Heparanase is a $\beta$-d-endoglucuronidase that cleaves heparan sulfate, an important structural component of the extracellular matrix (ECM) and vascular basement membrane (BM). The cleavage of heparan sulfate by heparanase-expressing cells, such as activated leukocytes, metastatic tumor cells, and proliferating endothelial cells, facilitates degradation of the ECM/BM to promote cell invasion associated with inflammation, tumor metastasis, and angiogenesis. In addition to its enzymatic function, heparanase has also recently been shown to act as a cell adhesion and/or signaling molecule upon interaction with cell surfaces. Despite the obvious importance of the mechanisms for the binding of heparanase to cell surfaces, the receptor(s) for heparanase remain poorly defined. In this study, we identify the 300-kDa cation-independent mannose 6-phosphate receptor (CIMPR) as a cell surface receptor for heparanase. Purified platelet heparanase was shown to bind the human CIMPR expressed on the surface of a transfected mouse L cell line. Optimal binding was determined to be at a slightly acidic pH (6.5–7.0) with heparanase remaining on the cell surface for up to 10 min at 37 °C. In contrast, mouse L cells or Chinese hamster ovary cells expressing the cation-dependent mannose 6-phosphate receptor (CDMPR) showed no binding of heparanase. Interestingly, the binding of heparanase to CIMPR was independent of Man-6-P moieties. Significantly, primary human T cells upon activation were shown to dramatically up-regulate levels of cell surface-expressed CIMPR, which showed a concomitant increase in their capacity to bind heparanase. Furthermore, the tethering of heparanase to the surface of cells via CIMPR was found to increase their capacity to degrade an ECM or a reconstituted BM. These data suggest an important role for CIMPR in the cell surface presentation of enzymatically active heparanase for the efficient passage of T cells into an inflammatory site and have implications for the use of this mechanism by other cell types to enhance cell invasion.

The extracellular matrix (ECM) and its specialized form known as basement membranes (BM) represent a major physical barrier to migrating cells (1, 2). An important ubiquitous structural component of the ECM/BM is heparan sulfate proteoglycan (HSPG). HSPGs are a diverse family of complex macromolecules that consist of a protein core to which are attached linear side chains of the glycosaminoglycan heparan sulfate (HS). They contribute to the assembly and stability of the ECM/BM by interacting with multiple matrix proteins, including collagen, laminin, nidogen, and fibronectin (3, 4). Heparanase is a $\beta$-d-endoglucuronidase that cleaves HS and has been proposed to have an important role in facilitating the disassembly of the ECM/BM by engaging in concert with the various matrix proteases. Heparanase is synthesized as a 65-kDa inactive proenzyme that is processed by removal of a 6-kDa linker fragment into an active form consisting of a heterodimer between the remaining 50- and 8-kDa polypeptide subunits (5). The proteolytic cleavage of heparanase is mediated by L-cathepsin and possibly other protease species (6), with the site of processing suggested as being localized to lysosomes (7, 8). Heparanase shows optimal catalytic activity in slightly acidic environments (pH 6.0–7.0); however, it retains its HS binding capacity at neutral pH (9) and has been proposed to also have a noncatalytic function as an adhesion or signaling molecule (7, 9–12). For many years it has been postulated that heparanase is utilized by cells such as activated leukocytes, metastatic tumor cells, and proliferating vascular endothelial cells to promote cell invasion associated with inflammation, tumor metastasis, and angiogenesis (14–16). In addition heparanase has been implicated in the liberation of HS-bound growth factors, e.g. basic fibroblast growth factor and vascular endothelial growth factor, from activated leukocytes (13).

3 The abbreviations used are: ECM, extracellular matrix; BM, basement membrane; HSPG, heparan sulfate proteoglycan; HS, heparan sulfate; TGF, transforming growth factor; $\beta$, uPA, urokinase plasminogen activator; CIMPR, cation-independent mannose 6-phosphate receptor; CDMPR, cation-dependent mannose 6-phosphate receptor; Man-6-P, mannose 6-phosphate; MPR, mannose 6-phosphate receptor; IGF-II, insulin-like growth factor II; PPM, polyphosphomannan ester; Strep-PE, streptavidin-phycoerythrin; MS9-II, mouse L cells stably transfected with human CIMPR; MS, mouse L cells stably transfected with expression vector; DMEM, Dulbecco’s modified Eagle’s medium; pgSA-745, Chinese hamster ovary cells unable to synthesize glycosaminoglycans; pgCDMPR, pgSA-745 cells stably transfected with human CDMPR; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; FCS, fetal calf serum; CIP, calf intestinal alkaline phosphatase; PE, phycoerythrin.
**Heparanase Binds the Cation-independent Man-6-P Receptor**

ECM deposits to initiate growth factor-dependent responses such as angiogenesis and wound healing (17, 18). The recent cloning of heparanase (19–23) has enabled experimental confirmation that the enzyme plays a key role in these processes. Heparanase overexpression strategies and mRNA knockdown approaches have demonstrated in experimental animal models a direct role for heparanase in tumor metastasis and angiogenesis (19, 24, 25), as well as inflammation (26). The clinical relevance of heparanase in tumor growth and metastasis is further supported by the comprehensive documentation of heparanase up-regulation in many human tumors, with heparanase expression correlating with increased metastasis, tumor vascularization, and reduced post-operative survival of patients (27–32). Furthermore, heparanase expression is up-regulated in inflammatory disease and diabetic nephropathy (33–37). The importance of heparanase in these disease settings, together with the identification of only a single heparanase, makes the enzyme a highly promising target for the development of anti-cancer and anti-inflammatory drugs.

