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A crosslinker-based identification of redox relay targets

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ABSTRACT

Thiol-based redox control is among the most important mechanisms for maintaining cellular redox homeostasis, with essential participation of cysteine thiols of oxidoreductases. To explore cellular redox regulatory networks, direct interactions among active cysteine thiols of oxidoreductases and their targets must be clarified. We applied a recently described thiol–ene crosslinking-based strategy, named divinyl sulfone (DVSF) method, enabling identification of new potential redox relay partners of the cytosolic oxidoreductases thioredoxin (TXN) and thioredoxin domain containing 17 (TXNDC17). Applying multiple methods, including classical substrate-trapping techniques, will increase understanding of redox regulatory mechanisms in cells.

1. Introduction

Thiol-mediated redox regulation, commonly mediated by thiol-oxidoreductases, is a crucial mechanism for maintaining cellular homeostasis. Thioredoxin (TXN) is representative, and one of the most frequently studied, of the oxidoreductases that play key regulatory roles in mammalian cells [1]. We recently found that, under acute redox stress, interactions among TXN and peroxiredoxin family proteins (PRDX1 or PRDX2) were augmented and their electron relays appeared to be accelerated to counteract the redox disturbances [2]. Thiol modifications, such as S-nitrosylation, S-glutathionylation and S-sulfination, are protein post-translational modifications (PTMs) induced by physiological signals or redox stress [3]. The state of such PTMs can alter the character of a protein, leading to changes in its activity, interaction partners or location. These regulatory processes are also mediated by redox active oxidoreductases [4]. Hence, direct thiol-based interactions are crucial for thiol modification and electron exchange to regulate cellular redox homeostasis.

Proteomics is a powerful technique and several applications were invented to explore the interaction partners of oxidoreductases. For detecting redox relay targets, one of the most conventional strategies is a substrate-trapping method, based on mutation of the oxidoreductase active site. TXN-related oxidoreductases have two vicinal catalytic cysteines, in a sequence known as the Cys-X-X-Cys (CXXC) motif, in their active sites (Fig. 1A). During cysteine thiol–disulfide exchange reactions, the N-terminal cysteine of the CXXC motif functions catalytically as a nucleophilic, transiently forming an intermolecular disulfide (Fig. 1B) [5]. The C-terminal cysteine of the CXXC motif then acts as a resolving cysteine, thereby catalyzing intermolecular disulfide formation. Hence mutation of the resolving cysteine to alanine (CXXA) or serine (CXXS) stabilizes the intermolecular disulfide state (Fig. 1B). This mutation-based technique has proven to be useful for identifying several TXN substrates, *in vivo* and *in vitro*, in mammals and plants [6–11]. This technique is also useful to characterize non-redox-based targets along with redox relay partners when the corresponding control mutants, such as double mutations (AXXA or SXXS) at the active sites, are analyzed in parallel (Fig. 1B). However, this technique has some constraints requiring consideration. For example, the mutants must be incorporated into cells and construction of the mutants might alter enzyme specificity. From a catalytic perspective, CXXA mutants trap primarily those substrates requiring reduction of a disulfide bond or isomerization and rarely trap those requiring

Abbreviations: TXN, Thioredoxin; TXNDC17, Thioredoxin domain containing 17; DVSF, Divinyl Sulfone.

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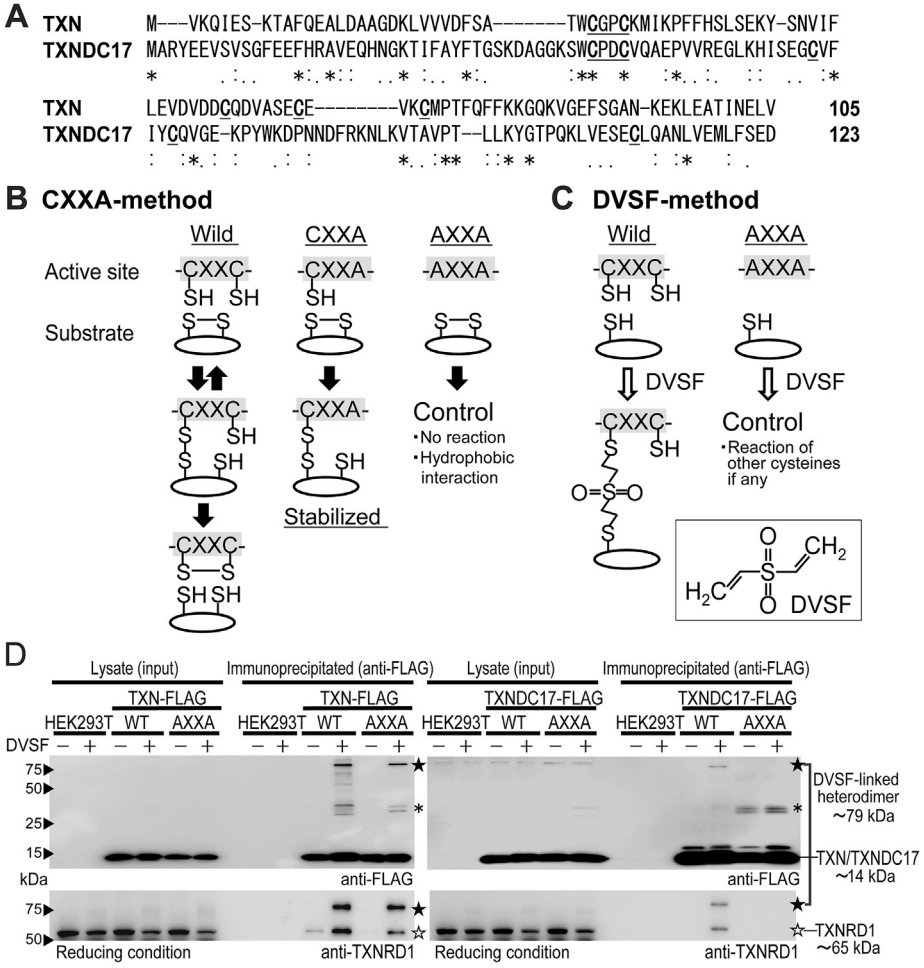

