Hepatocyte Growth Factor/Scatter Factor Induces Feedback Up-Regulation of CD44v6 in Melanoma Cells through Egr-1

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ABSTRACT

The hepatocyte growth factor/scatter factor (HGF/SF) receptor c-Met and variants of the CD44 family of surface adhesion molecules, including CD44v6, have been implicated in cancer progression and metastasis. CD44 isoforms bearing heparin sulfate chains can bind to HGF/SF and facilitate its presentation to c-Met. Here, we demonstrate that HGF/SF-Met binding up-regulates the expression of CD44v6 in murine melanoma cells, serving to compensate for loss by internalization. c-Met-mediated CD44v6 up-regulation was achieved through transcriptional activation of the immediate early gene egr-1. Enhanced egr-1 expression was apparent at the level of RNA 40 min after exposure to HGF/SF, and Egr-1 protein was detectable between 1 and 2 h post-treatment. CD44v6 RNA levels were correspondingly elevated 2 h after HGF/SF exposure. HGF/SF induced egr-1 activation via the Ras>Erk1/2 pathway but not through either phosphatidylinositol 3'-kinase or protein kinase C. Binding of NK2, a naturally occurring splice variant of HGF/SF, to c-Met failed to induce either Egr-1 or CD44v6, accounting at least in part for its antagonistic behavior. We also identified an Egr-1-binding site in the mouse CD44 gene promoter that accounts for its responsiveness to HGF/SF in melanoma cells. The compensatory up-regulation of both c-Met and CD44v6 in response to HGF/SF has important implications with respect to strategies used by cancer cells to sustain stimulation of growth- and metastasis-promoting pathways associated with tumor progression.

INTRODUCTION

HGF/SF is a pleiotropic cytokine/growth factor capable of inducing an extraordinary variety of biological activities, including cellular proliferation, migration, and invasiveness (1–5). This member of the TGF-α superfamily of growth factors, particularly by the Ras MAP kinase and ERK pathway (54–56), activates transcription factors such as Egr-1 and AP-1 (57–59). The immediate early gene egr-1 [also known as NGFI-A (60), Krox-24 (61), and Zif268 (62)] encodes a nuclear, zinc finger protein capable of binding to specific GC-rich DNA sequences (63, 64). The egr-1 gene is rapidly and transiently induced by growth factors and differentiation signals (60, 64) through different signal transduction pathways, including the PKC→Ras→MAP kinase and PI3K pathways (65–67).

In this study, we show that 37–32 mouse melanoma cells express the metastasis-associated protein CD44v6, which can facilitate presentation of HGF/SF to its receptor c-Met. Furthermore, we demonstrate that HGF/SF, but not NK2, promotes the feedback up-regulation of CD44v6 through the transcriptional activation of egr-1.
MATERIALS AND METHODS

Reagents. The antibodies used were obtained from the following commercial sources: (a) anti-c-Met (SP260), anti-AP-1/c-Jun, and anti-Egr-1 (C-19; Santa Cruz Biotechnology, Santa Cruz, CA); (b) anti-p-C-Met (Y1234/1235) (Biosource); (c) anti-actin (Chemicon International, Inc., Temecula, CA); and (d) anti-CD44v6 (Bender Medsystems). Western blot detection was accomplished using horseradish peroxidase-coupled anti-mouse, antirabbit (Amersham Pharmacia Biotech), and antirabbit secondary antibodies (Sigma Chemical Co., St. Louis, MO). Secondary antirabbit and antirat FITC antibodies and Texas red conjugates for fluorescence microscopy and FACS were purchased from Vector Laboratories, Inc. (Burlingame, CA). The Mek1/2 inhibitor PD98059 and U0126 were obtained from Cell Signaling Technologies. The P3K inhibitor LY294002 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Staurosporine was purchased from Roche Laboratories. Recombinant human HGF/SF was from R&D Systems, Inc. (Minneapolis, MN). The pBlueCD44 full cDNA was obtained from Dr. John Monroe (University of Pennsylvania). Purified NK2 was a generous gift from Dr. Ralph Schwall (Genentech).

