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Mouse Glutaredoxin – cDNA Cloning, High Level Expression in *E. coli* and its Possible Implication in Redox Regulation of the DNA Binding Activity in Transcription Factor PEBP2

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We have isolated a cDNA encoding glutaredoxin (GRX) from a mouse splenic cDNA library. This cDNA encoded a protein of 107 amino acids with a calculated molecular weight of 11.9 kDa. The deduced amino acid sequence of glutaredoxin in mouse was highly homologous with that in other mammals (81–89%), containing a putative active sequence of -Cys-Pro-Try-Cys-. Recombinant mouse glutaredoxin expressed in *E. coli* showed glutathione-disulfide oxidoreductase activity with β-hydroxyethyl disulfide as its substrate, whereas mutant glutaredoxin (Cys 22, Cys 25 to Ser) showed no activity. In electrophoretic mobility shift assay, we proved that wild type GRX, not mutant one, recovered the DNA-binding activity of a transcription factor, PEBP2, oxidized by diamide. This showed that GRX may be involved in the redox regulation of the DNA-binding activity of PEBP2 as is the case with thioredoxin.

Keywords: Glutaredoxin, redox regulation, transcription factor, PEBP2, DNA-binding activity

INTRODUCTION

There is accumulating evidence that redox regulation plays a crucial role in signal transduction. Thioredoxin is one of the pioneering molecules in redox regulation. Several proteins share the similar active site: -Cys-Xxx-Yyy-Cys- and are called the thioredoxin superfamily (Table I). Glutaredoxin is a member of the thioredoxin superfamily, but its biological functions remain unclear.

Glutaredoxin (GRX), also known as thioltransferase,[3] is a protein with an approximate molecular weight of 12,000 Dalton (Da). Its active center contains an amino acid sequence of -Cys-Pro-Try-Cys-, and this sequence is preserved among various species from *Escherichia coli* (*E. coli*) to mammals.[4–11] Coupled with reduced

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TABLE I Thioredoxin superfamily

	kDa	Location	Active site
Thioredoxin (TRX)	12	Cytoplasm	-Cys-Gly-Pro-Cys-
Thioredoxin 2 (TRX-2)	12	Mitochondria	-Cys-Gly-Pro-Cys-
TRX related protein-32 (TRP-32)	32	Cytoplasm	-Cys-Gly-Pro-Cys-
TRX-like protein (Txl)			
Glutaredoxin (GRX)	12	Cytoplasm	-Cys-Pro-Tyr-Cys-
Nucleoredoxin	48	Nucleus	-Cys-Pro-Pro-Cys-
Protein disulfide isomerase (PDI)	55	Endoplasmic reticulum	-[Cys-Gly-His-Cys]$_2$-
Ca binding protein 1 (CaBP 1)	49	Endoplasmic reticulum	-[Cys-Gly-His-Cys]$_2$-
Ca binding protein 2 (ERp 72)	72	Endoplasmic reticulum	-[Cys-Gly-His-Cys]$_3$-
Phospholipase C γ	61	Endoplasmic reticulum	-[Cys-Gly-His-Cys]$_2$-

β-nicotinamide adenine dinucleotide phosphate (NADPH), glutathione reductase, and glutathione (GSH), GRX has a GSH-disulfide oxidoreductase activity[12,13] and reduces low molecular weight disulfides and proteins.[14] GRX is a GSH-dependent electron donor for ribonucleotide reductase. It was originally identified in *E. coli*[15] as an essential enzyme for DNA synthesis. Thereafter, it has been shown that GRX can regenerate proteins inactivated by oxidative stress,[16–18] and that GRX may play a role in signal transduction.[19] Similar to another important redox protein, thioredoxin (TRX),[20] GRX may play various roles in the control of redox status *in vivo*. In mammals, the cDNA of GRX has been cloned in human[21,22] and pig,[23] and amino acid sequences of GRX protein have been determined in human,[5–8] pig,[9] calf,[10] and rabbit.[11] However, there is no report about the cDNA cloning and expression of mouse GRX.

