Identification of the Low Density Lipoprotein Receptor–related Protein (LRP) as an Endocytic Receptor for Thrombospondin-1

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Abstract. Thrombospondin-1 (TSP1) has potent biological effects on vasculature smooth muscle cells (SMCs) and endothelial cells. The regulation of extracellular accumulation of TSP1 is mediated by a previously obscure process of endocytosis which leads to its lysosomal degradation. Since members of the low density lipoprotein receptor (LDLR) family have been found to mediate endocytosis which leads to degradation of a diverse array of ligands, we evaluated their possible role in the uptake and degradation of TSP1 by vascular SMCs, endothelial cells and fibroblasts. ^{125}I-TSP1 was found to be internalized and degraded lysosomally by all these cell types. Both the internalization and degradation of ^{125}I-TSP1 could be inhibited by a specific antagonist of the LDLR family, the 39-kD receptor-associated protein (RAP). Antibodies to the LDLR-related protein (LRP) completely blocked the uptake and degradation of ^{125}I-TSP1 in SMCs and fibroblasts but not endothelial cells. Solid-phase binding assays confirmed that LRP bound to TSP1 and that the interaction was of high affinity (K_d = 5 nM). Neither RAP nor LRP antibodies inhibited the binding of ^{125}I-TSP1 to surfaces of SMCs. However, cell surface binding, as well as, endocytosis and degradation could be blocked by heparin or by pre-treatment of the cells with either heparitinase, chondroitinase or β-D–xyloside. The data indicates that cell surface proteoglycans are involved in the LRP-mediated clearance of TSP1. A model for the clearance of TSP1 by these cells is that TSP1 bound to proteoglycans is presented to LRP for endocytosis. In endothelial cells, however, the internalization of TSP1 was not mediated by LRP but since RAP inhibited TSP1 uptake and degradation, we postulate that another member of the LDLR family is likely to be involved.

THROMBOSPONDIN-1 (TSP1)[1] is a 450-kD trimeric, multifunctional glycoprotein present in platelet α-granules and expressed by a variety of cells and incorporated into their extracellular matrix (ECM) (for review see Lawler, 1986; Frazier, 1987; Asch and Nachman, 1989; Mosher, 1990; Bornstein, 1992). TSP1 is the prototypic member of a family that is currently composed of five proteins (Bornstein et al., 1991; LaBell et al., 1992; Laherty et al., 1992; Vos et al., 1992; Lawler et al., 1993). These proteins are structurally related and have similar arrangements of repeated modules such as EGF-like elements and calmodulin-like calcium-binding repeats as well as highly similar COOH-terminal domains. Although the function of TSP proteins are poorly defined, members of this family are believed to regulate a variety of processes that relate to vascular physiology including platelet aggregation, angiogenesis, and vascular cell growth (Frazier, 1987; Majack, 1988; Bouck et al., 1989; Iruela-Arispe et al., 1991; Dameron et al., 1994; Nicosia and Tuszynski, 1994).

Evidence for the regulation of cell growth by TSP1 has been found in endothelial cells, fibroblasts and smooth muscle cells (SMCs) (Majack, 1988; Phan et al., 1989; Taraboletti et al., 1990; Castle et al., 1993; Nicosia and Tuszynski, 1994). TSP1 inhibits the proliferation of endothelial cells, possibly by activation of latent transforming growth factor-β (TGF-β) (Taraboletti et al., 1990; Murphy-Ullrich et al., 1992; Schultz-Cherry and Murphy-Ullrich, 1993). In addition, TSP1 and fragments of TSP1 are able to directly inhibit tube formation by endothelial cells in vitro and neovascularization in vivo (Iruela-Arispe et al., 1991; Tolsma et al., 1993). In contrast, TSP1 is able to stimulate the migration and proliferation of SMCs and fibroblasts (Majack, 1988; Phan et al., 1989; Yabkowitz et al., 1993; Nicosia and Tuszynski, 1994). In vivo studies us-

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1. *Abbreviations used in this paper*: TSP1, thrombospondin-1; LDLR, low density lipoprotein receptor; LRP, low density lipoprotein receptor–related protein; VLDLR, very low density lipoprotein receptor; gp330, glycoprotein 330; RAP, receptor–associated protein; TGF–β, transforming growth factor–β; VN, vitronectin; FN, fibronectin; SMC, smooth muscle cells.

ing a rat balloon-catheter injury model indicate that increased expression of TSP by SMCs is an early response to injury and closely correlated with SMC proliferation (Raugi et al., 1990). Additionally, elevated levels of TSP have been detected in human and porcine atherosclerotic lesions that correlated with the presence of phenotypically modulated SMCs (Wight et al., 1985; Liau et al., 1993). These findings implicate TSP1 in the dynamic processes of tissue remodeling associated with the response to vascular injury.

Because of the potent biological activities of TSP1, it is reasonable to speculate that a mechanism to tightly regulate its expression at the level of synthesis as well as its extracellular half-life is required. In general, catabolism of ECM proteins occurs through a process of extracellular proteolysis (Tryggvason et al., 1987). For example, elastin and collagens are degraded by secreted proteases such as neutrophil elastase and type IV collagenase. Extracellular levels of proteins such as TSP1 and vitronectin (VN), however, have been found to be regulated by a cell-mediated process of endocytosis leading to lysosomal degradation (McKeown-Longo et al., 1984; Murphy-Ullrich and Mosher, 1987; Pannetti and McKeown-Longo, 1993*a*,*b*). The mechanism underlying the cell-mediated turnover of these proteins is poorly understood. In this manuscript we report findings of experiments that focused on characterizing the mechanism by which vascular cells (e.g., endothelial and SMCs) endocytose and degrade TSP1.

