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Processing of hepatocyte growth factor to the heterodimeric form is required for biological activity

Eva Gak, William G. Taylor, Andrew M.-L. Chan and Jeffrey S. Rubin

Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD 20892, USA

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Hepatocyte growth factor is a plasminogen-like molecule with diverse biological effects. Although it is synthesized as a single chain polypeptide, it was originally purified as a disulfide-linked heterodimer which was generated by an internal proteolytic event. Subsequent work indicated that preparations consisting largely of the monomeric form also exhibited potent activity. By using a combination of protease inhibition and site-directed mutagenesis, we established that conversion of the single chain polypeptide to the heterodimer occurred during the bioassay and was required for mitogenic and motogenic activity.

Hepatocyte growth factor; Scatter factor; Post-translational modification; Plasminogen

1. INTRODUCTION

Hepatocyte growth factor (HGF) was initially identified as a mitogen for hepatocytes [1–5], but subsequently was shown to stimulate the proliferation of a variety of cell types, including melanocytes, vascular endothelial cells and epithelial cells derived from many different organs [6–11]. It also has been reported to exhibit a cytotoxic effect on selected tumor cell lines [12]. HGF is indistinguishable from scatter factor (SF) [13–15], a motility agent that stimulates the dispersion of certain epithelial and endothelial cells [16–19]. Recently, branching morphogenesis from cysts of MDCK (Madin-Darby canine kidney) cells grown in collagen gels was attributed to HGF/SF [20]. The c-*met* proto-oncogene product, a membrane-spanning tyrosine kinase [21–23], is a high-affinity HGF/SF receptor [24,25], presumably involved in many, if not all, of these activities.

Structurally, HGF/SF resembles plasminogen in that they share a 38% identity in amino acid sequence and both are comprised of kringles [26] and a serine–protease domain. However, HGF/SF lacks two of the three residues which form the catalytic triad and, consequently, is thought to be devoid of proteolytic activity [27]. Molecular cloning revealed that HGF/SF, like plasminogen, is synthesized as a single chain polypeptide (p87) which can be cleaved to generate a disulfide-linked heterodimer consisting of a heavy (α, p60) and light (β, p32-34) chain [27,28]. Although HGF/SF was originally purified as a biologically active heterodimer [2–4], preparations consisting largely of the p87 monomer also exhibited potent mitogenic activity [6,29]. Thus, either the single chain form possessed intrinsic activity or its processing to the biologically active heterodimer occurred during the bioassay. If processing was required for biological activity, it might be an important mechanism for the regulation of HGF/SF function. To address this issue, we employed protease inhibition, as well as site-directed mutagenesis, and established that conversion to the heterodimer is necessary for mitogenic and scattering activity.

Correspondence address: J.S. Rubin, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD 20892, USA. Fax: (1) (301) 496-8479.

2. EXPERIMENTAL

2.1. *Site-directed mutagenesis*

The HGF/SF cleavage mutants were generated using a two-step PCR method [30] to modify the R494 codon. In the first step, two reactions were performed in parallel using as template a pCDV expression vector containing the entire HGF/SF coding sequence (nucleotides −27 to 2,199, according to the numbering scheme in [28]) [6] and the following primers: (i) a (+)primer spanning the wild-type sequence, 456–479 (5′-GAGTTCCATGATACCACACGAACA-3′) was coupled with a (−)primer corresponding to sequence 1,496–1,473 in which the R494 codon was substituted either with a K494 codon (5′-ATCCCATTTACAACCTTCAATTGT-3′) or E494 codon (5′-ATCCCATTTACAACCTGCAATTGT-3′); (ii) a (+)primer spanning the sequence, 1,473–1,496, in which the R494 codon was substituted either with a K494 codon(5′-ACAATTGAAGGTTGT-AAATGGGAT-3′) or a E494 codon (5′-ACAATTGCAGGTT-GTAAATGGGAT-3′) was coupled with a (−)primer corresponding to wild-type sequence, 2,112–2,089 (5′-ACCAGGACGATTTGGA-ATGGCACA-3′). PCR was performed for 25 cycles under low stringency conditions: 94°C, 1 min; 37°C, 2 min; 72°C, 3 min. The desired reaction products, 1.04 and 0.64 kb, respectively, were purified by agarose gel electrophoresis.

