Metabolism of Thrombospondin 2

BINDING AND DEGRADATION BY 3T3 CELLS AND GLYCOSAMINOGLYCAN-VARIANT CHINESE HAMSTER OVARY CELLS*

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Thrombospondin 1 (TSP1) and thrombospondin 2 (TSP2) are members of the thrombospondin family that have a similar structural organization but somewhat different functional activities. Iodinated recombinant mouse TSP2 bound to NIH 3T3 cells and was internalized and degraded through a chloroquine-inhibitable pathway. TSP2 degradation was saturable, specific, and similar to the kinetics of degradation of TSP1. Human platelet TSP1, recombinant mouse TSP1, and recombinant mouse TSP2 cross-competed with one another for degradation by 3T3 cells. Degradation of TSP2 was less sensitive to inhibition by heparin than degradation of TSP1. This is in agreement with differences in heparin-binding affinity of the two TSPs. Degradation of TSP2 was slower in cultures of Chinese hamster ovary (CHO) cells lacking heparan sulfate proteoglycans than in wild type CHO cells or in cultures of 3T3 cells treated with heparitinase than in untreated 3T3 cells. Degradation of TSP2 was inhibited by antibodies against the low density lipoprotein receptor-related protein (LRP) or by the 39-kDa receptor-associated protein, a known antagonist of LRP. This study indicates that TSP2 and TSP1 are metabolized by a common internalization and degradation pathway involving heparan sulfate proteoglycan and LRP. Competition for this pathway is a possible mechanism whereby cells can control the levels and ratio of TSP1 and TSP2 in the extracellular milieu.

Thrombospondins (TSPs) are a family of structurally related homologous glycoproteins. To date, five TSPs have been identified (1–16). These TSPs are divided into two subgroups, with TSP1 and TSP2 in one group and TSP3, TSP4, and TSP5 (also known as cartilage oligomeric matrix protein, COMP) in the other (17).

TSP1 is the best studied of the TSPs. It is a trimERIC, secreted modular glycoprotein. Each subunit contains an NH₂-terminal globular heparin-binding module (also called a PARP module), a domain that mediates disulfide-stabilized trimerization, a procollagen module, three type 1 (properdin) modules, three type 2 (epidermal growth factor) modules, a series of type 3 (Ca²⁺-binding) repeats, and a COOH-terminal globular domain (1, 17–20). Human platelet TSP1 has been shown to bind to cells, platelets, Ca²⁺, and many matrix and plasma proteins including fibronectin, collagens, laminin, heparan sulfate, fibrinogen, plasminogen, osteonectin, histidine-rich glycoprotein, and transforming growth factor-β1 (19–21). There are several possible cell surface receptors for TSP1, including GPIIb/IIIa (22, 23), the vitronectin receptor integrin αvβ₃ (23–26), heparan sulfate proteoglycans and sulfatides (27–32), GPIV (CD36) (33), integrin-associated protein (34), and possibly other receptors (35, 36).

TSP1 displays interesting biological activities. It modulates substratum adhesion of normal and tumor cells (25, 26, 31, 36–40) and has antiangiogenic activity in that it can inhibit endothelial cell proliferation, tube formation in vitro and neo-vascularization in vivo (40–43). In contrast to the effects on endothelial cells, TSP1 promotes growth and migration of smooth muscle cells and fibroblasts (44–47). TSP1 has also been shown to stimulate migration of keratinocytes (48) and stimulate chemotaxis and haptotaxis of neutrophils (49), smooth muscle cells (46), and several carcinoma and melanoma cells (50, 51).

TSP1, when secreted from platelets, can complex with activated TGF-β1. TSP1 also binds and activates latent TGF-β1 (52–55). Since most cell types secrete latent TGF-β and express TGF-β receptors on their surfaces, it is postulated that TSP1 is a key regulator of TGF-β1 activity under physiological conditions (52–55).

