Characterization of Mechanisms Involved in Secretion of Active Heparanase*

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Heparanase is an endo-β-D-glucuronidase involved in extracellular matrix remodeling and degradation and implicated in tumor metastasis, angiogenesis, inflammation, and autoimmunity. The enzyme is synthesized as a latent 65-kDa protein and is processed in the lysosomal compartment to an active 58-kDa heterodimer, where it is stored in a stable form. In contrast, its heparan sulfate substrate is localized extracellularly, suggesting the existence of mechanisms that trigger heparanase secretion. Here we show that secretion of the active enzyme is mediated by the protein kinase A and C pathways. Moreover, secretion of active heparanase was observed upon cell stimulation with physiological concentrations of adenosine, ATP, and ADP, as well as by the noncleavable ATP analogue adenosine 5′-O-(thiotriphosphate). Indeed, heparanase secretion was noted upon cell stimulation with a specific P2Y receptor agonist and was inhibited by P2Y receptor antagonists. The kinetics of heparanase secretion resembled the secretion of cathepsin D, a lysosomal enzyme, indicating that the secreted heparanase is of lysosomal origin. We suggest that secretion of active heparanase is initiated by extracellular cues activating the protein kinase A and C signaling pathways. The secreted enzyme(s) then facilitate cell invasion associated with cancer metastasis, angiogenesis, and inflammation.

Heparanase is an endoglycosidase that specifically cleaves heparan sulfate (HS)2 side chains of heparan sulfate proteoglycans (HSPG) (1, 2). HSPG consist of a protein core to which HS side chains are covalently attached. These complex macromolecules are highly abundant in the extracellular matrix (ECM) and are thought to play an important structural role, contributing to ECM integrity and insolubility (3). In addition, HS side chains can bind a variety of biological mediators, such as growth factors, cytokines, and chemokines, thus functioning as a readily available reservoir that can be liberated upon local or systemic cues. Moreover, HSPG on the cell surface participate in signal transduction cascades by potentiating the interaction between certain growth factors and their receptors (4–6). HS-degrading activity is thus expected to affect several fundamental aspects of cell behavior under normal and pathological settings and should therefore be kept tightly regulated. Traditionally, heparanase activity was implicated in cellular invasion associated with angiogenesis, inflammation, and cancer metastasis (7–11). This notion gained further support by employing small interfering RNA and ribozyme technologies, clearly depicting heparanase-mediated HS cleavage and ECM remodeling as critical requisites for metastatic spread (12). Since the cloning of the hepanase gene and the availability of specific molecular probes, heparanase up-regulation was documented in an increasing number of primary human tumors, correlating with enhanced local and distant metastasis, increased microvessel density, and reduced postoperative survival of cancer patients. Collectively, these studies provide compelling evidence for the clinical relevance of the enzyme, making it an attractive target for the development of anti-cancer drugs (1, 2, 13).

