Hepatocyte growth factor/scatter factor (HGF/SF) stimulates cell proliferation, motility, and morphogenesis by activation of its receptor, the c-Met tyrosine kinase. HGF/SF is structurally related to plasminogen, including an amino-terminal hairpin loop, four kringle domains, and a serine protease-like region. A truncated HGF/SF isoform, designated HGF/NK2, which extends through the second kringle domain and behaves as a competitive HGF/SF antagonist, was previously shown to be encoded by an alternative HGF/SF transcript. In this study, we describe a second naturally occurring HGF/SF variant, HGF/NK1, consisting of the HGF/SF amino-terminal sequence and first kringle domain. This product is encoded by a 2-kilobase alternative transcript containing intronic sequence that was contiguous with exon K1b. Analysis of baculovirus-expressed HGF/NK1 revealed that this isoform possesses the heparin binding properties of HGF/SF and modest mitogenic and scattering activity relative to HGF/SF. However, at a 40-fold molar excess, HGF/NK1 inhibited HGF/SF-dependent DNA synthesis. HGF/NK1 stimulated tyrosine phosphorylation of Met, and covalent affinity cross-linking demonstrated a direct HGF/NK1-receptor interaction. These findings establish that the HGF/SF gene encodes multiple alternative products, which include not only a mitogenic agonist (HGF/SF) and a pure antagonist (HGF/NK2) but also a molecule with partial agonist/antagonist properties.
ferent molecules, each unique in its structural and biological properties.

**EXPERIMENTAL PROCEDURES**

cDNA Library, Probes, and Restriction Enzymes—An M426 cDNA library (31) was screened with probes corresponding to either the heavy or light chain region of HGF/SF, as described previously (30). Restriction enzyme analysis of selected cDNA clones was performed with PstI, XbaI, and EcoRV (New England Biolabs, Inc.) according to the manufacturer's instructions.

Polymerase Chain Reaction—A series of polymerase chain reaction (PCR) experiments were conducted using pairs of oligonucleotide primers corresponding to different regions of the HGF/NK1 sequence and either human genomic DNA or pH46 as template. The sense primers were: P1, 5'-TGGTTGACAACTCCTGCCA-3' (in signal peptide domain); P3, 5'-GAGTGAGCTAGAAGTCTGTGAC-3' (in carboxyl-terminal portion of K1); P5, 5'-GCAATGGAGACCACCAAACT-GTCT-3' (in midregion of pH46 3'-untranslated sequence (3'-UT)); and P6, 5'-GCTTGTTGTCCTGAGCCC-3' (in midregion of 3'-UT, corresponding to sense primer 5); P6, 5'-CAATGAGGATGGTTAAGCAGAGGAAT-3' (at 3' end of pH46). For PCR (32), 0.3 μg of human genomic DNA or 5 ng of plasmid pH46 was subjected to 35 cycles of amplification with the following cycling conditions: 1 min at 94°C, 2 min at 58°C, and 3 min at 72°C. PCR products (5%) were electrophoresed in 1% agarose gels and detected by ethidium bromide staining.

Northern Blot Analysis—Poly(A) RNA was isolated from M426 and SK-LMS-1 cells as described (33). Samples (5 μg each) were electrophoresed and transferred to nitrocellulose filters. Filters were prehybridized for 2 h at 42°C in Hybriol (Oncor; 40% formamide, 10% dextran sulfate, 1% SDS, 6 × SSC, and blocking agents) and then hybridized for 15 h in the same solution with [125I]dCTP-labeled randomly primed probes corresponding either to the heavy chain of HGF/SF or to a segment of the 3'-UT of pH46. The heavy chain probe was generated by PCR using HGF/SF DNA corresponding to sense primer 5'-GGTAAATTCTGAGCCAATGAGAACAAAGAATGTTTAAATGAGCCA-3' and antisense primer 5'-ATTGTCAGCGCATGGTAAGGAATTTTATGAGGAAT-3' (coding for a segment of 3'-UT containing a polyadenylation signal (AATAAA). Hybridization was performed according to published methods (37). Autoradiographs were exposed to Kodak X-Omat AR film or phosphorstorage phosphorimager (Molecular Dynamics). Alternatively, cells were solubilized directly in SDS and boiled for 3 min in the presence of 100 mM 3-mercaptoethanol, and lysates were subjected to electrophoresis for autoradiography as described previously (37). Met Autophosphorylation—Confluent B5/589 cells were serum-starved for 24 h, exposed to HGF/SF or HGF/NK1 for 10 min at 37°C, lysed in Hepes solubilizer buffer, and immunoprecipitated with anti-phosphotyrosine monoclonal antibody 4G10 bound to agarose beads (50 μl of beads/lysate; Upstate Biotechnology, Inc.). Immunoprecipitated proteins were resolved by SDS-PAGE, were transferred to nitrocellulose filters, and autoradiographed using a phosphorimager (Molecular Dynamics). Alternatively, cells were solubilized directly in SDS and boiled for 3 min in the presence of 100 mM 3-mercaptoethanol, and lysates were subjected to electrophoresis for autoradiography as described previously (37).