Materials and Methods

Cells

Human saphenous vein smooth muscle cells were provided by Dr. Peter Libby (Brigham and Women's Hospital, Harvard Medical School, Boston, MA) and grown in M199 medium, 10% fetal calf serum (Intergen, Purchase, NY), 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO BRL, Gaithersburg, MD). Human WI38 lung fibroblasts (ATCC CCL 75) (American Type Culture Collection, Rockville, MD) were grown in DME (Mediatech, Washington, D.C.) supplemented with 10% iron-enriched bovine calf serum (Hyclone, Logan, UT), penicillin/streptomycin, and 1 mM sodium pyruvate. Human umbilical vein endothelial cells were obtained from Dr. Thomas Maciag (American Red Cross, Rockville, MD) and were grown on fibronectin-coated surfaces according to Maciag et al. (1981).

Proteins

TSP1 was purified from human platelets by adsorption to barium citrate followed by heparin–agarose chromatography according to Alexander and Detwiler (1984). Human VN (conformationally altered form) was provided by Dr. Paula McKeown-Longo (Albany Medical College, Albany, NY). Human FN was purified from plasma according to Miekka et al. (1982). LRP was isolated from human placenta by α_2-macroglobulin/methylamine–Sepharose affinity chromatography as previously described (Ashcom et al., 1990). Gp330 from porcine kidney brush border membrane extracts was isolated by RAP-Sepharose affinity chromatography as previously described (Kounnas et al., 1992*b*; Kounnas, et al., 1993). Human RAP, expressed as a glutathione S-transferase fusion protein in bacteria, was prepared (free of glutathione S-transferase) as outlined by Williams et al. (1992). Pro-urokinase (pro-uPA) was provided by Dr. Jack Henkin (Abbott Laboratories, Abbott Park, IL). BSA fraction V, heparitinase III, and chondroitinase ABC were purchased from Sigma Chemical Co. (St. Louis, MO). The synthetic peptides GRGDSP and GRGESP were made on a Milligen peptide synthesizer (model 9050) and purified by reverse phase HPLC using a C-18 Delta Pak column (Millipore, Bedford, MA).

Antibodies

IgG was isolated from rabbit polyclonal anti–LRP serum (rb777) (Kounnas et al., 1992*a*) by affinity chromatography on protein G-Sepharose (Pharmacia) followed by absorption on RAP-Sepharose and then affinity selection on LRP-Sepharose (1–2 mg protein/ml resin). IgG from the rabbit polyclonal antiserum raised against a synthetic peptide corresponding to the last 11 residues of the LRP cytoplasmic domain (rb704) (Kounnas et al., 1992*a*) was isolated by protein G-Sepharose chromatography. The mouse monoclonal antibody to rat gp330 designated 1H2 was provided by Dr. Robert McCluskey (Harvard/Massachusetts General Hospital, Boston, MA). The mouse monoclonal antibody to the 515-kD heavy chain of human LRP designated 8G1 has been described previously (Strickland et al., 1991). IgG was purified on protein G-Sepharose from each of the mouse ascitic fluids.

Solid-phase Binding Assays

Homologous ligand displacement assays were conducted according to methods previously outlined by Williams et al. (1992). Briefly, microtiter wells were coated with LRP, gp330 or BSA (3 μg/ml) in TBS, pH 8.0, 5 mM $CaCl_2$ for 4 h at 37°C, non-specific sites were blocked with 3% BSA, 5 mM $CaCl_2$ in TBS, pH 8.0. The wells were then incubated with radiolabeled TSP1 (5 nM) in the same buffer plus 0.05% Tween-20 and varying concentrations of unlabeled competitor for 18 h at 4°C. The binding data was analyzed and dissociation constants (K_d) were determined by using the computer program LIGAND (Munson and Rodbard, 1980).

Cell-mediated Ligand Binding, Internalization, and Degradation Assays

Cell assays were carried out according to procedures previously described (Kounnas et al., 1993) using subconfluent monolayers of cells grown in 24-well plates (1.9 cm^2) (Corning, Corning, NY). Prior to addition of radioactive ligands the cells were washed and incubated in either M199 or DME containing 10 mM Hepes, Nutridoma serum substitute (Boehringer Mannheim Biochemicals), penicillin/streptomycin, and 0.2% BSA (assay medium) for 0.5 h at 37°C. Radioiodination of ligands was performed using Iodogen (Pierce Chemical Co., Rockford, IL) and specific activities ranging from 2–5 μCi/μg protein were typically achieved. For cell surface binding assays, the cells were washed with ice cold assay medium and then incubated on ice for 15 min in a cold room. Radiolabeled TSP1 (2 nM) in assay medium (4°C) was then added to the cell layers in the presence of 1 mM concentrations of either TSP1, RAP, or pro-uPA. For RGD and RGE treatments, 2 mM doses of each peptide in assay medium was used as competitor. For heparin treatment, 10 mg/ml heparin (grade I; Sigma Chemical Co.) in assay medium (~660 nM) was used. For ligand internalization and degradation assays cells were preincubated for 0.5 h at 37°C, 5% CO_2, with the above mentioned doses of competitors. ^{125}I-TSP1 (2 nM) was then added and incubated with the cells for 4 h at 37°C, 5% CO_2. To inhibit lysosomal protease activity, the cells were treated with 0.1 mM chloroquine (Sigma Chemical Co.) for 0.5 h at 37°C, 5% CO_2 and throughout the duration of the uptake and degradation assays. Radioactivity in the cell medium that was soluble in 10% TCA was taken to represent degraded ligand. Total ligand degradation was corrected for the amount of degradation that occurred in radioligand-containing medium lacking cells. To determine the amount of ^{125}I-ligand that was internalized, the cells were washed three times with isotonic PBS and then treated with serum-free medium containing 0.5 mg/ml trypsin, 0.5 mg/ml proteinase K (Sigma Chemical Co.) and 0.5 mM EDTA for 2–4 min at rt. The cells were then centrifuged at 6,000 *g* for 4 min and the amount of radioactivity in the cell pellet was measured. For VN uptake and degradation experiments, the concentration of ^{125}I-VN added to the cells was 8 nM and the concentration of VN used as competitor was 1.3 mM.

