Heparanase Enhances the Insulin Receptor Signaling Pathway to Activate Extracellular Signal-regulated Kinase in Multiple Myeloma

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Anurag Purushothaman , Stephen K. Babitz , and Ralph D. Sanderson

From the Department of Pathology and Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama 35294

Background: ERK phosphorylation is enhanced by heparanase, an enzyme associated with aggressive behavior of multiple myeloma.

Results: Heparanase activates ERK by up-regulating insulin receptor phosphorylation and insulin receptor substrate-1.

Conclusion: Heparanase activates ERK by enhancing the insulin signaling pathway.

Significance: Targeting the insulin receptor signaling pathway may block the tumor-promoting effects of heparanase.

ERK signaling regulates proliferation, survival, drug resistance, and angiogenesis in cancer. Although the mechanisms regulating ERK activation are not fully understood, we previously demonstrated that ERK phosphorylation is elevated by heparanase, an enzyme associated with aggressive behavior of many cancers. In the present study, myeloma cell lines expressing either high or low levels of heparanase were utilized to determine how heparanase stimulates ERK signaling. We discovered that the insulin receptor was abundant on cells expressing either high or low levels of heparanase, but the receptor was highly phosphorylated in heparanase-high cells compared with heparanase-low cells. In addition, protein kinase C activity was elevated in heparanase-high cells, and this enhanced expression of insulin receptor substrate-1 (IRS-1), the principle intracellular substrate for phosphorylation by the insulin receptor. Blocking insulin receptor function with antibody or a small molecule inhibitor or knockdown of IRS-1 expression using shRNA diminished heparanase-mediated ERK activation in the tumor cells. In addition, up-regulation of the insulin signaling pathway by heparanase and the resulting ERK activation were dependent on heparanase retaining its enzyme activity. These results reveal a novel mechanism whereby heparanase enhances activation of the insulin receptor signaling pathway leading to ERK activation and modulation of myeloma behavior.

Heparanase, the only known mammalian endoglycosidase that cleaves heparan sulfate, is up-regulated in most human tumors (1–4). Clinically, increased heparanase levels are associated with increased tumor metastasis, high microvessel density, and reduced postoperative survival time of cancer patients (3, 5, 6). Although some of the tumor-promoting effects of heparanase can be attributed to its ability to remodel the extracellular matrix barrier by cleaving heparan sulfate chains, heparanase is also known to regulate cell signaling and gene transcription (3, 7, 8). For example, heparanase induces endothelial cell migration via protein kinase B/Akt activation (9) and VEGF expression via Src signaling (10). Heparanase enhances syndecan-1 shedding (11, 12), and we previously demonstrated that this occurs due to heparanase-driven activation of ERK, which increases expression of MMP-9, a syndecan-1 sheddase (13). Shed syndecan-1 in turn acts within the tumor microenvironment to stimulate further tumor growth, angiogenesis, and dissemination (14, 15). Other studies have shown that activation of the ERK signaling pathway plays an important role in the pathogenesis of myeloma by mediating cell proliferation, survival, drug resistance, and angiogenesis (16, 17). Moreover, inhibitors of the MAPK pathway inhibit myeloma cell growth and osteoclast differentiation (18, 19). Thus, activation of ERK by heparanase may promote myeloma progression via multiple mechanisms.

The primary mediators of signal transduction are receptor tyrosine kinases (RTKs) present on cell surfaces. RTKs such as the insulin receptor couple ligand binding to downstream intracellular signaling cascades and gene transcription. The insulin receptor is a plasma cell marker that is absent on memory B cells but induced upon differentiation into plasma cells (20). It is highly expressed in normal plasma cells and multiple myeloma cells and can associate with insulin-like growth factor 1 receptor (IGF-1R) and exist as an insulin/IGF-1 hybrid receptor on XG myeloma cell lines (20). The insulin receptor is not associated with myeloma disease progression, but the interaction of insulin receptor with its ligand insulin can induce growth of myeloma cells (20). Insulin is as potent as IGF-1 in inducing growth of myeloma cells and by binding to insulin receptor, insulin can trigger the phosphorylation of insulin receptor, AKT, and MAPK in myeloma cells (20). Recent studies support the role of insulin as an important growth factor.