The structural modules of TSP2 are similar to those of TSP1, with an increasing gradient of sequence identity from the NH₂-terminal module (38% identity) to the COOH-terminal domain (82% identity) (4, 6). The patterns of expression of TSP1 and TSP2 mRNAs are distinct in tissues of embryonic and developed mice (56, 57). To compare the structure and function of TSP1 and TSP2, we have expressed mouse TSP2 (mTSP2) in a baculovirus system as a disulfide-bonded homotrimer (58). mTSP2 supports adhesion for endothelial cells, osteosarcoma cells, and colon carcinoma cells by mechanisms similar but not identical to those of TSP1. Adherence to both TSPs appears to utilize heparan sulfate proteoglycans and αvβ₃ integrin, and is regulated by Ca²⁺ and reduction. One major difference between adhesive activities of TSP1 and TSP2 is the differential sensitivity to inhibition of adhesion by heparin (58). In another adhesion system where adrenocortical cells are used as a source, bovine TSP2 (also known as corticotropin-induced secreted protein, CISP) shows an antiadhesive activity (59). TSP2, like TSP1, binds TGF-β1 (55). TSP2 does not, however,
activate latent TGF-β1 (55). This lack of activity apparently is due to substitution of the activating RK sequence in TSP1 (55) with the trypsin-susceptible, nonactivating RR sequence in TSP2 (55, 58). TSP2 inhibits the activation of latent TGF-β1 by TSP1, presumably through the common TGF-β1 binding sequence GGWSHW present in both TSP1 and TSP2 (55). Therefore TSP2 may act as a buffer to the activation of latent TGF-β1 by TSP1.

Because TSP1 has potent biological activities, and TSP2, besides its own functions, may regulate TSP1 function, one may postulate that the ratio of TSP1 and TSP2, as well as their amount, is regulated by their expression and half-life. TSP1 is secreted from platelet α-granules upon activation (19, 60). It is also produced by a variety of normal and transformed cell lines (19, 61, 62). Whereas TSP1 production is up-regulated dramatically by serum or growth factors, TSP2 expression is constitutive (5, 6). It has been shown that platelet TSP1 can bind to cells and incorporate into the extracellular matrix (63, 64), or be cleared by cells via endocytosis and lysosomal degradation (30, 63–68). Cell surface heparan sulfate proteoglycans are required for binding and degradation of platelet TSP1 (30, 63–68). Low density lipoprotein receptor-related protein (LRP) has been shown recently to synergize with heparan sulfate proteoglycans in mediating internalization and degradation of platelet TSP1 (67, 68). One may hypothesize, based on the homology between TSP1 and TSP2, that their metabolism is similar. However, many homologous proteins have different receptors, and the NH₂-terminal region that mediates binding to heparin is the part with the lowest sequence identity between TSP1 and TSP2. Thus, one may also hypothesize that the metabolism of TSP1 and TSP2 is different. To evaluate these hypotheses, we carried out experiments to investigate whether recombinant mouse TSP2 is metabolized by a mechanism similar to that of platelet or recombinant TSP1 and whether TSP1 and TSP2 compete for the same degradation pathway.

MATERIALS AND METHODS

Proteins and Reagents—Human platelet TSP1 (hTSP1) and recombinant mTSP2 produced with baculovirus were purified as described previously (58). Production and purification of recombinant mTSP1 were similar to those used for mTSP2. Briefly, mouse TSP1 cDNA including bases 51–3751 flanked by MluI linkers was cloned into the pML-EMK plasmid (Pharmingen, San Diego, CA) linearized by MluI and EcoRI digestion and used to generate a 5' mouse TSP1 fragment containing bases 210-1404 which lacked excess 5' untranslated region. A 3' fragment was generated by EcoRI digestion and incomplete BamHI digestion which cut at the BamHI site in the pML multiple cloning region but not inside the mouse TSP1 3' cDNA. These two fragments were subcloned into the baculovirus transfer vector pAcSG2 (Pharmingen, San Diego, CA) linearized by NcoI and MluI and EcoRI digestion was used to generate a 5' mouse TSP1 fragment containing bases 210-1404 which lacked excess 5' untranslated region. A 3' fragment was generated by EcoRI digestion and incomplete BamHI digestion which cut at the BamHI site in the pML multiple cloning region but not inside the mouse TSP1 3' cDNA. These two fragments were subcloned into the baculovirus transfer vector pAcSG2 (Pharmingen, San Diego, CA) linearized by NcoI and BglII, utilizing the compatible cohesive ends of BamH1 and BglII. Recombinant mTSP1 virus was generated using the Baculovirus transfer system (Pharmingen) with Lipofectin per the manufacturer’s instructions. After transfection, recombinant viruses were plaque-puriﬁed once, and third passage virus in serum-free medium SF900 II (Life Technologies, Inc.) was used to infect Spodoptera frugiperda cells.