Similar to several other classes of enzymes, heparanase is first synthesized as a latent enzyme that appears as a ~65-kDa protein when analyzed by SDS-PAGE. The 65-kDa latent enzyme is directed to the ER by a C terminal 35-amino acid signal peptide and is readily detected in the culture medium of transfected cells (14). The latent heparanase form does not accumulate extracellularly, however, due to an efficient cellular uptake (14, 15), followed by intracellular proteolytic processing (15, 16), yielding an 8-kDa polypeptide at the N terminus and a 50-kDa polypeptide at the C terminus that heterodimerize to form the active heparanase enzyme (17–19). Likewise, heparanase was noted to reside primarily intracellularly within endocytic vesicles identified as endosomes and lysosomes (20–22). Applying a polyclonal antibody (number 733) that preferentially recognizes the 50-kDa heparanase subunit versus the 65-kDa latent enzyme, we have demonstrated that the 50-kDa active heparanase subunit similarly resides in endocytic vesicles, assuming a perinuclear localization (16). More recently, we have demonstrated heparanase processing by endosomal/lysosomal preparation (22), identified the lysosome as the heparanase-processing organelle (16), and identified cathepsin family members, mainly cathepsin D and L, as heparanase-activating proteases (23). Accumulation of heparanase in endocytic vesicles for a relatively long period of time (16, 21) led us to hypothesize that this compartment may serve as an intracellular enzyme pool.
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that can get secreted in response to a proper stimulus, ensuring a tightly regulated extracellular enzymatic function. We investigated this hypothesis by examining heparanase secretion and activity in the cell conditioned medium in response to exogenous stimuli. Here, we provide evidence that stimulation of tumor-derived cells with phorbol 12-myristate 13-acetate (PMA) and forskolin markedly enhances the secretion of active heparanase in a time- and dose-responsive manner. We further demonstrate that physiological concentrations of ATP and ADP similarly enhance the secretion of active heparanase and discuss the significance and possible implications of these findings.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Antibody 1453 was raised in rabbits against the entire 65-kDa latent heparanase isolated from the conditioned medium of heparanase-transfected 293 cells. This antibody was affinity-purified on immobilized, bacterially expressed 50-kDa heparanase-glutathione S-transferase fusion protein (16). Monoclonal anti-heparanase antibody was kindly provided by Dr. Hua-Quan Miao (ImClone Systems Inc., New York, NY). Monoclonal anti- cathepsin D antibody, forskolin, PMA, ATP, ATPγS, ADP, adenosine, 2-methylthioadenosine 5′-diphosphate (2Me-SADP), pyridoxal phosphate-6-azophenyl-2′-4′-disulfonic acid (PPADS), and 2′-deoxy-N6-methyladenosine-3′,5′-bisphosphate (MRS 2179) were purchased from Sigma. The inhibitory compounds H89, U-73122, and bisindolylmaleimide I (Bis) were purchased from Calbiochem. Inhibitors were dissolved in Me2SO as stock solutions, and Me2SO was added to cell cultures as control, without a noticeable effect.

Cell Culture and Transfection—Human MDA-MB-231, MDA-MB-435, and MDA-MB-468 breast carcinoma and HCT116 and HT29 colon carcinoma cells were purchased from the ATCC. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. For stable transfection, subconfluent MDA-468, MDA-435, MDA-231, and HT29 cells were transfected with the pSecTag2 vector (Invitrogen) containing the full-length heparanase cDNA, using the FuGENE 6 reagent, according to the manufacturer’s instructions (Roche Applied Science). The pSecTag2 vector is designed for efficient protein secretion driven by the vector’s signal peptide and contains c-Myc and His tags at the protein C terminus. Transfection proceeded for 48 h, followed by selection with Zeocin (Invitrogen) for 2 weeks. Stable transfectant pools were further expanded and analyzed.

Heparanase Secretion and Immunoblotting—Cells were grown to confluence, followed by incubation for 20 h in serum-free medium. Fresh serum-free medium was added, and the cells were incubated without or with the indicated reagent for an additional 20 h, unless stated otherwise. Conditioned medium was collected and applied onto 35S-labeled ECM-coated dishes to evaluate heparanase enzymatic activity (see below) or preabsorbed with concavalin A-Sepharose beads to concentrate the samples and reduce nonspecific reactivity, followed by SDS-PAGE under reducing conditions using 10% gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad) and probed with the appropriate antibody followed by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) and an enhanced chemiluminescent substrate (Pierce), as described (14, 16, 24).

Immunocytochemistry—Heparanase transfected HT29 colon carcinoma cells were left untreated or incubated with ATP (10 μM) for 2 h. Indirect immunofluorescence staining was then performed essentially as described (16, 25). Briefly, cells were fixed with cold methanol for 10 min, washed with phosphate-buffered saline, and subsequently incubated in phosphate-buffered saline containing 10% normal goat serum for 1 h at room temperature, followed by a 2-h incubation with monoclonal anti-heparanase antibodies. Cells were then extensively washed with phosphate-buffered saline and incubated with Cy2-conjugated secondary antibody (Jackson ImmunoResearch) for 1 h, washed, and mounted (Vectashield, Vector, Burlingame, CA). Nuclei were counterstained with propidium iodide (Vector), and staining was visualized by confocal microscopy.

