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Characterization of Protein Targets of Mammalian Thioredoxin Reductases

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Introduction

Mammalian thioredoxin reductases (TRs) are members of the pyridine nucleotide-disulfide oxidoreductase family. The main function of these enzymes is to maintain thioredoxins (Trxs) in the reduced state. Three TRs have been identified in mammals: TR1 (also known as TrxR1 or Txnrd1) localized in the cytosol/nucleus (Holmgren and Bjornstedt, 1995; Gladyshev *et al.*, 1996), TR3 (also known as TrxR2 or Txnrd2) targeted to mitochondria (Gasdaska *et al.*, 1999; Lee *et al.*, 1999), and thioredoxin/glutathione reductase TGR (also known as Txnrd3 or TR2) that is primarily expressed in testes (Sun *et al.*, 2001).

Mammalian TRs are homodimeric enzymes with a head to tail arrangement of subunits. The N-terminal redox-active dithiol in one subunit and the C-terminal selenolthiol site of the other subunit form a redox active center (Sandalova *et al.*, 2001; Biterova *et al.*, 2005). The proposed mechanism of mammalian TR involves the reduction of the N-terminal active site disulfide by NADPH via enzyme-bound FAD, the electron transfer from the N-terminal dithiol to the C-terminal selenenylsulfide, and finally reduction of the substrate by the C-terminal selenolthiol (Zhong *et al.*, 2000; Cheng *et al.*, 2009). TGR differs from other TRs in that it also has an N-terminal glutaredoxin domain, which can be reduced by the C-terminal selenolthiol as well as by glutathione. In addition, TRs occur in a variety of forms that differ in their N-terminal regions.

The accessibility and high reactivity selenocysteine (Sec) in the C-terminal tetrapeptide allows mammalian TRs to be active *in vitro* with a wide range of substrates from small molecules, such as selenite and lipid hydroperoxides, to proteins such as Trx, protein disulfide isomerase (PDI) and glutathione peroxidases (GPxs) (Gromer *et al.*, 2004; Arner, 2009). The major intracellular substrates of TRs are thought to be thioredoxins, which in turn deliver reducing equivalents to many cellular proteins. One example is ribonucleotide reductase, which is essential for DNA synthesis and converts ribonucleotides to deoxyribonucleotides. Thioredoxins also reduce methionine sulfoxide reductases and peroxiredoxins, and therefore are involved in the repair of oxidized proteins and in redox signaling via hydrogen peroxide (Holmgren, 1985; Arner and Holmgren, 2000). In addition, the Trx system participates in many cellular pathways by controlling the redox state of transcription factors via cysteines critical for DNA binding; these transcription factors include NF- κ B, AP-1, and p53 (Lillig and Holmgren, 2007). Therefore, TRs are involved in the control of cellular growth, proliferation, viability and apoptosis through the control of Trx activity and cellular redox state. Another major cellular redox system is the glutathione

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system, which is also powered by NADPH. Trx and glutathione systems work in parallel, but also may overlap in function.

Identification of substrates of TRs has been a slow process, with new targets identified mostly on a one-to-one basis. However, the reaction mechanism of TRs supports a method that could trap TR substrates in a covalent non-productive complex with TRs. Accordingly, attachment of TRs to affinity matrices allows isolation and identification of these targets. This method is described in this chapter with focus on targets for TR1 and TR3.

Preparation of TR-immobilized Affinity Resins

Preparation of TR-immobilized resins is based on an established procedure that employs cyanogen bromide (CNBr)-activated Sepharose 4B. CNBr-activated matrices, such as Sepharose- or agarose-based, are commonly used materials in proteins analysis. They are widely used for preparation of protein affinity resins to study protein-protein interactions, antibody isolation and protein purification. Such affinity resins were previously used to characterize targets of plant and animal Trxs, wherein the immobilized Trxs were used in which the resolving Cys in the active site was mutated to Ser or Ala (Motohashi *et al.*, 2001; Schwertassek *et al.*, 2007). Removal of the resolving Cys allowed trapping of Trx-interacting proteins due to formation of stable mixed disulfides between monothiol Trx and its substrate. Subsequently, Trx targets could be eluted from the Trx resin by reducing the mixed disulfide with a reducing agent such as dithiothreitol (DTT).

