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Expression of c-*met* proto-oncogene in COS cells induces the signal transducing high-affinity receptor for hepatocyte growth factor

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By transfection of the expression plasmid containing a human c-*met* cDNA into COS-7 cells, high-affinity binding sites specific for HGF with a K_d value of 30 pM were newly detected. Furthermore, only in the c-*met* transfected COS-7 cells, but not in the control COS-7 cells, DNA synthesis was markedly induced in response to HGF. Thus, transient expression of exogenous c-*met* cDNA resulted in the appearance of high-affinity receptor for HGF and conversion of the normally non-responsive COS-7 cells into the HGF-responsive cells. These results provide evidence for identifying the c-*met* product as a signal transducing high-affinity receptor for HGF.

Hepatocyte growth factor; HGF; HGF receptor; c-*met* Proto-oncogene; Mitogenic signal

1. INTRODUCTION

Hepatocyte growth factor (HGF), first discovered as a potent mitogen for adult rat hepatocytes in primary culture [1–3], is now thought to be a pleiotropic factor which acts as mitogen, motogen and morphogen for various epithelial cells [4–6]. HGF is a heterodimer composed of a 69 kDa α-chain and a 34 kDa β-chain [3]. Cloning of HGF cDNA revealed that HGF is derived from a single-chain precursor having 38% homology with plasminogen [7,8]. A high-affinity HGF receptor, with a dissociation constant (K_d) of 20–30 pM and an apparent molecular weight of 200–220 kDa, was identified on primary cultured rat hepatocytes and other epithelial cells [9–11].

Recent studies have suggested that the HGF receptor is the c-*met* proto-oncogene product, a transmembrane protein tyrosine kinase composed of a 50 kDa extracellular α-subunit and a transmembrane 145 kDa β-subunit with tyrosine kinase domain [12]. The c-*met* product was rapidly tyrosine phosphorylated in response to HGF treatment [13,14]. Affinity cross-linking experiments with ^{125}I-labeled HGF or its smaller form, followed by immunoprecipitation using the c-*met*-specific antisera suggested the specific interaction between the c-*met* product and HGF [13,15]. However, it has remained unknown whether the c-*met* protein could actually serve as a high-affinity receptor for HGF with a K_d value comparable to the effective dose for biological actions of HGF and whether it could transduce the mitogenic and/or motogenic signal in response to HGF. For elucidation, we have transfected the c-*met* cDNA into COS-7 cells, which are normally non-responsive to HGF and lack any detectable high-affinity receptor for HGF. We found that the transient expression of c-*met* product in COS-7 cells induced a high-affinity HGF receptor and a mitogenic responsiveness to HGF.

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2. MATERIALS AND METHODS

2.1. *Cell cultures*

COS-7 cells were routinely maintained in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum (FCS). COS-7 cells transfected with plasmids were maintained in DME medium with 4% FCS.

2.2. *Expression plasmids and transfection*

pSR2 was constructed by inserting the 5.0-kb full-size open reading frame of human c-*met* cDNA (the major form with 18 amino acid residues deleted as described in [16]) into the *Eco*RI site of mammalian expression vector pMB1 (S. Rong and G.F. Vande Woude, manuscript in preparation) (Fig. 1). pMB1 vector is derived from pMEX [17] by inclusion of the promoter of murine sarcoma virus–long terminal repeat (MSV-LTR), polyadenylation signal, and the multicloning site. 1 μg of each plasmid pool was used to transfect COS-7 monolayers grown on 24-well culture plates. Transfection was done using the DEAE-dextran protocol [18].

2.3. *RT-PCR and Southern blot analysis*

1 μg of total RNA prepared from COS-7 or COS-7^{met} cells by the AGPC method [19] was analyzed using the RT-PCR protocol, as described [20]. Using as primers HM2A (sense primer; 5'-ACTTCTT-GACGGTCCAAAGG-3') and HM2B (antisense primer; 3'-TCTA-GACAGACGTTAGATGT-5') derived from the human c-*met* cDNA sequence, 779–798 and 1,680–1,69, respectively, PCR was carried out for 30 cycles with 2 units of *Taq* polymerase. The reaction cycle was as follows: primer annealing at 55°C for 45 s, primer extension at 72°C