These products were used as the templates for a subsequent reaction using a (+)primer spanning the wild-type sequence, 658–687

(5'-CGAGGTCTCATGGATCATACAGAATCAGGC-3') and a (−)primer corresponding to wild-type sequence, 1,874–1,857 (5'-ATAGTTGATCAATCCAGT-3'). PCR was carried out for 35 cycles under the following conditions: 94°C, 1 min; 60°C, 2 min; 72°C, 3 min. The 1.21 kb product of this reaction was recovered from an agarose gel and digested with *Bgl*II to excise a 0.5 kb fragment containing the mutated sequence. This fragment was ligated into the original pCDV–HGF/SF construct from which the corresponding *Bgl*II fragment had been removed. The pCDV–HGF/SF mutant constructs were transformed in DH5 F'IQ competent bacteria (Gibco-BRL) and the sequence of the region between the two *Bgl*II sites was confirmed by nucleotide sequence analysis [31].

2.2. *Recombinant expression*

Transient expression of the wild-type and mutant HGF/SF proteins was carried out with COS-1 cells as previously described [6]. The conditioned medium was concentrated around 25-fold in a Centricon-30 microconcentrator (Amicon) prior to testing in the mitogenic or scatter bioassays.

2.3. *Biosynthetic studies*

Metabolic labeling and immunoprecipitation were performed as previously described [6] with a few modifications. Transfected COS-1 cells were incubated with cysteine-free, serum-free DMEM for 1 h prior to labeling with [^{35}S]cysteine (1 mCi/5 ml per dish, New England Nuclear) for 12 h. In some instances 0.1% fetal bovine serum was present during the labeling period. After the labeled medium was concentrated >10-fold in Centricon-30 microconcentrators, protein was immunoprecipitated with 10 μg of preimmune or immune antiserum raised against highly purified HGF/SF [6]. The samples were analyzed by autoradiography of gels dried following SDS/10% PAGE under reducing conditions [32].

2.4. *Plasmin treatment of HGF/SF*

Approximately 15 μl of concentrated medium containing wild-type HGF/SF generated in the absence of serum was incubated with human plasmin (0.15 U/ml, American Diagnostica) in DMEM containing 0.1 M HEPES, pH 7.4 (total reaction volume, 100 μl) for 1 h at 37°C.

2.5. *Assays of HGF/SF biological activity*

DNA synthesis by B5/589 human mammary epithelial cells was measured by [^{3}H]thymidine incorporation as described [33,34].

The scatter assay was performed according to published methods [16]. After 20 h, the cells were fixed for 20 min with 0.1 ml of methanol/formaldehyde/phosphate-buffered saline (7/2/1) and stained with hematoxylin and eosin.

3. RESULTS

3.1. *Serine protease inhibitor blocks HGF/SF mitogenic activity*

HGF/SF secreted by COS cells, transiently expressing the full-length coding sequence, exhibited potent activity in a [^{3}H]thymidine incorporation assay using quiescent B5/589 human mammary epithelial cells (Fig. 1). For these experiments, the growth factor was prepared such that it was predominantly in the single chain form, p87 (see Fig. 2, left panel). To determine whether the mitogenic activity was a direct effect of p87 itself or a consequence of proteolytic processing to the heterodimeric form, the bioassay was also performed in the presence of the serine protease inhibitor, aprotinin. As shown in Fig. 1, thymidine incorporation was significantly reduced when aprotinin was included in the assay. Under the same conditions aprotinin did not inhibit the mitogenic action of epidermal growth factor (data not shown), arguing against a toxic effect. To ensure that the inhibitory effect of aprotinin on the activity of p87 was due specifically to inhibition of HGF/SF processing, the assay was carried out in parallel with a portion of the same recombinant preparation previously treated with plasmin to generate the heterodimer before addition to the bioassay. The plasmin-treated sample stimulated a high level of DNA synthesis comparable to that of unprocessed HGF/SF but was not inhibited by aprotinin (Fig. 1). This implied that the serine protease inhibitor reduced the activity of the single chain form of HGF/SF by disrupting its conversion to the heterodimer in the mitogenic bioassay.

