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Specific Involvement of Glypican in Thrombin Adhesive Properties

Rachel Bar-Shavit, Miriam Maoz, Yoav Ginzburg, and Israel Vlodavsky

Department of Oncology, Hadassah-University Hospital, Jerusalem 91120, Israel

Abstract We have previously demonstrated that thrombin possesses an active yet cryptic Arg-Gly-Asp (RGD) site which upon exposure induces endothelial cell (EC) adhesion via $\alpha_v\beta_3$ integrin [Bar-Shavit et al. (1991): J Cell Biol 112:335]. This was achieved in the presence of cell surface–associated heparan sulfate proteoglycans (HSPG) and exceedingly low concentrations of plasmin [Bar-Shavit et al. (1993): J Cell Biol 123:1279]. A portion of the cell surface–associated HSPG (glypican) is anchored via a covalently linked glycosyl-phosphatidylinositol (PI) residue, which can be released by treatment with glycosyl-PI-specific phospholipase C (PI-PLC). We report here that exposure of either bovine aortic EC, smooth muscle cells (SMC), or wild-type CHO cells to PI-PLC released HSPG involved in the conversion of thrombin to an adhesive molecule. The adhesion-promoting activity of the released HSPG was abolished following treatment with heparinase but not chondroitinase ABC. Incubation of thrombin with heparan sulfate–deficient CHO cells or cells that were pretreated with PI-PLC failed to induce its conversion to an adhesive molecule, indicating that glypican was playing a major role in this conversion. Moreover, affinity-purified glypican, but not syndecan or fibroglycan, elicited efficient conversion of plasmin-treated thrombin into an adhesive molecule. Antibodies raised against the RGD site in thrombin failed to interact with native thrombin, prothrombin, or the RGD site in other adhesive proteins such as vitronectin, fibrinogen, or fibronectin. Anti-thrombin-RGD antibodies which blocked the adhesion-promoting activity of thrombin were also capable of recognizing thrombin that was first incubated with a suboptimal concentration of plasmin in the presence of PI-PLC-released HSPG. Heparin, heparan sulfate, and PI-PLC-released HSPG had no effect on other cellular properties of thrombin such as receptor binding and growth-promoting activity. Altogether we have demonstrated that the heparin binding domain in thrombin plays a specific role in promoting thrombin adhesive properties and that membrane-associated glypican is likely to be the major physiological inducer of this property.

Key words: endothelial cells, heparan sulfate, cryptic RGD, cell attachment, thrombin

Thrombin is a multifunctional serine protease generated at sites of vascular injury. Beside eliciting various cell-activating functions, thrombin is implicated in the maintenance of normal vessel wall dynamics as well as in pathological situations such as atherosclerosis and restenosis [Shuman, 1986; Fuster et al., 1992; Fitzgerald and Fitzgerald, 1989]. We have recently demonstrated that thrombin belongs also to a group of adhesive molecules which mediate cell adhesion through a common RGD-dependent mechanism [Bar-Shavit et al., 1991, 1993]. Different functional domains have been identified in thrombin, inducing monocyte chemotaxis (via a surface-expressed "Loop B" region) [Bar-Shavit et al., 1992], cell adhesion (via an RGD-containing site), and proteolysis (via the catalytic pocket of the enzyme composed of Ser^{95}, His^{195}, and Asp^{102}). Cloning of the platelet thrombin receptor has clarified how thrombin induces cell activation. Cleavage of the extracellular amino terminal extension of the receptor unmasks a new amino terminus that functions as an internal ligand to activate the receptor [Vu et al., 1991a,b; Coughlin et al., 1992]. As the concentration of thrombin in the vasculature increases it may become immobilized to the subendothelial basement membrane, exerting long-acting functional properties by virtue of being protected from inactivation by anti-thrombin III (ATIII) [Bar-Shavit et al., 1992]. In contrast, unbound,

Abbreviations used: EC, endothelial cells; ECM, extracellular matrix; ELISA, enzyme-linked Immunosorbent assay; GAGs, glycosaminoglycans; HSF, human skin fibroblasts; PI-PLC, phosphatidylinositol-specific phospholipase C; SMC, smooth muscle cells.

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Address reprint requests to Dr. Rachel Bar-Shavit, Ph.D., Department of Oncology, Hadassah-University Hospital, Jerusalem 91120, Israel.

fluid-phase thrombin undergoes rapid inactivation by plasma protease inhibitors to prevent thrombosis and systemic coagulation.

Heparin is a multifunctional, highly sulfated polysaccharide consisting of alternating uronic acid and D-glucosamine residues. The biological properties of heparin are usually ascribed to interactions between highly negatively charged glycosaminoglycan chains and positively charged residues of the protein. Specific interactions of heparin and ATIII have been described [Lindahl et al., 1984], resulting in the inactivation of thrombin by ATIII, possibly through the formation of an intermediatory ternary thrombin-heparin-ATIII complex [Gan et al., 1994]. The kinetics of inhibition are consistent with binding of both the inhibitor and thrombin to heparin in a template fashion. Because heparin binds with higher affinity to ATIII than thrombin, inhibition proceeds mainly through the interaction of ATIII–heparin complexes with free thrombin. X-ray crystalography of thrombin reveals two large electropositive patches (i.e., exosite I and II) located on the surface of thrombin, at opposite poles of the molecule. These exosites represent potential exosites for the binding of negatively charged glycosaminoglycan ligands. Among these cationic residues are Arg-245 and Lys-248, found within a sequence that is the inversion of the XBBXBX consensus sequence (where X and B are hydrophobic and basic residues, respectively) proposed for heparin binding domains [Kjellen and Lindahl, 1991; Gallagher et al., 1992; Lindahl, 1989; Hook et al., 1984].

Heparan sulfate proteoglycans (HSPG) are ubiquitous macromolecules associated with the cell surface and extracellular matrix (ECM) of a wide range of vertebrate and invertebrate tissues [Hook et al., 1984; Yanagishita and Hascall, 1992]. The basic HSPG structure consists of a core protein to which several linear heparan sulfate chains are covalently attached. A characteristic feature that distinguishes the core protein of cell surface proteoglycans from core proteins of secreted proteoglycans is their mode of anchorage to the plasma membrane. David et al. [1990] have cloned a glycosyl phosphatidylinositol (GPI)–anchored cell surface HSPG termed glypican whose 61 kDa core protein was susceptible to release by phosphatidylinositol-specific phospholipase C (PI-PLC) [Ishihara et al., 1987; Low and Saltiel, 1988; Yanagishita and McQuillan, 1988]. Specific release of glypican by PI-PLC generates soluble forms of HSPG that may interact in the vicinity of cell surfaces with ligands such as growth factors [Bashkin et al., 1992; Brunner et al., 1991] and alter intracellular cell functions [Low et al., 1988].

We have previously shown that thrombin contains a cryptic RGD sequence which may be surface exposed in the presence of low concentrations of plasmin and cell surface–associated HSPG [Bar-Shavit et al., 1991, 1993]. In the present study we show that glypican, released by PI-PLC from cell surfaces, is specifically involved in the conversion of thrombin to an adhesive protein. It appears that heparin/HS interactions with thrombin are specifically involved in the induction of thrombin adhesive properties, but do not affect its binding to cell surface receptors and its growth-promoting activity.

