Internalization but not Binding of Thrombospondin-1 to Low Density Lipoprotein Receptor-Related Protein-1 Requires Heparan Sulfate Proteoglycans

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Abstract   The amino-terminal domain of the extracellular matrix (ECM) protein thrombospondin-1 (TSP-1) mediates binding to cell surface heparan sulfate proteoglycans (HSPG) as well as binding to the endocytic receptor, low density lipoprotein-related protein (LRP-1). We previously found that recombinant TSP-1 containing the amino-terminal residues 1–214, retained both of these interactions (Mikhailenko et al. [1997]: J Biol Chem 272:6784–6791). Here, we examined the activity of a recombinant protein containing amino-terminal residues 1–90 of TSP-1 and found that this domain did not retain high-affinity heparin-binding. The loss of heparin-binding correlated with decreased binding to the fibroblast cell surface. However, both ligand blotting and solid phase binding studies indicate that this truncated fragment of TSP-1 retained high-affinity binding to LRP-1. Consistent with this, it also retained the ability to block the uptake and degradation of ^{125}I-TSP-1. However, TSP-1$_{1–90}$ itself was poorly endocytosed and this truncated amino-terminal domain was considerably more effective than the full-length heparin-binding domain (HBD) of TSP-1 in blocking the catabolism of endogenously expressed TSP-1. These results indicate that TSP-1 binding to LRP-1 does not require prior or concomitant interaction with cell surface HSPG but suggest subsequent endocytosis requires high-affinity heparin-binding. J. Cell. Biochem. 91: 766–776, 2004.   © 2004 Wiley-Liss, Inc.

Key words: thrombospondin; endocytosis; heparin-binding domain; heparan sulfate proteoglycan; low density lipoprotein-related protein

Thrombospondin-1 (TSP-1) is a high molecular weight (450 kDa) glycoprotein that is composed of three identical disulfide-linked polypeptide chains [Coligan and Slayter, 1984; Lawler et al., 1985; Lawler and Hynes, 1986; Adams, 1997]. It is stored in platelet α-granules, expressed by a variety of cell types and a component of the extracellular matrix (ECM) [Asch et al., 1986; Bornstein, 1992]. Like most ECM proteins, TSP-1 is modular and contains amino-terminal and carboxyl-terminal globular domains, a region similar to the procollagen domain, and repeat sequence motifs designated type 1, type 2, and type 3 repeats. By interacting with other extracellular proteins [Silverstein et al., 1985, 1990; Mosher et al., 1992], cell surface receptors [Asch et al., 1993;

Yabkowitz et al., 1993; DeFreitas et al., 1995; Gao et al., 1996; Lawler et al., 1998], and growth factors [Schultz-Cherry et al., 1994; Crawford et al., 1998], TSP-1 regulates the adhesive, motile, and proliferative behavior of cells [Mosher, 1990; Murphy-Ullrich et al., 1993; Adams, 1995; Patel et al., 1997; Ferrari do Outerio-Bernstein et al., 2002; Orr et al., 2003].

TSP-1 contains a high-affinity binding domain for heparin [Dixit et al., 1984], heparan sulfate proteoglycans (HSPG) [Roberts, 1988], and some sulfate glycolipids [Roberts et al., 1985]. This binding site has been localized to the amino-terminal globular domain of TSP-1 which can be released as a proteolytic fragment by chymotrypsin [Raugi et al., 1984], thermolysin [Dixit et al., 1984], or plasmin [Lawler et al., 1985]. Recombinantly produced amino-terminal heparin-binding domain (HBD) of TSP-1 retains high-affinity heparin-binding [Yabkowitz et al., 1989; Mikhailenko et al., 1997]. At least two regions critical for high-affinity interaction with heparin have been defined and they consist of the amino acid sequences ARKGSGRR (residues 22–29) and MKKTRG (residues 79–84) [Lawler et al., 1992; Merle et al., 1997]. Interestingly, the focal adhesion disassembly activity of TSP-1 has also been localized to residues 17–35, encompassing one of the HBDs [Murphy-Ullrich et al., 1993] and cell surface calreticulin has been identified as a binding partner [Goicoechea et al., 2000].

We previously generated and characterized a recombinant protein consisting of the HBD domain of TSP-1 fused to glutathione S-transferase (GST) and determined that residues 1–214 of TSP-1 is sufficient to mediate the binding and internalization of TSP-1 by the endocytic receptor, low density lipoprotein-related protein-1 (LRP-1) [Mikhailenko et al., 1997]. The ability of heparin and heparitinase treatment to block LRP-1 mediated uptake and degradation of TSP-1 [Godyna et al., 1995; Mikhailenko et al., 1995, 1997] suggests that cell surface HSPG binding may be a prerequisite for TSP-1 internalization. In addition to mediating the degradation of TSP-1, LRP also appears to be a critical player in the signaling events necessary for focal adhesion disassembly via the formation of a tri-molecular complex with calreticulin and TSP-1 [Orr et al., 2003]. The mechanism by which this complex signals focal adhesion dis-

assembly is currently unknown and will require further dissection of the molecular interactions between these molecules.