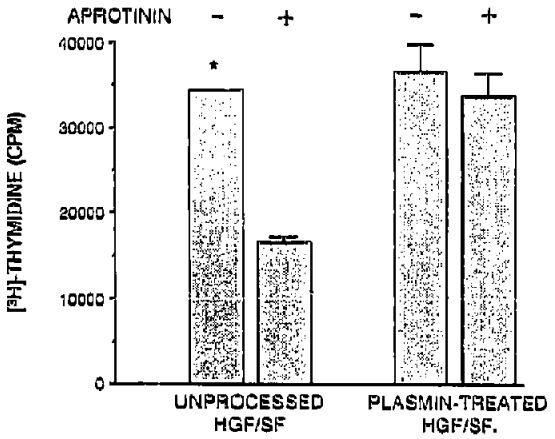

Fig. 1. Effect of aprotinin on DNA synthesis of B5/589 cells stimulated by unprocessed vs. plasmin-treated HGF/SF. Equal volumes of concentrated media were added to bioassay wells after a 1 h pre-incubation with (+) or without (−) aprotinin (100 trypsin U/ml; bovine lung aprotinin, Boehringer-Mannheim). Mean cpm ± S.D. of triplicate measurements are presented. Background with untreated cells was ~ 3,000 cpm; all mitogenic activity in the recombinant preparations could be neutralized by antiserum against HGF/SF [6]. *S.D. was too small to be shown on histogram.

3.2. *Biochemical analysis of wild-type and mutant HGF/SF molecules*

To confirm the hypothesis that internal cleavage of p87 was required for its biological activity, we prepared two mutants with alterations at the putative cleavage site. In both instances the arginine residue at position 494 was replaced, either conservatively with lysine (R494K) or non-conservatively with glutamic acid (R494E). The wild-type and mutant forms were expressed transiently in COS-1 cells metabolically labelled with [^{35}S]cysteine. The recombinant proteins were recovered from conditioned medium by immunoprecipitation with HGF/SF antiserum and visualized by autoradiography following SDS/PAGE. Similar levels of labeled protein were obtained following transfection with the three different constructs (Fig. 2). Cells transfected with the wild-type sequence in the anti-sense orientation did not produce any protein specifically recog-

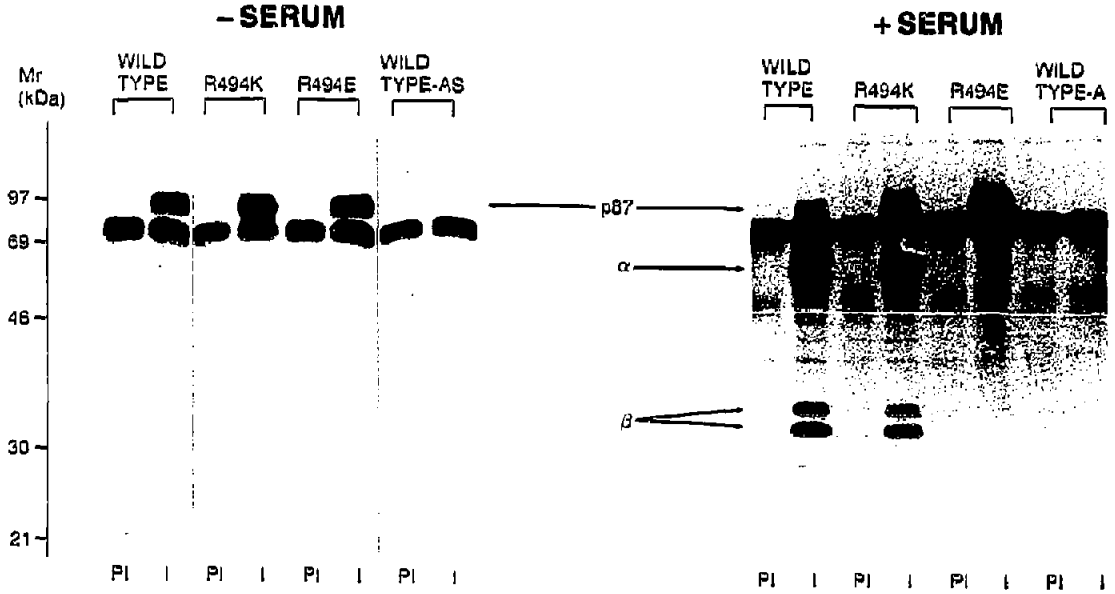

Fig. 2. Processing of HGF/SF wild-type and cleavage mutants in the absence or presence of 0.1% fetal bovine serum. Metabolically labeled protein from COS-1 transfectants was precipitated with preimmune (PI) or immune (I) serum and resolved by SDS/PAGE. Results from cells transfected with the wild-type sequence in the anti-sense orientation (WILD TYPE-AS) are also provided. Arrows indicate monomeric HGF/SF (p87) as well as the heavy (α) and light (β) chains of the heterodimer. The light chain frequently has been observed as a doublet [3,6,18]. Molecular mass markers are indicated (in kDa).