It is evident from recent studies that heparanase can bind efficiently to the surface of cells, a process that has been postulated as important in a number of key aspects of heparanase function (15, 16). Cell surface display of heparanase has been implicated in cell adhesion, whereby heparanase can bind to T cells and aid adhesion to ECM components under shear flow conditions (9–11). The interaction of heparanase with the surface of endothelial cells has also been shown to trigger signaling cascades, including the enhancement of protein kinase B/Akt signaling and stimulation of phosphatidylinositol 3-kinase- and p38-dependent cell migration and invasion (38), as well as activation of the Src pathway that induces vascular endothelial growth factor expression to promote an angiogenic response (39). In addition, the ability of cells to bind and internalize exogenous heparanase via cell surface receptors has been suggested as important for the cellular uptake, processing, and storage of the enzyme (7, 12, 40). Furthermore, the tethering of degradative enzymes to the surface of migrating cells promotes the efficient local and directed degradation of ECM/BM barriers (15, 41), and this is likely to also be the case with heparanase (42, 43). The identification of heparanase receptor(s) expressed on the surface of cells is of fundamental importance in understanding the above processes; however, in most instances, these receptors remain undefined. A number of lines of evidence indicate that HS is important in the uptake of heparanase (12, 44, 45). More recently, the low density lipoprotein receptor-related protein and an unidentified mannose 6-phosphate receptor (MPR) were also suggested to mediate heparanase uptake; however, with the latter receptor it was only shown to be the case when in cooperation with HSPGs and receptor-associated protein-sensitive receptors (45).

Here, we identify the 300-kDa CIMPR (CD222) as a novel receptor for heparanase. Interestingly, the binding of heparanase to CIMPR was independent of Man-6-P. The tethering of heparanase to cell surfaces via CIMPR was found to significantly enhance the ability of cells to degrade the ECM/BM. Human primary T cells upon stimulation were shown to up-regulate the cell surface expression of CIMPR, and concomitantly the binding of heparanase, suggesting that this is a mechanism utilized by T cells and possibly other cells types to mediate efficient ECM degradation.

**EXPERIMENTAL PROCEDURES**

**Proteins and Reagents**—Polyphosphomannan ester (PPME) and 5-polyporphosphomannan ester were the gifts of G. Bartell (John Curtin School of Medical Research, Canberra, Australia). Streptavidin-phycocerythrin (Strep-PE) and streptavidin-TRICOLOR were purchased from Caltag Laboratories ( Burlingame, CA). Heparanase was purified from human platelets as described previously (46) and consisted of the majority (~95%) in an active processed form. Purified heparanase was labeled with biotin using biotin-NHS (Sigma) according to the manufacturer’s instructions, and it retained an activity comparable with that of unlabeled heparanase. Methotrexate, heparin (from porcine intestinal mucosa; Mn ~ 15,000), the monosodium salts of Man-6-P and glucose-6-phosphate, mannose, and bacterial heparanase were purchased from Sigma. The anti-CIMPR antibody JT-CIMPR was a generous gift of J. Trapani (Peter MacCallum Cancer Centre, Melbourne, Australia), and the anti-CIMPR antibody MEM-328 was from Abcam (Cambridge, UK). The anti-heparanase antibody (Hpa1) was obtained from Insight Biopharmaceuticals (Rehovot, Israel). The iduronidase and anti-iduronidase antibody (47) were the kind gifts of D. Brooks (Women’s and Children’s Hospital, Adelaide, Australia). The anti-HS antibody (F58-10E4) was from Seikagaku Corp. (Tokyo, Japan).

**Cells and Cell Culture**—Mouse L cells stably overexpressing human CIMPR (MS9-II) or transfected with DNA vector alone (MS), have been described previously (48) (obtained by permission of W. Sly (Washington University, St. Louis) from J. Trapani (Peter MacCallum Cancer Centre, Melbourne, Australia)) and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) (CSL, Parkville, Victoria, Australia) and 3.2 μm methotrexate (Sigma). The MS9-II cells express similar levels of cell surface CIMPR when compared with physiological cell settings such as activated primary human T cells and endothelial cells.4 Xylosyltransferase-deficient pgSA-745 Chinese hamster ovary cells (express no cell surface heparan sulfate) (49) stably transfected with a mammalian expression vector for human CDMPR (pKC4-CDM6R) or vector alone (pKC4) were maintained in DMEM supplemented with 10% FCS and 0.4 mg/ml genetin (Invitrogen). Transient transfection of MS cells with pCR3.1-CDMPR or pCR3.1 was carried out using Genejuice transfection reagent as described by the manufacturer (Novagen). HR9 cells (50) (provided by A. Tester, University of Melbourne, Australia) were cultured on 1% gelatin-coated flasks and maintained in DMEM supplemented with 10% FCS and daily addition of 50 μg/ml ascorbic acid. Purified human primary T cells (see below) were maintained in RPMI 1640 medium supplemented with 10% FCS. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂.

**Cell Surface Binding Assays**—Cells were analyzed for the binding of heparanase by immunofluorescence flow cytometry. Typically 2 μg/ml of biotinylated heparanase was added to 2 × 10^6 cells/ml in 100 μl of supplemented medium. Cells were stained with streptavidin-PE and analyzed for 20,000 events using a flow cytometer (46).