1.3-kb alternative HGF/SF transcript, we detected several

**RESULTS**

Isolation and Characterization of Human Fibroblast cDNA Clones Encoding a New Truncated HGF/SF Variant—In the course of studies that identified HGF/NK2 as the product of a 1.3-kb alternative HGF/SF transcript, we detected several cDNA clones in an M426 library that hybridized to a HGF/SF heavy but not light chain oligonucleotide probe. Restriction enzyme analysis indicated multiple distinct patterns among these clones. One was typified by a 1.2-kb insert that encoded HGF/NK2, as previously reported (30). A different pattern was exhibited by three other clones that contained a 2-kb insert and lacked EcoRV and XbaI sites present in HGF/SF (Fig. 1A). Sequence analysis of one of these clones, designated pH46, revealed a distinct, 210-amino acid truncated version of HGF/SF consisting of the signal peptide and the amino-terminal and K1 domains. The coding sequence of HGF/NK1 terminated immediately downstream of the K1 domain with two additional amino acids and a translational stop codon not found in the corresponding region of HGF/SF. The open reading frame was flanked by 54 base pairs of 3'-untranslated sequence previously observed in HGF/SF transcripts as well as a unique 12-kb 3'-UT containing a polyadenylation signal (AATAAA). The presence of novel sequence downstream from the K1 domain suggested that HGF/NK1 mRNA resulted from alternative processing of the nascent HGF/SF transcript. To determine the mechanism responsible for the generation of HGF/NK1, we examined the genomic structure near exon K1b (38).
HGF/NK1 Is a Variant with Partial Agonist/ Antagonist Activity

39). PCR analysis of total cellular and pH46 DNA was performed with various pairs of oligonucleotide primers corresponding to different regions of the HGF/NK1 cDNA sequence. As shown in Fig. 1 (A and B), amplification of sequences extending from the 3′ end of K1 to the middle or downstream end of the 3′-UT (Fig. 1B, lanes 3–6) and within the 3′-UT (Fig. 1B, lanes 7 and 8) yielded fragments of the same apparent size when either genomic or HGF/NK1 cDNA was used as template. To rule out the possibility of plasmid contamination of the genomic DNA preparation, PCR was carried out with primers (P1 and P2) to exons spanning >10 kb of genomic sequence (Fig. 1A and Refs. 38 and 39). Although a fragment of the expected length was obtained with pH46 DNA as template, no product was observed with the genomic DNA preparation (Fig. 1B, lanes 1 and 2). These data indicated that the unique sequence at the 3′ end of the HGF/NK1 cDNA was contiguous with exon K1b in the human genome. This finding was reinforced by the fact that the first 10 nucleotides of the 3′-UT were identical to the published intrinsic sequence (38) located immediately downstream from exon K1b (Fig. 1C). As illustrated in Fig. 1C, the 3′-UT of pH46 corresponds to the 5′ end of the ~6.6-kb intron separating exons K1b and K2a in the HGF/SF gene (38).

Although retention of intrinsic sequence in pH46 raised the possibility that the HGF/NK1 clone might have resulted from incomplete or aberrant RNA processing, this cDNA possessed a polyadenylation signal approximately 30 base pairs upstream from a poly(A) tail. Moreover, using a probe derived from the unique 3′-UT, Northern blot analysis of poly(A) RNA from M426 fibroblasts and SK-LMS-1 cells revealed a 2-kb transcript, corresponding to a faint band seen with an HGF/SF heavy chain probe (Fig. 2). Taken altogether, these results established that HGF/NK1 was a naturally occurring variant encoded by an alternative HGF/SF transcript.