Receptor-associated protein (RAP) and R777 antibodies against LRP were prepared as described previously (67). Native and urea-treated vitronectin (70), platelet factor 4 (PF-4) (71), ﬁbronectin (72), ﬁbrinogen (73), and type I collagen (74) were puriﬁed as described elsewhere. Heparin was purchased from Sigma. Heparitinase, heparinase I, and chondroitinase ABC from Arthrobacter globiformis (Seikagaku, Tokyo, Japan) was also tested.

Generation of Polyclonal Antibodies against mTSP2—Puriﬁed mTSP2, 30 µg was transferred to nitrocellulose paper after electrophoresis in SDS. The nitrocellulose paper containing the mTSP2 band was cut out, washed with H₂O, air-dried, frozen in a dry ice/propylene glycol bath, and crushed into a ﬁne powder with a glass rod. The powder was emulsiﬁed with complete Freund’s adjuvant and injected subcutaneously into two New Zealand White male rabbits (30 µg each). The rabbits were boosted three times at 1-month intervals with electrophoretically repuriﬁed mTSP2, 35 µg, on nitrocellulose in incomplete Freund’s adjuvant, followed by boosts with puriﬁed soluble mTSP2 every 4–6 weeks. Antibody titers and antibody speciﬁcities were checked by enzyme-linked immunobead assay (ELISA) on 96-well plates coated with hTSP1, mTSP2, or BSA, blocked with 5% milk. mTSP1 and hTSP1 were produced by a similar protocol. The speciﬁcities and species cross-reactivities of rabbit antibodies to mTSP2 or hTSP1 were determined by direct ELISA. The anti-mTSP2 antisera had titers against mTSP2 of 120,000–140,000, against hTSP1 of 1,600 and against mTSP1 of 1,150. Anti-TSP1 antisera had a titer against mTSP1 of 11,500 and against mTSP2 of 1,120.

Iodination of Proteins—Puriﬁed TSP, 100 µg was iodinated with 0.5 mCi of Na¹²⁵I in the presence of 0.5 mM chloramine-T as described previously (63, 65). After 1 min, phenylmethylsulfonyl ﬂuoride-treated bovine albumin was added to a concentration of 10 mM. Na¹²⁵I–TSP was repuriﬁed by afﬁnity chromatography on heparin-agarose and eluted with 1 M NaCl in 0.3 mM CaCl₂ and 10 mM Tris, pH 7.4. Albumin was added to a ﬁnal concentration of 2 mg/ml, and Na¹²⁵I–TSPs were stored as small aliquots at −70 °C until use. Iodinated TSPs had the expected mobilities in autoradiograms of polyacrylamide gels after electrophoretic separation in SDS without and with reduction. Specific activities were 1.2–5.2 mCi/µg TSP. Iodinated TSPs had 2–7% trichloroacetic acid-soluble radioactivity.

Binding and Degradation of Na¹²⁵I–TSPs—NH₃ 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in high glucose Dulbecco’s modiﬁed Eagle medium (DMEM) containing 10% fetal bovine serum at 37 °C in an incubator containing 8% CO₂. Chinese hamster ovary (CHO) cells were maintained as described previously (66) in a 5% CO₂ incubator. Cells were grown to confluence on 24-well tissue culture plates (Costar, Cambridge, MA) and washed three times with DMEM before assays. Binding and degradation assays were carried out in DMEM with 0.2% bovine albumin containing 100 units/ml penicillin G and 150 µg/ml streptomycin sulfate according to procedures described before (63–66). Binding medium containing Na¹²⁵I–TSP was incubated with cells in the CO₂ incubator at 37 °C for various times. After the incubation, binding medium was removed and mixed with trichloroacetic acid at a ﬁnal concentration of 10%. After incubation on ice for 15 min, the precipitate was removed by centrifugation. The increase in trichloroacetic acid-soluble radioactivity above the baseline value during the incubation was taken as TSP that had been degraded by the cells. The negligible increase in trichloroacetic acid-soluble radioactivity during incubation in plates without cells was considered as the baseline. At the end of the incubation, cell layers were washed with cold Tris-buffered saline three times and dissolved in 0.2 N NaOH to determine the amount of total cell protein and Na¹²⁵I–TSP that was associated with the cell layers.