Recently, there have been increasing reports about reduction–oxidation (redox) sensitive transcription factors.[24–29] It has already been demonstrated that the signal transduction by Runt domain of Polyoma virus enhancer-binding protein 2 (PEBP2) is affected by the redox status,[30] and that both TRX and Ref-1[31] modulate this regulation.[32]

In the present report, we describe the cDNA cloning, sequencing and expression in *E. coli* of murine GRX. We prepared the mutant mouse GRX construct replacing two cysteine residues in the active center by serine, and compared the GSH-disulfide oxidoreductase activity between wild type and mutant recombinant GRXs. To investigate biological roles of GRX, we examined its effects on the DNA-binding activity of PEBP2 using an electrophoretic mobility shift assay with these recombinant mouse GRXs and recombinant Runt domain of PEBP2.

MATERIALS AND METHODS

Materials

E. coli BL21 (DE3) pLysS, XL1-blue, and the plasmid vector pBlue-script SK+ were purchased from Strategene (CA, USA). The altered site *in vitro* mutagenesis system was purchased from Promega (Wisconsin, USA), and the *E. coli* expression vector of pRSET from Invitrogen (Netherlands). Nickel-nitrilotriacetic acid (Ni-NTA) resin was purchased from Qiagen (Germany), the Protein Assay Kit from Biorad (CA, USA). GSH, glutathione reductase, and NADPH were purchased from Sigma (WI, USA). All the other chemicals were of at least reagent grade from standard sources.

cDNA Cloning and Sequencing

Preparation of the Probe

The primer for DNA synthesis was designed based on the cDNA sequence of GRX in other mammals. The human cDNA library was used as

the template and a part of human GRX cDNA was amplified in the polymerase chain reaction (PCR) (Hirota, unpublished data). The PCR product of about 300 bp was labeled with ^{32}P and used as a probe for screening a mouse cDNA library.

Screening and Sequencing

A mouse splenic cDNA library constructed in lambda GT11 was kindly provided by Dr. A. Shimizu (Center for Molecular Biology and Genetics, Kyoto University). The host *E. coli* Y1090r$^-$ transformed with 1×10^6 pfu of lambda GT11 was incubated, and the phage DNA was transferred to a nitrocellulose filter after the plaque growth. Hybridization was carried out overnight at 65°C using a reaction solution consisting of 6× SSC, 5× Denhardt's solution, 0.5% of sodium dodecyl sulfate (SDS), and 0.1 mg/ml salmon sperm DNA. After washing twice with 2× SSC/0.2% SDS at 65°C for 15 min and once with 0.1× SSC/0.15% SDS at 65°C for 30 min, the membrane was subjected to autoradiography.

After repeating these procedures three times, the phage DNA of one of the positive clones was purified, the insert was rescued and subcloned into the pBluescript SK+ vector. According to the manufacturer's instructions, this insert was sequenced in both directions by the ABI PRISM 377 DNA sequencer (Perkin Elmer Co., CA, USA).

Construction of Wild Type and Mutant Open Reading Frame of Murine GRX

To add restriction sites of BamH1 and EcoR1 at 5′ and 3′ ends respectively, mouse GRX open reading frame (ORF) was amplified in PCR using two primers; 5′-CGCGGATCCGCTCAGGAGTTTG-TGAACTG-3′, and 5′-CGGAATTCTTATAACT-GCAGAGCTCCAA-3′ (underlines show the sequence in each primer recognized by BamH1 or EcoR1). PCR was carried out using TP-480 (Takara Co., Japan) at 94°C, 52°C, and 72°C (for 1 min each) for 30 cycles. The recovered PCR fragments were digested with BamH1 and EcoR1, and subcloned into the pALTER-1 vector.

Site-directed mutagenesis was performed using the Altered Site *in vitro* mutagenesis system following the manufacturer's instructions. To induce mutation by replacing two amino acid residues of Cys 23 and Cys 25 in the active center with Ser, the mutagenetic oligo DNA of 5′-TTTCTTGGGTCTTTCTGGAGTAGGGGGAGG-TGGGCTTGATGAAC-3′ was used (the bases replaced to induce mutation are underlined). After the procedure, the inserts were sequenced in both directions to confirm the induction of mutation.

