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Chapter 4

Heparan sulfate-modified CD44 promotes hepatocyte growth factor/scatter factor-induced signal transduction through the receptor tyrosine kinase c-Met.

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SUMMARY

CD44 has been implicated in tumor progression and metastasis, but the mechanisms involved are as yet poorly understood. Recent studies have shown that CD44 isoforms containing the alternatively spliced exon v3 carry heparan sulfate side chains and are able to bind heparin-binding growth factors. In the present study, we have explored the possibility of a physical and functional interaction between CD44 and hepatocyte growth factor/scatter factor (HGF/SF), the ligand of the receptor tyrosine kinase c-Met. The HGF/SF-c-Met pathway mediates cell growth and motility and has been implicated in tumor invasion and metastasis. We demonstrate that a CD44v3 splice variant efficiently binds HGF/SF via its heparan sulfate-side chain. To address the functional relevance of this interaction, Namalwa Burkitt's lymphoma cells were stably co-transfected with c-Met and either CD44v3 or the isoform CD44s, which lacks heparan sulfate. We show that, as compared to CD44s, CD44v3 promotes: (i) HGF/SF-induced phosphorylation of c-Met, (ii) phosphorylation of several downstream proteins, and (iii) activation of the MAP kinases ERK1 and 2. By heparitinase treatment and the use of a mutant HGF/SF with greatly decreased affinity for heparan sulfate, we show that the enhancement of c-Met signal transduction induced by CD44v3 was critically dependent on heparan sulfate moieties. Our results identify heparan sulfate-modified CD44 (CD44-HS) as a functional co-receptor for HGF/SF which promotes signaling through the receptor tyrosine kinase c-Met, presumably by concentrating and presenting HGF/SF. As both CD44-HS and c-Met are over expressed on several types of tumors, we propose that the observed functional collaboration might be instrumental in promoting tumor growth and metastasis.

INTRODUCTION

The CD44 family of cell surface glycoproteins is broadly expressed by cells of epithelial, mesenchymal, and hematopoietic origin and is involved in cell-matrix adhesion, hematopoiesis, and lymphocyte homing and activation (1). Furthermore, a large body of experimental and clinical studies support a role for CD44 in tumor progression and metastasis (2-4). The CD44 gene consists of 19 exons (5). Due to alternative splicing, which involves at least 10 exons encoding domains of the extracellular portion of the CD44 molecule, a large number of CD44 isoforms is generated (6-10). Posttranslational modification generates further diversity, yielding both N- and O-linked glycan forms of CD44 in addition to proteoglycan variants containing chondroitin-, keratan-, or heparan sulfate (11-14). The expression pattern of these CD44 variants is tissue-specific. On lymphocytes the short 80-90 kDa standard form of CD44 (CD44s) is most abundant, while larger
variants (CD44v) predominate on some normal and neoplastic epithelia and are also found on activated lymphocytes and on malignant lymphomas (15-19). This selective expression suggests specific biological functions for the various splice variants, but at present, these are poorly defined. Similarly, the mechanism(s) through which CD44 functions in tumorigenesis is not known.

An obstacle towards understanding the functions of the CD44 family is the limited knowledge of its molecular partners. The cytoplasmic tail of the CD44 molecule has been shown to interact with the actin cytoskeleton via ankyrin and proteins of the ERM-family, and is associated with Src-family tyrosine kinases (20-23). This suggests a role in signaling as well as in the regulation of cell shape and motility. Although several potential CD44 ligands have been identified, the only interaction of the extracellular domain of CD44 that has been extensively studied is that with hyaluronate. CD44 acts as a major receptor for this glycosaminoglycan which is highly abundant in mesenchymal tissues and is believed to play a role in cell migration and differentiation (24, 25).

A novel and potentially highly significant function of CD44 is its ability to interact with heparin-binding growth factors (26, 27). These growth factors bind to a HS side chain attached to the evolutionary conserved consensus motif SGSG encoded by exon v3 (13, 27). Heparan sulfate proteoglycans (HSPGs) are believed to play an important regulatory role in cell growth and motility by binding growth factors and by presenting these factors to their high affinity receptors. This process has been particularly well explored for the fibroblast growth factors 1 and 2 (FGF-1 and 2). For these factors, binding to HSPGs has been shown to be required for their biological function, presumably by promoting FGF dimerization required for efficient receptor cross-linking and activation (28-32).

