Catalysis of Disulfide Isomerization in Thrombospondin 1 by Protein Disulfide Isomerase†

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ABSTRACT: Thrombospondin 1 is a multidomain glycoprotein from platelets and most cells that participates in diverse biological processes. The structure and some functional properties of thrombospondin 1 are regulated by disulfide interchange in the Ca^{2+}-binding repeats and C-globular domain. The recent identification of the enzyme, protein disulfide isomerase, on the platelet surface suggested that protein disulfide isomerase may catalyze disulfide isomerization in platelet thrombospondin 1. Protein disulfide isomerase was found to form disulfide-linked complexes with thrombospondin 1, which is consistent with protein disulfide isomerase-mediated rearrangement of disulfide bonds in thrombospondin 1. To quantitate disulfide interchange in thrombospondin 1, perturbation of the enzyme inhibitory properties of platelet thrombospondin 1 were measured, specifically changes in the apparent dissociation constant for inhibition of neutrophil cathepsin G by thrombospondin 1. The inhibition constant increased ≥10−14-fold following incubation of either Ca^{2+}-replete or Ca^{2+}-depleted thrombospondin 1 with protein disulfide isomerase and reduced glutathione. The rate of protein disulfide isomerase-catalyzed disulfide interchange in thrombospondin 1 increased linearly with protein disulfide isomerase concentration and the K_m for reduced glutathione was 0.4 ± 0.2 mM. Disulfide isomerization in both platelet and fibroblast thrombospondin 1 was probed by measuring perturbation in epitopes for two anti-thrombospondin 1 monoclonal antibodies. Antibody D4.6 binds to the C-terminal Ca^{2+}-binding domains which are involved in disulfide interchange, whereas antibody HB8432 binds toward the N-terminus of the thrombospondin 1 subunit. In accordance with the location of these epitopes, incubation of platelet thrombospondin 1 or fibroblast thrombospondin 1 with protein disulfide isomerase and reduced glutathione resulted in 2-fold enhancement of binding of D4.6, whereas binding of HB8432 did not significantly change. In summary, protein disulfide isomerase catalyzes disulfide interchange in thrombospondin 1 which alters binding of neutrophil cathepsin G and antibody D4.6 to thrombospondin 1.

Thrombospondin 1 (TSP1)[1] is a trimeric glycoprotein from platelets and most normal and transformed cells and is involved in several and diverse biological processes (Bornstein, 1995). The protein has a subunit molecular mass of 150 kDa and contains domains of the procollagen, properdin, and epidermal growth factor variety with unique N- and C-termini modules and 12 unique Ca^{2+}-binding loops (Lawler & Hynes, 1986). There is a single free thiol in each subunit of TSP1. Speziale and Detwiler (1990) have shown that this free thiol can reside on any one of 12 different cysteines in the carboxy-terminal Ca^{2+}-binding repeats and globular domain of TSP1, implying that there is a complex intramolecular disulfide interchange process operating in TSP1.

Disulfide interchange in a protein is usually very specific and may be catalyzed by disulfide isomerases. The enzyme protein disulfide isomerase (PDI) has recently been shown to be present on the platelet surface, which makes it well-positioned to catalyze disulfide interchange in platelet-derived TSP1 (Chen et al., 1995; Essex et al., 1995). PDI functions as a disulfide isomerase enzyme by virtue of two very reactive surface-exposed disulfide bonds (Freedman, 1994). These disulfide bonds catalyze thiol−disulfide interchanges that can lead to the net formation, the net reduction, or the net rearrangement of protein disulfide bonds depending on the nature of the protein substrate, the redox conditions, and the presence of other thiols and disulfides. The redox potential of the PDI active site dithiol/disulfide groups is −110 mV, which corresponds to an equilibrium constant with GSH (reduced glutathione)/GSSG of 50−60 μM.

PDI was found to form disulfide-linked complexes with TSP1, which is consistent with PDI-catalyzed isomerization of disulfide bonds in TSP1. The observation that TSP1 is a tight-binding competitive inhibitor of neutrophil serine proteinases (Hogg et al., 1993a,b; Hogg, 1994) formed the basis for an assay for PDI-catalyzed disulfide interchange in TSP1. Specifically, PDI-catalyzed disulfide interchange in TSP1 was probed by measuring changing affinity of TSP1 for neutrophil cathepsin G. Disulfide interchange in platelet and fibroblast-derived TSP1 was also probed by measuring effects of PDI catalysis on epitopes for two anti-TSP1 monoclonal antibodies (mAbs). The results indicate that both platelet- and fibroblast-derived TSP1 are substrates for PDI-catalyzed disulfide isomerization.

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[1] Abbreviations: AAPF-pNA, Suc-Ala-Ala-Pro-Phe *p*-nitroanilide; BSA, bovine serum albumin; ECM, extracellular matrix; GSH, reduced glutathione; mAb, monoclonal antibody; PDI, protein disulfide isomerase; TSP1, thrombospondin purified from human platelets using buffers containing 2 mM Ca^{2+}.

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EXPERIMENTAL PROCEDURES

Reagents and Chemicals. HEPES, GSH, iodoacetamide, and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) were purchased from Sigma Chemical Co. (St. Louis, MO). Suc-Ala-Ala-Pro-Phe *p*-nitroanilide (AAPF-pNA) was obtained from Calbiochem (San Diego, CA). Dulbecco's minimal essential medium and fetal calf serum were purchased from ICN Pharmaceuticals Australasia Pty Ltd. (Sydney, Australia). All other chemicals were of reagent grade.

