Truncated thioredoxin (Trx80) exerts unique mitogenic cytokine effects via a mechanism independent of thiol oxido-reductase activity

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Abstract **Recently we discovered that a naturally occurring C-terminally truncated thioredoxin (Trx80) is a potent mitogenic cytokine stimulating IL-12 production from CD40$^+$ monocytes. To further characterise Trx80 we have engineered cysteine to serine mutants of Trx80 corresponding to the active site cysteines of Trx (Trx80SGPS) and to the structural cysteine at position 72 (Trx80C72S). Trx80SGPS and Trx80C72S retained the cell stimulatory activity of Trx80 and increased peripheral blood mononuclear cell (PBMC) proliferation three- to five-fold in vitro ($P < 0.01$, $n = 18$). Both Trx80SGPS and Trx80C72S significantly stimulated IL-12 and IFN-γ secretion from PBMCs in the same manner as Trx80 ($P < 0.01$, $n = 9$ and 10). The previously described Trx80 dimer is caused by non-covalent interactions, and not by any intermolecular disulphide bonds.**

Key words: Thioredoxin; Redox; Cytokine; Monocyte

1. Introduction

Human cytosolic thioredoxin (Trx) is in its dithiol form the major intracellular disulphide reductase. Reduced Trx is not only an electron donor to many proteins but can also regulate the activity of other proteins by thiol redox control [1–3].

When cells are activated by various stimuli, Trx translocates from the cytoplasm to the nucleus [3–8]. In the nucleus, Trx regulates DNA binding and activity of transcription factors, such as AP-1, NF-κβ and glucocorticoid receptor [3,9–18]. Reduced Trx binds to the MAPKKK apoptosis signalling kinase 1 (ASK1). Binding of reduced Trx to ASK1 inhibits ASK1, thus preventing apoptosis [19]. Extracellular Trx is a co-cytokine with several interleukins, like IL-1, IL-4, IL-6 and TNF-α [12,20,21]. Plasma levels of Trx are increased in certain diseases where the immune system is involved, for example in HIV and rheumatoid arthritis [22,23]. Increased Trx plasma levels in HIV correlates with a shorter life-expectancy for the affected patient. It has been proposed that this is caused by the fact that extracellular Trx is a chemokine for T cells, monocytes and PMN cells [24–26].

In addition, an extracellular C-terminally truncated version of Trx was detected in human peripheral blood mononuclear cell (PBMC) cultures, in which monocytes were the major cellular source [27–32]. Truncated Trx stimulates eosinophilic cell cytotoxicity, an effect that Trx lacks. Moreover, truncated Trx lacks the capacity to catalyse dithiothreitol (DTT) reduction of insulin disulphides, which is a hallmark for Trx [32,33]. Recombinant truncated Trx with the 80 N-terminal residues (Trx80) has the same effects on eosinophilic cytotoxicity compared to endogenously purified truncated Trx. Moreover, Trx80, like endogenous truncated Trx, lacks redox activity with insulin-disulphides [32].

We have earlier described that human plasma levels of truncated Trx vary to a large degree between different individuals. In 12 donors levels of truncated Trx were between 1 and 171 ng/ml, with a median value of 20 ng/ml [33]. High expression of truncated Trx was found at the cell membrane of monocytes when sub-cellular localisation of truncated Trx was evaluated in several different cell lines [34]. Trx80 induces proliferation of human PBMCs and the primary target cell for Trx80 in PBMC cultures is the monocyte. Trx80 stimulates a Th1 response in human PBMC cultures by inducing secretion of the Th1 inducer cytokine IL-12 from CD40$^+$ monocytes. Trx80 also plays a possible role in innate immunity since monocytes stimulated with 100 nM Trx80 up-regulate their CD14 receptor five-fold. Moreover, Trx80 up-regulates CD40, C54 and CD86 on monocytes, an effect that Trx lacks. In addition, Trx80 stimulates IFN-γ secretion in synergy with IL-2 in PBMC cultures [33,35]. We have shown that Trx80 is a dimer in solution, with a secondary structure that resembles Trx measured by circular dichroic spectroscopy [33].

Since Trx80 possesses unique cell stimulatory functions on monocytes we examined if the function of Trx80 is dependent on oxido-reductase activity. Previous results showing that Trx80 is not a substrate for Trx reductase (TrxR) in vitro and does not catalyse insulin disulphide reduction by DTT [32,33] led us to believe that the function of Trx80 could be different from Trx and not dependent on thiol–disulphide exchange reactions. In order to further investigate how Trx80 exerts its cell stimulatory effects we performed site-directed mutagenesis of the active site cysteines, Cys31 and Cys34, and the structural cysteine at position 72 in Trx80 replacing these Cys residues with Ser residues. In cell experiments with

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Abbreviations: Trx, thioredoxin; Trx80, C-terminally truncated thioredoxin containing the 80 N-terminal amino acids of Trx; TrxR, thioredoxin reductase; PBMC, peripheral blood mononuclear cell; DTT, dithiothreitol

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human PBMCs we found that Trx80SGPS and Trx80C72S retained the cytokine properties of Trx80. Moreover, we found that Trx is able to reduce both Trx80 and Trx80C72S but not Trx80SGPS and that the Trx80 dimer is held together by non-covalent forces.