EXPERIMENTAL PROCEDURES

Materials

Plasmin was purchased from American Diagnostica Inc. (New York, NY). Heparin and low Mr heparin (Fragmin) were obtained from Kabi-Pharmacia (Uppsala, Sweden). Tissue culture dishes were obtained from Falcon Labware Division, Becton Dickinson and Co. (Oxnard, CA), four-well plates were from Nunc (Roskilde, Denmark), and 96-well plates from Costar Co. (Cambridge, MA). Dulbecco's Modified Eagle's Medium (DMEM, 4.5 g glucose/l), calf serum, fetal calf serum, penicillin, streptomycin, and saline containing 0.05% trypsin, 0.01 M sodium phosphate, and 0.02 EDTA (STV) were obtained from Biological Industries (Beit Haemek, Israel). Bacterial (flavobacterium heparinum) heparinase I (EC 4.2.2.7) was kindly provided by Dr. J. Zimmermann (IBEX Technologies, Montreal, Canada). The synthetic hexapeptides (GRGDSP, GRGESP) were obtained from Peninsula Laboratories, Inc. (Belmont, CA). Glypican, syndecan, and fibroglycan were affinity purified from detergent extracts of human fetal lung fibroblasts [David et al., 1990], and were kindly provided by Dr. Guido David (Center for Human Genetics, Campus Gasthuisberg, Leuven, Belgium), who also provided the anti-glypican monoclonal antibodies (mAb) IG11. Highly purified PI-PLC from *Bacillus thuringiensis* and recombinant PI-PLC isolated from *Bacillus subtillis* were a kind gift of Dr. Martin Low (College of Physicians and Surgeons, Columbia University, NY) [Low et al., 1988]. Similar results were obtained with both enzyme

preparations. Cetyl piridinium chloride (CPC) and all other reagent grade chemicals were purchased from Sigma (St. Louis, MO).

Cells

Cloned populations of adult bovine aortic endothelial cells (EC) and smooth muscle cells (SMC) were established as previously described [Gospodarowitcz et al., 1976; Bar-Shavit et al., 1990]. Cells were cultured in DMEM (1 g glucose/l) containing 10% bovine calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) at 37°C in a 10% CO_2 humidified incubator. Partially purified, brain-derived bFGF (100 ng/ml) was added to EC every other day during the phase of active cell growth. Cells were dissociated with STV and subcultured at a split ratio of 1:5. EC were characterized by indirect immunofluorescence using rabbit anti-human factor VIII antibodies (Behringwerke Ag, Mailburg, Germany). Wild-type Chinese hamster ovary cells (CHO-KI) and CHO mutant cells pgsA-745 and pgsD-677 (i.e., lacking both heparan sulfate and chondroitin sulfate, or lacking heparan sulfate but expressing threefold the normal level of chondroitin sulfate, respectively) were kindly provided by Dr. J.D. Esko (University of Alabama, Birmingham) and cultured as described [Esko, 1991; Esko et al., 1985]. SMC, human skin fibroblasts (HSF), and 3T3 cells were maintained in culture as described [Bar-Shavit et al., 1990].

Treatment of Cells With PI-PLC

Cultured cell monolayers were washed (four times) with serum-free medium to remove serum. The cells were detached by incubation (15 min, 37°C) with 0.5 mM EDTA in PBS, centrifuged, and washed twice in DMEM containing 0.05% BSA. Cells were suspended in this medium to a concentration of 3×10^6 cells/0.5 ml, and incubated (1 hr, 37°C) with PI-PLC (0.7–0.8 units/ml) with occasional shaking. The reaction was stopped by centrifugation of the cells ($1{,}000 \times g$, 10 min, 4°C) and the supernatant was subjected to further incubation (1 hr, 24°C) with thrombin (10 μg/ml) and plasmin (0.1 μg/ml). By the end of the incubation period, the various preparations were used to coat plastic surfaces for adhesion assays.

Preparation of Coated Surfaces

Thrombin and various modified thrombin preparations were diluted (10 μg/ml) in PBS containing 0.1% BSA and adsorbed onto the surface of 16 mm wells of four-well plates for 2 hr at 4°C. Unbound thrombin was removed and the dishes were washed three times with PBS. In representative experiments, ^{125}I-thrombin, iodinated by the Iodogen procedure [Bar-Shavit et al., 1989, 1990], was included in the incubation medium with plastic, cells, or ECM, together with unlabeled thrombin at a final concentration of 10 μg/ml. The concentration of plasmin was 2 μg/ml when incubated with thrombin alone or 0.1 μg/ml when incubated with thrombin in the presence of CHO cells or ECM. The amount of surface-associated thrombin was determined by γ counting of radioactive material solubilized with 1 N NaOH. These measurements revealed that regardless of the amount of cpm added (up to 1×10^6 cpm) approximately 1% of the thrombin was bound to plastic and each of the CHO cell types, as compared to about 7% that was bound to ECM.

Attachment Assay

Confluent endothelial cells were dissociated with trypsin–EDTA solution, washed once in growth medium, and resuspended in DMEM containing 0.2% BSA. Cells (1.3×10^5 cells/well) were added to each protein-coated well (Costar, Cambridge, MA) and incubated at 37°C for 2 hr. The plates were washed three times with PBS and the firmly attached cells were fixed with 3% paraformaldehyde. Fixed cells were rinsed with 0.1 M borate buffer (pH 8.5), stained (10 min at 22°C) with 0.1 ml/well Methylene blue (1% in 0.1 M borate buffer, pH 8.5), and washed four times in borate buffer. This procedure removed practically all noncell-bound dye. Cell-incorporated Methylene blue was dissolved with 0.1 N HCl (0.2 ml/well, 40 min, 37°C) and determined by its absorbance at 600 nm. Uptake of Methylene blue is linearly correlated to the number of viable cells [Goldman and Bar-Shavit, 1979]. Each experiment was performed at least three times and the variation between different experiments did not exceed ±20%.

Affinity-Purified Anti-Thrombin-RGD Antibodies

Rabbit anti-thrombin-RGD antibodies were obtained according to published procedures. In brief, rabbit anti-thrombin-RGD antiserum was obtained by subcutaneous injections of a KLH (Kyhole limpet hemocyanin)-conjugated 14-mer synthetic peptide representing residues 177–190 of thrombin B-chain. The antibodies in the

IgG fraction obtained by 50% ammonium sulfate precipitation and DEAE chromatography recognized in a radioimmunoassay the 14-mer peptide (ED_{50} = 2 ng/ml), but not native thrombin, prothrombin, fibrinogen, or fibronectin (at a concentration of up to 0.4 μg/ml).

ELISA

Ninety-six-well microtiter plates (Nunc-Immuno plate maxisorp) [Sheehan et al., 1989] were coated (5 μg/ml, 2–4 hr, 4°C, bicarbonate buffer, pH 8.0) with a 14 amino acid peptide representing residues 177–190 of thrombin B-chain, fibronectin, fibrinogen, vitronectin, thrombin, or prothrombin. The unbound material was washed away and the surface of the microtiter wells was saturated (1 hr, 24°C) with 5% dry milk in TTBS (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween-20). The wells were washed (0.5% dry milk in TTBS) three times, and incubated (2–16 hr, 24°C) with various dilutions of the test antiserum, followed by three washes and sequential incubation (1 hr, 24°C) with alkaline phosphatase–conjugated anti-rabbit antibodies (1:5,000; Promega, Madison, WI) and paranitrophenyl phosphate (PNPP) in diethanolamine buffer. Color development was determined by measuring the optical density at 405 nm.