In the present study, we explored the physical and functional interaction between heparan sulfate modified forms of CD44 (CD44-HS) and hepatocyte growth factor/scatter factor (HGF/SF). HGF/SF is a heparin-binding growth factor (33) that induces growth, motility, and morphogenesis of target epithelial and endothelial cells by binding to the receptor tyrosine kinase c-Met (34, 35). In addition, recently HGF/SF was shown to be involved in hematopoiesis, and lymphocyte adhesion and migration (36-42). Apart from these physiological functions, there is ample evidence for a key role of the HGF/SF - c-Met pathway in tumor growth, invasion and metastasis. For example, HGF/SF induces epithelial cells to invade collagen matrices in vitro, and NIH 3T3 cells co-transfected with c-met and HGF/SF acquire an invasive and metastatic phenotype (43-45). Furthermore, in HGF/SF transgenic mice, tumors develop in many different tissues including mammary glands, skeletal muscles and melanocytes (46). In human cancer, both HGF/SF and c-Met are often over expressed, and in hereditary renal cancer germline mutations in the c-met gene have recently been reported (47-52). Here, we show that CD44-HS strongly promotes signal transduction through the
HGF/SF - c-Met pathway, which is demonstrated to occur in a heparan sulfate
dependent fashion.

MATERIALS AND METHODS

**Antibodies.** Mouse monoclonal antibodies (mAbs) used were anti-pan CD44, 
NK-1(P1 (IgG1) (53) and Hermes-3 (IgG2a) (54) (a gift from S. Jalkanen, University 
of Turku, Turku, Finland); anti-HGF/SF, 24612.111 (IgG1) (R&D Systems, Abington, United 
Kingdom); anti-heparan sulfate, 10E4 (IgM) (55); anti-desaturated uronate from 
heparitinase treated heparan sulfate ("AHS stub"), 3G10 (IgG2b) (55), anti-
phosphotyrosine, PY-20 (IgG2b) (Affiniti, Nottingham, UK); and IgG1 and IgM control 
antibodies (ICN, Zoetermeer, The Netherlands). Polyclonal antibodies used were rabbit 
anti-c-Met, C-12 (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-phospho-
p44/42 MAP kinase (Thr202/Tyr204) (New England Biolabs, Beverly, MA); rabbit anti-
ERK1 (C-16) and anti-ERK2 (C-14) (Santa Cruz Biotechnology); RPL-conjugated goat 
antibody (Southern Biotechnology, Birmingham, AL); FITC-conjugated rabbit anti-
mouse (DAKO, Glostrup, Denmark); HRP-conjugated rabbit anti-mouse (DAKO); and 
HRP-conjugated goat anti-rabbit (DAKO).

**Cell lines and transfectants.** The Burkitt’s lymphoma cell line Namalwa was 
purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were 
cultured in RPMI 1640 (Life Technologies, Breda, The Netherlands) supplemented with 
10% Fetal Clone 1 serum (HyClone Laboratories, Logan, UT), 10% FCS (Integro, 
Zaandam, The Netherlands), 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml 
streptomycin (all Life Technologies). Namalwa cells transfected with CD44s (Nam-S), 
CD44v8-10 (Nam-V8) or CD44v3-10 (Nam-V3) were described previously (56). A second 
transfection of Namalwa cells, expressing either CD44s (Nam SM) or CD44v3-10 (Nam 
V3M), with c-Met was performed as described (41).

**Purification of wild type and mutant HGF/SF.** The construction of pVL1393 
vectors (Pharmingen, San Diego, CA) containing wild type or mutant HGF/SF (HP1) 
cDNA was described elsewhere (57).

