Brief Definitive Report

Molecular Behavior Adapts to Context: Heparanase Functions as an Extracellular Matrix–degrading Enzyme or as a T Cell Adhesion Molecule, Depending on the Local pH

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Summary

Migration of lymphocytes into inflammatory sites requires their adhesion to the vascular endothelium and subendothelial extracellular matrix (ECM). The ensuing penetration of the ECM is associated with the expression of ECM-degrading enzymes, such as endo-β-D glucuronidase (heparanase), which cleaves heparan sulfate (HS) proteoglycans. We now report that, depending on the local pH, a mammalian heparanase can function either as an enzyme or as an adhesion molecule. At relatively acidified pH conditions, heparanase performs as an enzyme, degrading HS. In contrast, at the hydrogen ion concentration of a quiescent tissue, heparanase binds specifically to HS molecules without degrading them, and thereby anchors $CD4^+$ human T lymphocytes. Thus, the local state of a tissue can regulate the activities of heparanase and can determine whether the molecule will function as an enzyme or as a proadhesive molecule.

Leukocyte-mediated inflammatory reactions and immune surveillance require the activation of leukocytes by specific antigens, cytokines, and chemokines, and the ensuing extravasation of circulating immune cells from blood vessels to sites of inflammation (1–3). The recognition and the subsequent interactions of the infiltrating immune cells, such as $CD4^+$ T lymphocytes, with glycoproteins of the subendothelial basement membrane and extracellular matrix (ECM) is mediated primarily by integrin receptors of the β1 subfamily (1). In addition, the physiologically programmed migration of T cells is associated with the secretion of various matrix-degrading enzymes, such as endo-β-D glucuronidase (heparanase) (4–6). Naive $CD4^+$ T lymphocytes respond to activation by synthesizing heparanase de novo, whereas memory T cells release heparanase from preformed stores within minutes of contact with antigen (4). The cell surface–, basement membrane–, or ECM-associated heparan sulfate (HS) proteoglycans, which serve as substrates for the catalytic activity of heparanase, determine the self-assembly and insolubility of the ECM, stabilize the matrix structure, and maintain the matrix integrity by interacting with ECM glycoproteins (4). The HS molecules also affect the proliferation of immune cells (7) and bind growth factors and cytokines (8, 9). Herein, we examined whether the performance of heparanase is affected by physiological alterations in the environment, specifically, whether in addition to displaying an enzymatic activity, heparanase can also regulate the accumulation of T cells in inflamed tissues.

Materials and Methods

Isolation and Purification of Mammalian Heparanase. Heparanase was purified from human placenta by a modification of a previously described method (10). Briefly, human placentas were homogenized and suspended in 10 mM phosphate–citrate buffer (PCB; pH 6.0), sonicated, and centrifuged (3000 *g*, 4°C, 15 min). Proteins were precipitated with 35–55% $AmSO_4$, resuspended in 10 mM PCB containing 0.1 M NaCl, and dialyzed against the same buffer. The dialyzed material was then subjected to cation exchange chromatography on CM–Sepharose (Kabi-Pharmacia, Stockholm, Sweden) equilibrated with PCB (pH 6.0) containing 0.1% 3-([3-cholamidopropyl]-dimethylammonio)-1-propane-sulfonate (CHAPS). The column was subjected to a 0.3–1.1 M NaCl gradient; heparanase eluted at 0.6–0.7 M NaCl. The enzymatically active heparanase fractions were pooled, dialyzed, and applied to heparin–Sepharose (Kabi-Pharmacia; 10 mM phosphate–citrate containing 0.2 M NaCl, 0.1% CHAPS, pH 7.2). Upon elution, active fractions eluted with a NaCl gradient (0.35–1.1 M) at 0.7–0.8 M NaCl. The pooled fractions were adjusted to 1 M NaCl in 10 mM PCB (pH 6.0; 0.1% CHAPS) and applied to a Con A–Sepharose column (Sigma Chemical Co., St. Louis, MO). Heparanase was eluted with the same buffer containing 0.25 M α-methyl mannoside (Sigma Chemical Co.), dialyzed, and subjected to fast protein liquid chromatography (FPLC) Mono-S cation exchange chromatography. Purified heparanase was eluted at 0.7 M NaCl of the NaCl gradient (0.25–1 M NaCl).