4 R. J. Wood and M. D. Hulett, unpublished observations.
Heparanase Binds the Cation-independent Man-6-P Receptor

10^5 cells in 1 ml of PBS, pH 6.0 to 7.2, containing 0.1% fraction V bovine serum albumin (BSA) on ice for 60 min, and cells were washed three times with ice-cold PBS and 0.1% BSA, pH 6.0 to 7.2. Biotinylated anti-rabbit Ig (Amersham Biosciences) was used as a control for background binding. Cell bound heparanase-biotin or anti-rabbit Ig-biotin was detected using Strept-PE (Caltag Laboratories) by flow cytometry using a FACScan (BD Biosciences) with Weasel software (Walter and Eliza Analysis Software Eclectic and Lucid version 2.2.2).

Assays to test the binding of heparanase to CDMPR were performed as above in the presence of 10 mM CaCl_2, pH 6.5. Binding assays were performed on transiently transfected MS cells 48 h post-transfection. As no antibody was available to human CDMPR, expression of the transfected gene was confirmed by reverse transcriptase-PCR with the primers 5’-ctccctgacctctcaagc-3’ and 5’-ctgggtagaatctgtgctg-3’ (data not shown), and cell surface expression of the receptor was demonstrated by binding of the Man-6-P containing molecule PPME (Fig. 1). Heparanase binding inhibition assays were carried out as above in the presence of 10 mM CaCl_2, pH 6.5. Binding assays were performed on transiently transfected MS cells 48 h post-transfection. As no antibody was available to human CDMPR, expression of the transfected gene was confirmed by reverse transcriptase-PCR with the primers 5’-ctccctgacctctcaagc-3’ and 5’-ctgggtagaatctgtgctg-3’ (data not shown), and cell surface expression of the receptor was demonstrated by binding of the Man-6-P containing molecule PPME (Fig. 1). Heparanase binding inhibition assays were carried out as above in the presence of 10 mM CaCl_2, pH 6.5. Binding assays were performed on transiently transfected MS cells 48 h post-transfection. As no antibody was available to human CDMPR, expression of the transfected gene was confirmed by reverse transcriptase-PCR with the primers 5’-ctccctgacctctcaagc-3’ and 5’-ctgggtagaatctgtgctg-3’ (data not shown), and cell surface expression of the receptor was demonstrated by binding of the Man-6-P containing molecule PPME (Fig. 1). Heparanase binding inhibition assays were carried out as above in the presence of 10 mM CaCl_2, pH 6.5. Binding assays were performed on transiently transfected MS cells 48 h post-transfection. As no antibody was available to human CDMPR, expression of the transfected gene was confirmed by reverse transcriptase-PCR with the primers 5’-ctccctgacctctcaagc-3’ and 5’-ctgggtagaatctgtgctg-3’ (data not shown), and cell surface expression of the receptor was demonstrated by binding of the Man-6-P containing molecule PPME (Fig. 1).
washing the culture wells with PBS followed by addition and removal of 2 ml of NH$_4$OH/Triton X-100 solution (100 mM NH$_4$OH, 0.5% Triton X-100). MS and MS9-II cells were incubated at 4°C with or without 2 μg/ml heparanase for 60 min and washed three times in PBS before being seeded in DMEM containing 10% FCS onto the labeled matrix at 1 × 10^4 cells/well. The cells were incubated at 37°C for 12, 24, and 48 h at which time the media were collected, and the cells were removed by centrifugation at 400 × g. Five hundred microliters of cleared media was then added to 3.5 ml of Ready Safe scintillation fluid (Beckman Coulter, Fullerton, CA), and the γ-radiation level was assessed with a 1500 Tri-Carb liquid scintillation analyzer (PerkinElmer Life Sciences).

Matrigel (BD Biosciences) was thawed overnight at 4°C and diluted 1:2 in ice-cold serum-free DMEM. Thirty microliters containing 10% FCS were aliquoted per well into a 96-well plate and incubated at room temperature for 60 min, washed three times in PBS containing 0.1% BSA, pH 7.2, and loaded into wells at 3 × 10^4 cells/well in DMEM containing 10% FCS. The plates were incubated for 12 or 48 h at 37°C. The media were removed, and the plates were washed twice in warm PBS to remove the cells, and the gel was then solubilized by the addition of 100 μl of ice-cold PBS per well and incubation at 4°C overnight. The protein concentration remaining in each well was then assayed using a Bradford protein assay as recommended by the manufacturer (Bio-Rad).

**Primary Human T Cell Purification and Stimulation**—Human primary T lymphocytes were isolated by negative selection using a modification of a protocol used to isolate human natural killer cells (52). Whole peripheral blood was collected from healthy human volunteers into heparinized tubes by informed consent under studies approved by the human ethics committee of the Australian National University. Ficoll-Paque Plus (Amersham Biosciences) was overlaid by blood diluted at a ratio of 1:2 in 0.5% BSA/PBS and centrifuged at 600 × g at room temperature for 20 min. Peripheral blood mononuclear cells were harvested and washed twice in 0.5% BSA/PBS. Peripheral blood mononuclear cells and freshly isolated red blood cells suspended in 0.5% BSA/PBS were then combined at a ratio of 1:100. RosetteSep human T cell enrichment antibody mixture (Stem Cell Technologies, Canada) was added in a ratio of 50 μl of mixture/ml of cells, which included monoclonal antibodies to the following cell surface antigens: CD16 (granulocytes, monocytes/macrophages), CD19 (B cells), CD36 (platelets, monocytes), and CD56 (NK cells). The suspension was incubated at room temperature for 60 min, diluted 1:2 in 0.5% BSA/PBS, overlaid on RosetteSep DM-L (Stem Cell Technologies), and then centrifuged at 600 × g for 20 min without braking. T cells were harvested from the interface and washed twice in 0.5% BSA/PBS. T cells were then resuspended at a concentration of 1.25 × 10^6 cells/ml in 10% FCS/RPMI supplemented with penicillin, streptomycin, neomycin, and 2 mM glutamine and seeded in a Linbro 12-well plate (ICN Biomedical, Aurora, OH). In activation experiments, the media were supplemented with either 20 ng/ml phorbol 12-myristate 13-acetate along with 0.1 μM ionomycin or 5 μg/ml concanavalin A (Sigma).