HGF/SF (wild type and HP1) was produced in a Baculovirus system as described 
previously (58). In brief, Sf9 insect cells were transduced with an amplified virus stock and 
after 3 days media were pooled and analysed for scattering activity in the MDCK 
dissociation assay (59). Then, HGF/SF was purified with Ni-NTA resin from the 
QIAexpress system (Qiagen, Hilden, Germany). HGF/SF concentrations were measured by 
ELISA as described previously (41). In addition, HGF/SF (wt and HP1) was analysed by 
Western blotting using goat anti-HGF/SF.

**Enzyme treatments.** For enzymatic cleavage of glycosaminoglycans, cells were 
treated with either heparitinase (Flaobacterium heparinum, EC 4.2.2.8, ICN Biomedicals, 
Aurora, OH) or chondroitinase ABC (Proteus vulgaris, EC 4.2.2.4, Boehringer Mannheim.
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Almere, The Netherlands) in PBS at 37°C for the periods indicated. Enzyme treatments were followed by FACS analysis or immunoprecipitation.

**FACS analysis.** For FACS analysis cells were blocked with 10% pooled human serum (CLB, Amsterdam, The Netherlands), 1% BSA (Fraction V) (Sigma, Bornem, Belgium) in PBS at 4°C for 15 min and washed with FACS buffer (1% BSA in PBS), respectively. Then, the cells were incubated with the primary antibodies for 1 h, washed, and incubated with the secondary antibody for 30 min. Incubations were in FACS buffer at 4°C, and cells were analyzed by using a FACScan (Becton Dickinson, Mountain View, CA).

For binding of recombinant human HGF/SF (wild type or HP1) R & D Systems or our own product), cells were incubated with this protein (18 nM or as indicated) for 1 h prior to the antibody incubations. This step was followed by washing with FACS buffer.

**Immunoprecipitation and Western blot analysis.** Immunoprecipitation was performed as described (41). The only modifications were that, for precipitation of CD44, cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% NP-40, 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Sigma), 1 mM sodium orthovandate (Sigma), 2 mM EDTA, and 5 mM NaF. For precipitation of c-Met, cells were lysed in 10 mM Tris-HCl (pH 8), 150 mM NaCl, 10% glycerol, 1% NP-40, 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Sigma), 2 mM sodium orthovandate (Sigma), 5 mM EDTA, and 5 mM NaF.

Western blotting of immunoprecipitates and total cell lysates was essentially performed as described previously (23). A single modification was that, for analysis of phosphorylated proteins, membranes were blocked and stained in 2% BSA, 20 mM Tris-HCl, 150 mM NaCl (pH 7.5), and 0.05% Tween-20 (Sigma). Films were scanned with an Eagle Eye II video system (Stratagene, La Jolla, CA) and band intensities were determined with ONE-D-Scan software (Stratagene). c-Met phosphorylation was expressed as the ratio of phosphorylated c-Met to c-Met precipitated.

For analysis of phosphorylation of the ERK1 and 2 MAP kinases, after the indicated treatments, 5 x 10^5 cells were directly lysed in sample buffer and analysed by 10% SDS-PAGE and blotted. Equal loading was confirmed by Ponceau S staining of the blot. The part of the blot below 50 kD was stained with anti-phospho-MAPK antiserum, the upper part with anti-phosphotyrosine PY-20. Primary antibodies were detected by HRP-conjugated goat anti-rabbit and HRP-conjugated rabbit anti-mouse, respectively. Identification of the ERKs was confirmed by staining with anti-ERK1 or anti-ERK2.

**RESULTS**

**Binding of HGF/SF to CD44 isoforms.** Binding of HGF/SF to different CD44 isoforms was assessed by using a panel of Namalwa Burkitt’s lymphoma cell lines
Figure 1. A. Schematic representation of the CD44 gene, and the CD44v3-10, CD44v8-10, and CD44s cDNAs used for transfection. Solid boxes represent constant exons while open boxes represent alternative exons. Note that, due to a stop codon, the variable exon 1 (v1) is not translated in the human. UT, untranslated region; EC, extracellular constant region; EV, extracellular variable region; TM, transmembrane region; CT, cytoplasmic region. B. Binding of HGF/SF to CD44 Namalwa transfectants. Using a FACs flow cytometer, one clone of mock transfected (Neo) Namalwa cells, and two independent clones of CD44s, CD44v8-10 or CD44v3-10-transfected Namalwa cells were analysed for their binding of HGF/SF. Bound HGF/SF was detected with mouse anti-HGF/SF followed by RPE-conjugated goat anti-mouse.
CD44 promotes c-Met activation

Table I. Surface expression of CD44 on Namalwa transfectants.