We adapted a similar catalytic mechanism-based method to identify targets of TRs. The following recombinant mammalian TR forms were prepared: (i) wild-type TR containing a C-terminal GCUG tetrapeptide (this sample was a 1:1 mixture of the Sec-containing form and the form truncated at the Sec UGA codon due to inefficiency of Sec insertion into recombinant TRs); (ii) a mutant in which Cys in the C-terminal tetrapeptide was replaced with Ser (GSUG) (this form also was a 1:1 mixture of the Sec-containing form and the form truncated at Ser); (iii) a mutant in which Sec was replaced with Cys (GCCG); (iv) a mutant, in which Sec in the C-terminal tetrapeptide was replaced with Cys and Cys was replaced with Ser (GSCG); (v) a mutant in which both Cys and Sec were replaced with Ser residues (GSSG); and (vi) a truncated mutant, in which the Sec codon functioned as a stop signal (GC-stop). Each TR form was then linked to a CNBr-activated Sepharose to prepare TR-immobilized affinity resins. It was previously found that Sec is essential for catalysis by mammalian TRs (Zhong and Holmgren, 2000; Gromer *et al.*, 2003). This is thought to act as the attacking group that reduced the active site disulfide in oxidized thioredoxins, forming an intermediate selenenylsulfide bond between TR and Trx. The Cys adjacent to Sec in the sequence is thought to act as a resolving residue, which reduces the intermediate selenenylsulfide (Arner, 2009). Alternatively, this Cys might be the attacking residue and Sec may be the resolving residue. In this case, one would expect to find differences in the ability of mutant TR forms to bind target proteins. In particular, GSUG, GC and GSCG mutants would be expected to stabilize the mixed selenenylsulfide or disulfide bonds between TR and Trx (or others targets), whereas GCUG, GCCG and especially GSSG mutants would be expected to either complete the reaction or not interact with the substrate. Thus, the target proteins present in cell lysates could be enriched through formation of mixed disulfide or selenenylsulfide intermediates with some immobilized TR, whereas other mutant TR forms could serve as controls. Schematic representation of our method is shown in Fig. 1.

Materials

Recombinant mouse or human TRs; various forms differing in their C-terminal regions (see examples of mutants in the section above)

HisBind HiTrap metal-affinity column (GE HealthCare)

2',5'-ADP-Sepharose (GE HealthCare)

CNBr-activated Sepharose 4B (Sigma)

Buffer A: 0.1 M NaHCO₃, 0.5 M NaCl, pH 8.5

Buffer B: 0.2 M glycine, pH 8.0

Method

1. The recombinant wild-type and C-terminal mutant form of human or mouse TR are prepared as described previously (Turanov *et al.*, 2006). Briefly, BL21(DE) *E. coli* cells transformed with pET-28-TR constructs are grown in LB medium and protein expression is induced with 0.5 mM IPTG when cells reach ~0.6 A_{600nm}. After the induction, cells are grown overnight at 30 °C, harvested by centrifugation and stored at –80 °C until used.
2. *E. coli* cells are resuspended at 4 °C in 50 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 8.0, containing Protease Inhibitor Cocktail EDTA-free (Roche) and sonicated. Following centrifugation for 30 min at 14,000 rpm at 4 °C, the supernatant is loaded onto a 5 ml HisBind column (GE HealthCare). Recombinant proteins containing N-terminal His-tag are eluted with a linear gradient of 20–250 mM imidazole in loading buffer. Fractions containing TR are pooled, dialyzed against PBS, pH 7.4, and applied to a 5 ml 2',5'-ADP-Sepharose (GE HealthCare) column equilibrated with PBS. The column is washed extensively with PBS and the bound proteins are eluted with 1 M NaCl in PBS, then fractions containing pure proteins are dialyzed against PBS and stored at –80 °C.
3. Different TR forms (10–20 mg) in 100 mM sodium carbonate buffer, pH 8.5, containing 0.5 M NaCl, (coupling buffer A) are incubated with freshly activated CNBr-activated Sepharose 4B according to the manufacturer's protocol. The resin is gently mixed using end-over-end mixer for two hours at room temperature or overnight at 4 °C.
4. Unbound protein ligand is washed from resin with buffer A and unreacted groups are blocked with 0.2 M glycine, pH 8.0 (buffer B) for two hours at room temperature or overnight at 4 °C.
5. After several washing steps with buffer A, the TR-affinity resin is equilibrated with PBS or another buffer suitable for following steps.
6. The bright yellow color of the resin and colorless protein solution after protein binding will indicate successful preparation of TR-affinity resin. The immobilized TR is quantified based on the difference between the amounts of TR initially used and remaining in solution after the coupling reaction. Typically, more than 90% of protein could be immobilized.

Identification of Targets of Mammalian TRs in Cell Lysates

To identify cellular targets, the immobilized TR forms are reduced with NADPH, briefly washed with a buffer to remove the excess reductant, and mouse or rat liver mitochondrial or cytosolic lysates are added to each resin. Following incubation and washing, the target proteins are then eluted by adding a DTT-containing buffer. Eluted proteins are concentrated and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Resulting protein gels are stained by Coomassie Blue or Silver staining. Analysis of stained gels may reveal several protein bands, which are present independent of the TR form used. The binding of

these proteins is either non-specific or does not involve the C-terminal tetrapeptide, and these proteins are deemed as non-specific TR targets, or at least they do not associate due to the redox chemistry of TR. Next, specific protein bands can be cut from the gel and subjected to protein identification by mass-spectrometry analysis, LC-MS/MS. Identification of TR targets is summarized in Scheme 1.