Iodination of Placental Heparanase. Purified heparanase (20 μl) was added to 60 μl of 0.2 M sodium phosphate, pH 7.2, containing 1 μCi $Na^{125}I$ and incubated (45 s, 22°C) with chloramine T (10 μl of 1 mg/ml). The reaction was terminated by the addition of

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0.05% Na-metabisulfide (50 μl) and 10 mM KI (50 μl). The reaction mixture was then applied onto a 0.3 ml heparin–Sepharose column equilibrated with 0.2 M NaCl in 20 mM phosphate buffer, pH 7.2. The column was washed with the same buffer, and the ^{125}I-heparanase was eluted with PCB (1.5 ml) containing 1.1 M NaCl, 0.3% CHAPS, and 0.2% gelatin. The specific activity of the ^{125}I-heparanase was 6–7 × 10^4 cpm/ng, its enzymatic activity was confirmed, and the labeled material was stored at −70°C until used.

Analysis of Heparanase-induced ECM Degradation. To obtain radiolabeled, cell-free ECM, bovine corneal endothelial cells were plated in flat-bottomed microtiter plates (Becton Dickinson & Co., Rutherford, NJ) and maintained as previously described (5, 11, 12), except that 5% dextran T-40 was included in the growth medium. $Na_2[^{35}S]O_4$ (40 μCi/ml; Amersham Corp., Amersham, UK) was added 3 and 7 d after seeding. 6–8 d after the cells reached confluence, the cell layer was dissolved by exposure (3 min, 22°C) to 0.5% (vol/vol) Triton X-100 and 0.025 N NH_4OH in PBS, and the plates were washed extensively with PBS. This procedure resulted in the underlying ECM being left intact, firmly attached to the entire area of the wells, free of cellular debris, and >85% of the total ECM-bound ^{35}S was incorporated into HS proteoglycans. The purified heparanase was incubated (different pHs, 37°C) with ^{35}S-labeled ECM (12). The supernatants were then collected, centrifuged (10,000 *g*, 5 min, 4°C), and 0.5-ml aliquots of the supernatants were applied to Sepharose 6B columns (Sigma Chemical Co.; 0.7 × 35 cm) equilibrated with PBS containing 0.1% sodium azide. Fractions (0.2 ml) were collected at a flow rate of 5 ml/h, and their radioactivity was determined. The excluded (Vo) and total included (Vt) volumes were marked by blue dextran and phenol red, respectively. Each experiment was performed at least four times, and the variations in elution positions (K_{av} values) were always <10%.

Cell Adhesion Assays. Human CD4$^+$ T cells were purified from the peripheral blood of healthy donors. The leukocytes were isolated on a Ficoll gradient, washed, and incubated at 37°C in a 10% CO_2-humidified atmosphere. After 2 h, the nonadherent cells were removed and incubated on nylon-wool columns, nonadherent cells were eluted and washed, and the CD4$^+$ T cells were selected (>94% purity) as previously described (13, 14). Adhesion of T cells to ECM-coated wells was assayed as previously described (13). Briefly, T cells were labeled with $Na_2[^{51}Cr]O_4$ (Amersham Corp.), washed, resuspended in adhesion medium (RPMI-1640 medium supplemented with 2% BSA, 1 mM Ca^{2+} and Mg^{2+}, 1% sodium pyruvate, glucose, and 1% Hepes), and added to the precoated wells. The microtiter plates were incubated at 37°C for 30 min in a humidified 10% CO_2 incubator and washed three times with PBS to remove nonadherent cells. The adherent ^{51}Cr-labeled T cells were lysed, and the radioactivity in the resulting supernatants was determined. For each experimental group, the results were expressed as the mean percentage (±SD) of bound T cells in quadruplicate wells.