In the current study, we performed further analysis to determine the relationship between HSPG binding and TSP-1 internalization by LRP-1. We found that the primary LRP-1 binding site resides within amino-terminal residues 1–90 of TSP-1. This domain did not retain high-affinity binding to heparin and was also unable to block TSP-1 binding to the cell surface. However, this truncated amino-terminal domain was able to block cell-mediated TSP-1 internalization and was a substantially more effective inhibitor of the catabolism of endogenously expressed TSP-1. These results provide additional insight into the cell surface binding and catabolism of TSP-1.

MATERIALS AND METHODS

Proteins

TSP-1 was purified from human platelets by adsorption to barium citrate followed by heparin-agarose chromatography according to Alexander and Detwiler [1984]. Receptor-associated protein (RAP) was prepared as described previously [Williams et al., 1992]. LRP was kindly provided by Dr. D. Strickland (American Red Cross, Rockville, MD). Amino-terminal domain of murine TSP-1 (amino acid residues 1–90 and 1–214) were expressed in bacteria as fusion proteins with GST using pGEX2T vector (Amersham Pharmacia Biotec, Piscataway, NJ). Fusion proteins were purified by glutathionine (G)-sepharose (Sigma Chemicals Co., St. Louis, MO) chromatography. After dialyzed in TBS (50 mM Tris, 150 mM NaCl, pH 7.4), these fragments were purified using a Detoxin gel column (Pierce Chemical Co., Rockford, IL) to remove endotoxin contaminants. TSP-1, GST–TSP$_{1-90}$, GST–TSP$_{1-214}$, and GST were labeled with [^{125}I] Iodine (Amersham Pharmacia Biotec) using Iodogen (Pierce Chemical Co.) to a specific activity of 2–10 μCi/μg (∼0.28 MBq/μg).

Antibodies

Rabbit anti-TSP-1 serum (rb98) was prepared by immunizing and boosting rabbits with polyacrylamide gel slices containing purified human platelet TSP-1. Antibody was purified by chromatography on protein G-sepharose. The

specificity of the antibody was confirmed by immunoblot analysis.

Cell Culture

A previously described normal mouse embryo fibroblast cell line (MEF) was used for all studies [Mikhailenko et al., 1997]. The cells were cultured in complete medium containing 10% fetal bovine serum (FBS) (Intergen, Purchase, NY), 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, and 25 µg/ml amphotericin B in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Gaithersburg, MD). MEF were made quiescent by changing to medium containing 0.5% FBS, 10 µmol/L insulin, and 5 µg/ml transferrin (Sigma Chemical Co.) for 48–72 h.

Affinity Co-Electrophoresis (ACE)

Porcine intestinal heparin (Sigma) was derivatized with tyramine, iodinated using Iodogen (Pierce Chemical Co.), and chromatographed on sephadex G-100 to produce a low molecular weight fraction ($\frac{3}{4}$ 6,000 Dalton) as described previously [Alexander and Detwiler, 1984]. Purified GST–TSP$_{1-90}$ and GST–TSP$_{1-214}$ were tested for binding to low molecular heparin using ACE. Protein concentrations were measured by binding to amidoblack, with crystalline BSA as a standard [Schaffner and Weissmann, 1973]. In these studies, the labeled heparin was migrated through the protein ligand embedded at various concentrations in a horizontal non-sieving agarose gel at physiological pH and ionic strength. The shifts in electrophoretic mobility of the heparin are used to determine the dissociation constant. ACE was carried out exactly as previously described [Isaacs et al., 1988; Lee and Lander, 1991; Lim et al., 1991], with 50 mM MOPS, pH 7.0, 125 mM sodium acetate as the gel preparation, and running buffer. ACE gels were dried and exposed to storage phosphor screens (Molecular Dynamics, Sunnyvale, CA). Measurements of heparin mobility were made and converted to retardation coefficients (the mobility shift in a given lane divided by the mobility of the heparin in a protein-free lane), as previously described [Isaacs et al., 1988]. Data were then fit, using a non-linear least squares approach (Kaleidagraph, Synergy Software, Reading, PA), to the equation $R = R^*/[1 + (K_d/[P_{tot}]^2)]$, where R = retardation coefficient, $[P_{tot}]$ = protein concentration in a given lane of an ACE gel. The variables that were fit simultaneously were K_d, the dissociation constant, and R^*, the maximum value of R.

