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Requirements for the Different Cysteines in the Chemotactic and Desensitizing Activity of Human Thioredoxin

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ABSTRACT

Thioredoxin (Trx) is a protein disulfide oxidoreductase that can be secreted and act as a chemoattractant for leukocytes. Like chemokines, it causes desensitization of monocytes against its chemotactic activity and that of monocyte chemoattractant protein-1 (MCP-1). To investigate the role of the redox properties of Trx, and particularly of some of its five cysteines, in its chemotactic and desensitizing action, we tested different mutants, including Trx80, a truncated form, and various mutants lacking specific cysteines: Trx C62S/C73S and the redox-inactive mutant Trx C32S/C35S. Of the mutants, only Trx80 maintained the chemotactic activity of wild-type Trx toward both monocytes and polymorphonuclear neutrophils, all of them desensitized monocytes against wild-type Trx or MCP-1, but not chemotactic peptide formyl-methionyl-leucil peptide. These data indicate that different redox-active cysteines are important for Trx chemotactic action, whereas its desensitizing action does not have these requirements, suggesting a redox-independent mechanism. *Antioxid. Redox Signal.* 7, 1189–1194.

INTRODUCTION

A FEW CHEMOTACTIC PROTEINS do not fall in the class of chemokines, a term that, although literally meaning "chemotactic cytokine," actually refers to a family of proteins with specific structural features (7, 8). These "unusual" molecules include proteins, like defensins (33), and small molecules, such as opiates (12). These molecules share with chemokines not only a chemotactic activity, but also another property of chemokines, desensitization, a phenomenon by which cells exposed to a chemokine will not respond to subsequent stimulation with the same chemokine (homologous desensitization) or with a different one (heterologous desensitization) (21, 29).

Thioredoxin (Trx) is a protein disulfide oxidoreductase catalyzing the reduction of disulfide bonds in proteins through the redox-active site CGPC (in human Trx, this is the $Cys^{32/35}$) (13, 14). We previously reported that Trx is chemotactic for various cell types (3). We also observed heterologous desensitization between Trx and specific chemokines. In fact, Trx desensitized human monocytes to monocyte chemotactic protein-1 (MCP-1), RANTES, and macrophage inflammatory protein-1β (MIP-1β), but not to MIP-1α, stromal cell-derived factor-1, fractalkine, or formyl-methionyl-leucil peptide (FMLP) (23). Although *in vitro* it does not desensitize polymorphonuclear leukocytes (PMNs) to interleukin-8 (IL-8) (23), its administration or transgenic overexpression *in vivo* diminishes PMN infiltration induced by bleomycin in the lung (15) or locally by lipopolysaccharide (22).

The interplay between the redox activity of Trx and its chemotactic action is far from being established, although in the case of the chemotactic action of Trx, we could show that a mutant with replacement of the two cysteines in the CGPC active site by serines was inactive (3). However, one article suggested that also Cys^{62} and Cys^{69} in the

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Trx sequence may contribute to the redox activity (32), and we also observed that Cys^{73} could undergo reversible glutathionylation (5).

In the present study, we investigate the role of the redox activity of Trx in its two effects on chemotaxis in monocytes: its direct chemotactic action, and its ability to desensitize against MCP-1 chemotaxis. To this purpose, we studied the effect of wild-type Trx, of the CGPC double mutant (C32S/C35S), and of the mutant C62S/C73S that lacks also Cys^{73}, previously shown to undergo oxidoreduction by glutathionylation or dimerization (5, 11). We also investigated a truncated form (Trx80), a 10-kDa C-terminally truncated form comprising the 80 N-terminal amino acids, that is a naturally occurring one (30), does not have the enzymatic activity of Trx as insulin disulfide reductase, and is not a substrate for Trx reductase (25), but has cytokine actions as an eosinophil activator (31) and is a mitogen and an IL-12 inducer for human mononuclear cells (25–27).

The results indicate that the chemotactic action and the desensitizing one of Trx are dissociated in terms of redox and cysteine requirements.