Heparanase Activity—Preparation of ECM-coated dishes and determination of heparanase activity were performed as described in detail elsewhere (19, 26). Briefly, cells (5 × 10^5 to 2 × 10^6) were plated on 35-mm dishes coated with 35S-labeled ECM. Cells were allowed to adhere for 30 min, medium was replaced with serum-free Dulbecco’s modified Eagle’s medium, and the cells were incubated (4–20 h, 37°C) in the absence or presence of the indicated reagents. Alternatively, conditioned medium was applied directly onto 35S-ECM-coated dishes. The incubation medium containing sulfate-labeled degradation fragments was subjected to gel filtration on a Sepharose CL-6B column. Fractions (0.2 ml) were eluted with phosphate-buffered saline, and their radioactivity was counted in a β-scintillation counter. Degradation fragments of HS side chains were eluted at 0.5 < Kav < 0.8 (peak II, fractions 15–30). Nearly intact HSPG were eluted just after the Vv (Kav < 0.2, peak I, fractions 3–15).

Cell Migration—Cells were incubated in serum-free medium for 20 h and applied on top of fibronectin-coated cell inserts (8 μm; Costar). Growth medium containing 10% fetal calf serum was added to the lower compartment, and the cells were allowed to migrate for 4 h. Inserts were subsequently fixed with 4% paraformaldehyde, and cells remaining on the upper side of the filter were removed by a cotton swab. Migrating cells were stained with crystal violet, visualized, and counted under a light microscope.

RESULTS

PMA and Forskolin Induce Secretion of Active Heparanase—Heparanase activity is strongly implicated in biological processes that require ECM remodeling, such as cancer metastasis, inflammation, and angiogenesis (1, 2, 13). Interestingly, however, heparanase was noted to reside primarily intracellularly within endocytic vesicles identified as endosomes and lysosomes (16, 20, 21). Thus, heparanase secretion from intracellular pools may be required in order to exert its enzymatic function extracellularly. We examined this possibility by exposing cells overexpressing heparanase to PMA, followed by immunoblot analysis of the culture medium. As demonstrated in Fig. 1, treatment of MDA-468 (Fig. 1A), MDA-435 (Fig. 1B), HT29 (Fig. 1C), and 293 cells (Fig. 1D) with PMA
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FIGURE 1. PMA and forskolin stimulate the secretion of enzymatically active heparanase. A–D, immunoblotting. Heparanase-transfected MDA-468 (A), MDA-435 (B), HT29 (C), and HEK293 (D) cells were serum-starved for 20 h. Serum-free medium was then replaced, and the cells were left untreated (Con) or incubated with vehicle control (Me2SO; DMSO), PMA (100 nM), or forskolin (10 μM) for 20 h in the presence of heparin (50 μg/ml) in order to prevent cellular uptake of the secreted heparanase. Cell conditioned medium (1 ml) was then collected, and secreted heparanase was evaluated by immunoblotting with anti-heparanase 1453 antigen. E and F, heparanase enzymatic activity. Heparanase-transfected HT29 (E) and HEK293 (F) cells were plated onto 35-mm dishes coated with 35S-labeled ECM and were left untreated or stimulated with PMA (E; 100 nM), forskolin (F); 10 and 100 μM) for 20 h in the absence of heparin. The conditioned medium was then collected, and sulfate-labeled HS degradation fragments were analyzed by gel filtration chromatography, as described under “Experimental Procedures.” G, inhibition. Heparanase-transfected HT29 (top) and MDA-435 cells (bottom) were left untreated (−) or stimulated with PMA in the absence (+) or presence of PKC (Bis; 20 μM) or PKA (H89; 5 μM) inhibitors (Inh). Secreted heparanase was evaluated by immunoblotting, as above.