Iodination and Binding of ^{125}I-Peptide Corresponding to the Internal Thrombin Receptor Ligand

Radiolabeling of the 14 amino acid peptide (SFLLRNPNDKYEPF) was performed using chloramine T, as described [McConahey and Dixon, 1980]. Briefly, the peptide (10 μg) was added to 60 μl of 0.2 M sodium phosphate, pH 7.2, containing 1 μCi Na ^{125}I. Chloramine T (10 μl of 1 mg/ml) was added for 45 sec at room temperature and the reaction was stopped by the addition of 50 μl 0.05% sodium metabisulfite and 50 μl of 10 mM KI. The reaction mixture was then applied onto a Sephadex G-10 column. Fractions were collected in phosphate buffer (0.1 M, pH 7.2). The specific activity was 0.8–1.1 × 10^5 cpm/ng peptide and the labeled material was kept for up to 4 weeks at −70°C.

Binding of ^{125}I-Peptide to SMC

Cells (5 × 10^5 cells/well) were grown to confluency in 60 mm culture wells. Confluent cultures were transferred to 4°C, washed once with PBS, and incubated (2 hr) with various concentrations of ^{125}I-peptide in the absence or presence of 1,000-fold excess unlabeled peptide. The cultures were washed (×3) with PBS, followed by incubation (30 min, 24°C) with the bifunctional crosslinker disuccinimidyl suberate (DSS; 4 mM) in DMEM containing 0.2% BSA. At the end of this incubation, cells were washed and dissolved in SDS-PAGE sample buffer for analysis by SDS-PAGE.

Cell Proliferation Assay

SMC and HSF (human skin fibroblasts) were plated (5 × 10^4 cells/16 mm well and 1 × 10^4 cells/16 mm well, respectively) in DMEM supplemented with 10% FCS. Twenty-four hours after seeding the medium was replaced with medium containing 0.2% FCS, and 48 hr later the cells were stimulated with thrombin in the presence of ^{3}H-thymidine (1 μCi/well). DNA synthesis was determined 48 hr afterward by measuring the radioactivity incorporated into 10% trichloroacetic acid–insoluble material, as described [Bar-Shavit et al., 1990].

RESULTS

PI-PLC Releases Cell Membrane–Associated HSPG Active in Converting Thrombin to an Adhesive Molecule

Exposure of the cryptic RGD site in thrombin involves interaction with cell surface HSPG and an exceedingly low concentration of plasmin. The role of cell surface heparan sulfate was demonstrated by using CHO cell mutants defective in various aspects of GAG synthesis [Esko, 1991; Esko et al., 1985]. In order to identify the type of naturally occurring cell surface HSPG involved in thrombin conversion to an adhesive molecule, CHO-KI cell monolayers were exposed (1 hr, 37°C) to increasing concentrations of PI-PLC. Glypican, representing a unique fraction of cell-associated HS, is anchored via a covalently linked glycosyl-phosphatidylinositol (PI) residue. This specific linkage can be cleaved by treatment with glycosyl-PI-specific phospholipase C (PI-PLC). CHO-KI cell monolayers were exposed (1 hr, 37°C) to increasing concentrations of PI-PLC. The released material was then incubated (1 hr, 24°C) with thrombin (10 μg/ml) and plasmin (0.1 μg/ml) and tested for its ability to promote EC adhesion. As demonstrated in Figure 1, a 6–7-fold stimulation in EC attachment was observed in the presence of

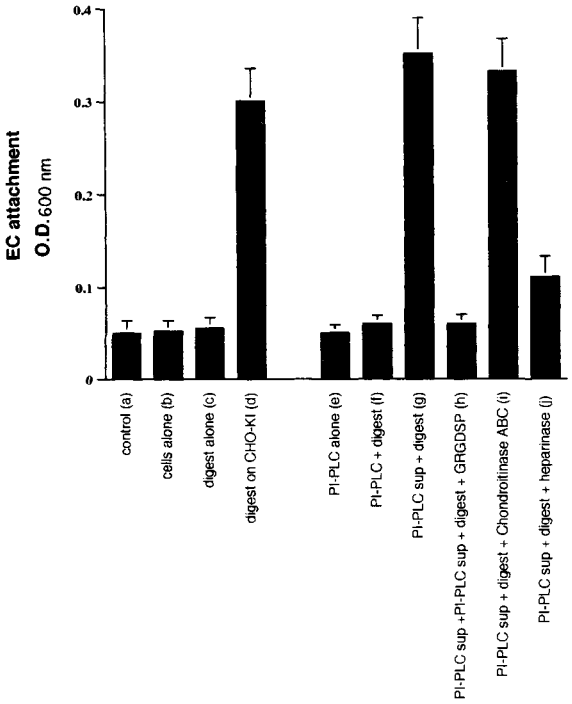
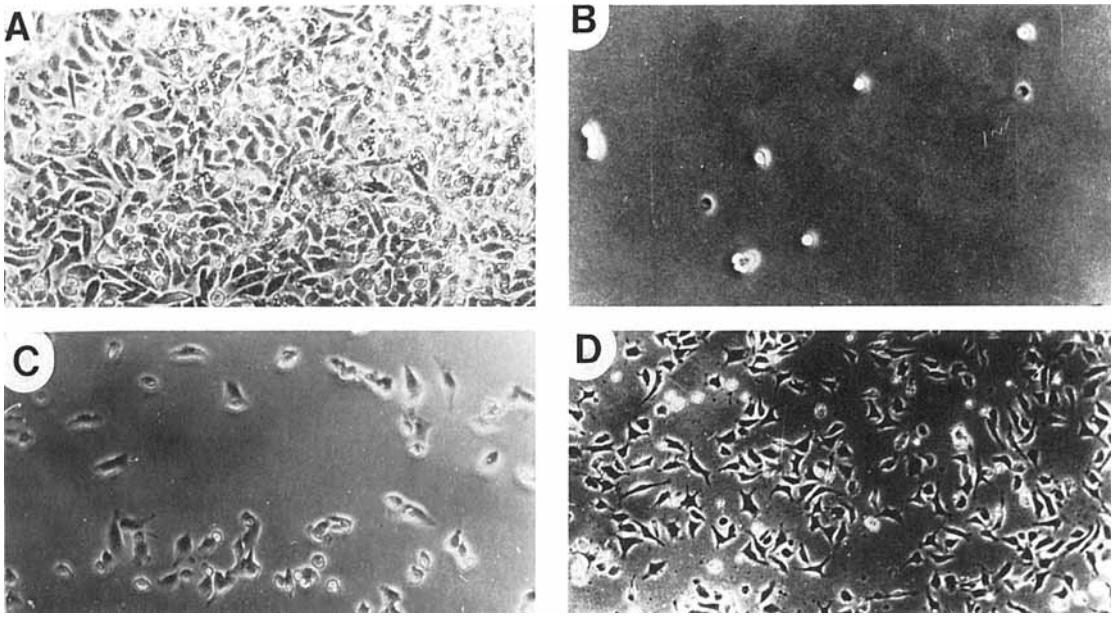


Fig. 1. Top: PI-PLC releases membrane HSPG that converts thrombin to an adhesive molecule. CHO-KI cell monolayers were treated with PI-PLC (0.3 U/ml DMEM, 1 hr, 37°C). The supernatant was collected and incubated (1 hr, 24°C) with thrombin (10 μg/ml) and plasmin (0.1 μg/ml). EC adhesion to dishes coated with this incubation mixture **(g)** was inhibited in the presence of 1 mg/ml GRGDSP **(h)** and compared to that observed on plates coated with reaction buffer alone as control **(a)**, reaction buffer incubated on top of fixed cells alone **(b)**, thrombin digested with plasmin (0.1 μg/ml) **(c)**, or plasmin-digested thrombin that was incubated on top of CHO-KI cells **(d)**. PI-PLC-released HSPG was further digested with either chondroitinase ABC (5 U/ml) **(i)** or bacterial heparinase I (5 hr, 0.2 U/ml) **(j)** before coating wells. The level of EC adhesion was determined as compared to coating with PI-PLC alone **(e)** or PI-PLC that was first incubated with minimally digested thrombin **(f)**. Each data point represents the mean of four culture wells, and the variation between different wells did not exceed ±15% of the mean. **Bottom:** Phase microscopy of EC adhesion and spreading. EC adhesion and flattening on surfaces coated with thrombin (10 μg/ml) that was first incubated with plasmin (0.1 μg/ml) and PI-PLC-released HSPG **(D)**, as compared to EC adhesion and spreading on surfaces coates with PI-PLC (0.8 U/ml) alone **(B)**, BSA alone **(C)**, or thrombin (10 μg/ml) that was preincubated with plasmin (0.1 μg/ml) on the surface of fixed CHO-KI cells **(A)**.