Recombinant Expression of HGF/NK1—In preliminary experiments, the HGF/NK1 coding sequence was placed into an MMTneo vector (40) and introduced into NIH/3T3 cells using standard calcium phosphate transfection methodology. Immunoblot analysis of conditioned medium from transfected cells revealed the presence of a ~20-kDa HGF/SF cross-reactive protein that was absent from the medium of control cells (data not shown). This protein bound avidly to heparin-Sepharose and eluted with 0.9–1.0 M NaCl, comparable with conditions employed for HGF/SF (4). A highly purified preparation was obtained by subsequent sizing chromatography and ion exchange or reverse-phase HPLC (data not shown). However, the amounts recovered (typically a few µg/liter conditioned medium) were not sufficient to perform extensive biological analysis.

As an alternative, we expressed the protein in Sf9 insect cells with a baculovirus vector. This approach had proven successful in generating HGF/SF with biological activity comparable with that of the naturally occurring factor (18). The chromatographic, electrophoretic, and immunologic properties of baculovirus-expressed HGF/NK1 matched those of the recombinant NIH/3T3-derived material. Benefitting from the higher level of expression in the baculovirus system, we were able to obtain a highly purified preparation of recombinant HGF/NK1 with a one-step purification process based on heparin affinity chromatography (Fig. 3). The yield was approximately 40 µg of HGF/NK1 from 1 liter of Sf9-conditioned medium. Like HGF/SF and HGF/NK2 (4, 30), HGF/NK1 migrates as a more compact mol-
HGF/NK1 is a Variant with Partial Agonist/ Antagonist Activity

Biological Properties of Purified, Recombinant HGF/NK1—HGF/NK1 exhibited mitogenic activity as determined by [3H]thymidine incorporation in B5/589 human mammary epithelial cells (Fig. 4A). However, it was signifi-cantly less potent than baculovirus-expressed HGF/SF tested under the same conditions. Even at a concentration as high as 8 nM, HGF/NK1 stimulated only 20–25% of the maximal DNA synthesis elicited by HGF/SF at 0.5 nM. Moreover, HGF/NK1 behaved as a specific antagonist of HGF/SF in the same assay. A 40-fold molar excess of HGF/NK1 reduced the mitogenic activity of 0.1 nM HGF/SF by ~70%, whereas no inhibition of epidermal growth factor activity was observed under the same conditions (Fig. 4B). At high concentrations (5–10 nM), HGF/NK1 also promoted scattering of Madin-Darby canine kidney cells, with an effect comparable with that of HGF/SF at a 50-fold lower molar concentration (data not shown).

Recombinant HGF/NK1 Binds Directly to Met and Stimulates Met Tyrosine Phosphorylation—To establish that the activities of HGF/NK1 were attributable to a direct interaction with the high affinity HGF/SF receptor, a series of covalent cross-linking experiments were performed. Following incubation of B5/589 cells with 125I-labeled HGF/NK1 and cross-linking agent, cell lysates were immunoprecipitated with a Met-specific peptide antiserum in the presence or the absence of competing synthetic peptide. When the immunoprecipitates were resolved by SDS-PAGE, autoradiography revealed a single major Met peptide-specific band corresponding in size to a complex consisting of 125I HGF/NK1 and the Met β subunit at a stoichiometry of 1:1 (Fig. 5A). Cross-linking in the presence of either excess unlabeled HGF/NK1 or HGF/SF suggested that the affinity of HGF/NK1 for Met was within an order of magnitude of that of the full-length growth factor (Fig. 5B).

To further study the interaction of HGF/NK1 with its receptor, we examined the ability of this ligand to stimulate tyrosine phosphorylation of Met. Using concentrations corresponding to those employed in the bioassays described above, a significant increase in Met tyrosine phosphorylation was detected in response to HGF/NK1 (Fig. 6). In fact, the intensity of the phosphotyrosine signal induced by HGF/NK1 at 8 nM was comparable with that observed with HGF/SF at 0.5 nM. These results demonstrated that HGF/NK1 and HGF/SF were capable of stimulating a similar level of Met tyrosine phosphorylation, even though HGF/NK1 elicited a weaker mitogenic response.