nized by the HGF/SF antiserum. With each of the other constructs, cells labeled in serum-free medium exhibited little or no evidence of HGF/SF processing. On the other hand, use of serum-containing medium during the labeling period resulted in conversion of most of the wild-type and R494K protein to the heterodimeric form (Fig. 2). In contrast, the R494E protein remained unprocessed, which indicated that this mutant could be used to test the requirement of proteolytic processing for biological activity.

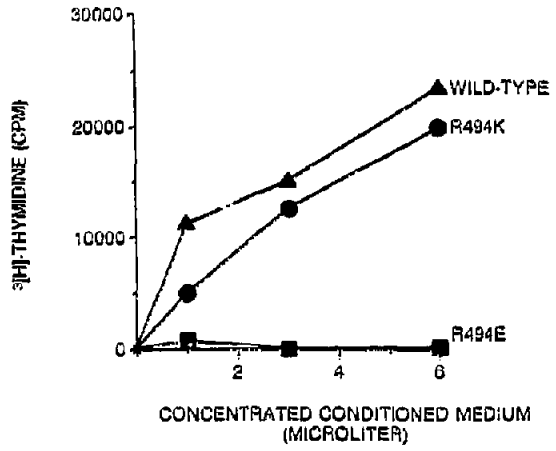

Fig. 3. Mitogenic stimulation by HGF/SF wild-type and cleavage variants of quiescent B5/589 mammary epithelial cells. Data from a representative experiment are presented as the mean cpm of duplicate measurements that varied <3,000 cpm.

3.3. *Biological activity of the site-directed mutants*

When the mitogenic activity of wild-type and variant HGF/SF proteins was compared in the B5/589 bioassay, dramatic differences were observed. While the R494K derivative was slightly less potent than the native molecule, the R494E protein failed to stimulate DNA synthesis at any of the concentrations tested (Fig. 3). This was the case despite the fact that similar amounts of immunologically cross-reactive proteins were present in the samples (data not shown). Furthermore, comparable results were obtained when the various HGF/SF analogs were monitored for their ability to induce scattering of the MDCK cells. Both the wild-type and R494K proteins stimulated the characteristic spreading and dispersion of cells associated with a fibroblastoid phenotype. In contrast, the R494E variant was devoid of such activity (Fig. 4). These differences in biological activity were not attributable to gross structural changes, as judged by similar heparin-binding properties (unpublished observations) and immunological cross-reactivity of the proteins (Fig. 2). Thus, these findings established that inhibition of HGF/SF conversion to the heterodimeric form eliminated both mitogenic and scattering activity.

4. DISCUSSION

In the present study we demonstrated that a aprotinin, a serine protease inhibitor, specifically blocked the ability of HGF/SF p87 to stimulate DNA synthesis in B5/589 cells, which provided strong evidence that proc-