MATERIALS AND METHODS

Materials

Human recombinant Trx and its mutants were prepared essentially as described previously (26–28). Recombinant human MCP-1 was from PeproTech (Rocky Hill, NJ, U.S.A.). FMLP was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ficoll/Hypaque, Percoll, and dextran were from Pharmacia. Hanks' balanced salt solution (HBSS) was from Irvine Scientific (Santa Ana, CA, U.S.A.). RPMI 1640 was from GIBCO (Grand Island, NY, U.S.A.). Diff-Quik was from Harleco (Gibbstown, NJ, U.S.A.). Micro Boyden chambers and polycarbonate filter were from Neuroprobe Inc. (Pleasanton, CA, U.S.A.).

Cells

Human mononuclear cells and PMNs were obtained from buffy coats of blood donors from normal volunteers through the courtesy of Centro Trasfusionale (Ospedale S. Salvatore, L'Aquila, Italy). Mononuclear cells were obtained by centrifugation on Ficoll/Hypaque. The human PMNs were prepared to 95% purity by dextran sedimentation, followed by hypotonic lysis of contaminating red blood cells (20) The cellular viability was >95% in all experiments, as measured by trypan blue dye exclusion.

Migration assay

Cell migration for human monocytes and PMNs was evaluated using a 48-well microchemotaxis chamber, as previously described (6, 10). Twenty-five microliters of control medium (phosphate-buffered saline for monocytes and HBSS for PMNs, with 0.2% bovine serum albumin) or chemoattractant solution was seeded in the lower compartment of the chemotaxis chamber. Desensitization experiments was performed essentially as described (23). In brief, 50 µl of cell suspension (1.5×10^6/ml) was preincubated at 37°C for 15 min in the presence or absence of different concentrations of the desensitizing agent (Trx or mutants), and then the cells were seeded in the upper compartment. The two compartments of the chemotactic chamber were separated by a 5-µm polycarbonate filter (polyvinylpyrrolidone-free for PMN chemotaxis). The chamber was incubated at 37°C in air with 5% CO_2 for 2 h (monocytes) or for 45 min (PMNs). At the end of incubation, filters were removed, fixed, and stained with Diff-Quik, and five oil immersion fields were counted after sample coding. Data are expressed as means ± SD of five fields.

RESULTS

Chemotactic activity of Trx mutants

In the experiments shown in Fig. 1, we compared the effect of the various mutants in a standard chemotaxis assay in a Boyden chamber with either monocytes (A) or PMNs (B). In both cells, we could observe a marked chemotactic activity of Trx, in accordance with previous results. The truncated form Trx80 and the C73S mutant retained this activity, whereas the active-site double mutant C32S/C35S and the double mutant C62S/C73S were devoid of chemotactic activ-

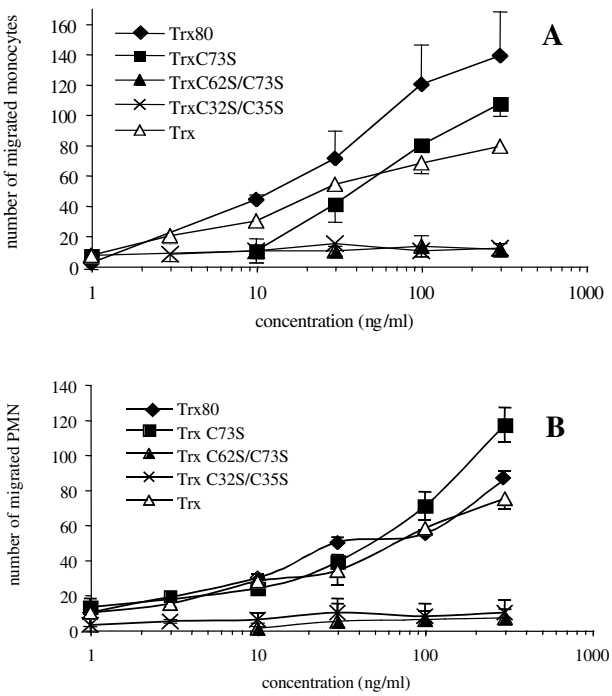

FIG. 1. Chemotactic activity of Trx mutants toward human monocytes (A) and PMNs (B). Chemotactic activity was assayed as described in Materials and Methods. The results are expressed as average number of cells migrated in five oil immersion fields ± SD.

ity. These figures summarize different experiments. In all these experiments, we also tested the same protein boiled for 30 min to make sure that its chemotactic activity was not due to heat-stable contaminants, especially endotoxins. We could confirm that the chemotactic activity of wild-type Trx, Trx80, and C73S, all tested at the concentration of 300 ng/ml, was lost upon boiling (data not shown). Also, all experiments, including those with the mutants that were found to be inactive, included samples with recombinant human MCP-1 or recombinant human IL-8 as positive controls (in experiments with monocytes and PMNs, respectively) to make sure the assay was running.