Heparan sulfate and chondroitin sulfate were enzymatically removed from cell surfaces by treatment with heparinase III (heparitinase) or chondroitinase ABC. To determine the optimal dose of each enzyme, cells were metabolically labeled with ^{35}S-labeled $NaSO_4$ (150 μCi/ml) for 18 h, washed with PBS, and incubated with different concentrations of enzyme (heparitinase, 0.25–10 U/ml or chondroitinase, 0.05–1 U/ml) for 1.5 h at 37°C in 5% CO_2. Both the amount of ^{125}I-ligand–derived radioactivity released into the medium and the amount of radioactivity remaining associated with the cell layer (extractable with 0.2 N NaOH) were measured by liquid scintillation counting.

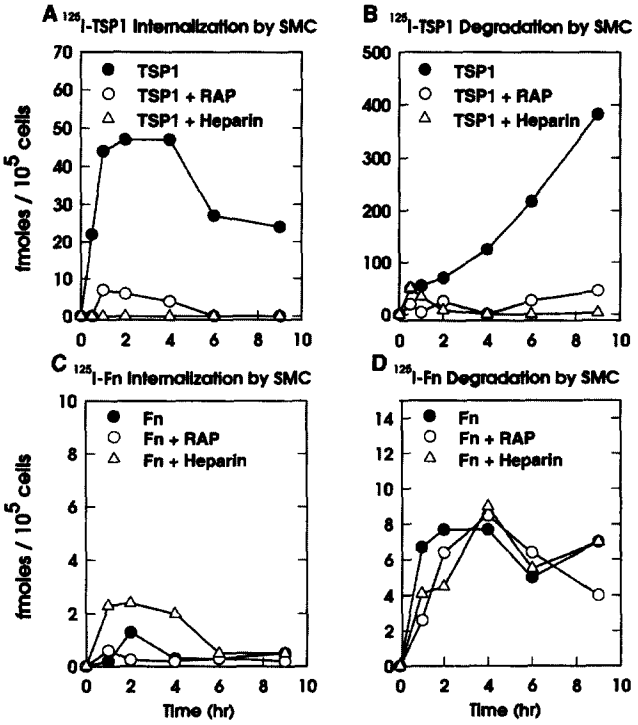

Figure 1. Kinetics of the internalization and degradation of ^{125}I-TSP1 and ^{125}I-FN by cultured human SMCs. ^{125}I-TSP1 (*A* and *B*) and ^{125}I-Fn (*C* and *D*) were incubated with cultured SMCs for various time periods at which the amount of each ligand internalized and degraded were determined. RAP (1 μM) or heparin (660 nM) were coincubated with the radiolabeled ligands throughout the course of the experiment. The plotted data is corrected for non-specific binding.

To inhibit the synthesis of proteoglycans, cultured cells were treated with *p*-nitrophenyl-β-D-xylopyranoside which competes with xylose-substituted core proteins as a substrate for galactosyltransferases (Schwartz, 1977). Cultured cells were grown for 3 d in complete medium containing 1 mM *p*-nitrophenyl-β-D-xylopyranoside (Sigma Chemical Co.) and then used for radioligand internalization and degradation assays.

Results

Smooth Muscle Cells Internalize and Degrade TSP1

Fibroblasts and endothelial cells have been previously demonstrated to internalize and degrade TSP1 (McKeown-Longo et al., 1984; Murphy-Ullrich and Mosher, 1987). However, evidence for the catabolism of TSP1 by the other major vascular cell type, SMCs has not been established. Given that TSP has potent biological effects on both SMCs as well as endothelial cells, we were interested in determining whether vascular SMCs were also capable of mediating TSP1 clearance. As shown in Fig. 1 *A*, ^{125}I-TSP1 was rapidly internalized by cultured SMCs, reaching a maximum level within 1 h. Fig. 1 *B* demonstrates the time course for SMC degradation of ^{125}I-TSP1 as measured by the appearance of TCA soluble radioactivity in the medium. The degradation of TSP1 was shown to be inhibitable by chloroquine (Fig. 2 *C*), a drug that blocks lysosomal protease activity through its ability to increase the pH of endocytic vesicles. The chloroquine affect suggested that TSP1 was delivered to lysosomes following its endocytosis. Both the internalization and degradation of ^{125}I-TSP1 by SMCs were completely inhibited by treatment with heparin which has been shown to block these processes in fibroblasts and endothelial cells (McKeown-Longo et al., 1984; Murphy-Ullrich and Mosher, 1987). The results show that SMCs are capable of efficiently endocytosing and then lysosomally degrading TSP1.