Cell Culture, Transfection, and Stimulation. The 37-32 mouse cell line was derived from a cutaneous melanoma arising in the HGF/SF transgenic mouse line MH37 (15). Cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD), supplemented with 10% fetal bovine serum (Harlan Sprague Dawley, Indianapolis, IN), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine (Life Technologies, Inc.), 5 μg/ml insulin (Sigma), and 5 ng/ml epidermal growth factor (Upstate Biotechnology), and incubated in 5% CO2 at 37°C. Cells were treated with 40 ng/ml HGF/SF (R&D Systems) and harvested at the times indicated.

Western Blot and Immunoprecipitation. After HGF/SF treatment, cells were lysed with M-PER (Pierce Chemical Co., Rockford, IL) in the presence of protease inhibitor cocktail (Roche). Fifty μg of protein from lysates were separated by SDS-PAGE using 4–20% gradient gels (Novex). Proteins were transferred to nitrocellulose membranes (Novex) and probed against primary antibodies. Proteins were visualized with secondary antibodies coupled to horseradish peroxidase and developed using SuperSignal-West Pico Chemiluminescent Substrate (Pierce). Immunoprecipitation of c-Met was performed by treating the cells with M-PER (Pierce) and incubating the resulting lysates with either antiphospho-antibodies or anti-c-Met overnight at 4°C. After the addition of protein A/G agarose PLUS (Santa Cruz Biotechnology) and washing in M-PER buffer, samples were fractionated by SDS-PAGE using 4–20% gradient gels (Novex). After transfer, blots were blocked and incubated overnight with an anti-c-Met antibody. Bands were visualized by incubation with a secondary antirabbit antibody conjugated to horseradish peroxidase and Super Signal-West Pico Chemiluminescent Substrate. After stripping, membranes were blocked and incubated overnight with other antibodies as indicated in the figure legends.

Northern Blots. Twenty μg of total RNA were resolved on a denaturing 1% agarose formaldehyde gel and transferred to a nitrocellulose membrane (Schleicher & Schuell; Refs. 23 and 68). Membranes were prehybridized and hybridized against CD44 using ultrahybridization solution (Sigma) following the manufacturer’s instructions and subjected to autoradiography.

cDNA Arrays. Mouse pathway finder array (Superarray, Inc.) was used to hybridize cDNA from 37-32 cells, treated as indicated with either HGF/SF or NK2. The array was performed following the manufacturer’s protocol using 5 μg of total RNA as a template for cDNA synthesis.

Nuclear Extracts and EMSA Assay. Gel mobility shift assays and nuclear extracts were as described by Andrews and Faller (69). Briefly, cells were lysed in buffer A [10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, and 0.5 mM DTT plus protease inhibitor cocktail] for 10 min at 4°C. After centrifugation, the pellet was resuspended in buffer B [20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibitor cocktail]. Samples were incubated in ice for 30 min and then subjected to centrifugation to recover the nuclear proteins. EMSAs were performed as described by Recio and Aranda (70). Five μg of nuclear extracts were added to a buffer containing ~30,000 cpm of 32P-labeled oligonucleotide, 0.1 μg of Poly (dl-dC), 40 mM HEPES (pH 7.0), 140 mM NaCl, 4 mM DTT, 0.01% NP40, 100 mg/ml BSA, and 4% Ficoll. After incubation, the samples were loaded onto a 6% nondenaturing polyacrylamide gel. AP-1, Egr-1, and Egr-1 mutant oligonucleotides were purchased from Santa Cruz Biotechnology.

RESULTS

CD44v6 Is Expressed in Mouse Melanoma Cells and Helps Present HGF/SF to c-Met. Melanoma cells isolated from HGF/SF transgenic mice (line 37-32) express high levels of c-Met and are very responsive to HGF/SF (15, 23). Recently, a role for CD44 in presenting HGF/SF to c-Met in colorectal cancer cells has been described (32, 71). We therefore explored a possible role for CD44 in HGF/SF-Met signaling in a melanoma cell line derived from an HGF/SF transgenic mouse. Preliminary data using confocal microscopy revealed that HGF/SF and NK2 promoted the colocalization and cointernalization of c-Met and the v6-containing isoform of CD44 (hereafter referred to as CD44v6) in 37-32 cells (data not shown). Western blot and FACS analyses then showed that these melanoma cells not only expressed the CD44v6 isoform but that the cell surface levels of this receptor were elevated 24 h after HGF/SF treatment (Fig. 1, A and B).

