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A novel mouse model for the identification of thioredoxin-1 protein interactions

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ABSTRACT

Thiol switches are important regulators of cellular signaling and are coordinated by several redox enzyme systems including thioredoxins, peroxiredoxins, and glutathione. Thioredoxin-1 (Trx1), in particular, is an important signaling molecule not only in response to redox perturbations, but also in cellular growth, regulation of gene expression, and apoptosis. The active site of this enzyme is a highly conserved C-G-P-C motif and the redox mechanism of Trx1 is rapid which presents a challenge in determining specific substrates. Numerous *in vitro* approaches have identified Trx1-dependent thiol switches; however, these findings may not be physiologically relevant and little is known about Trx1 interactions *in vivo*. In order to identify Trx1 targets *in vivo*, we generated a transgenic mouse with inducible expression of a mutant Trx1 transgene to stabilize intermolecular disulfides with protein substrates. Expression of the Trx1 "substrate trap" transgene did not interfere with endogenous thioredoxin or glutathione systems in brain, heart, lung, liver, and kidney. Following immunoprecipitation and proteomic analysis, we identified 41 homeostatic Trx1 interactions in perinatal lung, including previously described Trx1 substrates such as members of the peroxiredoxin family and collapsin response mediator protein 2. Using perinatal hyperoxia as a model of oxidative injury, we found 17 oxygen-induced interactions which included several cytoskeletal proteins which may be important to alveolar development. The data herein validates this novel mouse model for identification of tissue- and cell-specific Trx1-dependent pathways that regulate physiological signals in response to redox perturbations.

1. Introduction

Thiol switches, much like phosphorylation of serine, threonine, and tyrosine, regulate protein activity through a variety of oxidative cysteine modifications in response to intracellular and environmental signals. Thioredoxin-1 (Trx1), a fundamental oxidoreductase, is essential for cell growth and survival as deletion of this enzyme is embryonic lethal [1]. The redox action of Trx1 belies its function, a general antioxidant that maintains redox homeostasis in cellular compartments. However, Trx1 is also an important regulator of thiol switches, which indicates that it has the capacity to regulate signaling responses. Within its active site are two vicinal cysteines (Cys32/35) that lie in a cysteine-glycine-proline-cysteine orientation [2]. This "thioredoxin fold" is highly conserved throughout organismal taxa, from *E. coli* to modern humans [3]. The crystal structure of Trx1, resolved in the 1970s, demonstrated that the thioredoxin fold contains five pleated sheets in parallel and antiparallel orientation which are surrounded by four alpha-helices [4]. Additional crystallography studies have shown that Trx1 undergoes a conformation change when the reactive cysteines are oxidized [5]. It is the cyclical oxidation and reduction of vicinal thiols in the Trx1 active site which gives this enzyme its oxidoreductase activity. This mechanism is responsible for its intermolecular interactions with other dedicated redox cycling enzymes as well as protein substrates containing thiol switches [6]. Trx1 reduces oxidized cysteines, including disulfide bridges, in target substrates. The Cys32 residue, through nucleophilic attack, binds to the target substrate. Cys35 then reduces this bond which creates a disulfide bridge between the two cysteines within the active site of Trx1. This effectively inhibits the reducing activity of Trx1. Trx1 activity is restored when it is reduced by its partner enzyme, thioredoxin reductase-1 (TrxR1) [7]. This reaction mechanism is rapid which presents a challenge when studying Trx1 interactions both *in vitro* and *in vivo*.

One method that has been essential for elucidating novel Trx1 substrates is the use of Isotope-Coded Affinity Tags (ICAT). This