In some experiments, cells were treated with heparitinase (0.4 unit/ml), heparinase I and heparinase I (0.4 unit/ml) or chondroitinase ABC (0.1 or 0.05 unit/ml) in two separate experiments in the binding medium containing 25 mM HEPES, pH 7.0, for 45 min at 37 °C before Na¹²⁵I–TSPs were added, and the incubation was carried out for additional 4 h.

Immunoﬂuorescence Studies—Cells for immunofluorescence studies were grown on glass coverslips to subconﬂuence. Cells were washed three times with DMEM. Some coverslips with cells were incubated with 20 µg/ml TSP in DMEM containing 0.2% albumin for 1 h, with or without blocking reagents, before being washed three times and ﬁxed with 3.7% paraformaldehyde. Cells on one set of coverslips were permeabilized with chilled acetone for 5 min. Coverslips were stained with primary antibodies against TSP1 or TSP2 at 1:1,000 dilution for 1 h at 37 °C from rabbit anti-mouse IgG (goat anti-rabbit IgG) or goat anti-rabbit IgG at 1:100 dilution. Coverslips were rinsed three times with Tris-buffered saline before mounting on glass slides in glycerol gelatin (Sigma). Slides were observed and photographed on a Nikon microscope equipped with epifluorescence and phase contrast. Controls for speciﬁc staining included nonimmune serum at the same dilution and immune serum whose signals were blocked by preincubation with either hTSP1 or mTSP2.

RESULTS AND DISCUSSION

Internalization and Degradation of TSP2 by 3T3 Cells—When iodinated mTSP2 was incubated with NIH 3T3 cells, it became rapidly associated with the cell layers (Fig. 1A). Trichloroacetic acid-soluble radioactivity began to appear in the supernatant after about 30 min and increased linearly over the
4-h incubation period (Fig. 1). No intermediate degradation products were detected either in the culture medium or in the cell associated pool by polyacrylamide gel electrophoresis in SDS followed by autoradiography (data not shown). The increase in trichloroacetic acid-soluble radioactivity in the medium was blocked to near background by chloroquine, an inhibitor of endogenous acidification and the lysosomal degradation pathway (75, 76) (data not shown). We compared the metabolism of mTSP2 to that of human platelet TSP1 and, in order to account for any differences that might arise from species differences or cell of origin, recombinant full length mTSP1 expressed by the same methods used to express mTSP2. Iodinated hTSP1 or mTSP1 was metabolized with kinetics similar to that of mTSP2 (Fig. 1). From these data, we conclude that mTSP1 and mTSP2, like human platelet TSP1 (30, 63–68), bind to cells, become internalized, and are degraded through a lysosomal pathway.

Degradation of TSP2 by 3T3 Cells: Saturability and Specificity—When increasing concentrations of mTSP2 were incubated with 3T3 cells, degradation was saturable (Fig. 2). Replotting of the degradation data to a double reciprocal plot yielded a straight line, with an apparent “Kₘ” of 47 ± 21 μg/ml (104 ± 47 nM) and a maximum turnover rate of 9 ± 3 μg of mTSP2 degraded/mg cell protein per 4 h (data are expressed as mean ± S.E. of three separate experiments, each with duplicate numbers). The values for hTSP1 and mTSP1 were 130 ± 48 μg/ml (289 ± 106 nM) and 17 ± 6 μg of TSP degraded/mg of cell protein/4 h, and 15 ± 8 μg/ml (33 ± 18 nM) and 4 ± 2 μg of TSP degraded/mg of cell protein/4 h, respectively. Degradation of each 125I-TSP at 1 μg/ml was inhibited by approximately 50% by 50 μg/ml of the same unlabeled TSP in the binding medium (Table I). PF4, 15 μg/ml, inhibited degradation of 125I-labeled mTSP1 and TSP2 to 69 ± 12% and 68 ± 7% of control, respectively (data not shown). Degradation was not inhibited by 100 μg/ml fibronectin, fibrinogen, native or urea-treated vitronectin, or type I collagen (data not shown).