Expression and Purification of Wild Type and Mutant GRX Fusion Protein in E. coli

ORFs of wild-type or mutant GRX were subcloned into expression vectors of pRSET. The pRSET-transformed host bacteria of BL21(DE3)-pLysS were incubated at 37°C in Luria–Bertani medium containing ampicillin (100 μg/ml) until an optical density $(OD)_{600}$ of 0.7 was reached. Protein synthesis was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) at final concentration of 1 mM and the medium was shaken for 4 h additionally.

The cells were centrifuged at 15 000 rpm, 4°C for 30 min. The pellet was resuspended in the suspension buffer, which consisted of lysozyme 1 mg/ml, 10 mM mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.8 mM imidazole, 50 μM N α-p-tosyl-L-lysine choloromethyl ketone in phosphate buffer saline (PBS) and lysed by sonication.

After centrifugation at 15 000 rpm, 4°C for 30 min, the supernatant was passed through a Ni-NTA resin column. Washing the column with PBS containing 20 mM and 80 mM imidazole, histidine (His)-tagged GRX fusion protein was eluted with PBS containing 200 mM imidazole. The purity of wild-type and mutant GRX fusion protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Measurement of the Activity of Recombinant GRX

GSH-disulfide oxidoreductase activity of recombinant GRX was determined with β-hydroxyethyl disulfide (HED) as the substrate, as described previously.[10] The measurement was done in a 96-well plate reader. In each well, 5 µl of sample solution and 100 µl of reaction mixture, composed of 0.1 M Tris-HCl pH 8.0, 2 mM ethylene diamine tetraacetic acid (EDTA) pH 8.0, 0.1 mg/ml bovine serum albumin, 1 mM GSH, 0.2 mg/ml NADPH, and 6.0 µg/ml yeast glutathione reductase, were applied. The measurement was started after adding 0.7 mM of β-hydroxyethyl disulfide. Absorbance at 340 nm was measured at every 10 s for 2 min, and maximal NADPH consumption rate (V_{max}) was recorded as the activity of GRX. Wells containing the mixture without samples were used as backgrounds. This negative kinetics assay was performed with a microplate reader and SOFTmax Version 2.31 software (Molecular Device, Menlo Park, CA).

Preparation of Recombinant Runt Domain

His-tagged recombinant Runt domain was expressed in *E. coli* and purified through a Ni-NTA resin column as previously described.[27]

Electrophoretic Mobility Shift Assay (EMSA)

The DNA probe containing the PEBP2-binding site was prepared as previously reported.[30] In the indicated lanes, Runt domain was pre-treated with 2 mM diamide at 30°C for 10 min. Then, the proteins were treated at 37°C for 15 min with reducing agents, such as 100 mM dithiothreitol (DTT), GSH system (0.1 mM GSH + 0.0002 unit/µl glutathione reductase + 1 mM NADPH), wild type or mutant GRX, wild type or mutant GRX + GSH system.

The DNA-binding reaction was carried out for 10 min at 30°C in 10 µl of reaction solution. This solution contained 20 mM N-[2-Hydroxyethyl]-piperazine-N′-[2-ethanesulfonic acid] (HEPES)-KOH pH 8.0, 4% (w/v) Ficoll 400, 2 mM EDTA, 100 mM KCl, 0.1 µg poly (dI-dC), 6% glycerol, 0.2 mg/ml bovine serum albumin, 0.04% bromophenol blue, 10 fmol of ^{32}P-labeled probe, and 5 ng of purified His-tagged Runt domain.

The reaction solution was loaded on 10% non-denaturing polyacrylamide gel (acrylamide : bisacrylamide = 39 : 1) in 0.25× Trisborate-EDTA buffer. After drying, the gel was analyzed by phosphorimager (Fujifilm BAS 2000) or autoradiography.

RESULTS

cDNA Cloning and Sequencing of Murine GRX

With ^{32}P labeled DNA probe, screening of murine GRX cDNA was performed from the lambda GT11 cDNA library. Figure 1 shows the cDNA sequence of mouse GRX and the corresponding amino acid sequence. The ORF coded a protein consisting of 107 amino acids, and the calculated molecular weight of mouse GRX was 11.9 kDa, which is similar to those of GRX in other mammals.

Figure 2 shows a comparison of amino acid sequences among mammalian species. The amino acid sequence of -Cys-Pro-Try-Cys- in the active center was preserved also in murine GRX. Homology of the deduced amino acid sequence of mouse GRX to those of human, pig, calf, and rabbit was 89%, 81%, 81%, and 83%, respectively.

