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Selective redox regulation of cytokine receptor signaling by extracellular thioredoxin-1

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The thiol-disulfide oxidoreductase thioredoxin-1 (Trx1) is known to be secreted by leukocytes and to exhibit cytokine-like properties. Extracellular effects of Trx1 require a functional active site, suggesting a redox-based mechanism of action. However, specific cell surface proteins and pathways coupling extracellular Trx1 redox activity to cellular responses have not been identified so far. Using a mechanism-based kinetic trapping technique to identify disulfide exchange interactions on the intact surface of living lymphocytes, we found that Trx1 catalytically interacts with a single principal target protein. This target protein was identified as the tumor necrosis factor receptor superfamily member 8 (TNFRSF8/CD30). We demonstrate that the redox interaction is highly specific for both Trx1 and CD30 and that the redox state of CD30 determines its ability to engage the cognate ligand and transduce signals. Furthermore, we confirm that Trx1 affects CD30-dependent changes in lymphocyte effector function. Thus, we conclude that receptor–ligand signaling interactions can be selectively regulated by an extracellular redox catalyst.

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Introduction

Disulfide bonds have long been recognized as structural elements stabilizing proteins in harsh extracellular environments. More recently, an additional concept has emerged: some disulfide bonds operate as dynamic scaffolds capable of regulated rearrangement into a variety of functional forms (Jordan and Gibbins, 2006). Consistent with this notion, various cell surface processes have long been known to depend on catalyzed thiol-disulfide exchange including cell adhesion (Essex, 2004), uptake of bacterial toxins (de Paiva *et al*, 1993) and viral fusion with the host membrane (Sanders, 2000). Moreover, a variety of cell surface signaling receptors appear to exist in more than one thiol-disulfide configuration, for example CD28 (Greene *et al*, 1996). However, in most cases, neither the catalyst driving thiol-disulfide exchange nor the functional differences between the redox forms have been elucidated.

A number of thiol-disulfide oxidoreductases are known to be secreted and to act on the cell surface. One of these redox catalysts is protein disulfide isomerase (PDI), a member of the thioredoxin (Trx) superfamily. Cell surface-PDI has been found to act on transmembrane and surface-associated proteins, including the envelope protein of HIV-1, to cause its fusogenic conformation (Markovic *et al*, 2004) and integrins, to mediate platelet adhesion (Lahav *et al*, 2003). Another thiol-disulfide oxidoreductase associated with extracellular functions is Trx1. Best known for its intracellular roles, Trx1 reduces transiently formed disulfide bonds of cytosolic and nuclear target proteins and thereby participates in a multitude of fundamental processes, ranging from oxidant scavenging and DNA synthesis to regulation of apoptosis and cell proliferation (Powis and Montfort, 2001). In addition, Trx1 is released to the extracellular environment by a variety of normal and neoplastic cells (Rubartelli *et al*, 1992). Human Trx1 was first purified as a cytokine-like factor from supernatants of virally transformed lymphocytes and initially named adult T-cell leukemia-derived factor (Tagaya *et al*, 1988), Tac-inducing factor (Tagaya *et al*, 1989), B-cell stimulatory factor or 'B cell IL-1' (Wakasugi *et al*, 1990). Extracellular Trx1 is present in the circulation of healthy subjects and its levels increase under inflammatory conditions, including viral infection (Nakamura *et al*, 2001a). Circulatory Trx1 acts as a chemoattractant for monocytes, neutrophils and lymphocytes (Bertini *et al*, 1999), and inhibits neutrophil migration into inflammatory sites both *in vitro* and *in vivo* (Nakamura *et al*, 2001b). More recently, Trx1 was found to be secreted by dendritic cells upon cognate T-cell recognition and to contribute to subsequent T-cell activation (Angelini *et al*, 2002).

At present, the mechanism(s) and pathway(s) by which extracellular Trx1 influences cellular behavior remain unknown. As many of its reported extracellular activities depend on a functional active site, it appears likely that Trx1 catalyzes thiol-disulfide exchange in one or more cell surface target proteins through its enzymatic activity. However, thiol-disulfide exchange reactions, even if highly specific, are too transient to be detected by conventional techniques. To date, only a single cell surface receptor, CD4, a member of the immunoglobulin superfamily, has been

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shown to be susceptible to Trx1 redox activity (Matthias *et al*, 2002). Other cell surface proteins targeted by the enzymatic activity of Trx1 await identification.

In this study, we address the question as to which cell surface receptors expressed on lymphocytes specifically interact with extracellular Trx1 by way of disulfide bond exchange. Using a kinetic trapping technique that enables the detection and isolation of otherwise short-lived reaction intermediates on the surface of intact cells, we identify and validate the tumor necrosis factor receptor superfamily member CD30 (TNFRSF8) as the principal target molecule for Trx1 on infected and transformed lymphocytes. The cell surface activity of Trx1 is highly selective, discriminating between different members of the TNFR superfamily. Trx1-mediated thiol-disulfide exchange leads to a structural change in the CD30 ectodomain that can be detected with conformation-sensitive antibodies. We demonstrate that disulfide exchange between Trx1 and CD30 interferes with binding of the CD30 ligand (CD30L) to its cognate receptor and that Trx1 affects CD30-dependent changes in lymphocyte effector function. As CD30 is implicated in both stimulatory and apoptotic signaling, our findings suggest that Trx1 interacts with CD30 to modulate lymphocyte behavior and survival under conditions of infection and inflammation.

Results

To identify candidate Trx1 target proteins on the cell surface of lymphoid cells, we applied a trapping technique based on the reaction mechanism. This approach makes use of the finding that mutant thiol-dependent oxidoreductases lacking the C-terminal cysteine of the CXXC active site motif form long-lived mixed disulfide intermediates with target proteins. Thus, target proteins remain covalently linked to the mutant oxidoreductase and become amenable to isolation and analysis (principle shown in Figure 1A). Kinetic trapping has been applied previously to identify interaction partners of Trx family members in plants (Motohashi *et al*, 2001) and in the secretory pathway of human lymphocytes (Dick and Cresswell, 2002; Dick *et al*, 2002). In these studies, the CXXC-based trapping technique identified both established and novel target proteins, subsequently confirmed by independent techniques, demonstrating the competence of this technique to identify bona fide interaction partners.

Mechanism-based kinetic trapping can be applied to human Trx1

To determine whether kinetic trapping can be applied to human Trx1, we created recombinant wild-type and mutant Trx proteins, each equipped with a C-terminal dual affinity tag composed of a streptavidin-binding peptide (SBP) and a hexahistidine tag. To create a trapping mutant, the second cysteine of the ^{32}CXXC^{35} motif was replaced by serine (C35S). Trx1 harbors three additional cysteine residues distal to the active site (cysteines 62, 69 and 73). As these residues are dispensable for catalytic activity but may cause oxidative inactivation by either intra- or intermolecular disulfide bond formation (Casagrande *et al*, 2002; Watson *et al*, 2003), we also created mutants containing amino-acid substitutions for those additional cysteines (Figure 1B; CCCCC, CCAAA, CSCCC, CSAAA and SSAAA annotate the identity of residues 32, 35, 62, 69 and 73).

To test whether Trx1(C35S)-based trapping is capable of identifying known Trx1 target proteins, Trx1(CSAAA) was allowed to react with cytosolic proteins released from digitonin-permeabilized cells. Incubation led to the formation of a reproducible pattern of distinct mixed disulfide conjugates as visualized by silver staining of a SDS–PAGE gel under non-reducing conditions (Figure 1C, lane 7). In accordance with the trapping mechanism, conjugation strictly depended on the availability of the N-terminal thiol (Cys-32) and the concurrent absence of the C-terminal thiol (Cys-35), as wild-type or cysteine-free Trx1 did not capture any proteins (Figure 1C, lanes 3, 5 and 9). The pattern of trapped proteins was not significantly influenced by the presence or absence of the non-catalytic cysteines (data not shown). The main cytosolic interaction partner of Trx1(CSAAA) was identified as peroxiredoxin-1 (Prx1) by liquid chromatography tandem mass spectrometry (LC-MS/MS). The Trx1–Prx1 association was further confirmed by immunoblotting (data not shown). The Trx1–Prx1 disulfide-linked conjugate is maintained under non-reducing conditions (Figure 1C, lane 7) and cleaved into its monomer constituents under reducing conditions (Figure 1C, lane 8).