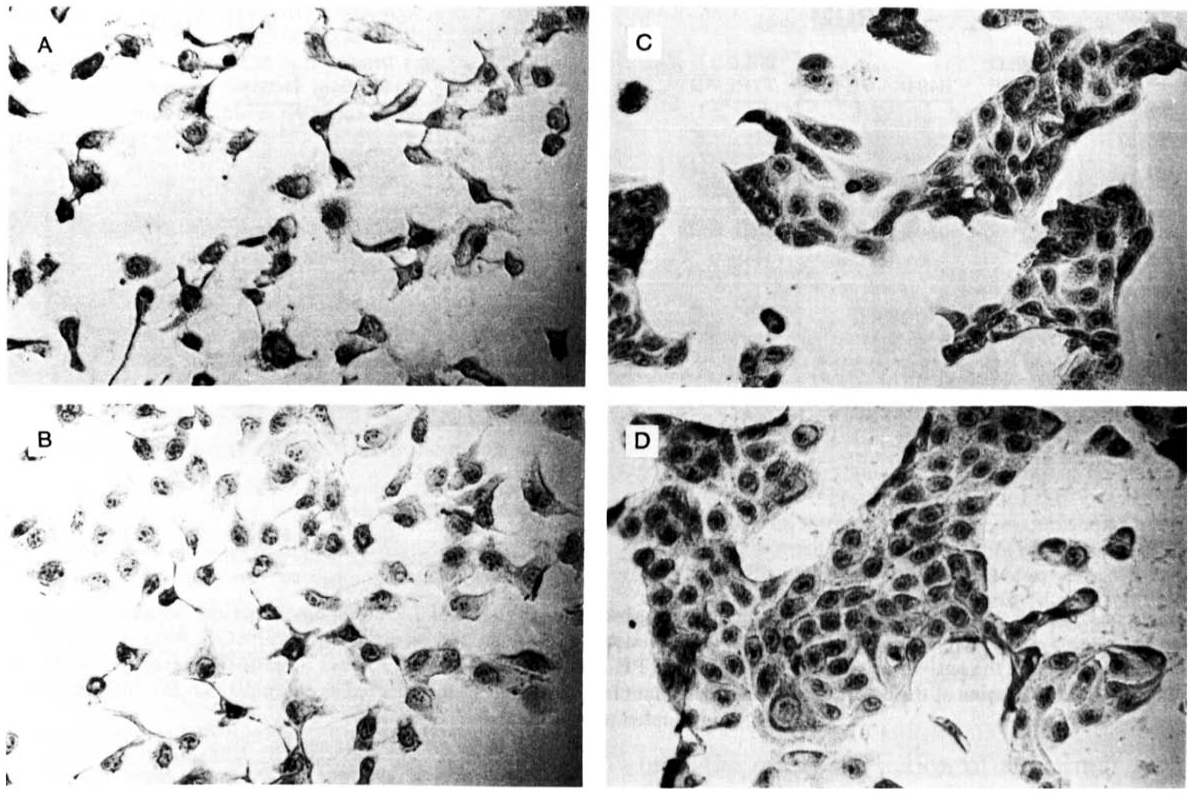

Fig. 4. Effect of HGF/SF wild-type and cleavage mutants in scatter assay with MDCK cells. Photomicrographs of cells treated with a 1:250 dilution of concentrated, conditioned medium containing similar quantities of wild-type HGF/SF (A), R494K (B), R494E (C) or no addition (D). Magnification ×400. These pictures are representative of the fields in multiple wells and reflect consistent differences in serial dilutions of the samples.

essing occurred during the bioassay and was required for mitogenesis. The persistence of some growth factor activity in this experiment was presumably due to incomplete inhibition of the endogenous protease(s). To confirm this interpretation, we prevented proteolytic cleavage entirely by site-directed mutagenesis of the cleavage site. The non-conservative substitution of glutamic acid for arginine 494 rendered the molecule resistant to processing without altering other physical attributes, as indicated by its binding both to heparin and a specific HGF/SF antiserum. We further showed that this analog was totally lacking in mitogenic and scattering activity. Thus, we conclude that an internal cleavage step is required for HGF/SF biological activity.

The location and identity of the protease(s) responsible for processing are unknown, but existing data offer some basis for speculation. Because the factor is readily secreted in the monomeric form, cleavage presumably occurs outside the cells producing HGF/SF. The fact that processing takes place in our bioassays implies that the active protease(s) may reside on the target cell's surface or in its extracellular matrix. Insofar as the cells in our bioassays are routinely exposed to serum prior to testing, either serum nor the cells could be the source of a converting enzyme. Preliminary efforts to reproduce the cleavage event in a cell-free system suggest that neither serum nor plasminogen activators, proteases commonly associated with the cell surface and extracellular matrix [35,36], are solely capable of HGF/SF processing (unpublished observations). Nonetheless, the plasmin/plasminogen activator system is an attractive candidate for HGF/SF activation. The structural homology of HGF/SF and plasminogen extends to their internal cleavage site [27], raising the possibility of a common processing mechanism. Even if the plasminogen activators are not directly responsible for the processing of HGF/SF, they might activate cell-associated plasminogen absorbed from serum (or the bloodstream in vivo) which in turn could cleave the HGF/SF precursor, just as plasmin mediates the processing of latent TGF-β in fibroblast culture fluid [37]. The coordinated activation of plasminogen and HGF/SF in the context of cell proliferation, migration or tissue remodelling is a compelling scenario which warrants further investigation.

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