The LDLR family of receptors are involved in the uptake and degradation of a variety of functionally diverse ligands (Kounnas et al., 1994; Williams et al., 1994). To evaluate the potential role of members of the LDLR family in the catabolism of TSP1, RAP, an antagonist of ligand

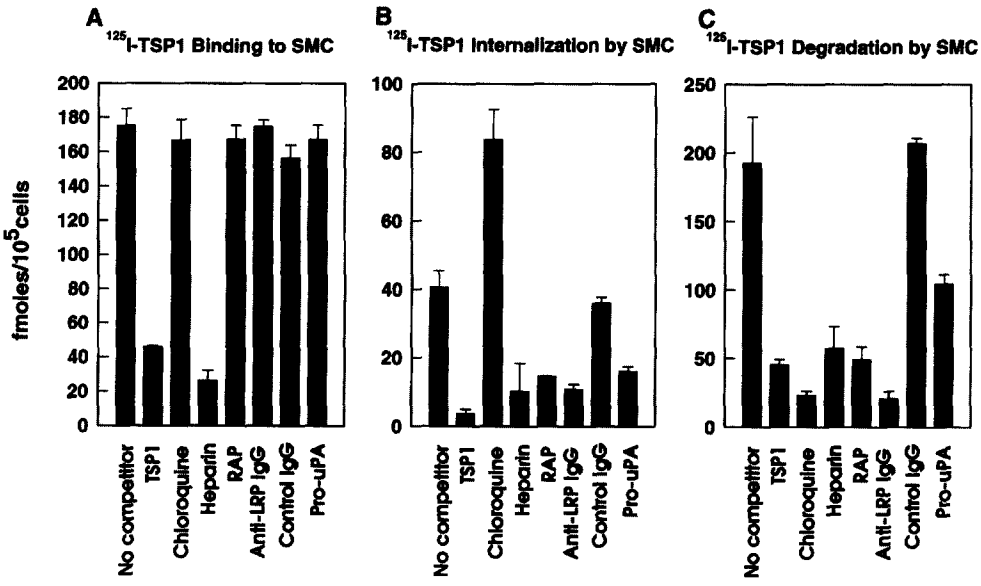

Figure 2. ^{125}I-TSP1 binding, internalization and degradation by human vascular SMCs. ^{125}I-TSP1 (2 nM) was incubated with SMCs at 4°C for 3 h. Competition of binding was done by co-incubating radiolabeled TSP1 with 1 μM concentrations of either TSP1, RAP, and prourokinase (pro-uPA). For antibody treatment, 100 μg/ml of blocking anti-LRP IgG (rb777) or control IgG (rb704) were used. For heparin treatment, a concentration of 660 nM was used. For cell internalization and degradation experiments SMCs were pre-incubated for 0.5 h at 37°C, 5% CO_2 with the above mentioned doses of competitors. ^{125}I-TSP1 (2 nM) was then added and incubated with cells for 4 h at 37°C, 5% CO_2. To inhibit lysosomal protease activity, SMCs were treated with 0.1 mM chloroquine for 30 min at 37°C, 5% CO_2 prior to the addition of radiolabeled ligand and the drug was present throughout the duration of the internalization and degradation assays.

binding to members of the LDLR family, was used in the ^{125}I-TSP1 uptake and degradation assays. As shown in Fig. 1, ^{125}I-TSP1 uptake and degradation were both blocked by RAP. In comparison with TSP1, the catabolism of another extracellular matrix protein, FN was also evaluated. As shown in Fig. 1, *C* and *D*, little if any ^{125}I-FN was internalized and degraded by SMCs and neither heparin nor RAP were inhibitory. The results indicate that TSP1 but not FN is internalized and degraded by SMCs and that a member of the LDLR family is likely to be involved in this catabolic process.

LRP Mediates Internalization and Degradation of TSP1

We next examined SMCs for the presence of LDLR family members that might mediate TSP1 uptake. Immunoblotting of SMC extracts showed that LRP was present, however, gp330 was not detectable (Fig. 3 *A*). Polyclonal LRP antibodies that have been shown to block the function of LRP in other cell types (Kounnas et al., 1993; Chappell et al., 1993) were then used in an attempt to perturb ^{125}I-TSP1 uptake and degradation in SMCs. As shown in Fig. 2, *B* and *C*, the LRP antibodies inhibited the internalization and degradation of ^{125}I-TSP1. As a negative control for these experiments, polyclonal antibodies elicited against the cytoplasmic domain of LRP (indicated as control IgG in Fig. 2) showed no effect. The LRP ligand pro-uPA, also effectively competed for the internalization and degradation of ^{125}I-TSP1 by SMCs. The results indicate that LRP is expressed by SMCs and mediates endocytosis of TSP1. However, since neither LRP antibodies, RAP or pro-uPA inhibited cell layer-binding of TSP1 (Fig. 2 *A*), LRP is apparently not the initial or the predominant binding moiety on the cell surface.

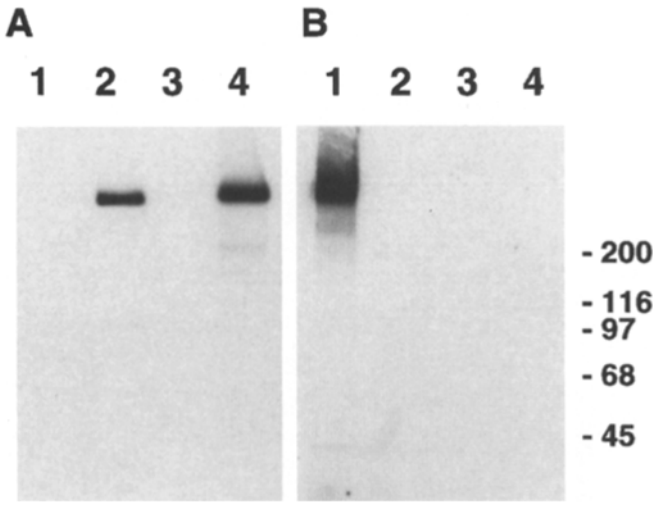

Figure 3. Immunological detection of LRP and gp330 in cell extracts. Detergent extracts of F9 teratocarcinoma cells (grown in retinoic acid and dibutylcyclic AMP containing medium) (lane *1*), WI38 lung fibroblasts (lane *2*), human umbilical vein endothelial cells (lane *3*), and human saphenous vein smooth muscle cells (lane *4*) were electrophoresed on 4–12% polyacrylamide gels in the presence of SDS, transferred to nitrocellulose membranes, and immunologically stained using polyclonal LRP antibody (affinity selected on LRP-Sepharose) (*A*) or gp330 antibody (monoclonal antibody 1H2) (*B*). The migration positions of molecular mass standards are indicated on the right in kD.