Competition of TSP2 and TSP1 for Degradation by 3T3 Cells—Incubation with mTSP2 at 50 μg/ml reduced degradation of labeled hTSP1 and mTSP1 to 49 ± 8%, and 71 ± 6%, respectively (Table I). When 50 μg/ml unlabeled hTSP1 was incubated with 125I-TSPs, degradation of labeled mTSP1 and mTSP2 by 3T3 cells was reduced to 64 ± 4% and 50 ± 9% of control, respectively (Table I). Incubation with 50 μg/ml of mTSP1 reduced degradation of labeled hTSP1 and mTSP2 to 51 ± 11% and 45 ± 11% of control, respectively (Table I). Due to limited amounts and solubility of proteins, we were not able to test higher concentrations of mTSP2 or mTSP1 for cross inhibition studies. Incubations with hTSP1 at 100 μg/ml resulted in greater inhibition of degradation of labeled mTSP1 or mTSP2. 3T3 cells appeared necrotic after incubation with hTSP1 at concentrations higher than 150 μg/ml. The fact that TSP2 and TSP1 cross-inhibited degradation of each other in rough approximation to the calculated Kₘ values of degra-
tion indicates that the two proteins are internalized and degraded through similar pathways.

Inhibition of TSP2 Degradation by Heparin—It was shown previously that high capacity binding and degradation of hTSP1 by cells in culture is inhibited by heparin (30, 63–68). TSP2 differs most from TSP1 in the NH2-terminal PARP module that mediates binding to heparin (77, 78) and binds heparin less avidly than TSP1 as assessed by salt concentration re-

Effect of Heparitinase on Degradation of TSP2 by 3T3 Cells—Inhibition of degradation by PF4, a heparin-binding protein like TSP1 and TSP2, and by heparin itself suggests that TSP2 interacts with a heparan sulfate proteoglycan at some point in the degradative pathway. 3T3 cells treated with heparitinase degraded less mTSP2 (Fig. 4). The decrease in degradation of mTSP1 and hTSP1 by 3T3 cells, in contrast, was inhibited by heparin at a 6-10-fold lower concentration. Greater than 50% inhibition was achieved at a heparin concentration of 0.4–0.6 μg/ml or roughly 1:1 molar concentrations of heparin and TSP1 subunit (Fig. 3).

Metabolism of TSP2 by CHO Cells Defective in Heparan Sulfate Proteoglycans—CHO cells defective in heparan sulfate have been shown to be defective in adhesion to (28) or degradation of (66) platelet TSP1. The following cells were used to probe further the role of heparan sulfate proteoglycans in the metabolism of TSP2: K1 wild type cells, mutant 745 cells defective in xylosyltransferase and deficient in several glycosaminoglycans, mutant 803 cells specifically lacking heparan sulfate chains, and mutant 677 cells lacking heparan sulfate and having increased levels of chondroitin sulfate. Wild type K1 cells degraded TSP2 (Table II). Mutant 745 cells and 803 cells degraded much less TSP2 compared to the wild type cells. The decrease in degradation of TSP2 by the mutant 677 cells was less profound (Table II), suggesting that the excess chondroitin sulfate chains could compensate for the deficiency of heparan

**TABLE I**

Cross-inhibition of TSP degradation by each other

| Radioactive ligand | Competitor   | Degradation (% control) |
|--------------------|-------------|-------------------------|
| hTSP1              | hTSP1       | 54 ± 10                 |
|                    | mTSP1       | 51 ± 11                 |
|                    | mTSP2       | 49 ± 8                  |
| mTSP1              | hTSP1       | 64 ± 4                  |
|                    | mTSP1       | 44 ± 8                  |
|                    | mTSP2       | 71 ± 6                  |
| mTSP2              | hTSP1       | 50 ± 9                  |
|                    | mTSP1       | 45 ± 11                 |
|                    | mTSP2       | 49 ± 6                  |