<table>
<thead>
<tr>
<th>CD44 isoform</th>
<th>clone</th>
<th>MFI</th>
<th>% positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>none (Neo)</td>
<td>A</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>CD44s</td>
<td>A</td>
<td>83</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>96</td>
<td>97</td>
</tr>
<tr>
<td>CD44v8-10</td>
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<td>126</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>142</td>
<td>88</td>
</tr>
<tr>
<td>CD44v3-10</td>
<td>A</td>
<td>137</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>82</td>
<td>87</td>
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*Mean fluorescence intensity after staining with the anti-pan CD44 mAb NKI-P1 followed by FITC-conjugated rabbit anti-mouse.

stably transfected with cDNAs encoding either CD44s, CD44v8-10 or CD44v3-10 (Fig. 1A) (56). Prior to transfection, the cells were negative for CD44 and c-Met expression at both the protein and mRNA level (data not shown). All transfectants used for HGF/SF binding studies expressed comparable levels of CD44 (Table I). HGF/SF binding to the CD44 transfectants was measured by FACS analysis using an anti-HGF/SF mAb, an approach that avoids chemical modification of the ligand. As shown in Fig. 1B, CD44 negative control cells as well as CD44s and CD44v8-10 transfectants showed a low saturable binding of HGF/SF. In contrast, cells expressing CD44v3-10 bound much larger quantities of HGF/SF. These results suggest that CD44v3-10 contains (a) binding site(s) for HGF/SF.

*Binding of HGF/SF to CD44 is heparan sulfate-dependent.* We next conducted a series of experiments aimed at determining the role of HS-side chains in the binding of HGF/SF. First, the presence of total HS on the different transfectants was assessed by FACS analysis using the HS-specific mAb 10E4 (Fig. 2A), and the mAb 3G10 (Fig. 2B) which recognizes the ΔHS-stubs remaining on HSPG core proteins after treatment with heparitinase (55). Both figures show that cells transfected with CD44v3-10 express approximately 20-fold higher levels of HS compared to those transfected with other CD44 isoforms. Next, we investigated the presence of HS on CD44 itself. This was done by using mAb 3G10. With this mAb, a single major HS band was detected in Western blots of CD44 precipitates from the CD44v3-10 cells, but not from the other transfectants (Fig. 2C). Staining the blot with an anti-pan CD44 mAb demonstrated that this band corresponded to CD44v3-10 (Fig. 2C).

To assess the role of HS in the interaction between HGF/SF and CD44v3-10, we studied the effect of heparitinase treatment and performed binding studies
Figure 2. Presence of heparan sulfate on CD44 isoforms. A. Heparan sulfate expressed on representative mock, CD44s, CD44v8-10, or CD44v3-10 Namalwa transfectants that were treated with either PBS (filled histogram), 25 mU/ml heparitinase (solid line), or 25 mU/ml chondroitinase ABC (dotted line) at 37°C for 3 h. Heparan sulfate was detected by FACS analysis using the mAb 10E4, followed by RPE-conjugated goat anti-mouse. B. A similar FACS analysis as shown in A, but with the use of mAb 3G10 which recognizes ΔHS-stubs which remain on HSPG core proteins after treatment with heparitinase. C. Western blot of CD44 immunoprecipitates. CD44 was precipitated from CD44 Namalwa transfectants using the anti-pan CD44 mAb Hermes-3. Precipitates were then treated with either PBS (-), 200 mU/ml heparitinase (HT), or 1 U/ml chondroitinase ABC (CH) at 37°C for 2 h. The Western blot was stained with the anti-pan CD44 mAb Hermes-3 (upper panel), stripped, and re-stained with the mAb 3G10 (lower panel) which recognizes ΔHS-stubs after treatment of HS with heparitinase.
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with HP1, a HGF/SF mutant which has a greatly decreased (more than 50-fold) affinity for heparan sulfate and heparin (57). As shown in Fig. 3A, heparitinase treatment resulted in a near complete loss of HGF/SF binding, while treatment with chondroitinase ABC had no effect. The essential role of HS-moieties on CD44v3-10 in HGF/SF binding was further confirmed by the observation that HP1 did not bind to CD44v3-10 (Fig. 3B). These data demonstrate that CD44v3-10 is a heparan sulfate modified CD44 isoform (CD44-HS), that binds HGF/SF via its HS side chain.

