Binding and degradation of thrombospondin-1 mediated through heparan sulphate proteoglycans and low-density-lipoprotein receptor-related protein: localization of the functional activity to the trimeric N-terminal heparin-binding region of thrombospondin-1

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Thrombospondin-1 (TSP-1) is a multimodular trimeric protein involved in cell adhesion, motility and growth. TSP-1 binds to cells and is internalized and degraded in a process that requires the presence of heparan sulphate proteoglycan; the process is inhibited by heparin or receptor-associated protein (RAP), an antagonist of the low-density-lipoprotein receptor (LDLR) family. We characterized the attributes of TSP-1 that mediate the process. TSP277, which is truncated at Gln-277 of TSP-1 and contains the heparin-binding domain and the heptad repeat region that mediates trimerization, bound to and was degraded

by a variety of cells with kinetics similar to those of the binding and degradation of intact TSP-1. Degradation of TSP277 was inhibited by heparin or RAP with dose responses similar to those for inhibition of degradation of TSP-1. Binding and degradation of TSP277 were decreased in Chinese hamster ovary cells lacking heparan sulphate. These results indicate that the N-terminal heparin-binding domain in a trivalent configuration is sufficient to mediate binding and degradation of TSP-1 via the proteoglycan-LDLR family pathway.

INTRODUCTION

Thrombospondin-1 (TSP-1) is a trimeric protein held together by interchain disulphide bonds at Cys-252 and Cys-256 [1]. TSP-1 was originally identified as a platelet alpha granule protein [2,3]. It was subsequently found to be produced and secreted by a number of cell types in culture [4–7]. Proposed functions for TSP-1 include an involvement in platelet aggregation [8,9], cell adhesion [10–14], cell growth [15–17], angiogenesis [18], binding and activation of latent transforming growth factor- β [19–22], and regulation of protease activities [23–25]. Several TSP-1 receptors have been identified, including cell-surface proteoglycans [13,26,27] and sulphatides [28–30], CD36 [31,32], integrins [12,33–36], integrin-associated protein [37] and receptors that are perhaps yet to be characterized [38,39].

The subunit of TSP-1 is composed of an N-terminal heparin-binding proline- and arginine-rich protein (PARP) module (residues 1–258), a heptad repeat region that mediates trimerization (residues 259–289), a procollagen module (residues 290–378), three properdin modules (residues 379–548), three epidermal growth factor-like modules (residues 549–691), Ca²⁺-binding (or type III) repeats (residues 692–950) and a C-terminal globular domain (residues 951–1170) [40,41]. Binding sites for cell-surface receptors have been localized to several regions within TSP-1. Binding of TSP-1 to proteoglycans involves the N-terminal heparin-binding region [26,42]. However, peptides based on sequences within both the PARP module [43,44] and the properdin modules [29,30] have heparin-binding activity [29]. The Arg-Gly-Asp sequence located in the type III region is

involved in binding to integrins [12,33]. The properdin modules and the C-terminal globular region both mediate attachment to melanoma cells [11,39,45]. Integrin-associated protein binds to the C-terminal globular domain [37].

Binding of soluble TSP-1 to a number of cell types is followed by its internalization and degradation [26,46–52]. Several lines of evidence suggest that the binding and degradation involve cell-surface heparan sulphate proteoglycans [26,46–51] and low-density-lipoprotein receptor-related protein (LRP), a member of the low-density-lipoprotein receptor (LDLR) family [50,51]. We sought to define further the structural features of TSP-1 that are important for its binding and degradation. We report that truncated N-terminal trimers of TSP-1 lacking everything but the PARP modules and the heptad repeat region are bound to and degraded by cells in a manner similar to that of intact TSP-1.

METHODS

Production and purification of proteins

TSP277, which comprises the N-terminal heparin-binding PARP module and the heptad repeat region including Cys-252 and Cys-256, which participate in interchain disulphide bonds, was produced by using a baculovirus expression system and purified by heparin-agarose affinity chromatography [1]. Unreduced TSP277 was predominantly trimeric when analysed by SDS/PAGE [53] with an apparent molecular mass of 110 kDa,

Abbreviations used: CHO, Chinese hamster ovary; LDLR, low-density-lipoprotein receptor; LRP, low-density-lipoprotein receptor-related protein; PARP, proline- and arginine-rich protein; RAP, receptor-associated protein; TSP, thrombospondin.

