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Proteolytic processing of the hepatocyte growth factor/scatter factor receptor by furin

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The hepatocyte growth factor/scatter factor (HGF/SF) receptor consists of an α- and a β-subunit, which are derived from a single-chain precursor by endoproteolytic processing. The precursor is not proteolytically processed in LoVo colon carcinoma cells The uncleaved receptor immunopurified from the cells was cleaved in vitro by furin. Furthermore, the HGF/SF receptor was proteolytically processed in LoVo cells transfected with furin cDNA. These results indicate that furin is a processing endoprotease for the HGF/SF receptor. Tyrosine autophosphorylation of the uncleaved receptor was induced by HGF/SF, and the growth of the cells expressing the uncleaved receptor was stimulated by HGF/SF, indicating that the proteolytic processing of the receptor is not essential for the signal transduction of HGF/SF.

Hepatocyte growth factor; Scatter factor; c-Met receptor; Proteolytic processing; Furin

1. INTRODUCTION

Hepatocyte growth factor/scatter factor (HGF/SF) [1,2] is a heparin-binding glycoprotein with a broad spectrum of biological activities. It stimulates the growth of endothelial cells, melanocytes, and epithelial cells including hepatocytes [3–7]. It also dissociates epithelial cell colonies into individual cells by causing a breakdown of intercellular junctions and stimulates the motility of epithelial cells [8,9]. Furthermore, it inhibits the growth of some tumor cell lines [10].

The HGF/SF receptor has been identified as the c-*met* proto-oncogene product [11]. It belongs to the receptor tyrosine kinase family [12] and consists of a 50 kDa α-subunit and a 145 kDa β-subunit, which are linked by a disulfide bond [13,14]. The α-subunit and the N-terminal region of the β-subunit are extracellular and the C-terminal tyrosine kinase domain of the β-subunit is cytoplasmic [12,13].

The mature 190 kDa heterodimeric HGF/SF receptor is produced from a 170 kDa single-chain precursor by proteolytic processing and terminal glycosylation [15]. There is a tetrabasic sequence, Arg-Lys-Lys-Arg, in the extracellular domain of the receptor [12], and, like the cleavage site of the insulin receptor [16,17], this region has been considered to be a proteolytic processing site [13]. As Arg-X-Arg/Lys-Arg is a consensus sequence required for cleavage by furin [18], it is possible that furin is a processing protease for the HGF/SF receptor. Furin is a mammalian homolog of the yeast Kex2 protease [19,20]. It is expressed in all tissues and cell lines tested [21,22], and is considered to be a processing protease for a variety of protein precursors with Arg-X-Arg/Lys-Arg sequences at the cleavage sites, such as those for growth factors, serum proteases, receptors, and viral glycoproteins [18]. Some of them have been reported to be cleaved by furin [23–31].

Mondino et al. reported that the HGF/SF receptor in the LoVo human colon carcinoma cell line is not proteolytically processed but terminally glycosylated and transported to the cell surface [32]. They also found that the coding-region of the HGF/SF receptor of LoVo cells was not mutated and the uncleaved receptor was cleaved by mild trypsin digestion to generate α- and β-subunits of the correct size [32]. They therefore suggested that the processing endoprotease for the HGF/SF receptor is lost in LoVo cells [32].

In this study, using the uncleaved HGF/SF receptor of LoVo cells, we showed that the HGF/SF receptor is proteolytically processed by furin in vitro and in living cells. We also examined the effect of the proteolytic processing of the HGF/SF receptor on the signal transduction of HGF/SF.

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2. MATERIALS AND METHODS

2.1. *Materials*

Reagents were obtained as follows: Na[^{125}I] and [α-^{32}P]dCTP from

Amersham International; Iodobeads from Pierce Chemical Co., Geneticin (G418) from Gibco Laboratories, anti-phosphotyrosine monoclonal antibody PY20 from ICN ImmunoBiologicals; anti-HGF/SF receptor polyclonal antibody from Santa Cruz Biotechnology Inc. and protein A-Sepharose from Pharmacia LKB Biotechnology Inc. Recombinant human HGF/SF was provided by the Research Center, Mitsubishi Kasei Co. [33]. C-terminally truncated soluble furin was purified from the conditioned medium of the Chinese hamster ovary cell line stably expressing mutant furin (Δ704) as described previously [34] To construct the furin expression vector pCMVFur, a mouse furin cDNA fragment covering the entire coding sequence [21] was inserted downstream of the cytomegalovirus promoter of pRc/CMV (Invitrogen). Anti-HGF/SF receptor antiserum was prepared by immunizing a rabbit with the chemically synthesized C-terminal 28 amino acid peptide of the human HGF/SF receptor conjugated to keyhole limpet hemocyanin (Calbiochem Co) using succinimidyl 4-(*p*-maleimidophenyl)butyrate (Pierce Chemical Co).