CD44-HS promotes c-Met activation. To explore the functional impact of HGF/SF bound to CD44-HS on the c-Met signaling pathway, we generated double transfectants expressing c-Met in combination with either CD44v3-10 or CD44s. We selected stable transfectants expressing equal amounts of c-Met to be used in the subsequent studies (Fig. 4). Using these cell lines, we assessed in the first instance HGF/SF induced c-Met phosphorylation. As shown in Fig. 5, triggering with HGF/SF led to a vast and rapid increase in the phosphorylation of c-Met on tyrosine residues in the cells expressing CD44v3-10. By contrast, phosphorylation

Figure 3. The role of heparan sulfate in the binding of HGF/SF to CD44 Namalwa transfectants. A, FACS analysis to detect HGF/SF bound to CD44 Namalwa transfectants that were treated with either PBS, 10 mU/ml heparitinase, or 50 mU/ml chondroitinase ABC at 37°C for 2 h prior to incubation with 18 nM HGF/SF at 4°C for 1 h. B, FACS analysis of wild type or mutated (HP1) HGF/SF bound to CD44 Namalwa transfectants. HGF/SFs were detected with mouse anti-HGF/SF followed by RPE-conjugated goat antimouse. Results are expressed as relative mean fluorescence intensity (MFI) (as compared with PBS treated mock transfectants). Error bars represent the standard deviation from three independent experiments.
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Figure 4. Expression of c-Met in CD44 or CD44/c-Met Namalwa transfectants. CD44s and CD44v3-10 Namalwa transfectants with or without c-Met were lysed and analysed for the expression of c-Met by Western blotting. The Western blot was stained with rabbit anti-c-Met followed by HRP-conjugated goat anti-rabbit. The epidermoid carcinoma cell line A431 was used as a positive control. The c-Met precursor (pre c-Met) and β-chain (c-Met (β)) are indicated.

The expression of c-Met was only weakly increased in the cells with CD44s (Fig. 5) and was absent in the parental cell line (data not shown), confirming the lack of endogenous c-Met in these cells. The dose-response studies demonstrated that CD44v3-10 promotes c-Met phosphorylation over a broad dose range (Fig. 5A) with an approximately 7-fold relative increase at plateau level. The time curve (Fig. 5B) showed that phosphorylation was maximal between 2 and 10 min after addition of the growth factor and declined thereafter. Moreover, this strong enhancing effect of CD44v3-10 on c-Met phosphorylation was dependent on HS moieties since it was lost upon heparitinase treatment (Fig. 6A). The importance of HS for HGF/SF signaling was further strengthened by studies using the HGF/SF heparin-binding domain mutant HP1. This mutant induced an equal (weak) phosphorylation of c-Met in both the CD44v3-10 and CD44s transfectants (Fig. 6B). Thus, these data suggest that CD44v3-10 binds HGF/SF via its HS side chains and then presents it to the high affinity receptor c-Met.

