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Enhanced Tumorigenicity and Invasion-Metastasis by Hepatocyte Growth Factor/Scatter Factor-Met Signalling in Human Cells Concomitant with Induction of the Urokinase Proteolysis Network

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Hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic effector of cells expressing the Met tyrosine kinase receptor. Although HGF/SF is synthesized by mesenchymal cells and acts predominantly on epithelial cells, we have recently demonstrated that human sarcoma cell lines often inappropriately express high levels of Met and respond mitogenically to HGF/SF. In the present report we show that HGF/SF-Met signalling in the human leiomyosarcoma cell line SK-LMS-1 enhances its in vivo tumorigenicity, an effect for which the mitogenicity of this signalling pathway is likely to play a role. In addition, we found that HGF/SF-Met signalling dramatically induces the in vitro invasiveness and in vivo metastatic potential of these cells. We have studied the molecular basis by which HGF/SF-Met signalling mediates the invasive phenotype. A strong correlation has previously been demonstrated between the activation of the urokinase plasminogen activator (uPA) proteolysis network and the acquisition of the invasive-metastatic phenotype, and we show here that HGF/SF-Met signalling significantly increases the protein levels of both uPA and its cellular receptor in SK-LMS-1 cells. This results in elevated levels of cell-associated uPA and enhanced plasmin-generating ability by these cells. These studies couple HGF/SF-Met signalling to the activation of proteases that mediate dissolution of the extracellular matrix-basement membrane, an important property for cellular invasion-metastasis.

Hepatocyte growth factor/scatter factor (HGF/SF) is an effector of cells expressing the Met tyrosine kinase receptor (16, 24, 40). It is produced by mesenchymal cells and acts predominantly on cells of epithelial origin in an endocrine and/or paracrine fashion (45, 48). As its name implies, HGF/SF promotes the growth and/or scattering of various cell types. HGF/SF has also been shown to mediate other biological activities, including the formation of tubules (26) and lumens (51), the promotion of angiogenesis (9, 18), the inhibition of cell growth (22), and the conversion from a mesenchymal to an epithelial phenotype (52). In vivo, this ligand-receptor pair is believed to play a role in neural induction (50), kidney development (43, 57), tissue regeneration (25), and wound healing (28) and is required for normal embryological development (6, 44, 54).

While HGF/SF-Met signalling clearly plays a role in normal cellular processes, this signalling pathway has also been implicated in tumor development and progression. Met was originally isolated as the product of a human oncogene, *trp-met*, which encodes an altered Met protein possessing constitutive, ligand-independent tyrosine kinase activity and transforming ability (12, 31). The coexpression of unaltered Met and HGF/SF molecules in the same cell, which generates an autocrine stimulatory loop, is also oncogenic (3, 34). The oncogenic capability of HGF/SF-Met signalling is likely due to the inappropriate expression of mitogenic and angiogenic signals.

In addition to transforming cells, deregulated Met signalling in NIH 3T3 cells increases their invasiveness in vitro (17, 38) and metastatic potential in vivo (38). HGF/SF-Met signalling also induces the invasiveness and metastatic potential of other cell types (3, 39, 56). The detection of significant levels of HGF/SF in the pleural effusion fluid of patients whose cancer had metastasized to the pleura (23) suggests the involvement of HGF/SF-Met signalling in promoting metastasis in humans. The molecular mechanism(s) by which HGF/SF-Met signalling promotes the invasive-metastatic phenotype is largely unknown but probably involves the induction of proteases which mediate the degradation of the extracellular matrix-basement membrane (ECM/BM) (47). In this regard, we recently demonstrated that HGF/SF-Met-transformed NIH 3T3 cells exhibit an elevated level of collagenase activity compared with that of control cells (38).

The urokinase type plasminogen activator (uPA) is a serine protease of limited specificity (8, 14, 41). Active uPA is found predominantly at the cell surface, where it is retained by a high-affinity receptor (uPAR). One uPA substrate is HGF/SF, which is secreted as an inactive monomer that may be cleaved into its biologically active heterodimeric form by uPA (27). In addition, uPA is involved in the degradation of the ECM. Although uPA can cleave some ECM proteins (e.g., fibronectin) directly, most of its ECM/BM-degrading properties are believed to come through indirect means by virtue of its ability to activate plasminogen to the broad-specificity serine protease plasmin. Like uPA, active plasmin is predominantly a cell surface-associated protease, but its broader specificity enables it to play a more direct role in ECM/BM degradation than uPA. In addition, plasmin can activate the metalloproteinases, a class of proteases with potent ECM/BM-degrading capability (5). Since uPA plays a central role in catalyzing ECM/BM degradation, it is not surprising that a strong association between uPA expression and the invasive-metastic phenotype has been demonstrated.

Although HGF/SF is synthesized by mesenchymal cells and acts predominantly on Met-expressing epithelial cells, we have

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recently demonstrated that human sarcoma cell lines often inappropriately express high levels of Met and may respond mitogenically to HGF/SF (35, 36). We (35, 36) and others (15) have also shown that clinical sarcoma samples may overexpress the Met receptor. Thus, this receptor-ligand pair has been implicated in human sarcomagenesis. In the present report, we have extended our analysis of HGF/SF-Met signalling in human sarcoma cells and have examined the effect of this signalling pathway on the in vitro invasiveness and the in vivo tumorigenicity and metastatic potential of these cells. We have also investigated the activation of the urokinase proteolysis network by HGF/SF-Met signalling in these cells.

MATERIALS AND METHODS

Cell lines. The SK-LMS-1 human leiomyosarcoma cell line was obtained from American Type Culture Collection (Rockville, Md.) and grown in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Gaithersburg, Md.) containing 10% fetal bovine serum (FBS; Life Technologies) in 10% CO_2.

Growth factors. Human HGF/SF was purified, as previously described, from the supernatant of transformed NIH 3T3 cells that overproduce the factor (37). HGF/SF concentrations are presented as scatter units per milliliter; 5 scatter units is equivalent to approximately 1 ng of protein. Purified human NK2, a naturally occurring truncated HGF/SF variant, was a gift from D. Bottaro (National Cancer Institute, Bethesda, Md.).

Transfections. Transfections were performed using a calcium phosphate transfection system according to the manufacturer's protocol (Life Technologies). Cells in 100-mm-diameter dishes were cotransfected with 0.25 μg of a plasmid (pSV2neo) (46) conferring resistance to G418 (Life Technologies) and 19.75 μg of a plasmid carrying the human *hgf/sf* gene (34) or with 1 μg of pSV2neo and 19 μg of carrier DNA. Cells were selected for 2 weeks in 600 μg of G418 per ml and then grown as pools of ~50 clones or as individual clones.

In vivo tumorigenicity and metastasis assays. (i) Tumorigenicity. The tumorigenicity assay was performed as previously described (7). Briefly, cells were washed with DMEM, and 0.1 ml of a cell suspension was injected subcutaneously into the backs of weanling athymic nude mice (BALB/c *nu/nu*). Tumor formation was monitored weekly.

(ii) Spontaneous metastasis assay. The spontaneous metastasis assay was performed as previously described (38). Briefly, animals were sacrificed when they appeared distressed, or 12 weeks after the initial injection of cells if they did not appear distressed, and examined for the presence of lung metastases.

(iii) Experimental metastasis assay. The experimental metastasis assay was performed as previously described (38). Briefly, cells were washed with DMEM, and 0.1 ml of a cell suspension was injected into the tail vein of weanling athymic nude mice (BALB/c *nu/nu*). Animals were sacrificed when they appeared distressed, or after 12 weeks if they did not appear distressed, and examined for the presence of lung metastases.

In vitro invasion assays. (i) Invasion through Matrigel-coated filters. SK-LMS-1 cells (0.2 ml) at a density of 20,000 cells per ml in DMEM-BSA (DMEM containing 1 mg of bovine serum albumin [BSA] per ml) were added to a Matrigel invasion chamber (Becton Dickinson, Bedford, Mass.). The chamber was placed into a well of a 24-well culture plate containing 0.5 ml of DMEM-BSA alone or supplemented with various factors and incubated for 20 or 40 h, as indicated in the legend to Fig. 1 and below. At the end of the incubation period the noninvasive cells, which remain on the top side of the filter, were removed with a cotton swab and the invasive cells, which are attached to the underside of the filter, were stained with Diff-Quik staining solutions (Baxter, McGaw Park, Ill.). The stained cells were then photographed at a magnification of ×17.5 and counted. All cell numbers reported in the text represent the average from six samples for each experimental condition examined and have been normalized to reflect the increase in cell number that occurs during the incubation period because of the mitogenic activity of HGF/SF (as was determined by counting cells grown in parallel on Matrigel-coated dishes).