Fig. 1. Sequence comparisons of TXN and TXNDC17 and reaction schemes describing the CXXA and DVSF methods. (A) Alignment of the amino acid sequences of human TXN and TXNDC17. Cysteine residues, including CXXC motifs, are shown in bold and underlined text. (B) Reaction schemes for the CXXA method. (Left) Example of a reaction scheme for reduction by wildtype TXN family proteins. A wildtype active site cysteine forms a mixed disulfide through nucleophilic attack of a substrate thiol. After disulfide bond formation, active site residues exchange electrons or return to their original state. (Middle) Reaction scheme for the CXXA mutant. After a disulfide is formed through the nucleophilic cysteine in the active site, reduction of this disulfide would require a second cysteine, also known as a resolving cysteine. Mutation of the resolving cysteine, therefore, leads to stabilization of the covalent complexes. (Right) Reaction scheme for the AXXA mutant. This mutant is used as a negative control for detecting noncovalently associated biological targets. (C) Reaction schemes for the DVSF method. (Left) The bifunctional electrophile crosslinker DVSF crosslinks redox enzymes with their redox relay partners. (Right) Reaction scheme for the AXXA mutant. This mutant is used as a control for detecting the reaction of DVSF with cysteine residues outside of the active site. The structure of DVSF is shown in the box. (D) After immunoprecipitation using the DVSF method, Protein lysates and eluents were subjected to immunoblotting with anti-FLAG and anti-TXNRD1 antibodies under reducing conditions. Heterodimeric proteins, containing redox proteins (TXN or TXNDC17, ~14 kDa) and TXNRD1 (~65 kDa), were detected at ~79 kDa. TXNRD1 monomeric protein was also detected, probably because of direct disulfide-linked interactions with bait proteins. Even though the active site cysteines of TXN were mutated, the remaining three cysteine residues appeared to form disulfide bonds with TXNRD1. Because TXNRD1 formed homodimers, which can sometimes form inter-disulfide bonds, monomeric TXNRD1 was also detected with DVSF treatment [30]. The asterisk indicates DVSF-linked dimer formations or non-specific bands.

oxidation. This is because the redox state of the CXXA mutant is regarded as being reduced [12,13]. Other strategies, like cis-proline mutation, were investigated but have not become widely accepted, probably because of their lack of versatility [14].

In order to elucidate new potential redox relay partners of oxidoreductases, here we introduced use of newly reported bifunctional electrophile crosslinker, divinyl sulfone (DVSF), which mediate thiol-ene click reaction, in an application referred to as the “DVSF method” [15]. This reagent was reported to crosslink TXN to peroxiredoxin family proteins [16–18]. As a reference, we also employed the conventional substrate-trapping CXXA mutant, referring to this application as the “CXXA method” (Fig. 1B). In both methods, AXXA mutants were used as negative controls for detecting noncovalently associated biological targets (Fig. 1B and C). We applied these two strategies to identify the redox relay and interaction partners of TXN, as a well-documented positive control, and of the less studied cytosolic oxidoreductase, thioredoxin domain containing 17 (TXNDC17).

2. Materials and methods

Human embryonic kidney (HEK239T) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Wako, Kyoto, Japan) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, Inc. Lawrenceville, GA, USA) at 37 °C in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO_2. Details of plasmid construction and interaction analyses are described in the Supporting Information [19,20]. All identified lists are shown in Supplemental Tables 1 and 2. Note that nonspecific contaminants are inevitable in immunoprecipitation experiments, even using harsh conditions like SDS-containing buffers (Supplemental Table 2). Hence, we performed parallel control experiments