MCP-1-desensitizing activity of Trx mutants in monocytes

We then investigated whether chemotactic or nonchemotactic Trx mutants cross-desensitized monocytes to the chemotactic action of wild-type Trx or to the chemokine MCP-1.

As shown in Fig. 2A, Trx desensitizes monocytes against the chemotactic activity of MCP-1, as we previously reported. Similar desensitizing activity was achieved by the chemotactic mutant Trx80 (Fig. 2B), but also by the nonchemotactic mutants Trx C62S/C73S (Fig. 2C) and Trx C32S/C35S (Fig. 2D). The less effective in terms of desensitization to MCP-1 was probably the mutant C62S/C73S, with the caveat that it is always difficult to make strong conclusions from the comparison of the potency of different recombinant preparations of cytokines.

All Trx mutants tested, at 100 ng/ml, including the chemotactic Trx80 and the nonchemotactic Trx C62S/C73S and Trx C32S/C35S, desensitized against the chemotactic activity of wild-type Trx (Fig. 3), as we previously reported for wild-type Trx.

Also in this case, we tested boiled preparations of the mutants, and this treatment destroyed their desensitizing activity even when they were tested at the highest concentration of 300 ng/ml (data not shown). As a further proof that the

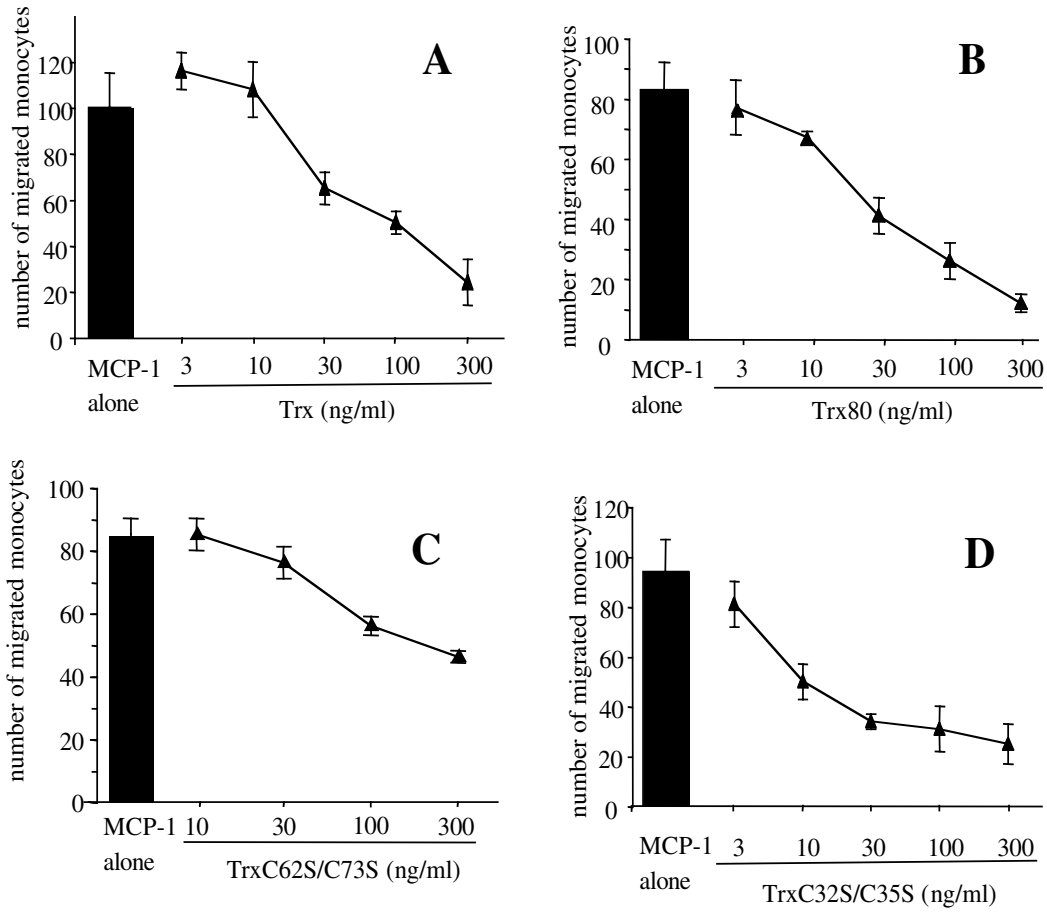

FIG. 2. Effect of Trx mutants on monocyte chemotaxis toward MCP-1. Monocytes were preincubated 15 min with different concentrations of wild-type Trx (**A**), Trx80 (**B**), TrxC62S/C73S (**C**), TrxC32S/C35S (**D**), or vehicle (filled bars in each panel). Then the cells were put in the upper compartment of Boyden chambers, and MCP-1, at the concentration of 25 ng/ml, was added to the lower compartment. The results are expressed as average number of cells migrated in five oil immersion fields ± SD.

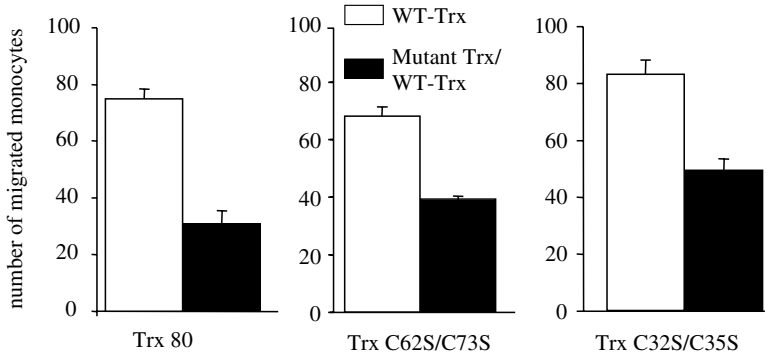

FIG. 3. Effect of Trx mutants on monocyte chemotaxis toward wild-type Trx. Monocytes were preincubated 15 min with vehicle (open bars) or 100 ng/ml Trx80, TrxC62S/C73S, or TrxC32S/C35S (filled bars). Then the cells were put in the upper compartment of Boyden chambers, and wild-type Trx (WT-Trx), at the concentration of 30 ng/ml, was added to the lower compartment. The results are expressed as average number of cells migrated in five oil immersion fields ± SD.