(ii) Invasion in three-dimensional Matrigel plugs. SK-LMS-1 cells at a density of 50,000 cells per ml in DMEM were mixed with an equal volume of Matrigel (Becton Dickinson), plated at 0.1 ml per well in a 96-well culture plate, and incubated for 20 min at 37°C in 10% CO_2 to facilitate gel (plug) formation. At the end of this incubation period, 0.1 ml of DMEM–10% FBS alone or supplemented with various factors was placed on top of each Matrigel plug, and the plate was returned to the incubator. The medium above each plug was changed after 2 days, and representative cell clusters were photographed at a magnification of ×300 after 4 days.

Western blotting. Western analysis (immunoblotting) was performed according to standard procedures (42). Briefly, supernatants were prepared for Western analysis by centrifugation (5 min; 2,000 × *g*) followed by the addition of an equal volume of 2× sodium dodecyl sulfate (SDS) gel-loading buffer (without dithiothreitol) to the clarified supernatant and subsequent boiling (10 min). Cell extracts were prepared for Western analysis by washing the cell monolayers with Dulbecco's phosphate-buffered saline (Life Technologies), lysing the cells in hot (85°C) 1× SDS gel-loading buffer (with or without dithiothreitol), and then boiling (10 min), shearing (four times with a 25⅝-gauge needle), centrifuging (5 min; 16,000 × *g*), and quantitating (DC protein assay; Bio-Rad, Melville, N.Y.) the extracts. Samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose filters (Schleicher & Schuell, Keene, N.H.), and incubated for 18 h at 4°C with the following primary antibodies at the concentrations listed: (i) anti-human uPA, a rabbit polyclonal antibody, 2 μg/ml (catalog number 389; American Diagnostica, Greenwich, Conn.); (ii) anti-human uPAR, a rabbit polyclonal antibody, 1 μg/ml (catalog number 399R; American Diagnostica); (iii) antiactin, a mouse monoclonal antibody, 1 μg/ml (catalog number 1378996; Boehringer Mannheim, Indianapolis, Ind.); (iv) anti-Met, a rabbit polyclonal antibody, 1 μg/ml (catalog number sc-161; Santa Cruz Biotechnology, Santa Cruz, Calif.), and (v) anti-P-Tyr, a mouse monoclonal antibody (4G10), 5 μg/ml (a gift of Deborah Morrison, ABL-Basic Research Program, Frederick, Md.).

At the end of the incubation period the filters were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:1,000; Boehringer Mannheim) for 1 h at room temperature. The filters were then washed and exposed to the enhanced chemiluminescence detection system (Amersham, Arlington Heights, Ill.).

Immunoprecipitation. (i) Analysis of HGF/SF levels in *hgf/sf*-transfected SK-LMS-1 cells. Cells were grown in DMEM–10% FBS in six-well culture plates at 100,000 cells per well for 3 days, washed with methionine-cysteine-free DMEM (Life Technologies), and incubated for 15 h in methionine-cysteine-free DMEM–5% dialyzed FBS (Life Technologies) supplemented with 300 μCi of Tran^{35}S-Label (ICN, Costa Mesa, Calif.) per ml. At the end of this incubation, 0.5 volume of triple-detergent lysis buffer (42) was added to the supernatants, which were then centrifuged (5 min; 4°C; 16,000 × *g*). Net-gel buffer (0.67 volume) (42) and anti-HGF/SF antibody (goat polyclonal antibody, 20 μg/ml [catalog number AB-294-NA; R&D Systems, Minneapolis, Minn.]) were then added to the clarified supernatants, which were incubated for 18 h at 4°C. At the end of this incubation period, 50 μl of protein G-agarose (Life Technologies) was added to the samples, which were incubated with rotation for an additional 1 h at 4°C. The samples were then washed three times with a cold 1:1 mixture of triple-detergent lysis buffer and net-gel buffer and one time with cold 10 mM Tris (pH 7.5)–0.1% Nonidet P-40. Fifty microliters of 1× SDS gel-loading buffer (with dithiothreitol) was then added to each sample. After boiling (5 min) and centrifugation (5 min; 16,000 × *g*), the supernatants were resolved by SDS-PAGE. The gels were treated with Amplify (Amersham), dried, and exposed to film.

(ii) Phosphotyrosine analysis of the Met receptor. SK-LMS-1 cells were grown in DMEM–10% FBS in 100-mm-diameter culture dishes at 300,000 cells per dish for 30 h and then washed and fed with DMEM-BSA. After an additional 16 h of incubation, the cells were fed with fresh DMEM-BSA alone or supplemented with various factors. The cells were incubated for 10 min, washed with cold TBS (25 mM Tris [pH 7.5], 150 mM NaCl, 1 mM sodium orthovanadate), and incubated for 15 min on ice with lysis buffer (1 ml per dish; 25 mM Tris [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1% SDS, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 0.1 mg of phenylmethylsulfonyl fluoride per ml, 2 μg of aprotinin per ml). The lysates were centrifuged (5 min; 4°C; 16,000 × *g*), and anti-Met antibody (rabbit polyclonal antibody, 1.6 μg/ml [catalog number sc-161; Santa Cruz Biotechnology]) was then added to the clarified supernatants, which were subsequently incubated for 18 h at 4°C. At the end of this incubation period, 50 μl of protein G-agarose (Life Technologies) was added to the samples, which were incubated with rotation for an additional 1 h at 4°C. The samples were washed three times with cold lysis buffer and one time with cold TBS. Fifty microliters of 1× SDS gel-loading buffer (with dithiothreitol) was added to each sample. After boiling (5 min) and centrifugation (5 min; 16,000 × *g*), the supernatants were resolved by SDS-PAGE and subjected to Western analysis.

Plasminogen activation assay. SK-LMS-1 cells were grown in DMEM–10% FBS in six-well plates at 30,000 cells per well for 20 h. At the end of this incubation period, the cells were fed with fresh DMEM–10% FBS alone or supplemented with 200 scatter units of HGF/SF per ml and incubated for an additional 32 h. After this incubation the cells were washed with DMEM-BSA and treated with an acidic buffer (two times for 5 min each; 50 mM glycine [pH 3.0], 100 mM NaCl) to remove cell-associated uPA (49). After further washing with DMEM-BSA, the cells were fed with fresh DMEM-BSA alone or supplemented with 200 scatter units of HGF/SF per ml and incubated for an additional 16 h. At the end of this incubation period, the cells were washed with DMEM-BSA and fed with fresh DMEM-BSA supplemented with 30 ng of ^{125}I-human plasminogen per ml. The plasminogen (American Diagnostica) was iodinated to a specific activity of 41 μCi/μg by the chloramine T method (2, 19). After an additional 8 h of incubation, the supernatants and cell extracts were harvested as described above under the Western blotting protocol, using SDS gel-loading buffer containing dithiothreitol. The samples were resolved by SDS-PAGE, and the gels were dried and exposed to film. Where indicated, 0.2 mM amiloride (Sigma, St. Louis, Mo.), a uPA-neutralizing chemical (55), was added during the final 8-h incubation, and a uPA-neutralizing antibody (mouse monoclonal antibody [catalog number 394; American Diagnostica]) was added during the final 16- and 8-h incubations.