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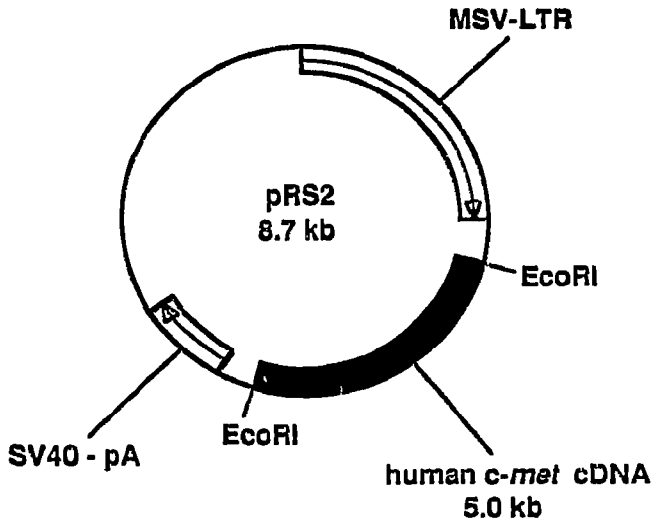

Fig. 1. Structure of pRS2, expression plasmid of human c-*met* cDNA. pRS2 was constructed as described in Materials and Methods.

for 2 min, and denaturation at 90°C for 1 min. After the reaction, 2.5 μl of the 50 μl sample was electrophoresed in a 1% agarose gel and transferred to a Nylon membrane filter (Hybond-N, Amersham). Hybridization with ^{32}P-labeled human c-*met* cDNA and washing was carried out as described [20].

2.4. *Western blotting*

Plasma membranes of COS-7 and COS-7^{met} cells were prepared by the Percoll gradient method, as described elsewhere [21]. 20 μg was solubilized with boiling Laemmli sample buffer and separated on a 7.5% SDS-polyacrylamide gel. Following the transfer of proteins to a nitrocellulose membrane, the membrane was blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS) and probed with a 1:75 dilution of polyclonal rabbit antisera directed against C-terminal 28 amino acids of the human c-*met* coding region [22]. The membrane was then washed three times with 0.2% Tween-20 in PBS, and the c-*met* protein was detected with ^{125}I-labeled Protein A (Amersham).

2.5. *Radioiodination and binding assay for HGF receptor*

Recombinant human HGF was radioiodinated by the chloramine-T method as described elsewhere [9]. Specific activity ranged from 20,000 to 30,000 cpm/fmol. Binding assay was carried out as follows: monolayers of COS-7 cells or COS-7^{met} cells cultured for 48 h, were washed once with binding buffer (Hank's solution containing 20 mM HEPES, 2 mg/ml bovine serum albumin, pH 7.0) and equilibrated for 30 min at 10°C. Ice-cold binding buffer containing various concentrations of [^{125}I]HGF, with or without excess amounts of unlabeled HGF, was added and the cells were incubated for 1 h at 10°C. Cultures were washed three times with ice-cold binding buffer and [^{125}I]HGF bound to cells was measured in a γ-counter after detaching the cells with 1 N NaOH. All binding assays were done in triplicate.

2.6. *Measurement of DNA synthesis*

Cells were seeded at a density of 1 × 10^5 cells/well on 24-well culture plates. After 24 h, cultures were made quiescent by incubation in serum-free DME medium for 48 h. After addition of various concentrations of recombinant human HGF, cells were incubated for different times and then 0.15 μCi of ^{125}I-labeled uridine deoxyribose ([^{125}I]UdR, Amersham) were added. Cells were incubated for additional 2 h, washed once with cold PBS, and solubilized by 1 N NaOH. The amounts of [^{125}I]UdR incorporated into DNA were determined by a γ-counter.

3. RESULTS

Using the DEAE–dextran method, COS-7 cells were transiently transfected with plasmid pRS2 which contains a 5.0 kb full-size coding sequence of human c-*met* cDNA downstream of the MSV-LTR promoter (Fig. 1). Expression of c-*met* mRNA was analyzed by RT-PCR protocol, that is, reverse transcription (RT) of RNA followed by polymerase chain reaction (PCR), using primers derived from the human c-*met* sequence. Analysis of the PCR products by Southern hybridization with the ^{32}P-labeled human c-*met* probe revealed a band of predicted size (921 bp) in mRNA derived from the COS-7 cells transfected with pRS2 (COS-7^{met} cells), but hybridization was not evident with mRNA from the untransfected COS-7 cells (Fig. 2A). To verify expression of the c-*met* protein at the cell surface, Western blot analysis of plasma membranes was performed using antisera against c-*met* protein. As shown in Fig. 2B, two protein species with an apparent molecular weight of 185 and 150 kDa, which may correspond to a heterodimeric α–β complex and a β-chain of c-*met* product, respectively, were detected in the COS-7^{met} cells. There were no such bands in the untransfected COS-7 cells. Taken toghether, the expression of c-*met* mRNA and