Results and Discussion

Purification of Heparanase and Analysis of Function. Heparanase was isolated from human placenta and purified >3.4 × 10^5-fold by ammonium sulfate precipitation followed by sequential chromatographies on carboxymethyl–, heparin–, and Con A–Sepharose columns. The molecular weight, pH dependency, and substrate specificities of partially purified heparanase

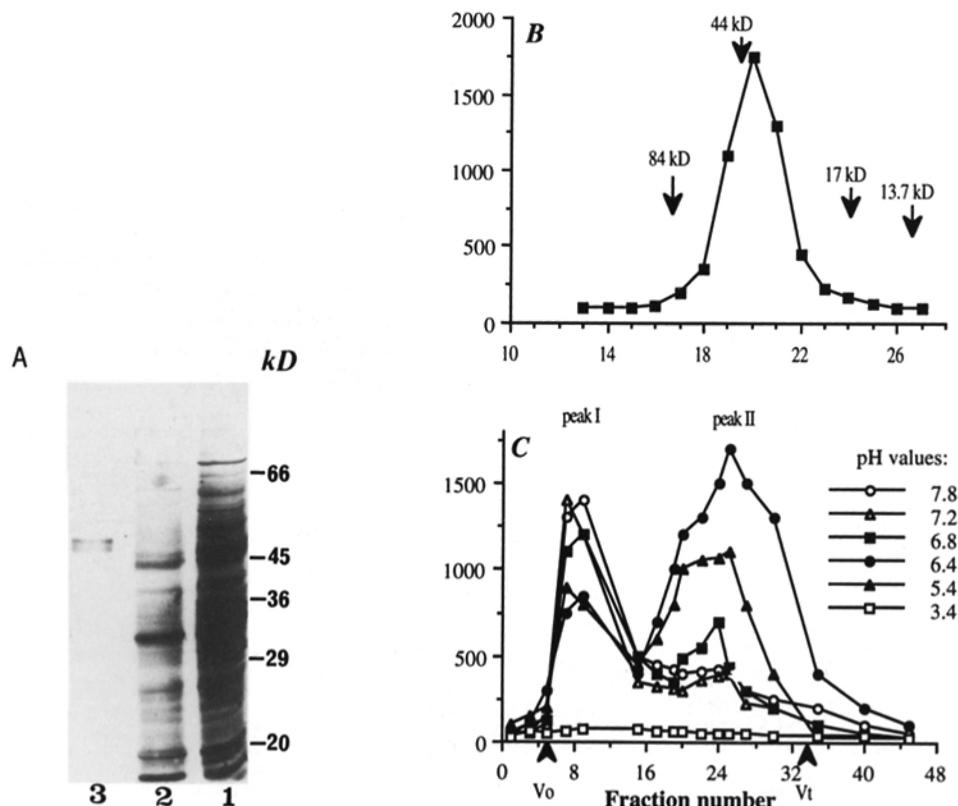

Figure 1. Purification of heparanase and pH dependence of its enzymatic activity. (*A*) SDS-PAGE analysis of preparations obtained during the purification of heparanase. Samples were concentrated by precipitation with 10% TCA and subjected to SDS-PAGE. (Lane *1*) 35–50% $AmSO_4$ precipitate of the placental homogenate. (Lane *2*) Pooled enzymatically active fractions eluted from CM-Sepharose. (Lane *3*) Pooled active fractions eluted from Con A–Sepharose. (*B*) Determination of the molecular weight of heparanase by gel filtration. Heparanase was eluted from Con A–Sepharose and subjected to FPLC gel filtration on Superdex-75. The heparanase activity during a 1-h incubation at 37°C was determined for each fraction (12). Transferrin, ovalbumin, myoglobulin, and ribonuclease, with molecular masses of 84, 44, 17, and 13.7 kD, respectively, were used as marker proteins. (*C*) pH dependency of heparanase activity. FPLC-purified heparanase was diluted in 10 mM PCB at different pH values, and its ability to release low molecular weight ^{35}S-labeled (peak II) material from the ECM is tested. The experiments depicted in *B* and *C* were performed four times; the variations in elution positions (K_{av} values) were <10%.