Because the variant CD44v6 is associated with HS, we determined if HS chains were required for HGF/SF responsiveness by treating the 37-32 cells with heparitinase III before HGF/SF stimulation. Fig. 1C shows that pretreatment of 37-32 cells with heparitinase III totally blocked the ability of HGF/SF to phosphorylate c-Met tyrosines at the positions Y1234/1235. This result, although not conclusive standing alone, supports a role for the HS-modified CD44v6 isoform in the presentation of HGF/SF to c-Met, in agreement with data reported previously by several authors (10, 32, 34–36).

HGF/SF, but not NK2, Up-Regulates CD44v6 RNA Transcripts via the Erk1/2 Pathway. Next, Northern blot hybridization was used to determine whether HGF/SF induced the expression of CD44v6 at the level of RNA and when this induction occurred. Fig. 2A shows that CD44v6 transcript levels were elevated by exposure to HGF/SF, reaching a 2-fold peak at 2 h and decreasing thereafter. At 6 h, the levels of CD44v6 RNA were below basal expression. The Mek1/2-specific inhibitor, U0126, was able to inhibit HGF/SF-mediated up-regulation of CD44v6, demonstrating a role for the Erk1/2 pathway (Fig. 2B). In contrast to HGF/SF, the variant NK2 could not activate
against the indicated antibodies. Immunoprecipitated, and samples were separated by SDS-PAGE. Blots were probed were treated with 25 mU/ml heparitinase III for 3 h before HGF/SF treatment. c-Met was c, CD44v6 by HGF/SF (40 ng/ml) are demonstrated by FACS analysis. In melanoma cells. Twenty ng/ml) or NK2 (120 ng/ml). The Mek1/2-specific cells were treated for 2 h either with HGF/SF (40 HGF/SF induction. Twenty ng/ml HGF/SF for 2 h. Northern blot analysis showed that the 2-fold egr-1 expression returned to basal levels by 4.5 h (Fig. 3A).

Examination of Egr-1 expression by Western blot analysis of total lysates showed that Egr-1 protein was elevated after 30 min, reaching a peak by 2 h and thereafter becoming undetectable (Fig. 3B). These data indicate that, like CD44v6, the Egr-1 transcription factor is up-regulated by HGF/SF but not NK2.

HGF/SF-induced Up-Regulation of Egr-1 Is Mediated through the Ras>Erk1/2 Pathway. Egr-1 can be regulated through the PKC, Erk1/2, and PI3K signaling pathways (61–63). Western blot analysis of nuclear protein extracts from 37-32 cells after 1.5 h treatment with HGF/SF in the presence or absence of specific inhibitors for PI3K or Mek1/2 (LY294002 or PD98059, respectively) showed that Egr-1 up-regulation was mediated through the Erk1/2 pathway and not the PI3K pathway (Fig. 4A). These results were corroborated by immunofluorescent staining of 37-32 cells with an Egr-1 antibody (Fig. 4B) and by Western blot analysis of total protein extracts (data not shown).

To determine whether PKC was involved in the up-regulation of Egr-1 by HGF/SF, melanoma cells were treated with the PKC-specific inhibitor staurosporin (100 nm) for 1 h before HGF/SF treatment. Western blot analysis of nuclear extracts from these cells showed that staurosporin could not block HGF/SF-mediated Egr-1 up-regulation (Fig. 4C). These results demonstrate that, like CD44v6, the regulation of Egr-1 by HGF/SF is mediated through the Ras>Erk1/2 pathway. Moreover, up-regulation of Egr-1 preceded that of CD44v6.