To test whether Trx1(CSAAA) would also undergo authentic interactions under conditions more typical of an extracellular environment, we allowed Trx1(CSAAA) to react with human plasma proteins. To avoid nonspecific absorbance to high-abundance serum proteins, we applied Trx1(CSAAA) to a <30 kDa plasma ultrafiltrate, leading to the capture of a small number of proteins, as visualized by colloidal Coomassie staining (Figure 1D). Using LC-MS/MS, the principal interaction partner from the plasma ultrafiltrate was identified as peroxiredoxin-2 (Prx2), a well-established target protein of Trx1, recently found to be present in human plasma (Chen *et al*, 2004). These experiments provided proof-of-principle evidence that kinetic trapping is capable of capturing and identifying proven target proteins of human Trx1 from both intra- and extracellular environments.

Trx1 kinetic trapping is mediated by specific protein–protein interactions

To further investigate whether the capture of proteins by Trx1(CSAAA) is Trx-specific, we directly compared Trx1(CSAAA) with the corresponding trapping mutant of another member of the Trx superfamily, glutaredoxin-1 Grx1(CSAAA) (CSAAA annotates the identity of residues 22, 25, 7, 78 and 82). Grx1, like Trx1, uses its active site thiol to act as a disulfide reductase in the cytosolic environment. However, in contrast to Trx1, Grx1 is specialized in the recognition and reduction of protein–glutathione mixed disulfide bonds and forms mixed disulfide intermediates with glutathione rather than with proteins (Yang *et al*, 1998; Peltoniemi *et al*, 2006). As expected, we did not detect trapping of peroxiredoxins (or other Trx1-interacting proteins) by Grx1(CSAAA), neither on silver gels (Figure 1E, lanes 3 and 4) nor by immunoblotting (data not shown). The activity and thiol reactivity of the Grx1 trapping mutant was confirmed in independent experiments (data not shown), thus demonstrating that the mere availability of an active site thiol does not explain the profile of proteins captured by the Trx1 trapping mutant. Instead, our results support the notion that Trx-mediated reducing activity is steered toward

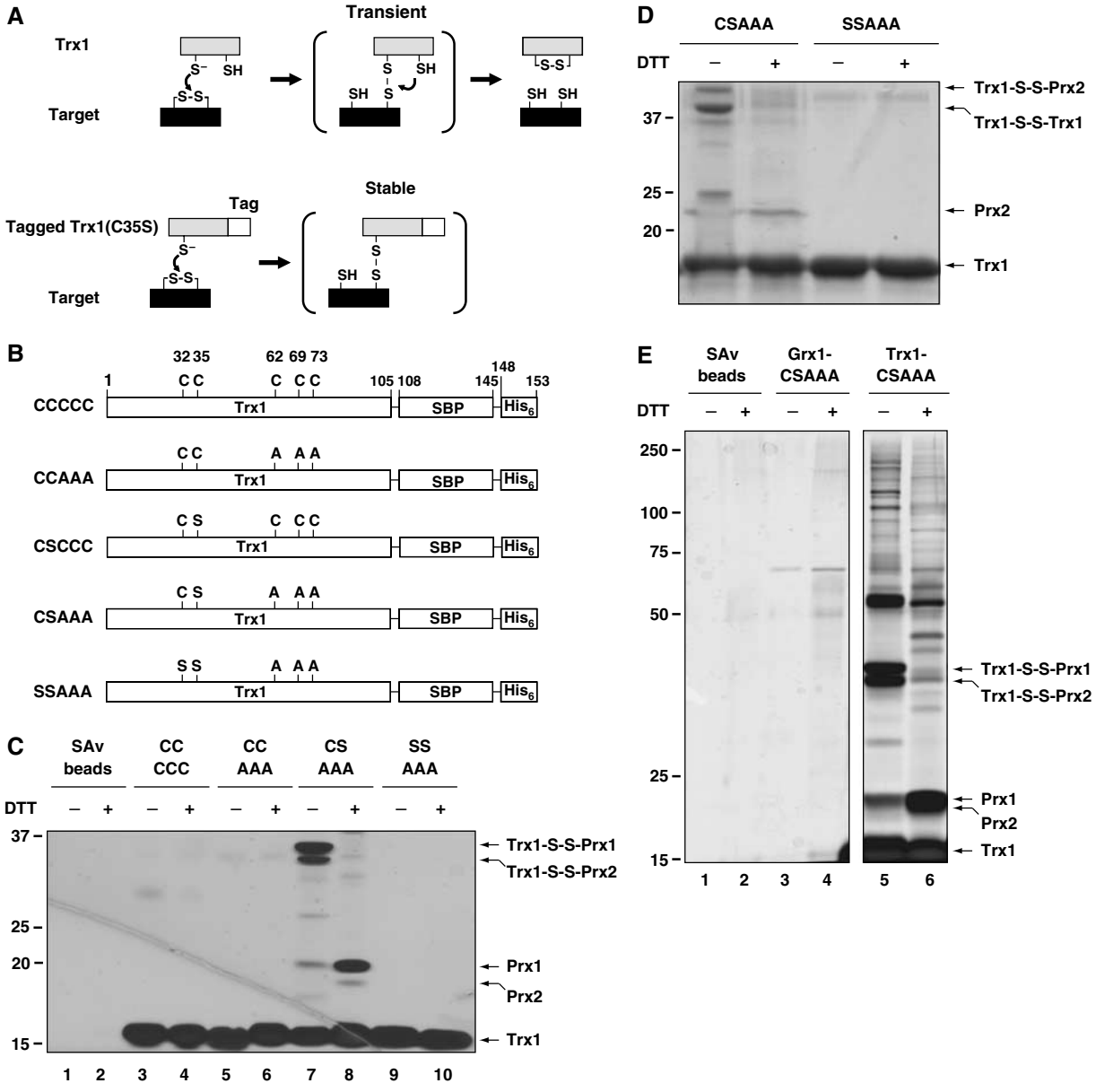

Figure 1 Mechanism-based kinetic trapping identifies target proteins of human Trx1. (**A**) Catalytic mechanism of wild-type Trx1 (upper panel) and the principle of substrate trapping (lower panel). The mixed disulfide intermediate is normally resolved by cysteine-35. Replacement of cysteine-35 by serine (C35S) stabilizes the covalent intermediate. The affinity tag enables purification of resulting enzyme–substrate complexes. (**B**) Schematic representation of recombinant Trx1 constructs. Catalytic (C^{32}, C^{35}) and structural cysteines (C^{62}, C^{69}, C^{73}) are indicated. His_6: hexahistidine tag; SBP: streptavidin-binding peptide. (**C**) Trapping of cytosolic Trx1 target proteins. Cytosolic proteins released from digitonin-permeabilized Jurkat cells were incubated with different recombinant Trx1 proteins. Disulfide-linked Trx1 complexes were analyzed by silver staining under non-reducing and reducing conditions. Trx1 complexes involving Prx1 and Prx2 are indicated; SAv streptavidin. (**D**) Trapping of extracellular Trx1 target proteins. Trapping (CSAAA) and non-trapping (SSAAA) mutants of Trx1 were incubated with a <30 kDa fraction prepared from fresh human serum. Disulfide-linked complexes were analyzed by colloidal Coomassie staining under non-reducing and reducing conditions. The Trx1–Prx2 conjugate and the Trx1 dimer as well as monomeric Prx2 and Trx1 are indicated. (**E**) Kinetic trapping is mediated by specific protein–protein interactions. Cytosolic proteins from digitonin-permeabilized Jurkat cells were incubated with SAv sepharose, Grx1(CSAAA) or Trx1(CSAAA). Disulfide-linked complexes were analyzed by silver staining under non-reducing and reducing conditions. Trx1 conjugates formed with Prx1 and Prx2 are indicated. Other bands correspond to additional cytosolic proteins interacting with Trx1.

distinct target disulfide bonds by specific protein–protein interactions.