Mutagenesis of Cys22 and Cys25 to Ser

The Altered Site *in vitro* mutagenesis system was used to replace two residues of Cys (Cys22 and Cys25) with Ser in the active center of mouse GRX. cDNA sequencing confirmed the mutagenesis corresponding to the replacement of Cys22 and Cys25 with Ser (data not shown).

```
1                                                                    60
ATG GCT CAG GAG TTT GTG AAC TGC AAG ATC CAG TCT GGG AAG GTG GTC GTG TTC ATC AAG
Met Ala Gln Glu Phe Val Asn Cys Lys Ile Gln Ser Gly Lys Val Val Val Phe Ile Lys

21                                                                  120
CCC ACC TGC CCC TAC TGC AGA AAG ACC CAA GAA ATC CTC AGT CAA CTG CCT TTC AAA CAA
Pro Thr Cys Pro Tyr Cys Arg Lys Thr Gln Glu Ile Leu Ser Gln Leu Pro Phe Lys Gln

121                                                                 180
GGT CTT CTG GAG TTT GTG GAC ATC ACA GCC ACT AAC AAC ACC AGT GCG ATT CAA GAT TAT
Gly Leu Leu Glu Phe Val Asp Ile Thr Ala Thr Asn Asn Thr Ser Ala Ile Gln Asp Tyr

181                                                                 240
TTA CAA CAG CTC ACC GGA GCG AGA ACA GTT CCT CGG GTC TTC ATA GGT AAA GAC TGC ATA
Leu Gln Gln Leu Thr Gly Ala Arg Thr Val Pro Arg Val Phe Ile Gly Lys Asp Cys Ile

241                                                                 300
GGC GGA TGC AGT GAT CTA ATC TCC ATG CAA CAG ACT GGG GAG CTG ATG ACT CGG CTG AAG
Gly Gly Cys Ser Asp Leu Ile Ser Met Gln Gln Thr Gly Glu Leu Met Thr Arg Leu Lys

301                          324
CAG ATT GGA GCT CTG CAG TTA TAA
Gln Ile Gly Ala Leu Gln Leu ***
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FIGURE 1 Mouse GRX cDNA. The nucleotide sequence and deduced amino acid sequence of mouse glutaredoxin cDNA.

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          1                                                          50
Mouse     MAQEFVNCKIQSGKVVVFIKPTCPYCRKTQEILSQLPFKQGLLEFVDITA
Human     MAQEFVNCKIQPGKVVVFIKPTCPYCRRAQEILSQLPIKQGLLEFVDITA
Pig       MAQAFVNSKIQPGKVVVFIKPTCPFCRKTQELLSQLPFKEGLLEFVDITA
Calf      -AQAFVNSKIQPGKVVVFIKPTCPYCRKTQELLSQLPFKQGLLEFVDITA
Rabbit    -AQEFVNSKIQPGKVVVFIKPTCPYCRKTQEILSELPFKQGLLEFVDITA

          51                                                         100
Mouse     TNNTSAIQDYLQQLTGARTVPRVFIGKDCIGGCSDLISMQQTGELMTRLK
Human     TNHTNEIQDYLQQLTGARTVPRVFIGKDCIGGCSDLVSLQQSGELLTRLK
Pig       TSDTNEIQDYLQQLTGARTVPRVFIGKECIGGCTDLESMHKRGELLTRLQ
Calf      AGNISEIQDYLQQLTGARTVPRVFIGQECIGGCTDLVNMHERGELLTRLK
Rabbit    TSDMSEIQDYLQQLTGARTVPRVFLGKDCIGGCSDLIAMQEKGELLARLK

          101    107
Mouse     QIGALQL
Human     QIGALQ-
Pig       QIGALK-
Calf      QMGALQ-
Rabbit    EMGALRQ
```

FIGURE 2 Mammalian GRX sequences. Comparison of deduced amino acid sequence of mouse GRX with those of human,[6] pig,[9] calf[10] and rabbit.[11] The amino acid sequences other than in mouse were determined directly from purified proteins. The sequences of CPYC in the active centers are boxed.