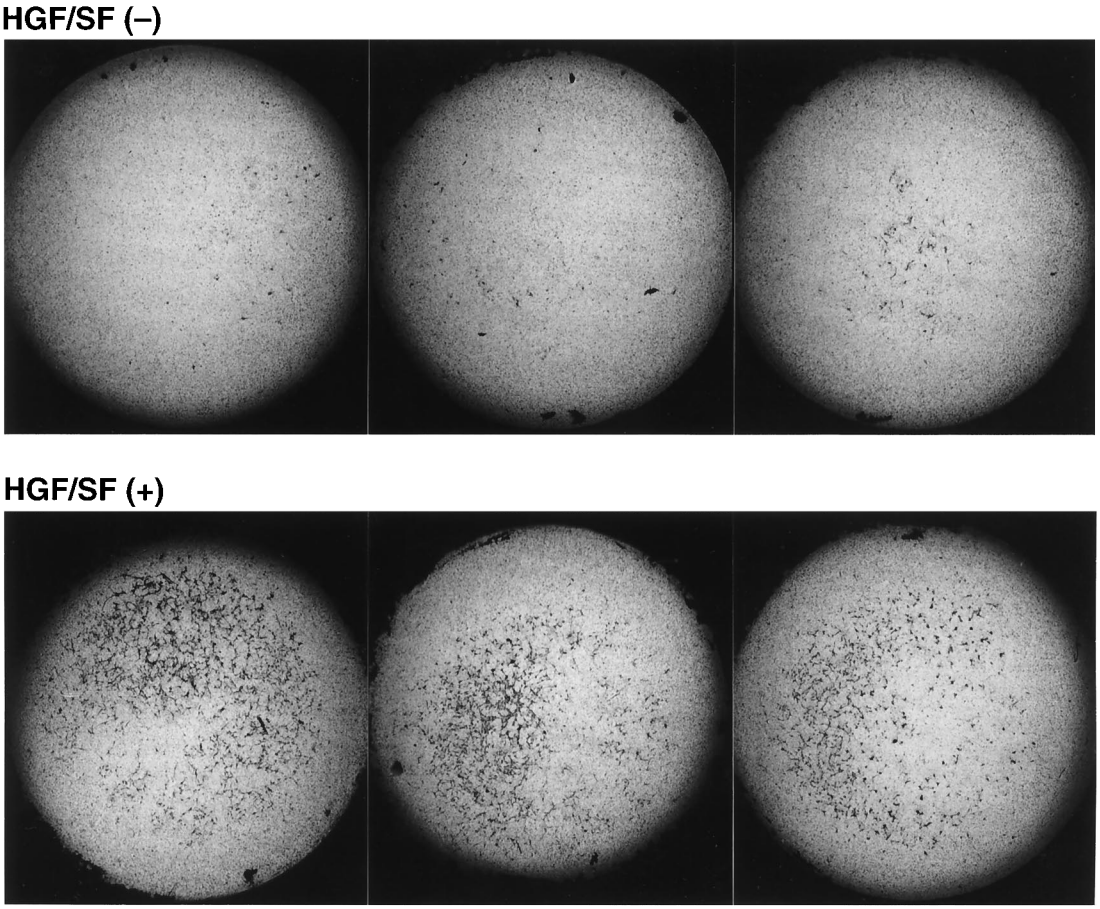

FIG. 1. Effect of HGF/SF on the in vitro invasiveness of SK-LMS-1 cells: invasion through Matrigel-coated filters. Cells were placed in a chamber containing a Matrigel-coated filter at its base. The chambers were immersed in DMEM-BSA alone (−) or supplemented with 200 scatter units of HGF/SF per ml (+) as indicated. After a 40 h incubation, the noninvasive cells, which remain on the top side of the filter, were removed and the invasive cells, which attach themselves to the underside of the filter, were stained. Three replicate filters are shown for each experimental condition. The filters were photographed at a low magnification (×17.5) to permit the visualization of the entire surface of each filter.

Plasmin binding assay. SK-LMS-1 cells were grown in the absence of HGF/SF as previously described for the plasminogen cleavage assay. At the end of the 16-h incubation, the cells were washed with DMEM-BSA and incubated for 2 h with DMEM-BSA alone or supplemented with 5 μg of human plasmin (American Diagnostica) per ml. At the end of this incubation period, the cells were washed with cold binding buffer (DMEM-BSA containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5]) and fed with binding buffer supplemented with 100 ng of ^{125}I-human plasmin or ^{125}I-human HGF/SF per ml in the presence or absence of 1 mg of ε-amino-*n*-caproic-acid (ε-ACA; Sigma) per ml. The plasmin (American Diagnostica) was iodinated to a specific activity of 17 μCi/μg, while the HGF/SF was iodinated to a specific activity of 10 μCi/μg, by the chloramine T method (2, 19). After a 3-h incubation at 4°C, the cells were washed with binding buffer; solubilized with a solution composed of 2% SDS, 2 mM EDTA, and 10 mM $NaHCO_3$, and quantitated by liquid scintillation counting.

RESULTS

Induction of the in vitro invasive phenotype in human sarcoma cells via HGF/SF-Met signalling. The SK-LMS-1 cell line is derived from a human leiomyosarcoma (smooth-muscle tumor). We have previously shown that these cells express a high level of the Met receptor but only a small quantity of its ligand, HGF/SF, and respond mitogenically to exogenous HGF/SF (36). To examine whether HGF/SF-Met signalling could induce the invasive phenotype in these cells, we performed an in vitro invasion assay that assesses a cell's ability to migrate through a BM (Matrigel)-coated filter in response to a given stimulus, in this case HGF/SF. When the cells were allowed 20 h to invade the filter, we found an average of 4 (±1) invasive cells in the absence of HGF/SF and 158 (±24) invasive cells in the presence of HGF/SF. Thus, HGF/SF induced a 40-fold increase in invasiveness. When the cells were allowed 40 h to invade the filter, we again found only a small number of invasive cells in the absence of HGF/SF (Fig. 1, top row) but a large number of invasive cells (too numerous to count) in the presence of this factor (Fig. 1, bottom row).

We also cultured the SK-LMS-1 cells in three-dimensional Matrigel plugs and found that while cells grown in the absence of HGF/SF form compact cyst-like structures (Fig. 2, top row), those grown in the presence of HGF/SF exhibit extensive branching and invasion into the matrix (Fig. 2, middle row). Similar results were obtained with HGF/SF at 60, 200, and 600 scatter units/ml. Thus, our findings clearly indicate that HGF/SF-Met signalling induces the in vitro invasive phenotype in this human sarcoma cell line.

HGF/SF signalling enhances the in vivo tumorigenicity and metastatic capability of human sarcoma cells. We next explored the possibility that HGF/SF-Met signalling affects the in vivo tumorigenicity and metastatic potential of these cells. Since SK-LMS-1 cells express little or no HGF/SF (36) and since human Met-expressing cells respond more efficiently to human HGF/SF than to murine HGF/SF (4, 34), we wanted to