resulted in a marked increase of the 50-kDa active heparanase subunit in the culture medium. In contrast, the latent 65-kDa protein was readily detected in the culture medium of control untreated cells (Con) due to its secreted nature (1), and its levels were not significantly changed upon PMA treatment. In order to confirm the immunoblotting results, conditioned medium from control and PMA-treated HT29 cells was applied onto 35S-labeled ECM, and heparanase enzymatic activity was evaluated. Heparanase activity was not detected in medium conditioned by nontransfected HT29 cells (data not shown) but was clearly evident in medium conditioned by heparanase-transfected cells (Fig. 1E, Con). Heparanase activity was markedly increased in the conditioned medium of heparanase-transfected HT29 cells in response to treatment with PMA (Fig. 1E, PMA), in agreement with the elevated levels of the 50-kDa heparanase subunit detected by immunoblotting (Fig. 1C). Treatment of heparanase-transfected MDA-468, MDA-435, and HT29 cells with forskolin did not result in a significant increase of heparanase secretion (Fig. 1, A–C) and activity (data not shown). In contrast, treatment of heparanase-transfected 293 cells with forskolin stimulated a significant increase in secretion of the 50-kDa heparanase subunit (Fig. 1D), correlating with enhanced heparanase activity in the cell culture medium (Fig. 1F). Since PMA is a strong PKC inducer, we next examined the ability of PKC inhibitors to block heparanase secretion induced by PMA. To this end, heparanase-transfected HT29 (Fig. 1G, upper panel) and MDA-435 (Fig. 1G, lower panel) cells were left untreated (−) or stimulated with PMA in the absence or presence of PKC (Bis) or PKA (H89) inhibitors. Heparanase stimulation, peaked at 2 h, and gradually decreased (Fig. 2A, top). A similar kinetic was noted upon treatment of heparanase-transfected 293 cells with forskolin (Fig. 2B, top). The decline in extracellular heparanase levels at later time points following PMA or forskolin treatment is most probably due to HS-mediated heparanase uptake (14).

Since the active 50-kDa heparanase subunit was mainly detected in endocytic vesicles (16), we rationalized that the secreted heparanase found in the culture medium following treatment with PMA and forskolin originated from such an intracellular pool. In order to support this notion, we compared the secretion kinetics of the lysosomal protein cathepsin D with that of the 50-kDa heparanase subunit. Notably, secretion of the low molecular weight forms of cathepsin D, typically processed and residing in lysosomes, was not only induced by PMA and forskolin but also resembled the kinetics of heparanase secretion (Fig. 2, lower panels), suggesting that both proteins indeed originate from similar cellular compartments.

Nucleotides Induce Secretion of Enzymatically Active Heparanase—The ability of PMA and forskolin to induce the secretion of enzymatically active heparanase as well as of a typical lysosomal enzyme, such as cathepsin D, supports the notion that heparanase is stored in the lysosomal compartment in a stable form (16, 21) and is secreted in response to local or systemic cues, thus maintaining its extracellular availability tightly regulated. Since both PMA and forskolin are not considered physiological, we sought inducers more relevant to biological systems in which heparanase activity is implicated, mainly cancer

secretion was examined by immunoblotting. PMA treatment elicited a marked increase in secretion of the 50-kDa heparanase subunit, an increase that was practically blocked (Fig. 1G, top) or markedly reduced (Fig. 1G, bottom) in the presence of the PKC inhibitor Bis. In contrast, the PKA inhibitor H89 only slightly reduced the effect of PMA, indicating, as expected, activation of PKC rather than of PKA. The inverse situation was noted upon treatment of heparanase-transfected 293 cells with forskolin. In these cells, forskolin was found to effectively induce secretion of active heparanase (Fig. 1D), and this effect was significantly inhibited by H89 but not by Bis (not shown).

Next, we examined the kinetics of heparanase secretion elicited by PMA and forskolin (Fig. 2). Heparanase-transfected MDA-435 cells were incubated with PMA for the time indicated, conditioned medium was collected, and heparanase secretion was examined by immunoblotting. The 50-kDa heparanase subunit was first detected in the culture medium 1 h following PMA
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Intracellular signaling in the regulation of heparanase secretion by nucleotides, ATP, ADP, UTP, and adenosine, was studied to elucidate the mechanisms involved in the secretion of active heparanase.