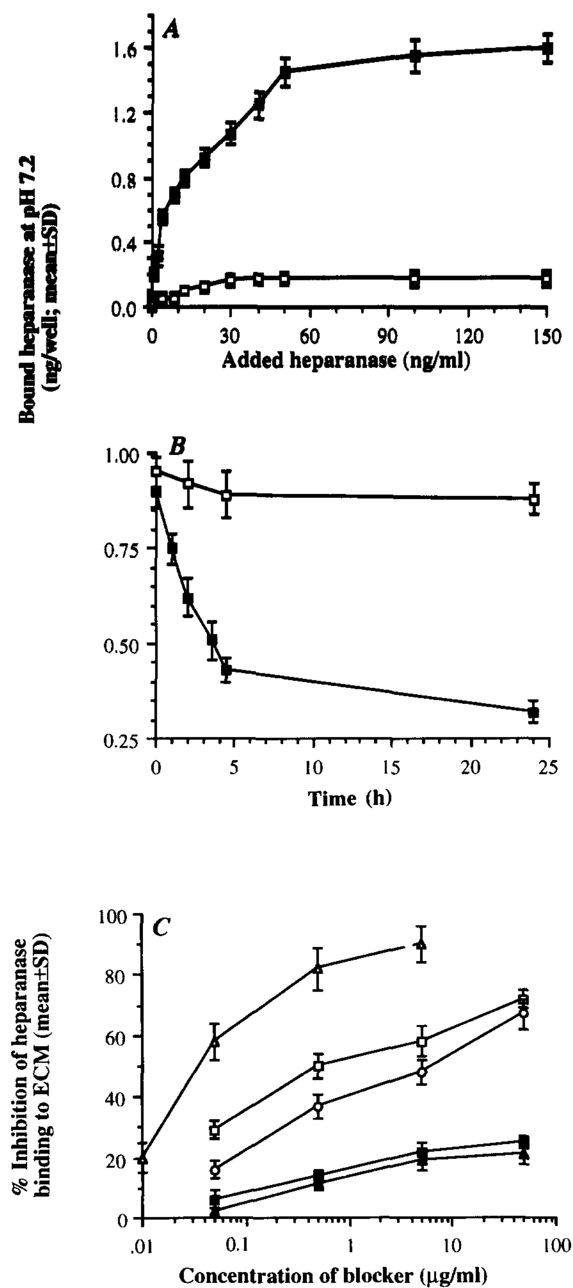

Figure 2. Analysis of heparanase binding to ECM. (*A*). Binding of heparanase to wells coated with ECM (■) or BSA (□). Different concentrations of ^{125}I-heparanase were added to the immobilized substrates. After 2 h at 37°C, unbound ^{125}I-heparanase was aspirated, the wells were washed, and bound heparanase was solubilized and quantitated by gamma counting. (*B*) Dissociation of heparanase from immobilized ECM (■) or heparin–Sepharose (□). Purified heparanase (0.1 μg/ml) was incubated (2 h, 37°C, in PBS at pH 7.2) with ECM-coated wells or heparin beads (0.2 ml), and then the unbound material was removed. The coated wells were then incubated with PBS for the time indicated, washed, and the remaining ECM-bound ^{125}I-heparanase was solubilized and quantitated by gamma counting. (*C*) Blockage by glycosaminoglycans of ^{125}I-heparanase binding to intact ECM. Labeled enzyme (0.1 μg/ml) was preincubated (2 h, 37°C, pH 7.2) with chondroitin sulfate (Chemicon International, Inc., Temecula, CA), heparin, hyaluronic acid, or HS (Sigma Chemical Co.) and these mixtures were added to ECM-coated microtiter wells. ECM-coated plates were also preincubated (2 h, 37°C, pH 7.2) with unlabeled heparanase. In *A–C*, the results are depicted for triplicate wells and are representative of four experiments. –□–, Heparin; –○–, heparan sulfate; –■–, chondroitin sulfate; –▲–, hyaluronic acid; –△–, unlabeled heparanase.

isolated from human blood cells, such as lymphocytes, platelets, and neutrophils, are similar to those of the purified placental enzyme (not shown). To assess the purity of the placental heparanase preparations, proteins recovered from the sequential purification steps were subjected to SDS-PAGE (Fig. 1 *A*). The final preparation (lane *3*) consisted of a ~45-kD doublet protein. To verify that heparanase activity was associated with this doublet protein, material eluted from Con A–Sepharose was subjected to FPLC gel filtration, and the fractions were tested for heparanase activity by measuring the release of low molecular weight ^{35}S-labeled HS degradation products from the ECM-coated wells (12). Maximal heparanase activity was associated with the 45-kD protein (Fig. 1 *B*). Degradation of HS by the purified placental heparanase, as by heparanases from other cell sources (5), was inhibited by preincubation of the enzyme with heparin (not shown).