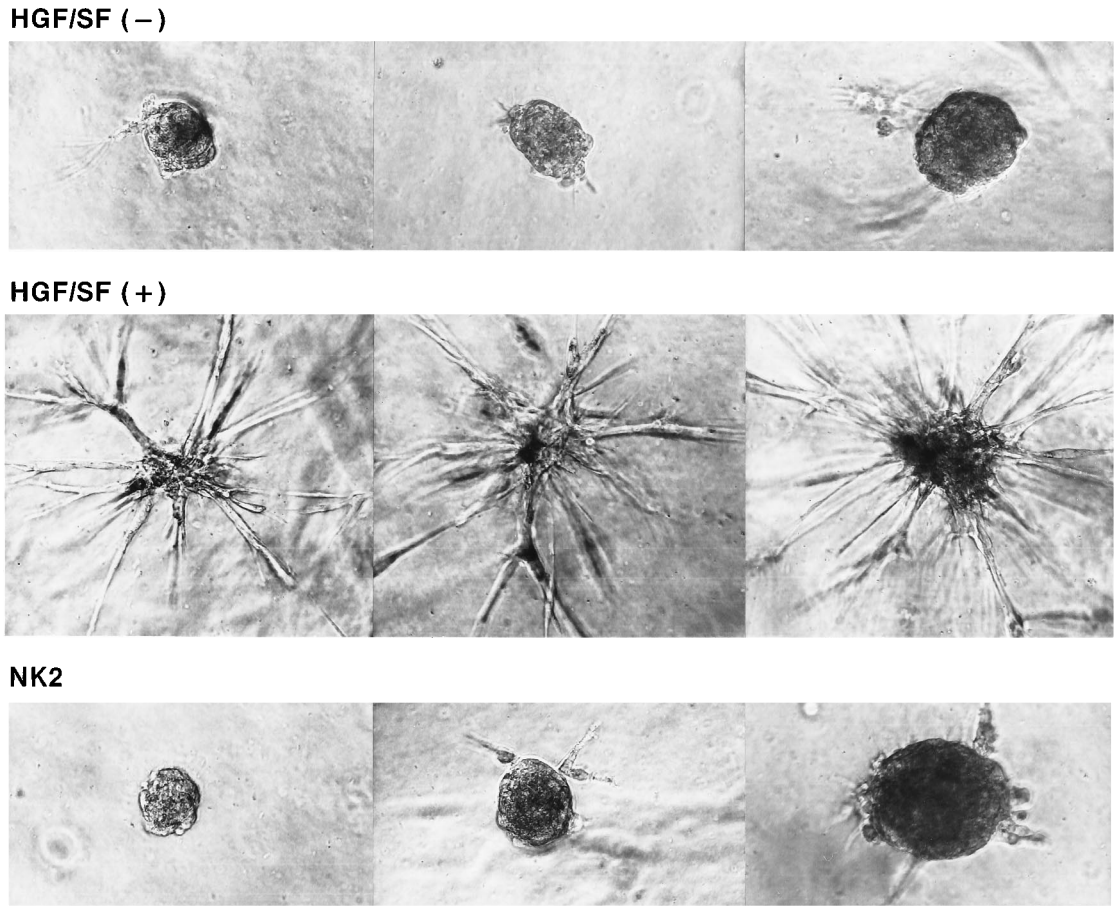

FIG. 2. Effect of HGF/SF on the in vitro invasiveness of SK-LMS-1 cells: invasion in three-dimensional Matrigel plugs. Cells were suspended in a Matrigel solution and transferred to tissue culture plates. After a brief incubation at 37°C (which causes the Matrigel solution to gel, thereby enclosing the cells in a three-dimensional matrix), the cells were fed with DMEM–10% FBS alone (−) or supplemented with 200 scatter units of HGF/SF per ml (+) or 100 ng of NK2 per ml as indicated. Following a 4-day incubation, three representative cell clusters for each experimental condition were photographed at a magnification of ×300.

provide the SK-LMS-1 cells with an autocrine source of human HGF/SF. To this end, SK-LMS-1 cells were cotransfected with a plasmid carrying the human *hgf/sf* gene, together with a plasmid conferring resistance to G418. After selection in G418, cells expressing elevated levels of HGF/SF were obtained (Fig. 3), and the ability of these cells to generate tumors and metastases in athymic nude mice was assessed (Tables 1 and 2). Our findings indicate that the generation of an HGF/SF-Met autocrine loop in the SK-LMS-1 cells significantly enhances their tumorigenicity, since tumors formed with a two- to threefold higher frequency and a twofold shorter latency in mice injected with HGF/SF-expressing SK-LMS-1 cells relative to those in mice injected with control ($G418^R$) SK-LMS-1 cells (Table 1). In addition, while control ($G418^R$) SK-LMS-1 cells are nonmetastatic, SK-LMS-1 cells expressing elevated levels of HGF/SF are highly metastatic in both spontaneous and experimental metastasis assays (Table 2).

Induction of the urokinase proteolysis network in human sarcoma cells via HGF/SF-Met signalling. Since the invasive-metastatic phenotype of the SK-LMS-1 cells can be tightly regulated by the addition of exogenous HGF/SF, we employed these cells to examine the downstream effectors of this phenotype, focusing initially on the uPA proteolysis network. We began by investigating whether HGF/SF affects the expression of the uPAR. Figure 4A shows an anti-uPAR Western blot of lysates from SK-LMS-1 cells that were treated for 24 h with various concentrations of HGF/SF in the presence of serum. As little as 4 scatter units of HGF/SF per ml induced the expression of uPAR in these cells, with maximal stimulation occurring at ~64 scatter units/ml. This up-regulation of uPAR by HGF/SF is more pronounced in the presence of serum than in its absence (data not shown). We also examined this induction with respect to the length of time of HGF/SF treatment and found that HGF/SF causes a rapid and sustained increase in uPAR protein levels in these cells, with an increase evident by 8 h, peaking by 24 h, and remaining elevated for at least 48 h after HGF/SF addition (Fig. 4B). Reprobing of the filters shown in Fig. 4 with an antiactin antibody demonstrated that equal amounts of protein were loaded and transferred for all samples. As an independent verification that HGF/SF-Met signalling increases the expression of the uPAR in these cells, we found that HGF/SF-treated cells bound significantly more exogenously added ^{125}I-uPA than untreated control cells (data not shown).

We also examined the effect of HGF/SF treatment on the expression of uPA in the SK-LMS-1 cells. Cell lysates and supernatants from cells that had been exposed to HGF/SF for 48 h were analyzed by Western blotting using an anti-uPA antibody (Fig. 5A). These results indicate that HGF/SF induces a significant increase in the level of both cell-associated

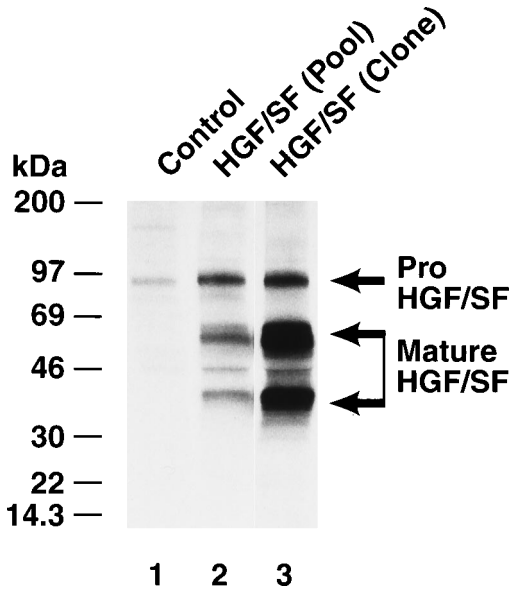

FIG. 3. HGF/SF expression in control and *hgf/sf*-transfected SK-LMS-1 cells. Lysates from cells that had been metabolically labeled with [^{35}S]methionine-cysteine were immunoprecipitated with an anti-HGF/SF antibody, resolved by SDS-PAGE on a 4 to 15% polyacrylamide gel under reducing conditions, and visualized by autoradiography. Lysates from cells that had been cotransfected with a plasmid carrying the human *hgf/sf* gene together with a plasmid conferring resistance to G418 are presented in lanes 2 and 3 (a pool of such cells is presented in lane 2, and an individual clone of such cells is shown in lane 3); a lysate from a pool of control cells that had been transfected only with the plasmid conferring resistance to G418 is presented in lane 1. Since the metabolic labeling of each sample was performed in the presence of serum, most of the HGF/SF detected is in the mature, heterodimeric form.

(compare lanes 1 and 2) and soluble (compare lanes 5 and 6) uPA when these cells are stimulated for 48 h in the presence of serum.

We noticed that in addition to uPA, a protein of ~100 kDa which is recognized by the anti-uPA antibody is also increased by HGF/SF treatment (Fig. 5A; compare lane 1 with lane 2 and lane 5 with lane 6). This protein apparently represents a complex which forms between uPA and a serum-derived protein, since it is no longer present when the cells are incubated in the absence of serum (Fig. 5A, lanes 3, 4, 7, and 8). A likely candidate for this serum-derived uPA-binding protein is the type 1 plasminogen activator inhibitor, an ~50-kDa molecule that has been shown to form a covalent, SDS-stable complex with uPA (1).

When the level of cell-associated uPA is examined with respect to the length of time of HGF/SF treatment, an increase is evident by 8 h, peaks by 24 h, and remains elevated for at least 48 h after HGF/SF addition (Fig. 5B), thus paralleling our previous observations on the kinetics of the increase of the

TABLE 1. In vivo tumorigenicity of control (G418^R) and *hgf/sf*-transfected SK-LMS-1 cells

Cells injected	No. of mice with tumors/no. injected (%)	Mean latency ± SD (wk)
Control (G418^R)	5/14 (36)	9.2 ± 0.45
HGF/SF (pool)	9/14 (64)	4.7 ± 1.9
HGF/SF (clone)	17/19 (89)	4.2 ± 1.4

[a] Cells (10^6) were injected subcutaneously into the backs of athymic nude mice.