supernatants of PI-PLC-treated CHO-KI cells (Fig. 1f), as compared to thrombin treated with 0.1 μg/ml plasmin alone (Fig. 1a). In fact, EC adhesion obtained in the presence of PI-PLC-released material was similar to that observed following incubation of thrombin on top of intact wild-type CHO-KI monolayers (Fig. 1c) as determined by the Methylene blue uptake assay (Fig. 1, top) and phase microscopy (Fig. 1, bottom, A), but there was no reaction to PI-PLC alone (Fig. 1, bottom, B). It appears that conversion of thrombin to an adhesive protein was induced by cell surface material released by PI-PLC, since PI-PLC alone, or thrombin that was incubated with PI-PLC and 0.1 μg/ml plasmin, did not promote cell adhesion (Fig. 1, top, d, e, and bottom, B). EC adhesion was mediated by the RGD site in thrombin as indicated by its

inhibition in the presence of excess GRGDSP peptide (Fig. 1g). Exposure of the PI-PLC-released material to chondroitinase ABC (5 U/ml) did not abolish its ability to promote the conversion of thrombin into an adhesive molecule (Fig. 1h). In contrast, PI-PLC-released material that was pretreated with heparinase (0.2 U/ml) was no longer capable of promoting thrombin adhesive properties (Fig. 1i), indicating that the active component released by PI-PLC from cell surfaces is heparan sulfate. The same adhesion profile was obtained when thrombin was initially immobilized to the plastic surface and then treated with a low concentration of plasmin and PI-PLC-released cell surface HSPG (data not shown). A 1 hr exposure of CHO-KI cells to PI-PLC was sufficient to induce a maximal EC adhesion to thrombin that was pretreated with the PI-PLC-released material and 0.1 μg/ml plasmin (Fig. 2a). EC adhesion to this thrombin preparation reached a nearly maximal value following 1 hr exposure of the CHO cells to 0.3 U/ml PI-PLC (Fig. 2b). In other experiments, cells were metabolically labeled (48 hr, 37°C) with $Na_2{}^{35}SO_4$ (25 μCi/ml), washed free of unincorporated radioactivity and exposed to PI-PLC (1 hr, 37°C). More than 90% of the released material was precipitated by 0.05% CPC in 0.6 M NaCl, under conditions which precipitate mostly HS glycosaminoglycans [Roden et al., 1972]. Treatment of the CHO-KI cells with bacterial heparinase released about threefold more sulfate-labeled material as compared to PI-PLC (data not shown).

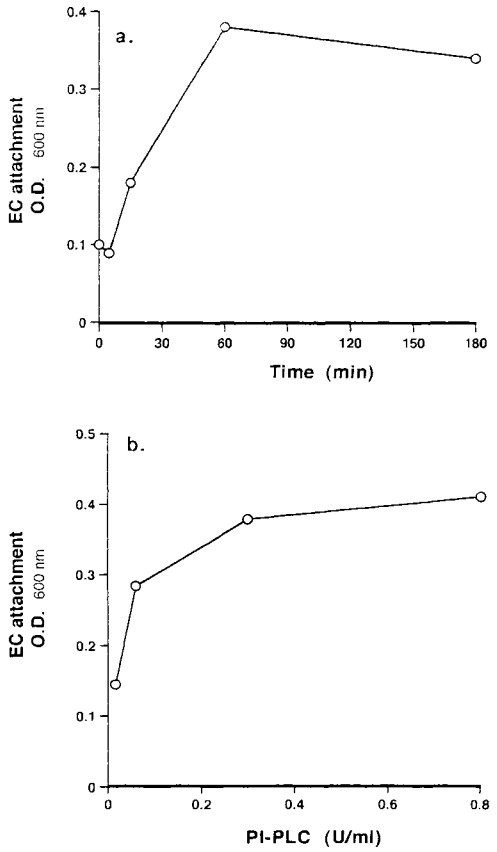


Fig. 2. Effect of PI-PLC-released HSPG on thrombin adhesive properties. **a:** Time course. CHO-KI cells were treated with PI-PLC (0.3 U/ml) for various time periods. The released material was further incubated (1 hr, 24°C) with thrombin (10 μg/ml) and plasmin (0.1 μg/ml), and used to coat four-well plates. EC (0.5×10^6 cells/well) were then seeded in medium containing 0.2% BSA and unattached cells removed after 2 hr incubation at 37°C. The number of attached cells was evaluated by Methylene blue uptake. **b:** Dose response. CHO-KI cell monolayers were exposed (1 hr, 37°C) to increasing concentrations of PI-PLC (0.2–0.8 U/mL). The supernatants were collected, further incubated with thrombin (10 μg/ml) and plasmin (0.1 μg/ml), and used to coat four-well plates. The extent of EC adhesion was evaluated by the Methylene blue uptake assay, as described in Materials and Methods. The variation between triplicate determinations did not exceed ±15% of the mean.

Adhesion-Promoting Activity of Material Released by PI-PLC From Various Cell Types

We examined different cell types for their ability to convert thrombin to an adhesion molecule upon incubation with plasmin and material released by PI-PLC. PI-PLC effectively released glycosyl-PI-anchored HSPG from CHO-KI cells, and the released material promoted EC attachment to thrombin, correlated with the concentration of PI-PLC used (Fig. 3e,f). In fact, the extent of EC adhesion observed in the presence of PI-PLC-released material was similar to the maximal level of adhesion obtained upon incubation of thrombin on fixed monolayers of CHO-KI cells (Figs. 1, 3d). No stimulation of EC adhesion above that observed on 0.2% BSA alone (Fig. 3a) was obtained when EC were plated on native thrombin (Fig. 3b) or thrombin that was first digested with plasmin (Fig. 3c). Material released by PI-PLC from CHO-KI mutant cells defective in GAG synthesis (pgsA-745 and pgsD-677) failed to promote thrombin adhesive properties (Fig. 3g,h,i,j, respectively). The gel filtration pattern (FPLC Superdex 75 column, Pharmacia, Uppsala) of cleavage products, obtained by treatment of thrombin with PI-PLC-released HSPG and 0.1 μg/ml plasmin, was similar to the profile obtained following incubation of thrombin on top of CHO-KI wild-type cell