**CD44-HS promotes downstream signaling through c-Met in a heparan sulfate-dependent fashion.** The pivotal role of CD44-HS in promoting the action of HGF/SF was further supported by analysing the cell lysates of HGF/SF-stimulated cells for tyrosine phosphorylated proteins. We observed tyrosine phosphorylation of several substrates, the two most prominent phosphoproteins of unknown identity are found at 115-125 kD. A minor phosphoprotein is found at 145 kDa which likely represents c-Met (Fig. 6C). In addition, several smaller
Figure 5. CD44v3-10 strongly promotes c-Met activation. A. Dose-kinetics of the tyrosine phosphorylation of c-Met in CD44v3-10/c-Met and CD44s/c-Met double transfectants. Transfectants were stimulated with increasing concentrations HGF/SF for 10 min at 37°C. c-Met was immunoprecipitated with rabbit anti-c-Met and the Western blot was stained with the anti-phosphotyrosine mAb PY-20 followed by HRP-conjugated rabbit anti-mouse (upper panel). Then, the blot was stripped and re-stained with rabbit anti-c-Met followed by HRP-conjugated goat anti-rabbit (lower panel). The ratios of tyrosine-phosphorylated c-Met to precipitated c-Met, as determined by densitometric scanning of the blots, are shown in a diagram. B. Time-kinetics of the tyrosine phosphorylation of c-Met in CD44v3-10/c-Met and CD44s/c-Met double transfectants that were stimulated with 2.2 nM HGF/SF for increasing periods at 37°C. c-Met was precipitated and analysed as in (A). The ratios of tyrosine-phosphorylated c-Met to precipitated c-Met, as determined by densitometric scanning of the blots, are shown in a diagram. The c-Met precursor (pre c-Met) and β-chain (c-Met (β)) are indicated. Several independent clones were tested and gave comparable results.

phosphoproteins of unknown origin were observed (not shown) including a 42 kD phosphoprotein which may represent the p42 ERK2 MAP kinase.

In order to establish whether signal transduction by c-Met is potentiared by the HS moieties on CD44v3-10, we further investigated the activation of
Figure 6. HGF/SF binding to heparan sulfate moieties on CD44v3-10 potentiates signal transduction through c-Met. A. CD44v3-10/c-Met (v3) and CD44s/c-Met (s) double transfectants were treated with 10 mU/ml heparitinase at 37°C for 3.5 h, and subsequently incubated in the presence or absence of 2.2 nM HGF/SF. Then, c-Met was precipitated with rabbit anti-c-Met and the Western blot was stained with anti-phosphotyrosine (PY-20) followed by HRP-conjugated rabbit antimouse (upper panel). Next, the blot was stripped and stained with rabbit anti-c-Met followed by HRP-conjugated goat anti-rabbit (lower panel). The c-Met precursor (pre c-Met) and β-chain (c-Met (β)) are indicated. B. CD44v3-10 does not promote c-Met phosphorylation by a HGF/SF heparin-binding domain mutant. CD44s/c-Met (s) and CD44v3-10/c-Met (v3) double transfectants were incubated in the presence or absence 2.2 nM wild type HGF/SF or with the heparin-binding domain mutant HGF/SF (HP1) for 10 min at 37°C. Then, c-Met was precipitated with rabbit anti-c-Met and the Western blot was stained with anti-phosphotyrosine (PY-20) followed by HRP-conjugated rabbit antimouse (upper panel). Next, the blot was stripped and re-stained with rabbit anti-c-Met followed by HRP-conjugated goat anti-rabbit (lower panel). C. Western blot from total cell lysates from equal numbers of the cells described in (A). The upper part of the blot was stained with the anti-phosphotyrosine mAb PY-20, followed by HRP-conjugated rabbit anti-mouse. The lower part of the same blot was stained with anti-phospho-MAPK antibody, followed by HRP-conjugated goat anti-rabbit. The arrows indicate a phosphorylated protein at 145 kDa and two major phosphoproteins at 115-125 kD (upper panel), and the phosphorylated ERK1 and ERK2 MAP kinases (lower panel). Several independent clones were tested and gave comparable results.
downstream targets of c-Met signaling. Since HGF/SF has been shown to activate the ERK MAP kinases in MDCK, HT29 and A549 cells (60-64), we assessed whether HGF/SF is also able to induce MAP kinase activation in Namalwa B cells. For this purpose we used an antibody recognizing only the active, phosphorylated, form of the ERK 1 and 2 (p44 and p42) MAP kinases. As shown in Fig. 6C, HGF/SF treatment results in phosphorylation of the MAP kinases ERK1 and 2 in Namalwa transfectants expressing c-Met. The phosphorylation of the ERK2 MAP kinase upon HGF stimulation of the cells was also confirmed by MAP kinase gel-shift analysis. We observed stronger phosphorylation of ERK1 and 2 in the CD44v3-10 expressing cells as compared to the CD44s expressing cells (Fig. 6C, bottom panel). Moreover, heparitinase treatment resulted in a decrease of HGF/SF-induced ERK phosphorylation in the CD44v3-10 cells, resulting in a level of ERK phosphorylation that is similar to the level of HGF/SF-induced ERK phosphorylation in CD44s transfectants. HGF/SF-induced phosphorylation of the ERKs in CD44s transfectants remained unaffected by heparitinase treatment. Taken together, our data demonstrate that signal transduction elicited by HGF/SF-induced c-Met activation is strongly promoted by CD44-HS, and depends on the presence of the HS moiety on CD44-HS.