**Results:**
- **Figure 1** illustrates the kinetics of heparanase secretion induced by PMA and forskolin in HEK293 cells stably overexpressing heparanase-transfected 293 cells. Secretion of the 50-kDa heparanase protein was noted to enhance heparanase secretion.
- **Figure 2** shows the effect of nucleotides on heparanase secretion, demonstrating that ATP, ADP, UTP, and adenosine, have the ability to function extracellularly and to initiate signal transduction cascades mediated by a family of purinergic receptors that play important roles in development, differentiation, and cell proliferation.
- ATP and ADP were found to be more effective activators of heparanase secretion compared to UTP and adenosine.
- **Figure 3** presents qualitative evidence indicating that ATP and ADP, but not UTP or adenosine, were capable of stimulating heparanase secretion.

**Conclusion:** These findings suggest that ATP and ADP play crucial roles in regulating heparanase secretion. Further studies are needed to elucidate the specific mechanisms underlying these effects.
Similarly, treatment of these cells with ADP (10 μM) markedly stimulated heparanase secretion that was practically blocked by the PKC inhibitor Bis, whereas PKA inhibition by H89 was less effective (Fig. 5B). These results were further corroborated by evaluating heparanase activity in the cell conditioned medium. Clearly, heparanase activity was markedly increased upon ADP treatment, whereas PKC inhibitor (Bis) reduced heparanase activity to the level of control untreated cells, and PKA inhibition resulted in an about 50% decrease in heparanase activity (Fig. 5C), nicely correlating with the immunoblotting results (Fig. 5B). Furthermore, ATPγS, a nonhydrolyzed ATP analogue that cannot be consumed by cells as an energy source, was as effective as ATP in eliciting heparanase secretion (data not shown), supporting the notion that heparanase secretion induced by ATP, and probably by other nucleotides, is a receptor-mediated response.

In order to better characterize cell surface receptors that mediate heparanase secretion, we utilized more specific P2Y receptor agonists and antagonists. Treatment of heparanase-transfected 293 cells with 2Me-SADP (2Me), a P2Y1 receptor-specific agonist (30), elicited a marked elevation of heparanase secretion (Fig. 5D), comparable in magnitude with the stimulatory effect of ATP and ADP on heparanase secretion (Figs. 3 and 4). Moreover, the effect of 2Me-SADP was abolished by PPADS, an antagonist of P2Y1, -4, and -6 receptors, and even more so by MRS2179, an antagonist specific for P2Y1 receptors, clearly implicating the P2Y1 receptors and PKC as mediators of heparanase secretion.

Since heparanase activity has long been correlated with acquired cellular motility, we questioned whether elevated levels of extracellular heparanase activity upon nucleotide stimulation enhance cell migration. Significantly, ATP and ADP markedly enhanced 293 (Fig. 6, left panels) and MDA-231 cell migration (Fig. 6, right panels), ascribing a cellular effect to the above described molecular and biochemical alterations induced by nucleotides.

**DISCUSSION**

HSPGs are thought to play key roles in numerous biological settings, including cytoskeleton organization, cell migration, wound healing, inflammation, cancer metastasis, and angio-
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the active enzyme in lysosomes, due to the relatively long half-life of the 50-kDa heparanase subunit (14, 21), led us to hypothesize that this intracellular enzyme pool could be secreted in response to exogenous stimuli. Inflammatory cytokines, such as tumor necrosis factor-α and interleukin 1β, as well as fatty acids were shown to stimulate secretion of heparanase by endothelial cells (39), although activity data were not provided. Notably, we could not demonstrate enhanced heparanase secretion by tumor necrosis factor-α in any of the tumor-derived cell lines included in the present study (data not shown), suggesting that effective stimuli vary among cell types and biological settings. PMA and forskolin effectively stimulated the secretion of enzymatically active heparanase in a PKC- and PKA-dependent manner, respectively (Fig. 1). Importantly, the 50-kDa heparanase subunit was detected in the culture medium by 1 h and peaked 2 h after PMA stimulation, followed by a decline. This elimination of extracellular active heparanase is mediated, among other receptors (15), by cell surface HS and was prevented by the addition of heparin (data not shown), in agreement with our previous identification of HSPG as mediators of heparanase uptake (14). Thus, the availability of extracellular heparanase is tightly regulated by at least two independent mechanisms, one that mediates secretion of the enzyme from intracellular pools and a second that limits accumulation of the secreted enzyme by efficient cellular uptake, together ensuring a limited retention of active heparanase in the extracellular environment.