Kinetic trapping can be applied to detect Trx1 interactions on the cell surface

Having established the Trx1 kinetic trapping approach for soluble target proteins, we asked whether the kinetic trapping technique can also be applied to the surface of intact cells in culture. Given previous indications of disulfide bond exchange between CD4 and wild-type Trx1 (Matthias *et al*, 2002), we asked whether kinetic trapping would enable us to detect this interaction on the surface of the CD4 positive promyelocytic cell line U937. In brief, we allowed mutant Trx1 to interact with the surface of live cells, removed unreacted oxidoreductase by washing and collected disulfide-linked Trx1 complexes from cellular lysates by

streptavidin (SAv) affinity purification. We found that cell surface CD4 forms a mixed disulfide with exogenously added Trx1(CSAAA) (Supplementary Figure S1, left panel), which could be dissociated by DTT treatment (Supplementary Figure S1, right panel). This result confirmed that kinetic trapping can indeed be used to identify specific Trx1-reactive cell surface proteins and should therefore allow *de novo* identification of previously unknown cell surface target proteins.

Kinetic trapping on the surface of lymphoid cell lines identifies a prominent Trx1 interaction partner

Human Trx1 was first identified as an autocrine factor secreted by and acting on virally transformed lymphoid cell lines. We therefore applied cell surface trapping to a human EBV-transformed lymphoblastoid B-cell line (LCL-721.220) derived from the same parental cell line (LCL-721) as the 3B6 cell line, originally used in the description of the costimulatory factor '3B6-IL1', later identified as Trx1 (Wakasugi *et al*, 1990). Mixed disulfide complexes, which formed on the surface of LCL-721.220 cells were isolated and analyzed by Trx1-specific immunoblotting to visualize overall mixed disulfide conjugates. Interestingly, we found that Trx1 predominantly engages a single protein on the lymphoblastoid surface, suggesting a highly selective interaction (Figure 2A, lane 3). As expected, the trapping product, a mixed disulfide conjugate of about 160 kDa, was susceptible to reduction (Figure 2A, lane 4). The 160 kDa conjugate did not form on the surface of the EBV-negative Burkitts lymphoma cell line BL-41 (Figure 2B). In contrast, a conjugate of the same size was found to be formed on the surface of CCRF-CEM T cells (Figure 2C) and YT large granular lymphoma cells (data not shown). Pretreatment of the cell surface with the alkylating agent iodoacetamide (IAA) did not interfere with the formation of the 160 kDa mixed disulfide conjugate, thus confirming that the conjugate was formed by the expected disulfide exchange mechanism (rather than by *de novo* disulfide bond formation between two thiol groups).

CD30/TNFRSF8 is the principal Trx1 sensitive receptor on various lymphoid cell lines

To identify the unknown Trx1 target protein, we performed cell surface trapping on a larger scale (5×10^9 LCL-721.220 cells), purified the Trx1-interacting surface protein by SAv affinity purification and visualized the protein by colloidal Coomassie staining (Figure 3A, left panel). The 160 kDa band was absent in the control precipitation with Trx1(CCAAA). Corresponding bands from non-reducing and reducing lanes were subjected to tryptic digestion and LC-MS/MS analysis. From both samples the unknown protein was identified as TNFRSF8 (CD30), a member of the TNFR superfamily. To validate the direct covalent interaction between Trx1(CSAAA) and CD30, an aliquot of trapped complexes from the same experiment was separated under non-reducing and reducing conditions and subjected to immunoblotting analysis with anti-Trx1 (Figure 3A, middle panel) and anti-CD30 antibodies (Figure 3A, right panel), respectively. The observed mobility difference between non-reducing (NR) and reducing (R) lanes demonstrated the formation of a mixed disulfide conjugate (Figure 3A, right panel). Additional immunoblotting experiments demonstrated that low nanomolar concentrations of Trx1(CSAAA) are sufficient to detect the interaction with CD30 (Figure 3B) and also confirmed that trapping of CD30 depends on the N-terminal cysteine of the CXXC motif (Figure 3C, lanes 1–8). Application of the Grx1 trapping mutant under the same conditions did not lead to its conjugation to CD30 (Figure 3C, lanes 9 and 10). Immunoblotting and flow cytometry experiments confirmed that BL-41 cells, unlike the other lymphocytic cell lines, do not express CD30 (Supplementary Figure S2), thus explaining the absence of the 160 kDa conjugate band (Figure 2B).

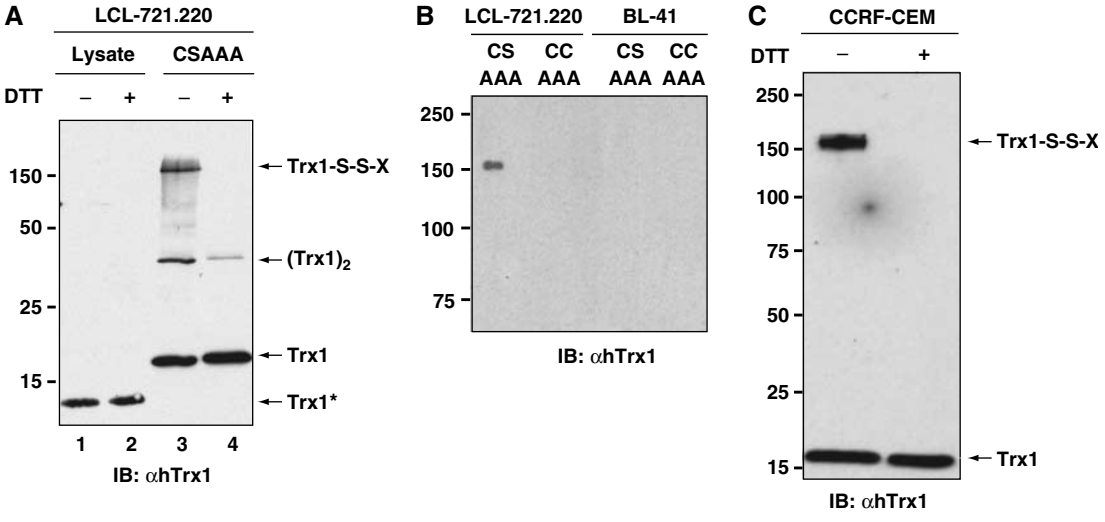

Figure 2 Trx1 targets a single principal interaction partner on the surface of lymphocytic cell lines. (**A**) LCL-721.220 cells were incubated with Trx1(CSAAA) and disulfide-linked complexes analyzed by anti-Trx1 immunoblotting under non-reducing and reducing conditions. Cellular lysate was included as an immunoblotting control (Trx1*: endogenous Trx1). Dimerization of exogenously added Trx1 is indicated. (**B**) LCL-721.220 or BL-41 B cells were treated as in (A). Trx1(CCAAA) was included as a control. Disulfide-linked complexes were analyzed as in (A). (**C**) CCRF-CEM T cells were treated as in (A) and complexes analyzed by anti-Trx1 immunoblotting under non-reducing and reducing conditions. Conjugation of the unknown cell surface protein is indicated (Trx1-S-S-X).