2 2 *Cell surface iodination and immunoprecipitation*

Subconfluent cells were detached from a 90-mm dish using phosphate-buffered saline containing 0.01% EDTA, recovered by centrifugation, and resuspended in 0 5 ml phosphate-buffered saline containing 0.49 mM $MgCl_2$ and 0.68 mM $CaCl_2$ The suspended cells were iodinated with 0 5 mCi Na[^{125}I] using Iodobeads, lysed, and immunoprecipitated with anti-HGF/SF receptor antibody (2 μg) as described previously [35] The immunoprecipitate was assayed for in vitro cleavage by furin or separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7% polyacrylamide). The HGF/SF receptor was detected by autoradiography.

2.3. *In vitro assay of cleavage of the HGF/SF receptor by furin*

The iodinated uncleaved HGF/SF receptor of LoVo cells which bound to the anti-HGF/SF receptor antibody coupled to protein A-Sepharose beads, was incubated with 0 025 to 0.4 units/ml soluble furin in 25 μl 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid/NaOH (pH 7 0) containing 0 5% Triton X-100 and 1 mM $CaCl_2$ for 5 h at 37°C. The HGF/SF receptor was analyzed by SDS-PAGE (7% polyacrylamide), followed by autoradiography.

2.4. *Cell culture and DNA transfection*

LoVo cells were provided by the Japanese Cancer Research Resources Bank and cultured in Ham's F-12 medium containing 10% fetal calf serum Cells (1×10^6) were transfected with 20 μg pCMVFur as described previously [36] and cultured in the presence of 1 mg/ml G418. Three weeks later, G418-resistant colonies were selected and expanded. For control, pRc/CMV was transfected instead of pCMVFur and a cell line was obtained (neo cells).

2.5 *Northern blot analysis*

Cellular total RNA was prepared by the procedure described by Chomczynski and Sacchi [37]. Fifteen micrograms of the RNA was denatured by formaldehyde, electrophoresed on a 1% agarose gel, and transferred to a nylon membrane (Biodyne). The 0.9 kbp *Bam*HI-*Bam*HI fragment of the mouse furin cDNA [21] was labeled with [α-^{32}P]dCTP by the random primer labeling method [38] and used as a hybridization probe. Hybridization and washing were carried out according to the method of Alwine et al. [39].

2.6. *Immunoblotting*

Confluent cells in a 90-mm dish were incubated with 1 nM HGF/SF for 5 min at 37°C. The cells were lysed and immunoprecipitated with anti-HGF/SF receptor antibody as described above. The immunoprecipitate was separated by SDS-PAGE (6% polyacrylamide) under reducing conditions. Immunoblotting with anti-phosphotyrosine antibody and anti-HGF/SF receptor antiserum was performed as described previously [35].

2.7. *Cell growth assay*

Cells (1×10^4) were plated on a 6-well plate and incubated without or with 0 2 nM HGF/SF for 6 days The medium was changed at day 3 to supplement HGF/SF. The cells were trypsinized and counted using a hemocytometer.

3. RESULTS

3.1. *Cleavage of the HGF/SF receptor by furin in vitro*

First, we examined whether purified furin cleaved the immunopurified uncleaved HGF/SF receptor (p190^{NC}) of LoVo cells in vitro. The cell surface of LoVo cells was iodinated using Iodobeads. The cells were lysed and immunoprecipitated by anti-HGF/SF receptor antibody. The immunoprecipitate was incubated with various concentrations of purified C-terminally truncated soluble furin, and analyzed by SDS-PAGE under reducing conditions. The HGF/SF receptor was uncleaved in LoVo cells (Fig. 1, lane 1). Furin dose-dependently cleaved the p190^{NC} to generate the heterodimer of the correct size (p50$^{\alpha}$ and p145$^{\beta}$) (Fig. 1, lane 2–6).