2. Materials and methods

2.1. Materials

AIM V cell culture medium and L-glutamine were purchased from Life Technologies, Paisley, UK; isopropyl thiogalactoside was from Saveen Biotech, Malmö, Sweden; primers were provided by Kebo Lab, Stockholm, Sweden; Nco 1 and Sal 1 were purchased from Promega, Madison, WI, USA; Ficoll–Paque and [^{3}H]thymidine were from Amersham Pharmacia Biotech, Uppsala, Sweden; Polymyxin B sulphate was provided by Sigma Chemical, St. Louis, MO, USA; Spin-X filters were from Corning Costar, Cambridge, MA, USA; penicillin/streptomycin were purchased from Bio Whittaker Europe, Verviers, Belgium; IL-12 (p40 and p70 reactive) and IFN-γ sandwich enzyme-linked immunosorbent assay (ELISA) antibodies, human recombinant IL-12 and alkaline phosphate-linked streptavidin were from Mabtech, Nacka, Sweden; human recombinant IFN-γ was from PreproTech, London, UK; IL-18 sandwich ELISA antibodies and human recombinant IL-18 were provided by R&D Systems, Minneapolis, MN, USA.

2.2. Construction and purification of C31S, C34S Trx80 mutant named Trx80SGPS

In order to introduce the cysteine to serine mutations of the active site cysteines at positions 31 and 34 of Trx80 two oligonucleotides were synthesised having the sequences 5′-GCC ACG TGG TCT GGG CCT TCC AAA ATG ATC AAG CC 3-′ and 5′-GG CTT GAT CAT TTT GGA AGG CCC AGA CCA CGT GGC 3-′, with the underlined nucleotides introducing the desired point mutations. A polymerase chain reaction (PCR) was run with pET 3d/Trx80 [33] as template using 25 cycles of 95°C 1 min, 55°C 1 min, and 68°C 12 min. The resulting PCR product was purified and ligated into a pGEM T vector. This was transformed to *Escherichia coli* strain DH-5α, amplified and the sequence was verified using an ALF DNA sequenator (Pharmacia, Uppsala, Sweden). The pGEM T vector insert carrying the active site mutant of Trx80 was then cut with restriction enzymes Nco 1 and Sal 1 and subsequently ligated to a pET 24d expression plasmid. The resulting pET 24d-Trx80SGPS expressed in an *E. coli* BL-21 (DE_3) strain, grown in Luria–Bertani (LB) medium containing 30 μg/ml kanamycin at 37°C to an optical density of 0.5 at 595 nm and induced with 0.5 mM isopropyl thiogalactoside. Cells were harvested after 4 h and the Trx80SGPS protein was then purified using the same purification scheme as described earlier for Trx80 [33].

2.3. Construction and purification of the Trx80C72S mutant

A PCR was run with pACA/Trx-C72S [36] as template with the same oligonucleotides used when designing recombinant Trx80 [33]. The resulting PCR product was purified and ligated into a pGEM T vector, transformed into *E. coli* DH-5α strain, amplified and subsequently the sequence was verified using an ALF DNA sequenator (Pharmacia). The pGEM T vector with the Trx80C72S insert was subsequently cut with Nco 1 restriction enzyme and ligated into a pET 24d expression plasmid. The pET 24d-Trx80C72S plasmid was transformed to *E. coli* BL-21 (DE_3) strain, grown in LB medium, induced and purified as described above.

The Trx80SGPS and Trx80C72S were pure when run on an 8–25% gradient sodium dodecyl sulphate (SDS)-gel and silver-stained (Fig. 1). Protein concentrations of Trx80, Trx80SGPS and Trx80C72S were estimated as described before [33]. Trx was over-expressed and purified as described before [36].

2.4. SDS–polyacrylamide gel electrophoresis (PAGE)

Trx, Trx80, Trx80SGPS and Trx80C72S were kept at 25–45 μM in phosphate-buffered saline (PBS) buffer at room temperature overnight to allow oxidation [36]. Subsequently, one set of protein samples was incubated at 95°C with a loading buffer containing SDS and DTT, whereas the other set of protein samples was incubated with a loading buffer lacking DTT. This procedure was performed to investigate if SDS and heating alone would make the proteins appear as monomers on the gel, showing that the dimer of Trx80 is caused by non-covalent forces, or if DTT was needed to make the proteins monomeric, suggesting that the Trx80 dimer is held together by intermolecular disulphide bonds, which are not accessible to DTT when the protein is folded [33]. Protein samples were run on an 8–25% gradient SDS-gel on a Phastsystem (Pharmacia LKB, Uppsala, Sweden) and subsequently silver-stained (Fig. 1).