DISCUSSION

We observed that cells transfected with CD44v3-10 efficiently bind HGF/SF (Fig. 1) and that this CD44 isoform is decorated with HS moieties (Fig. 2). By contrast, transfectants that express CD44s or CD44v8-10, CD44 isoforms which are not modified with HS (Fig. 2), were not able to bind HGF/SF above background (parental) levels (Fig. 1). This selective HS-modification of CD44v3-10 is in line with the recent study by Jackson et al. (13) which demonstrated that HS side chains bind to CD44 at the SGS motif encoded by exon v3. Indeed, we demonstrated that the interaction of HGF/SF with CD44v3-10 is HS-dependent. Binding was completely abrogated by heparitinase treatment, and HP1, a HGF/SF mutant with greatly decreased affinity for heparan sulfate and heparin (57), failed to bind CD44v3-10 (Fig. 3). Interestingly, it has been demonstrated that specific chemical modifications of HS side chains on proteoglycans appear to regulate their affinity for selected heparin-binding growth factors, including HGF/SF and FGF-2, and hence determine growth factor binding specificity (65-69). This suggests that the HS moiety covalently attached to CD44v3-10 contains specific binding sites for HGF/SF.

The key finding of our study is that CD44-HS has a major functional effect on HGF/SF-induced signal transduction. Expression of CD44-HS at the cell surface led to a vast increase in HGF/SF induced phosphorylation of c-Met on
tyrosine residues (Fig. 5). Furthermore, it resulted in a strong tyrosine phosphorylation of two as yet unidentified 115-125 kDa proteins that were hardly phosphorylated in the absence of CD44-HS (Fig. 6C). One of these proteins might represent p110/115-Grb2 associated binder (Gab)-1, an adaptor protein that has recently been found to associate with the multifunctional docking site of c-Met (70). Alternatively, the observed bands might be p120-Cbl and/or p125-FAK. Both protein tyrosine kinases participate in signal transduction via receptor protein tyrosine kinases and integrins (71, 72). This is particular interesting given our previous results that HGF/SF-stimulation of Namalwa Burkitt's lymphoma cells results in enhanced integrin α4β1-mediated adhesion (41) and the recent observation that Cbl is involved in integrin activation and spreading of

Figure 7. Model for the presentation of HGF/SF to c-Met. A. HGF/SF molecules, which are largely monomers, only weakly activate the c-Met pathway in (tumor) cells that lack cell surface expression of CD44-HS. B. By upregulating CD44-HS, (tumor) cells acquire a greatly increased sensitivity to HGF/SF, which might result in a growth and motogenic/metastatic advantage. Presumably, CD44 acts by concentrating HGF/SF at the cell surface and by presenting HGF/SF to c-Met. This presentation may involve ligand multimerization by HS side chains, resulting in increased c-Met dimerization. Alternatively, HGF/SF - CD44-HS interaction might lead to a conformational changes of the c-Met receptor promoting signal transduction.
promotes c-Met activation