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Figure 1 SDS/PAGE of input and bound ligands

Autoradiography of ^{125}I -labelled TSP-1 and ^{125}I -labelled TSP277. (**A**) ^{125}I -labelled TSP-1 (lanes 1 and 3) and ^{125}I -labelled TSP277 (lanes 2 and 4), before binding assays, were analysed by SDS/PAGE in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of 5% (v/v) 2-mercaptoethanol. (**B**) Proteins bound to fibroblast cell layers after a 1 h incubation at 37 °C without (—) or with (+) 10 µg/ml heparin were analysed without reduction. Lanes 1 and 2, bound ^{125}I -labelled TSP-1; lanes 3 and 4, bound ^{125}I -labelled TSP277. In (**B**) the positions of non-reduced trimeric TSP-1 and trimeric (upper band) and dimeric (lower band) TSP277 are shown. The arrowhead indicates the top of the 3.5% stacking gel. ^{14}C -labelled molecular mass standards are (horizontal lines at the left, from top to bottom): myosin (200 kDa), phosphorylase b (92.5 kDa), BSA (69 kDa) and ovalbumin (46 kDa). The positions of trimeric TSP-1 in the two gels were different owing to the relative heights of the stacking gels.

although some TSP277 was disulphide-bonded only as dimers [1] (see also Figure 1A). We presume that the apparent dimers are trimeric in the absence of SDS by virtue of association of the heptad repeat region [1]. TSP-1 from platelet releasate [33], receptor-associated protein (RAP) and affinity-purified polyclonal antibodies against the 600 kDa LRP (R777) [50] were prepared as described.

Iodination of proteins

TSP277 and TSP-1 (approx. 100 µg of each) were iodinated with 0.5 mCi of Na^{125}I by the chloramine-T method, and repurified on heparin-agarose columns as described [26]. The specific radioactivities of labelled proteins were 1–5 mCi/mg of protein. Iodinated proteins were analysed by SDS/PAGE and autoradiography and had the expected mobilities (Figure 1A). After digestion of ^{125}I -labelled TSP277 with trypsin (20 µg/ml for 1 min at room temperature), approximately half of the radioactivity was associated with a 28 kDa fragment that bound to heparin-agarose and was monomeric when assessed by SDS/PAGE without reduction. This fragment is presumed to represent residues Asn-1 to Arg-237 [1]. The remainder of the radioactivity migrated close to the dye front, was soluble in 10% (w/v) trichloroacetic acid and probably represent fragments comprising amino acids Thr-238 to Gln-277. The 1:1 distribution of radioactivity between these bands is compatible with the positions of the two tyrosines, Tyr-150 and Tyr-240, in TSP277.

Figure 2 Time course of binding and degradation of TSP277 and TSP-1 by fibroblasts

Equal molar concentrations of ^{125}I -labelled TSP277 (0.24 µg/ml; □) and ^{125}I -labelled TSP-1 (1 µg/ml; ○) were added in the presence (solid lines) or absence (dotted lines) of 250 µg/ml heparin and incubated for various lengths of time at 37 °C. After the incubation, binding medium was removed and cell layers were washed. Bound proteins were then solubilized in 0.2 M NaOH and counted (top panel). Binding medium was incubated with trichloroacetic acid at a final concentration of 13% (w/v). Increases in trichloroacetic acid-soluble radioactivity were considered to represent degradation (bottom panel). Results are the averages and ranges of duplicate determinations.

Binding and degradation assays

Binding and degradation assays were adapted from previously described methods [26]. Cells were seeded and used 3–4 days later when newly confluent. ^{125}I -labelled TSP277 and TSP-1 in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, U.S.A.) containing 0.2% bovine albumin were added to monolayers of cells and incubated for various lengths of time in the presence or absence of potential competitors. Cells were washed, then solubilized in sample buffer [50 mM Tris/HCl, pH 6.8, 1% SDS, 10% (v/v) glycerol, 0.01% Bromophenol Blue] for analysis by SDS/PAGE or in 0.2 M NaOH for γ counting. Data represent the average of duplicate determinations; variation between duplicates was less than 20% for all experiments.

Degradation was quantified by measuring the increase in trichloroacetic acid-soluble radioactivity. After incubation of the radiolabelled ligand with the cells, the binding medium was taken out and precipitated with 13% (v/v) trichloroacetic acid (final concentration), and material not precipitated by trichloroacetic acid was determined with a γ counter.

Cell culture

Human foreskin fibroblasts and Chinese hamster ovary (CHO) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. CHO wild-

type K1 and mutant cells were a gift from J. Esko (University of Alabama, Birmingham, AL, U.S.A.) and have been characterized elsewhere [47,54–56]. CHO pgsA (745) mutants are defective in xylosyl transferase activity and are glycosaminoglycan-deficient; pgsX (803) mutants are defective in glucuronic acid transferase and are heparan sulphate-deficient; and pgsY (677) mutants are defective in *N*-acetylglucosamine transferase and are heparan sulphate-deficient with excess chondroitin sulphate.

