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Biochemical, structural, and biological properties of human thioredoxin active site peptides

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Abstract

The human redox protein thioredoxin is an autocrine growth factor for some cancer cells. Redox activity is essential for this function but other required structural features of thioredoxin are not known. Two 8-mer peptides (I and II) and one 14-mer peptide (III) were designed based on the amino acid sequence of the redox active site of thioredoxin. Peptide I and peptide III contained the wild-type sequence of thioredoxin while peptide II contained serine residues in place of the catalytically active cysteines. Circular dichroism spectroscopy indicated that all three peptides were comprised mainly of random coil, with peptide III containing slightly more ordered secondary structure. Peptides I and III were substrates for thioredoxin reductase with K_M values of 890 and 265 μM, respectively. The redox inactive peptide II could not compete with thioredoxin for reduction by thioredoxin reductase in a coupled insulin reduction assay. However, peptide II was a competitive inhibitor for the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) by thioredoxin reductase. All three peptides gave only background levels of stimulation of the proliferation of Swiss 3T3 murine fibroblasts when compared to the stimulation caused by thioredoxin. These results suggest that while the ability of thioredoxin to stimulate cellular proliferation is redox-dependent, more information than that contained in the redox active site domain alone defined by 14 amino acids is required.

Key words: Thioredoxin; Thioredoxin reductase; Redox; Growth stimulation; Human; Peptide

1. Introduction

Regulation of the intracellular redox state is critical for cell viability. Thioredoxin (Trx) and the flavoenzyme TR (EC 1.6.4.5) comprise a redox system found in nearly all cells and the active site amino acid sequence of Trx is highly conserved between bacterial and mammalian cells [1]. Two half-reactions occur in the reduction of Trx by TR, in which the reduction of the FAD prosthetic group of TR by NADPH and electron transfer to the active site cysteines in TR occurs first. The second half-reaction is the reduction of bound oxidized Trx [2]. Trx functions in vitro as a co-factor for reduction of proteins [3,4], as a protective system against oxidizing species [5,6], and can catalyze the in vitro folding of proteins [7]. Trx has also been shown to modulate the activity of transcription factors such as AP-1 [8,9], TFIIIC [10], BZLF1 [11], and NF-κB [12] and steroid receptors [13,14], suggesting a role for Trx in the redox-regulation of gene expression.

Trx is also a growth factor and is identical to the previously described leukemic cell autocrine growth factor, ADF [15–17]. Some human solid cancers have high levels of Trx mRNA compared to normal tissue [17] and cell lines overexpressing Trx have been shown to secrete Trx through a non-classical leaderless secretory pathway [18]. This suggests that Trx may be an autocrine growth factor for solid tumors as well as hematopoietic tumors. We have found that recombinant human Trx has mitogenic activity for fibroblasts which is dependent on a redox active form of the protein [19]. However, the structural requirements outside of the cysteines in the catalytic domain of Trx for reduction by TR and the Trx-mediated stimulation of cellular proliferation are unknown.

Two peptides were synthesized which were exact duplicates of the 8 and 14 residues containing the active site of Trx, and another 8 residue peptide containing substitutions of the active site cysteines with serines. We report the ability of these peptides to interact with TR, and their mitogenic properties.

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Abbreviations: ADF, adult T-cell derived leukemic factor; CD, circular dichroism; DMEM, Dulbecco's modified Eagles medium; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; FAD, flavin adenine dinucleotide; FBS, fetal bovine serum; GSH, glutathione (reduced); hFSH, human follicle stimulating hormone; Trx, thioredoxin; TR, thioredoxin reductase.