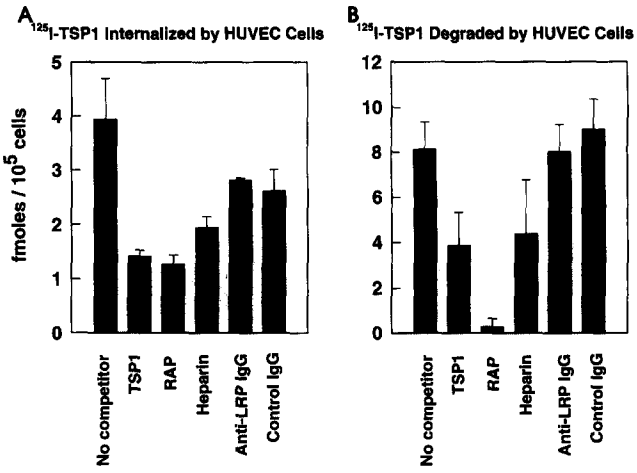

Figure 4. ^{125}I-TSP1 internalization and degradation by human saphenous vein endothelial cells. For cell internalization and degradation experiments, endothelial cells were pre-incubated for 0.5 h at 37°C, 5% CO_2 with 1 μM concentrations of either TSP1, RAP or 660 nM of heparin. For antibody treatment, 100 μg/ml of blocking anti-LRP IgG (rb777) or control IgG (rb704) were used. ^{125}I-TSP1 (2 nM) was then added and incubated with cells for 4 h and the amount of TSP1 internalized or degraded was subsequently measured.

The uptake and degradation of ^{125}I-TSP1 by fibroblasts and endothelial cells were also investigated. Fibroblasts efficiently internalized and degraded ^{125}I-TSP1 with kinetics similar to those observed in SMCs (data not shown). In addition, heparin, RAP and antibodies to LRP blocked both the internalization and degradation of ^{125}I-TSP1 by fibroblasts. In contrast, endothelial cells internalized ~10-fold lower levels of ^{125}I-TSP1 as compared to fibroblasts or SMCs and antibodies to LRP had no effect (Fig. 4). The latter results were consistent with the immunoblotting data (Fig. 3 *A*) indicating that LRP was not detected in extracts of endothelial cells. However, the finding that RAP inhibited ^{125}I-TSP1 internalization and degradation in endothelial cells (Fig. 4) suggests that some LDLR family member might be involved. Since neither LRP nor gp330 seem to be expressed by endothelial cells (Fig. 3, *A* and *B*) the remaining members of the family, VLDLR and LDLR, must be considered as potential mediators of the process.

LRP and gp330 Bind to TSP1 in Solid-phase Binding Assays

Purified LRP and gp330, two members of the LDLR family, were evaluated for their ability to bind TSP1 in solid-phase binding assays. ^{125}I-TSP was found to bind to microtiter wells coated with LRP or gp330 but not BSA (Fig. 5). The binding was competitively inhibited by increasing concentrations of unlabeled TSP1. A dissociation constant (K_d) of 12 nM (n = 2) was derived for the binding of TSP1 to LRP from the best-fit of the data to a single class of sites. Similarly, a K_d of 5 nM (n = 2) was determined for the binding of TSP1 to gp330. In addition, the LRP and

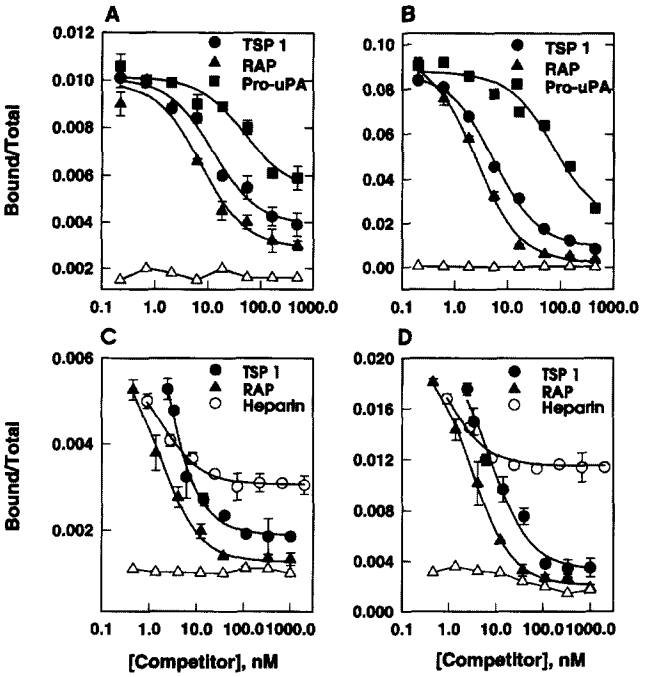

Figure 5. TSP binds to LRP and gp330 in solid phase assay. ^{125}I-TSP1 (2 nM) was incubated in the presence of the indicated concentrations of unlabeled TSP1 (●), RAP (▲), pro-uPA (■), or heparin (○) with microtiter wells coated with LRP *(A* and *C)* or gp330 *(B* and *D).* The binding of ^{125}I-TSP1 in the presence of increasing concentration of unlabeled TSP to wells coated with BSA is shown as a control (△). The data are expressed as the quotient of the amount of radioactivity bound over the total radioactivity added per well and averaged from duplicate determinations. The curves represent the best-fit of the data to a single class of sites. The K_d value measured for the binding of TSP1 to LRP was 12 nM. The K_i values measured for the inhibition of TSP1 binding to LRP by RAP and pro-uPA were 7 and 49 nM, respectively.