The abbreviations used are: MMP-9, matrix metalloproteinase 9; HPSE, heparanase; IGF-1, insulin like growth factor-1; IGF-1R, insulin like growth factor-1 receptor; IR, insulin receptor; IRS-1, insulin receptor substrate-1; RTK, receptor tyrosine kinase.
acting through the tyrosine kinase growth factor cascade to enhance tumor cell proliferation (21). Even hyperinsulinemia linked to obesity is shown to be associated with an increased risk of multiple myeloma (22).

The insulin receptor substrate 1 (IRS-1) protein is the principal intracellular substrate of insulin receptor tyrosine kinase activity and is the most upstream molecule in the signal transduction cascade mediated by insulin, interleukin 4, and IGF-1 stimulation (23, 24). IRS-1 docks with both the IGF-1R and the insulin receptor (25). IRS-1 expression and phosphorylation is often increased in human cancers and over expression of IRS-1 can induce cellular transformation. Although little is known about the role of IRS-1 in myeloma, a study looking at the variation in genes related to the IGF-1 signaling pathway identified IRS-1 as a major candidate associated with the risk of myeloma (26).

Because heparanase stimulates ERK signaling and because this signaling is known to enhance myeloma progression, we sought to determine the mechanism whereby heparanase was activating ERK. We discovered that heparanase expression leads to stimulation of insulin receptor phosphorylation and protein kinase C (PKC) activity. PKC up-regulates the expression of IRS-1, which is then phosphorylated by the kinase activity of the insulin receptor. High levels of phospho-IRS-1 in turn activate ERK signaling. These findings suggest a prominent role for heparanase in initiating the insulin signaling cascade and subsequent activation of ERK signaling in multiple myeloma.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—RPMI-8226, U266, and CAG myeloma cells were cultured in RPMI 1640 growth medium supplemented with 10% fetal bovine serum. CAG cells were transfected as previously described with empty vector or vector containing the cDNA for human heparanase to generate heparanase-low and heparanase-high cells, respectively (12). CAG cells were also transfected with a vector containing a mutated cDNA (mutated at amino acid 225) that codes for enzymatically inactive heparanase (12).

**Western Blot**—Western blotting was performed as described previously (13). Membranes were incubated with primary antibodies that recognize phospho-ERK (Santa Cruz Biotechnology), total ERK (Santa Cruz Biotechnology), phospho-insulin receptor (which detects the phosphorylation of tyrosine at 1162 and 1163 of β-subunit) (R&D Systems), insulin receptor (which detects the α-subunit) (Abcam), phospho-IRS-1 (Millipore), IRS-1 (Millipore), and actin (Sigma). Secondary antibody conjugated with horseradish peroxidase (HRP) (Vector Laboratories, Inc.) was used at 1:2000 dilution to detect primary antibodies, and enzymatic signals were visualized by chemiluminescence. For some experiments, cells were treated with recombinant human heparanase (250 ng/ml; provided by Dr. Israel Vlodavsky) and incubated at 37 °C for 2 h. To examine the potential role of insulin in activating ERK signaling, serum-starved CAG cells were treated with different doses of insulin (Roche Applied Science) for 15 min. For some experiments, CAG heparanase-high cells grown in complete medium were treated with anti-insulin antibody (which prevents insulin binding to the α-subunit of insulin receptor) (Abcam) or an isotype-matched control antibody. In some experiments, cells were treated with 10 μM of AG1024 (Santa Cruz Biotechnology) (an inhibitor of insulin receptor tyrosine kinase activity) (27) or 10 μM of picropodophyllin (Santa Cruz Biotechnology) (an IGF-1R inhibitor). For experiments using recombinant enzyme, serum-starved RPMI-8226 or CAG cells were pretreated with or without 30 μM AG1024 for 2 h followed by exposure to recombinant heparanase (250 ng/ml) for another 2 h. Cells were then lysed and analyzed for ERK signaling. Band densities of Western blots were quantified using NIH ImageJ software.

**Immunohistochemistry**—Sections from formalin-fixed tumor tissues formed from heparanase-low or heparanase-high cells were stained for phospho-ERK (Cell Signaling), phospho-IRS-1 (Millipore), and total-IRS-1 (Millipore), as described previously (13).

**Phospho-RTK Array**—Arrays were purchased from R&D Systems. Array membranes were incubated with cell lysates and processed as recommended by the manufacturer’s instructions using a phospho-tyrosine-specific antibody conjugated to HRP. Approximately 10^7 CAG heparanase-high or heparanase-low cells were maintained in full serum overnight and lysed with a buffer containing 1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 30 min. Protein assay (Pierce) was performed before incubating the lysates with the RTK membranes, and signals were visualized using chemiluminescence.