2.5. Enzymatic assays

The reduced nicotinamide adenine dinucleotide phosphate (NADPH) consumption assay was carried out at 20°C in a total volume of 500 μl with 200 μM NADPH in TE buffer. The reaction cuvette contained 10 μM of either oxidised Trx80, Trx80SGPS or Trx80C72S. The reaction was initiated by adding 10 μg of calf thymus TrxR to both the reference and reaction cuvettes, and $A_{340\,nm}$ was recorded. This was followed by addition of 1 μM Trx to both the reference and reaction cuvettes, and NADPH consumption was followed at 340 nm using an Ultrospec© 3000 spectrophotometer (Amersham-Pharmacia Biotech) [37]. Trx80, Trx80SGPS and Trx80C72S were also tested for their ability to catalyse DTT reduction of insulin disulphides [38].

2.6. Cell experiments

Human PBMCs were purified from healthy blood donors (Blood Bank, Karolinska Hospital) by standard Ficoll–Paque centrifugation. The purified PBMCs were resuspended at 1×10^6 cells/ml in serum-free AIM V medium supplemented with 2 mM L-glutamine, penicillin at 100 units/ml and streptomycin at 100 μg/ml for all cell experiments. All cell experiments were performed at 37°C and 5% CO_2 in a humidified cell incubator. Before addition to cell cultures Trx80, Trx80SGPS and Trx80C72S were passed over a column with polymyxin B-linked agarose according to the manufacturer's recommendation to remove any possible endotoxin contamination, and subsequently reduced with excess DTT, desalted and sterile-filtered.

The [^{3}H]thymidine assays were performed in 96-well flat-bottomed plates for 3 days in a final volume of 200 μl. Cells were stimulated with Trx80, Trx80SGPS, Trx80C72S or Trx at concentrations from 10 nM to 1 μM, IL-2 at 5 U/ml or 20 U/ml. 9 h prior to terminating the cultures each well was pulsed with 0.5 μCi of [^{3}H]thymidine. Cells were then harvested onto fibre-glass filters using a multi-channel cell harvester (Tomtec, Wallac) and counted with a MicroBeta Plus Scintillation counter (Tomtec, Wallac).

PBMC cultures that were assayed for IL-12 and IFN-γ production were grown in 24- or 48-well flat-bottomed plates for 3 days at 1×10^6 cells/ml in serum-free AIM V medium supplemented as described above. PBMC cultures assayed for IL-18 were cultured for 1, 2 and 3 days. All cell cultures which were assayed for cytokine production were in a final volume of 500 μl to 1 ml. Cells were cultured in the presence of Trx80, Trx80SGPS, Trx80C72S or Trx at 100 nM or 1 μM with or without IL-2 at 20 U/ml or with only IL-2 at 20 U/ml. When terminating cultures cells were centrifuged $400\times g$ for 10 min and supernatants were collected in three different aliquots and immediately frozen at −20°C. Cell medium was then analysed in duplicate wells using sandwich ELISA for IL-12, IL-18 and IFN-γ. The sandwich ELISA for IL-12 detected both IL-12 heavy chain (p40) and the heterodimer (p70). The detection limit of the IL-12 ELISA was 0.2 ng/ml; IL-18 ELISA, 0.3 ng/ml; and for IFN-γ ELISA the detection limit was 0.03 ng/ml.

2.7. Statistics

[^{3}H]thymidine incorporation and cell medium levels of cytokines in PBMC cultures were compared using the non-parametric Wilcoxon matched-pairs test. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Enzymatic activity of Trx80, Trx80SGPS and Trx80C72S

Trx80, Trx80SGPS and Trx80C72S did not act as substrates for TrxR (Fig. 2). However, when 1 μM of Trx was added both to the reaction cuvettes and the reference cuvette a change in absorbance at 340 nm was seen in cuvettes containing Trx80 and Trx80C72S. Hence, as shown in Fig. 2, Trx

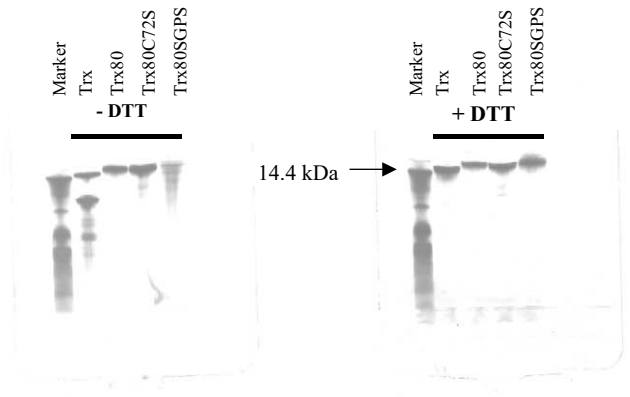

Fig. 1. SDS–gel electrophoresis of Trx, Trx80, Trx80C72S and Trx80SGPS under reducing and non-reducing conditions. Trx, Trx80, Trx80SGPS and Trx80C72S were oxidised overnight at room temperature at 25–45 μM in PBS. Proteins were then incubated in 2% SDS buffer with and without 10 mM DTT at 95°C. Subsequently, protein samples were loaded upon an 8–25% gradient SDS–PAGE and silver-stained. As indicated in the figure, Trx forms a disulphide-linked dimer upon oxidation. In contrast, Trx80, Trx80C72S and Trx80SGPS dimers seen upon gel filtration are broken by SDS treatment, indicating that the dimer forms by non-covalent interactions.

was able to reduce disulphides in Trx80 and Trx80C72S in a similar way as described earlier for Trx reduction of Trx80 [33]. However, Trx was not able to reduce disulphides in Trx80SGPS (data not shown). This indicates that the disulphide that Trx reduces in oxidised Trx80 is located between Cys31 and Cys34.

