, only the *TrxR1* gene has been successfully amplified by PCR with an intense signal appeared on the agarose-gel electrophoresis (Fig 1B). Primers designed for cloning *TrxR2* gene failed to amplify the corresponding gene even by the touch-down PCR.

Both the open reading frames of normal and cancer *TrxR1*s predicted polypetides of 499 amino acids with a COOH-terminal Gly-Cys-Secys-Gly motif provided that an in-frame TGA codon encodes Secys. Deduced amino acid sequences of NCI-H441 *TrxR1* and human placenta *TrxR1* (access #S79851) revealed three point mutations,

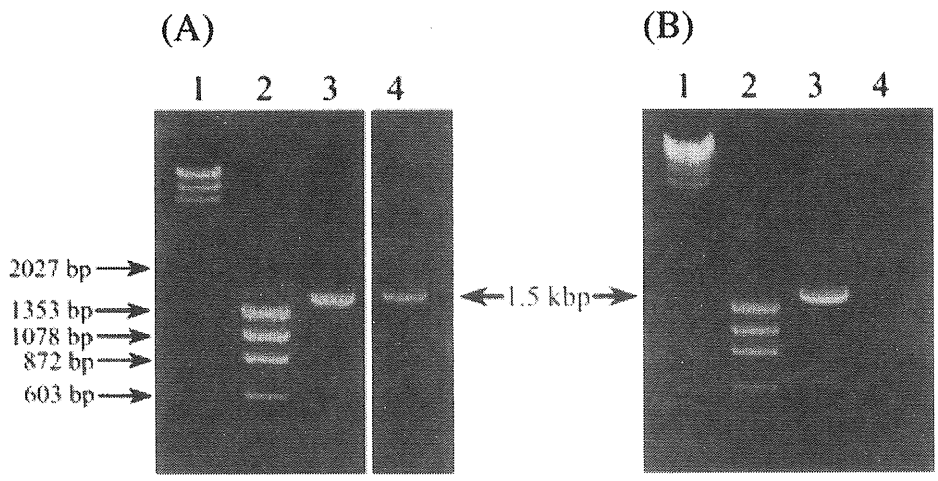

Fig. 1 Agarose gel electrophoresis of amplified *TrxR* genes.
(A) NCI-H441 cDNA was amplified by primers of *TrxR1* (lane 3) and *TrxR2* (lane 4). *TrxR1* was diluted 20 times before loaded on the agarose gel but still gave a signal which is as strong as the *TrxR2* gene, which required touch-down PCR for its specific amplification. (B) Normal lung cDNA was amplified with primers of *TrxR1* (lane 3) and *TrxR2* (lane 4). Lane 4 gives no detectable signals. *TrxR2* was not successfully obtained.

Met101Thr, Ser156Arg, and Gly215Asp. All the three mutations were caused by single base changes. Among the three mutation, Ser156Arg and Gly215Asp were also observed in the sequence of *TrxR1* gene cloned from the normal lung cells. The Met101Thr point mutation remained as the adenocarcinoma-specific mutation (Fig. 2). Swiss MODEL has been used to locate the ternary position of the M101T mutation. The predicted structure indicated that the Met101 was located over the redox active site, Cys59 and Cys64, which transfers the pair of electrons from NADPH via FAD (Fig. 3).

The gene encoding *TrxR2* was cloned from NCI-H441 but not successfully from the cDNA prepared from normal lung cells. The open reading frame predicts a polypeptide of 521 amino acids with a COOH-terminal Gly-Cys-Secys-Gly motif as *TrxR1*. The deduced amino acid sequence of *TrxR2* shows 56.1 % identity to that of *TrxR1* except for the 36 additional residues upstream of the NH2-terminal sequence (Fig. 2). The sequence of this 36-residue region is typical of that of a mitochondrial leader peptide. Three amino acid substitutions by single base substitutions were indicated through comparison with the sequence of human *TrxR2* (AF171054), that were A65S, I370T, and G384S. It is not clear whether those mutations were organ specific like Ser156Arg and Gly215Asp found in *TrxR1* genes or one of those were carcinogenesis-specific.

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ID   TrxR1(NCI-H441) Amino Acid Seq
SQ   SEQUENCE   499 AA;  54573 MW;  1282118 CN;
MNGPEDLPKSYDYDLIIIGGGSGGLAAAKEAAQYGKKVMVLDFVTPTPLGTRWGLGGTCVNVGCIPK
KLMHQAALLGQALQDSRNYGWKVEETVKHDWDRTIEAVQNHIGSLNWGYRVALREKKVVYENAYGQ
FIGPHRIKATNNKGKEKIYSAERFLIATGERPRYLGIPGDKEYCISSDDLFSLPYCPGKTLVVGAS
YVALECAGFLAGIGLDVTVMVRSILLRGFDQDMANKIGEHMEEHGIKFIRQFVPIKVEQIEAGTPG
RLRVVAQSTNSEEIIEGEYNTVMLAIGRDACTRKIGLETVGVKINEKTGKIPVTDEEQTNVPYIYAI
GDILEDKVELTPVAIQAGRLLAQRLYAGSTVKCDYENVPTTVFTPLEYGACGLSEEKAVEKFGEENI
EVYHSYFWPLEWTIPSRDNNKCYAKIICNTKDNERVVGFHVLGPNAGEVTQGFAAALKCGLTKKQLD
STIGIHPVCAEVFTTLSVTKRSGASILQAGCXG

