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# An alternatively spliced form of Met receptor is tumorigenic

**Jae-Ho Lee[1,2,4], Chong Feng Gao[3], Chong Chou Lee[3], Myung Deok Kim[1,2] and George F. Vande Woude[3]**

[1]Department of Biochemistry
School of Medicine
[2]Department of Molecular Science and Technology
Graduate School, Ajou University
Suwon 443-721, Korea
[3]Laboratory of Molecular Oncology
Van Andel Research Institute
Grand Rapids, MI 49503, USA
[4]Corresponding author: Tel, 82-31-219-5053;
Fax, 82-31-219-5059; E-mail, jhlee64@ajou.ac.kr

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Abbreviations: BCA, bicinchoninic acid; HGF/SF, hepatocyte growth factor/scatter factor

## Abstract

**The Met tyrosine kinase receptor is a widely expressed molecule, which mediates pleiotropic cellular responses following activation by its ligand, hepatocyte growth factor/scatter factor (HGF/SF). Previously, one of the authors identified an alternatively spliced form of Met (Met-SM) that lacked a single exon of a 47-amino-acid segment in the juxtamembrane domain. Here we report that Met-SM is a potent transforming gene in NIH3T3 mouse fibroblast cells. Met-SM-transfected NIH3T3 cells show stronger foci-forming activity than wild type-Met-transfected ones. In addition, Met-SM-transfected NIH3T3 cells form colonies in soft agar and are tumorigenic in athymic nu/nu mice. Furthermore, HGF/SF significantly increases the focus-forming activity of Met-SM comparing to wild type Met. The amount of protein and of tyrosine kinase activity of Met-SM accumulates to a high level following HGF/SF treatment. The accumulation of Met-SM correlated well with its delayed ubiquitination and increased stability. These results are consistent with the important role of the juxtamembrane domain in protein stability of Met receptor and suggest that the alternatively-spliced form may contribute to the development and progression of human cancer.**

**Keywords:** alternative splicing; hepatocyte growth factor; neoplasms; proto-oncogene proteins c-met

## Introduction

The Met tyrosine kinase is a high-affinity receptor for hepatocyte growth factor/scatter factor (HGF/SF) (Bottaro *et al.*, 1991; Naldini *et al.*, 1991). Both Met and HGF/SF are expressed in numerous tissues, although their expression is confined predominantly to cells of epithelial and mesenchymal origin, respectively (Stoker *et al.*, 1987). Signaling via this receptor-ligand pair has been shown to affect a wide range of biological activities, including angiogenesis (Bussolino *et al.*, 1992), cellular motility (Stoker *et al.*, 1987), cell growth (Nakamura *et al.*, 1986; Higashio *et al.*, 1993), and morphogenic differentiation (Montesano *et al.*, 1991; Tsarfaty *et al.*, 1992).

In addition to mediating a variety of normal cellular processes, Met-HGF/SF signaling has been implicated in the development and progression of various malignant tumors (reviewed by Jeffers *et al.*, 1996; Trusolino *et al.*, 2002). Oncogenic form of Met includes the native receptor itself in an autocrine loop with its ligand HGF/SF (Bellusci *et al.*, 1994; Rong *et al.*, 1994). In addition to mediating transformation in model systems, there is evidence showing that autocrine Met-HGF/SF signaling plays a role in human cancer (reviewed by Jeffers *et al.*, 1996). Met can be oncogenically activated via a number of specific point mutations. Mutations originally identified in human papillary renal carcinomas (Schmidt *et al.*, 1997) were found to generate Met molecules possessing constitutive kinase activity and transforming ability (Jeffers *et al.*, 1997a). In addition to papillary renal carcinoma, mutations in Met have been reported in ovarian cancer (Tanyi *et al.*, 1999), in early-onset hepatocellular carcinoma (Park *et al.*, 1999), gastric carcinoma (Lee *et al.*, 2000) and in several cases they are found in metastatic lesion originated from the primary tumor (Otsuka *et al.*, 1998; Di Renzo *et al.*, 2000).

Whereas all the mutations reported in hereditary papillary renal cancer and hepatocellular carcinoma are missense mutations of the tyrosine kinase domain of Met, the missense mutation found in gastric carcinoma by the authors was located in the juxtamembrane domain and showed transforming activity when overexpressed in NIH 3T3 mouse fibroblasts (Lee *et al.*, 2000). Along with the presence of a protein kinase C (PKC) regulatory site (Gandino *et al.*, 1994) and a protein tyrosine phosphatase-binding site in the juxtamembrane domain (Villa-Moruzzi *et al.*, 1998), there was a clear demonstration of Y1003 as the target of c-Cbl binding,

which leads ubiquitination and protein degradation (Peschard *et al*., 2001). An activating mutation in the juxtamembrane in gastric cancer raised the question of biological properties of previously reported alternatively splicing form of Met (Met-SM) by one of the authors, which lacked exon 14 that encoded a significant portion of Met juxtamembrane domain (Lee *et al*., 1994). Here, we show that Met-SM was different biochemically from wild type Met and has enhanced oncogenic activity.