Egr-1 Binds to the CD44 Mouse Promoter and Is Responsible for CD44v6 Up-Regulation by HGF/SF. The HGF/SF-induced up-regulation of Egr-1 protein and CD44v6 RNA is consistent with a role for Egr-1 in the transcriptional activation of CD44 (Fig. 2A). Maltzman et al. (57) have demonstrated previously a functional binding site for Egr-1 in the human CD44 gene promoter in response to the B-cell antigen receptor. To prove that a comparable Egr-1-binding site functions in the CD44 mouse promoter, several experiments were designed. The 37-32 melanoma cells were transfected either with egr-1 sense or antisense oligonucleotides and then treated with 40 ng/ml HGF/SF for 2 h. Northern blot analysis showed that the 2-fold increase in CD44v6 RNA induced by HGF/SF was partially blocked by the presence of the egr-1 antisense, but not the egr-1 sense, oligonucleotide (Fig. 5A), indicating that Egr-1 is required for HGF/ SF-mediated CD44v6 up-regulation. Next, we attempted to detect the direct binding of Egr-1 to the mouse CD44 promoter using a ChIP assay. The 37-32 mouse melanoma cells were treated with HGF/SF for 1 h, and then specific chromatin–protein complexes were immunoprecipitated using an anti-Egr-1 antibody. The region homologous to the Egr-1-binding site was then subjected to PCR amplification using as a template the DNA from the Egr-1-specific immunoprecipitated complexes. The appropriate band (451 bp) corresponding to the fragment containing the predicted Egr-1-binding site was detected only in the HGF/SF-treated cells (Fig. 5B). As a control, the amplification was performed on complexes obtained without exposure...
to Egr-1 antibody and from the untreated 37-32 melanoma cells (Fig. 5B).

An EMSA was also performed using an Egr-1 consensus motif oligonucleotide. Nuclear extracts generated from HGF/SF-treated cells showed an increase in binding to the labeled Egr-1 consensus sequence oligonucleotide (Fig. 5C). As expected, this increase in binding was blocked in cells treated with the Mek1/2 inhibitor PD98059 but not in those treated with the PI3K inhibitor (Fig. 5C). There was no binding when the nuclear extracts from the HGF/SF-treated cells were assayed using a mutant Egr-1-binding site oligonucleotide. Others have described the participation of the AP-1 transcription factor in the transcriptional up-regulation of the CD44 receptor (58, 59). Fig. 5D shows a time course of the activation of AP-1 after HGF/SF stimulation. Maximum activation occurred 1 h after triggering, and the binding was inhibited by the presence of an anti-AP-1 antibody.

Fig. 5E displays the sequence of the mouse CD44 promoter from -685 to the transcriptional start site. Putative transcription factor-binding sites are indicated, including the transcriptional start site identified using the TESS program (59, 72). The Egr-1-binding site for the mouse promoter is located at a position 235 upstream of the transcriptional start site (Fig. 5E).

DISCUSSION

HS proteoglycans have been shown to be crucial for ligand–receptor interactions (27–33). Although their role in HGF/SF-Met interaction is less well defined, several in vitro studies have indicated that these proteoglycans may play an important regulatory role in HGF/SF-Met signaling (10, 32, 34–36). Some isoforms of the CD44 family...
have been implicated in the presentation of HGF/SF to c-Met (32, 73). These HS proteoglycans may help to localize HGF/SF to specific cells or extracellular matrix components within the microenvironment and may be required for the establishment of a chemotactic gradient (38, 42, 74). In this study, we show that 37-32 murine melanoma cells, which already possess a strong HGF/SF-Met autocrine loop, also express the variant 6 isoform of CD44. It has been proposed that the presence of the v6 and v7 regions promotes CD44 oligomerization, which in turn facilitates binding to ligands (reviewed in Ref. 40); these ligands include GAGs and growth factors, such as HGF/SF. We further demonstrated that expression of the CD44v6 variant is up-regulated by HGF/SF through a feedback loop requiring the presence of Egr-1.

A number of studies has demonstrated that enhanced activity of oncogenic Ras-associated MAP kinase pathways in different cellular systems regulates the expression and alternative splicing of the CD44 family proteins (54–56). The expression of CD44v6 in melanoma cells and its relationship to HGF/SF-Met signaling are significant because the CD44v6 isoform has been implicated in metastasis and tumor progression (47, 48, 72). In some cases, such as gastric carcinoma, colorectal adenocarcinoma, pancreatic cancer, and breast cancer, the expression of CD44v6 has been proposed as a marker for poor prognosis (47, 49, 75, 76). In other cases, such as gastric carcinoma, colorectal adenocarcinoma, pancreatic cancer, and breast cancer, the expression of CD44v6 has been proposed as a marker for poor prognosis (47, 49, 75, 76). Aberrant c-Met expression has also been documented in a variety of human cancers (reviewed in Refs. 5, 77, and 78). Moreover, HGF/SF is known to up-regulate the expression of c-Met in normal and malignant cells (79–81). Thus, our data forge an intriguing regulatory link between activation by the multifunctional cytokine HGF/SF, and up-regulation of those receptors required to sustain its cellular activities. Interestingly, we found that NK2, which can behave as an HGF/SF antagonist in a number of cell types (4, 6, 13, 14, 82), is unable to up-regulate CD44v6 expression in 37-32 mouse melanoma cells, although both ligands induce colocalization and internalization of c-Met and CD44v6 (data not shown). As a consequence, NK2 cannot replenish cell surface levels of CD44v6, offering at least a partial explanation for its antagonistic behavior.