desensitizing action against MCP-1 and Trx was not an aspecific effect of a contaminant, we studied the effect of these mutants on the chemotactic activity of FMLP. As shown in Fig. 4, none of them, even at the highest concentration of 300 ng/ml, desensitized monocytes to FMLP.

DISCUSSION

In the present study, we used mutants lacking different cysteines to study their importance in the chemotactic activity of Trx, and its relation to other cytokine-like actions and to the oxidoreductase activity of Trx. Our early work, showing that the C32S/C35S mutant that is redox-inactive also lost its chemotactic activity, has suggested that chemotaxis depends on the protein disulfide oxidoreductase activity (3).

Of the mutants tested in this study, only the double mutants C32S/C35S and C62S/C73S have clearly lost their chemotactic activity on both monocytes and PMNs. Although only Cys^{32} and Cys^{35} are clearly implicated as the redox center of Trx, so that only the first double mutant should be regarded as "redox dead," there has been a report suggesting that also Cys^{62} can participate in the redox functions of Trx in a second redox motif with Cys^{69} (32).

We also show that Trx80, which has no insulin disulfide reductase activity and is not a substrate for Trx reductase (25), is also chemotactic. It should be noted, however, that Trx80 still has the CGPC motif, which could mediate other oxidoreduction reactions independent of Trx reductase and for other substrates than insulin.

But the data shown so far in our chemotaxis experiment clearly dissociate the chemotactic activity of Trx from other cytokine-like activities that are displayed by Trx80, but not wild-type Trx, including eosinophil cytotoxicity stimulation, IL-12 induction, and mitogenesis in monocytes (for review, see 24). It should be noted that the above-mentioned activities are also displayed by the C32S/C35S mutant of Trx80, and thus are clearly independent of the redox functions mediated by the CGPC active group.