3.2. Characterisation of the Trx80 dimer

Upon Sephadex G-75 chromatography with 1 mM DTT present, reduced Trx80 eluted before Trx with an M_r of 25 000, demonstrating that Trx80 is a dimer in solution [33].

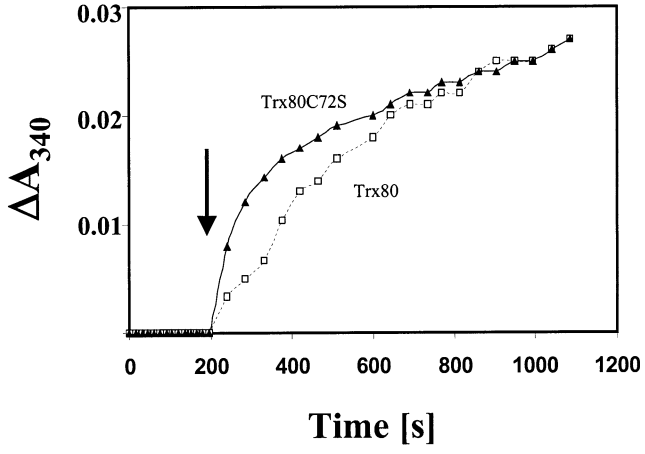

Fig. 2. Reduction of Trx80 and Trx80C72S by Trx, NADPH and TrxR. Both reference and reaction cuvettes contained TE buffer with 200 μM NADPH. To the respective reaction cuvettes 10 μM of Trx80, Trx80C72S and Trx80SGPS was added. Reactions were then initiated by adding 10 μg of TrxR to both the reference and reaction cuvettes. Absorbance at 340 nm was recorded. At the time point indicated with an arrow, 1 μM of Trx was added to each cuvette. Time-dependent consumption of NADPH when Trx is reduced by TrxR and recycles by reducing Trx80 and Trx80C72S is illustrated by changes of absorbance at 340 nm; (□) indicates Trx80; (▲) indicates Trx80C72S.

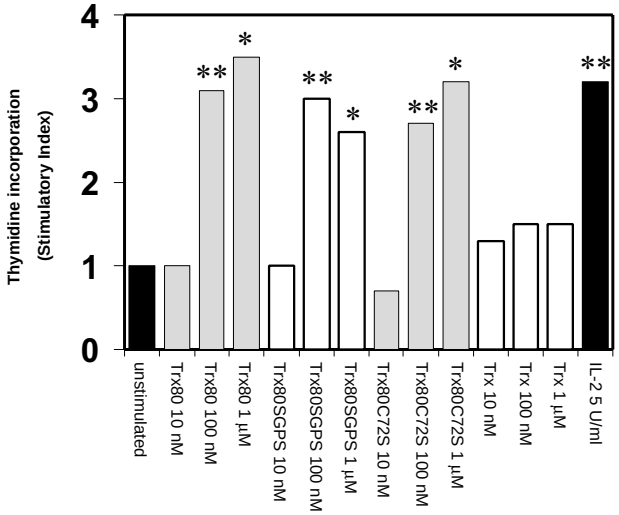

Fig. 3. Trx80, Trx80SGPS and Trx80C72S induce proliferation in PBMCs. Human PBMCs at 1×10^6 cells/ml were cultured in the presence of different concentrations of Trx80, Trx80SGPS, Trx80C72S, Trx, as indicated in the figure, or IL-2 at 5 U/ml in AIM V medium for 3 days. 9 h prior to harvesting cultures 0.5 μCi [^{3}H]thymidine was added. The mean of [^{3}H]thymidine incorporation from triplicate wells in one representative donor of 18 is shown. There was a significant increase in proliferation when cells were cultured in the presence of 100 nM or 1 μM Trx80, Trx80SGPS and Trx80C72S, or IL-2 at 5 U/ml compared to unstimulated cells. * = $P<0.05$; ** = $P<0.01$ ($n=18$).