2. Materials and methods

2.1. Peptide synthesis

Peptides I and III were synthesized off the active site of Trx (Table 1) and represent amino acids 30–37 and 27–40 of human Trx, respectively [17]. The third peptide, peptide II, was similar to peptide I with

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the exception that it contained serine residues substituted for the cysteine residues, rendering it redox inactive. Peptides were synthesized at a 25 μmol scale using a double coupling protocol on a Gilson 422 automated multiple peptide synthesizer and purified by HPLC to greater than 98% purity by Dr. Ron Jasensky (The University of Arizona Biotechnology Resource Facilities). Peptides were purified using a gradient Rainin HPLX system with a C-18 RP 21.4 mm × 25 cm Dynamax column packed with 5 μm diameter 300 Å pore size silica. Analytical HPLC entailed a similar HPLC system with a 4.6 mm column, a flow-rate of 1 ml/min, and mobile phase gradients between 0.1% TFA in water and 0.1% TFA in acetonitrile. Amino acid analysis was performed using an ABI A420 with automated hydrolysis. Capillary zone electrophoresis was performed on a Beckman P/ACE 2100. Molecular ions were detected using cesium ion induced liquid SIMS performed on a AMD Intectra double focusing magnetic sector mass spectrometer.

2.2. Thioredoxin reductase assay

Recombinant human Trx and human placental TR were prepared as previously described [17,20]. Two assays were performed to monitor TR activity. The first assay monitored the change in absorbance at 412 nm due to the NADPH-dependent reduction of DTNB by TR [2]. The second assay monitored the oxidation of NADPH by the change in absorbance at 340 nm in the presence of Trx and insulin [2]. Peptides I and III were reduced with an excess of immobilized DTT (Reductacryl Reagent, Calbiochem, La Jolla, CA) for a minimum of 10 min at room temperature prior to addition into the spectroscopic assays. GSH at 2 mM had no activity in the insulin reduction assay.

2.3. Cell growth conditions

Swiss murine 3T3 fibroblast cells (2×10^5) were grown to approximately 70% confluence in DMEM containing 10% FBS. The cells were then incubated in DMEM containing 0.5% FBS for 24 h to arrest cell growth. To each culture was added either DTT, GSH, Trx, or the peptides as indicated and allowed to incubate for 48 h. Trx and peptide samples were reduced with a 3-fold excess of DTT or with immobilized DTT prior to addition to the cultures. Cellular proliferation was measured by the increase in cell number relative to control cultures with time using a hemacytometer following detachment of the cells with 0.025% trypsin.

2.4. CD spectroscopy

A stoppered 1-cm pathlength quartz cuvette was used in an Aviv Model 60DS spectropolarimeter and 2 μM peptide samples reduced with 6 μM DTT were scanned in 5 mM potassium phosphate (pH 7.0). A total of four scans were signal-averaged from which a buffer blank was subtracted to generate the final CD spectra. The estimated percentages of secondary structure were calculated from the CD spectra with the program PROSEC (PROtein SECondary structure estimator v2.1, Aviv Associates, Lakewood, NJ) [21].

3. Results

CD spectra of all three peptides reduced with DTT were recorded (Fig. 1). The calculated secondary structure of peptide I was random coil (71.1%) and turn content (25.4%) with a minor proportion of α-helix (3.5%). Peptide II was comprised of random coil (81.3%), turn content (18.7%) and negligible α-helical content while peptide III was comprised of random coil (70.2%), turn content (21.5%), and α-helix (8.3%).

The K_M values for reduction of the peptides and Trx by human TR in the Trx-specific insulin reduction assay are shown in Table 2. The K_M value of peptide III was 265 μM, two orders of magnitude higher than the K_M value for human Trx, and one order of magnitude higher than the K_M for *E. coli* Trx. A relatively high K_M value