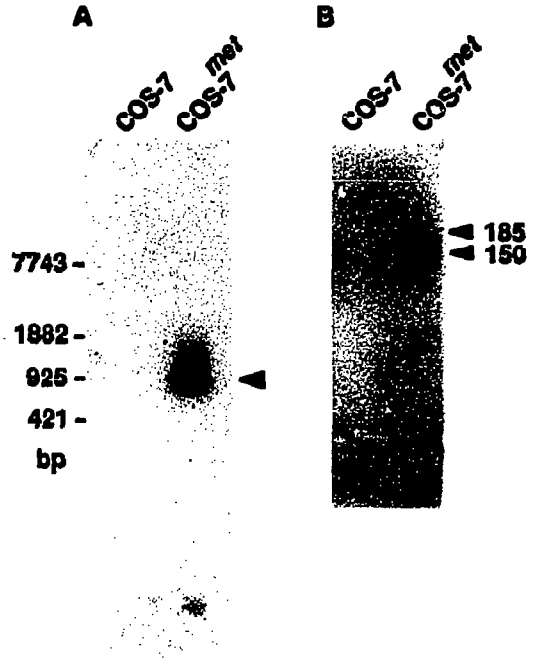

Fig. 2. (A) RT-PCR analysis of human c-*met* mRNA in COS-7 and COS-7^{met} cells. 1 μg of each total RNA was subjected to RT-PCR and 1/20 of reaction mixture was loaded on an agarose gel and analysed by Southern blotting with the ^{32}P-labeled c-*met* probe. (B) Immunoblot analysis of c-*met* protein expressed on COS-7 cells or COS-7^{met} cells. 20 μg of plasma membrane proteins prepared from COS-7 and COS-7^{met} cells were fractionated on a 7.5% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with polyclonal antisera directed against the human c-*met* product. Detection was performed with [^{125}I]Protein A.

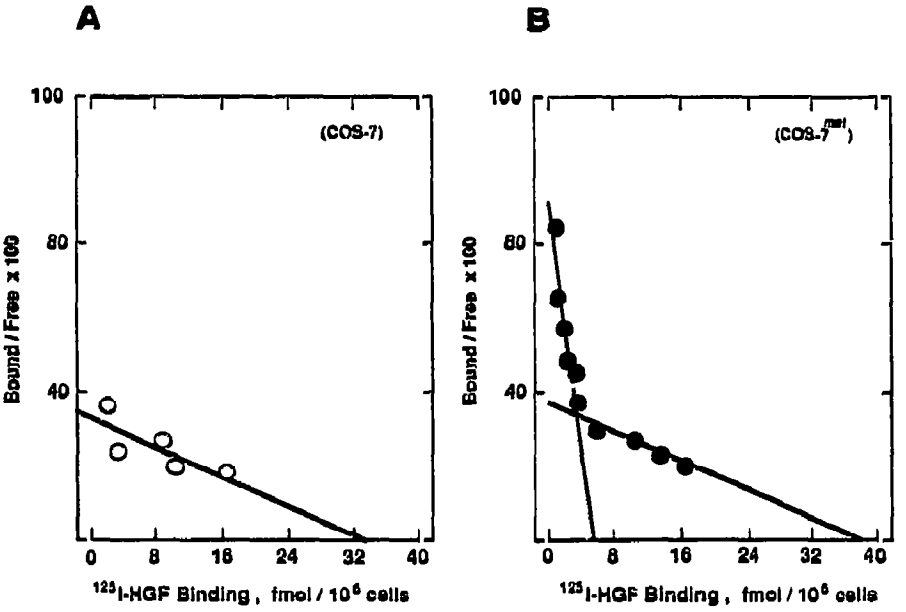

Fig. 3. Scatchard plot analysis of [^{125}I]HGF specific binding on COS-7 cells (A) or COS-7^{met} cells (B). Cells were incubated with various concentrations of [^{125}I]HGF (4–300 pM), in the presence or absence of a 100-fold excessive concentration of unlabeled HGF. Specific binding of [^{125}I]HGF was determined by subtracting the counts of samples incubated with unlabeled HGF from the counts bound in the absence of unlabeled ligand and plotted according to Scatchard [26].

c-*met* protein only in the c-*met* transfected COS-7^{met} cells was evident.