Expression and Purification of GRX Fusion Protein

The cDNA of wild-type or mutant murine GRX was subcloned into the pRSET vector, and protein synthesis in the host bacteria BL21(DE3)pLysS was induced with IPTG. With an Ni-NTA column, the His-tagged recombinant protein was purified, and the expression of each protein was confirmed with SDS-PAGE (Figure 3). The level

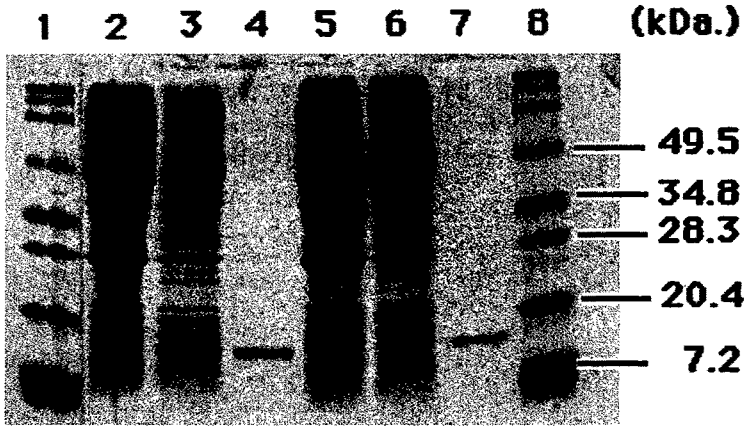

FIGURE 3 Analysis of purified recombinant GRX in mouse. The open reading frames of wild-type and mutant glutaredoxin were subcloned in expression vectors of pRSET. His-tagged fusion proteins with approximate molecular weights of 15 kDa (glutaredoxin; 12 kDa + Tag; 3 kDa) were observed. Lanes 1, 8: Molecular weight standards (kDa). Lanes 2–4: wild type, lanes 5–7: mutant. Lanes 2, 5: lysate of *E. coli* (before adding IPTG). Lanes 3, 6: lysate of *E. coli* (after adding IPTG). Lanes 4, 7: purified recombinant GRX.

of expression was relatively low and recombinant proteins were not apparent in crude extracts. However, in the elution with PBS and 200 mM imidazole, purified His-tagged fusion proteins with approximate molecular weights of 15 kDa (GRX; 12 kDa + Tag; 3 kDa) were observed.

Measurement of the Activity of Wild Type and Mutant Mouse GRX

The GSH-disulfide oxidoreductase activity of recombinant mouse GRX was measured with HED as the substrate. The activity of wild-type GRX was observed in a dose-dependent manner. On the other hand, mutant mouse GRX showed no activity suggesting that the amino acid sequence including Cys22 and Cys25 contributed to the activity of mouse GRX (Figure 4).

Effects of GRX on the DNA-binding Activity of PEBP2

Pre-treatment of recombinant Runt domain with 2 mM diamide resulted in the loss of its DNA-binding activity. This activity was recovered with 100 mM of DTT. These results suggested that the DNA-binding activity of recombinant Runt domain was redox-regulated.

Treatment with the GSH system had no effect on the DNA-binding activity of Runt domain, whereas wild-type mouse GRX alone slightly increased the DNA-binding activity of Runt domain. The combination of GSH system and wild-type GRX significantly restored the attenuated binding activity of Runt domain. Mutant GRX or the combination of the GSH system and mutant GRX had no effects on its binding activity.

These findings showed that the GRX–GSH system may increase the DNA-binding activity of recombinant Runt domain through a redox regulatory mechanism (Figure 5).

DISCUSSION

This work is the first report of the cDNA cloning, sequencing, and expression in *E. coli* of mouse GRX. In the present study, wild-type mouse GRX showed β-hydroxyethyl disulfide reducing activity in a dose-dependent manner. Disappearance of the activity in mutant GRX (which has Ser in place of Cys22 and Cys25) indicated that the amino acid sequence of -Cys-Pro-Try-Cys- preserved