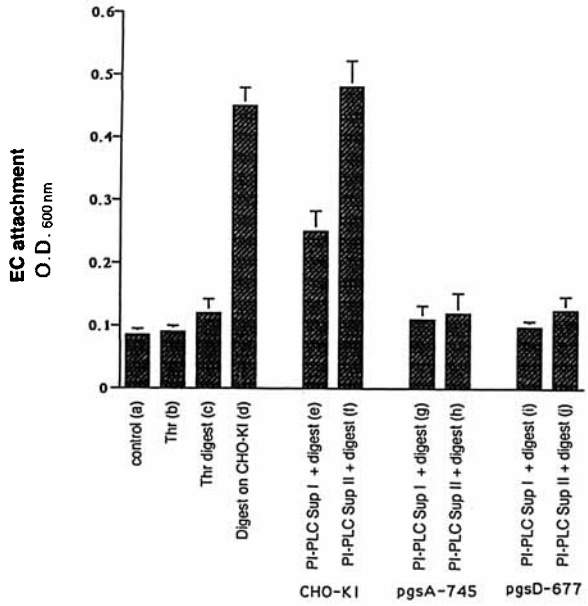


Fig. 3. Effect of HSPG released by PI-PLC from CHO-KI cells and GAG-deficient CHO cell mutants on thrombin adhesive properties. PI-PLC was applied (1 hr, 37°C) to CHO-KI cells (**e,f**), pgsA-745 cells (**g,h**), or pgsD-677 cells (**i,j**). The released material was then collected and incubated (1 hr, 24°C) with thrombin (10 μg/ml) and plasmin (0.1 μg/ml) prior to coating four-well plates. Control wells were also coated with either 0.2% BSA alone (**a**), native thrombin (**b**), or thrombin that was first digested with plasmin (**c**). The concentrations of PI-PLC were 0.3 U/ml (e,g,i), or 0.8 U/ml (d,f,h,j). The ability of PI-PLC (either 0.3 U/ml or 0.8 U/ml)-released material to convert thrombin to an adhesive molecule was compared to that observed on top of CHO-KI monolayers (**d**). The level of EC adhesion was evaluated by the Methylene blue uptake assay as described in Materials and Methods. The variation between duplicate determinations did not exceed ±12% of the mean.

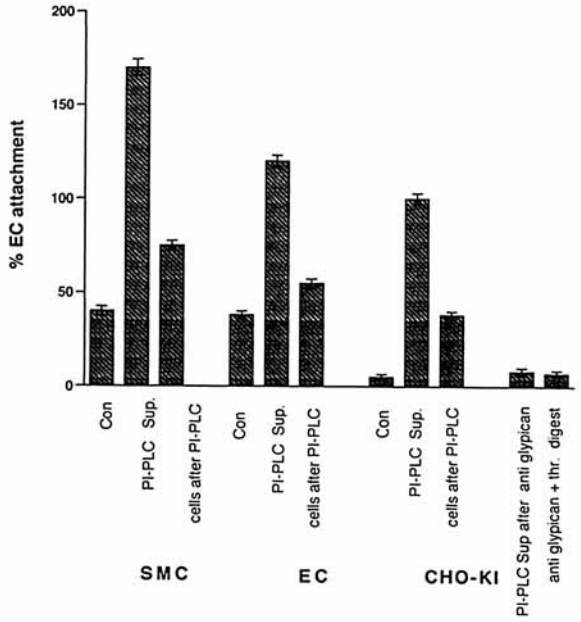


Fig. 4. Effect of material released by PI-PLC from various cell types on thrombin adhesive properties. Thrombin (10 μg/ml) was incubated (1 hr, 24°C) with plasmin (0.1 μg/ml) and supernatants of various cell types (i.e., SMC, EC, and wild-type CHO-KI cells) that were first treated (1 hr, 37°C) with 0.3 U/ml PI-PLC (PI-PLC Sup). The cell monolayers were washed (×2) and further incubated with thrombin and plasmin, as described above (cells after PI-PLC). Four-well plates were then coated with the respective supernatants (with or without PI-PLC-released material), or with DMEM containing 0.2% BSA alone (Con). The supernatant of CHO-KI cells that were first treated (1 hr, 37°C) with 0.3 U/ml PI-PLC was further incubated with anti-glypican (IG11, 10 μg/0.25 ml), immunoprecipitated (4 hr, 24°C), and absorbed onto Protein-A Sepharose beads. The resulting supernatant (PI-PLC Sup after anti glypican) was further incubated with thrombin (10 μg/ml) and plasmin (0.2 μg/ml) prior to coating. Antibodies (10 μg/0.25 ml) were also incubated (4 hr, 24°C) with plasmin-digested thrombin prior to coating (anti glypican + thr. digest). The extent of EC adhesion was evaluated as described in Materials and Methods. One hundred percent cell attachment represents the value of EC adhesion to dishes coated with thrombin that was first incubated with plasmin and material released by PI-PLC from CHO-KI cell monolayers (100% = 0.455 O.D. 600). The variation between triplicate determinations did not exceed ±12% of the mean.

monolayers in the presence of 0.1 μg/ml plasmin (data not shown).

We were especially interested in the prospect that cells of the vessel wall may participate in converting thrombin to an adhesive protein. For this purpose, cultured vascular SMC and EC were applied. As demonstrated in Figure 4, adhesion of EC to thrombin that was first exposed to the supernatant of PI-PLC-treated (1 hr, 37°C, 0.8 U/ml) SMC was nearly twice that induced following a similar incubation of thrombin with the supernatant of PI-PLC-treated CHO-KI cells or EC. This result correlates with previous observations, indicating that PI-PLC released from SMC a 2–3-fold higher mitogenic activity toward EC as compared to PI-PLC-treated EC or 3T3 fibroblasts. This mitogenic activity was exerted by PI-PLC-released glypican-bFGF complexes [Bashkin et al., 1992; Brunner et al., 1991]. Our data (Fig. 4) suggest that the level of glycosyl-PI-anchored HSPG is high in SMC compared to EC and CHO-KI cells. It appears that nearly the entire membrane-associated HSPG involved in the conversion of thrombin to an adhesive molecule resides in glycosyl-PI-anchored HSPG. First, immunodepleted supernatant (using antibodies directed toward glypican) of PI-PLC-released HSPG failed to induce EC adhesion (data not shown); and second, incuba-

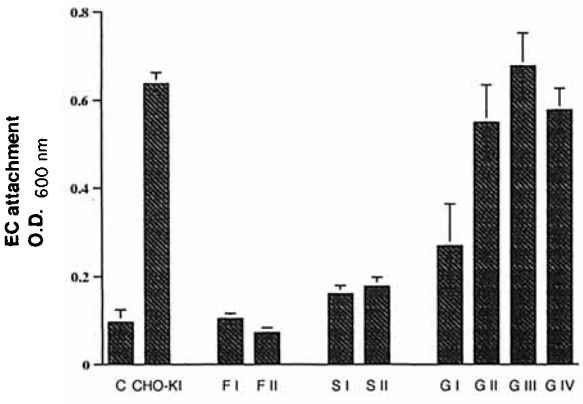


Fig. 5. Top: Effect of various affinity-purified species of HSPG on thrombin adhesive properties. Increasing concentrations (0.1–100 ng/m) of affinity-purified fibroglycan (FI and FII; 10 and 100 ng/ml), syndecan (SI and SII; 10 and 100 ng/ml), or glypican (GI-IV; 0.01, 0.1, 10, and 100 ng/ml) were incubated (1 hr, 24°C) with thrombin (10 μg/ml) and plasmin (0.1 μg/ml) prior to coating plastic dishes. The level of EC adhesion was determined by the Methylene blue uptake assay in comparison to that obtained on dishes coated with BSA alone (C), or with thrombin and plasmin incubated on top of CHO-KI cells (CHO-KI). The variation between triplicate determinations did not exceed ±15% of the mean. **Bottom:** Phase microscopy of EC adhesion and spreading. Thrombin (10 μg/ml) was incubated (1 hr, 24°C) with plasmin (0.1 μg/ml) on top of CHO-KI cells **(B)** or in the presence of 100 ng/ml fibroglycan **(C)**, syndecan **(D)**, or glypican **(E)**, prior to coating plastic dishes. EC (0.5×10^6 cells/ml) were then seeded and cell adhesion and flattening was visualized as compared to cells seeded on plastic coated with BSA alone **(A)**.

tion of minimally digested thrombin on top of cell monolayers that were first treated with PI-PLC and washed free of the released material was not associated with a gain in cell adhesion properties (Fig. 4).