Proteins. TSP1 was purified from human platelet concentrates as described previously (Hogg et al., 1993b). Buffers containing 2 mM $CaCl_2$ were used throughout the chromatographic purification of TSP1. TSP1 concentration was determined using a $A_{1cm}^{1\%}$ at 280 nm of 10.9 and a M_r of 450 000. Human neutrophil cathepsin G was purified from white cells of patients with chronic myeloid leukemia and characterized as described previously (Hogg et al., 1993a; Saklatvala & Freedman, 1980). PDI was purified from human placenta according to the method of Lambert and Freedman (1983) for the isolation of bovine liver PDI. The only modification was that the homogenizing buffer contained the proteinase inhibitors 10 mM EDTA, 10 μM leupeptin, 1 μM trasylol, 2 mM phenylmethanesulfonyl fluoride, and 100 μM benzamidine. PDI was judged to be homogeneous by SDS−polyacrylamide gel electrophoresis and its concentration was determined by protein assay (BCA protein assay; Pierce, Rockford, IL).

Rabbit polyclonal antibodies against human placenta PDI were produced in NZ white rabbits. The murine anti-human TSP1 mAb HB8432 was produced in mouse ascites from the HB8432 hybridoma (American Type Cell Culture; Jaffe et al., 1983). The murine anti-human TSP1 mAb D4.6 was a gift from Dr. William Frazier (Dixit et al., 1986). The mAbs were purified from mouse ascites fluid by affinity chromatography on protein A−Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Biotin-linked goat anti-mouse IgG antibody and streptavidin−horseradish peroxidase-conjugated antibody were from Amersham Australia (Sydney, Australia), and swine anti-rabbit IgG horseradish peroxidase-conjugated antibody was from Dako (Carpinteria, CA). All proteins were aliquoted and stored at −80 °C until use.

Interaction of PDI with TSP1. TSP1 (100 nM) was incubated with GSH (0.7 mM) or purified placenta PDI (300 nM) or both GSH (0.7 mM) and PDI (300 nM) for 1 min at 37 °C, followed by quenching of the reactions with iodoacetamide (50 mM) for 5 min at 37 °C. The reaction buffer was 20 mM HEPES, 0.14 M NaCl, and 2 mM EDTA, pH 7.4. Samples were resolved on SDS−6% polyacrylamide gel electrophoresis according to Laemmli (1970). Proteins were transferred to poly(vinylidene difluoride) (PVDF) membranes, developed according to the manufacturer's instructions (DuPont), and visualized using chemiluminescence. Rabbit anti-PDI polyclonal antibody serum was used at a final dilution of 1:5000 and swine anti-rabbit HRP at 1:1000.

Probing PDI-Catalyzed Disulfide Interchange in TSP1 by Measuring Changing Affinity of TSP1 for Cathepsin G. PDI-catalyzed disulfide isomerization in TSP1 was measured by monitoring the change in the apparent dissociation constant, K_i, for inhibition of cathepsin G by TSP1. TSP1 (150 nM) was incubated in 50 mM HEPES, 0.125 M NaCl, and 1 mg/mL PEG 6000 buffer, pH 7.4, containing EDTA at 37 °C, and either GSH, PDI, or both GSH and PDI were added for discrete time periods. In some cases the EDTA in the incubation buffer was substituted for 2 mM $CaCl_2$. Refer to figure legends for individual reaction conditions. The apparent dissociation constant for binding of TSP1 to cathepsin G was quantitated by measuring the competitive inhibition of hydrolysis of the cathepsin G chromogenic substrate AAPF-pNA by TSP1 (Hogg et al., 1993b). Chromogenic substrate reactions were in 50 mM HEPES, 0.125 M NaCl, and 1 mg/mL PEG 6000 buffer, pH 7.4, containing 5 mM EDTA at 25 °C. TSP1 from the disulfide isomerization reactions was diluted into buffer containing 300 μM AAPF-pNA and inhibition assays were initiated by the addition of cathepsin G to a final concentration of 46 nM. The initial rates of hydrolysis of AAPF-pNA by cathepsin G were analyzed using the model for inhibition of cathepsin G by TSP1 described by Hogg et al. (1993b). The rates were linear over the 20 min of the assay, implying that the dilution of TSP1 in the inhibition assays was sufficient to quench further disulfide interchange in TSP1 over this time course.

Probing PDI-Catalyzed Disulfide Interchange in TSP1 by Measuring Changes in Epitopes for Anti-TSP1 mAbs. PDI-catalyzed disulfide isomerization was measured in purified platelet TSP1 adsorbed to plastic or TSP1 made endogenously by human foreskin fibroblasts and deposited in extracellular matrix (ECM).

TSP1 (100 μL of 2 μg/mL in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 0.02% NaN_3, pH 9.6) was adsorbed to wells of 96-well plates (Linbro/Titertek, Flow Laboratories) overnight at 4 °C in a humid environment. The weight of TSP1 adsorbed to plastic was quantitated by measuring binding of iodinated TSP1. TSP1 was iodinated as described previously (Owensby et al., 1991) to a specific activity of 3.4×10^7 cpm/μg. Cold TSP1 was spiked with 4 ng of iodinated TSP1/well. Wells were washed three times with adsorption buffer, separated, and counted in a Packard Crystal II γ counter. The weight of TSP1 bound per well was calculated from the total TSP1 added per well and the ratio of bound versus total iodinated TSP1.