**RESULTS**

**CIMPR but Not CDMPR Binds Heparanase**—To determine whether heparanase is able to bind the CIMPR and/or CDMPR, transfected cell lines expressing either receptor on their cell surfaces were analyzed by immunofluorescence flow cytometry. Biotinylated human platelet heparanase bound the human CIMPR-expressing mouse L cell line (MS9-II) at levels 5–6-fold higher than control mouse L cells transfected with an expression vector alone (MS). A negative control biotinylated protein showed no binding (Fig. 1, A and C). The optimal binding of heparanase to the MS9-II cells was determined to be at pH 6.5–7.0 (data not shown). In contrast, CDMPR-expressing pgSA-745 cells showed only a background level of heparanase binding when compared with the vector alone control transfected cells (Fig. 1, B and C). As expected, the CDMPR-expressing pgSA-745 cells were capable of binding the Man-6-P containing sugar PPME (Fig. 1B). The observation of the inability of CDMPR-expressing pgSA-745 cells to bind heparanase was further examined by expression of CDMPR in MS cells. MS cells transiently transfected with a CDMPR expression construct showed no heparanase binding when compared with vector alone control transfected MS cells. The CDMPR-expressing MS cells bound PPME at similar levels to the CIMPR-expressing MS9-II cells, suggesting equivalent levels of cell surface expression of the CDMPR and CIMPR in these cell systems (Fig. 1D). These data indicate that heparanase is able to bind the CIMPR but not the CDMPR.

To further investigate the interaction of heparanase with the CIMPR, purified human platelet heparanase was incubated with MS9-II cells, and binding to the CIMPR was assessed by immunoprecipitation of the CIMPR followed by Western blot analysis for heparanase. Heparanase incubated with MS9-II cells was found in association with the CIMPR as demonstrated by the detection of heparanase in Western blots of immunoprecipitates of the CIMPR generated with two different anti-human CIMPR monoclonal antibodies, but not with an isotype-matched control antibody (Fig. 1E). Using an identical approach, no heparanase was detected binding to the non-CIMPR-expressing MS cells (data not shown).

**Cell Surface Localization and Internalization of Heparanase and CIMPR**—Confocal microscopy was used to visualize the binding of heparanase to the MS9-II and MS cells. MS9-II cells showed strong heparanase binding that was localized to the cell surface, whereas MS cells showed only weak binding (Fig. 2). The analysis of these cells for expression of the CIMPR using an anti-CIMPR-specific monoclonal antibody showed a very similar pattern of fluorescent staining as that for heparanase binding when compared with the vector alone control transfected cells (Fig. 2). Using an identical approach, no heparanase was detected binding to the non-CIMPR-expressing MS cells (data not shown).

**Heparanase Binds the Cation-independent Man-6-P Receptor**
Heparanase Binds to MS9-II Cells Is Not Dependent on HS—It has been shown previously that heparanase can bind to HS expressed on the surface of cells (12, 53). Although a significant difference was observed in the binding of heparanase by the CIMPR-expressing MS9-II and the vector control MS cell lines, it remained important to determine that the observed binding of heparanase to the MS9-II cells was not simply because of the interaction with HS. Initially, the expression levels of cell surface HS on MS9-II and MS cells were determined by immunofluorescence flow cytometry and found to be very similar between both cell lines (data not shown). The binding of heparanase to the MS9-II and MS cells was then assessed as described above, however, in the presence of various concentrations of heparin. At concentrations of 1 μg/ml and above, heparin inhibited some of the binding of heparanase to the MS9-II cells; however, the cells still retained significant heparanase binding that was about 60% that of untreated cells (Fig. 3). The binding of heparanase to the negative control MS cells was inhibited with heparin essentially to background levels of fluorescence. In further support, the pretreatment of MS9-II cells with bacterial heparinase to remove cell surface HS reduced heparanase binding to levels similar to that observed by blocking with heparin, with 60% of binding retained (data not shown).

Heparanase Binding to the CIMPR Is Man-6-P-independent—As the name suggests, the interaction of the majority of the ligands for the CIMPR is dependent on Man-6-P moieties. To determine whether the interaction of heparanase with CIMPR is Man-6-P-dependent, binding inhibition studies were performed with various sugar inhibitors. Man-6-P showed no inhibitory effect on the binding of heparanase at a range of concentrations known to inhibit other Man-6-P-dependent ligands, even at high nonphysiological concentrations (Fig. 4A). Similar results were observed for a number of other simple sugars, including mannose, glucose, and glucose 6-phosphate (data not shown). Interestingly PPME, which is a large and highly Man-6-P-rich polysaccharide of ~2000 kDa, significantly inhibited the binding of heparanase (Fig. 4, A and B). The inhibition of heparanase binding by PPME is likely to be a result of steric hindrance rather than as acting as an inhibitor of heparanase binding by direct blocking of the Man-6-P-binding sites. This possibility was further suggested by the use of the much smaller Man-6-P-containing sugar 5-polymannosidomannan ester that had no effect on heparanase binding at similar
Heparanase Binds the Cation-independent Man-6-P Receptor