CD44 promotes c-Met activation

macrophages (73). Furthermore, Cbl was recently reported to be required for efficient cellular transformation through the Tpr-Met oncoprotein (74). In addition to the 120-125 kDa proteins, we demonstrated for the first time that HGF/SF induces phosphorylation of the MAP kinases ERK1 and 2 in B cells (Fig. 6C). Even more intriguing was the observation that CD44-HS promoted the HGF/SF-induced phosphorylation of ERK1 and 2. ERK1 and 2 are intermediates in signaling pathways linking extracellular signals to gene transcription in the nucleus and have been implicated in a wide variety of biological responses including cell proliferation. Interestingly, several recent studies have implicated the ERKs in integrin activation (75) as well as in HGF/SF-induced motility (i.e. scattering), and tubulogenesis of the epithelial Madin-Darby canine kidney cell line (60, 62, 63). Because of our previous data concerning the involvement of HGF/SF in integrin-mediated adhesion of B cells (41), we are currently investigating the possible role of the ERKs in B cell adhesion and migration.

We demonstrated that the enhancing effects of CD44-HS on signal transduction via c-Met were critically dependent on the interaction of HGF/SF with the HS moieties on CD44-HS, as they were not observed after heparitinase treatment, or when the cells were triggered with the heparin-binding domain HGF/SF-mutant HP1 (Fig. 6). Importantly, the specific effects of the heparitinase treatment and the mutations in HP1 on HGF/SF-induced signal transduction in the CD44v3-10 expressing cells as compared to the CD44+ cells demonstrates that the difference in HGF/SF-elicted responses in these cells is not due to any possible clonal variation in these stable cell lines. We speculate that CD44-HS promotes the action of HGF/SF through concentration of HGF/SF on the cell surface and by presenting it to the high affinity receptor c-Met (Fig. 7). Similar mechanisms were proposed for the role of high and low affinity receptors in FGF functioning (32, 76, 77). In addition, CD44-HS might also protect HGF/SF from proteolytic degradation as endothelial cell-derived HS was shown to do for FGF-2 (78).

It should be noted, that, apart from growth factor presentation, CD44 may have additional functions in HGF/SF - c-Met mediated signaling. For example, CD44 might recruit molecular partners into a multi-molecular complex with c-Met. This possibility is suggested by the fact that two recently identified cytoplasmic molecules associated with CD44 have also been implicated in c-Met signaling. First, studies by Ponzetto et al. (64) have shown that c-Met is a substrate for Src-family tyrosine kinases, while our own studies have revealed a physical and functional association between CD44 and Src-family member p56^k^{k} (23). Second, studies by Jiang et al. (79) and Crepaldi et al. (80) have demonstrated that HGF/SF stimulates the tyrosine phosphorylation of the ERM-protein ezrin. As reported by Tsukita et al. (22), ERM-proteins serve as molecular linkers between CD44 at the cell surface and the actin cytoskeleton. This interaction is believed to be involved in the regulation of cell shape and motility.
We propose that collaboration between CD44-HS and growth factor receptors, *viz.* e-Met, as shown in our present study, might be an important factor in tumor growth and metastasis. By over expressing CD44-HS, tumor cells would acquire a strongly increased sensitivity to HGF/SF mediated growth signals, leading to a growth advantage and promoting metastasis (Fig. 7). This hypothesis is supported by the fact that e-Met and HGF/SF are (over)expressed in conjunction with CD44 in several types of tumors. In colorectal cancer, for example, e-Met is frequently over expressed (48, 49, 81), while HGF/SF is expressed within the tumor tissue microenvironment. Interestingly, in these tumors CD44 splice variants, including variants decorated with HS, are often over expressed and predict metastatic spread and tumor related death (82, 83). A similar scenario may hold for breast cancer and non-Hodgkin's lymphoma, as in these tumor types over expression of CD44v3 as well as e-Met has also been reported (19, 42, 51, 84).

In conclusion, we demonstrated that through binding and presenting HGF/SF, CD44-HS promotes signal transduction via the receptor tyrosine kinase e-Met. Consequently, over expression of CD44-HS might give tumor cells a growth and metastatic advantage and, in this way, might influence disease outcome.

**FOOTNOTES**

1 M. Spaargaren and G.J.T. Zwartkruis, unpublished observation.


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