Supradex75 chromatography of Trx, Trx80, Trx80C72S and Trx80SGPS under the same conditions as above gave similar results and Trx80, Trx80C72S and Trx80SGPS all eluted with an M_r of 25 000. However, in contrast to Trx80C72S and Trx80, Trx80SGPS also showed higher molecular weight species, indicating aggregation (data not shown). To further elucidate the characteristics of the Trx80 dimer we performed reducing and non-reducing gel electrophoresis of oxidised Trx80, Trx80C72S, Trx80SGPS and Trx. Trx formed a dimer upon oxidation as described before [36]. This dimer was resistant to heating and SDS treatment but not to DTT, showing that it was held together by a disulphide bond (Fig. 1). The fact that Trx upon oxidation forms an intermolecular disulphide between Cys72 has been described earlier [36,39]. In contrast, the dimer of Trx80, Trx80C72S and Trx80SGPS seen upon gel filtration [33] was not resistant to SDS and heating. This shows that the dimer was held together by non-covalent interactions (Fig. 1). These are probably hydrophobic interactions, as suggested before [33]. The addition of DTT did not change the result for Trx80 and Trx80C72S. However, the aggregation seen on gel filtration and on non-reducing SDS–gel electrophoresis for Trx80SGPS disappeared when the Trx80SGPS sample was treated with DTT and incubated at 95°C in the presence of SDS (Fig. 1).

3.3. Trx80SGPS and Trx80C72S have the same effect on cell proliferation as Trx80

Human PBMCs were cultured in serum-free AIM V medium in the presence of Trx80, Trx80C72S, Trx80SGPS or Trx at 10 nM to 1 μM for 3 days (Fig. 3). At 100 nM and 1 μM Trx80 gave a median three-fold increase in DNA synthesis measured by [^{3}H]thymidine incorporation ($P<0.01$, $n=18$ for 100 nM and $P<0.05$, $n=8$ for 1 μM). The mutants

Trx80SGPS and Trx80C72S also had their maximum effect at 100 nM ($P < 0.01$, $n = 18$), and their stimulation of DNA synthesis was similar compared to Trx80. The increase in DNA synthesis by Trx80 and the mutants at 100 nM were approximately the same as for 5 U/ml of IL-2. Trx gave no effect on cell proliferation (Fig. 3).

3.4. *Trx80, Trx80SGPS and Trx80C72S induce secretion of IL-12 and IFN-γ in human PBMCs*

Trx80, Trx80SGPS and Trx80C72S all induced secretion of IL-12 from human PBMCs cultured in serum-free AIM V medium for 3 days (Fig. 4). In unstimulated cells, or in cells cultured in the presence of Trx at 100 nM or 1 μM and/or IL-2 at 20 U/ml, IL-12 was not detected. Trx80, Trx80SGPS and Trx80C72S at 1 μM gave a significant increase in IL-12 secretion ($P < 0.05$ for Trx80 and $P < 0.01$ for Trx80SGPS and Trx80C72S, $n = 9$) (Fig. 4). The levels of IL-12 varied among the different donors between 0.2 and 2 ng/ml measured by sandwich ELISA. When Trx80 and the mutant proteins were cultured together with IL-2 at 20 U/ml an enhanced effect was seen and 1 μM of either Trx80, Trx80SGPS or Trx80C72S cultured in combination with 20 U/ml of IL-2 gave IL-12 levels of 0.2–15 ng/ml in the different donors ($P < 0.01$, $n = 9$; Fig. 4). Also, Trx80, Trx80SGPS and Trx80C72S at 100 nM in combination with IL-2 at 20 U/ml gave a significant increase in IL-12 secretion ($P < 0.05$ for Trx80 an $P < 0.01$ for Trx80SGPS and Trx80C72S, $n = 10$; Fig. 4).

Neither Trx80 nor Trx80SGPS, Trx80C72S, Trx or IL-2 could induce secretion of IFN-γ from PBMCs cultured in serum-free AIM V medium for 3 days (Fig. 5). However, when PBMCs were cultured in the presence of both IL-2 at

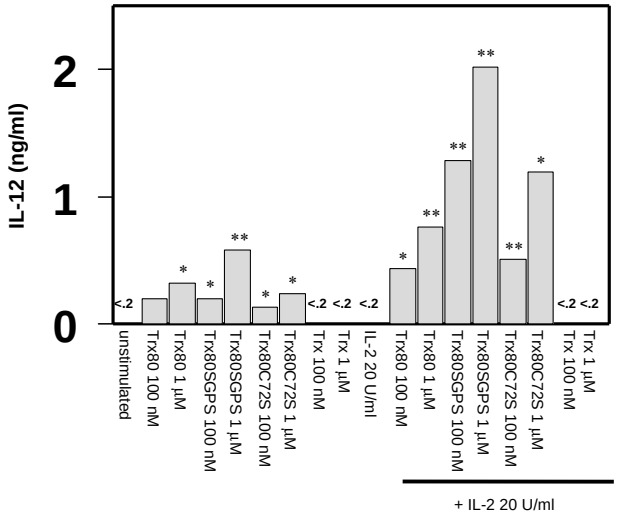

Fig. 4. Trx80, Trx80SGPS and Trx80C72S induce secretion of IL-12 from PBMCs. Human PBMCs at 1×10^6 cells/ml were cultured in AIM V medium for 3 days in the presence of Trx80, Trx80SGPS, Trx80C72S and Trx at 100 nM and 1 μM, with or without IL-2 at 20 U/ml or in the presence of only IL-2 as indicated in the figure. IL-12 secreted to the medium was analysed in duplicate wells with sandwich ELISA. The figure shows the median result of nine experiments with different blood donors. There was a significant increase in the treatments indicated with * = $P < 0.05$ and ** = $P < 0.01$ compared to unstimulated cells. The detection limit of the ELISA was 0.2 ng/ml of IL-12.