Fibroblasts were extracted and cultured from human foreskin as described previously (Martin, 1973). Fibroblasts were seeded at 15 000 per well in 96-well tissue culture plates (Corning) and cultured for 4 days in Dulbecco's minimal essential medium, 10% (v/v) fetal calf serum, 2 mM *L*-glutamine, 10 units/mL penicillin G, and 10 μg/mL streptomycin sulfate. Fibroblasts were dislodged from the ECM using 0.02 M NH_4OH according to Gospodarowicz and Lui (1981). The ECM-coated wells were processed immediately as described below.

Wells containing plastic-adsorbed TSP1 or fibroblast-derived TSP1 in ECM were washed three times with 200 μL of 20 mM HEPES and 0.14 M NaCl buffer, pH 7.4, and incubated in 100 μL of the same buffer containing 2 mM Ca^{2+} in the absence or presence of GSH (0.7 mM), PDI (180 nM), or both GSH (0.7 mM) and PDI (180 nM) for 60 min at 37 °C. Wells were washed three times with 200 μL of 20 mM HEPES, 0.14 M NaCl, and 2 mM EDTA buffer, pH 7.4, and nonspecific plastic binding sites were blocked by incubating with 2% BSA in 200 μL of the same buffer for 90 min at 37 °C. Wells were washed three times with 200 μL of 20 mM HEPES, 0.14 M NaCl, 2 mM EDTA, and

0.05% (v/v) Tween 20 buffer, pH 7.4. All subsequent washing steps (200 μL) and incubation steps (100 μL) were performed using this buffer. The murine anti-human TSP1 mAbs, HB8432 and D4.6, were added to wells at a concentration of 5 μg/mL and incubated with orbital shaking for 2 h at room temperature. Wells were washed three times, and biotinylated goat anti-mouse IgG was added at 1:1000 dilution and incubated with orbital shaking for 90 min at room temperature. Wells were washed three times, and streptavidin−HRP-conjugated antibody was added at 1:500 dilution and incubated with orbital shaking for 45 min at room temperature. After the wells were washed four times, color was developed with 100 μL of 0.003% H_2O_2 and 1 mg/mL 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) in 50 mM citrate, pH 4.5, for 5 min with orbital shaking. Absorbances at 405 nm were measured using a Molecular Devices Thermomax kinetic microplate reader (Molecular Devices Corp). Results were corrected for control wells containing no primary antibody. No binding of murine anti-TSP1 mAbs to control wells not coated with TSP1 but blocked with 2% BSA was observed.

RESULTS

Interaction of PDI with TSP1. It was anticipated that catalysis of disulfide isomerization in TSP1 by PDI would result in the formation of transient mixed disulfides between the active-site thiols of PDI and the thiol groups of TSP1. To test this hypothesis, TSP1 was incubated briefly with GSH, PDI, or both GSH and PDI, and the reactions were quenched with iodoacetamide. The samples were separated on SDS−6% polyacrylamide gel electrophoresis and Western blotted using an anti-PDI polyclonal antibody. Covalent PDI−TSP1 complexes formed in the absence or presence of GSH (data not shown). PDI−TSP1 complexes dissociated under reducing conditions, confirming that complexes of PDI and TSP1 involved disulfide linkages. However, only a very small fraction of the total PDI in the reaction formed stable covalent complexes with TSP1, ≪1% in the presence or absence of GSH. This was not unexpected considering that PDI−substrate mixed disulfides are very unstable. These complexes are probably resolved by nucleophilic attack from a substrate sulfhydryl or the second active-site cysteine.

PDI-Catalyzed Disulfide Interchange in TSP1 Perturbs Inhibition of Cathepsin G by TSP1. Ca^{2+}-depleted TSP1 is a potent competitive inhibitor of cathepsin G with a site-binding inhibition constant in the range 0.9−4 nM, while Ca^{2+}-replete TSP1 has approximately 600-fold weaker affinity for cathepsin G (Hogg et al., 1993b). PDI-catalyzed disulfide interchange in TSP1 has been characterized by measuring the change in affinity of the isomerized TSP1 for cathepsin G.

The inhibition of cathepsin G amidolytic activity by TSP1 was measured as described in *Experimental Procedures* and is shown in Figure 1. The dependence on the magnitude of the steady-state rate of substrate hydrolysis on TSP1 concentration is consistent with hyperbolic tight-binding inhibition of cathepsin G activity by TSP1. This has been described in detail previously (Hogg et al., 1993b). The inhibition model assumes that six equivalent and independent sites on TSP1 bind cathepsin G and the Michaelis cathepsin G−substrate complex with a dissociation constant, K_i, and weakens the K_m for the chromogenic substrate by a factor