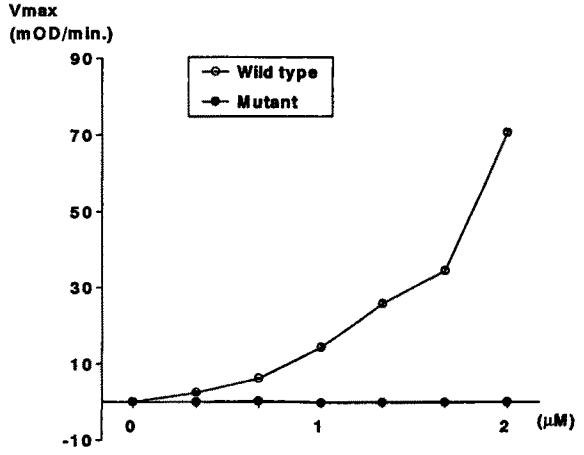

FIGURE 4 Measurement of the activity of wild-type and mutant mouse GRX. The GSH-disulfide oxidoreductase activity of recombinant mouse GRX was measured with β-hydroxyethyl disulfide as the substrate. The activity of wild-type GRX was observed in a dose-dependent manner. On the other hand, mutant mouse GRX (Cys 22, Cys 25 to Ser) showed no activity.

FIGURE 5 Effects of GRX on the DNA-binding activity of PEBP2. Lane 1: without diamide. Lane 2: Pretreatment of recombinant Runt domain with 2 mM of diamide resulted in the attenuation of the DNA-binding activity. Lane 3: The activity was recovered with 100 mM of DTT. Lanes 4–6: Wild-type mouse GRX alone (Lane 4: 5 μM, lane 5: 10 μM, lane 6: 15 μM) slightly increased the DNA-binding activity of Runt domain. Lane 8: Treatment with GSH system (0.1 mM GSH + 0.0002 unit/μl glutathione reductase + 1 mM NADPH) had no effects on the DNA-binding activity of Runt domain. Lane 9: The combination of GSH system and 5 μM of wild-type GRX greatly restored the binding activity of Runt domain. Mutant GRX (lane 7) or the combination of GSH system and mutant GRX (lane 10) had no effects on the binding activity.

also in mouse GRX was the active center, similar to what has been reported in other mammals.[33,34]

PEBP2 is a murine heteroduplex protein composed of α and β subunits.[35] A human homolog of PEBP2, AML1, is involved in the transcriptional control of various genes, such as T-cell receptor, CD3, macrophage colony stimulating factor, and interleukin-3.[36] The α-subunit of PEBP2 combines with the specific DNA sequence of RACCR-CA. On the other hand, the β-subunit is known not to directly combine with DNA, but to potentiate the signal transduction through the α-subunit.[35,37] All the Runt family members, such as Drosophila segmentation gene *runt*,[38] human *AML1* gene,[39] and the α-subunit of *PEBP2* gene, contain a highly preserved region named 'Runt domain', which is involved in the DNA binding as well as in the formation of dimer with the β-subunit.[30,40]

Recently, there have been many reports about reduction–oxidation (redox) control of transcription factors, principally in relation to thioredoxin.[24,29] It has already been demonstrated that the signal transduction by Runt domain is affected by redox status,[30] and that both TRX and Ref-1[31] modulate the signal transduction by Runt domain[32]. Interestingly, it has recently been reported that GRX regulates the activity of some transcription factors, Nuclear Factor 1[41] and OxyR.[42]

In our present study, we showed that mouse GRX restored the DNA-binding activity of PEBP2 oxidized by diamide. This reactivation was enhanced when coupled with a GSH system (GSH, GSH reductase and NADPH). In contrast, mutant GRX in which the Cys-residues in the active center were replaced with Serine had no effects on the DNA-binding activity even if it was coupled with GSH system. Further study is necessary to clarify this regulation, but GRX may contribute to the redox regulation of PEBP2, similar to TRX that plays an important role in regulation of several transcription factors, such as NF-κB,[24–26] AP-1,[27,28] glucocorticoid receptor.[29] GRX has been considered to reduce specifically disulfide bonds of protein-GSH mixed disulfides (protein SSG).[43] However, our results showed that GRX reactivated Runt domain (previously inactivated by diamide) without GSH. This finding indicated that not only protein SSG but disulfides of proteins could be a substrate for GRX.

In conclusion, we have demonstrated here the cDNA cloning of mouse GRX and possible involvement of GRX in redox regulation of PEBP2 DNA binding activity. GRX as well as TRX may play crucial roles in redox regulation of signal transduction.

Note: The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB013137.

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