FIGURE 2. Confocal microscopy of heparanase binding and internalization by CIMPR-expressing cells. A, heparanase and the CIMPR are expressed on the cell surface. MS9-II and MS cells were cultured as adherent monolayers on coverslips and incubated with biotinylated heparanase (2 μg/ml) or a mouse anti-human CIMPR antibody (JT-CIMPR). Heparanase binding was detected by incubation with streptavidin-TRICOLOR (red), and the localization of CIMPR was detected using an anti-mouse Ig FITC secondary antibody. All binding incubations were performed on ice. Images were taken in sequence using a Bio-Rad Radiance 2000 confocal microscope and the images processed with LaserSharp 2000 (Bio-Rad). The CIMPR and heparanase images were overlaid to give the merged image. B, heparanase internalization by CIMPR-expressing cells. Biotinylated heparanase (2 μg/ml) was incubated with MS9-II cells and detected with streptavidin-TRICOLOR on ice. The kinetics of heparanase internalization was then assessed by raising the temperature of the cells to 37 °C and capturing images using a confocal microscope as described above at times 0, 5, 10, 15, and 20 min. Control cells are MS9-II cells that were incubated with streptavidin-TRICOLOR only and shown at the 20-min time point. Because of bleaching of fluorescence, different fields are shown for the images at 0/5, 10/15, and 20 min.

concentrations of Man-6-P showed increased inhibition (Fig. 5B). This finding was confirmed by treatment of the enzyme with CIP that ablated over a third of the binding of the enzyme (Fig. 5C). It should be noted that it was expected that CIP treatment of heparanase, PPME, or iduronidase would not be completely efficient in removing all phosphate moieties from these molecules; however, their partial removal resulted in the clearly quantifiable reduced binding of PPME and iduronidase but not heparanase to CIMPR. These data, together with inability of the CDMPR to bind heparanase, collectively demonstrate that the binding of heparanase to the CIMPR is Man-6-P-independent.

Heparanase Binding by MS9-II Cells Increases Matrix Degradation—The tethering of heparanase to cell surfaces through the CIMPR may represent a mechanism to enhance the capacity of a cell to degrade the ECM. This was investigated by examining the ECM-degrading capacity of heparanase bound to the surface of the CIMPR-expressing cell line MS9-II versus the nonexpressing cell line MS. Initially, the ability of heparanase-exposed and nonexposed cells to degrade a specialized BM-like matrix produced by HR9 cells was assessed. MS9-II and MS cells were found to have low degradative capacity in their native state; however, upon exposure to exogenous heparanase the MS9-II cells showed a 3–4-fold increased capacity to degrade the HR9-produced BM when compared with MS cells exposed to heparanase (Fig. 6A). The increased capacity of MS9-II cells exposed to heparanase to degrade an ECM was also demonstrated using a Matrigel solubilization assay. MS9-II cells exposed to heparanase showed an increased capacity to solubilize the commercial ECM reagent Matrigel after 12 h when compared with nonexposed MS9-II cells or MS cells exposed/nonexposed to heparanase (Fig. 6B). This trend was more pronounced following a 48-h incubation period; however, it was interesting to note that MS cells exposed to heparanase over this longer period also demonstrated the abil-
Primary Human T Cells Up-regulate Surface Expression of CIMPR upon Activation and Increase Their Ability to Bind Exogenous Heparanase—Heparanase has been implicated in the ability of lymphocytes to extravasate to sites of inflammation (33, 54, 55). As described above, the tethering of heparanase to the cell surface via the CIMPR may aid this process. We therefore investigated whether resting primary human T cells were able to increase cell surface expression of CIMPR following activation by stimulation with the pharmacological agents phorbol 12-myristate 13-acetate and ionomycin, agonists that mimic ligand-induced T cell activation. An increase in the level of cell surface-expressed CIMPR was observed at 24 h following post-stimulation (2-fold), with levels further increasing over longer periods to 96 h (~6-fold) (Fig. 7A). The increase in the cell surface levels of CIMPR by the activated T cells corresponded with an increased ability of the cells to bind heparanase (Fig. 7B). Similar results were obtained upon stimulation of T cells with concanavalin A (data not shown). These data suggest that the activation of primary human T cells results in the up-regulation of cell surface CIMPR and thus an increased capacity to bind heparanase.

DISCUSSION

Heparanase has long been implicated in promoting cell invasion, particularly in the context of tumor metastasis, angiogenesis, and inflammation (15, 16, 56). The cell surface expression of heparanase has been described on a range of tumor cells (28, 57, 58), activated leukocytes (42, 43), and endothelial cells (18, 59), where it has been suggested as important in mediating efficient degradation of ECM barriers. Despite initial suggestions that heparanase may contain a C-terminal transmembrane region or a motif for glycosylphosphatidylinositol anchoring, it is now clear that heparanase is not anchored directly to cell membranes, and instead is tethered to cell surfaces via specific receptors (16). However, the receptors for heparanase are poorly defined. In this study for the first time we identify the CIMPR as a novel cell surface receptor for heparanase. The CIMPR was found to bind heparanase independently of Man-6-P moieties and to display enzymatically active heparanase on cell surfaces to promote ECM degradation.