**Flow Cytometry**—The expression of insulin receptor was evaluated by incubating cells with anti-human insulin receptor antibody (Abcam) in PBS on ice for 1 h. The cells were then washed and incubated with secondary antibody conjugated to phycoerythrin (BD Biosciences) on ice for 30 min. Following incubation, the cells were washed, resuspended in PBS, and subjected to flow cytometry analysis using a Becton Dickinson FACSCalibur.

**Quantification of IRS-1**—Endogenous levels of IRS-1 in cells were measured using a commercially available IRS-1 sandwich ELISA kit (Cell Signaling), according to the manufacturer’s instructions. The magnitude of absorbance for the color developed is proportional to the quantity of total IRS-1.

**Knockdown of IRS-1 by shRNA**—IRS-1 knockdown was performed using MISSION lentiviral transduction particles from Sigma. Lentiviral transduction particles are produced from a lentiviral plasmid vector containing the following two different shRNA sequences for the human IRS-1 gene: IRS-1 shRNA 1, CCGGGGCTAAGAACAATATCTGCATCTCGAGATGCA-GATATGTTGCTTTTTTTTTT; IRS-1 shRNA 2, CCGG-GCTATCTACTTGGCCAGATCTGATCTGGCAATGAGTAGTTTTTTT. The non-target shRNA control transduction particles containing the sequence CCGGCAAC-AAGATGAGAGGACCAAACCTCGAGTTGGTGCCTTCTCA-TCTTTGTTTTTT does not target any human gene but will activate the RNAi pathway. Briefly, to 10^5 CAG heparanase-high cells, 25 μl of lentiviral particles were added and incubated for 18–20 h at 37 °C in a humidified incubator. The next day, the medium containing the lentiviral particles was removed, and fresh complete RPMI medium was added to each well. The
cells were then selected with puromycin (5 µg/ml) and assessed for IRS-1 knockdown by ELISA and Western blotting.

Gelatin Zymography—After cells were incubated with serum-free medium for 48 h, supernatants were collected and concentrated in Centriplus columns with a 30-kDa cut-off value (Millipore). Protein in the concentrated media was quantified using the BCA protein assay reagent kit (Pierce), and an equal amount of protein (50 µg) was mixed with non-reducing sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 4% SDS, and 0.01% bromophenol blue) and analyzed by SDS-PAGE using 10% polyacrylamide gels co-polymerized with gelatin (Bio-Rad). Electrophoresis was carried out at 10 mA for 2 h. The SDS in the acrylamide gel was extracted by incubation with 2.5% Triton X-100 solution for 2 h at room temperature, and gelatinolytic activities were developed in a buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl$_2$, and 0.02% Brij 35 at 37 °C overnight. The gel was then stained with Coomassie Blue. Following destaining, sites of proteolytic activity were visualized as clear bands against the blue background of stained gelatin.

Quantification of Syndecan-1—Medium conditioned for 48 h by CAG cells (10$^6$ cells/ml) was collected, and the level of shed syndecan-1 present in the conditioned medium was determined by ELISA as described (12). In some experiments, the cells were incubated with either the PKC inhibitor staurosporine (100 nM) (Sigma) or dimethyl sulfoxide control for 24 h, the cell culture medium was collected, and levels of shed syndecan-1 were assessed.

Protein Kinase C Assay—PKC activity was measured using a commercially available, nonradioactive protein kinase assay kit (Calbiochem). The kit utilizes a peptide pseudosubstrate pre-coated on a 96-well plate. Following exposure of wells to whole cells extracts, the extent of reaction is measured using a biotinylated monoclonal antibody that recognizes the phosphorylated form of the peptide pseudosubstrate. The biotinylated monoclonal antibody is detected using HRP-conjugated streptavidin. The absorbance was read at 492 nm. The PKC activity is directly proportional to the color intensity.