To determine whether the c-*met* product could serve as a high-affinity receptor for HGF, specific binding of [^{125}I]HGF to the transfected COS-7^{met} cells was examined and the findings compared with the binding to the untransfected COS-7 cells. Scatchard plot analysis of the specific binding of [^{125}I]HGF revealed the presence of two classes of receptors with high affinity (K_d-30 pM, B_{max}=3,540 sites/cell) and low affinity (K_d=513 pM,

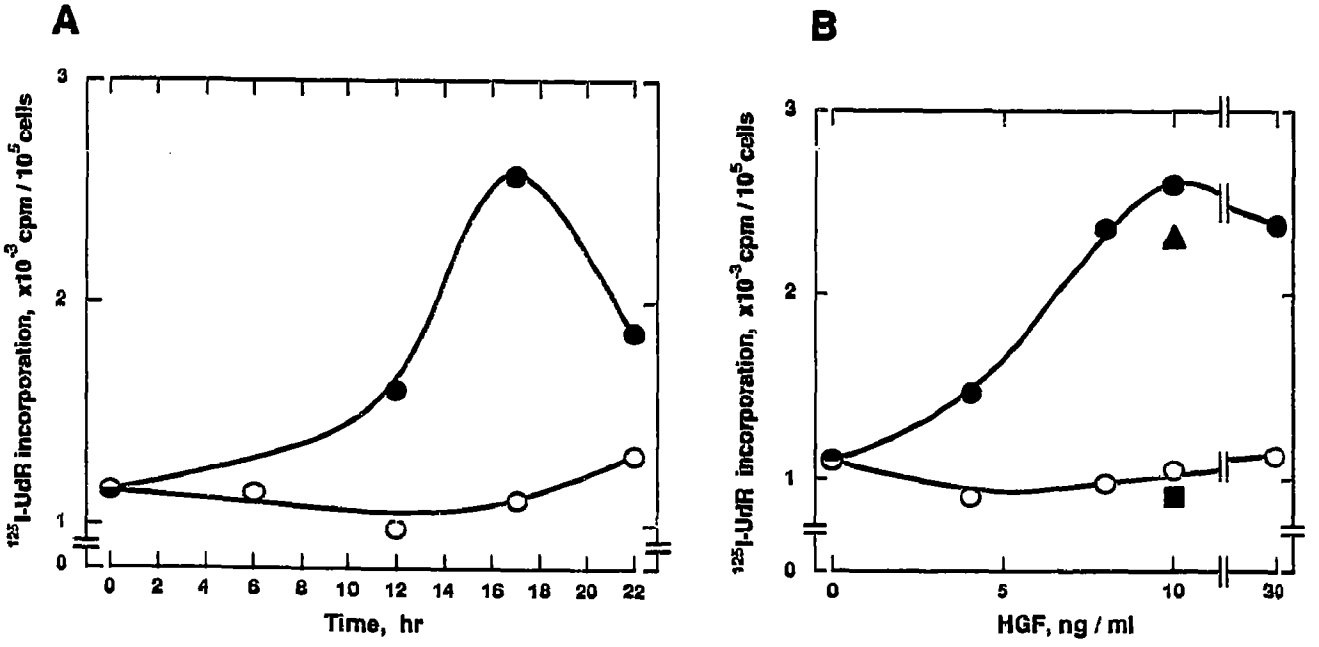

Fig. 4. Stimulation of [^{125}I]UdR incorporation in COS-7 and COS-7^{met} cells induced by HGF. (A) COS-7 cells (○) and COS-7^{met} cells (●) were cultured in serum-free media for 48 h and quiescent cells were stimulated by 10 ng/ml of HGF. At the indicated time, [^{125}I]UdR incorporation for 2 h into each cell was determined. (B) COS-7 cells (○) and COS-7^{met} cells (●) cultured for 48 h in serum-free media were stimulated by the indicated amounts of HGF for 17 h. COS-7^{met} cells were stimulated by HGF in the presence of anti-HGF rabbit antiserum (■) or preimmune serum (▲).

B_{max}=22,800 sites/cell) in the COS-7^{met} cells (Fig. 3B). On the contrary, only low-affinity binding sites (K_d=479 pM, B_{max}=20,100 sites/cell) were detected in the control COS-7 cells (Fig. 3A). The K_d value of the high-affinity binding sites detected in the COS-7^{met} cells is consistent with values obtained with HGF responsive cells, such as rat hepatocytes [9] and renal tubular epithelial cells [10]. These results strongly suggest that the c-*met* proto-oncogene encodes a high affinity receptor for HGF.