RESULTS

We tested whether TSP277 could bind to and be degraded by cells. After incubation with monolayers of fibroblasts for 1 h at 37 °C, 125 I-labelled TSP277, like 125 I-labelled TSP-1, was found to be associated with the cell layers as assessed by washing the cell layers, extracting with SDS, and analysing by SDS/PAGE followed by autoradiography. The major discrepancy between the patterns of input and bound ligands was the enrichment of TSP-1 in the high-molecular-mass multimers in the bound fraction of intact TSP-1 analysed without reduction; these multimers failed to enter the stacking gel (Figure 1B). No degraded fragments were obvious. Degradation of 125 I-labelled TSP277 to trichloroacetic acid-soluble radioactivity was inhibited by the presence of chloroquine (results not shown). The inhibition by chloroquine and the absence of any detectable degradative

intermediates suggest that TSP277, like intact TSP-1 [26,50,51], is degraded by fibroblasts through a lysosomal pathway.

To determine whether TSP277 was bound and degraded by cells with the same kinetics as TSP-1, we compared binding and degradation of labelled TSP277 and intact TSP-1 over a 4 h time course at 37 °C (Figure 2). TSP277 rapidly associated with the cell layers. A similar time course was found with intact TSP-1. Trichloroacetic acid-soluble radioactivity was detected as early as 20 min after addition of labelled proteins, and increased over the 4 h time course. The degradative capacities of cells for TSP277 and TSP-1 were similar (Figure 2). Binding and degradation of both TSP277 and TSP-1 were less in the presence of 250 μ g/ml heparin (Figure 2). Similar results were obtained when heparin was present at 10 μ g/ml. At both heparin concentrations, binding and degradation of TSP277 were not blocked as completely as binding and degradation of TSP-1, even though heparin was present in approx. 10- and 250-fold excess of the ED_{50} (concentration causing 50% inhibition) of both proteins (see Figure 3).

Mutant CHO cells defective in various aspects of heparan sulphate glycosaminoglycan synthesis are defective in binding and degradation of TSP-1 [47]. We compared the metabolisms of TSP277 and TSP-1 by these cells. Mutant CHO cells bound less TSP-1 and TSP277 than non-mutant CHO cells (Table 1). Most of the residual binding of TSP277 to 745 and 803 cells was not inhibited by heparin (results not shown). Mutant CHO cells degraded 70–80% less TSP277 and 80–95% less TSP-1 than non-mutant cells (Table 1).

It has been shown previously that heparin blocks the binding of TSP-1 to cells [26] and, after binding, TSP-1 is internalized and degraded through a mechanism that is inhibited by RAP, a ligand-binding antagonist of members of the LDLR family including, in fibroblasts, the 600 kDa LRP [50,51]. Degradation of TSP277 showed similar sensitivities to inhibition by heparin (Figure 3) or RAP (Figure 4) as degradation of intact TSP-1. R777 polyclonal antibodies against LRP [50,51] at 100 μ g/ml inhibited the degradation of TSP-1 and TSP277 by fibroblasts to $27 \pm 1\%$ and $49 \pm 5\%$ of the control value respectively.

The results suggest that TSP-1 and TSP277 bind to the same heparan sulphate proteoglycan and LDLR family sites on the cell surface. Competitive studies were done to learn whether excess TSP-1 inhibits degradation of TSP277 and vice versa. In the presence of increasing concentrations of unlabelled TSP-1 or TSP277, less 125 I-labelled TSP-1 or TSP277 was degraded by fibroblasts. The limited solubility of TSP277 and TSP-1 did not allow full dose-response curves. TSP-1 at 100 μ g/ml inhibited the degradation of TSP-1 and TSP277 to 44% and 59% of control values respectively. In the presence of unlabelled 40 μ g/ml

Figure 3 Inhibition of degradation of TSP277 by heparin

Fibroblasts were incubated with equal molar concentrations of 125 I-labelled TSP277 (0.24 μ g/ml; $\square$) or 125 I-labelled TSP-1 (1 μ g/ml; $\circ$) in the presence of increasing concentrations of heparin for 4 h at 37 °C. The degradation assay was performed as in Figure 2. Results are the averages and ranges of duplicate experiments.

Table 1 Binding and degradation of TSP277 and TSP-1 by various CHO cells

CHO cells were seeded at $(2-4) \times 10^4$ cells per well in 12-well cluster dishes to achieve approximately the same cell density at the time of the assay. Equal amounts of radioactivity in the form of iodinated proteins were added to cells. After 4 h, proteins bound and degraded were quantified as c.p.m. per μ g of cell-layer protein as determined by the Lowry assay [58]. Data were normalized to binding and degradation of wild-type K1 cells, which were taken as 100%. Results are the averages and ranges of duplicate wells.