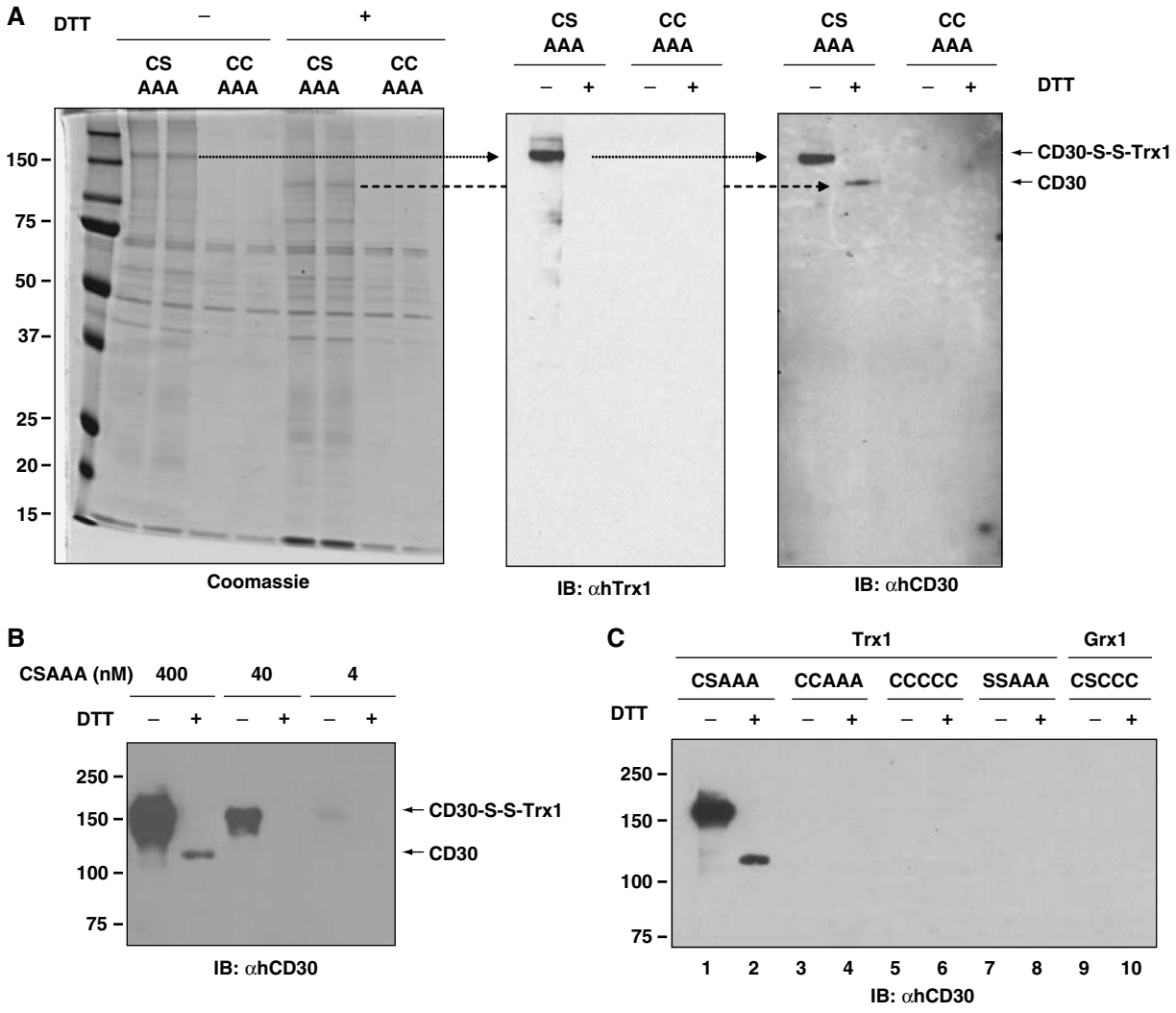

Figure 3 TNFRSF8/CD30 is the main Trx1 interaction partner on the surface of lymphoid cell lines. (**A**) A total of 5×10^9 LCL-721.220 cells were incubated with Trx1(CSAAA) or Trx1(CCAAA) as control. Disulfide-linked Trx1 complexes were purified by SAv affinity purification and analyzed by colloidal Coomassie staining under non-reducing and reducing conditions (left panel). Indicated bands were subjected to tryptic digestion and LC-MS/MS analysis. In parallel, part of the same sample was analyzed by anti-Trx1 immunoblotting (middle panel). The blot was stripped and reprobed with anti-human CD30 antibody (right panel). (**B**) CCRF-CEM T cells were incubated with different amounts of Trx1(CSAAA) and disulfide-linked Trx1 complexes were analyzed by anti-CD30 immunoblotting under non-reducing and reducing conditions. (**C**) CCRF-CEM T cells were incubated with recombinant Trx1 constructs or the Grx1(C25S) trapping mutant as indicated and complexes were analyzed as described in (B). The difference in signal intensity between non-reduced and reduced form of CD30 is due to less efficient recognition of the reduced form by the CD30-specific antibody.

Trx1 discriminates between CD30 and other members of the TNFR superfamily

Ectodomains of TNFR superfamily proteins typically are composed of one to four cysteine-rich domains (CRDs), each normally harboring three disulfide bonds (Bodmer *et al*, 2002). To exclude the possibility that Trx1 interacts with CRDs uniformly, we tested whether Trx1 discriminates between distinct members of the TNFR superfamily. As shown in Figure 4A, CD95 (TNFRSF6) did not form a mixed disulfide conjugate with Trx1(CSAAA) on the same cells under identical conditions. The same result was obtained for the epidermal growth factor receptor (EGFR), which contains a total of 25 ectodomain disulfide bonds, and is expressed at substantial levels on A431 cells (Gill and Lazar, 1981) (Figure 4B). These findings indicate that Trx1 reactivity of cell surface receptors is selective and is not determined by the presence of CRDs or the number of ectodomain disulfide bonds. To further exclude that Trx1 reactivity of receptors is determined or limited by surface expression levels, we ectopically overexpressed CD30 or other TNFR superfamily members under control of the same promoter in HeLa cells and analyzed Trx1 cell surface trapping by indirect immunofluorescence. While mock-transfected HeLa cells did not capture Trx1(CSAAA) on their surface (Figure 4C, lower row), expression of CD30 led to a strong Trx1 surface association and colocalization of both proteins (Figure 4C, upper row). In contrast, expression of CD95 (Figure 4D), TNFR1 or NGFR (data not shown) did not promote Trx1 interactions with the cell surface, further strengthening the notion that Trx1 reactivity is a specific property of CD30.

The domain structure of human CD30 differs from other members of the TNFR superfamily and from its murine homologue by the presence of two additional CRDs, arising