TABLE 2. In vivo metastasis of control (G418^R) and *hgf/sf*-transfected SK-LMS-1 cells

Metastasis type	Cells injected	No. of mice with metastases/no. with tumors (%)
Spontaneous[a]	Control (G418^R)	0/5 (0)
	HGF/SF (pool)	2/9 (22)
	HGF/SF (clone)	8/17 (47)
Experimental[b]	Control (G418^R)	0/10 (0)
	HGF/SF (pool)	2/12 (17)
	HGF/SF (clone)	6/13 (46)

[a] Athymic nude mice with subcutaneous tumors were examined for lung metastases.

[b] Cells (10^6) were injected intravenously into athymic nude mice, which were subsequently examined for lung metastases.

uPAR by HGF/SF (Fig. 4B). We also find that HGF/SF treatment increases the amount of uPA that is released from cells following a brief exposure to an acidic buffer that dislodges uPA from its cellular receptor (49), thus demonstrating that HGF/SF stimulation increases the amount of endogenous uPA bound to the surface of the SK-LMS-1 cells (data not shown).

Since HGF/SF treatment induces both uPA and the uPAR in SK-LMS-1 cells, HGF/SF-treated cells may possess greater uPA activity than untreated cells. To investigate this possibility, we compared the ability of HGF/SF-treated and untreated control cells to cleave the uPA substrate plasminogen to plasmin (Fig. 6). Plasminogen is an inactive monomeric protease of 791 amino acids which is converted by uPA to its active, heterodimeric form by the cleavage of a single peptide bond, between residues 561 and 562 (33). Control cells and cells that had been stimulated with HGF/SF for 48 h were incubated with ^{125}I-plasminogen substrate for 8 h, after which time the cell pellets (lanes 1 to 4) and supernatants (lanes 5 to 8) were harvested and analyzed by SDS-PAGE and autoradiography. These results demonstrate that HGF/SF-treated cells possess a significantly enhanced plasminogen-cleaving and plasmin-forming ability compared with that of untreated control cells (compare lanes 1 and 2) and that the plasmin formed is associated with the cell pellet, which contains cells and ECM components (compare lanes 1 and 5). The HGF/SF-induced enhancement in plasminogen-cleaving and plasmin-forming ability is abolished by simultaneous treatment with either the uPA-neutralizing chemical amiloride (lane 3) (55) or a uPA-neutralizing antibody (lane 4), thus demonstrating that it is uPA which is responsible for the enhanced plasminogen-cleaving and plasmin-forming ability of HGF/SF-treated SK-LMS-1 cells relative to that of untreated control cells.

As was previously noted, the plasmin that is generated in response to HGF/SF treatment remains associated with the cells and ECM (Fig. 6). The association of plasmin with the cell surface is believed to be physiologically relevant since soluble plasmin is rapidly inactivated by its principal inhibitor (α2-plasmin inhibitor), whereas surface-associated plasmin is protected from inhibition, and because the localization of this powerful protease at the cell surface may enhance the cell's invasiveness (8, 14, 41). We therefore investigated the role that HGF/SF might play in enhancing the binding of plasmin to cells. We found that while HGF/SF treatment increased the generation of plasmin from plasminogen (Fig. 6), it did not directly enhance the plasmin-binding ability of the SK-LMS-1 cells, since both HGF/SF-treated and untreated control cells bound similar amounts of ^{125}I-plasmin (data not shown). We then considered the possibility that plasmin enhances its own

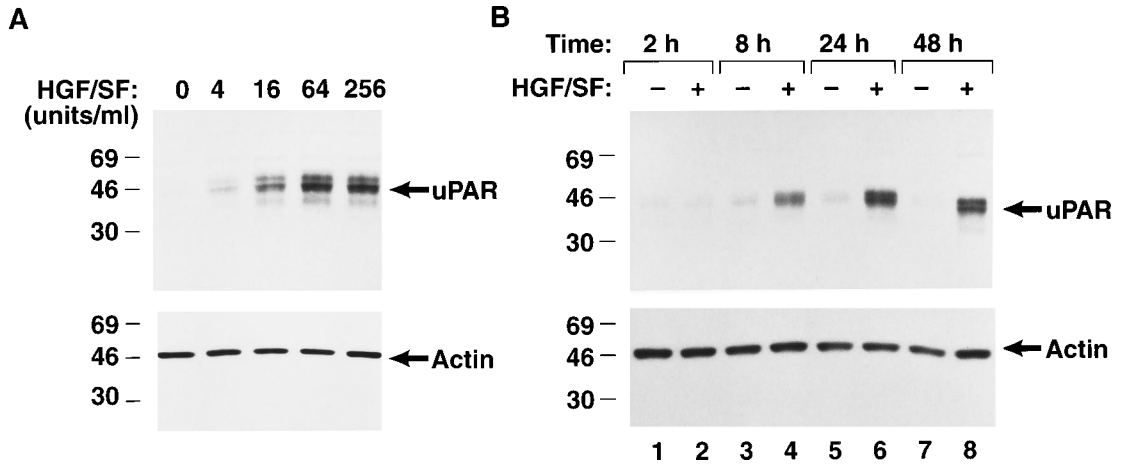

FIG. 4. Effect of HGF/SF on the expression of the uPAR in SK-LMS-1 cells. (A) Cells were grown in DMEM–10% FBS in six-well culture dishes at 60,000 cells per well for 20 h. At the end of this incubation period, fresh DMEM–10% FBS alone or supplemented with various concentrations of HGF/SF (as indicated above each lane) was added to each well, and the cells were incubated for an additional 24 h and then processed for Western analysis under nonreducing conditions. The samples were resolved by SDS-PAGE on a 4 to 15% polyacrylamide gel and blotted with an anti-uPAR antibody (top panel). The filter was then stripped and reprobed with an antiactin antibody (bottom panel). (B) Cells were grown in DMEM–10% FBS in six-well culture dishes at 75,000 cells per well for 20 h. At the end of this incubation period, fresh DMEM–10% FBS alone (−) or supplemented with 200 scatter units of HGF/SF per ml (+) (as indicated above each lane) was added to each well, and the cells were incubated for the times indicated above each lane and then processed for Western analysis under nonreducing conditions. The samples were resolved by SDS-PAGE on a 4 to 15% polyacrylamide gel and blotted with an anti-uPAR antibody (top panel). The filter was then stripped and reprobed with an antiactin antibody (bottom panel).

binding to the SK-LMS-1 cells and ECM. To this end, we examined the effect that pretreating cells with cold plasmin had on their ability to subsequently bind ^{125}I-plasmin. This analysis showed a significant enhancement (four- to fivefold) of ^{125}I-plasmin binding to cells that had been pretreated with cold plasmin relative to that to untreated control cells (Fig. 7). Furthermore, this binding is strongly inhibited by the lysine analog ε-ACA, indicating that the lysine binding site(s) in plasmin mediates its binding to the cells and ECM. Thus, plasmin plays an active role in enhancing its own binding to human sarcoma cells. In contrast to the results obtained with ^{125}I-plasmin, the binding of ^{125}I-HGF/SF to the SK-LMS-1 cells and ECM is neither significantly enhanced by pretreatment with cold plasmin nor inhibited by ε-ACA (Fig. 7).