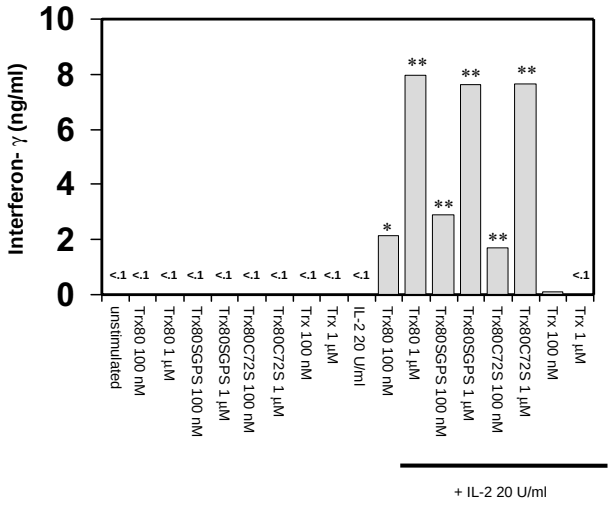

Fig. 5. Trx80, Trx80SGPS and Trx80C72S in synergy with IL-2 induce secretion of IFN-γ. Human PBMCs at 1×10^6 cells/ml were cultured in AIM V medium for 3 days in the presence of Trx80, Trx80SGPS, Trx80C72S and Trx at 100 nM and 1 μM alone or in combination with IL-2 at 20 U/ml as indicated in the figure. IFN-γ secreted to the medium was analysed in duplicate wells with sandwich ELISA. One representative experiment out of nine is shown. There was a significant increase in the treatment indicated with * = $P < 0.05$ and ** = $P < 0.01$ ($n = 9$) compared to unstimulated cells. The detection limit of the ELISA was 0.03 ng/ml of IFN-γ.

20 U/ml and Trx80, Trx80SGPS or Trx80C72S at 100 nM or 1 μM a synergistic effect on IFN-γ secretion was seen (Fig. 5). This effect was not seen when PBMCs were cultured with Trx and IL-2 in combination (Fig. 5). The effect of all three Trx80 proteins was similar on IFN-γ secretion and the absolute IFN-γ values in different donors varied between 5 and 40 ng/ml when cultured in the presence of 20 U/ml IL-2 and Trx80 or mutant Trx80 at 1 μM ($P < 0.01$ for all proteins, $n = 9$ for Trx80C72S and $n = 10$ for Trx80 and Trx80SGPS). Also, Trx80, Trx80SGPS and Trx80C72S at 100 nM in combination with 20 U/ml of IL-2 gave a significant increase in IFN-γ secretion, being between 0 and 12 ng/ml in different donors ($P < 0.01$ for all proteins, $n = 10$ for Trx80SGPS and $n = 9$ for Trx80 and Trx80C72S; Fig. 5).

Trx80, Trx80 SGPS, TrxC72S or Trx alone or in combination with IL-2 did not stimulate secretion of IL-18 in PBMC cultures (data not shown).

4. Discussion

In this study we have conclusively shown that the mechanism of the mitogenic cytokine Trx80 does not depend on the active site Cys residues of Trx, since the mutant protein Trx80SGPS showed the same effects as Trx80 in stimulating PBMC proliferation and IL-12 and IFN-γ production in PBMC cultures (Figs. 3–5). This is a surprising result since for all described effects of Trx as an extracellular co-cytokine and chemokine the Cys residues and the associated oxidoreductase activity of the protein are essential [3,26]. In addition, when Trx modulates intracellular signalling pathways by activating transcription factors and Jun N-terminal kinase or inactivating the MAPKKK ASK1, the active site Cys residues are essential [3,8,16,17,40,41].

Trx80 was previously shown to lack disulphide reductase

activity with insulin [32,33], which is a test of general dithiol–disulphide oxido-reductase activity of Trx. However, Trx was able to reduce disulphides in Trx80 and we speculated that this interaction could occur at the cell surface and mediate the cytokine activities of Trx80 [33,35]. Here we show that Trx reduces disulphides in Trx80 and Trx80C72S but not in Trx80SGPS (Fig. 2), although all three Trx80 proteins show the same cytokine effects on PBMCs (Figs. 3–5). Hence, this study does not support the earlier suggested mechanism of Trx80, involving thiol–disulphide exchange reactions between Trx80 and Trx at the cell surface, and instead suggests that the mechanism whereby Trx80 exerts its cytokine effects is redox-independent.