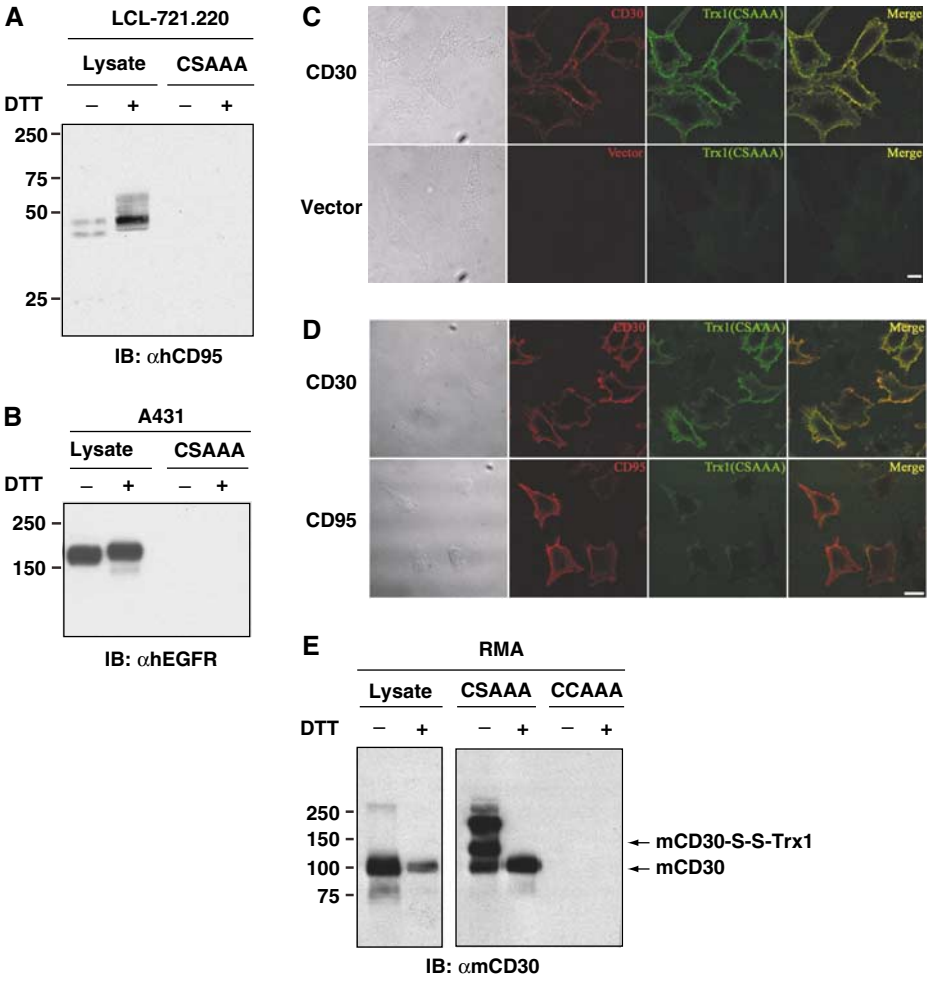

Figure 4 Trx1 discriminates between CD30 and other receptors with CRDs. (**A**) LCL-721.220 B cells were incubated with Trx1(CSAAA) and disulfide-linked Trx1 complexes were analyzed by anti-CD95 immunoblotting. (**B**) A431 epithelial carcinoma cells were incubated with Trx1(CSAAA) and complexes were analyzed by anti-EGFR immunoblotting. Cellular lysate was included as control. (**C**) HeLa cells were transiently transfected with an expression construct for human CD30 or empty vector incubated with Trx1(CSAAA) and analyzed by immunofluorescence microscopy using CD30- and Trx1-specific antibodies (scale bar, 20 μm). (**D**) HeLa cells were transiently transfected with expression constructs for human CD30 or CD95, treated as described in (C) and analyzed by immunofluorescence microscopy using CD30-, Trx1- and CD95-specific antibodies (scale bar, 20 μm). (**E**) RMA mouse lymphoma cells were incubated with Trx1(CSAAA) or Trx1(CCAAA). Disulfide-linked Trx1 complexes were analyzed by anti-mouse CD30 (mCD30) immunoblotting under non-reducing and reducing conditions. The disulfide-linked Trx1-mCD30 complex and monomeric mCD30 are indicated. The additional band of higher molecular weight has not been further characterized but might represent a conjugate between Trx1 and a dimer of mCD30.

from the internal duplication of two exons (Burgess *et al*, 2004). We asked whether this unusual feature might confer Trx1 reactivity to human CD30 and tested whether the shorter murine CD30 could also interact with Trx1. As shown in Figure 4E, murine CD30 expressed on the Rauscher murine leukemia virus-induced T-cell lymphoma line RMA is efficiently targeted by Trx1(CSAAA), thus demonstrating that the additional CRDs in human CD30 are not required for Trx1 reactivity. The result also suggests that the enzymatic affinity of Trx1 for CD30 has been conserved during mammalian evolution.

Trx1 catalyzes disulfide bond reduction in CD30 and induces a conformational change disrupting the Ki-1 epitope

To demonstrate by an independent method that Trx1 attacks and breaks a disulfide bond in CD30, we used thiol-specific cell surface biotinylation to verify that Trx1 activity generates free thiols within the CD30 ectodomain (Supplementary Figure S3). Subsequent analysis of CD30 cell surface expression by flow cytometry and fluorescence microscopy revealed that a brief treatment of $CD30^+$ cells with wild-type Trx1 led to the complete loss of CD30 recognition by the Ki-1 antibody (Figure 5A, upper panel and Figure 5B, second column). Similar results were obtained when another monoclonal antibody against CD30, MAB229 (R&D Systems; Clone 81337), was used to examine CD30 expression (Figure 5A, middle panel). Under the same conditions, recognition by the Ber-H2 antibody was only slightly affected (Figure 5A, lower panel and Figure 5B, third column), indicating that Trx1-mediated disulfide bond reduction (and possibly rearrangement) induces a structural alteration in the CD30 ectodomain, which disrupts or conceals the Ki-1 epitope.

Pursuant to the observation that recognition of CD30 by antibodies Ki-1 and MAB229 is affected by CD30 redox state, we used flow cytometry to analyze the response of CD30 to

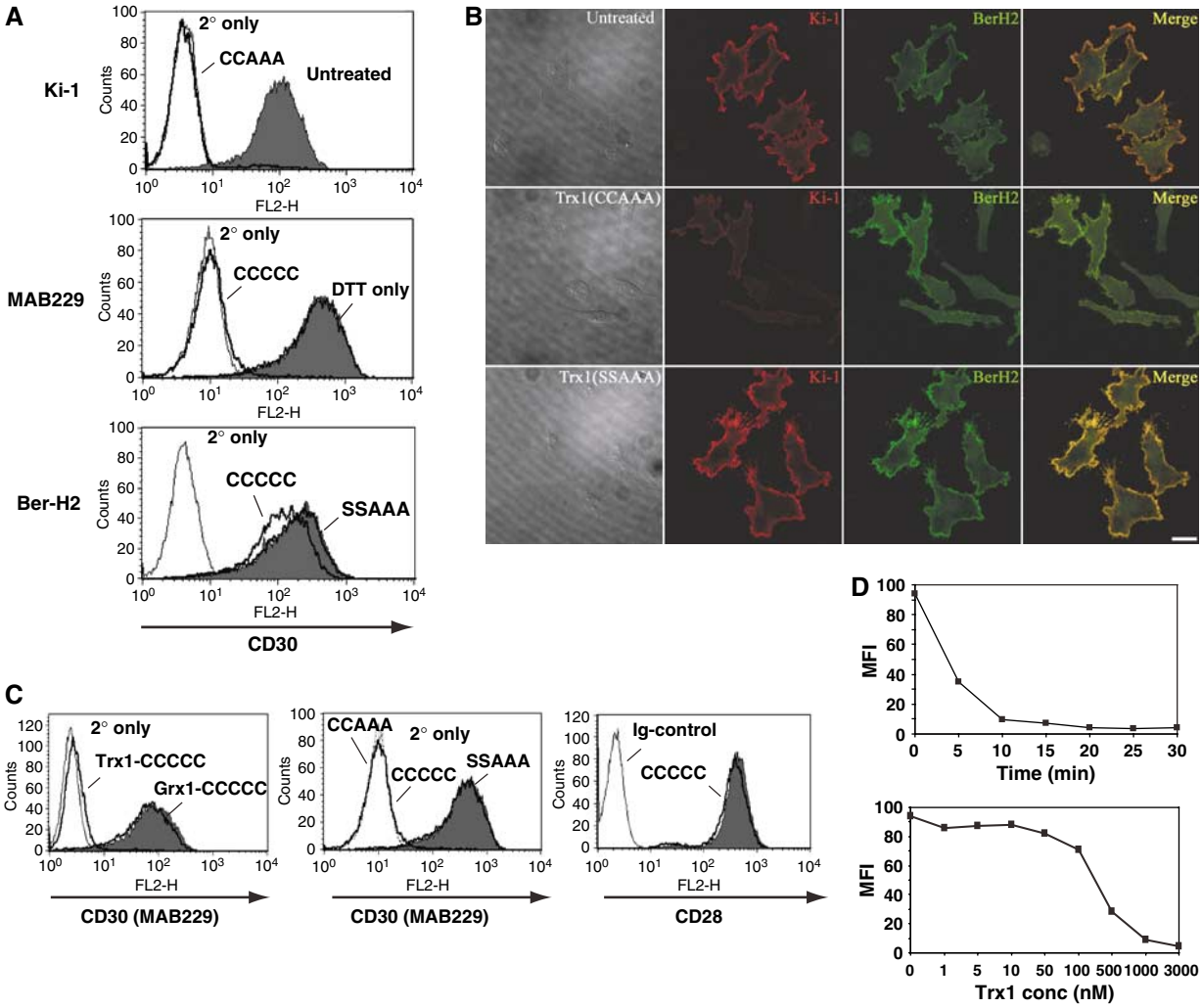

Figure 5 Trx1 catalyzes disulfide bond reduction within the CD30 ectodomain. (**A**) CCRF-CEM T cells were left untreated or treated with active (CCAAA/CCCCC) or inactive Trx1 (SSAAA) in the presence of DTT as a regenerating system or with DTT only. Cell surface CD30 was analyzed by flow cytometry using three different anti-CD30 antibodies (Ki-1: upper panel; MAB229: middle panel; Ber-H2: lower panel). PE-labeled secondary antibody only was included as control. Untreated cells are shown in gray. (**B**) HeLa cells were transiently transfected with a CD30 expression vector and either left untreated or treated with active (CCAAA) or inactive Trx1 (SSAAA) in the presence of DTT. Cells were analyzed by immunofluorescence microscopy using CD30-specific antibodies Ki-1 and Ber-H2 (scale bar, 20 μm). (**C**) CCRF-CEM cells were left untreated or treated with different Trx1 constructs or wild-type Grx1 as indicated. Cell surface CD30 was analyzed by anti-CD30 antibody MAB229 (left and middle panel). Alternatively, cells treated with wild-type Trx1 were analyzed by PE-conjugated anti-CD28 antibody (right panel). Untreated cells are shown in gray. (**D**) CCRF-CEM cells were treated with 5 μM active Trx1 for different time points (upper graph) or with different Trx1 concentrations (lower graph) for 30 min. Cells were analyzed as described in (C).