Cell Binding, Internalization, and Degradation

The assays were conducted as described previously [Kounnas et al., 1993]. MEF were seeded into 12-well culture dishes (Corning, New York, NY) at 1×10^5 cells/well and allowed to grow for 24 h. Prior to addition of radioactive ligand, cells were washed with assay medium containing 10 mM HEPES, 1% Nutridoma serum substitute, and 1% BSA in DMEM medium and incubated in the same medium for 1 h at 37°C. For cell binding assays, the medium was replaced with ice-cold assay medium and the cells were incubated at 4°C for 30 min. ^{125}I-TSP-1 (5 nM) was added to the cell layers in the presence of increasing concentrations of GST, GST–TSP$_{1-214}$, and GST–TSP$_{1-90}$ and allowed to bind for 3 h at 4°C. Following binding, the cells were washed with ice-cold PBS, and solubilized in 0.1% NaOH for γ counting. For internalization and degradation assays, the cells were pretreated with 0.1 mM chloroquine (Sigma) for 0.5 h at 37°C, 5% CO$_2$ to inhibit the lysosomal protease activity. The preincubation medium was removed, and medium containing ^{125}I TSP-1 (5 nM) plus competitors was added and incubated for 5 h at 37°C, 5% CO$_2$. The conditioned culture medium was treated with trichloroacetic acid (final concentration 10%) and centrifuged at 10,000g for 10 min. The amount of radioactivity present in the supernatant was taken to represent the amount of degraded TSP-1. The cell layers were washed with cold PBS and then treated with 5 µg/ml trypsin, 0.5 mg/ml proteinase K, and 5 mM EDTA (Sigma Chemical Co.) in PBS for 2–4 min at 4°C. The released cells were pelleted by centrifugation at 1,300g for 15 min, and the amount of radioactivity in the pellet was measured.

Solid Phase Binding Assays

Enzyme-linked immunosorbent assays (ELISA) were performed as described [Ashcom et al., 1990]. Briefly, microtiter wells were coated with LRP or BSA (3 µg/ml) in TBS with 5 mM CaCl$_2$ (pH 8.0) for 4 h at 37°C, then non-specific sites were blocked with 3% BSA in the same buffer. The wells were then incubated with increasing concentrations of

TSP-1, GST, GST–TSP$_{1-214}$, or GST–TSP$_{1-90}$ in the absence or presence of RAP (4 µM) in the same buffer at 4 °C overnight. Polyclonal anti-TSP-1 antibody and goat anti-rabbit IgG conjugated to horseradish peroxidase were used to detect the bound proteins. The ELISA data were fit by non-linear regression analysis to the equation as described [Kounnas et al., 1992].

Ligand Blotting

Ligand blotting was carried out according to methods described previously [Winkles et al., 1993]. Three micrograms of purified LRP was separated on 4−20% Tris-glycine gels (Novex, San Diego, CA) under non-reducing conditions and transferred to nitrocellulose membrane. The membrane was cut into stripes. After blocking with 3% non-fat milk, the filters were incubated with ^{125}I GST–TSP$_{1-214}$ (1 nM), ^{125}I GST–TSP$_{1-90}$ (1 nM), ^{125}I TSP-1 (1 nM), and ^{125}I GST (1 nM) in the absence or presence of RAP (1 µM) at 4°C overnight. After washing, the filters were dried and exposed to autoradiographic film. In the case of binding to unlabeled TSP-1 and TSP-1 fragments, the filters were subsequently incubated sequentially with polyclonal anti-TSP antibody and goat anti-rabbit IgG horseradish peroxidase conjugate, then ECL^{TM} Western Blotting Detection reagents (Amersham Life Sci, Piscataway, NJ) were used to visualize the bound proteins.

Western Blot Analysis

MEF were cultured and made quiescent for 72 h. Cells were then treated with 1 µM GST, RAP, GST–TSP$_{1-90}$, and GST–TSP$_{1-214}$ in serum free media for 30 h. Conditioned media were collected and protein concentration was measured using Bio-Rad protein assay (Bio-Rad laboratory, Hercules, CA). Equal amount of protein in conditioned media was concentrated using StrataClean^{TM} resign (Stratagene, La Jolla, CA) and subjected to 4−20% Tris-glycine gel under reducing conditions. After electrophoretic transfer to nitrocellulose membranes and blocking, the membranes were incubated with polycolonal anti-TSP-1 antibody for 2 h at room temperature. After intensive washing, secondary antibody was used for the detection of immunoreactive bands with the enhanced chemiluminescence detection system (Pierce Chemical Co.). Equal loading and transfer of protein samples were assayed by staining the blots with Ponceau S.

RNA Preparation and Northern Blot Hybridization

Cells seeded as above were incubated with GST–TSP$_{1-214}$, GST–TSP$_{1-90}$, and RAP for 4 h at 37°C, 5% CO_2, then total cellular RNA was isolated using RNeasy Mini Kit (QIAGEN, Valencia, CA). RNA was separated in 1% agarose-formaldehyde gels and transferred to Zetabind nylon membranes (Cuno, Inc., Meriden, CT). The immobilized RNA was hybridized overnight with $3-5 \times 10^6$ cpm/ml of ^{32}P-labeled cDNA probe prepared by random primer synthesis (Boehringer–Mannheim, Germany). A 1.1 kb TSP-1 cDNA was used as a probe. An 800 bp human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA was used as a control probe. Hybridization and membrane wash conditions have been described previously [Williams et al., 1992].

Statistical Analysis

Data were expressed as the mean $\pm$ SD. The significance of difference between the values was estimated by Student's t-test. P values of <0.05 were considered significant difference.