The CIMPR is a ubiquitously expressed 250-kDa type 1 integral membrane protein that has a predominantly intracellular location (90–95%); however, 5–10% is constitutively expressed on the surface of most cell types. The CIMPR is a multifunctional receptor that interacts with a diverse array of ligands. A key function of the receptor is the transport of newly synthesized lysosomal enzymes containing Man-6-P moieties from the Golgi apparatus to lysosomes. The CIMPR also has an important role as a membrane-bound extracellular recycler of exogenous insulin-like growth factor II (IGF-II) and is therefore an important regulator of cell growth (60–62). The receptor also binds a range of other important ligands, including retinoic acid, plasminogen, and the uPA receptor (63–65). The extracellular region of the CIMPR contains 15 P-type lectin domains that share 14–38% amino acid identity with the single extracellular domain of the CDMPR (66). Three distinct binding sites for Man-6-P have been mapped to domain 3 and domain 9 (67) with a low affinity site on domain 5 (68), whereas the IGF-II-binding site has been mapped to domain 11 (69–71). Plasminogen and uPA binding have been located to the N-terminal half of domain 1 (72). The binding domain(s) of retinoic acid remains to be elucidated.
Heparanase Binds the Cation-independent Man-6-P Receptor

FIGURE 5. The binding of iduronidase to CIMPR is inhibited by Man-6-P and alkaline phosphatase treatment. A, iduronidase binds CIMPR expressed on the surface of cells. MS9-II and MS cells were incubated in the presence (solid line) and absence (filled histogram) of iduronidase (1 μg/ml), and cell surface binding was detected using a mouse anti-iduronidase antibody followed with an anti-mouse Ig-PE conjugate by immunofluorescence flow cytometry. The histograms are representative of three independent experiments. B, Man-6-P inhibits the binding of heparanase to the CIMPR. MS9-II (black boxes) or MS (gray boxes) were preincubated with various concentrations of Man-6-P (0, 1, 5, 50 mM) for 60 min on ice, and the binding of iduronidase (1 μg/ml) was determined by immunofluorescence flow cytometry as above. Iduronidase binding is expressed as a fold increase in median fluorescence relative to background fluorescence. Error bars represent S.E. (n = 3, where n represents three independent experiments). C, alkaline phosphatase treatment inhibits the binding of iduronidase to the CIMPR. Iduronidase was pretreated with or without calf intestinal alkaline phosphatase for 60 min at 37 °C in either phosphate buffer (PO4) or normal saline (NS), and the effect of binding to MS9-II (black squares) and MS (gray squares) cells was determined by using immunofluorescence flow cytometry as above. Error bars represent S.E. (n = 3, where n represents three independent experiments).

although it is known to be independent of the Man-6-P and IGF-II-binding sites (63).

It is well established that the expression and/or tethering of degradative enzymes to the surface of cells enhances their invasive capacity by promoting efficient local and directional dismantling of the ECM (41, 73–75). Examples include the urokinase-type plasminogen activator-receptor system (41, 76) and the membrane-type matrix metalloproteinases (77, 78). In this context it is significant that the CIMPR binds uPA and plasminogen/plasmin, a degradative enzyme system that plays an important role in maintaining vascular homeostasis by degrading fibrin clots (79) and also aids cell invasion associated with inflammation, angiogenesis, and tumor metastasis (80). In addition, cell surface expressed CIMPR can also bind secreted lysosomal degradative enzymes (81, 82). These observations, together with that reported herein for heparanase binding to the CIMPR, suggest that this receptor may well provide a mechanism for tethering a number of ECM-degrading enzymes, possibly simultaneously, on cell surfaces to promote efficient cell invasion. The inducible cell surface expression of CIMPR on T cells upon activation, described in this study for the human and suggested previously in the rat (83), implies that this cell type uses this mechanism to aid migration to sites of inflammation. It remains of significant interest to determine whether other invasive cell types, e.g. other leukocytes, endothelial cells, and metastatic tumor cells, also use this mechanism of up-regulating cell surface expression of CIMPR to enhance migration. To this end, we have recent evidence to suggest that certain metastatic tumor cell lines express high levels of CIMPR on their cell surfaces and demonstrate a concomitant increased capacity to bind heparanase. Furthermore, some recent studies have reported that heparanase is translocated to the surface of activated macrophages and monocyte-derived dendritic cells upon maturation; however, the receptor(s) mediating this tethering were not identified (42, 43). The CIMPR represents an attractive candidate receptor for heparanase in these settings.

Our results convincingly demonstrate that the binding of heparanase to the CIMPR is Man-6-P-independent. We have provided a number of lines of supporting evidence using different experimental approaches, including (i) Man-6-P did not block the interaction of heparanase with CIMPR-expressing cells (Fig. 4A), (ii) the removal of Man-6-P moieties on heparanase using CIP did not affect binding to CIMPR (Fig. 4C), and (iii) heparanase did not bind the structurally related Man-6-P-binding receptor CDMPR (Fig. 1C). In contrast, parallel control experiments with iduronidase, an enzyme that is well characterized as binding CIMPR in a Man-6-P-dependent manner, clearly demonstrated that the interaction of iduronidase and CIMPR-expressing cells was blocked with Man-6-P or CIP treatment of the enzyme (Fig. 5). The observed Man-6-P-independent binding of heparanase to the CIMPR is different from two previous reports that present data that can be interpreted as heparanase binding to an unidentified MPR in a Man-6-P-dependent manner (45, 84). However, it should be noted that in both of these studies no direct evidence was provided that heparanase binds MPRs. Bartlett et al. (84) suggested that Man-6-P inhibited the in vitro degradation of a35SO4-labeled ECM by human lymphocytes, and they speculated that this could be due to Man-6-P displacement of heparanase bound to cell surface MPRs. In a recent study, Vreys et al. (45) proposed that the cellular uptake
of secreted precursor heparanase involved MPRs, however, only when in cooperation with HSPGs and receptor-associated protein-sensitive receptors. The basis of this finding was that Man-6-P was shown to partially block the uptake of heparanase by mouse embryonic fibroblast cells, an effect only observable when receptor-associated protein-sensitive receptors were either blocked or absent, with the readout for these studies being the appearance of processed heparanase in cell extracts. As heparanase appears to be processed in the lysosomal compartment (7, 8), a possible explanation for the effect of Man-6-P observed in both of these studies is that rather than Man-6-P