Lack of induction of the invasive phenotype and the urokinase proteolysis network in human sarcoma cells by the naturally occurring truncated HGF/SF variant NK2. Mature HGF/SF is an ~90-kDa heterodimeric protein possessing four "kringle" domains. A naturally occurring ~30-kDa monomeric variant (NK2) contains only two of the four kringle domains (11). NK2 binds to the Met receptor and can mediate some, but not all, of the biological activities of HGF/SF (11, 21). For example, while both NK2 and HGF/SF can induce the scattering of epithelial cells, only HGF/SF is mitogenic. Thus, we

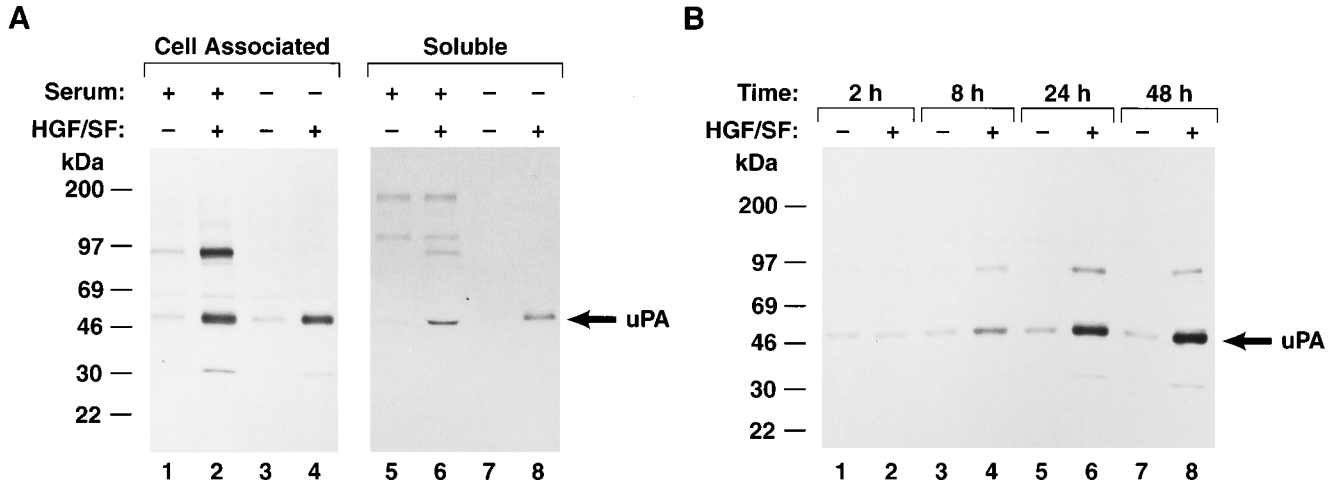

FIG. 5. Effect of HGF/SF on uPA expression in SK-LMS-1 cells. (A) Cells were grown in DMEM–10% FBS in six-well culture dishes at 30,000 cells per well for 20 h. At the end of this incubation period, fresh DMEM–10% FBS alone (−) or supplemented with 200 scatter units of HGF/SF per ml (+) (as indicated above each lane) was added to each well, and the cells were incubated for an additional 48 h, after which time the cells (lanes 1 to 4) and supernatants (lanes 5 to 8) were processed for Western analysis under nonreducing conditions. The samples were then resolved by SDS-PAGE on a 4 to 15% polyacrylamide gel and blotted with an anti-uPA antibody. For some samples (lanes 3, 4, 7, and 8), the 48-h incubation was interrupted at 32 h, at which time the cells were washed with DMEM, fed with fresh DMEM-BSA alone or supplemented with 200 scatter units of HGF/SF per ml, and then incubated for an additional 16 h. (B) The filter presented in Fig. 4B was stripped and reprobed with an anti-uPA antibody.

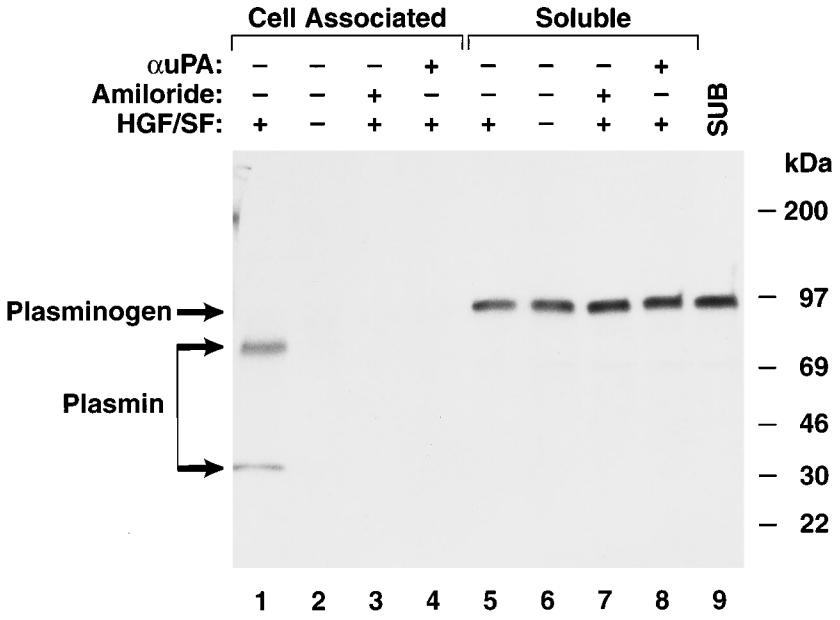

FIG. 6. Effect of HGF/SF on the plasmin-generating ability of SK-LMS-1 cells. Cells were cultured for 32 h in DMEM–10% FBS alone (−) or supplemented with 200 scatter units of HGF/SF per ml (+) (as indicated above each lane), briefly washed with acidic buffer to remove cell-associated uPA, and then cultured for an additional 16 h in DMEM-BSA alone or supplemented with 200 scatter units of HGF/SF per ml. The cells were then incubated for 8 h in fresh DMEM-BSA supplemented with 30 ng of ^{125}I-plasminogen per ml. After this time, cell extracts (lanes 1 to 4) and supernatants (lanes 5 to 8) were harvested, resolved by SDS-PAGE on a 4 to 15% polyacrylamide gel under reducing conditions, and visualized by autoradiography. Where indicated, amiloride (0.2 mM) was added during the final 8-h incubation and a uPA-neutralizing antibody (αuPA; 50 μg/ml) was added during both the 16- and 8-h incubations. The ^{125}I-plasminogen substrate is presented in lane 9.

were interested in determining if NK2 could induce the expression of uPA and its receptor in human sarcoma cells.

In Fig. 8A, lysates from cells that had been stimulated for 10 min with medium alone (lane 5) or medium supplemented with NK2 (lanes 1 to 4) or HGF/SF (lane 6) were immunoprecipitated with an anti-Met antibody, resolved by SDS-PAGE, and subjected to Western analysis using an antiphosphotyrosine antibody (top panel). These results indicate that both NK2 and HGF/SF are able to induce the phosphorylation of the Met receptor, presumably by stimulating its intrinsic tyrosine kinase activity. The filter was stripped and reprobed with an anti-Met antibody (lower panel) to demonstrate that equal quantities of protein were immunoprecipitated, loaded, and transferred for all samples.

The ability of NK2 to induce the expression of uPA (Fig. 8C, lanes 1 to 4 and 7 to 10) and its receptor (Fig. 8B, lanes 1 to 4) was then assessed via Western analysis. The results demonstrate that NK2 stimulation does not increase the level of either protein under conditions in which HGF/SF stimulation (Fig. 8B lane 6, and C, lanes 6 and 12) clearly does.

We next examined the ability of NK2 to induce the invasive phenotype in the SK-LMS-1 cells. In a 20-h in vitro invasion assay, NK2 induced 34 (±16) and 22 (±16) cells to migrate through a Matrigel-coated filter when used at 225 and 45 ng/ml, respectively. While these numbers are higher than the number of cells that spontaneously invaded in the absence of any stimulus (7.3 [±4.1] cells), they are substantially lower than the invasion induced (300 [±107] cells) by 200 scatter units (equivalent to ~40 ng/ml) of HGF/SF per ml. We also investigated whether NK2 could induce SK-LMS-1 cells grown in three-dimensional Matrigel plugs to form the branching and invasive structures that are characteristic of HGF/SF stimulation and found that it could not, even when examined over a wide range of concentrations (33, 100, 300, and 900 ng/ml; Fig. 2, bottom row). Taken together, these results demonstrate that NK2 exhibits a much reduced capacity to stimulate the invasion of human sarcoma cells, relative to that of HGF/SF.