gp330 ligands, RAP and pro-uPA (Kounnas et al., 1992, 1993), were found to both compete for binding of ^{125}I-TSP to LRP or gp330 (Fig. 5). Inhibition constants (K_i) of 7 and 2 nM were determined for the inhibition of ^{125}I-TSP binding to LRP or gp330 by RAP, respectively. For the inhibition of ^{125}I-TSP1 binding to LRP and gp330 by pro-uPA, the K_is were 49 and 72 nM, respectively. Given that heparin blocked TSP1 cell surface binding, uptake and degradation, we evaluated its effects on the binding of ^{125}I-TSP1 to LRP and gp330 in solid phase assay. As shown in Fig. 5, *C* and *D*, heparin had a partial inhibitory effect as compared to RAP and excess unlabeled TSP1. The findings indicate that the receptors LRP and gp330 can bind to TSP1 with high affinity and that RAP can completely block ligand binding whereas heparin is a poor inhibitor.

RGD Peptide Does Not Inhibit TSP1 Uptake and Degradation by SMCs or Fibroblasts

Given that the vitronectin receptor $\alpha_v\beta_3$ has been implicated as a receptor for TSP1 (Lawler et al., 1988; Adams and Lawler, 1993), we examined the effect of an antagonist of its ligand binding activity, an Arg-Gly-Asp (RGD) containing synthetic peptide, on the processes of ^{125}I-TSP1 endocytosis and degradation. As shown in Table I, neither the RGD peptide nor the Arg-Gly-Glu (RGE) peptide inhibited ^{125}I-TSP1 internalization or degradation in SMCs. Similarly, the uptake and degradation of ^{125}I-TSP1 by lung fibroblasts was not affected by the RGD peptide (Table I.). To verify the functionality of the RGD peptide, it was used to perturb the cellular internalization and degradation of ^{125}I-VN. VN has been previously shown to be internalized and degraded by lung fibroblasts and both processes could be blocked by RGD-containing peptides as well as by heparin (Panetti and McKeown-Longo, 1993). As shown in Table II, the internalization and degradation of ^{125}I-VN by lung fibroblasts were inhibited by RGD but not by the control RGE-containing peptide. These results demonstrate the efficacy of the RGD peptides that failed to inhibit ^{125}I-TSP1 catabolism by SMCs. The findings indicate that TSP1 internalization and degradation by SMCs and fibroblasts is not dependent on an RGD interaction as is the case for the internalization and degradation of VN.

Proteoglycans Have a Role in Binding, Internalization, and Degradation of TSP1

Heparin has been previously reported to inhibit the binding and degradation of ^{125}I-TSP1 by fibroblasts (McKeown-Longo et al., 1984) and endothelial cells (Murphy-Ullrich and Mosher, 1987). In our experiments, heparin also inhibited cell-binding, internalization and degradation of ^{125}I-TSP1 by SMCs (Figs. 1 and 2, and Table I). Similar results were observed in lung fibroblasts and saphenous vein endothelial cells (Table I and Fig. 4). The inhibitory effects of heparin have been postulated to indicate that a heparin-like molecule (e.g., proteoglycan) might mediate cell-binding of TSP1 which leads to its endocytosis and degradation (Murphy-Ullrich and Mosher, 1987; Murphy-

Table I. Effects of Competitors on the Internalization and Degradation of ^{125}I-TSP by Human Lung Fibroblasts and Smooth Muscle Cells

	Lung fibroblasts		Smooth muscle cells	
	Internalized	Degraded	Internalized	Degraded
Control	38 ± 0.1	140 ± 16	28 ± 1.6	160 ± 30
TSP	6 ± 1 (83%)	7 ± 1 (95%)	11 ± 2 (60%)	17 ± 2 (90%)
RAP	8 ± 1 (79%)	36 ± 2 (75%)	7 ± 0.4 (75%)	45 ± 4 (72%)
Heparin	1 ± 0.1 (96%)	1 ± 2 (99%)	1 ± 0.1 (96%)	2 ± 1 (98%)
RGD	33 ± 0.2 (11%)	122 ± 3 (13%)	24 ± 0.7 (14%)	153 ± 14 (5%)
RGE	32 ± 3 (14%)	132 ± 15 (6%)	28 ± 0.7 (*)	165 ± 5 (*)
Anti-LRP IgG	16 ± 3 (57%)	52 ± 8 (63%)	14 ± 0.1 (50%)	55 ± 7 (65%)
Control IgG	27 ± 0.6 (28%)	137 ± 7 (3%)	25 ± 0.1 (10%)	152 ± 5 (5%)
VN	18 ± 0.2 (52%)	78 ± 0.1 (44%)	17 ± 2 (40%)	100 ± 9 (37%)

Values represent means ± SD of the amount of fmol of ^{125}I-TSP internalized or degraded/10^5 cells/4 h incubation in the absence (control) or presence of the indicated competitors. Values in parentheses represent inhibition as compared to results obtained from cells without added competitor. An asterisk indicates a negative percent inhibition value.