Real Time PCR—Approximately 10$^6$ cells were treated with the PKC inhibitor staurosporine (100 nM) for 12 h. RNA was extracted (RNeasy mini kit, Qiagen), and cDNA was synthesized (Clontech, Mountain View, CA). Real-time PCR was conducted using the following primers: MMP-9 (F), TGCACGCCAAAGAAGTG; MMP-9 (R), CAGTGAAGCCTCATAGG; IRS-1 (F), CCACCTCAGAAACTTCTTCTCAT; IRS-1 (R), AGAGTCATCCACTGTCACTTCA; and SYBR Green Supermix (Bio-Rad). Expression was determined relative to 28S rRNA.

Statistical Analysis—All results were representative of at least three independent experiments. Except where noted, comparisons between two groups were analyzed by Student’s $t$ test, and a $p$ value $\leq$ 0.05 was considered statistically significant. Data are means ± S.D.

RESULTS

Heparanase Induces ERK1/2 Activation in Myeloma—Activation of the ERK1/2 signaling cascade mediates human multiple myeloma growth, drug resistance, and survival (18, 28, 29).

[Image 366x692 to 455x716]

FIGURE 1. Heparanase promotes ERK phosphorylation in myeloma. A, protein lysates from CAG human myeloma cells expressing either low or high levels of heparanase (HPSE-low or HPSE-high) or mutant heparanase that lacks enzyme activity were subjected to Western blot analysis. Membranes were probed with anti-phospho-ERK (p-ERK) antibody or total ERK (t-ERK) antibody. B, serum-starved wild-type CAG, RPMI 8266, or U226 cells were treated with or without recombinant heparanase (rHPSE) for the indicated time points, and cell lysates were immunoblotted for p-ERK and total ERK. C, heparanase enhances ERK activation in myeloma tumors growing in vivo. Subcutaneous tumors in SCID mice formed by heparanase-low or heparanase-high cells were removed and immunostained with antibody to p-ERK. Original magnification, 1300×.

Here, using three different models, we examined the effect of heparanase on ERK activation. In the first model, CAG myeloma cells engineered to express low or high levels of heparanase or a mutated form of heparanase that lacks heparan sulfate-degrading enzyme activity were utilized. Western blot analysis demonstrates that heparanase-high cells have significantly higher levels of phospho-ERK1/2 compared with heparanase-low or mutant cells lacking enzyme activity (Fig. 1A). It is important to note that the level of heparanase expression and activity in the heparanase-high CAG cells is similar to that found in some myeloma patient tumors (6, 30). Thus, the increase in ERK activation is not due to an enhancement of heparanase expression beyond levels that are likely to be present in the human cancer microenvironment. In addition, a role for heparanase in ERK activation is supported by our previous finding that treatment of heparanase-high cells with SST0001, an inhibitor of heparanase, lowers ERK activation and blocks the aggressive tumor growth of heparanase-high cells (31).

To confirm and extend these findings, we used recombinant human heparanase, which when introduced to cells is taken up by cells and remains biologically active (12, 13, 32). 2 h after addition of recombinant heparanase, levels of phosphorylated ERK were elevated in three different myeloma cell lines compared with cells not receiving exogenous heparanase (Fig. 1B).
This indicates that exogenous heparanase can enhance a relatively rapid up-regulation of ERK activation and that the effect on ERK activation seen in heparanase-high myeloma cells is not simply an artifact related to their transfection.

Because heparanase promotes tumor progression (6) and because MEK/ERK signaling plays a central role in the pathogenesis of myeloma (18), we investigated whether the heparanase mediated up-regulation of ERK activation seen in vitro also occurs within tumors growing in vivo. Immunohistochemistry revealed that tumors formed in mice by heparanase-high cells have high levels of phospho-ERK compared with cells within tumors formed by heparanase-low cells (Fig. 1C).

**Heparanase Promotes Insulin Receptor Activation**—We used an antibody array system that simultaneously examines the relative tyrosine phosphorylation level of 42 different RTKs (supplemental Table 1) to identify the upstream signaling events driving ERK phosphorylation. Results demonstrate that heparanase evoked an increase in phosphorylation of the insulin receptor in the heparanase-high cells compared with heparanase-low cells (Fig. 2A). Western blotting confirmed that there was more phosphorylated insulin receptor present in the heparanase-high cells than in the heparanase-low cells (Fig. 2B).