DISCUSSION

We have demonstrated that HGF/SF-Met signalling enhances the tumorigenicity, invasiveness, and metastatic capability of human cells. Our in vitro results show that the ability of SK-LMS-1 leiomyosarcoma cells (which express Met but little or no HGF/SF) to invade through a BM (Matrigel)-coated filter or to form branching and invasive structures in three-dimensional Matrigel plugs is significantly enhanced by HGF/SF treatment. Our in vivo findings demonstrate that control SK-LMS-1 cells, which are weakly tumorigenic and nonmetastatic in nude mice, become highly tumorigenic and metastatic when they are engineered to produce their own supply of HGF/SF. While HGF/SF-Met signalling has previously been shown to enhance the in vivo tumorigenicity (3, 34) and metastatic capability (38, 39) of rodent cells, this is the first demonstration that human cells are affected in a similar manner by this signalling pathway.

We also showed that one of the molecular changes that occur in response to HGF/SF-Met signalling in the SK-LMS-1 cells is the activation of the uPA proteolysis network. It is likely that the activation of this network contributes to the generation of the invasive-metastatic phenotype by HGF/SF-Met signalling in these cells, since a strong correlation between the acquisition of the invasive-metastatic phenotype and the activation of this proteolysis network has been demonstrated in other systems (8, 14). This correlation is believed to be due primarily to the establishment of a proteolytically active cell surface, which facilitates invasion by mediating the degradation of ECM/BM components. One of the cell surface-associ-

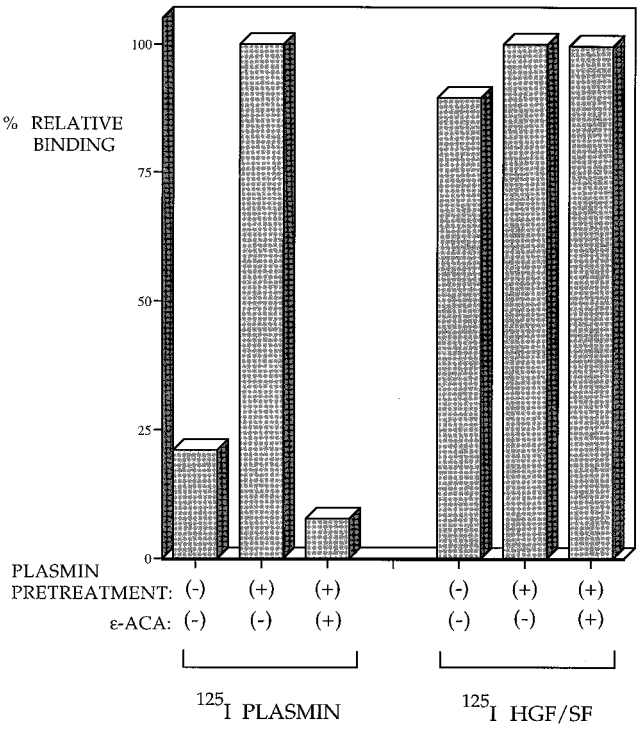

FIG. 7. Effect of plasmin pretreatment on the binding of ^{125}I-plasmin to SK-LMS-1 cells. Cells were cultured in DMEM–10% FBS, briefly washed with an acidic buffer, and cultured for an additional 16 h in DMEM-BSA. The cells were pretreated for 2 h at 37°C with DMEM-BSA alone (−) or supplemented with 5 μg of plasmin per ml (+), washed, and incubated for 3 h at 4°C in DMEM-BSA supplemented with 100 ng of ^{125}I-plasmin or ^{125}I-HGF/SF per ml (as indicated). After this time the cells were washed and solubilized, and bound radioactivity was quantitated in a liquid scintillation counter. Where indicated, the binding assay was performed in the presence of 1 mg of ε-ACA per ml. Each sample represents an average of two replicates and is illustrated as a percentage of the maximum binding (i.e., the binding which occurs on plasmin-pretreated cells in the absence of ε-ACA).

ated proteases that is able to directly participate in ECM/BM degradation is uPA itself, but the more potent cell surface-associated protease is plasmin, which is generated from plasminogen in a reaction that may be catalyzed by uPA. We find that HGF/SF-Met signalling significantly increases the protein levels of both uPA and its cellular receptor in sarcoma cells, resulting in elevated cell-associated uPA protein levels and enhanced plasmin-generating capability. We also find that the plasmin generated by HGF/SF-treatment remains associated with the cells and ECM. Thus, HGF/SF-Met signalling increases the level of cell-associated proteases (uPA and plasmin) displayed on sarcoma cells, with the expected effect of enhancing the ECM/BM-degrading capability, invasiveness, and metastatic potential of these cells. Although HGF/SF has previously been shown to enhance uPA activity in several cell types (18, 32, 39) and to up-regulate uPA and its receptor at the RNA level in Madin-Darby canine kidney (MDCK) epithelial cells (32), this is the first investigation that directly demonstrates the HGF/SF-mediated induction of uPA and its receptor at the protein level and the first study to show that HGF/SF up-regulates the uPA proteolysis network in metastatic human tumor cells.

Our finding that the plasmin generated by incubating HGF/SF-treated SK-LMS-1 cells in the presence of plasminogen remains associated with the cells and ECM prompted us to investigate the nature of this interaction. Our data indicate that plasmin plays an active role in mediating its own binding to the cells and ECM, since we detected a significant increase (four- to fivefold) in the binding of ^{125}I-plasmin to cell and ECM components in samples that had been pretreated with cold plasmin. This finding can be explained by the fact that plasmin cleaves its substrates on the C-terminal side of lysine and arginine residues and thus in some cases generates a C-terminal lysine on the newly cleaved substrate molecule; this lysine can in turn be recognized and bound by plasmin, which possesses an intrinsic ability to bind to proteins via their exposed C-terminal lysine residues. Once bound, plasmin is protected from inactivation by its principal inhibitor, α2-plasmin inhibitor (8, 14, 41). In this fashion, molecules which have been cleaved by plasmin and possess a C-terminal lysine residue may provide a scaffolding which localizes and concentrates plasmin at distinct locations while at the same time protecting it from inactivation. In our system, a likely scenario is that HGF/SF treatment enhances the plasmin-generating capability of the SK-LMS-1 cells by up-regulating uPA and its receptor. The plasmin thus formed would cleave substrate molecules residing at the cell surface and in the ECM, in some cases exposing a C-terminal lysine residue on the newly cleaved substrate. The C-terminal lysine residue would in turn provide a docking site for the plasmin molecule, thereby protecting it from inactivation and localizing it to distinct locations. The expected biological consequence of the localization of plasmin to the cell surface would be increased invasiveness. The enhanced binding of plasmin to previously cleaved substrates has been described by others and has been proposed to play a role in fibrinolysis (33) and in the binding of plasmin(ogen) to endothelial cells (10, 20). Our finding that the binding of plasmin to SK-LMS-1 cells and ECM is inhibited by the lysine analog ε-ACA is consistent with the results obtained in these other systems and indicates that the lysine binding sites in plasmin mediate its binding to SK-LMS-1 cells and ECM.

We also investigated whether the naturally occurring truncated HGF/SF variant (NK2), which has previously been shown to scatter epithelial cells (21) and to be devoid of mitogenic activity (11, 21), could induce the invasive phenotype and the uPA proteolysis network in SK-LMS-1 cells. Our finding that it could not is consistent with the view that these two activities are integrally related. The reason that NK2 is unable to induce the invasive phenotype and the uPA proteolysis network (this study) or mitogenesis (11, 21) under conditions in which HGF/SF clearly does so is unknown but is apparently not NK2's inability to engage and activate the Met receptor, since we have demonstrated that Met becomes phosphorylated in response to NK2 stimulation of SK-LMS-1 cells. It is possible that HGF/SF and NK2 induce qualitatively different autophosphorylation patterns on the cytoplasmic tail of the Met receptor, which differentially influences their ability to interact with secondary signalling molecules.

In addition to uPA and plasmin(ogen) and their respective receptors, other molecules that participate in the regulation of the uPA proteolysis network include the naturally occurring inhibitors of uPA (type 1 and 2 plasminogen activator inhibitors; protease nexin I) and plasmin (α2-antiplasmin; α2-macroglobulin) (8, 14, 41). In fact, by down-regulating the levels of plasmin produced, these inhibitors may actually enhance invasion, since uncontrolled ECM degradation may decrease a cell's invasiveness by interrupting its ability to interact with the ECM (53). We have been unable to detect the production of type 1 plasminogen activator inhibitor by SK-LMS-1 cells (data not shown) and have not yet investigated the potential production of the other inhibitors by these cells.