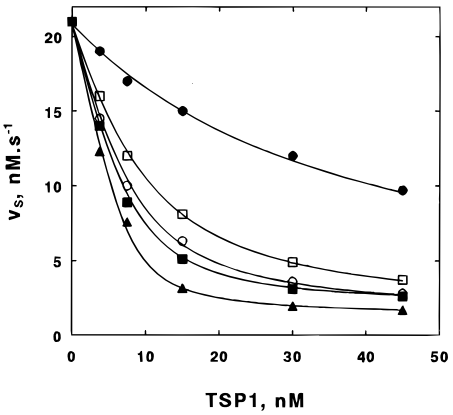

FIGURE 1: Effect of EDTA and either GSH, PDI, or both GSH and PDI on TSP1 inhibition of cathepsin G. TSP1 (150 nM) was incubated with either Ca^{2+} (2 mM) or EDTA (2 mM) in the absence or presence of either GSH (0.7 mM), PDI (180 nM), or both GSH (0.7 mM) and PDI (180 nM) at 37 °C for 60 min. The inhibition of cathepsin G amidolytic activity by the treated TSP1 was measured as described in Experimental Procedures. The data points are for Ca^{2+} alone (▲), EDTA alone (■), EDTA and PDI (○), EDTA and GSH (□), and EDTA, GSH, and PDI (●). The solid lines represent the best fit of the data to eq 1 of Hogg et al. (1993b) with K_i the unknown parameter (see Results). Values for K_i were 4 ± 2 nM (▲), 10 ± 2 nM (■), 14 ± 3 nM (○), 25 ± 4 nM (□), and 141 ± 15 nM (●).

α. The value for α under the experimental conditions described herein is 21 (Hogg et al., 1993b).

Disulfide interchange in TSP1 is very complex and the details of the process are not understood. The work of Speziale and Detwiler (1990) suggests that the TSP1 used in our studies is probably a heterogeneous population of molecules in which the free thiol in the TSP1 subunit resides on any one of 12 Cys residues in the Ca^{2+}-binding repeats and C-globular domain. Disulfide interchange in this TSP1 preparation will probably be manifested as a redistribution of the relative proportion of the different isomeric species in the population. The measured K_i, therefore, will be the average of K_i values for all the TSP1 isoforms. The average K_i has been designated as apparent K_i in these studies. The apparent K_i estimates were calculated from the steady-state rate versus TSP1 concentration values by fitting the data to eq 1 of Hogg et al. (1993b) using nonlinear regression analysis. Number of binding sites and α were fixed at 6 and 21, respectively, and K_i was the unknown parameter.

TSP1 was incubated with EDTA in the absence or presence of GSH, PDI, or both GSH and PDI at 37 °C for 60 min. Incubation of TSP1 for 60 min at 37 °C with EDTA alone increased the apparent K_i for inhibition of cathepsin G by TSP1 from 4 ± 2 to 10 ± 2 nM, incubation with PDI alone increased K_i to 14 ± 3 nM, incubation with EDTA and GSH increased K_i to 25 ± 4 nM, whereas incubation with EDTA, GSH, and PDI increased K_i to 141 ± 15 nM.

It is noteworthy that incubation of TSP1 with EDTA for 60 min weakened the apparent K_i 2.2-fold. This implies that removal of Ca^{2+} from TSP1 is in itself sufficient to promote disulfide interchange in TSP1. Incubation of TSP1 with EDTA for discrete times up to 24 h resulted in a half-maximal change in apparent K_i at approximately 2.5 h and a maximal K_i of 50 ± 12 nM (data not shown).

No effects of GSH and PDI on the gross structure of TSP1 were observable when untreated TSP1 was compared to GSH and PDI-treated TSP1 on SDS−5% polyacrylamide gel

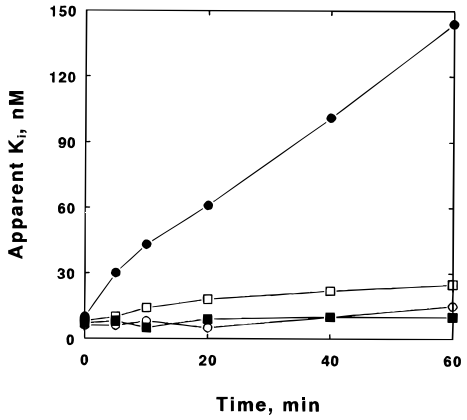

FIGURE 2: Analysis of PDI-catalyzed disulfide interchange in TSP1 as a function of time. PDI-catalyzed disulfide isomerization in TSP1 was measured by monitoring changes in the apparent dissociation constant for inhibition of cathepsin G by TSP1 as described in Experimental Procedures. TSP1 (150 nM) was incubated with EDTA (6.7 mM) in the absence or presence of either GSH (0.7 mM), PDI (180 nM), or both GSH (0.7 mM) and PDI (180 nM) at 37 °C for discrete times up to 60 min. Incubation of TSP1 with either EDTA alone (○) or EDTA and PDI (■) weakened the apparent K_i for inhibition of cathepsin G by TSP1 2−3-fold at 60 min. Incubation of TSP1 with EDTA and GSH (□) weakened the apparent K_i 6-fold at 60 min. In contrast, incubation of TSP1 with EDTA, GSH, and PDI (●) weakened the apparent K_i 35-fold at 60 min.