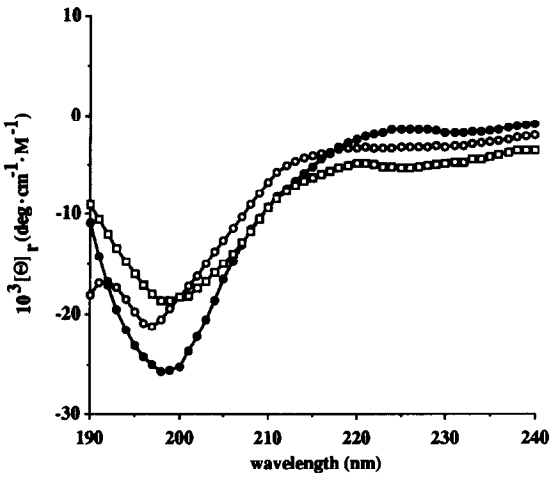

Fig. 1. Circular dichroism spectra of the three peptides. CD scans of peptide I (open circles), peptide II (closed circles), and peptide III (open squares) at 2 μM concentrations in 5 mM potassium phosphate buffer and 6 μM DTT were recorded as described in section 2. The ellipticity is given as the molar ellipticity per residue.

of 890 μM was determined for peptide I. The redox inactive peptide II at 2 mM was not a substrate for TR in the insulin reduction assay and was unable to compete with 45 μM Trx for the reduction of insulin, but was a competitive inhibitor for reduction of DTNB by human TR with a K_i of 3.8 mM.

Cell growth studies showed that peptides I and II at concentrations of 100 μM stimulated the proliferation of Swiss 3T3 murine fibroblasts to 112 and 116% ($P > 0.05$ in both cases) of the levels obtained with control cultures, respectively, compared to 151% ($P < 0.05$) by Trx at 0.5 μM (Fig. 2). When the concentrations of peptides I and II were increased to 1000 μM the level of growth stimulation did not increase above those attained with 100 μM (data not shown). Peptide III at 10 μM stimulated proliferation to 129% of control levels ($P < 0.05$) and at 100 μM the level was decreased to 105% of control levels (Fig. 2). Peptide III at 1 μM concentrations did not stimulate cellular proliferation (data not shown). GSH at 100 μM stimulated cellular proliferation to 119% of control levels ($P > 0.05$) (Fig. 2). Neither 3 nor 100 μM DTT change the final cell number relative to control cultures with only 0.5% FBS (data not shown).

Table 1
Amino acid sequence of human thioredoxin active site synthetic peptides

Peptide I	Thr-Trp-Cys-Gly-Pro-Cys-Lys-Met
Peptide II	Thr-Trp-Ser-Gly-Pro-Ser-Lys-Met
Peptide III	Phe-Ser-Ala-Thr-Trp-Cys-Gly-Pro-Cys-Lys-Met-Ile-Asn-Pro

The two active-site cysteines in thioredoxin are underlined.

4. Discussion

Trx has an identical predicted amino acid sequence to the leukemic cell autocrine growth factor ADF [17]. We have shown that recombinant human Trx is capable of stimulating both DNA synthesis and cellular proliferation of murine fibroblasts [19]. Furthermore, site-directed mutagenesis of the active-site cysteines to serine residues resulted in loss of the mitogenic properties. The present study was designed to elucidate which other features of Trx were important for enzymatic reduction and mitogenesis.

The CD spectra indicated that the three peptides were very similar in structure. They contained a high propensity for random coil structure, as evidenced by characteristic negative ellipticities below 200 nm [22], and minor turn content, which is the simplest element of most peptides in solution [26]. While the active site of intact human Trx is β-sheet and random coil [25], peptides in solution are comprised of numerous interconverting conformations and, more often than not, do not assume any particular structural preference [26].

Peptides I and III used in this study were catalytically active in a Trx-specific reduction assay with human TR, albeit with a 62-fold higher K_M value for peptide III and a 207-fold higher value for peptide I. Bacterial Trx, which has 26% identity in primary structure with human Trx, was still a better substrate for reduction by human TR than peptide III. The K_M value of peptide I in the insulin reduction assay was decreased 3-fold by the addition of 6 amino acid residues (3 at each end) to the sequence of peptide I to produce peptide III. The redox inactive peptide II was neither a substrate nor a competitive inhibitor for TR in the insulin reduction assay, suggesting that any interaction of peptide II with TR could easily be overcome by Trx. However, peptide II could compete with DTNB for reduction by TR with a K_i of 3.8 mM, compared to the previously reported K_M value for DTNB of 365 μM for reduction by TR [20]. This suggests that the redox-inactive peptide II is recognized, if only very weakly, by TR.