RESULTS

GST–TSP$_{1-90}$ and GST-TS$_{1-214}$ Specifically Bind to Purified LRP-1 With Similar Affinity

We previously localized the domain responsible for cell-mediated endocytosis to the first 214 amino acids of TSP-1 [Mikhailenko et al., 1997]. In the current study a further truncated protein, containing the identical GST domain fused to the first 90 amino acids of TSP-1, was generated to define the relationship between cell surface HSPG binding and LRP-1-mediated uptake and degradation. We first examined the ability of truncated GST–TSP$_{1-90}$ to interact with LRP-1. As shown in Figure 1A, ^{125}I-labeled TSP-1, GST–TSP$_{1-214}$, and GST–TSP$_{1-90}$ were all able to bind to immobilized LRP-1. The specificity of binding was demonstrated by the ability of RAP to abrogate the binding to LRP-1 (Fig. 1A, lanes 5–8). Similar binding results were obtained when unlabeled TSP-1 and recombinant TSP-1 fragments were visualized by immunoblotting after binding to immobilized LRP-1 (results not shown). An ELISA assay was
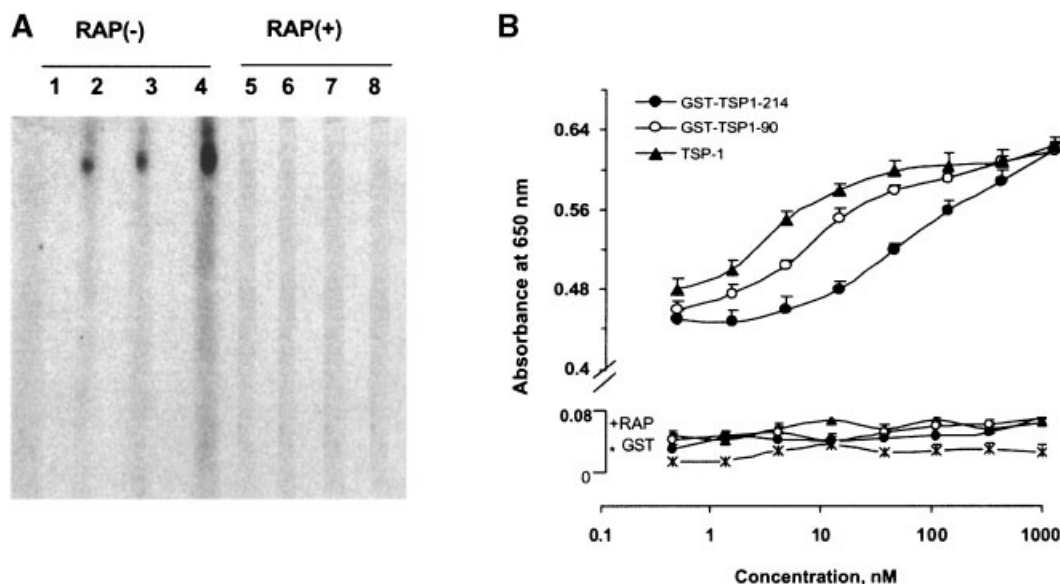
Fig. 1. Both GST–TSP$_{1-214}$ and GST–TSP$_{1-90}$ bind to purified LRP and their binding is inhibited by receptor-associated protein (RAP). **Panel A** shows ligand blotting of a membrane containing purified LRP that was transferred to nitrocellulose membrane and probed in the absence or presence of 1 μM RAP with 1 nM ^{125}I-GST (**lane 1,5**), 1 nM ^{125}I-GST–TSP$_{1-214}$ (**lane 2,6**), 1 nM ^{125}I-GST–TSP$_{1-90}$ (**lane 3,7**), and 1 nM ^{125}I-TSP-1 (**lane 4,8**), and exposed to autoradiographic film. The blot is representative of four independent experiments. **Panel B** shows TSP-1, GST–TSP$_{1-90}$, or GST–TSP$_{1-214}$ binding to purified LRP immobilized on microtiter wells, and RAP (4 μM) inhibited the binding. The K$_d$ of TSP-1, GST–TSP$_{1-214}$, and GST–TSP$_{1-90}$ were 5, 70, and 29 nM, respectively.

next used to determine the relative affinity of TSP-1, GST–TSP$_{1-214}$, and GST–TSP$_{1-90}$ binding to LRP-1. TSP-1, GST–TSP$_{1-214}$, and GST–TSP$_{1-90}$ all bound to microtiter wells coated with LRP-1 in a dose-dependent manner (Fig. 1B). The apparent affinity for these molecules was determined by non-linear regression analysis. A K$_d$ of 5 nM was obtained for TSP-1. For GST–TSP$_{1-214}$ and GST–TSP$_{1-90}$, K$_d$ of 70 and 29 nM were obtained, respectively (Fig. 1B). Thus, using two distinct assays that further truncation of the HBD domain to only 90 amino acids of TSP-1 did not decrease LRP-1 binding. We conclude that the major high-affinity LRP-1 binding domain resides within the first 90 amino acids of TSP-1.