ID   TrxR2(NCI-H441) Amino Acid Seq
SQ   SEQUENCE   521 AA;  56227 MW;  E0AEAA9B CRC32;
MAAMAVALRGLGGRFRWRTQAVAGGVRGAARGAAAGQRDYDLLVVGGGSGGLACAKEAAQLGRKVS
VVDYVEPSPQGTRWGLGGTCVNVGCIPKKLMHQAALLGGLIQDAPNYGWEVAQPVPHDWRKMAEAVQ
NHVKSLNWGHRVQLQDRKVKYFNIKASFVDEHTVCGVAKGGKEILLSADHIIIATGGRPRYPTHIEG
ALEYGITSDDIFWLKESPGKTLVVGASYVALECAGFLTGIGLDTTIMMRSIPLRGFDQQMSSMVIEH
MASHGTRFLRGCAPSRVRRLPDGQLQVTWEDSTTGKEDTGTFDTVLWAIGRVPDTRSLNLEKAGVDT
SPDTQKILVDSREATSVPHIYAIGDVVEGRPELTPTAIMAGRLLVQRLFSGSSDLMDYDNVPTTVF
TPLEYGCVGLSEEEAVARHGQEHVEVYHAHYKPLEFTVAGRDASQCYVKMVCLREPPQLVLGLHFLG
PNAGEVTQGFALGIKCGASYAQVMRTVGIHPTCSEEVVKLRISKRSGLDPTVTGCXG
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Fig. 2 Deduced amino acid sequences of *TrxR1* and *TrxR2* amplified from cDNA of human lung adenocarcinoma cells NCI-H441.
Deduced amino acid sequence of *TrxR1* (upper) indicated the occurrence of three amino acid substitutions, Met101Thr, Ser156Arg, and Gly215Arg when compared with the corresponding sequence of human placenta *TrxR1*. The latter two substitutions of Ser156Arg and Gly215Arg were also observed in the sequence of normal lung *TrxR1*. Deduced amino acid sequence of *TrxR2* (lower) indicated the occurrence of Ala65Ser, Ile370T, and Gly384Ser when compared with the sequence of human *TrxR2*.

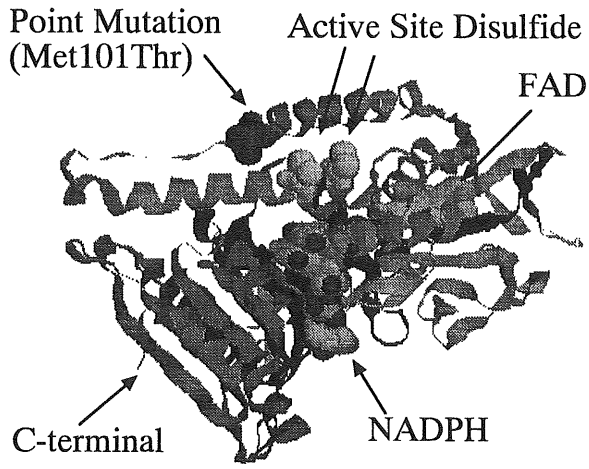

Fig. 3 SWISS-MODEL prediction of ternary structure of normal lung *TrxR1*.
The M101 was found located above the active-site disulfide formed between Cys59 and Cys64. The Met residue would be accessible by reactive oxygen species and thus oxidized to methionine sulfoxide.

Discussion

Thioredoxin-*TrxR* redox system is one of the major redox systems in animal cells, which, together with the glutathione-glutathione reductase system, participates in the redox control of a great variety of biological processes involved in cell life and death[6-8]. Mammalian cells contain two distinct forms of thioredoxin (*Trx*): *Trx1* is located in the cytosol and nucleus and is also secreted[9], whereas *Trx2* is restricted to mitochondria[10]. Most of the reactive oxygen species generated in mammalian cells are generated as a result of the univalent reduction of molecular oxygen to the superoxide anion (O_2-) by electrons that leak from the mitochondrial respiaratory chain; the O_2- then undergoes spontaneous or enzyme-mediated dismutation to H_2O_2. These species can react with various targets including proteins. In particular, methionine residues can be oxidized into methionine sulfoxide (MetSO). Such modifications can alter the biological properties of the targeted proteins[11-14]. Swiss-Model has predicted the ternary position of Met-101 near the active site cysteines, and the location may be closer for the selenocysteine residue when the COOH terminal sequence to carry the electrons from the active site to the surface of the enzyme molecule. Met to Thr mutation in the lung cancer may confer the immunity to the Methionine oxidation, which would prevent excessive NADPH consumption and oxidative destruction of selenocysteine.

TrxR2 was amplified from the cDNA of NCI-H441 cells but not from the cDNA of normal lung cDNA. This might reflect the increased *TrxR2* expression in the mitochondria of the lung adenocarcinoma cells. Increased oxidative stress in mitochondria would result in collapse of the mitochondrial membrane potential, and, ultimately, cell death[15-16]. Oxidative stress in mitochondria also promotes the calcium-dependent, nonspecific permiabilization of the inner membrane as a result of oxidation and cross-linking of thiol groups in membrane proteins[17,18]. Such increased nonspecific permeabilzation has been suggested to lead to the release of mitochondrial constituents, including cytochrome c, into the cytosol, which in turn induces cell death by apoptosis[19-23]. Thus, the line of defense system by *TrxR2* and *Trx2* may be reinforced in transformed lung cells against H_2O_2 and be likely to plays a critical role in the rapid growth of the adenocarcinoma cells.

Acknowledgements We appreciate Dr. Thressa C. Stadtman for encouragement and helpful discussion. The present work was supported in part by a grant-in-aid for Scientific Research (11780449) from the Ministry of Education, Science and Culture of Japan, and by MISHIMA KAIUN MEMORIAL FOUNDATION, THE JAPAN SCIENCE SOCIETY, and KUROZUMI MEDICAL FOUNDATION in the very early stage of the research to T.T.

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