It is known that human Trx upon oxidation forms dimers with intermolecular disulphides involving Cys72 [36,39]. However, this intermolecular disulphide is reduced when DTT is present [36]. We have previously shown that Trx80 is a dimer in solution also in the presence of DTT and we proposed that upon truncation of Trx to Trx80 a hydrophobic surface area should be exposed that causes the dimer formation of Trx80 [33]. Moreover, we have seen that Trx80SGPS, Trx80C72S and Trx80 have similar elution profiles upon gel chromatography with 1 mM DTT, eluting before Trx with a M_r of 25 000. The finding that the Trx80 dimer is broken upon treatment with SDS and heating supports the idea that the dimer is held together by hydrophobic interactions rather than covalent intermolecular bonds (Fig. 1).

Trx80, Trx80SGPS and Trx80C72S have the same activity when stimulating IFN-γ secretion. In PBMCs the main cellular source of IFN-γ are the T cells. We have previously shown that Trx80 does not stimulate the T cells directly. Trx80 first stimulates IL-12 production from monocytes, which in turn presumably induces IFN-γ secretion from T cells [35]. In addition, it is clear that IL-2 is a more potent inducer of PBMC proliferation compared to Trx80 (Fig. 3). However, Trx80 is superior to IL-2 in inducing IL-12 secretion (Fig. 4). The effects of Trx80 on PBMC proliferation are probably caused by two mechanisms. Firstly, Trx80 directly stimulates proliferation of purified monocytes [35]. Secondly, the secretion of IL-12 from $CD40^+$ monocytes will induce proliferation of T cells in the PBMC cultures. However, IL-12 is less potent compared to IL-2 in inducing T cell proliferation [35,42].

The data that have accumulated around Trx80 raise several important issues. The in vitro [43,44] studies suggest that Trx80 can be important in: (i) protecting monocytes from apoptosis and promoting phagocytosis of apoptotic cells by monocytes by enhancing the expression of the CD14 receptor; and (ii) inducing a Th1-type immune response in T cells by stimulating IFN-γ and IL-12 production [35,45–48]. These effects should be important for the individual to clear viral infections, but could also be harmful by eliciting autoimmune responses [49].

Since we have now shown that Trx80 exerts its effects via a mechanism independent of thiol–disulphide reactions we propose that the effects of Trx80 are receptor-mediated. The receptor is probably located at the cell surface on monocytes since Trx80 mediates its effects via monocytes and since truncated Trx is localised at the cell surface on monocytes [31,32,34,35]. The effects of Trx80 are unique and important in pathophysiology, and therefore it is of great importance to further elucidate the identity of a possible receptor for Trx80 and the mechanism by which Trx80 is produced.

The great variation of Trx80 levels in apparently healthy blood donors should also be elucidated, and this might be a prognostic factor for the risk to develop certain autoimmune diseases such as rheumatoid arthritis and diabetes.

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References

[1] Arner, E.S. and Holmgren, A. (2000) Eur. J. Biochem. 267, 6102–6109.
[2] Holmgren, A. (1985) Annu. Rev. Biochem. 54, 237–271.
[3] Nakamura, H., Nakamura, K. and Yodoi, J. (1997) Annu. Rev. Immunol. 15, 351–369.
[4] Ohira, A., Honda, O., Gauntt, C.D., Yamamoto, M., Hori, K., Masutani, H., Yodoi, J. and Honda, Y. (1994) Lab. Invest. 70, 279–285.
[5] Sachi, Y., Hirota, K., Masutani, H., Toda, K., Okamoto, T., Takigawa, M. and Yodoi, J. (1995) Immunol. Lett. 44, 189–193.
[6] Sasada, T., Sono, H. and Yodoi, J. (1996) J. Toxicol. Sci. 21, 285–287.
[7] Wei, S.J. et al. (2000) Cancer Res. 60, 6688–6695.
[8] Harper, R., Wu, K., Chang, M.M., Yoneda, K., Pan, R., Reddy, S.P. and Wu, R. (2001) Am. J. Respir. Cell. Mol. Biol. 25, 178–185.
[9] Tanaka, H., Makino, Y. and Okamoto, K. (1999) Vitam. Horm. 57, 153–175.
[10] Qin, J., Clore, G.M., Kennedy, W.M., Huth, J.R. and Gronenborn, A.M. (1995) Structure 3, 289–297.
[11] Qin, J., Clore, G.M., Kennedy, W.P., Kuszewski, J. and Gronenborn, A.M. (1996) Structure 4, 613–620.
[12] Schenk, H., Vogt, M., Droge, W. and Schulze-Osthoff, K. (1996) J. Immunol. 156, 765–771.
[13] Okamoto, T., Ogiwara, H., Hayashi, T., Mitsui, A., Kawabe, T. and Yodoi, J. (1992) Int. Immunol. 4, 811–819.
[14] Meyer, M., Schreck, R. and Baeuerle, P.A. (1993) EMBO J. 12, 2005–2015.
[15] Matthews, J.R., Wakasugi, N., Virelizier, J.L., Yodoi, J. and Hay, R.T. (1992) Nucleic Acids Res. 20, 3821–3830.
[16] Makino, Y. et al. (1996) J. Clin. Invest. 98, 2469–2477.
[17] Makino, Y., Yoshikawa, N., Okamoto, K., Hirota, K., Yodoi, J., Makino, I. and Tanaka, H. (1999) J. Biol. Chem. 274, 3182–3188.
[18] Hayashi, T., Ueno, Y. and Okamoto, T. (1993) J. Biol. Chem. 268, 11380–11388.
[19] Saitoh, M. et al. (1998) EMBO J. 17, 2596–2606.
[20] Rosen, A., Lundman, P., Carlsson, M., Bhavani, K., Srinivasa, B.R., Kjellstrom, G., Nilsson, K. and Holmgren, A. (1995) Int. Immunol. 7, 625–633.
[21] Tagaya, Y. et al. (1989) EMBO J. 8, 757–764.
[22] Yoshida, S., Katoh, T., Tetsuka, T., Uno, K., Matsui, N. and Okamoto, T. (1999) J. Immunol. 163, 351–358.
[23] Nakamura, H., De Rosa, S., Roederer, M., Anderson, M.T., Dubs, J.G., Yodoi, J., Holmgren, A. and Herzenberg, L.A. (1996) Int. Immunol. 8, 603–611.
[24] Nakamura, H., De Rosa, S.C., Yodoi, J., Holmgren, A., Ghezzi, P. and Herzenberg, L.A. (2001) Proc. Natl. Acad. Sci. USA 98, 2688–2693.
[25] Miller, L.A., Usachenko, J., McDonald, R.J. and Hyde, D.M. (2000) J. Leukoc. Biol. 68, 201–208.
[26] Bertini, R. et al. (1999) J. Exp. Med. 189, 1783–1789.
[27] Balcewicz-Sablinska, M.K., Wollman, E.E., Gorti, R. and Silberstein, D.S. (1991) J. Immunol. 147, 2170–2174.
[28] Dessein, A.J., Lenzi, H.L., Bina, J.C., Carvalho, E.M., Weiser, W.Y., Andrade, Z.A. and David, J.R. (1984) Cell. Immunol. 85, 100–113.