Table II. Effects of Competitors on the Internalization and Degradation of ^{125}I-VN by Human Lung Fibroblasts and Smooth Muscle Cells

	Lung fibroblasts		Smooth muscle cells	
	Internalized	Degraded	Internalized	Degraded
Control	279 ± 1	1088 ± 52	54 ± 8	412 ± 65
VN	15 ± 0.1 (95%)	101 ± 0.1 (90%)	9 ± 1 (83%)	22 ± 6 (95%)
RAP	295 ± 5 (*)	1264 ± 42 (*)	42 ± 0.8 (22%)	368 ± 11 (11%)
Heparin	73 ± 2 (74%)	236 ± 5 (78%)	18 ± 1 (66%)	40 ± 0.2 (90%)
RGD	18 ± 1 (94%)	180 ± 5 (61%)	42 ± 2 (22%)	230 ± 2 (44%)
RGE	265 ± 5 (*)	958 ± 51 (2%)	55 ± 2 (*)	514 ± 54 (*)
Anti-LRP IgG	270 ± 3 (4%)	1065 ± 26 (3%)	53 ± 1.6 (2%)	430 ± 19 (*)
Control IgG	269 ± 4 (4%)	983 ± 33 (10%)	51 ± 2.3 (5%)	390 ± 27 (5%)
TSP1	99 ± 5 (64%)	189 ± 25 (82%)	29 ± 1.6 (46%)	36 ± 2 (91%)

Values represent means ± SD of the amount of fmol of ^{125}I-VN internalized or degraded/10^5 cells/7 h in the absence (control) or presence of the indicated competitors. Values in parentheses represent percent inhibition as compared to results obtained from cells without added competitor. An asterisk indicates a negative percent inhibition value.

Ullrich et al., 1988). In our study, heparitinase or chondroitinase treatment of SMCs resulted in reduced binding, internalization and degradation of ^{125}I-TSP1 (Table III). However, the magnitude of inhibition of binding and internalization achieved with each enzyme treatment alone was not as great as that obtained with heparin treatment. In control experiments, the doses of heparitinase and chondroitinase used were shown to be optimal (data not shown). Thus incomplete digestion can be ruled out for the difference in inhibitory activity between the enzyme and heparin treatment. The data suggests that both chondroitin sulfate and heparin sulfate proteoglycans can contribute to the clearance process. As further evidence for the involvement of proteoglycans in the uptake and degradation of ^{125}I-TSP1, cells treated with β-xyloside to inhibit

Table III. Effect of Heparitinase and Chondroitinase Treatment on the Binding, Internalization, and Degradation of ^{125}I-TSP1 by SMC

	Control	Heparitinase treated	Chondroitinase treated	Heparin treated
Surface bound*	111 ± 1	80 ± 1 (30%)	80 ± 10 (30%)	0 (100%)
Internalized	48 ± 3	28 ± 4 (42%)	25 ± 1 (48%)	0 (100%)
Degraded	143 ± 21	13 ± 3 (91%)	38 ± 10 (74%)	0 (100%)

Values represent means ± SD of triplicate determinations of the amount of fmol of ^{125}I-TSP1 bound, internalized or degraded/10^5 cells. (*) For ^{125}I-TSP1 cell surface binding experiments, ^{125}I-TSP1 was incubated with the cells for 3 h at 4°C. For internalization and degradation experiments ^{125}I-TSP1 was incubated with the cells for 4 h at 37°C in 5% CO_2. All values have been corrected for non-specific binding. Cells were treated with either enzyme for 2 h at 37°C in 5% CO_2 prior to the addition of the radioligand. Values in parentheses represent percent inhibition as compared to results obtained from untreated control cells.

Table IV. Effects of β-D-Xylopyranoside on the Internalization and Degradation of ^{125}I-TSP by Human Lung Fibroblasts and Smooth Muscle Cells

	Lung fibroblasts		Smooth muscle cells	
	Internalized	Degraded	Internalized	Degraded
Control	51 ± 0.3	131 ± 0.7	36 ± 0.8	150 ± 27
TSP	6 ± 0.1 (89%)	26 ± 1 (80%)	12 ± 2 (66%)	37 ± 2 (75%)
RAP	11 ± 0.7 (78%)	38 ± 6 (71%)	12 ± 0.7 (66%)	34 ± 3 (77%)
Heparin	8 ± 1 (84%)	33 ± 4 (75%)	7 ± 1 (80%)	15 ± 2 (90%)
β-Xyloside	13 ± 1 (75%)	44 ± 3 (66%)	20 ± 0.6 (44%)	13 ± 6 (91%)

Values represent means ± SD of triplicate determinations of the amount of fmol of ^{125}I-TSP internalized or degraded/10^5 cells/4 h in the absence (control) or presence of competitors or in β-D-xyloside treated cells. Values in parentheses represent percent inhibition as compared to results obtained from cells without added competitor.

proteoglycan synthesis did not internalize and degrade ^{125}I-TSP1 (Table IV). Taken together, the results suggest that an intermediate and possibly obligatory step in the catabolism of TSP1 via LRP involves TSP1 interaction with proteoglycans. It has been shown that heparan sulfate proteoglycans are also involved with the catabolism of VN (Panetti and McKeown-Longo, 1993*a*,*b*). The ability of VN to inhibit the TSP1 uptake and degradation and vice versa (Tables I and II) may be the result of competition for binding to proteoglycans, a step that seems to precede the endocytosis of each ligand via distinct receptors.