Table 1: Effect of Ca^{2+} on PDI-Catalyzed Disulfide Interchange in TSP1[a]

reactants	apparent K_i[b] (nM)	fold increase in K_i
	+2 mM EDTA	
TSP1	10 ± 2	
TSP1 + GSH	25 ± 4	2.5
TSP1 + PDI	14 ± 3	1.4
TSP1 + GSH + PDI	141 ± 15	14.1
	+2 mM Ca^{2+}	
TSP1	4 ± 2	
TSP1 + GSH	7 ± 3	1.8
TSP1 + PDI	5 ± 1	1.3
TSP1 + GSH + PDI	38 ± 6	9.5

[a] TSP1 was incubated with either 2 mM EDTA or 2 mM Ca^{2+} alone or with the addition of either 0.7 mM GSH, 180 nM PDI, or 0.7 mM GSH and 180 nM PDI for 60 min at 37 °C. [b] The apparent inhibition constant for interaction of the treated TSP1 with cathepsin G was measured using buffer containing 5 mM EDTA as described in *Experimental Procedures*.

electrophoresis run under nonreducing conditions (data not shown). This indicates that PDI is not catalyzing reduction of the disulfide bonds that link the three subunits but that it is catalyzing rearrangement of disulfide bonds within the subunits.

Analysis of PDI-Catalyzed Disulfide Interchange in TSP1 as a Function of Time and of PDI and GSH Concentrations. The time dependence for catalysis of disulfide interchange in TSP1 is shown in Figure 2. The apparent K_i increased linearly up to 60 min in the presence of PDI and GSH. EDTA, GSH, or PDI alone had only small effects on K_i at 60 min, as also shown in Figure 1.

As expected for an enzyme-catalyzed reaction, the rate of PDI-catalyzed disulfide isomerization in TSP1 increased linearly with PDI concentration (Figure 3A). The K_m for GSH in PDI-catalyzed disulfide isomerization in TSP1 was determined from experiments shown in Figure 3B. Fit of

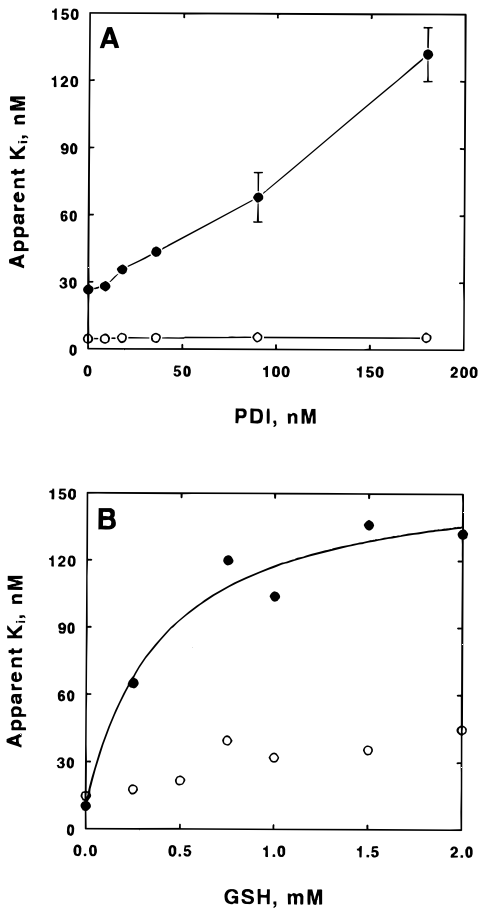

FIGURE 3: Analysis of PDI-catalyzed disulfide interchange in TSP1 as a function of PDI and GSH concentrations. PDI-catalyzed disulfide isomerization in TSP1 was measured by monitoring changes in the apparent dissociation constant for inhibition of cathepsin G by TSP1 as described in Experimental Procedures. (A) PDI-catalyzed disulfide interchange in TSP1 as a function of PDI concentration. TSP1 (150 nM) was incubated with EDTA (6.7 mM) and PDI up to 180 nM in the absence (○) or presence (●) of GSH (0.7 mM) for 60 min at 37 °C. The data points and error bars represent the mean and range, respectively, of duplicate experiments. (B) PDI-catalyzed disulfide interchange in TSP1 as a function of GSH concentration. TSP1 (150 nM) was incubated with EDTA (6.7 mM) and GSH up to 2 mM in the absence (○) or presence (●) of PDI (180 nM) for 60 min at 37 °C. The solid line represents the best fit of the data to the Michaelis−Menten equation. The concentration of GSH required for half-maximal PDI activity (K_m) is 0.4 ± 0.2 mM and the apparent K_i at saturating GSH is 150 ± 16 nM.

the data to the Michaelis−Menten equation estimated a K_m for GSH of 0.4 ± 0.2 mM and an apparent K_i at saturating GSH of 150 ± 16 nM.

Effect of Ca^{2+} on PDI-Catalyzed Disulfide Interchange in TSP1. TSP1 binds up to 36 mol of Ca^{2+}/mol of TSP1 trimer (Misenheimer & Mosher, 1995), which has been shown to markedly influence the structure of TSP1 observed using electron microscopy (Lawler et al., 1985) and its susceptibility to proteolysis [for example, Misenheimer and Mosher (1995)]. It is likely that Ca^{2+} is bound to TSP1 *in vivo*, so it was of interest to determine whether Ca^{2+}-replete TSP1 is also a substrate for PDI.