**FIG. 3.** Effect of heparin on binding and degradation of TSPs. NIH 3T3 cells were incubated with binding medium containing 1 μg/ml 125I-TSP with increasing concentrations of heparin. After 4 h of incubation at 37 °C, binding medium was taken out, and A, TSP associated with cell layer and B, TSP degradation were assayed as described in Fig. 1. Results are shown as mean ± S.E. of at least four values for each heparin concentration tested and related to control values from cells incubated with no heparin.

**FIG. 4.** Effect of heparitinase on degradation of TSPs. NIH 3T3 cells were incubated with either heparitinase alone or chondroitinase ABC at 37 °C for 45 min. 125I-TSP, at a final concentration of 1 μg/ml, were then added to cells without removal of the enzyme and the incubation was carried out for additional 4 h. Degradation was measured as described for Fig. 1. To relate effects on TSP1 and TSP2, data with the two proteins are plotted against one another normalized to control (no enzyme). Error bars indicate mean ± S.D. of these determinations.
sulfate chains as previously shown for hTSP1 (66). Parallel studies of platelet hTSP1 and recombinant mTSP1 yielded similar results to those obtained with mTSP2 (Table II). These results, along with the heparitinase experiments, suggest that heparan sulfate proteoglycans are required for degradation of TSP2.

Role of Low Density Lipoprotein Receptor-related Protein in Degradation of TSP2—LRP is an endocytic receptor for the internalization and subsequent degradation of apolipoprotein E, lipoprotein lipase-enriched b-very low density lipoprotein, very low density lipoprotein, plasminogen activators, and a2-macroglobulin-proteinase complexes (79). LRP was also recently shown to be involved in the internalization and degradation of hTSP1 as assessed by the ability of RAP, a known antagonist of LRP ligand binding, to inhibit hTSP1 degradation by human lung fibroblasts and human smooth muscle cells (67, 68). RAP blocked degradation of TSP2 by 3T3 cells with an EC50 of 40–60 nM, similar to the concentrations required to block degradation of TSP1s (Fig. 5). Polyclonal anti-LRP IgG, when used at 75 μg/ml, inhibited TSP2 degradation by 3T3 cells, whereas a control IgG did not show any inhibition (data not shown). The cells did not show any obvious morphological change in the presence of RAP or the anti-LRP antibodies. These data indicate that LRP synergizes with heparan sulfate proteoglycans to mediate the degradation of TSP2 by 3T3 cells.

Immunofluorescence Studies—Immunofluorescence studies were carried out to look for microscopic evidence of the binding and degradative events found with iodinated TSPs.

**Fig. 6. Indirect immunofluorescence of the turnover of TSPs bound to NIH 3T3 cells.** Subconfluent NIH 3T3 cells were incubated with TSP in the presence of either heparin, 250 μg/ml (row C); RAP, 1 μM (row D); or chloroquine, 0.1 mM (row E). At the end of incubation, cells were washed with DME, fixed, permeabilized with chilled acetone, and stained with antibodies against hTSP1 (for hTSP1 and mTSP1 sets) or mTSP2 (for mTSP2 set) as described under “Materials and Methods.” No staining was seen if nonimmune serum was used.

| Cells | TSP degradation | Degradation | | | | |
|-------|-----------------|-------------|-----------------|-----------------|-----------------|-----------------|
|        | TSP degradation | Degradation | | | | |
|       | hTSP1 | mTSP1 | mTSP2 | hTSP1 | mTSP1 | mTSP2 |
| Wt K1 | 299 ± 28 | 422 ± 19 | 336 ± 60 | 100 | 100 | 100 |
| Mut 677 | 76 ± 9 | 177 ± 20 | 141 ± 27 | 26 | 42 | 42 |
| Mut 745 | 27 ± 6 | 121 ± 6 | 91 ± 19 | 9 | 29 | 27 |
| Mut 803 | 27 ± 10 | 119 ± 13 | 90 ± 32 | 9 | 28 | 27 |