DISCUSSION

In the present study, we identified a new naturally occurring truncated form of HGF/SF. This molecule, designated HGF/NK1, is encoded by a 2-kb alternative HGF/SF transcript, which results from retention of a portion of the intron separa-
cases involve transcripts that are expressed at relatively high levels in placenta: a soluble form of the HLA-G antigen (52), a variant of human growth hormone-V encoding a unique 104-amino acid carboxyl terminus (51), and an isoform of human gonadotropin-releasing hormone (50). The relative preponderance of cases in placenta led to the hypothesis that this organ may contain specific factors that facilitate export of mRNA with introns into the cytoplasm (52). This may be relevant to the expression of HGF/NK1. Miyazawa et al. detected a relatively abundant ~2-kb alternative HGF/SF transcript in placenta (29). Although the extent of truncation had not been fully determined, this mRNA hybridized to the coding sequence of HGF/NK2 but not to probes for K3, K4, or the serine-protease domain. Given its size and hybridization pattern, we surmise that it probably corresponded to the HGF/NK1 transcript described here. Recently, gene targeting experiments revealed that loss of the HGF/SF gene resulted in death in utero due to placental insufficiency (54, 55). This finding demonstrated that the HGF/SF gene is crucial for placental development and, in view of the observed expression pattern in placenta, raises the possibility that HGF/NK1 might participate in the development of this organ.

Our results concerning HGF/NK1 activity differ in some respects from the analysis of an artificially engineered version of HGF/NK1 (56). In the latter instance, HGF/NK1 behaved as a pure antagonist of HGF/SF mitogenic activity, showing no agonist activity at concentrations up to 100 nM in an assay using rat hepatocyte primary cultures. It also barely stimulated Met tyrosine phosphorylation when tested at 20 and 100 nM. There are several possible reasons for these differences. The artificially constructed version of HGF/NK1 was expressed in bacteria as a fusion protein containing a 10-amino acid FLAG epitope at its amino terminus to target secretion of the protein into the periplasmic space. The engineered protein also lacked the two amino acid extension to the K1 domain that is present in the naturally occurring isoform. Conceivably, either of these structural differences could affect biological activity by modifying receptor-ligand interactions. Recently, we expressed the naturally occurring HGF/NK1 sequence in bacteria and observed biological activity very similar to that described above for the baculovirus-expressed protein. Thus, bacterial expression per se does not result in a molecule having activities at variance with the results obtained in the present study. However, our bacterial expression strategy included a series of steps to optimize protein refolding and fidelity of disulfide bond formation that could influence the activity of the final product. Independent of differences in HGF/NK1 preparations, the discrepancies in our data relative to the earlier report might be attributable to the cells used in our respective bioassays. For instance, proteoglycan composition, which varies enormously among cells (57) and affects signaling by fibroblast growth factors (58–60) and HGF/SF (61, 62) might account for the contrasting responses of different cell types to HGF/NK1.

HGF/NK1 should prove to be a useful tool in the structure-function analysis of HGF/SF. Although its affinity for both heparin and Met are similar to that of HGF/SF and it retains biological activity, the smaller size and lack of glycosylation sites render HGF/NK1 particularly suitable for crystallographic and NMR structural analysis. Systematic modification of HGF/NK1 should provide additional insight into the interaction of HGF/SF with its receptors. This, in turn, may lead to the development of more potent, clinically useful agonists or antagonists of HGF/SF signaling.

2 D. P. Bottaro and J. S. Rubin, manuscript in preparation. 
3 J. S. Rubin, unpublished observations.

**Fig. 6. Tyrosine phosphorylation of Met by HGF/SF or HGF/NK1.** Serum-starved B5/589 cells were treated with varying concentrations of HGF/SF or HGF/NK1 for 10 min at 37°C. Total cellular lysates (2 mg) were immunoprecipitated with phosphotyrosine antibody bound to agarose beads, resolved by 7.5% SDS-PAGE, blotted, and probed with Met antiserum. The arrow indicates the Met protein; the positions of the molecular mass markers are at the right.
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