GST–TSP$_{1-214}$ but not GST–TSP$_{1-90}$ Retains High-Affinity Binding for Heparin

The amino-terminal domain of TSP-1 mediates TSP-1 binding to LRP-1, which mediates the subsequent endocytosis [Chen et al., 1996; Mikhailenko et al., 1997]. The ability of heparin and heparitinase treatment to block LRP-1 mediated uptake and degradation of TSP-1 [Godyna et al., 1995; Mikhailenko et al., 1995] suggests that cell surface HSPG binding is a prerequisite for TSP-1 internalization. GST–

TSP$_{1-90}$ was compared with TSP$_{1-214}$ for heparin-binding by ACE. As shown in Figure 2A, heparin-binding to GST–TSP$_{1-214}$ was demonstrated by the gradual shift in electrophoretic mobility of the ^{125}I-labeled heparin at increasing concentrations of GST–TSP$_{1-214}$. Measurement of electrophoretic retardation in each lane implied an apparent K$_d$ of 1.1 μM for GST–TSP$_{1-214}$ binding to heparin. On the other hand, GST–TSP$_{1-90}$ demonstrated no discernible binding to heparin, even at the highest concentration tested of 4.7 μM (Fig. 2B). Purified GST also showed no binding to heparin in ACE with protein concentrations up to 9.9 M (data not shown). We conclude that the first 90 amino acids of TSP-1 are not sufficient to retain high-affinity heparin-binding.

Binding of ^{125}I TSP-1 to the Cell Surface of Fibroblasts Is not Inhibited by GST–TSP$_{1-90}$

We previously determined that GST–TSP$_{1-214}$ directly bound to MEF and was a potent inhibitor of ^{125}I TSP-1 binding to the cell surface [Mikhailenko et al., 1997]. Since recombinant GST–TSP$_{1-90}$ retained the ability to interact with LRP-1 but was unable to bind to heparin, we next examined whether its ability
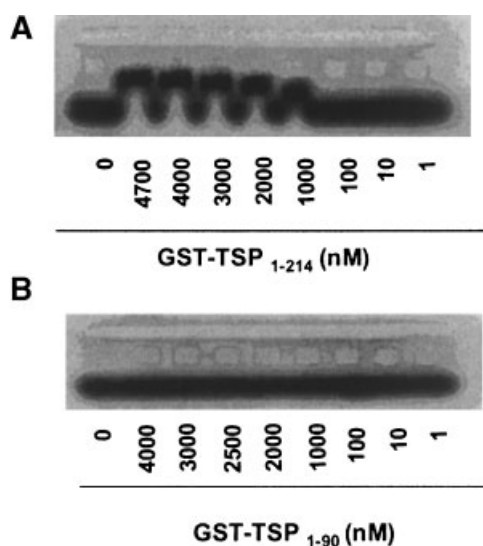
Fig. 2. GST–TSP$_{1-214}$ shows higher heparin-binding affinity than GST–TSP$_{1-90}$. ^{125}I-labeled low molecular weight heparin was subjected to electrophoresis through zones containing the indicated concentrations (nM) of purified recombinant GST fusion proteins. **Panel A**, electrophoretogram of a dried ACE gel where binding to GST–TSP$_{1-214}$ was tested. The direction of electrophoresis was from **top** to **bottom** in both panels. Heparin is progressively shifted with increasing concentrations of GST–TSP$_{1-214}$. Derived dissociation constant is 1.06 µM for GST–TSP$_{1-214}$ (see text for details). **Panel B**, heparin-binding to GST–TSP$_{1-90}$ was tested by identical methods as in Panel A. Flat line pattern indicated no discernible binding at these protein concentrations.

to inhibit TSP-1 binding to MEF was altered. Cell surface binding of ^{125}I TSP-1 was performed at 4°C to prevent cell-mediated catabolism of the labeled TSP-1. Consistent with

previous results, GST–TSP$_{1-214}$ was able to effectively inhibit TSP-1 binding to cells (Fig. 3A). However, GST–TSP$_{1-90}$ had almost no effect on the binding of TSP-1 to the cell surface (Fig. 3A). These results indicate that the majority of the cell surface binding activity was not retained within amino acids 1–90 of TSP-1 and the loss of heparin-binding correlated with the inability to block TSP-1 binding to fibroblasts.

Both GST–TSP$_{1-214}$ and GST–TSP$_{1-90}$ can Effectively Inhibit ^{125}I TSP-1 Catabolism