The presence of the 50-kDa heparanase subunit can also potentially be explained by extracellular processing of the 65-kDa latent enzyme. This, however, does not seem to be the case, since accumulation of the 50-kDa subunit preceded the appearance of the 65-kDa protein, best demonstrated in the kinetics studies for ATP (Fig. 4B). Moreover, the addition of protease inhibitors, including cathepsin inhibitors, to cell cultures had no influence on the levels of the 50-kDa protein found in the medium (data not shown), in
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FIGURE 7. A schematic model summarizing the main results of the current study. Heparanase mainly resides perinuclearly in endocytic vesicles that assume a polar localization (left). Stimulation of P2Y receptors by nucleotides leads to activation of the PKC signaling pathway, resulting in apparently larger, and a more diffused pattern of, heparanase-positive vesicles (right). Activation of PKC by PMA or by physiological concentrations of nucleotides elicits secretion of enzymatically active heparanase, leading to enhanced cell migration.

agreement with Rodriguez et al. (40), who have found that secretion of β-hexosaminidase, a lysosomal marker, typically reaches ~10% of the total enzyme content of the cell in response to ionomycin treatment. Similarly, ionomycin elicited rapid secretion of the 50-kDa heparanase subunit (data not shown), as would be expected from PMA effectiveness. Moreover, immunofluorescence staining revealed a clear change in the localization of heparanase-positive vesicles toward the cell periphery upon stimulation with ATP (Fig. 4). Thus, although not regarded as typical secretory vesicles, lysosomes may function as such and secrete their content under certain conditions and in response to the proper stimuli (41), in agreement with elevated levels of cathepsins found in several human malignancies (42, 43). Regarded as a universal source of metabolic energy, extracellular ATP as well as other nucleotides are capable of initiating signaling cascades through two classes of P2 receptors: P2X, which has an intrinsic activity of ion channel; and P2Y, a G-protein-coupled receptor (44). P2Y receptor activation is coupled to phospholipase C and adenylate cyclase activation, leading to PKC and PKA activation (28, 44, 45). Remarkably, each and every cell line examined in this study responded to nucleotides by a stimulated secretion of active heparanase, comparable in magnitude and kinetics with PMA and forskolin (Figs. 3 and 4). The differential responsiveness of cell lines to different nucleotides (ATP, ADP, and adenosine) is probably due to different receptor profiles, expressed by each cell type. Importantly, ATP exerts its maximal effect at a physiological concentration (1 μM; Fig. 4B) (27), emphasizing the biological relevance of this mediator. Extracellular nucleotides at low micromolar concentrations can influence a number of biological processes, including vascular tone, neurotransmission, cardiac function, and muscle contraction (28, 44, 46), but their significance in tumor progression and cancer metastasis is unclear. High levels of ATP and ADP were documented during platelet aggregation and at sites of inflammation, where their plasma concentration can transiently reach up to 50 μM (46), processes that appear to play important roles in cancer initiation and progression (47). Thus, nucleotides, by inducing P2Y- and PKC-dependent secretion of heparanase, cathepsins, and most likely other lysosomal resident enzymes, may facilitate cancer progression and tumor metastasis (Fig. 7).

Taken together, our results support the notion that heparanase resides and accumulates in the lysosomal compartment, where it may participate in normal turnover of HS. Lysosomal enzymes, including heparanase, can get secreted in response to a proper stimulus, thus maintaining the extracellular levels of ECM-degrading enzymes tightly regulated, preventing tissue damage that may result from an excess of proteolytic and endoglycosidic activities.

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