PDI-catalyzed disulfide interchange in Ca^{2+}-depleted versus Ca^{2+}-replete TSP1 is shown in Table 1. Although TSP1 incubated with PDI and/or GSH in the presence of 2 mM Ca^{2+} inhibited cathepsin G with 2−3-fold higher affinity than TSP1 incubated with PDI and/or GSH in the absence

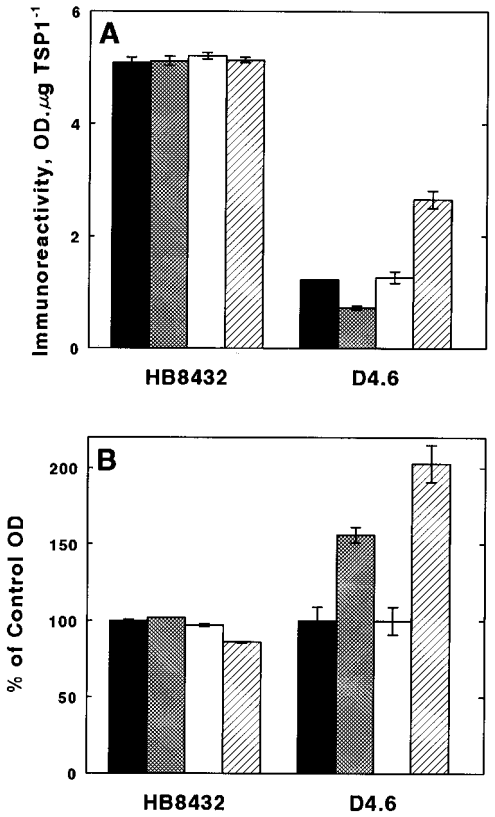

FIGURE 4: Probing PDI-catalyzed disulfide interchange in TSP1 by measuring changes in epitopes for anti-TSP1 mAbs. The source of TSP1 in these experiments was either purified platelet TSP1 adsorbed to plastic wells (A) or TSP1 produced endogenously by human foreskin fibroblasts and deposited into ECM (B). Binding of two different anti-TSP1 mAbs, HB8432 and D4.6, to TSP1 was measured by enzyme-linked immunosorbent assay as described in Experimental Procedures. Bound antibody was measured using biotin-linked anti-mouse IgG antibody and streptavidin−peroxidase. The platelet or fibroblast ECM TSP1 was incubated with either 2 mM Ca^{2+} (black bars), Ca^{2+} and GSH (gray bars), Ca^{2+} and PDI (open bars), or Ca^{2+}, GSH, and PDI (hatched bars) for 60 min at 37 °C. The GSH and PDI concentrations were 0.7 mM and 180 nM, respectively. The data in panel A are expressed as the OD value per microgram of TSP1 adsorbed to plastic (see *Results*). The OD values in panel B are expressed as the percentage of the OD value of wells not incubated with either GSH or PDI (black bars). The error bars represent the mean and range of duplicate experiments.

of Ca^{2+}, the relative changes in K_i for reactions containing either GSH, PDI, or both GSH and PDI are approximately equivalent. This indicates that PDI catalyzes disulfide interchange in Ca^{2+}-replete TSP1, which is the most likely state of TSP1 released from platelets and perhaps from cells.

Probing PDI-Catalyzed Disulfide Interchange in TSP1 by Measuring Changes in Epitopes for Anti-TSP1 mAbs. PDI-catalyzed disulfide isomerization in TSP1 was probed by measuring changes in epitopes for anti-TSP1 mAbs. This assay made it possible to measure PDI-catalyzed changes in the structure of TSP1 made by human foreskin fibroblasts and deposited in ECM. Most TSP1 *in vivo* is bound in ECM, and therefore, it was important to determine whether TSP1 is a substrate for PDI in this complex environment.

Purified platelet TSP1 adsorbed to enzyme-linked immunosorbent assay (ELISA) plate wells was used for comparison with fibroblast ECM TSP1 (Figure 4). The platelet TSP1 or fibroblast ECM TSP1 was incubated with Ca^{2+} in the absence or presence of either GSH, PDI, or both GSH and PDI for 60 min at 37 °C. Binding of the anti-human TSP1 murine mAbs, HB8432 and D4.6, to TSP1 was measured by enzyme-linked immunosorbent assay. The weight of TSP1 adsorbed to the plastic wells was measured using radiolabeled TSP1 as described in *Experimental Procedures*. Of the 500 ng of TSP1 added/well, 450 ± 18 ng adsorbed to the plastic. The data for binding of the mAbs to plastic-bound TSP1 is expressed as the OD value per microgram of TSP1 adsorbed. Because antibody binding was measured under identical conditions, this value is a quantitative assessment of the immunoreactivity of the plastic-bound TSP1.

Incubation of platelet TSP1 or fibroblast ECM TSP1 with either GSH, PDI, or both GSH and PDI in the presence of Ca^{2+} had no significant effect on binding of HB8432. In contrast, incubation of platelet TSP1 with GSH inhibited binding of D4.6 by 40%, and incubation with GSH and PDI enhanced binding 2.2-fold. Binding of D4.6 to fibroblast ECM TSP1 incubated with GSH was enhanced 1.6-fold, while incubation with GSH and PDI caused a 2-fold enhancement in binding of D4.6. Incubation of fibroblast ECM TSP1 with PDI alone did not perturb binding of D4.6. These results indicate that TSP1 produced endogenously by fibroblasts and deposited into ECM is a substrate for PDI-catalyzed disulfide isomerization.