reduction in greater detail. Addition of oxidized Trx1 did not influence antibody reactivity of CD30 (data not shown). When reduced Trx1 was applied in the absence of a regenerating system, a conformational change in CD30 could be observed, but remained incomplete (data not shown). Complete and sustained loss of antibody reactivity required a source of reducing equivalents for the oxidoreductase. Both DTT and Trx reductase (TrxR)/NADPH were found to be effective as regenerating systems. Importantly, neither DTT nor TrxR/NADPH had an effect when applied in the absence of Trx1 (Figure 5A, middle panel and data not shown). Wild-type Grx1 (Figure 5C, left panel) and the redox-inactive mutant of Trx1 (Figure 5C, middle panel) did not alter the redox-sensitive CD30 epitopes. Other cell surface receptors, for example CD28, analyzed in parallel on the same cells were unaffected by Trx1 treatment (Figure 5C, right panel). Loss of CD30 antibody recognition typically occurred within minutes (Figure 5D, upper panel). Testing the influence of Trx1 concentration under the same conditions, consistent effects on CD30 conformation became evident at concentrations around 100 nM (Figure 5D, lower panel).

Trx1-mediated disulfide exchange interferes with CD30 receptor–ligand interactions

The observation that antibody binding to CD30 is influenced by Trx1 suggested a redox-dependent conformational change within the CD30 ectodomain. To test whether Trx1-mediated reduction of CD30 influences binding of CD30 to its ligand (CD30L), we analyzed the interaction between CD30 and recombinant soluble CD30L (sCD30L) on the cell surface by flow cytometry. A brief incubation of $CD30^+$ Hodgkin's lymphoma HDLM-2 cells with wild-type Trx1 led to a substantial loss in CD30L binding to the cell surface (Figure 6A). A similar result was obtained for the large granular lymphoma

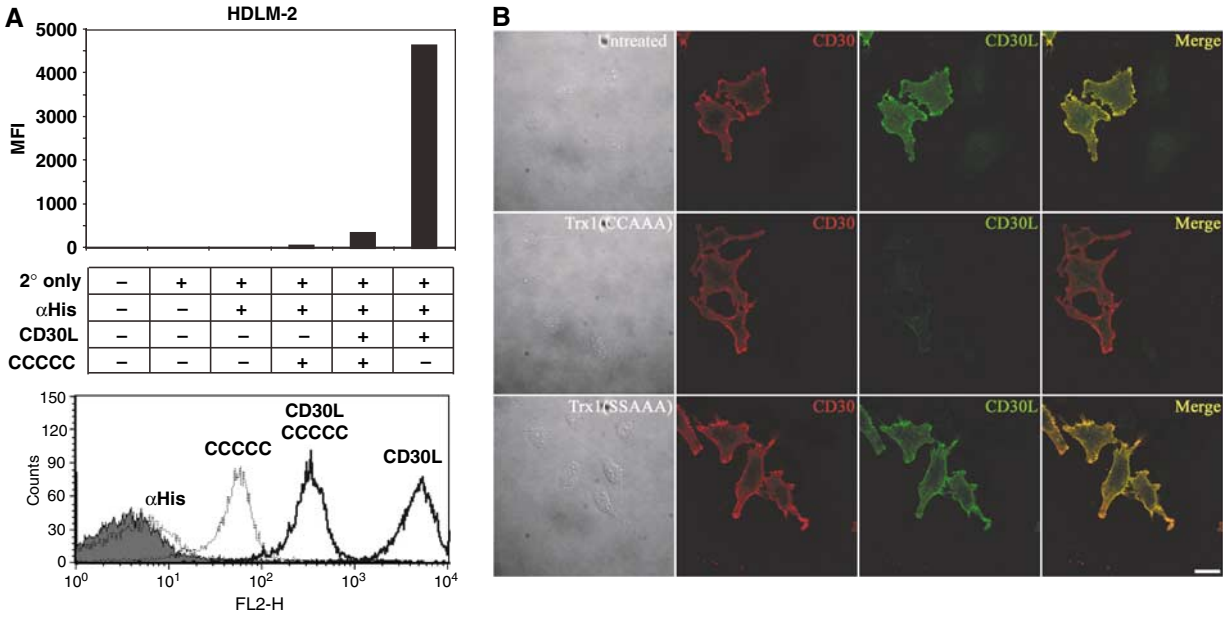

Figure 6 Trx1-mediated disulfide bond reduction in CD30 prevents binding of the CD30L. (**A**) HDLM-2 Hodgkin cells were either left untreated or treated with wild-type Trx1 in the presence of DTT as a regenerating system. After washing, cells were incubated with recombinant CD30L-His_{10} as indicated. Bound CD30L was detected by flow cytometry using anti-polyHis antibody. Cells incubated with anti-polyHis antibody only were used as control. Unstained cells are shown in gray. (**B**) HeLa cells were transiently transfected with an expression construct for CD30 and either left untreated or treated with active (CCAAA) or inactive Trx1 (SSAAA) in the presence of DTT. After washing, cells were incubated with recombinant CD30L-His_{10}. CD30L bound to the cell surface was analyzed by immunofluorescence microscopy using anti-CD30L antibody (scale bar, 20 μm).

cell line YT (Supplementary Figure S4). The same effect was evident in the absence of an exogenously added reducing system (Supplementary Figure S5), thus demonstrating that under the given conditions Trx1–substrate interactions are not limited by oxidative inactivation.

As shown by fluorescence microscopy, sCD30L binds to the surface of CD30-transfected HeLa cells and colocalizes with its receptor (Figure 6B, upper row). Treatment of HeLa cells with Trx1(CCAAA) (Figure 6B, middle row) but not a redox-inactive mutant (Figure 6B, lower row) strongly interferes with CD30L binding and colocalization.

Trx1-mediated reducing activity interrupts agonist-induced CD30 signaling in YT cells

Given its influence on ligand binding, we reasoned that Trx1 might interfere with CD30-mediated signaling. To test whether Trx1-mediated conformational alteration of CD30 affects CD30-dependent cellular responses, we made use of the YT large granular lymphoma line that has previously been used to study signals emanating from CD30 and to define the genes regulated by such signals (Muta *et al*, 2000). Consistent with previous results (Bowen *et al*, 1993), we observed that stimulation of CD30 with either agonistic antibodies or sCD30L led to upregulation of the IL-2Rα chain (CD25) within 24 h (Figure 7A, lower panel, compare columns 3, 4 and 7). Treatment of YT cells with Trx1(CCAAA) (but not with redox-inactive Trx1) prevented CD25 upregulation (Figure 7A, lower panel, columns 5 and 8), concomitant with the redox change in CD30 (Figure 7A, upper panel, column 4), thus demonstrating that the redox interaction between Trx1 and CD30 has a pronounced influence on CD30-mediated gene expression.