It has been demonstrated that the insulin receptor is a plasma cell marker, and its expression is increased throughout normal differentiation of plasma cells (20). FACS analysis using an antibody specific for the α-subunit of insulin receptor demonstrated that in all three multiple myeloma cell lines tested (CAG, RPMI 8226, and U266), there were high levels of cell surface insulin receptor (Fig. 2C). Interestingly, insulin receptor expression goes down slightly in heparanase-high CAG cells compared with heparanase-low CAG cells. However, there does not appear to be a consistent correlation between levels of insulin receptor expression and heparanase expression because U266 cells, which express high levels of heparanase,3 have high levels of insulin receptor (Fig. 2C). Three different approaches were then utilized to investigate whether triggering the insulin receptor can induce ERK signaling in myeloma cells. In the first approach, we added varying doses of insulin to serum-starved CAG cells. Western blot analysis revealed that insulin stimulates ERK activation and the levels of phospho-ERK increased with increasing doses of insulin (Fig. 2D). In the second approach, to heparanase-high cells growing in complete medium, we added either an anti-insulin receptor antibody (which binds to the α-subunit of insulin receptor and blocks insulin binding) or AG1024 (a specific inhibitor of IGF-1 and insulin receptor tyrosine kinase activity). After an overnight culture, the cells were harvested and analyzed for phospho-ERK by Western blot. Results demonstrate that both anti-insulin receptor antibody and AG1024 significantly reduced ERK phosphorylation (Fig. 3, A and B). Treatment of heparanase-high cells with picropodophyllin (a specific inhibitor for IGF-1R) did not affect ERK activation (Fig. 3B), confirming that the ERK activation is occurring via insulin receptor signaling. In a third approach, serum-starved CAG or RPMI 8226 wild-type cells were pretreated with or without AG1024 (30 μM) for 2 h.

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**FIGURE 3.** Blocking insulin receptor signaling inhibits ERK activation by heparanase. A, blocking IR decreases ERK activation in heparanase (HPSE)-high CAG cells. IR function blocking antibody (clone 47-9) or an isotype-matched control antibody was added to heparanase-high CAG cells. After overnight incubation at 37 °C, whole-cell lysates were prepared and subjected to immunoblotting for p-ERK and total (t) ERK. The two bands represent p44 and p42 MAPK (Erk1 and Erk2), respectively. The two blots represent replicates of the same experiment. B, HPSE-high CAG cells were treated overnight with an IGF-1R inhibitor picropodophyllin (PPP) or an insulin receptor tyrosine kinase activity inhibitor AG1024 (10 μM). After overnight incubation at 37 °C, whole-cell lysates were prepared and subjected to immunoblotting for p-ERK and total ERK. C, serum-starved wild-type CAG cells or RPMI 8226 cells were pretreated with or without AG1024 (30 μM) for 2 h followed by addition of recombinant HPSE (rHPSE) (250 ng/ml). After 2 h of recombinant HPSE treatment, cell lysates were prepared and were immunoblotted for p-ERK and total ERK.

FIGURE 4. Heparanase up-regulates IRS-1 levels in myeloma cells. An equal number of heparanase (HPSE)-low or heparanase-high or heparanase-mutant CAG cells were harvested, lysed, and subjected to Western blot analysis for phosphorylated IRS-1 (p-IRS-1) or total IRS-1 (t-IRS-1) protein level by ELISA. A, *, p < 0.05 versus heparanase-low cells. B and C, heparanase-high cells show intense staining for both p-IRS-1 and total IRS-1. Original magnification, 1300×.

followed by treatment with recombinant heparanase for additional 2 h. Western blot analysis revealed that activation of ERK by recombinant heparanase is inhibited in the presence of AG1024 (Fig. 3C). These results support the previously published studies demonstrating that insulin is a potent multiple myeloma cell growth factor (20) and that the MEK/ERK signaling pathway mediates myeloma cell survival and growth (18).

Heparanase Enhances the Expression of IRS-1 in Myeloma Cells—IRS-1 is the principle intracellular substrate of insulin receptor tyrosine kinase activity and is the most upstream molecule in the signal transduction cascade mediated by insulin and IGF (23, 24). It has also been proposed that IRS-1 is essential for insulin-induced mitogenic effects (33, 34). During insulin signaling, IRS-1 functions as an insulin receptor-specific docking protein to engage multiple downstream signaling molecules, including ERK (35). Because tyrosine phosphorylation of IRS-1 is involved in transmitting signals downstream to occupation of IGF-1 and insulin receptors, we sought to determine whether the enhanced insulin receptor tyrosine kinase activity mediated by heparanase is coupled with enhanced phosphorylation of IRS-1. By Western blot analysis, we found that phospho-IRS-1 levels were significantly elevated in heparanase-high cells compared with heparanase-low cells (Fig. 4A). To examine whether the difference in phosphorylated IRS-1 levels between these cells was secondary to an increase of IRS-1 content in heparanase-high cells, Western blot analysis of IRS-1 was performed. Results demonstrate that the amount of total IRS-1 was also up-regulated by heparanase expression in these cells (Fig. 4A). This was further confirmed by ELISA measuring the amount of total IRS-1 in whole cell extracts (Fig.