DISCUSSION

TSP1 is a multidomain glycoprotein that functions in diverse biological processes (Bornstein, 1995). A large number of properties have been attributed to TSP1 and in some cases the results are conflicting. For example, TSP1 can be both adhesive and nonadhesive, can stimulate and inhibit angiogenesis, and can inhibit and enhance proteolytic enzyme activity. These diverse functions of TSP1 cannot be explained by the related TSPs (Bornstein, 1995). A possible explanation for at least some of these conflicting results could be the existence of different structural forms of TSP1. The first hard evidence for this proposal came from the work of Speziale and Detwiler (1990). They found that a single free thiol exists on each of the three subunits of TSP1 and that this free thiol can reside on any one of 12 different cysteines in the carboxy-terminal Ca^{2+}-binding repeats and globular domain of TSP1. This finding implies that there is a complex intramolecular disulfide interchange process operating in TSP1 and suggested that disulfide isomerization in TSP1 might be a means by which certain functions of TSP1 are regulated.

This proposal is supported by the work of Sun et al. (1992), who showed that the cell adhesive activity of TSP1 depended on the Ca^{2+} concentration used in the purification of TSP1. They demonstrated that the Arg-Gly-Asp sequence in the last Ca^{2+}-binding repeat was variably accessible to integrins on cell surfaces and they proposed that thiol−disulfide isomerization in TSP1 regulates TSP1−integrin interactions. Sun et al. (1992) showed that the free thiol in the TSP1 subunit was at position Cys974 when TSP1 was purified in buffers containing 0.1 mM Ca^{2+}, while the work of Speziale and Detwiler (1990) suggests that TSP1 purified in buffers containing 2 mM Ca^{2+} is probably a heterogeneous population of molecules in which the free thiol resides on one of any 12 Cys. Another function of TSP1 that is most likely regulated by intramolecular disulfide interchange is its enzyme inhibitory activity. TSP1 is a tight-binding competi-

tive inhibitor of cathepsin G and neutrophil elastase (Hogg et al., 1993a,b), and the potency of inhibition of the neutrophil enzymes markedly depended on whether the TSP1 was purified in buffers containing either 0.1 or 2 mM Ca^{2+} (Hogg, 1994). Reactive centers for the neutrophil enzymes were localized to the TSP1 Ca^{2+}-binding repeats (Hogg et al., 1994), which is the region of TSP1 that undergoes intramolecular disulfide interchange reactions. We have also found that platelet-derived growth factor (PDGF) binds tightly to TSP1 and that the affinity of this interaction varies ≥90-fold depending on the Ca^{2+} concentration used in the purification of TSP1.[2] Finally, Misenheimer and Mosher (1995) showed that TSP1 purified in buffers containing 20 μM Ca^{2+} bound 9 mol of Ca^{2+}/mol of TSP1, while TSP1 purified in buffers containing 300 μM Ca^{2+} bound 15 mol of Ca^{2+}/mol of TSP1. This probably reflects changes in the structure of the Ca^{2+}-binding loops induced by thiol−disulfide interchange.

Disulfide interchange in proteins is usually very specific and quite slow unless it is catalyzed by a disulfide isomerase. A candidate disulfide isomerase that may regulate TSP1 structure *in vivo* is PDI, which has recently been identified on the platelet surface. PDI has traditionally been studied as an endoplasmic reticulum resident protein that assists in the correct folding of nascent proteins. Chen et al. (1995) and Essex et al. (1995) have shown that PDI is bound to the platelet surface and that mixtures of unactivated platelets and GSH can catalyze renaturation of scrambled ribonuclease.

Considering the potentially important role for PDI in regulating the biological functions of TSP1, we have investigated PDI-catalyzed disulfide interchange in TSP1 *in vitro*. The reactive centers of PDI contain two easily reduced surface-exposed disulfide bonds that function by catalyzing thiol−disulfide interchanges in protein substrates (Freedman et al., 1994). In view of this active-site chemistry, the reactive thiol groups of PDI were expected to form transient mixed disulfides with the thiol groups of TSP1. Complexes of this type between PDI and TSP1 were observed. To quantitate PDI-catalyzed disulfide isomerization in TSP1, changes in the apparent K_i for inhibition of cathepsin G by TSP1 were measured. Briefly, the assay entailed incubating TSP1 with GSH, PDI, or both GSH and PDI and then immediately assaying for TSP1 inhibition of cathepsin G in competitive chromogenic substrate reactions (Hogg et al., 1993b). GSH was used to activate PDI in the assays because it is probably the physiological cofactor for PDI (Darby et al., 1994). Catalysis of mixed disulfide formation by PDI shows a striking specificity for GSH/GSSG (Hwang et al., 1992). The results showed that PDI can catalyze disulfide interchange in Ca^{2+}-depleted or Ca^{2+}-replete TSP1. The apparent K_i for inhibition of TSP1 by cathepsin G increased ≥10−14-fold. The K_m for GSH was 0.4 ± 0.2 mM, which is comparable to the K_m for GSH in PDI-catalyzed renaturation of scrambled ribonuclease, 0.62 mM (Lambert & Freedman, 1983).