We next examined whether the decreased cell surface binding of GST–TSP$_{1-90}$ translates to a decreased ability to inhibit cell-mediated catabolism of TSP-1. [125]I TSP-1 catabolism by MEF was measured in the presence of increasing concentrations of GST–TSP$_{1-214}$ and GST–TSP$_{1-90}$. As shown in Figure 3B, GST–TSP$_{1-214}$ and GST–TSP$_{1-90}$ were both effective inhibitors of TSP-1 internalization by cultured fibroblasts. Similarly, both GST fusion proteins were able to inhibit the degradation of ^{125}I TSP-1 by MEF (Fig. 3C). The binding, internalization, and degradation results are summarized in Table I and indicate that while there was a 10-fold difference in the ability to inhibit TSP-1 binding to the cell surface between GST–TSP$_{1-214}$ and GST–TSP$_{1-90}$, there was only a modest difference in their ability to block internalization and degradation. We conclude that despite an inability to block the majority of TSP-1 binding to mouse fibroblasts,
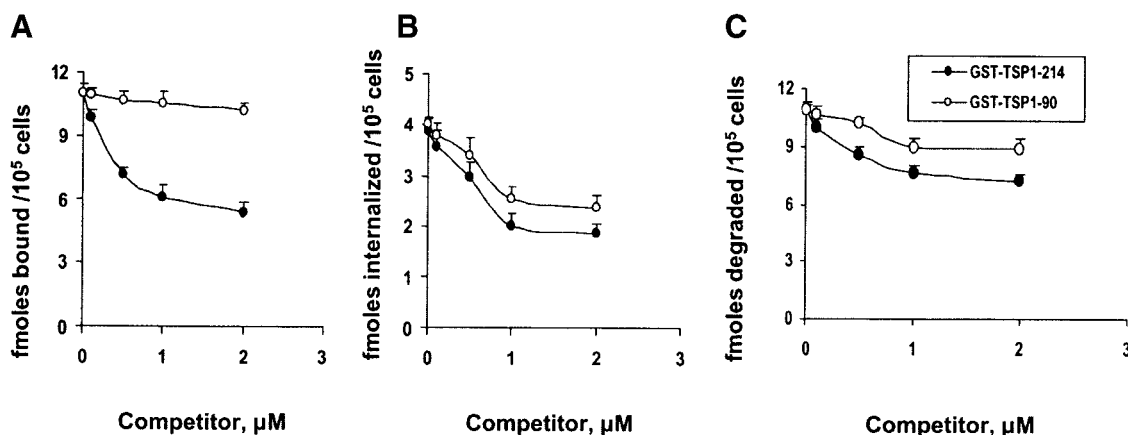

Fig. 3. Both GST–TSP$_{1-214}$ and GST–TSP$_{1-90}$ inhibit the binding, internalization, and degradation of ^{125}I-TSP-1 by MEF. MEF were seeded in 12-well culture dishes (10^5 cells/well) 1 day before the assay. ^{125}I-TSP-1 (0.5 ml, 5 nM) was added to each well in the presence of increasing concentrations of GST–TSP$_{1-214}$ and GST–TSP$_{1-90}$. Following incubation, the extent of binding (**Panel A**), internalization (**Panel B**), and degradation (**Panel C**) of ^{125}I-TSP was measured as described under "Materials and Methods." Each data point represents the average of triplicate determinations.

TABLE I. Inhibition of ^{125}I-TSP-1 Binding and Catabolism

	Binding (% inhibition)	Internalization (% inhibition)	Degradation (% inhibition)
Heparin (0.66 μM)	72.3 ± 4.5	81.5 ± 6.8	85.9 ± 8.4
GST–TSP$_{1-214}$	45 ± 6.2	49 ± 5.7	31 ± 2.7
GST–TSP$_{1-90}$	$4.2 \pm 1.05*$	37 ± 4.9	17 ± 3.2

Values represent inhibition compared to cells without competitor (n = 3).
*$P < 0.05$ as GST–TSP$_{1-90}$ versus GST–TSP$_{1-214}$ (by using Student's t-test).

GST–TSP$_{1-90}$ was an effective inhibitor of ^{125}I TSP-1 catabolism.

GST–TSP$_{1-90}$ Is More Resistant to Cell-Mediated Catabolism

We next compared the direct binding and catabolism of ^{125}I-GST–TSP$_{1-214}$ and ^{125}I-GST–TSP$_{1-90}$ by MEF. As shown in Figure 4, GST–TSP$_{1-214}$ bound to the cell surface of cultured fibroblasts and was effectively internalized and subsequently degraded. However, GST–TSP$_{1-90}$ was not effectively degraded and MEF binding, internalization, and degradation of the truncated protein all decreased by approximately two third. These data indicate that while GST–TSP$_{1-90}$ can block TSP-1 catabolism, the fragment itself is considerably more resistant to cell-mediated endocytosis.

GST–TSP$_{1-90}$ but not GST–TSP$_{1-214}$ Enhances Accumulation of Endogenously Expressed TSP-1 in the Conditioned Medium

We next compared the ability of the two recombinant GST fusion proteins to accumulate endogenously expressed TSP-1. MEF were incubated with GST–TSP$_{1-90}$ or GST–TSP$_{1-214}$ in serum-free culture medium for 30 h and endogenously expressed TSP-1 was detected by Western blotting. Interestingly, we found that a substantially greater increase in full-length TSP-1 was only observed in conditioned medium of cells treated with GST–TSP$_{1-90}$ (about 4.5 fold increase) (Fig. 5A). Northern blotting analysis indicated that there was a minimal change in TSP-1 mRNA expression in cells treated with either GST–TSP$_{1-214}$ or GST–TSP$_{1-90}$ (Fig. 5B) at various time points (4, 8, 12, 24, and 36 h with only 4 h data shown), indicating that the increased level of TSP-1 protein in the conditioned media was not due to an increase in TSP-1 mRNA level. These results indicate that GST–TSP$_{1-90}$ was a considerably more effective inhibitor of endogenously expressed TSP-1 catabolism than GST–TSP$_{1-214}$.