3.2. *Cleavage of the HGF/SF receptor by furin in living cells*

Northern blot analysis revealed that furin mRNA of the correct size (4.0 kb) was expressed in LoVo cells (Fig. 2). However, the in vitro experiment suggested that furin is a processing protease for the HGF/SF receptor (Fig. 1). Thus, it is possible that active furin is not produced in the cells. To examine whether p190^{NC} is processed by furin in living cells, we transfected the furin cDNA expression vector into LoVo cells. G418-resistant clones were screened by Northern blot analysis and two clones (Fur1 and Fur2) stably expressing the

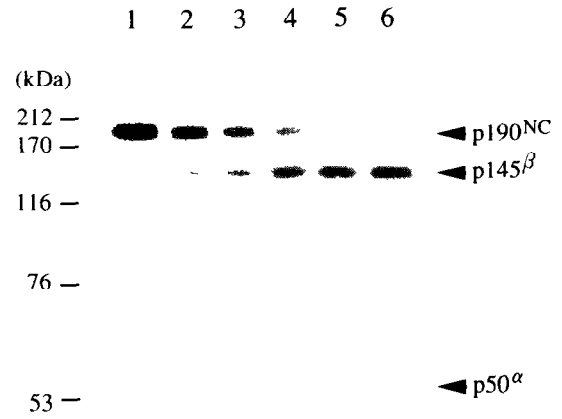

Fig. 1. Proteolytic processing of the uncleaved HGF/SF receptor by furin in vitro. The cell surface of LoVo cells were iodinated and the cell lysate was immunoprecipitated by anti-HGF/SF receptor antibody. The immunoprecipitate absorbed to protein A-Sepharose beads were incubated without (lane 1) or with 0 025 (lane 2), 0 05 (lane 3), 0.1 (lane 4), 0.2 (lane 5), and 0 4 units/ml (lane 6) soluble furin, then analyzed by SDS-PAGE under reducing conditions. The HGF/SF receptor was detected by autoradiography The molecular mass standards were myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa)

Fig. 2. Northern blot analysis of the RNA from the LoVo cells transfected with the furin cDNA. Total RNA (15 mg) from LoVo, neo, Fur1, and Fur2 cells were hybridized with mouse furin cDNA. White and black arrows indicate the endogenous and exogenous furin mRNAs, respectively.

furin mRNA were obtained (Fig. 2). The exogenous furin mRNA was smaller than the endogenous mRNA because the expression vector did not cover the entire 3′-non coding region of furin mRNA. The HGF/SF receptor in these clones and the control neo cells were analyzed by cell surface iodination and immunoprecipitation with anti-HGF/SF receptor antibody. In Fur1 and Fur2 cells, $p190^{NC}$ was proteolytically processed to the heterodimer consisting of the $p50^{\alpha}$ and $p145^{\beta}$ subunits linked by a disulfide bond (Fig. 3). A small amount of $p190^{NC}$ was detected in Fur2 cells (Fig. 3). This could be due to low levels of furin expression, because its mRNA was expressed at much lower levels in Fur2 than in Fur1 cells (Fig. 2). This proteolytic processing did not occur in neo cells (Fig. 3).

3.3. *HGF/SF-induced tyrosine autophosphorylation of the cleaved and uncleaved HGF/SF receptor*

Binding of HGF/SF to its receptor induces rapid tyrosine phosphorylation of the receptor in HGF/SF-responsible cells [11,35,40]. Mark et al. reported that the uncleaved HGF/SF receptor binds HGF/SF with an affinity similar to that of the cleaved receptor [41]. We examined whether proteolytic processing of the receptor was required for ligand-dependent tyrosine phosphorylation. Control neo cells, Fur1, and Fur2 cells were incubated with HGF/SF, lysed, and immunoprecipitated with anti-HGF/SF receptor antibody. The immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody or anti-HGF/SF receptor antiserum. The tyrosine phosphorylation of not only the cleaved receptor of Fur1 and Fur2 cells but also the uncleaved receptor of control neo cells was induced by HGF/SF (Fig. 4A), whereas the amount of the HGF/SF receptor was unchanged (Fig. 4B).