Most studies of CD44 promoter regulation have focused on the methylation state of the promoter. However, Maltzman et al. (57) have reported that the Egr-1 transcription factor is essential for the regulation of the human CD44 promoter in B-lymphocytes. Data presented in this study are the first to demonstrate the up-regulation of the Egr-1 transcription factor in response to HGF/SF. Transcriptional regulation of Egr-1 by HGF/SF-Met signaling is driven through Ras-Erk1/2 but is independent of PI3K and PKC (65–67). Because NK2 does not activate Erk1/2 in 37-32 cells (15), it was not surprising to find that NK2 cannot up-regulate either Egr-1 or CD44v6.

We anticipate that other transcription factors will participate in regulating the activity of the mouse CD44 promoter (see Fig. 5D). It has been shown that transcriptional activation of c-met by HGF/SF is mediated through AP-1 complexes (81). Additional data from our cDNA array (data not shown) and EMSA analysis implicate other early genes, such as c-Jun and c-fos, as HGF/SF-Met targets; c-Jun

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**Fig. 5.** Egr-1 binds to the CD44 mouse promoter and regulates CD44v6 transcription. A, Northern blot of 37-32 melanoma cells transfected either with the Egr-1 sense or antisense oligonucleotide. CD44 RNA expression was quantified 2 h after treatment with HGF/SF (40 ng/ml). Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. B, ChIP assay showing the amplification of the 451-bp fragment containing the Egr-1-binding site within the CD44 promoter only after HGF/SF treatment. In C, nuclear extracts from 37-32 cells treated as indicated were analyzed by EMSA for in vitro binding of the Egr-1 transcription factor to a labeled consensus sequence (Santa Cruz Biotechnology). D, time course of AP-1 transcription factor activation after HGF/SF treatment in 37-32 cells. Activation was inhibited by the presence of an anti-AP-1 antibody or 100-fold excess of cold oligonucleotide. E, nucleotide sequence of the mouse promoter fragment, 685 nucleotides upstream of the transcription initiation site. Several putative transcription factor-binding sites are shown (55). The homologous human Egr-1-binding site is located 235 bp upstream of the initiation site.
and c-fos are part of the AP-1 complex (83). These data, and the fact that AP-1-binding sites have been identified in the human CD44 promoter (57, 59), suggest that in addition to Egr-1, AP-1 may be involved in the transcriptional regulation of CD44.

Two different groups have demonstrated the functionality of the Egr-1-binding site in the human CD44 promoter (57, 58); however, the homologous site in the mouse promoter has not been reported. The antisense experiment and ChIP assay described here demonstrated not only the presence of the binding site in the homologous mouse promoter but its functionality as well. In the human CD44 promoter, the Egr-1-binding site is located at position −301 overlapping an SP1-binding site (57). In the mouse promoter, the Egr-1-binding site is located at position −235, also overlapping an SP1-binding site. Although the mouse Egr-1-binding site sequence is not identical to the human, the three nucleotides essential for the binding of Egr-1 to the homologous human site are conserved (57).

In conclusion, here we describe the novel up-regulation of the immediate early response gene, egr-1, by HGF/SSF in melanoma cells. In contrast to HGF/SSF, the antagonist NK2 was unable to up-regulate this transcription factor. We have further demonstrated that Egr-1 mediates enhanced CD44v6 expression in response to HGF/SSF treatment. The feedback loop in which expression of both c-Met and CD44v6 is up-regulated in melanoma cells in response to receptor-depleting exposure to HGF/SSF represents an important mechanism whereby cancer cells establish and maintain constitutive stimulation of growth- and metastasis-promoting pathways.

ACKNOWLEDGMENTS

We thank Dr. Ralph Schwall for providing purified NK2 and Dr. John Monroe for the full-length CD44 cDNA.

REFERENCES


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