4B). Interestingly, levels of phospho-IRS-1 and total-IRS-1 were not elevated in cells expressing the mutated form of heparanase that lacks enzymatic activity, suggesting that elevation in IRS-1 levels is dependent on heparanase-mediated degradation of heparan sulfate chains. Together, these results indicate that heparanase stimulates the insulin signaling cascade in myeloma cells by both triggering insulin receptor phosphorylation and by up-regulating IRS-1 levels.

Previously, we demonstrated that elevation of heparanase expression in CAG myeloma cells enhances their growth and metastasis *in vivo* as compared with control cells (6). Because IRS-1 is up-regulated in many cancers and plays an important role in tumor progression, we investigated whether the heparanase-mediated up-regulation of IRS-1 expression also occurs in
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IRS-1 Induces ERK Activation in Heparanase-high Cells—We have demonstrated that elevation of heparanase expression causes both enhanced ERK activation (Fig. 1) and increased IRS-1 expression, which together induce ERK activation, thereby promoting aggressive behavior of myeloma cells (13, 15). As a biological readout of ERK activity, we monitored ERK-mediated up-regulation of MMP-9 expression and subsequent MMP-9 mediated shedding of syndecan-1 (13). To determine whether IRS-1 levels influence MMP-9 expression, serum-free conditioned media from IRS-1 knockdown and control cells were subjected to zymography. IRS-1 knockdown cells exhibited low gelatinolytic activity corresponding to pro-MMP-9 (92-kDa gelatinase) as compared with control cells (Fig. 5A). To determine whether IRS-1 knockdown decreases syndecan-1 shedding, IRS-1 knockdown cells were plated at equal density for 24 h, conditioned media were harvested, and the level of syndecan-1 was quantified by ELISA. Values represent means of triplicate determination ± S.D. *p ≤ 0.05 versus control shRNA.

High PKC activity. PKC activity was assayed in heparanase-high and low cells using an ELISA-based detection method. Results demonstrate that heparanase-high cells had significantly elevated levels of PKC activity compared with heparanase-low cells (Fig. 6A).

Next, we determined whether the enhanced PKC activity seen in heparanase-high cells contributes to up-regulation of expression of IRS-1. CAG heparanase-high cells were grown in the presence or absence of PKC inhibitor staurosporine (37). Because PKC is known to regulate IRS-1 expression at the gene transcriptional level, we examined IRS-1 expression at the mRNA level. Following a 12-h exposure, staurosporine significantly reduced IRS-1 mRNA levels in heparanase-high cells (Fig. 6B). Staurosporine treatment did not reduce CAG cell viability. These results indicate that maintenance of the PKC pathway is necessary to maintain the IRS-1 levels contained in CAG heparanase-high cells. We do not know which isoform or iso-
forms of PKC are involved in the regulation of IRS-1 expression, but a previous study has shown that PKC-δ isoform regulates the mRNA expression of IRS-1 in breast cancer cell lines (36).

Because IRS-1 induces ERK activation (Fig. 5A) and because heparanase enhances MMP-9 expression by regulating ERK phosphorylation (13), we explored whether inhibiting PKC activity inhibits MMP-9 expression. Treatment of cells with staurosporine significantly decreased the mRNA levels of MMP-9 in heparanase-high cells (Fig. 6B), indicating that their high level of expression is dependent on PKC activity.

Because MMP-9 expression is regulated by PKC activity (Fig. 6B), we determined whether inhibiting PKC activity would decrease shedding of syndecan-1. The accumulation of the syndecan-1 ectodomain in cell culture media from heparanase-high cells in the presence or absence of staurosporine was quantified by ELISA. Staurosporine significantly blocked shedding of syndecan-1 by the heparanase-high cells (Fig. 6C).