3.4. *The effect of HGF/SF on the growth of LoVo cells expressing the cleaved or uncleaved HGF/SF receptor*

We then examined the effect of HGF/SF on the growth of LoVo cells expressing the cleaved or uncleaved HGF/SF receptor. Control neo cells, Fur1, and Fur2 cells were cultured in the presence or absence of HGF/SF for 6 days and the cells were counted. The numbers of not only the cells expressing the cleaved receptor (Fur1 and Fur2 cells) but also those expressing the uncleaved receptor (neo cells) were increased by HGF/SF (1.5- to 2-fold of those of untreated cells), although the growth rates of the untreated cells were different among the clones (Fig. 5). None of the clones exhibited scattered morphology after exposure to HGF/SF (not shown). These results indicate that the proteolytic processing of the HGF/SF receptor is not essential for the biological signal transduction of HGF/SF.

4. DISCUSSION

The HGF/SF receptor has a tetrabasic sequence at the extracellular domain and has been thought to be cleaved at this site [13], as is the insulin receptor [16,17]. Recently, Mark et al. reported that none of the HGF/SF receptor mutants with a single amino acid replacement of Arg, Lys, or Arg at the fourth, second, or first residue upstream of the putative cleavage site, were proteolyti-

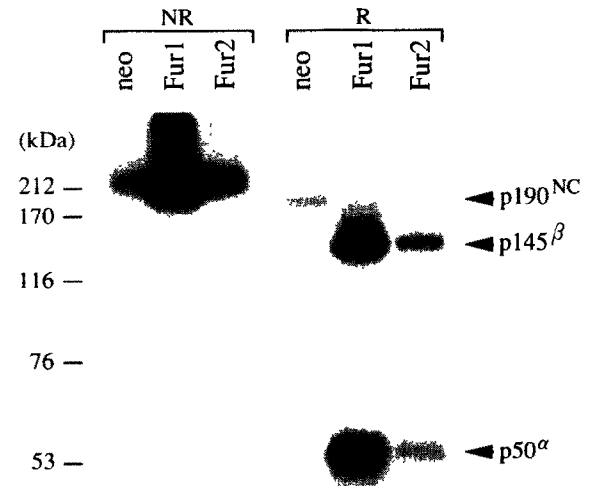

Fig. 3. Proteolytic processing of the HGF/SF receptor in the LoVo cells transfected with furin cDNA . The surfaces of neo, Fur1, and Fur2 cells were iodinated and the cell lysates were immunoprecipitated with anti-HGF/SF receptor antibody. The immunoprecipitates were analyzed by SDS-PAGE under non-reducing (NR) or reducing (R) conditions, and the HGF/SF receptor was detected by autoradiography.

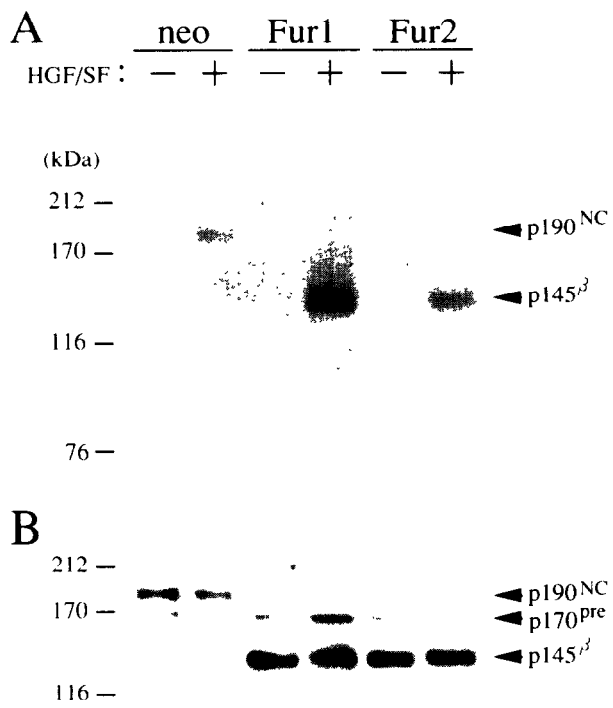

Fig. 4. HGF/SF-induced tyrosine phosphorylation of the cleaved and uncleaved HGF/SF receptor. Neo, Furl, and Fur2 cells were incubated without (−) or with (+) 1 nM HGF/SF for 5 min, lysed, and immunoprecipitated with anti-HGF/SF receptor antibody. The immunoprecipitates were separated by SDS-PAGE under reducing conditions and immunoblotted with anti-phosphotyrosine antibody (A) or anti-HGF/SF receptor antiserum (B). The 170 kDa protein ($p170^{pre}$) detected in (B) is the HGF/SF receptor precursor [15].

cally processed when their cDNAs were transfected into a fetal kidney cell line, and they suggested that the processing endoprotease for the HGF/SF receptor is furin or a furin-like molecule [41] because the sequence Arg-X-Arg/Lys-Arg is the recognition sequence of furin [18]. In this study, we showed that furin cleaves the HGF/SF receptor in vitro and in living cells, using LoVo cells in which the HGF/SF receptor is not cleaved. This is the direct evidence that furin is a processing endoprotease for the HGF/SF receptor.