DISCUSSION

We have determined that the LRP-1 binding activity of TSP-1 is completely retained within the first 90 amino acids. Furthermore, while
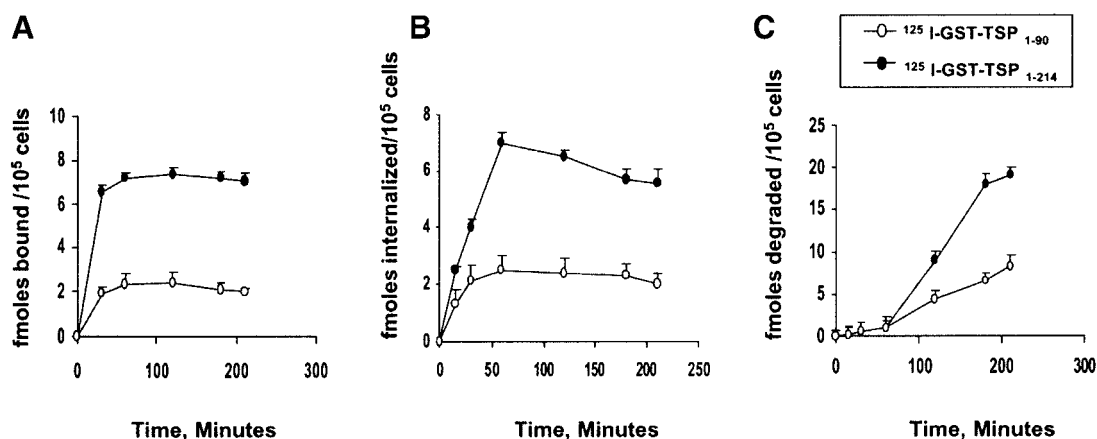

Fig. 4. Time course for the binding (**A**), internalization (**B**), and degradation (**C**) of ^{125}I-GST–TSP$_{1-214}$ and ^{125}I-GST–TSP$_{1-90}$ in MEF MEF were seeded in 12-well culture dishes (10^5 cells/well) 1 day before the assay. ^{125}I-GST–TSP$_{1-214}$ and ^{125}I-GST–TSP$_{1-90}$ (0.5 ml, 2 nM) was added to each well. At the indicated times, the extent of binding (**panel A**), internalization (**panel B**), and degradation (**panel C**) of ^{125}I-GST–TSP$_{1-214}$ and ^{125}I-GST–TSP$_{1-90}$ were measured as described under "Materials and Methods." Each data point represents the average of triplicate determinations.
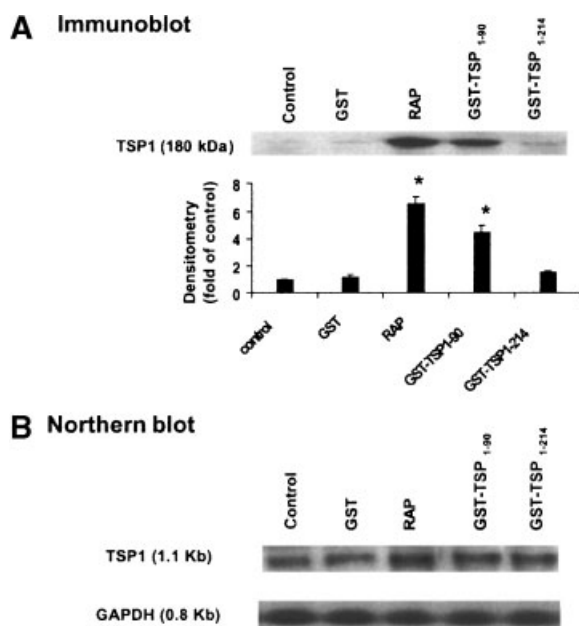
A Immunoblot

TSP1 (180 kDa)

B Northern blot

TSP1 (1.1 Kb)

GAPDH (0.8 Kb)

Fig. 5. Effects of GST–TSP$_{1-214}$ and GST–TSP$_{1-90}$ on extracellular accumulation of TSP-1 by Western blotting (**A**) and on TSP-1 mRNA expression by Northern blotting (**B**) in MEF. **Panel A**: TSP-1 levels in the conditioned culture medium of MEF treated with 1 μM GST, RAP, GST–TSP$_{1-214}$, or GST–TSP$_{1-90}$ in serum free medium for 30 h were analyzed by immunoblotting as described in "Materials and Methods." The data shown are representative of three separate experiments. Relative TSP-1 levels were determined by scanning densitometry of immunoblots. The level of TSP-1 expressed under control conditions was set to 1. Results are the mean ± SD of three separate experiments. *$P < 0.05$ as RAP or GST–TSP$_{1-90}$ treatment versus control group. **Panel B**: Quiescent MEF were treated with 1 μM GST, RAP, GST–TSP$_{1-214}$, or GST–TSP$_{1-90}$ for 4 h at 37°C, 5% CO_2, and subsequently harvested for RNA isolation. The mRNA level of TSP-1 was analyzed by Northern blotting as described in "Materials and Methods." GAPDH mRNA level was also determined and served as a control.