Differential Capacity of Cellular Species of HSPG to Promote Thrombin Conversion to an Adhesive Molecule

We have screened several naturally occurring species of cell surface HSPG for their ability to convert thrombin to an adhesive protein. A variety of affinity-purified species of syndecan, fibroglycan, and glypican were utilized. Toward this, detergent extract of human lung fibroblasts was subjected to ion exchange chromatography and gel filtration. The resulting highly enriched preparation of HSPG was subjected to sequential affinity chromatography on specific affinity columns for purification of syndecan, fibroglycan, and glypican [David et al., 1990; Aviezer et al., 1994]. As demonstrated in Figure 5, conversion of thrombin to an adhesive molecule was obtained, in a dose-dependent manner, in the presence of glypican (G) (Fig. 5, Top GI-GIV), but there was little or no reaction to syndecan (S) (Fig. 5, Top SI, SII; Bottom D) or fibroglycan (F) (Fig. 5, Top FI, FII; Bottom C) as observed by the Methylene blue uptake assay (Fig. 5, Top) and microscopic visualization of cell attachment and spreading (Fig. 5, Bottom). In fact, affinity-purified glypican incubated with plasmin-digested thrombin (Fig. 5, Top GI-GIV; Bottm E) was nearly as active as intact CHO-KI cell monolayers (Fig. 5, Top CHO-KI, Bottom B) in inducing EC attachment and spreading, while glypican alone failed to promote EC adhesion. These results indicate that conversion of thrombin to an adhesive molecule is mediated by both membrane-associated and soluble HSPG and is confined primarily to glypican.

Antibodies Directed Against the RGD Domain of Thrombin Selectively Recognize Thrombin Following Exposure to PI-PLC-Released HSPG

In order to gain insight to the possible mode of interaction between glypican and thrombin, we tested whether incubation of thrombin with glypican and a suboptimal concentration of plasmin exposes the RGD epitope in thrombin. For this purpose, we tested the ability of antibodies raised against a synthetic peptide corresponding to the RGD site of thrombin B-chain (i.e., a 14 amino acid peptide containing the RGD sequence) to interact with thrombin before and after treatment with plasmin and PI-PLC-released material. These antibodies were capable of recognizing the RGD site in the treated thrombin and were found to block its cell adhesion–promoting activity (Fig. 6b). As demonstrated in Figure 6, the antibodies failed to interact with either native thrombin (Fig. 6a,c) or its zymogen prothrombin (Fig. 6c). Furthermore, the antibodies did not recognize RGD