Cell line	Phenotype	Binding (% of wild-type K1 cells)		Degradation (% of wild-type K1 cells)	
		TSP-1	TSP277	TSP-1	TSP277
745	Glycosaminoglycan-deficient	31 ± 1	60 ± 1	< 8	28 ± 1
803	Heparan sulphate-deficient	30 ± 6	33 ± 1	< 5	19 ± 1
677	Heparan sulphate-deficient, excess chondroitin sulphate	31 ± 1	48 ± 1	22 ± 2	32 ± 1

Figure 4 Inhibition of degradation of TSP277 by RAP

Fibroblast cells were incubated with equal molar concentrations of ^{125}I -labelled TSP277 (0.24 $\mu\text{g}/\text{ml}$; $\square$) or ^{125}I -labelled TSP-1 (1 $\mu\text{g}/\text{ml}$; $\circ$) in the presence of increasing concentrations of RAP for 4 h at 37 °C. The degradation assay was performed as in Figure 2. Results are the averages and ranges of duplicate experiments.

TSP277 the degradative rates of ^{125}I -labelled TSP-1 and TSP277 were 52 % and 74 % of control values respectively.

DISCUSSION

TSP277, which lacks the procollagen, properdin, epidermal growth factor-like, type III and C-terminal globular regions, bound to and was degraded by cells in a manner similar to intact platelet TSP-1. Binding and degradation were inhibited by heparin, and little degradation occurred when TSP277 was incubated with mutant CHO cells lacking heparan sulphate glycosaminoglycans. These results are evidence that heparan sulphate proteoglycans are important for binding and degradation. The observation that heparin inhibited both TSP277 and intact TSP-1 degradation to a similar extent with similar dose-response curves suggests that the heparin-binding PARP module, of which there are three in intact TSP-1 and TSP277, represents the major heparan sulphate proteoglycan interaction site on TSP-1 and mediates the binding and degradation of intact TSP-1.

After binding to fibroblasts, intact TSP-1 is internalized and degraded through a pathway involving members of the LDLR family including LRP [50,51]. It has been shown that TSP-1 can interact with the 600 kDa LRP directly in a solid-phase binding assay [51]. Such binding is blocked by RAP, the LRP antagonist, but poorly by heparin, suggesting that interactions of TSP-1 with heparan sulphate and LRP might be mediated by different sites [51]. Degradation of TSP277 was inhibited by RAP in the same way that degradation of intact TSP-1 was inhibited. Degradation was also inhibited by antibodies to LRP. In addition to the heparan sulphate interaction site, therefore, TSP277 contains the structures responsible for interaction with LRP and is sufficient to mediate the binding and degradation of TSP-1 through the heparan sulphate proteoglycan-LRP pathway. A chimera of glutathione S-transferase and residues 1–214 of TSP-1, which comprises most of the heparin-binding PARP module, binds to LRP directly in a solid-phase binding assay (I. Millailenko and D. K. Strickland, unpublished work).

After binding, TSP277 is presumably passed on to or becomes associated with LRP through an interaction between TSP277 and LRP. Like intact TSP-1 and TSP277, proteoglycans are actively metabolized by cells. Some proteoglycans are internalized and degraded in the lysosomes as well as being released into the

medium by proteolysis [57]. This suggests the possibility that heparan sulphate proteoglycan interacts directly or indirectly with LRP and becomes internalized and degraded along with bound TSP-1 or TSP277. Co-internalization of heparan sulphate proteoglycan, LRP and TSP1 would explain the discrepancy between the ability of heparin to block TSP-1 internalization and degradation and yet only poorly inhibit the direct interaction between TSP-1 and LRP.

Gold beads coated with N-terminal heparin-binding fragments of TSP-1 are bound and taken up by endothelial cells in a pattern similar to that of intact TSP-1, whereas beads coated with a large TSP-1 fragment devoid of the heparin-binding region bind to cells very poorly [52]. These results, along with our demonstration of the ability of cells to bind and degrade fluid-phase TSP277, and the equivalent sensitivities of TSP277 and TSP-1 degradation to inhibition by heparin and RAP indicate that: (1) the major structural attributes of TSP-1 involved in the binding and degradation processes are in the N-terminal heparin-binding domain; (2) proteoglycans and receptors of the LDLR family are responsible for these activities; and (3) other known TSP-1 receptors are not required for these processes. Future experiments are needed to identify the critical sites for interactions of TSP277 with heparan sulphate proteoglycan and receptors of the LDLR family.

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