Using TR-affinity columns, we identified cytosolic and mitochondrial Trxs as major TR targets in mouse and rat liver (Turanov *et al.*, 2006). We found that the TR-C form (truncated TR with only one Cys-residue in C-terminal active site) was particularly efficient in binding Trx, whereas the TR-SS form (double Ser mutant form, used as negative control) did not show significant Trx binding, as expected (Fig. 2).

Materials

Mouse and rat tissues (liver)

TR-affinity resins

Phosphate-buffered saline (PBS) Buffer A: 20 mM Hepes, pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM KCl

Buffer B: 50 mM Tris-HCl, pH 8.0, 200 mM NaCl

Other reagents: NADPH, DTT (Sigma), Complete Protease Inhibitor Cocktail (Roche Applied Science)

Method

1. Preparation of cellular fractions is carried out by differential centrifugation. Rat livers (10–20 g) or mouse livers (2–5 g) are washed twice in ice-cold PBS and lysed on ice with 60 Dounce strokes with a tight fitting pestle in buffered sucrose (buffer A) containing 20 mM Hepes, pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, and a tablet/10 ml of Complete Protease Inhibitor Cocktail (Roche Applied Science). After two centrifugation runs at 1,000 g for 30 min to discard nuclei, mitochondria were pelleted at 10,000 g for 30 min, washed once, resuspended in buffer A and stored at -80°C . The cytosolic fraction was obtained by ultracentrifugation of the post-mitochondrial supernatant at 100,000 g for 1 hour. To obtain mitochondrial lysate, an aliquot of the mitochondrial fraction is thawed and resuspended in an appropriate buffer or PBS, sonicated, and the mitochondrial lysate is collected by centrifugation at 10,000 g. Protein content is determined by the Bradford assay (Bio-Rad).
2. TR-immobilized affinity resins are prepared as described above using CNBr-Sepharose. The immobilized TR is initially reduced with 0.2 mM NADPH for 30 min in buffer B. In order to search for TR targets, rat or mouse liver mitochondrial or cytosolic lysates in buffer B or PBS (10–50 mg of total protein) are incubated with 1–2 ml of TR-immobilized resin containing 5–10 mg of TR at room temperature for 1 hour under gentle stirring. The resins are washed with 50 mM Tris-HCl, pH 8.0, and 200 mM NaCl (buffer B) to remove nonspecifically bound proteins. The washing is repeated until the absorbance of the wash fraction at 280 nm is negligible. Finally, the resin is suspended in 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, and 10 mM DTT and the mixture is incubated for 30 min at room temperature. Eluted proteins are separated from the resin by centrifugation. The proteins are concentrated and separated on SDS-PAGE gels, visualized by western blot analyses or subjected to staining with Coomassie or Silver Staining Kit (Bio-Rad) and to identification of proteins by LC-MS/MS.

Concluding Remarks and Future Perspectives

The described method can be used to search for TR targets in different tissues and cellular compartments, such as the ER, cytosol and mitochondria. Lysates prepared from other organisms as well as biological fluids containing Trxs should also be amenable to this method. We further suggest that this mechanism-based affinity method can be used to isolate TR targets such as Trx from a variety of biological samples for further proteomic analyses, e.g., posttranslational modifications and binding partners. Fig. 2 shows a typical result with mouse TR1 and illustrates specificity of this method. Trx substrates could also be efficiently isolated on the TR3 affinity columns. Therefore, we suggest that this should be a general method of affinity isolation of Trxs and other TR substrates. Ultimately, the proteomic method including the efficient 2D SDS-PAGE and LC-MS/MS analysis can be developed for high-throughput analysis of TR-interacting proteins. The concept behind this method can also be transferred to certain other pyridine nucleotide disulfide oxidoreductases for identification of their respective targets.

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Figure 1. Schematic representation of the mechanism-based method for identification of TR targets

Upper panel, Putative mechanism of Trx (shown in gray) reduction by TR (shown in black), in which the reduced selenolthiol group in TR provides, in a two-step process, reducing equivalents to the active site disulfide in Trx. TR-CU designates the wild-type form of protein containing Cys and Sec in the C-terminal tetrapeptide. *Lower panel*, Mechanism-based procedure for identification of TR targets. A C-truncated mutant of an immobilized TR (designated as TR-C) allows trapping of the target via disulfide bond. Subsequent addition of DTT elutes the target, which is then subjected to tryptic digest and an MS/MS sequencing procedure for protein identification.

Figure 2. Identification of Trx1 as the major target of TR1

Rat liver cytosolic fractions were incubated with different TR1-immobilized resins. Bound proteins were eluted with DTT and separated by SDS-PAGE. SC, C and SS indicate immobilized TR mutant forms. The gel was stained with Coomassie Blue. Molecular weights of protein standards (in kDa) are shown on the *right*. *Arrow* shows migration of Trx1. Lower panel shows western blotting with anti-Trx1 antibodies.

Figure 3.