this domain contains the two regions previously identified as necessary for high-affinity heparin-binding [Lawler et al., 1992], it was incapable of high-affinity binding to heparin. These results are consistent with a previous report that a 218 amino acid NH$_2$-terminal domain retained heparin-binding but a shorter 164 amino acid polypeptide was unable to bind to heparin-sepharose [Prochownik et al., 1989]. The two truncated TSP-1 GST fusions proteins examined here have similar binding affinity for LRP-1 indicating that the truncated proteins retained the conformation necessary for interaction with the endocytic receptor. Therefore, current data favor the possibility that a third region between amino acids 164 and 214 is necessary for high-affinity heparin-binding. One possibility is that the potential

heparin-binding sequence TRDLASIARLRIA-KGVNDNE (residues 170–189) is also necessary for high-affinity binding. Alternatively, there may be sequences between 90 and 214 which are necessary for appropriate presentation of the two amino-terminus proximal HBDs.

ACE is an ideal method to measure affinity for heparin when dissociation rates are fast and it is a highly sensitive assay for heparin-binding [Schaffner and Weissmann, 1973]. The K$_d$ of 1.1 μM obtained for GST–TSP$_{1-214}$ is similar to the K$_d$ obtained for heparin-binding to a 25 kDa trypsin-derived HBD fragment of TSP-1 (K$_d$ ∼850 nM) [Herndon et al., 1999]. In contrast, we were unable to demonstrate heparin-binding with recombinant TSP$_{1-90}$. Considering that in general, GAG-binding proteins bind heparin more strongly than they bind to cellular GAGs such as HSPG and chondroitin sulfate proteoglycans [Herndon et al., 1999], it appears unlikely that any biological activity of TSP$_{1-90}$ is related to binding to cell surface HSPG. This is consistent with the results that TSP$_{1-90}$ was minimally active in blocking TSP-1 binding to the fibroblast cell surface, since previous data showed cell surface proteoglycans as mediators of the initial binding of TSP-1 to cells [Kaesberg et al., 1989; San Antonio et al., 1993; Murphy-Ullrich et al., 1993]. However, these conclusions are in contrast to those of Clezardin et al. [1997] in which a centrifugal cell-attachment assay was used to demonstrate an apparent requirement for the 1–90 amino acids of N-terminal domain of TSP-1 to bind to cell surface HSPG. This difference might be due to (1) different assay system: direct cell binding versus centrifugal cell-attachment assay. The later may magnify the relatively weak HSPG binding; (2) different cell types used in the two assays: Chinese hamster ovary (CHO) cells versus fibroblasts.

We previously determined that heparin and heparitinase treatment blocked LRP-1 mediated uptake and degradation of TSP-1 [Godyna et al., 1995; Mikhailenko et al., 1995]. One interpretation of these results is that cell surface HSPG binding is a prerequisite for TSP-1 internalization. The current data clearly indicate that the LRP-1 binding site of TSP-1 can be distinguished from the high-affinity HBD. TSP$_{1-90}$ retained the ability to effectively inhibit TSP-1 internalization and degradation and was more effective in blocking the

degradation of endogenously expressed TSP-1. Thus, it is clear that HSPG binding is not necessary for TSP-1 to bind to LRP-1 on the cell surface. Furthermore, we determined that TSP-1_{1-90} itself was poorly catabolized, presumably due to loss of binding to HSPG. The inability to be effectively catabolized could explain the puzzling observation that GST–TSP$_{1-90}$ was the most effective fragment in blocking LRP-1 mediated degradation of endogenously expressed TSP-1 yet was slightly less effective than GST–TSP$_{1-214}$ in the 5 h internalization and degradation assay. We speculate that this difference reflects the 30 h incubation period used to detect endogenously secreted TSP-1. Thus, we hypothesize that the inability of GST–TSP$_{1-90}$ to bind to cell surface HSPG may render this molecule more resistant to direct degradation by the cell, resulting in a higher extracellular concentration over time and consequently render a more effective blockade of TSP-1 catabolism.

It has long been recognized that the amino-terminal domain of TSP-1 is susceptible to release as an intact fragment by proteases [Dixit et al., 1984; Raugi et al., 1984; Lawler et al., 1985; Damas et al., 2001]. Thus, in a protease-rich environment such as during wound healing and cancer metastasis there could be increased release of various forms of the amino-terminal domain of TSP-1. We have shown that one such fragment can substantially alter the catabolism of full-length TSP-1. This may represent a novel means of regulating the availability of TSP-1 for TGF-β activation [Schultz-Cherry et al., 1994; Crawford et al., 1998], for modulating angiogenesis [Jimenez et al., 2000] and for regulation of focal adhesion dissembly [Orr et al., 2003]. These fragments of TSP-1 may also have distinct cell modulating activity [Ferrari do Outerio-Bernstein et al., 2002].

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