We next investigated whether the c-*met* product could transduce the mitogenic signal in response to HGF when expressed on COS-7 cells; these cells are normally non-responsive to HGF. As shown in Fig. 4A, the addition of HGF to the resting COS-7 cells cultured in serum-free medium elicited no measurable mitogenic response. However, when HGF was added to the COS-7^{met} cells cultured under similar conditions, there was a marked increase in their potential to incorporate [^{125}I]UdR into DNA. HGF stimulated DNA synthesis of COS-7^{met} cells with a dose-dependency similar to that found in hepatocytes and other epithelial cells (Fig. 4B). Stimulation of DNA synthesis in COS-7^{met} cells in response to HGF was abolished by adding polyclonal antisera raised against human recombinant HGF, but not with the pre-immune sera, thereby supporting specificity of the mitogenic action exhibited by HGF. It would thus appear that the c-*met* product expressed on COS-7^{met} cells is sufficient to mediate the mitogenic signal induced by HGF.

4. DISCUSSION

HGF stimulates growth and mobility for a variety of epithelial cells, but mesenchymal-derived cells such as blood cells and fibroblasts lack any measurable response to HGF. In parallel with the biological responsiveness, high-affinity binding sites for HGF with K_d values of 20–30 pM, in good accord with the half-maximal dose for mitogenic or motogenic activity of HGF, were detected only in epithelial cells, thereby indicating that specific binding on HGF to a high-affinity HGF receptor is required for biological responsiveness of target cells. We obtained evidence that expression of the c-*met* product in COS-7 cells induced both a high-affinity receptor for HGF and a mitogenic responsiveness to HGF. Since the fibroblast-derived COS-7 cells are normally deficient in a high-affinity HGF receptor and lack any response to HGF, we conclude that the c-*met* product primarily participates in expression of the high-affinity HGF receptor and in HGF signal transduction. The c-*met* product contains an intracellular tyrosine kinase domain as do a number of other growth factor receptors [12], and was recently found to be rapidly autophosphorylated by HGF stimulation [13–15]. Thus, it seems likely that the c-*met* tyrosine kinase is an entity of a high-affinity HGF receptor capable of transducing HGF signalling.

Hempstead et al. reported that the high-affinity receptor for nerve growth factor (NGF) requires formation of an heteromeric complex composed of the *trk* tyrosine kinase proto-oncogene product and another molecule of low-affinity receptor for NGF [23]. Low affinity binding sites for HGF were present in both HGF-responsive epithelial cells and also in HGF-non-responsive mesenchymal cells. There has heretofore been no evidence that biological responses could be mediated by the low affinity HGF binding sites. Moreover HGF has never been affinity cross-linked with any molecules, except for the p190 c-*met* gene product. Our findings that HGF tightly bound to a heparin column [2,3] and heparin dose-dependently replaced the binding of HGF to low affinity sites (data not shown), suggest that the low affinity HGF binding sites may be heparin-like extracellular matrixes. Thus, it is probable that the c-*met* encodes the functional high-affinity receptor for HGF by itself.

While high-affinity HGF binding sites are present in various epithelial cells, mesenchymal-derived cells synthesize and secrete HGF. Thus, it seems likely that HGF acts as a mediator for epithelial–mesenchymal interaction. As demonstrated in this study, the fibroblast-derived COS-7 cells normally non-responsive to HGF acquired the mitogenic responsiveness when transfected with c-*met* cDNA, thereby indicating that expression of the c-*met* product on the cell surface is the only requisite for HGF non-responsive COS-7 cells to exhibit a mitogenic response to HGF. In this respect, the unusual expression of the c-*met* gene in some mesenchymal-derived cells producing HGF may lead to the development of some tumors as a result of a continuous stimulation of c-*met* tyrosine kinase through an autocrine mechanism.

HGF is a multi-functional regulator stimulating cell growth and motility [24,25]. Since the mitogenic or motogenic signal of HGF is presumably transduced by the common high-affinity receptor of c-*met* tyrosine kinase [15], it is of interest to elucidate differences in signal transduction systems between cells exhibiting mitogenic or motogenic response. For this purpose, target proteins for tyrosine phosphorylation and other activated proteins for cell proliferation or locomotion will need to be identified.

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