YT cells respond to CD30 signals by downregulating the expression of cytotoxic effector molecules, including FasL, thus decreasing their cytotoxicity against Fas-expressing target cells (Bowen *et al*, 1993; Muta *et al*, 2000). To test if Trx1 influences CD30-mediated suppression of cytotoxicity, we treated YT cells with Trx1(CCAAA) or Trx1(SSAAA) before stimulation with agonistic anti-CD30 antibody and quantified cytotoxicity against Cr-labeled Raji cells. Upon CD30 stimulation, cytotoxicity was markedly reduced (Figure 7B, compare columns 1 and 4). The decrease in cytotoxicity was completely reversed by Trx1(CCAAA) but not the catalytically inactive mutant (SSAAA) (Figure 7B, columns 5 and 6). As judged by RT–PCR, changes in cytotoxicity correlated with changes in FasL mRNA expression (Figure 7B, lower panel). These results confirm that the catalytic activity of Trx1 modulates CD30-dependent changes in cellular behavior and function.

Discussion

Accumulating evidence indicates that the reduction and rearrangement of disulfide bonds constitutes a mechanism controlling protein function on the cell surface (Hogg, 2003). The idea that disulfide bonds can act as dynamical redox switches, specifically operated by secreted redox catalysts, represents a novel concept in signal transduction (Jordan and Gibbins, 2006). However, technical difficulties in detecting and analyzing individual disulfide rearrangements on the cell surface have made progress slow.

Trx1 is recognized as one of the most important regulators of cellular and organismal redox homeostasis (Gromer *et al*, 2004). In particular, intracellular Trx1 counteracts oxidative stress, promotes cell growth and inhibits apoptosis. Under

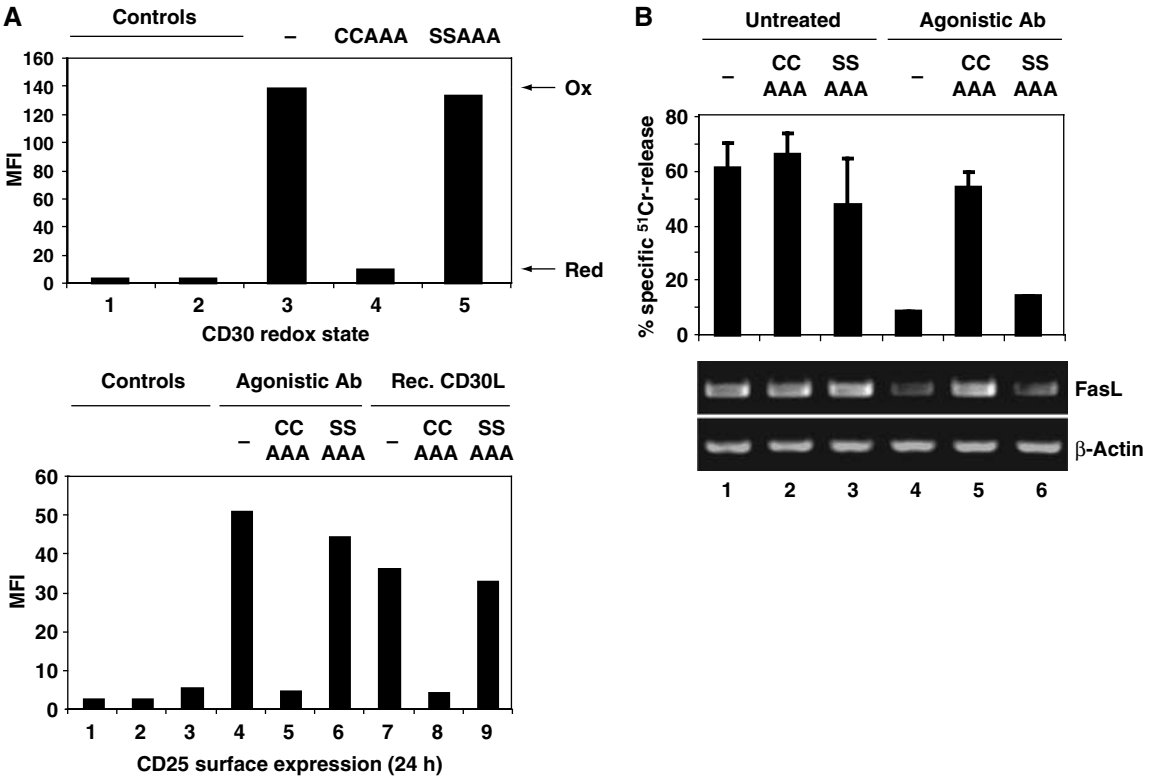

Figure 7 Trx1 controls CD30-mediated functional changes in lymphoma cells. (**A**) Trx1 inhibits CD30-mediated changes in gene expression. YT large granular lymphoma cells were left untreated or treated with active (CCAAA) or inactive Trx1 (SSAAA) in the presence of TrxR/NADPH as a regenerating system. After treatment, the change in CD30 redox state was verified by flow cytometry using antibody MAB229 (upper panel). Cells were then stimulated with agonistic anti-CD30 antibody (MAB229) or recombinant CD30L, or left unstimulated (column 3). After 24 h, cell surface expression of the IL-2Rα chain (CD25) was analyzed by flow cytometry using anti-CD25 antibody. As control, unstained cells and cells stained with 2° antibody only (columns 1 and 2) were used. (**B**) Trx1 inhibits CD30-mediated changes in effector function. YT cells were treated as described in (A). After 24 h, YT cells were harvested and analyzed for cytotoxicity on ^{51}Cr-labeled Raji cells (E:T ratio of 25:1). The experiment was performed in triplicates. Expression levels of FasL-mRNA were analyzed by RT–PCR. β-Actin levels are shown as control.

conditions of oxidative stress, Trx1 is released by cells and accumulates at sites of inflammation (Nordberg and Arner, 2001). Numerous studies have reported that secretory Trx1 influences effector functions and proliferation of lymphocytes (Nakamura *et al*, 1997). However, proteins and pathways coupling extracellular Trx1 redox activity to defined cellular responses have remained unknown. In this study, we addressed the question regarding which lymphocyte surface receptors are targeted and regulated by the redox activity of extracellular Trx1.

For this purpose, we made use of a mechanism-based kinetic trapping approach to capture mixed disulfide intermediates formed between exogenous Trx1 and its target proteins on the cell surface of living cells. Activity-based techniques offer the opportunity to identify interactions too short-lived to be detectable by conventional methods. To our knowledge, this is the first reported application of kinetic trapping to identify novel target proteins of mammalian Trx1 and the first application of this technique to the surface of intact cells. We demonstrate that Trx1 interacts with intra- and extracellular target proteins in a highly selective manner, guided by specific protein–protein recognition rather than random encounters with disulfide bonds.

Applying the approach to the surface of cell lines representative of the lymphoid lineage, we observed that Trx1 basically targets a single cell surface protein, subsequently identified as TNF receptor superfamily member 8, also known as CD30. The pronounced preference of Trx1 for one particular target protein might seem surprising, but could be due to the fact that we assessed Trx1 reactivity of proteins as they are embedded in their natural microenvironment, namely the intact surface of the active plasma membrane of living cells. It is conceivable that protein disulfide exchange interactions are limited and controlled by their native context and location.

To scrutinize the specificity of the observed interaction, we asked if the preference of Trx1 for CD30 might be caused by an unusual density of disulfide bonds within CD30 and/or exceptional cell surface expression levels. Although the CD30 ectodomain harbors a significant number of predicted disulfide bridges within CRDs, it does not appear to be unusual in terms of disulfide bond composition/density when compared to other members of the superfamily. When tested experimentally, Trx1 failed to interact with other CRD-containing proteins, including the EGFR featuring a total of 25 ectodomain disulfide bonds. The preference for CD30 could not be explained by exceptional surface expression levels either. While Hodgkin's disease cell lines typically express high levels of CD30, other cell lines including LCL-721.220 or CCRF-CEM show at least 20- to 100-fold lower expression as determined by flow cytometry, yet the same selective targeting was observed. Conversely, ectopic overexpression of several related TNFR superfamily members in HeLa cells did not lead to their interaction with Trx1, yet CD30 strongly

interacted on the same cells under the same conditions. Consistent with these findings, recent experiments demonstrate that Trx1 targets a particular site within the CD30 ectodomain (Y Balmer and TP Dick, unpublished data).