The pH-dependency of HS degradation by heparanase was determined by diluting the enzyme in buffer equilibrated to different pH values, incubating the diluted enzyme with ^{35}S-ECM, and analyzing the degradation products released into the incubation media. At pH 3.4, the heparanase is not active; therefore, no peaks of degraded ECM are observed (Fig. 1 *C*). At pH values >6.8 or <5.4, the heparanase exhibited little or no activity, as indicated by a low release of low molecular weight ^{35}S-labeled material characteristic of HS degradation fragments (Fig. 1 *C*; $0.5 \leq K_{av} \leq 0.8$ [peak II, fractions 20–35]) (11). The high molecular weight ^{35}S-labeled material eluting near the void volume of the column (peak I, fractions 5–15, $K_{av} < 0.3$), probably results from the activities of ECM-resident proteases (6). Although the purified heparanase is partially active at pH 7.2 or 7.8, maximal release of low molecular weight HS fragments occurred at pH values ranging from 5.4 to 6.8. Heparanase from activated $CD4^+$ rat T cells (5, 12), like HS-specific heparanase from tumor cells (15), exhibited its maximal enzymatic activity on ECM at pH 6.2–6.8, whereas heparinase counterparts in bacteria appear to be optimally active at pH 7.2 (16). Thus, heparanase-mediated cleavage of the HS scaffold of ECM is a pH-dependent process, which should be able to occur in vivo at the sites of inflammation and tumors where relatively acidic pH values have been detected (17–21).

Heparanase Can Associate with the ECM at Physiological pH. The direct binding of heparanase to ECM or BSA was examined by adding dilutions of ^{125}I-heparanase to microtiter wells coated with either ECM or BSA. At pH 7.2 and 37°C, heparanase bound to ECM in a saturable and specific manner (Fig. 2 *A*). However, during a 24-h period, most of the ECM-bound heparanase spontaneously dissociated from the ECM (Fig. 2 *B*). The high spontaneous dissociation could be due to the activity at 37°C of ECM-associated enzymes (6), such

as collagenase IV or plasminogen activators (22). If the enzymes cleave ECM ligands, the released heparanase should be intact; however, if these enzymes also cleave the heparanase, the conformational intactness of heparanase should be affected. Binding of ^{125}I-heparanase to ECM was inhibited if the ECM was preincubated with an excess of unlabeled heparanase, or if the heparanase was preincubated with heparin or HS (p <0.05), but not with hyaluronic acid or chondroitin sulfate (Fig. 2 *C*).

pH Dependence of the Enzymatic Activity of ECM-bound Heparanase. Heparanase was allowed to bind to ^{35}S-ECM at a pH that was not permissive to its enzymatic activity (pH 7.2, 37°C), and the unbound enzyme was removed. The pH of the medium was then maintained at 7.2 or decreased to 6.4, and the ensuing release of degraded HS was assessed. Minimal release of HS degradation fragments occurred if the pH was kept at 7.2 (Fig. 3). However, at pH 6.4, the enzymatic activity of heparanase was restored, as indicated by the release of HS moieties from the matrix. Hence, at a physiological pH , the ECM can retain heparanase in an inactive form; but if the pH decreases, the temporarily stored enzyme (Fig. 2 *B*) can regain its catalytic activity (Fig. 3).

The ECM-bound Heparanase Induces the Adhesion of CD4$^+$ T Lymphocytes. Heparanase was diluted in incubation medium at different pH levels, added to ECM-coated microtiter wells, and the unbound heparanase was removed. ^{51}Cr-labeled resting human CD4$^+$ T cells were added to the precoated wells, and their binding to the substrates was assessed. The T cells adhered well to ECM that had been preincubated with heparanase at pH 7.2–7.4 (p <0.05), and only slightly to ECM exposed to heparanase at pH 6.8 and 6.4. Nonactivated T cells did not bind to the intact ECM, regardless of its exposure to various pH values, or if the ECM had not been treated with heparanase (Fig. 4 *A*). In contrast, phorbol ester–activated T cells bound to ECM irrespective of whether