Although PDI catalyzes disulfide interchange in purified platelet TSP1 in solution, most TSP1 *in vivo* is bound in ECM and there is evidence that the structure of TSP1 in ECM is different from the structure of TSP1 in solution (Dixit et al., 1986). PDI-catalyzed disulfide isomerization in fibroblast ECM TSP1 was probed by measuring changes in epitopes for anti-TSP1 mAbs, D4.6 and HB8432. Antibody D4.6 was developed and characterized by Dixit et al. (1986). They showed that D4.6 binds to the Ca^{2+}-binding repeats of TSP1, a region of the molecule where thiol−disulfide interchange occurs. Therefore, it was anticipated that the epitope for D4.6 would be perturbed by disulfide isomerization in TSP1. Antibody HB8432 was developed by Jaffe et al. (1983). The epitope for HB8432 is in the vicinity of the properdin or type 1 repeats of TSP1 which are toward the amino terminus (D. F. Mosher, personal communication), a region of the molecule not expected to be markedly influenced by disulfide interchange in the carboxy-terminal part of the protein. In accordance with the location of the epitopes for these mAbs, incubation of platelet or fibroblast ECM TSP1 with PDI and GSH resulted in a 2-fold enhancement in binding of mAb D4.6 but did not significantly affect binding of mAb HB8432.

Hogg (1994) suggested that connective tissue cells at inflammatory sites may regulate neutrophil-mediated degradation of their extracellular matrix by specifically expressing and depositing TSP1 in their microenvironment. Considering the results presented herein, it is tempting to hypothesize that platelet-derived PDI may function in inflamed tissue to temporally and spatially regulate the activities of cathepsin G by controlling the inhibitory potency of TSP1. Quantification of the effects of PDI on the enzyme inhibitory properties of matrix-bound TSP1 are required before one can speculate as to whether PDI is likely to be a negative or positive regulator of cathepsin G activity. Interestingly, PDI has also been shown to be released from activated neutrophils (Bassuk et al., 1990), which is another potentially important source of PDI at sites of inflammation.

In summary, PDI can catalyze disulfide interchange in platelet and fibroblast TSP1 which alters binding of neutrophil cathepsin G and an anti-TSP1 mAb to TSP1. The presence of PDI on the surface of platelets and perhaps other cells suggests that cell-surface PDI may function to regulate the disulfide-bonded structure and some biological functions of TSP1.

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REFERENCES

Bassuk, J. A., Capodici, C., & Berg, R. A. (1990) *J. Cell. Physiol. 144*, 280−286.
Bornstein, P. (1995) *J. Cell Biol. 130*, 503−506.
Chen, K., Detwiler, T. C., & Essex, D. W. (1995) *Br. J. Haematol. 90*, 425−431.
Darby, N. J., Freedman, R. B., & Creighton, T. E. (1994) *Biochemistry 33*, 7937−7947.
Dixit, V. M., Galvin, N. J., O'Rourke, K. M., & Frazier, W. A. (1986) *J. Biol. Chem. 261*, 1962−1968.
Essex, D. W., Chen, K., & Swiatkowska, M. (1995) *Blood 86*, 2163−2173.
Freedman, R. B., Hirst, T. R., & Tuite, M. F. (1994) *Trends Biochem. Sci. 19*, 331−336.
Gospodarowicz, D., & Lui, G. M. (1981) *J. Cell. Physiol. 109*, 69−81.
Hogg, P. J. (1994) *Thromb. Haemostasis 72*, 787−792.

[2] P. J. Hogg, B. M. Jiménez, P. Stathakis, and C. N. Chesterman, submitted for publication.

Hogg, P. J., Owensby, D. A., Mosher, D. F., Misenheimer, T. M., & Chesterman, C. N. (1993a) *J. Biol. Chem. 268*, 7139−7146.
Hogg, P. J., Owensby, D. A., & Chesterman, C. N. (1993b) *J. Biol. Chem. 268*, 21811−21818.
Hogg, P. J., Jiménez, B. M., & Chesterman, C. N. (1994) *Biochemistry 33*, 6531−6537.
Hwang, C., Sinskey, A. J., & Lodish, H. F. (1992) *Science 257*, 1496−1502.
Jaffe, E. A., Ruggiero, J. T., Leung, L. L., Doyle, M. J., McKeown-Longo, P. J., & Mosher, D. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 998−1002.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lambert, N., & Freedman, R. B. (1983) *Biochem. J. 213*, 225−234.
Lawler, J., & Hynes, R. O. (1986) *J. Cell Biol. 103*, 1635−1648.
Lawler, J., Derick, L. H., Connolly, J. E., Chen, J.-H., & Chao, F. C. (1985) *J. Biol. Chem. 260*, 3762−3772.
Martin, G. M. (1973) in *Tissue Culture: Methods & Applications* (Kruse, P. F., Jr., & Patterson, M. K., Jr., Eds.) pp 39−43, Academic Press, New York.
Misenheimer, T. M., & Mosher, D. F. (1995) *J. Biol. Chem. 270*, 1729−1733.
Owensby, D. A., Morton, P. A., Wun, T.-C., & Schwartz, A. L. (1991) *J. Biol. Chem. 266*, 4334−4340.
Saklatvala, J., & Barrett, A. J. (1980) *Biochim. Biophys. Acta 615*, 167−177.
Speziale, M. V., & Detwiler, T. C. (1990) *J. Biol. Chem. 265*, 17859−17867.
Sun, X., Skorstengaard, K., & Mosher, D. F. (1992) *J. Cell Biol. 118*, 693−701.
Yu, X. C., Wang, C. C., & Tsou, C. L. (1994) *Biochim. Biophys. Acta 1207*, 109−113.

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