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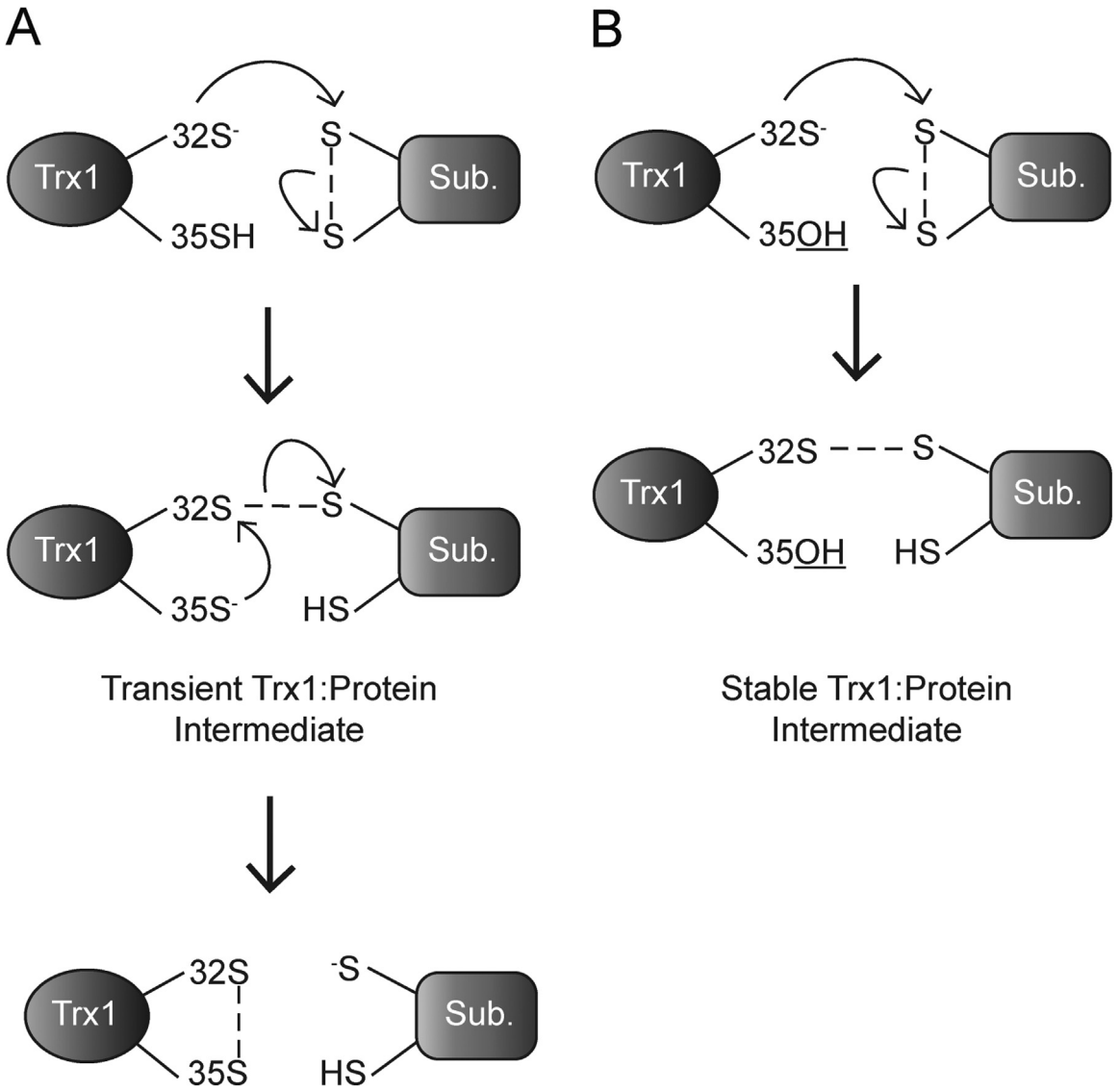

Fig. 1. Mechanism of the hTrx1^{C35S} substrate trap. (A) Mechanism of human Trx1 oxidoreductase function. Nucleophilic attack of the deprotonated Cys32 of Trx1 on the substrate disulfide results in an intermolecular disulfide intermediate. This disulfide intermediate is rapidly attacked by the Cys35 thiolate, releasing the reduced substrate with an intramolecular disulfide in the catalytic site of Trx1. (B) Mutating the resolving Cys35 to serine causes incomplete catalytic activity of Trx1 with the substrate and stabilizes the intermolecular disulfide. Following flag immunoprecipitation of the Trx1:substrate complex, the complexes are boiled off the beads into Laemmli's buffer for subsequent identification *via* mass spectrometry (Sub=substrate).

method was first developed using protein lysates from fertilized barley seeds [8]. Iodoacetamide was used to block free thiols generated by Trx1-dependent reduction of disulfides in target proteins. "Light" and "heavy" carbon ICAT reagents were added to the control and experimental samples, respectively, which allowed for the measurement of Trx1-dependent reduction based upon the ratio of light to heavy ICAT carbon (control *vs.* experimental) *via* liquid chromatography-mass spectrometry. The ICAT method has also been applied to additional models, including eukaryotic cells [9] and ventricle tissue from mice with cardiac-specific overexpression of Trx1 [10]. This method has as a distinct advantage in that not only can Trx1-dependent disulfide reduction of target substrates can be measured, but additional posttranslational modifications, such as nitrosylation by Trx1, can also be assessed [11]. However, while Trx1 substrates have been identified using ICAT, these interactions were found *in vitro* and may not have biological relevance.

The Trx1 substrate trap is a bona fide method to directly identify Trx1 interactions (Fig. 1) [12]. For human Trx1, mutation of Cys35 to serine (C35S) replaces the highly reactive resolving thiol with a hydroxyl group thereby interrupting the oxidoreductase reaction. Therefore, the substrate trap mutation stabilizes the intermolecular disulfide between Trx1 and substrate. The Trx1 substrate trap, including variations utilizing an alanine substitution, has been used in a variety of systems to identify Trx1-dependent thiol switches. This approach identified targets of the thioredoxin-related protein DsbG in *E. coli* [13] and Trx1: peroxiredoxin complexes in yeast [14]. It has also been used with plant-derived recombinant thioredoxin in a test tube-based system [15,16]. Studies from our laboratory have successfully used the Trx1 substrate trap in lung adenocarcinoma cells [17]. Novel nuclear targets, such as the transcription factor PC4 and SFRS1 interaction protein 1 (PSIP1), have been identified by fusing a canonical nuclear localization signal to the substrate trap transgene [18]. However, there are caveats with using this substrate trap *in vitro* or in cellular models since they do not recapitulate complex physiologies of multicellular organisms. Additionally, immortalized tumor cell lines or primary cells requiring extensive