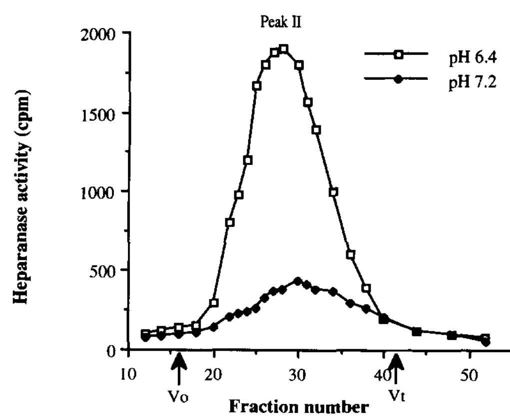

Figure 3. Restoration of the enzymatic activity of ECM-bound heparanase in response to a change in pH. Heparanase (0.1 μg/ml) was incubated (2 h, 37°C, pH 7.2) with ^{35}S-labeled ECM. Unbound heparanase was then removed by washing, and the ^{35}S-ECM plus bound heparanase was incubated (24 h, 37°C) at pH 6.4 or 7.2. Release of ^{35}S-labeled low molecular weight (peak II) HS degradation fragments was analyzed by gel filtration on Sephadex 6B. The figure shows one experiment representative of three.

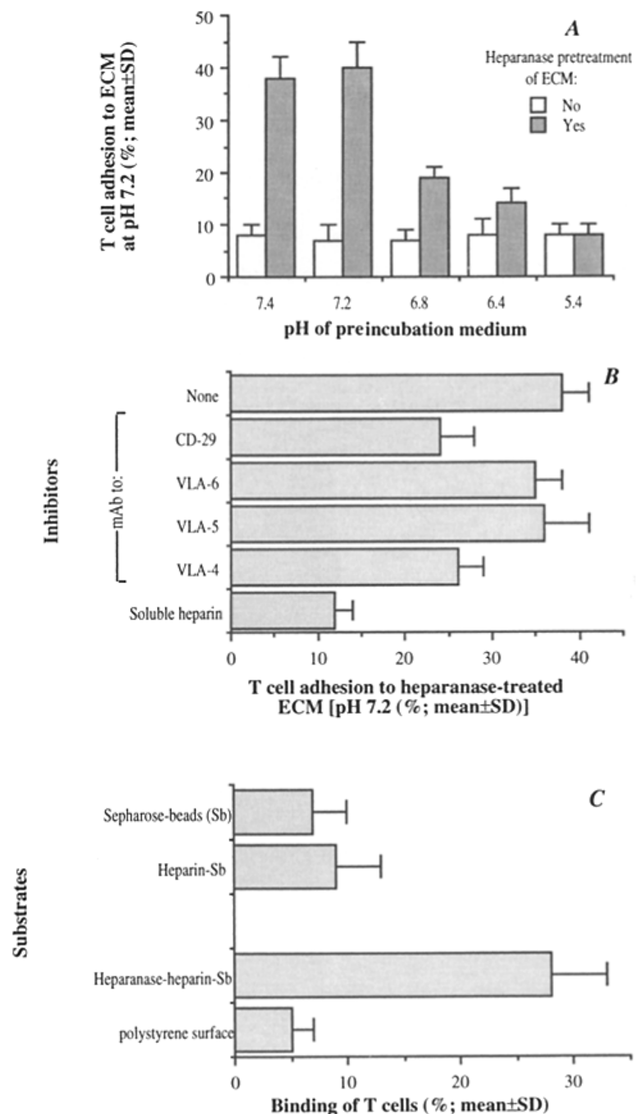

Figure 4. Adhesion of purified human CD4$^+$ T cells to ECM at pH 7.2. ^{51}Cr-labeled human CD4$^+$ T cells were added to ECM-coated microtiter wells that had been preincubated (2 h, 37°C, pH 7.2) with buffer or heparanase (0.1 μg/ml), followed by aspiration, washing, and blocking with 1% BSA in PBS. (*A*) Dependence of heparanase-mediated adhesion of T cells to ECM on the pH at which the heparanase was bound to the ECM. Cell binding to the wells was determined after 30 min at 37°C. (*B*) Involvement of integrins in heparanase-mediated binding of T cells to ECM. mAbs (15–25 μg/ml) to the human integrins, VLA-4-6, and CD29 (Serotec, Oxford, UK), were added to the ECM-coated wells simultaneously with the T cells. Where indicated, the T cells were preincubated with heparin (10 μg/ml) before seeding into the wells containing ECM and ECM-bound heparanase. In *A* and *B*, the results from quadruplicate wells are depicted of four experiments. (*C*) ^{51}Cr-labeled T cells (2 × 10^5 cells) were incubated (2 h, 37°C, pH 7.2) with 0.2 ml of Sepharose beads (*Sb*), heparin-Sb (0.2 ml), or heparanase bound to heparin-Sb (0.2 μg heparanase per sample). To measure T cell binding to the beads, the contents of the tubes were passed through disposable polypropylene columns (Pierce Chemical Co., Rockford, IL), which allow the passage of unbound, but not bound, cells. The columns were washed extensively, and the radioactivity associated with the columns was determined. In addition, the binding of T cells (2 × 10^5 cells/well) to heparanase-treated (1 μg/well; 2 h, 37°C, pH 7.2) microtiter wells (polystyrene; Falcon, Oxnard, CA) was examined 30 min after cell addition to the plates. The figure shows one experiment representative of four for *A–C*.

the ECM had been exposed to heparanase (not shown). The binding of T cells to heparanase-bound ECM was inhibited by heparin (Fig. 4 *B*), which competes with HS for heparanase recognition (6). Thus, analogous to proteoglycan-bound chemokines (3, 16, 23, 24), non–enzymatically active heparanase can act as a proteoglycan-associated proadhesive molecule for resting T cells.