[29] Lenzi, H.L., Mednis, A.D. and Dessein, A.J. (1985) Cell. Immunol. 94, 333–346.
[30] Silberstein, D.S., Dessein, A.J., Elsas, P.P., Fontaine, B. and David, J.R. (1987) J. Immunol. 138, 3042–3050.
[31] Silberstein, D.S., Ali, M.H., Baker, S.L. and David, J.R. (1989) J. Immunol. 143, 979–983.
[32] Silberstein, D.S., McDonough, S., Minkoff, M.S. and Balcewicz-Sablinska, M.K. (1993) J. Biol. Chem. 268, 9138–9142.
[33] Pekkari, K., Gurunath, R., Arner, E.S. and Holmgren, A. (2000) J. Biol. Chem. 275, 37474–37480.
[34] Sahaf, B., Soderberg, A., Spyrou, G., Barral, A.M., Pekkari, K., Holmgren, A. and Rosen, A. (1997) Exp. Cell Res. 236, 181–192.
[35] Pekkari, K., Avila-Carino, J., Bengtsson, A., Gurunath, R., Scheynius, A. and Holmgren, A. (2001) Blood 97, 3184–3190.
[36] Ren, X., Bjornstedt, M., Shen, B., Ericson, M.L. and Holmgren, A. (1993) Biochemistry 32, 9701–9708.
[37] Luthman, M. and Holmgren, A. (1982) Biochemistry 21, 6628–6633.
[38] Holmgren, A. (1979) J. Biol. Chem. 254, 9627–9632.
[39] Weichsel, A., Gasdaska, J.R., Powis, G. and Montfort, W.R. (1996) Structure 4, 735–751.
[40] Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K. and Yodoi, J. (1997) Proc. Natl. Acad. Sci. USA 94, 3633–3638.
[41] Das, K.C. (2001) J. Biol. Chem. 276, 4662–4670.
[42] Germann, T. and Rude, E. (1995) Int. Arch. Allergy Immunol. 108, 103–112.
[43] Devitt, A., Moffatt, O.D., Raykundalia, C., Capra, J.D., Simmons, D.L. and Gregory, C.D. (1998) Nature 392, 505–509.
[44] Heidenreich, S. (1999) J. Leukoc. Biol. 65, 737–743.
[45] Aste-Amezega, M. and al, e. (1994) Cell. Immunol. 156, 480–492.
[46] Chan, S.H. and Perussia, B. (1991) J. Exp. Med. 173, 869–879.
[47] Naume, B., Gately, M. and Espevik, T. (1992) J. Immunol. 148, 2429–2436.
[48] Sornasse, T., Larenas, P.V., Davis, K.A., de Vries, J.E. and Yssel, H. (1996) J. Exp. Med. 184, 473–483.
[49] Constant, S.L. and Bottomly, K. (1997) Annu. Rev. Immunol. 15, 297–322.