Discussion

The goal of this research effort was to elucidate the mechanism underlying the cell-mediated turnover of TSP1. The endocytosis and degradation of TSP1 by SMCs, endothelial cells and fibroblasts were studied and the data presented herein indicate that members of the LDLR family (Kounnas et al., 1994; Williams et al., 1994) are responsible for this process. Evidence for their participation was demonstrated by the ability of RAP, a specific antagonist of the ligand binding function of receptors belonging to the LDLR family, to inhibit TSP1 internalization in all the cell types examined. In SMCs and fibroblasts, LRP, a member of the LDLR family, mediates the endocytosis which leads to degradation of exogenously added TSP1. This conclusion was supported by the ability of antagonists of LRP function such as, LRP antibodies, RAP, and the LRP ligand, pro-uPA, to compete for TSP1 uptake and degradation. In addition, solid-phase binding assays confirmed that LRP could bind to TSP1 with high affinity. In endothelial cells much lower levels of TSP1 were internalized and degraded as compared to SMCs or fibroblasts. Results from immunoblotting showed that LRP was not detectable in extracts of endothelial cells and LRP antibodies had no effect on TSP1 internalization and degradation. These results suggest that endothelial cells endocytose and degrade TSP1 via some receptor other than LRP. However, given that RAP acts as an antagonist for TSP1 uptake and degradation in endothelial cells, the endocytic receptor is likely to be a member of the LDLR family.

We have demonstrated that at least one other member of the LDLR family, gp330, is capable of binding to TSP1 in solid-phase binding assays. Our preliminary evidence indicates that gp330 can mediate uptake of TSP1 in cultured F9 embryonal carcinoma cells (Argraves, Liau and Stefansson, unpublished observation). As of yet we do not know if or where gp330-mediated clearance of TSP1 is physiologically relevant. Gp330 expression in vivo is restricted to specialized absorptive epithelia such as in lung alveoli, kidney proximal tubules and brain ependyma (Kounnas et al., 1994; Zheng et al., 1994). It is not immunologically detectable in blood vessels or in cultured vascular cells, ruling out the likelihood that the observed clearance of TSP1 by endothelial cells is mediated by this receptor. Other candidates for the receptor responsible for mediating TSP1 clearance in endothelial cells are the LDLR and VLDLR. The ability of multiple members of the LDLR family to bind to a common ligand is a general functional property of members of this family. We have established that at least three members of the LDLR family are involved with TSP1 clearance, suggesting that this family is the principle means by which TSP1 is catabolized.

A role for proteoglycans in TSP1 endocytosis had previously been proposed based on the ability of heparin to inhibit both TSP1 uptake and degradation by cultured cells (McKeown-Longo et al., 1984). Furthermore, heparitinase digestion of endothelial cells was shown to reduce TSP1 binding (Murphy-Ullrich and Mosher, 1987) and, Chinese Hamster Ovary cells, deficient in glycosaminoglycan biosynthesis, were shown to be unable to bind and degrade TSP1 (Murphy-Ullrich et al., 1988). In our experiments, heparin as well as heparitinase, chondroitinase or β-D-xyloside treatment of cells reduced the uptake and degradation of ^{125}I-TSP1. Additionally, heparin completely blocked cell surface binding of TSP1 yet had only a modest effect on TSP1 binding to LRP. We interpret the results to indicate that the binding of TSP1 to glycosaminoglycan moieties of proteoglycans is a primary step in LRP-mediated internalization of TSP1. A similar model of endocytosis involving initial binding to proteoglycans and subsequent endocytosis via LRP has been proposed for three other LRP ligands, lipoprotein lipase (Chappell et al., 1993) and apolipoprotein E (Ji et al., 1993, 1994) and hepatic lipase (Kounnas et al., 1995). Additionally, the binding of basic fibroblast growth factor and TGF-β to their respective signalling receptors (Andres et al., 1989; Yayon et al., 1991; Massague, 1991; Lopez-Casillias, 1993) also involves initial binding to cell surface proteoglycans. The fact that RAP completely blocks cellular uptake of TSP1 suggests that the TSP1-binding proteoglycans are themselves unable to mediate ligand internalization. It remains to be determined whether the proteoglycans are internalized along with the LRP ligands or whether the two dissociate prior to LRP-mediated endocytosis.

TSP1 is an example of a matrix protein present in transitional matrices such as wounds, clots and areas undergoing developmental morphogenesis (Wight et al., 1985; Raugi et al., 1987, 1990; O'Shea and Dixit, 1988; Reed et al., 1993; Lahav, 1993). Our results suggest that a component of its dynamic expression is the control of its extracellular accumulation through the process of LRP-mediated clearance. The cellular clearance of proteins, like TSP1, that have potent biological activity is likely a general in vivo phenomenon. For example, VN, which regulates the activity of thrombin and plasminogen activator and the terminal complement complex C5b-9 (Salonen et al., 1989; Tomasini and Mosher, 1990; Ciambrone and McKeown-Longo, 1992; Keijer et al., 1991) is endocytosed via sequential receptor binding involving heparan sulfate proteoglycans and the integrin $\alpha_v\beta_5$ (Panetti and McKeown-Longo, 1993*a*,*b*). In agreement with the findings of Panetti and McKeown-Longo (1993*b*) we have shown that the internalization of ^{125}I-VN by fibroblasts is entirely mediated via an RGD-inhibitable pathway. On the other hand, in SMCs a component of the ^{125}I-VN internalized and degraded can be inhibited by RAP, suggesting a potential role for members of the LDLR family in its catabolism by SMCs. It remains to be established whether other transitional ECM proteins including other members of the TSP family are cleared via the action of LDLR family members.

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