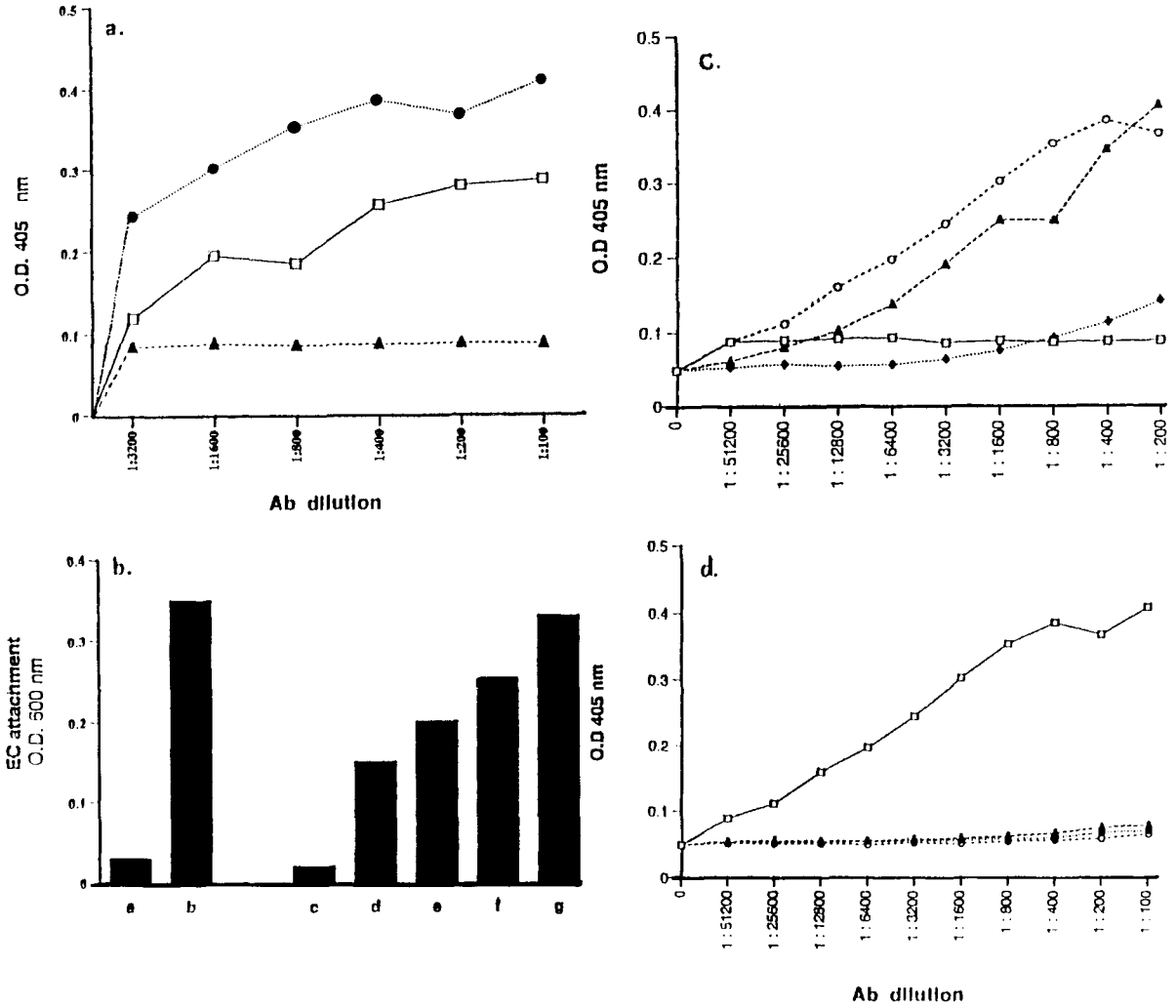


Fig. 6. Anti-thrombin-RGD antibodies that inhibit thrombin adhesion recognize thrombin following exposure to glypican. **a:** Thrombin (5 μg/ml) (▲), thrombin digested with a high concentration (2 μg/ml) of plasmin (●), or thrombin treated (1 hr, 24°C) with PI-PLC (0.8 U/ml)-released CHO-KI HSPG and plasmin (0.1 μg/ml) (□) were used to coat ELISA plates, followed by incubation (1 hr, 24°C) with various dilutions of anti-thrombin-RGD antibodies directed against residues 177–190. Detection of specifically bound antibodies was performed by sequential incubations with alkaline phosphatase–conjugated anti-rabbit IgG (Promega, 1:5,000) and the appropriate substrates, according to the manufacturer's instructions. **b:** Anti-thrombin-RGD antibodies (dilutions: 1:50, 1:100, 1:200, 1:400, and 1:800; c–g) were incubated (30 min, 24°C) with thrombin (10 μg/ml) that was pretreated with plasmin (2 μg/ml) prior to coating wells. The level of EC attachment was compared to that obtained on uncoated wells (a) and wells coated with thrombin (10 μg/ml) digested with plasmin (2 μg/ml) (b). Attachment activity was evaluated by the Methylene blue uptake assay, as described in Materials and Methods. **c,d:** Characterization of anti-RGD-thrombin antibodies. c: Synthetic peptides containing the RGD site (amino acids 177–190) (○), thrombin (10 μg/ml) digested with plasmin (2 μg/ml) (▲), prothrombin (□), or native thrombin (◆) were used (5 μg/ml) to coat ELISA plates. The plates were then incubated with various dilutions of the anti-RGD-thrombin antibodies and processed as described in Materials and Methods. d: Various RGD-containing proteins such as vitronectin (▲), fibrinogen (●), fibronectin (○), and thrombin digested with a high concentration (2 μg/ml) of plasmin (□) were used (5 μg/ml) to coat ELISA plates prior to adding the anti-thrombin-RGD antibodies. The plates were then processed for color detection as described above.

sequences in other adhesive molecules such as fibronectin, fibrinogen, or vitronectin (Fig. 6d), indicative of their selective recognition of the RGD region in thrombin. When supernatants of PI-PLC-treated CHO-KI cells were incubated (1 hr, 24°C) with thrombin (10 μg/ml) and plasmin (0.1 μg/ml) and used to coat ELISA plates, the antibodies recognized thrombin in a dose-dependent manner (Fig. 6a), but failed to interact with plasmin or PI-PLC alone.

Effect of Heparan Sulfate/Heparin on Thrombin Receptor Binding and Mitogenic Activity

We investigated the effect of heparin, fragmin (a low Mr heparin), and PI-PLC-released cell surface HSPG on thrombin-induced proliferation of SMC and HSF, as well as on the binding of ^{125}I-α-thrombin to its seven transmembrane domain receptor. As shown in Figure 7, no effect on ^{3}H-thymidine incorporation was observed when thrombin was preincubated with increasing concentrations of heparin and fragmin up to 200 μg/ml (Fig. 7d,e). Likewise, the mitogenic activity of thrombin was not affected by its exposure to PI-PLC-released cell surface material and 0.1 μg/ml plasmin (Fig. 7h). With both cell types, thrombin stimulated ^{3}H-thymidine incorporation by 5–10-fold. Glypican had also no effect on the catalytic activity of thrombin measured by the chromogenic substrate (D-Phe-L-Pipecolyl-Arg-Nitroanilide-Triacetate) assay (data not shown). In other experiments, binding of thrombin to its seven transmembrane domain receptor on SMC was not affected in the presence of either heparin or low Mr heparin (Table I). Similar results were obtained regardless of whether the binding studies were performed using ^{125}I-α-thrombin (with or without preincubation with heparin or fragmin) or iodinated 14 amino acid peptide (SFLLRNPNDKYEPF) representing the internal thrombin receptor ligand. Next, SMC monolayers were pretreated with GAG-degrading enzymes and incubated with ^{125}I-peptide in the presence and absence of excess unlabeled peptide. By the end of the binding period the cells were washed, the bound peptide crosslinked (4 mM DSS) to its putative receptor, and cell lysates subjected to SDS-PAGE. As shown in Figure 8, pretreatment of SMC with heparinase had no effect on the level of receptor crosslinking (Fig. 8, lane 3). Similarly, predigestion with chondroitinase ABC had no effect on receptor binding (Fig. 8, lane 6). Thus, it appears that heparin and cell surface heparan sulfate or chondroitin sulfate do not affect the level of thrombin receptor binding and mitogenic activity. A similar conclusion was obtained when wild-type CHO-KI cells and HS-deficient mutant cells were compared for the level of thrombin receptor binding (data not shown).

TABLE I. ^{125}I-α-Thrombin Binding to Monolayers of SMC*

^{125}I-α-thrombin	cpm/bound
1 nM	5,100 ± 78
5 nM	25,050 ± 550
25 nM	49,992 ± 880
25 nM + 200 μg heparin	49,540 ± 750
25 nM + 200 μg fragmin	48,990 ± 788

*^{125}I-α-thrombin (1 nM, 5 nM, and 25 nM) was incubated (1 hr, 4°C) with confluent SMC monolayers. ^{125}I-α-thrombin (5×10^6 cpm/well, 25 nM) was also preincubated (30 min, 4°C) with heparin (200 μg/ml) and low Mr heparin (200 μg/ml) prior to incubation with the cells. Specific binding was determined as described in Materials and Methods. Nonspecific binding was determined in the presence of 1,000-fold excess α-thrombin and was subtracted from the total binding.

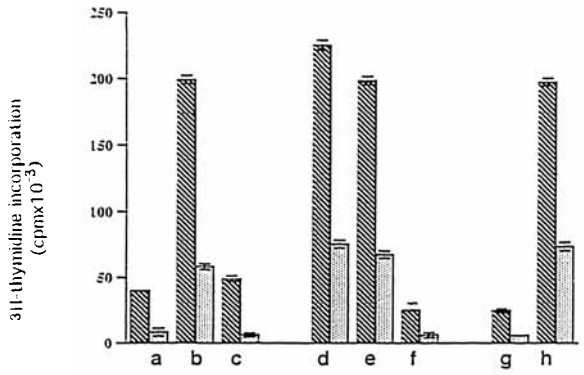


Fig. 7. Effect of heparin and low Mr heparin (fragmin) on thrombin receptor binding and mitogenic activity. Growth-arrested SMC (▨) or HSF (□) (10^5 cells/well, 0.2% FCS, 48 hr) (**a**) were incubated with plasmin (0.1 μg/ml) alone (**c**) or with thrombin (10 μg/ml) that was first digested with plasmin (0.1 μg/ml) (**b**). Thrombin (10 μg/ml) was also digested with plasmin (0.1 μg/ml, 30 min, 24°C) in the presence of heparin: 200 μg/ml (**d**), low Mr heparin (fragmin): 200 μg/ml (**e**), or PI-PLC (0.8 U/ml)-released material (**h**), followed by incubation (48 hr in the presence of ^{3}H-thymidine) with growth-arrested SMC or HSF. SMC and HSF were also incubated with fragmin alone (**f**), or PI-PLC alone (**g**). ^{3}H-thymidine incorporation was determined following TCA precipitation, as described in Materials and Methods.