HGF/SF-Met signalling is not unique in its ability to regulate

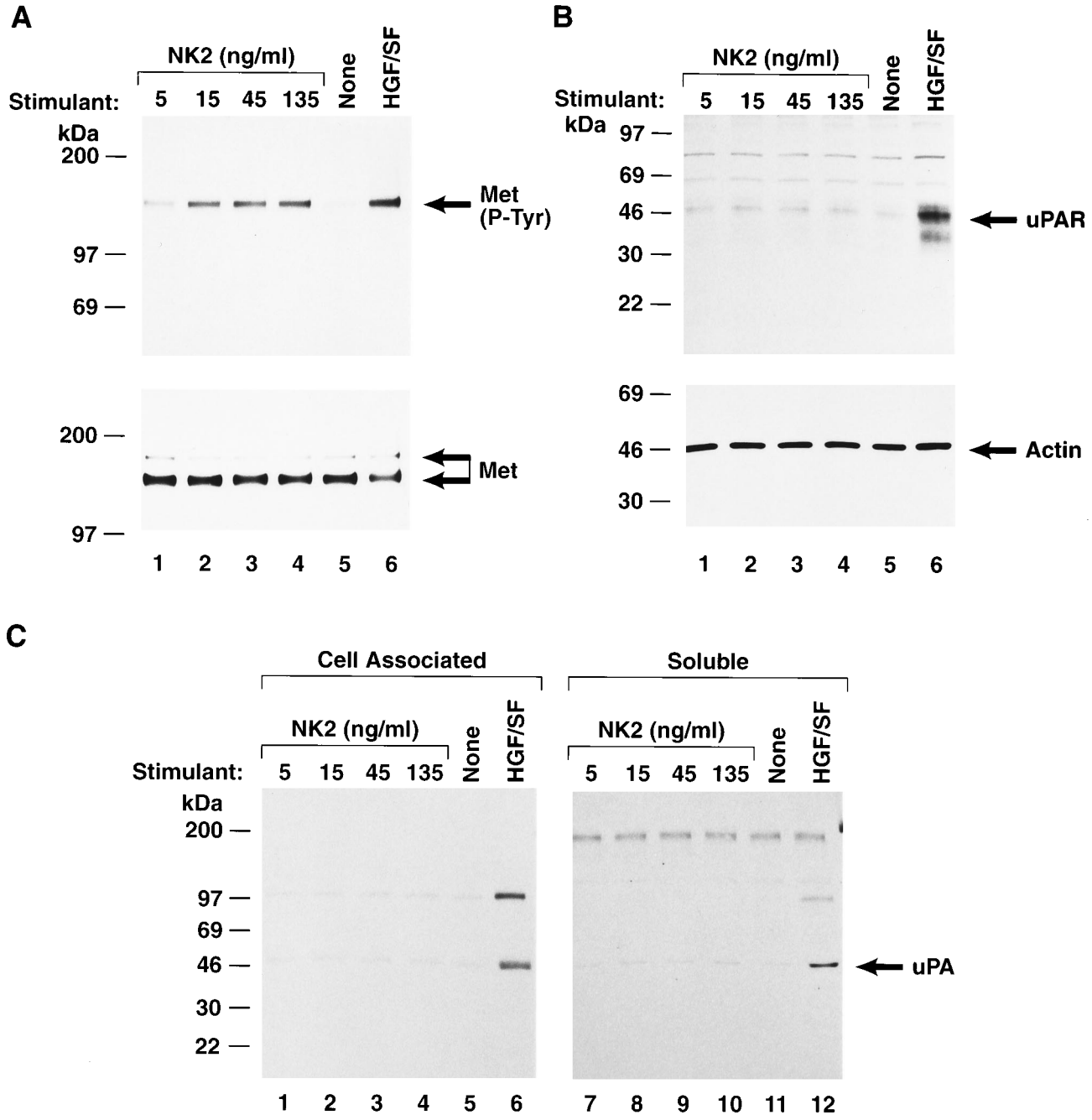

FIG. 8. The effect of NK2 on Met activation and uPA and uPAR expression in SK-LMS-1 cells. (A) Cells were cultured in DMEM–10% FBS, washed, and then incubated for an additional 16 h in DMEM-BSA. The cells were then stimulated for 10 min at 37°C with DMEM-BSA alone or supplemented with 200 scatter units of HGF/SF per ml or various concentrations of NK2 (as indicated above each lane). After this time cell lysates were prepared and immunoprecipitated with an anti-Met antibody, resolved by SDS-PAGE on a 7.5% polyacrylamide gel under reducing conditions, and analyzed by Western blotting using an antiphosphotyrosine antibody (top panel). The blot was then stripped and reprobed with an anti-Met antibody (bottom panel). (B and C) Cells were grown in DMEM-10% FBS in six-well culture dishes at 30,000 cells per well for 20 h. At the end of this incubation period, fresh DMEM–10% FBS alone or supplemented with 200 scatter units of HGF/SF per ml or various concentrations of NK2 (as indicated above each lane) was added to each well, and the cells were incubated for an additional 32 h. At this time the cells were washed with DMEM, fed with fresh DMEM–10% FBS alone or supplemented with 200 scatter units of HGF/SF per ml or various concentrations of NK2, and incubated for an additional 16 h. The cells and supernatants were then processed for Western analysis under nonreducing conditions and resolved by SDS-PAGE on a 4 to 15% polyacrylamide gel. (B) Cell extracts were blotted with an anti-uPAR antibody (top panel). The filter was then stripped and reprobed with an antiactin antibody (bottom panel). (C) Cell extracts (lanes 1 to 6) and supernatants (lanes 7 to 12) were blotted with an anti-uPA antibody.

the expression of genes related to the uPA proteolysis network, since other molecules, including phorbol esters, growth factors, hormones, and retinoids, have also been shown to either increase or decrease the expression of these genes (41). We have not yet investigated the effect of factors other than HGF/SF on the regulation of the uPA proteolysis network in SK-LMS-1 cells, but our finding that these cells exhibit a very low basal expression of uPA and its receptor in growth medium containing 10% FBS which can be strongly induced by adding HGF/SF to the same medium argues in favor of at least some specificity in this response.

Although, as discussed above, one of the expected effects of the activation of the uPA proteolysis network in response to HGF/SF-Met signalling is the enhancement of invasiveness via the activation of plasmin, other potential effects of uPA which may or may not influence invasiveness also deserve consideration. For example, uPA is mitogenic for some cells, including human fibroblasts (13), and it is possible that it is mitogenic for SK-LMS-1 cells as well. Also, uPA induces the migration of endothelial cells via a mechanism that is independent of its proteolytic activity (30), and the possibility that it produces a similar effect on SK-LMS-1 cells deserves consideration. In addition, since uPA cleaves monomeric pro-HGF/SF into its active, heterodimeric form (27), it may enhance HGF/SF-Met signalling by generating increased levels of active HGF/SF (which would in turn generate more uPA). uPA also plays a role in the activation of other factors, for example, transforming growth factor β (29), and thus may enhance other signalling pathways as well.

In addition to the activation of the uPA proteolysis network, other consequences of HGF/SF-Met signalling may contribute to the ability of this receptor-ligand pair to generate the metastatic phenotype in human sarcoma cells. For example, we have previously shown that HGF/SF is mitogenic for some human sarcoma cell lines, including the one used in the present investigation (36), and it is possible that this feature of HGF/SF-Met signalling plays a role in the generation of metastasis by sarcoma cells expressing both Met and HGF/SF, since a reduced dependency on exogenous growth factors could allow these cells to colonize in organs which may not otherwise be able to sustain their growth. In addition, others have shown that HGF/SF is angiogenic in vivo (9, 18), and it is likely that this property influences the metastatic capability of HGF/SF-expressing sarcoma cells. Further investigation will be necessary to determine the degree to which these and perhaps other, unknown properties of HGF/SF-Met signalling contribute to generation of the metastatic phenotype by this signalling pathway.

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