We found that the ability of the two Trx active-site peptides to stimulate cellular proliferation was severely decreased compared to Trx, despite the presence of active-site cysteines. The small stimulation observed with 100 μM of peptides I were most likely non-specific since 100 μM GSH was able to stimulate to the same low level. While the level of cellular proliferation attained with 10 μM peptide III was statistically significant, at 100 μM concentrations the levels dropped to control levels.

Table 2
K_M values of human placental thioredoxin reductase

Substrate	K_M value (μM)	V_{max}[a]
Peptide I	890.0	2.44
Peptide II	N.D.	N.D.
Peptide III	265.0	10.08
Human thioredoxin[b]	4.3	3.10
E. coli thioredoxin[b]	20.0	3.99
DTNB[b]	365.0	1.53

[a] nmol NADPH oxidized/minute [2]. N.D.-not determined.
[b] As previously reported [20].

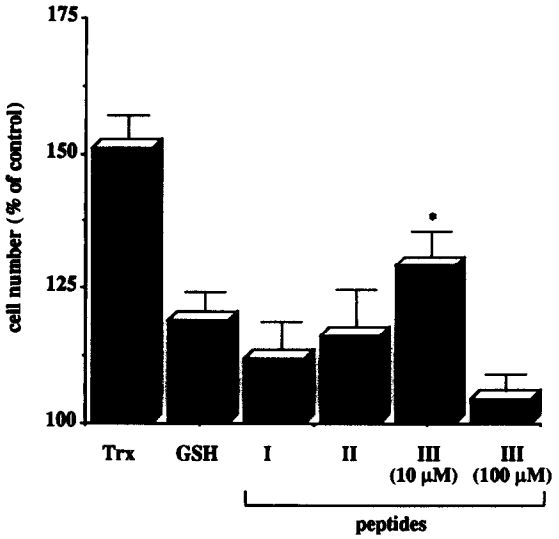

Fig. 2. Cellular proliferation of Swiss 3T3 murine fibroblasts with the peptides. Trx (0.5 μM), GSH (100 μM), peptide I and II (100 μM) and peptide III (10 and 100 μM) were added to cell culture dishes containing 2×10^5 fibroblast cells in DMEM with 0.5% FBS. After 48 h, the total cell number was determined as described in section 2 and data presented as a percentage of control cultures containing 3 μM DTT. The asterisk (*) denotes that the stimulation by peptide III at 10 μM was statistically significant above the control levels with 3 μM DTT alone ($P < 0.05$).

The results of our study suggest that the informational content in 8 and 14 amino acids of the Trx active site alone is insufficient for eliciting the full mitogenic effect. It has been reported that the β-subunit of hFSH contains a Trx-like active site in the receptor-binding domain [23]. In subsequent work, peptides of this domain were shown to bind to FSH receptors and propagate the signalling effect characteristic of FSH. Substitution of the active site cysteines with serines did not block the ability of the peptides to bind to the receptors but did abolish the transduction of the signal across the membranes [24]. Thus, there is precedent for Trx-like structural motifs present in growth factors. However, it is difficult to compare the results with hFSH and the present study directly since the peptides of the Trx-like domain in FSH were 20 amino acids long, and their enzymatic activity in an insulin reduction assay were not determined. However, the work does suggest that the Trx-like domains within the 20 amino acids may contain sufficient structural information for propagating signal(s) across the cell membrane. Even though peptide III could be reduced by TR, the 14 amino acid peptides spanning the active site of Trx are not sufficient to stimulate cellular proliferation. These results suggest that in addition to redox-activity

the presence of other structural domains in Trx than that contained in the 14 amino acids may be necessary for eliciting the same extent of growth signal as that induced by wild-type Trx.

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