* Wt K1 cells as 100%.

as judged by indirect immunofluorescence of permeabilized cells with anti-TSP1 or anti-TSP2 antibodies (Fig. 6, row A). This staining was relatively weak compared to staining of cells exposed to exogenous TSPs. When 20 μg/ml of mTSP2, mTSP1, or hTSP1 was incubated with intact 3T3 cells and cells were examined without permeabilization of membrane, TSP was detected bound to cell surfaces (not shown). When cells were permeabilized, a bright punctate staining pattern for the exogenously added TSP was evident that was not seen on nonpermeabilized cells incubated with exogenous TSP or permeabilized cells without incubation with exogenous TSP (Fig. 6, row B). This result indicates that TSP2 and TSP1 are endocytosed by the cells. Heparin inhibited binding and internalization of TSP2 and TSP1 to near background (Fig. 6, row C).

When binding was done in the presence of 1 μM RAP, the process of...
internalization of TSP from the cell surface bound pool to the intracellular pool was inhibited, as shown by a decrease in the punctate intracellular staining with a concomitant increase in cell surface staining (Fig. 6, row D). When chloroquine was included in the binding medium, the intracellular punctate staining was more intense and the labeled structures were larger, presumably because chloroquine inhibited the lysosomal degradation but not the internalization of TSPs (Fig. 6, row E). When cells were incubated with TSPs for 45 min and then chased for 3 h, the intracellular punctate staining disappeared, whereas if chloroquine was included in the chase medium, the punctate staining persisted (not shown).

Conclusion—Binding and degradation of TSP2 by 3T3 cells were inhibited by heparin and RAP. Heparitinase-treated 3T3 cells and CHO cells defective in synthesis of heparan sulfate proteoglycan were slower in degradation of TSP2. Binding and degradation of TSP1 and TSP2 by 3T3 cells showed different sensitivities to inhibition by heparin, consistent with sequence differences in the heparin-binding sequences of the PARP module of the two proteins (4, 6), the difference in salt concentration needed to displace TSP1 and TSP2 from immobilized heparin (58, 69), and the differential effects of heparin on cell adhesion to TSP1 and TSP2 (58). The effect of soluble heparin is probably only of mechanistic importance. Heparin potentially can exert its inhibitory effect at several steps in the TSP binding and degradation pathway. It could block the initial binding of TSPs to cell surface heparan sulfate proteoglycans, and thus inhibit the subsequent steps of uptake and degradation. Consistent with this hypothesis, heparin caused less 125I-TSP to be associated with cell layers and less cell surface TSP binding in the immunofluorescence studies. The fact that sensitivities of degradation of the two TSPs correlate with the other measures of the TSP-heparin interaction indicates that this is the major mechanism of inhibition. Heparin could also inhibit the association of cell surface bound TSPs with LRP. It was shown previously that degradation of TSP1 was inhibited by heparin oligosaccharides of 8 to 10 units that had no inhibitory effect on binding (30). One possibility is that the short heparin oligosaccharides act like RAP (67, 68) to inhibit association of TSP or heparan sulfate proteoglycan with LRP.

In addition to demonstrating that TSP1 and TSP2 have a similar degradation pathway, we found that TSP1 and TSP2 could compete with each other for degradation. This degradation is inhibited by RAP, which probably is an important physiological modulator. It was shown previously that TSP2, when existing alone, has potentially important adhesive functions for the same degradation pathway provides a mechanism for the fine control of the levels of the two TSPs in wounds and in tissues in which expression of TSP1 and TSP2 may overlap. This competition suggests that coexpression of TSP2 by neoplastic cells is a contributing factor compared to the dramatic regulation of TSP1 expression by serum or growth factors (5, 6), binding and degradation of TSP2 by cells may be the primary mechanism for control of the extracellular concentration of TSP2. One can envision a scenario whereby newly expressed or secreted TSP1, in the process of being degraded, blocks the degradation of constitutively expressed TSP2, thus buffering the cells from the effect of TSP1.

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