A different pattern emerges from our experiments of desensitization from the chemotactic activity of Trx. In our earlier work, as there are no known membrane receptors for Trx, we had embarked in studies of cross-desensitization as they may suggest which receptors are used by Trx for its chemotactic activity, although the results did not lead to clear indications in this sense (23). The results reported in the present article clearly demonstrate that desensitization, toward both MCP-1 and wild-type Trx, is observed with nonchemotactic mutants, including the C32S/C35S lacking the CGPC group. Therefore, in this case the independency on the redox activities of Trx is strongly suggested.

Interestingly, some similarities were noted between Trx and macrophage migration inhibitory factor (MIF). MIF is an inflammatory cytokine originally described as an inhibitor of macrophage migration and has enzymatic activity as a tautomerase/isomerase (for reviews, see 2, 4). MIF also has the CXXC redox-active site typical of protein disulfide oxidoreductases and shows thiol oxidoreductase activities, *i.e.,* reduces the disulfide bridge of insulin (an activity typical of Trx) and of smaller disulfides (an activity typical of glutaredoxin) (1, 17–19). Mutations of specific cysteines affect its cytokine actions, indicating that some of the latter are redox-mediated. As MIF is an inhibitor of macrophage migration, we had initially suspected that these redox functions might indicate a common mechanism with Trx. However, the lack of need for the CXXC site for the desensitizing action of Trx shown in the present article would suggest that, if there were

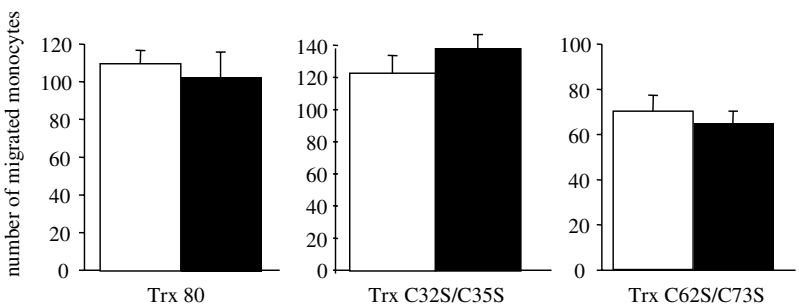

FIG. 4. Trx mutants do not affect monocyte FMLP chemotaxis. Monocytes were preincubated 15 min with vehicle (open bars) or 300 ng/ml of Trx80, TrxC62S/C73S, or TrxC32S/C35S (filled bars). Then the cells were put in the upper compartment of Boyden chambers, and FMLP, at the concentration of $10^{-7}M$, was added to the lower compartment. The results are expressed as average number of cells migrated in five oil immersion fields ± SD.

any similarities in the activities of MIF and Trx, these are mediated by other regions of these proteins.

The present study seems to rule out the disulfide-reducing redox activity at the active site of Trx in its homologous and heterologous desensitizing activities on monocyte chemotaxis, suggesting some classical (*i.e.,* receptor-mediated) mechanism. On the other hand, the chemotactic action seems to be more dependent on the proteins' cysteine residues, the redox state of which will give rise to molecular species with different conformations. As human Trx has five cysteines that are all contained in Trx80, the redox state of the cysteines following oxidation can give rise to a number of molecular species. The protein stored without a reductant has two disulfides and one free -SH group as has been described (32) and is the species we have used in our studies. In contrast, the C32S/C35S mutant (often referred to as SGPS mutant in the literature) of human Trx should be more similar to the reduced conformation of Trx as has been demonstrated for the corresponding mutant of *Escherichia coli* Trx (9, 16). Obviously, characterizing the redox state of Trx and Trx80 in plasma and further attempts to find receptors are future goals.

ABBREVIATIONS

FMLP, formyl-methionyl-leucil peptide; HBSS, Hanks' balanced salt solution; IL, interleukin; MCP-1, monocyte chemotactic protein-1; MIF, macrophage migration inhibitory factor; MIP-1, macrophage inflammatory protein-1; PMN, polymorphonuclear leukocyte; Trx, thioredoxin.

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