**DISCUSSION**

Our previous work has implicated heparanase as a master regulator of the aggressive phenotype in myeloma, which is due, at least in part, to heparanase up-regulation of ERK signaling (1, 13). In the present work, we have demonstrated that heparanase-mediated ERK activation occurs via the insulin receptor signaling pathway. The data support a model as shown in Fig. 7, whereby 1) heparanase triggers the phosphorylation/kinase activity of IR and PKC activity. Step 2, elevated PKC activity up-regulates IRS-1 expression. Step 3, high levels of IRS-1 and high IR tyrosine kinase activity up-regulates the phosphorylated levels of IRS-1. Step 4, high levels of phospho-IRS-1 up-regulate ERK activation.

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previous study reported that insulin can stimulate the growth of myeloma cells as potently as does IGF-1 (20). Our data showing that heparanase expression in myeloma cells can enhance the phosphorylation of the insulin receptor and that addition of an insulin receptor function blocking antibody can reduce ERK phosphorylation in heparanase-high cells strongly support the role of insulin receptor in regulating the ERK signaling pathway in myeloma. This is further indicated by our finding that triggering the insulin receptor in myeloma cells by addition of insulin enhances ERK activation. Although the role of IGF-R in myeloma has been studied extensively, little is known regarding the function of the insulin receptor in myeloma. By flow cytometry analysis, we found that the insulin receptor is expressed at high levels by myeloma cells, consistent with the previous report demonstrating the presence of the insulin receptor on myeloma cell lines and almost all myeloma cells from patients (20).

Mechanistically, we do not yet know how heparanase enhances the phosphorylation of the insulin receptor. One possibility is that heparanase, which is known to have multiple heparan sulfate-binding motifs, binds to the cell surface heparan sulfate proteoglycan syndecan-1, triggers clustering of syndecan-1 with the insulin receptor, and induces its dimerization and autophosphorylation. A previous study has shown that heparanase can induce the clustering of syndecan-1, thereby initiating signaling cascades that involve Rac1, Src, and the PKC pathways, resulting in enhanced cell adhesion and spreading (38). Similar to this finding, we also observed high PKC activity in heparanase-high cells and have found that heparanase-high cells spread much more avidly than do heparanase-low cells.3 Another study has shown that syndecan-1 clusters both IGF-1R and integrins resulting in integrin activation in carcinoma and endothelial cells (39). Similar to this finding, we recently observed the association of syndecan-1 with IGF-1R in CAG myeloma cells.4 Because insulin receptor and IGF-1R share 60% overall amino acid sequence homology and 84% homology in their tyrosine kinase domains, it is possible that heparanase promotes a syndecan-1/IGF-1R-insulin receptor complex on myeloma cells. In support of this notion, it was recently shown that insulin receptor associates with IGF-1R to form an insulin/IGF-1 hybrid receptor at the cell membrane of myeloma cells (20).

Our finding that heparanase up-regulates PKC and that this enhances IRS-1 expression is consistent with previous reports that IRS-1 expression is transcriptionally regulated by PKC activity (36). Activation of RTKs can enhance PKC activity, and we speculate that heparanase-induced insulin receptor activation initiates PKC activation, which, in turn, up-regulates the level of IRS-1. IRS-1 expression is often increased in human cancer (40, 41), and overexpression of IRS-1 and an increase in its phosphorylation has been shown to induce cellular transformation with activation of potent oncogenic signal transduction pathways such as Grb2-SOS-Ras and MAPK cascades (42, 43). Similar to this finding, we found that inhibiting IRS-1 by knock-downing its expression decreases the levels of phosphorylated ERK in heparanase-high cells. Interestingly, studies have shown a strong association of several IRS-1 single nucleotide polymorphisms with increased risk of multiple myeloma, indicating that IRS-1 expression and/or activity may play an important role in this cancer (26).

Overall, our studies for the first time demonstrate that heparanase expression and insulin signaling act in tandem to play a major role in regulating ERK signaling in myeloma. Interestingly, insulin is known to stimulate heparanase secretion from cells (44), raising the possibility that insulin plays a dual role in activating ERK signaling via insulin receptor regulation and by enhancing secretion of heparanase, which further drives insulin receptor signaling and ERK activation. These findings underscore the potential of heparanase inhibitors and insulin receptor pathway inhibitors as anti-cancer drugs and the possibility that they could be used to reverse the aggressive phenotype of some myeloma tumors.

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