To facilitate identification of low-abundance cell surface proteins, *in vitro* trapping experiments were typically performed using Trx1 concentrations of 1–3 μM. However, when disulfide exchange was subsequently tested at lower concentrations, Trx1(CSAAA) concentrations in the low nanomolar range (4–40 nM) were found to give rise to the formation of proportional amounts of Trx1-CD30 mixed disulfide intermediates (Figure 3B), thus demonstrating that the observed interaction is compatible with the expected physiological concentration range of secretory Trx1 (see below).

Wild-type Trx1 is known to act as a multiple-turnover catalyst if a suitable reducing system and electron source is provided for its regeneration. In agreement with these considerations, we observed that sustained reduction of CD30 in cell culture requires a Trx1 regenerating system. Using flow cytometry to monitor conformational changes in the CD30 ectodomain, CD30 was found to respond to Trx1 concentrations in the nanomolar range, starting at around 100 nM (Figure 5E, lower panel). However, the minimal Trx1 concentration required for sustained CD30 reduction might be substantially lower in specific environments, which are efficient in delivering reducing equivalents and preventing oxidative inactivation of Trx1.

At present, it is not clear how extracellular Trx1 is regenerated *in vivo*. Despite the overall oxidizing character of the extracellular compartment, reductive processes are known to take place on the cell surface. On the one hand, there is long-standing evidence for the existence of transplasma membrane redox systems delivering electrons to the cell surface (Crane *et al*, 1985). On the other hand, Trx1 may be regenerated by co-secreted reductants, as Trx1 secretion in DC-T co-culture is accompanied by the release of reduced cysteine and the creation of a reducing microenvironment between interacting cells (Angelini *et al*, 2002). In addition, TrxR was found to be secreted by activated monocytes and might be part of an extracellular Trx1 reducing system (Soderberg *et al*, 2000).

The concentration of Trx1 in human plasma is in the low nanomolar range (1–5 nM), and is found to be elevated several-fold under inflammatory conditions (Yoshida *et al*, 1999). However, plasma Trx1 is oxidized and appears to represent systemic dilution of Trx1 previously released within tissues. Accordingly, local tissue concentrations of secretory Trx1, for example, within activated lymph nodes, are expected to be markedly higher than in plasma. Overall Trx1 concentrations in mammalian tissues can be as high as 20 μM (Gromer *et al*, 2004). Certain Trx1-secreting cell types, including macrophages and dendritic cells, distinctly upregulate expression of Trx1 upon activation (Angelini *et al*, 2002). *In vitro* studies of Trx1 secretion suggest that a substantial fraction of intracellular Trx1 can be released within a few hours (Rubartelli *et al*, 1992). Although direct measurements of extracellular Trx1 within tissues are not available, physiologically relevant extracellular Trx1 concentrations may well reach into the upper nanomolar, if not lower micromolar range.

We found that Trx1-mediated disulfide reduction changes the conformation and functional properties of the CD30 ectodomain. In the reduced state, CD30 lost its ability to interact with its cognate ligand CD30L or agonistic antibodies. The presence of catalytically active Trx1 impeded CD30-dependent signaling in the YT lymphoma cell line, as demonstrated by its effect on CD25 and FasL expression, as well as its influence on cytotoxicity against Fas-expressing target cells.

The physiological role of the CD30-CD30L system has remained unclear. *In vitro* studies focusing on $CD30^+$ lymphoid malignancies showed that triggering of CD30 signaling can induce either proliferation, activation, growth arrest or apoptosis, depending on cell type and stimulatory conditions (Schneider and Hubinger, 2002). *In vivo*, cell surface expression of CD30 appears to be tightly regulated and restricted to B and T lymphocytes undergoing activation in lymphoid tissues. It has been proposed that CD30 provides proliferation and/or survival signals during lymphocyte responses (Croft, 2003).

Under inflammatory conditions, CD30 expression is markedly induced. *In vivo* activation of CD30 can be monitored by the release of sCD30, shed from the plasma membrane upon CD30L binding (Hansen *et al*, 2000). Similar to serum Trx1, serum sCD30 is increased in infection, autoimmunity and allergy, for example systemic lupus erythematosus, rheumatoid arthritis and atopic dermatitis (Horie and Watanabe, 1998). Both Trx1 and CD30 appear to play a role in the regulation of the antiviral inflammatory response. Both Trx1 secretion and CD30 expression have been associated with virally transformed lymphocytes. Elevated levels of sCD30 occur during viral infection. Likewise, viral infection leads to elevated Trx1 plasma levels and several studies indicate that secreted Trx1 modulates the antiviral inflammatory process (Nakamura *et al*, 2001b, 2002).

In this study, we have identified an enzyme–substrate relationship between Trx1 and CD30, a receptor of activated lymphocytes involved in the regulation of inflammation. As lymphocytes migrate between different microenvironments, it is conceivable that Trx1 catalyzes disulfide exchange dynamically, activating or inactivating the CD30 pathway in response to the redox environment. The interaction between Trx1 and CD30 might represent a regulatory link between oxidative stress and lymphocyte function. Understanding of this relationship *in vivo* awaits the generation of suitable experimental tools.

Materials and methods

Cell culture

BL-41, CCRF-CEM, HDLM-2, Jurkat, RMA and U937 cells were cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). LCL-721.220 and YT cells were cultured in IMDM (Gibco), HeLa and A431 cells in DMEM (Gibco) with the same supplements.

Substrate trapping

Depending on the type of experiment, recombinant trapping mutant was applied to cytosolic preparations, human serum ultrafiltrate or intact cells. A detailed description of the different substrate trapping protocols is provided as Supplementary information.

Analysis of CD30 redox state by flow cytometry

For reduction of cell surface CD30, 2.5×10^5 cells were incubated with 5 μM Trx1 together with 200 μM DTT or 100 nM human Trx reductase (TrxR)/500 μM NADPH for 30 min at 4°C. To monitor reduction of cell surface CD30, cells were stained with anti-human

CD30 monoclonal antibody MAB229 (R&D Systems), anti-human CD30 monoclonal antibody Ki-1 (Santa Cruz) or anti-human CD30 monoclonal antibody Ber-H2 (DakoCytomation) followed by incubation with R-PE-conjugated goat $F(ab')_2$ anti-mouse Ig's (Biosource). For control, cells were stained with R-PE-conjugated anti-human CD28 monoclonal antibody (BD Pharmingen). Cells were analyzed using a FACSCalibur (Becton Dickinson) and CellQuest software.

CD30L binding assay

A total of 2.5×10^5 cells were incubated with 5 μM Trx1 (SBP-CCCCC) and 200 μM DTT for 30 min at 37°C, washed three times and incubated with 250 ng/ml recombinant CD30L-His_{10} (R&D Systems) for 10 min at RT. After washing, cells were stained with anti-polyHis monoclonal antibody (Sigma) followed by incubation with R-PE-conjugated goat $F(ab')_2$ anti-mouse Ig's (Biosource).

Immunofluorescence microscopy

HeLa cells were seeded on coverslips and transfected with expression constructs using $CaCl_2$ precipitation. After 2 days, transfected cells were fixed with 3% formaldehyde and 2% sucrose in PBS for 7 min at RT. Fixed cells were washed three times with PBS and incubated with different Trx1 constructs or recombinant CD30L (R&D Systems). Proteins were visualized using appropriate primary antibodies (Anti-CD30L polyclonal antibody (R&D Systems), anti-CD30 monoclonal antibodies Ki-1 (Santa Cruz) or Ber-H2 (DakoCytomation), anti-CD95 monoclonal antibody (a kind gift from Dr P Krammer), anti-Trx1 polyclonal antibody (M Preuss and TP Dick, unpublished) followed by FITC-conjugated anti-goat IgG, FITC-conjugated anti-rabbit IgG or TRITC-conjugated anti-mouse IgG and analyzed with a Nikon C1Si confocal microscope.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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