Furin is a member of the mammalian endoprotease family homologous to the yeast Kex2 protease [42]. Among the members of the family, PC2 and PC1/PC3 are expressed only in neuroendocrine tissues [22] and PC4 is expressed only in the testis [43]. On the other hand, PACE4 [44] and PC6 [45] as well as furin [21,22] are ubiquitously expressed in various tissues. So, the possibility that PACE4 and/or PC6 are also responsible for the proteolytic processing of the HGF/SF receptor can not be excluded.

In LoVo cells, furin mRNA of the correct size was expressed at a level similar to those of other cell lines in which the HGF/SF receptor is cleaved (not shown). The HGF/SF receptor was cleaved in LoVo cells transfected with furin cDNA. Thus, active furin is not produced in the parental LoVo cells. A mutation in the furin gene may result in a defect of active furin in the cells.

Mondino et al. reported that the uncleaved HGF/SF receptor in LoVo cells is constitutively phosphorylated on tyrosine [32]. We also observed this (not shown). However, the uncleaved HGF/SF receptor in control neo cells was not tyrosine-phosphorylated in the absence of a ligand. Thus, the constitutive tyrosine phosphorylation of the HGF/SF receptor in parental LoVo cells may not be due to the loss of the proteolytic processing.

The tyrosine phosphorylation of not only the cleaved HGF/SF receptor of Furl and Fur2 cells, but also the uncleaved receptor of neo cells, was induced by HGF/SF. HGF/SF also stimulated the growth of neo cells as well as Furl and Fur2 cells. Although we did not determine the ligand-binding affinity of the uncleaved HGF/SF receptor in this study, it should not be drastically different from that of the cleaved receptor, because the sub-nanomolar (0.2 nM) HGF/SF, which is required for the maximal activity through the cleaved receptor [3–5], was also effective in stimulating the growth of neo cells in which the HGF/SF receptor was not cleaved. Mark et al. reported that the HGF/SF receptor mutants which were resistant to proteolytic processing bound HGF/SF with an affinity similar to that of the cleaved receptor (K_d 0.2 nM) [41]. Thus, the proteolytic processing of the HGF/SF receptor is not essential for the biological signal transduction of HGF/SF. Further investigation is

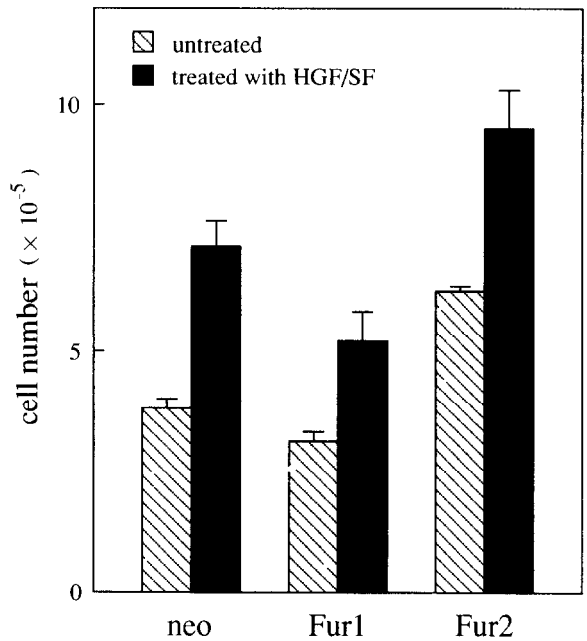

Fig. 5. Effect of HGF/SF on the growth of LoVo cells expressing the cleaved or uncleaved HGF/SF receptor. Neo, Furl, and Fur2 cells (1×10^4) were cultured without (hatched bars) or with 0.2 nM HGF/SF (black bars) for 6 days, and the cells were counted. Each value represents the mean ± S.D. ($n = 3$).

required to elucidate the biological significance of the proteolytic processing of the HGF/SF receptor.

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