FIGURE 6. Cell surface heparanase enhances degradation of the ECM. A, heparanase binds to CIMPR-expressing cells and aids degradation of an HR9 cell-produced ECM. An $^{35}$S-labeled extracellular matrix deposited by HR9 cells cultured in 12-well plates was seeded with $1 \times 10^5$ cells/well of MS9-II or MS cells that had been incubated with or without heparanase (2 $\mu$g/ml) and then washed. The cells were incubated for 24 h on the labeled matrix, and the amount of labeled $^{35}$SO$_4$ released into the media was assessed. Error bars represent S.E. ($n = 3$, where $n$ represents three independent experiments). * indicates $p < 0.05$ to all other treatments, determined using a two-tailed Student’s $t$ test. B, protein concentration (mg/ml) of MS, MS+HPSE, MS9, and MS9+HPSE after 6, 24, 48, and 96 h of incubation. C, protein concentration (mg/ml) of MS, MS+HPSE, MS9, and MS9+HPSE after 6, 24, 48, and 96 h of incubation. Error bars represent S.E. ($n = 3$, where $n$ represents three independent experiments).

FIGURE 7. Activated primary human T cells up-regulate cell surface expression of CIMPR and increase their capacity to bind heparanase. A, human primary T cells were stimulated with 20 ng/ml phorbol 12-myristate 13-acetate, 0.1 $\mu$M ionomycin (for 0, 6, 24, 48, 72, and 96 h), and the cell surface expression of CIMPR was determined by immunofluorescence flow cytometry using a mouse anti-human CIMPR antibody (JT-CIMPR) followed by an anti-mouse Ig-PE antibody. CIMPR binding is expressed as a fold increase in median fluorescence relative to background fluorescence. Error bars represent S.E. ($n = 5$, where $n$ represents five independent experiments). B, human primary T cells were stimulated as in A and were assayed for both surface expression of CIMPR and ability to bind heparanase. CIMPR expression was determined as above, whereas heparanase binding was carried out by incubating with biotinylated heparanase (2 $\mu$g/ml) and detected with streptavidin-PE. CIMPR expression (●) and heparanase binding (●) are shown. Error bars represent S.E. ($n = 3$, where $n$ represents three independent experiments).

of secreted precursor heparanase involved MPRs, however, only when in cooperation with HSPGs and receptor-associated protein-sensitive receptors. The basis of this finding was that Man-6-P was shown to partially block the uptake of heparanase by mouse embryonic fibroblast cells, an effect only observable when receptor-associated protein-sensitive receptors were either blocked or absent, with the readout for these studies being the appearance of processed heparanase in cell extracts. As heparanase appears to be processed in the lysosomal compartment (7, 8), a possible explanation for the effect of Man-6-P observed in both of these studies is that rather than Man-6-P

reflects the degree of matrix degradation) was determined after 12 (6) or 48 h (C) by the Bradford protein assay. Error bars represent S.E. ($n = 3$, where $n$ represents three independent experiments). * indicates $p < 0.05$; ** indicates $p < 0.01$; and *** indicates $p < 0.001$, determined using a two-tailed Student’s $t$ test.
Heparanase Binds the Cation-independent Man-6-P Receptor

blocking or displacing the binding of heparanase to MPRs, it may actually be inhibiting the processing of heparanase by impairing the delivery of critical lysosomal degradative enzymes to the lysosome. Indeed, cathepsin L and D, which have been shown to process the latent precursor form of heparanase to its active form (6), are both lysosomal enzymes, and their function is normally dependent on their interaction with the CIMPR via Man-6-P moieties for the appropriate trafficking from the Golgi compartment to lysosomes (60, 85).

The ability of heparanase to bind CIMPR on the surface of cells also has implications for the uptake of the enzyme. It is well characterized that cell surface CIMPR can efficiently bind and internalize a range of exogenous ligands, including Man-6-P-characterized that cell surface CIMPR can efficiently bind and internalize a range of exogenous ligands, including Man-6-P-containing ligands, e.g. secreted lysosomal enzymes (85, 86), as well non-Man-6-P-containing ligands such as IGF-II (61, 62). It is clear that the uptake of exogenous heparanase can be mediated by cell surface HSPGs such as syndecan family members (12); however, recent studies have also implicated the low density lipoprotein-related protein and an unidentified MPR (45). Using confocal microscopy we have shown CIMPR expressed on the surface of fibroblasts can mediate the internalization of exogenous heparanase (Fig. 2). The internalization of heparanase was not blocked by heparin in transfected fibroblasts expressing cell surface CIMPR, strongly suggesting an HSPG-independent uptake mechanism mediated via CIMPR. It should be noted that our demonstration of the ability of heparanase to bind the CIMPR independently of Man-6-P makes it unique among the lysosomal degradative enzymes. Thus, in contrast to Man-6-P-dependent lysosomal enzymes that can no longer bind the CIMPR following loss of Man-6-P moieties, the Man-6-P-independent binding of heparanase to the CIMPR would be expected to allow a continual recycling of heparanase. Thus, secreted heparanase could be captured/recaptured by cell surface CIMPR and internalized for storage and/or release. The ability of CIMPR-expressing cells to bind increased levels of heparanase was found to mediate enhanced ECM degradation. Significantly, heparanase was determined to remain on the surface of CIMPR-expressing MS9-II cells for up to 10 min at 37 °C before being internalized. We have recently observed that heparanase internalized by the CIMPR can be represented on the cell surface.9 This mechanism of heparanase uptake and recycling for cell surface display may represent a cellular process to enable efficient degradation of the ECM. It is interesting to note that CIMPR-negative MS cells, which can bind heparanase via cell surface HSPGs, also showed the capacity to degrade an ECM but only after longer incubation times and at significantly lower levels than CIMPR-expressing MS9-II cells. These data suggest that cell surface HSPGs, like the CIMPR, can bind and internalize heparanase that is likely to be stored intracellularly and represented over time to increase ECM degradation.