T cell recognition of adhesive glycoproteins present in ECM is mediated primarily by β_1 integrins (very late antigens [VLA]; 1, 2). Whether the heparanase-induced T cell binding to ECM at pH 7.2 involves integrins was examined in blocking experiments with mAb. Adhesion of T cells to ECM to which heparanase was bound was partially inhibited by mAb to the common β_1 chain (CD-29) of the VLA integrins and by mAb to the VLA-4 molecule ($p < 0.05$; Fig. 4 *B*). Hence, the heparanase-mediated adhesion of $CD4^+$ T lymphocytes to ECM appears to involve integrin binding.

Whether the $CD4^+$ T cells can interact directly with heparanase at physiological pH was assessed by examining their adhesion to immobilized heparanase. T cells bound preferentially to heparanase attached to heparin–Sepharose beads, but not to intact heparin–Sepharose beads or to heparanase-treated microtiter wells (Fig. 4 *C*). At pH 6.4, heparanase cleaves the heparin from the Sepharose beads and thus the cell binding to heparin–Sepharose beads could not be assessed at this pH. These results suggest that the conformation acquired by the heparanase complexed to heparin-like molecules or HS in the ECM is accessible for T cell recognition, and that pH-dependent conformational changes in heparanase may account for its versatile roles.

Heparanase, like growth factors, chemokines, and cytokines, is secreted from activated $CD4^+$ T cells (2, 12, 25). The secreted heparanase can bind to HS in the ECM and can perform different functional programs, depending on the local conditions. Our results suggest that at relatively low pH values, heparanase, as a pivotal element of the invading cell's ECM-specific enzymatic repertoire, can actively participate in ECM degradation, and thereby facilitates cell migration. In fact, significant amounts of heparanase have been observed in inflamed tissues, such as in synovial fluids of rheumatoid arthritis patients (Vlodavsky, I. et al., manuscript in preparation), where the pH values are mildly acidic (26). At a physiological pH, the relatively quiescent enzyme appears to act as a lectin-like proadhesive molecule that can organize the recruitment of resting T cells in extravascular loci. Therefore, the heparanase-mediated ECM-anchored $CD4^+$ T cells could readily respond to costimulatory signals elicited by specifically activated adjacent immune cells. Besides the particular relevance of our findings for interactions between immune cells and the ECM, the pH-dependent modification of heparanase function illustrates a general principle that biologically important molecules can adapt their behavior to context (27).

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