DISCUSSION

The differential capacity of membrane-associated heparan sulfate proteoglycans to promote thrombin adhesive properties was investigated. The crucial role of cell surface HS in the conversion of thrombin to an adhesive molecule was revealed by the abrogation of this conversion following incubation of thrombin with CHO mutant cell lines defective in their metabolism of glycosaminoglycans, or with wild-type CHO-KI cells pre-digested with heparinase. Optimal induction of thrombin adhesive properties was observed following its incubation with CHO-KI

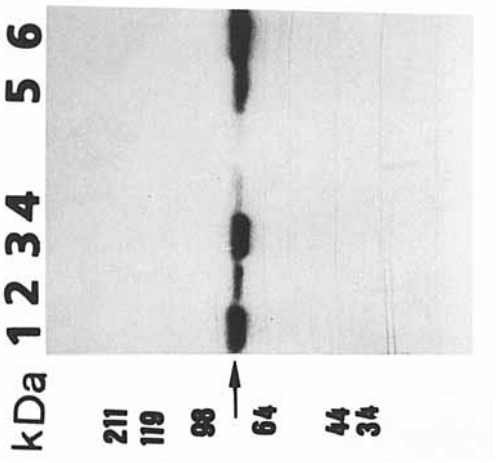


Fig. 8. Crosslinking of ^{125}I-thrombin receptor agonist peptide to SMC. SMC (1×10^6 cells/well) were either untreated (*lanes 1, 2*) or pretreated with heparinase (0.25 U/ml, 2 hr, 37°C) (*lanes 3, 4*) or chondroitinase ABC (0.5 U/ml, 2 hr, 37°C) (*lanes 5, 6*). The cells were washed free of digested material and incubated (2 hr, 4°C) with 14 amino acid receptor agonist ^{125}I-peptide (10^7 cpm, 5 ng/ml) in the presence (lanes 2, 4, 5) or absence (lanes 1, 3, 6) of 1,000-fold excess unlabeled peptide. Cells were then extensively washed, incubated (30 min, 24°C) with 4 mM DSS, solubilized in SDS-PAGE sample buffer, and the supernatant subjected to SDS-PAGE and autoradiography as described in Materials and Methods.

cells or exogenously added HS/heparin in the presence of a suboptimal concentration of plasmin [Bar-Shavit et al., 1993].

The greatest diversity in structure and function of membrane-associated proteoglycans is in those bearing heparan sulfate [Yanagishita and Hascall, 1992]. A single cell may simultaneously display seven or more species of heparan sulfate proteoglycans anchored in the plasma membrane. The most important characteristic that distinguishes the core proteins of cell surface HSPG from the core proteins of secreted HSPG is their anchorage in the plasma membrane, either through a hydrophobic amino acid domain or via a glycosyl-phosphatidylinositol (GPI) anchor, covalently bound to the core protein lipid "tail." GPI anchorage mechanism is a signal for sorting of proteins to the apical membrane domain of polarized cells [Lisanti and Rodriguez-Boulan, 1990; Lisanti et al., 1990]. The aim of the present study was to identify the type of membrane-associated HSPG involved in the conversion of thrombin to an adhesive molecule. Our data clearly demonstrate that this conversion was effectively induced by either PI-PLC-released cell surface material, or a preparation of purified glypican. No exposure of the cryptic RGD region in thrombin was observed following its incubation on top of cells that were first treated with PI-PLC, or in the presence of unrelated purified species of HSPG such as syndecan and fibroglycan. The fact that the total cell surface HS was highly active in promoting thrombin conversion to an adhesive molecule, while affinity-purified syndecan or fibroglycan were not active, points to the possibility that glypican may exert the entire specificity toward thrombin, most likely through glypican GAG residue steric interactions, which may be different from the GAGs present on either syndecan or fibroglycan. Although GAGs consist of a highly negative-charged polymer, thus suggesting interactions primarily via electrostatic association, a steric fit is likely to be required for interaction of heparin/HS with various heparin binding proteins. Indeed, a motif of correctly spaced basic amino acids in a structure of either an α-helix or β-structure was suggested by several groups to be involved in protein–GAG interactions [Cardin and Weintraub, 1989; Sobel et al., 1992]. Specific protein–HS/heparin interactions are implicated in the regulation of various activities such as anticoagulation through ATIII [Lindahl et al., 1984], a conformational modulation of bFGF receptor binding site, resulting in stimulation of bFGF action [Yayon et al., 1991], enhancement of cell adhesion through interaction with fibronectin [Le Baron et al., 1988], and of lipid transport via binding to apolipoproteins [Cardin et al., 1984] and release of lipoprotein lipase from EC surfaces [Eckle, 1989]. Likewise, specific interaction between thrombin and heparin/HS may lead, in conjunction with plasmin, to exposure of the RGD-containing region, resulting in thrombin-mediated cell adhesion [Bar-Shavit et al., 1991, 1993].

The anion binding exosite located on the surface of thrombin corresponds to a cluster of basic amino acids along the active site. The function of anion exosite I, the fibrinogen recognition site, has been well documented [Bode et al., 1992]. Anion binding exosite II, located near the carboxy terminus of the molecule, has been proposed to bind heparin, based on several single amino acid substitutions [Sheehan and Sadler, 1994]. It is of interest that Arg-245 and Lys-248 are within a sequence (FRLKKW) that is an inversion of the XBBXBX consensus sequence proposed for heparin binding domains [Cardin and Weintraub, 1989]. Thrombin/heparin inter-

actions have been proposed to be involved in the inhibition of thrombin by serpins, ATIII and heparin cofactor II (HCII) [Sheehan et al., 1993]. We have demonstrated a role for thrombin–heparin/HS interaction in the induction of thrombin adhesive properties and identified the type of cell surface–associated HSPG involved in this induction. Whether this interaction involves thrombin anion binding exosite I or II remains to be determined. The specific involvement of glypican in the conversion of thrombin to an adhesive molecule suggests that efficient conversion might take place preferentially on the apical cell surface, mediated by membrane-anchored or PI-PLC-released glypican [Lisanti and Rodriguez-Boulan, 1990; Lisanti et al., 1990]. The notion that PI-PLC-released glypican interacts with thrombin in a manner that exposes the hindered RGD site was supported by obtaining antibodies that selectively recognize the RGD-containing site in thrombin that was first exposed to PI-PLC-released cell surface material. These antibodies failed to recognize the native nonadhesive form of thrombin. In fact, immunohistochemical staining with anti-thrombin-RGD antibodies revealed abundant and specific localization of the RGD-containing region of thrombin in both the luminal surface and medial region of human carotid artery (Bar-Shavit et al., in preparation). This may indicate that the RGD site in thrombin is physiologically relevant and abundantly distributed throughout the vessel wall.

A close look at the amino acid sequence of the thrombin receptor reveals a highly acidic sequence (i.e., EPFWEDEEKNES) located 13 residues carboxy to the receptor cleavage site [Vu et al., 1991a,b]. This highly acidic region may be crucial in further association of thrombin with the negatively charged sequences of heparin/HS. The possible association between the thrombin receptor and membrane-associated heparan sulfate is not known. Our data show that the level of thrombin binding to its receptor was not affected by heparin. Moreover, selective degradation of membrane-associated proteoglycans (i.e., heparan sulfate, chondroitin sulfate) by heparinase or chondroitinase ABC did not affect the level of thrombin receptor binding, as revealed by crosslinking followed by SDS-PAGE. Likewise, preincubation of thrombin with heparin did not affect the mitogenic activity induced by thrombin or thrombin digested with a suboptimal concentration of plasmin, on fibroblasts and SMC. Our results demonstrate for the first time that glypican is specifically involved in thrombin conversion to an adhesive molecule, but has no effect on thrombin receptor binding and mitogenic activity. Site-directed mutagenesis of the putative heparin binding site in thrombin is likely to elucidate the role of thrombin–heparin interaction in the induction of cell adhesion and integrin activation.

In mammals, the only well characterized GPI-specific phospholipase is the plasma GPI–specific phospholipase D (GPI-PLD), whereas a GPI-PLC activity similar to that applied in the present study was not detected. Recently, it has been demonstrated that GPI-anchored bFGF binding heparan sulfate is released from cells by GPI-PLD [Metz et al., 1994], thus ascribing a potential physiological function to the enzyme in the control of bFGF availability and mitogenic activity. In the present study we have demonstrated that conversion of thrombin to an adhesive protein is brought about by both soluble and surface-associated GPI-anchored heparan sulfate. Release of GPI-anchored glypican could serve to increase the local concentration of glypican in the vicinity of thrombin and plasmin in areas of clot formation and tissue injury.

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