It should be considered that heparanase nonexpressing cells may utilize CIMPR to capture exogenous heparanase released by heparanase overexpressing cell types. This could represent an important mechanism for a number of cell types to participate in various heparanase-mediated physiological and pathological processes. Of interest in this respect is a recent study by Edovitsky et al. (26) that implicated endothelial cells as important for the expression and secretion of heparanase in delayed type hypersensitivity-associated inflammation. It was further proposed that the vascular endothelium might be a critical source of heparanase activity in the remodeling of the vascular BM for extravasation of leukocytes in inflammation. Traditionally, activated T lymphocytes have been regarded as the primary cellular source of heparanase in inflammation (33, 54, 55, 87). Although it is well characterized that activated T lymphocytes express their own heparanase (37, 55, 88, 89), the observation herein that these cells up-regulate cell surface expression of CIMPR suggests that T lymphocytes have the capacity to also capture and utilize heparanase produced by other cell types, e.g. endothelial cells at sites of inflammation. It remains of major interest to determine whether T lymphocytes and other cell types utilize this mechanism.

The binding of heparanase by the CIMPR raises the question as to whether the receptor is also involved in the direct sorting of the synthesized enzyme from the Golgi apparatus to lysosomes. The current model of heparanase biosynthesis, processing, and trafficking is that pro-heparanase is secreted via vesicles from the Golgi whereby it interacts with cell surface receptors (e.g. HSPG) and internalized where it accumulates in endosomes and eventually in lysosomes. The pro-heparanase is then processed in lysosomes into the active form of the enzyme. The fact that heparanase is a lysosomal enzyme that binds CIMPR suggests that the receptor is likely to play an important role in heparanase trafficking to the lysosomal compartment. Studies are currently underway to investigate this proposal.

As described above, there is strong evidence to suggest that heparanase is processed in lysosomes (7, 8). However, it is conceivable that in specific settings the processing of pro-heparanase may also occur on the surface of cells, possibly mediated by the concomitant binding by CIMPR of L-cathepsin (Man-6-P-dependent) together with pro-heparanase (Man-6-P-independent). It has been shown that under normal conditions a small percentage of active lysosomal enzymes escape lysosomal delivery and are secreted whereby they can be recaptured by cell surface CIMPR (85, 86). Furthermore, in disease states such as breast cancer, lysosomal enzymes such as cathepsin D are sometimes secreted at high levels (90). Importantly, the appropriate acidic conditions that are required for lysosomal enzyme activity would occur in the tissue microenvironment at sites of inflammation, tissue injury, and tumor growth, the very settings where heparanase is expected to be enzymatically active. In this context, it interesting to note that plasminogen/plasmin, like heparanase, binds the CIMPR in an Man-6-P-independent manner (65). Of particular relevance to the hypothesis that the CIMPR promotes the processing of latent heparanase is that the CIMPR plays a pivotal role in bringing together on the cell surface, plasminogen, the urokinase-type plasminogen activator receptor and latent TGFβ, to facilitate plasmin production and subsequently TGFβ activation (65, 72, 91). Thus, the CIMPR through specific interactions with multiple proteins, may well be central in bringing together a number of proteolytic enzymes and target substrates to mediate the activation of latent proteins, such as pro-heparanase. Work is in progress to determine whether the CIMPR plays a direct role in the processing of heparanase.
As discussed, heparanase can function as a signaling molecule independently of its enzymatic activity (38, 39). The receptor on the surface of cells that bind heparanase and mediate these signaling events are unknown, and their identification is clearly of major importance in understanding heparanase function. It is possible that the CIMPR upon binding heparanase is able to initiate signal transduction events. The role of CIMPR in the intracellular trafficking of lysosomal enzymes and the endocytosis-mediated uptake of secreted lysosomal enzymes and other ligands such as IGF-II is well characterized (62). In contrast, the role of CIMPR in transmembrane signaling remains unclear. It is known that CIMPR is able to mediate Na\(^+\)/H\(^+\) exchange and inositol triphosphate production following the binding of IGF-II (13). Furthermore, a number of studies have suggested a possible role for G-proteins in ligand-induced responses of CIMPR (62), including a recent study that demonstrates the receptor is coupled to a G-protein in the brain and activates a G-protein-sensitive protein kinase C-dependent pathway (92). Whether CIMPR has a role in heparanase-mediated signaling awaits further investigation.

In summary, our studies have identified the CIMPR as a novel receptor for heparanase. It should be noted that the interaction of heparanase with the CIMPR could be direct or indirect, something that is currently being investigated. However, it is clear that heparanase binds the CIMPR, and regardless of the nature of the interaction, the physiological outcomes as described in this study would be conserved. Interestingly, the binding of heparanase to the CIMPR was determined to be independent of Man-6-P. Significantly, heparanase tethered to primary T cells up-regulate levels of cell surface-expressed CIMPR that corresponds with an enhanced capacity to tether cell surface heparanase. This mechanism has implications for the passage of T cells into inflammatory sites, and possibly by cell surface heparanase. This mechanism has implications for the uptake, processing, and utilization of heparanase by non-heparanase-expressing cells. The identification of the CIMPR as a receptor for heparanase may provide a novel target for the development of new strategies to block the action of heparanase in tumor metastasis and inflammatory disease.

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