## Materials and Methods

### Cell lines and reagents

NIH 3T3 cells (CRL1658) were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM (Life Technologies) supplemented with 10% calf serum (Life Technologies). These cells produce only two scatter units/ml of HGF/SF, which is significantly lower than other NIH 3T3 sublines we have analyzed (Lee *et al*., 2000).

### Plasmid and constructs

The wild-type Met expression vector (PMB11) contains the murine Met cDNA in PMB1 vector as described previously (Lee *et al*., 2000). The Met-SM was subcloned from the original clone (Lee *et al*., 1994) into the same expression vector as wild type. To construct other Met mutants, the QuickChange site-directed mutagenesis kit (Stratagene) was used according to the manufacturer's instructions with PMB11 as the template. Mutations were verified by sequencing both strands of DNA in the region of interest.

### Transfection and focus-forming assay

Transfections and focus-forming assays were performed as described previously (Lee *et al*., 2000). Half of the transfected cells were cultured in DMEM/10% CS supplemented with 800 μg/ml G-418 (Life Technologies). These cells were used to assess the transfection efficiency and were grown as pools of cells consisting of at least 100 colonies and used for expression, phosphorylation, and *in vitro* and *in vivo* tumorigenesis experiments. Focus-forming activity assays were performed as described previously (Jeffers *et al*., 1997a).

### Soft agar colony generation assay

Noble agar solution (3.5% in PBS) prewarmed at 40°C was added to DMEM containing 10% FBS pre-warmed at 37 °C to make 0.7% agar. After rapid mixing by inversion, the resultant solution was added to 6-well plates (1 ml/well). The cells reaching 70-80% confluence were trypsinized, washed with PBS three times and diluted in Noble agar solution (0.3 % Noble agar in DMEM with 10% FBS) at 37°C. Then the cell suspensions were added into the 6-well plate with 0.7% agar layer (5000 cells in 1 ml). Plates were incubated at 37°C, with 5% $CO_2$ condition for three weeks. Twice a week, DMEM with 10% FBS with or without 100 units/ml HGF/SF were added (1 ml/well) to provide required nutrition and growth factors.

### Western blot analysis

Western blot analysis was performed essentially as described (Baek *et al*., 2004) under reducing conditions using the following primary antibodies: rabbit anti-Met polyclonal antibody (SP260; Santa Cruz Biotechnology), anti-phosphotyrosine monoclonal antibody (clone 4G10; Upstate Biotechnologies Inc.), and anti-ubiquitin monoclonal antibody (clone 1B3; MBL).

### Immunoprecipitation

Monolayers of stably transfected cells were washed twice with ice-cold PBS, lysed in ice-cold RIPA buffer consisting of 10mM sodium phosphate (pH 7.2), 150 mM NaCl, 1%(V/V) Nonidet P-40, 0.1% SDS supplemented with 10 mM sodium fluoride, 5 mM sodium orthovanadate, and complete protease inhibitor cocktail (Boehringer Mannheim). The cell lysates were centrifuged at 4°C for 15 min (14,000 *g*) and the supernatants were collected for further analysis. When samples were supposed to be used for the analysis of ubiquitination, *N*-ethylmaleimide, an inhibitor of deubiquitinating enzyme, was added at a concentration of 20 mM. After quantitation by using BCA protein assay reagent (Pierce), 400 μg of each lysate was precleared with protein A-Sepharose and then incubated with anti-Met antibody (SP260; Santa Cruz Biotechnology) and protein A-Sepharose for overnight at 4°C with rotation. The samples then were washed three times with ice-cold RIPA buffer. SDS gel-loading buffer (containing reducing agent) was added to each sample. After boiling for 5 min and centrifuging at 14,000 *g* for 5 min, the resulting supernatants were resolved by SDS/PAGE and examined by Western blot analysis.

### Thymidine incorporation assay

The thymidine incorporation assay was carried out as follows. Cells were plated in 96-well plates ($2 \times 10^3$ cells/well) and cultured in DMEM supplemented with 10% CS for 24 h. The cells were starved in serum-free DMEM medium for 30 h and then stimulated with various concentrations of HGF/SF for

12 h. [Methyl-$^3$H]thymidine (1 μCi, Perkin-Elmer Life Science) was added 4 h before analysis. Thymidine incorporation into DNA was measured by cold 5% trichloroacetic acid precipitation, followed by extraction in lysis buffer (0.02 N NaOH, 0.1% SDS), and then counted in the $^3$H channel of a liquid scintillation counter (TRI-CARB 3100TR, Packard).

## Pulse-chase analysis

Cells were seeded at a density of 1 $\times 10^6$ cells/100-mm dish in DMEM containing 10% calf serum. The next day, cells were cultured in serum-free DMEM in the presence or absence of HGF/SF (100 scatter units/ml) for 24 h. Then the cells were rinsed twice in labeling medium (Life technologies, Inc) and incubated in serum-free labeling medium for 3 h. The

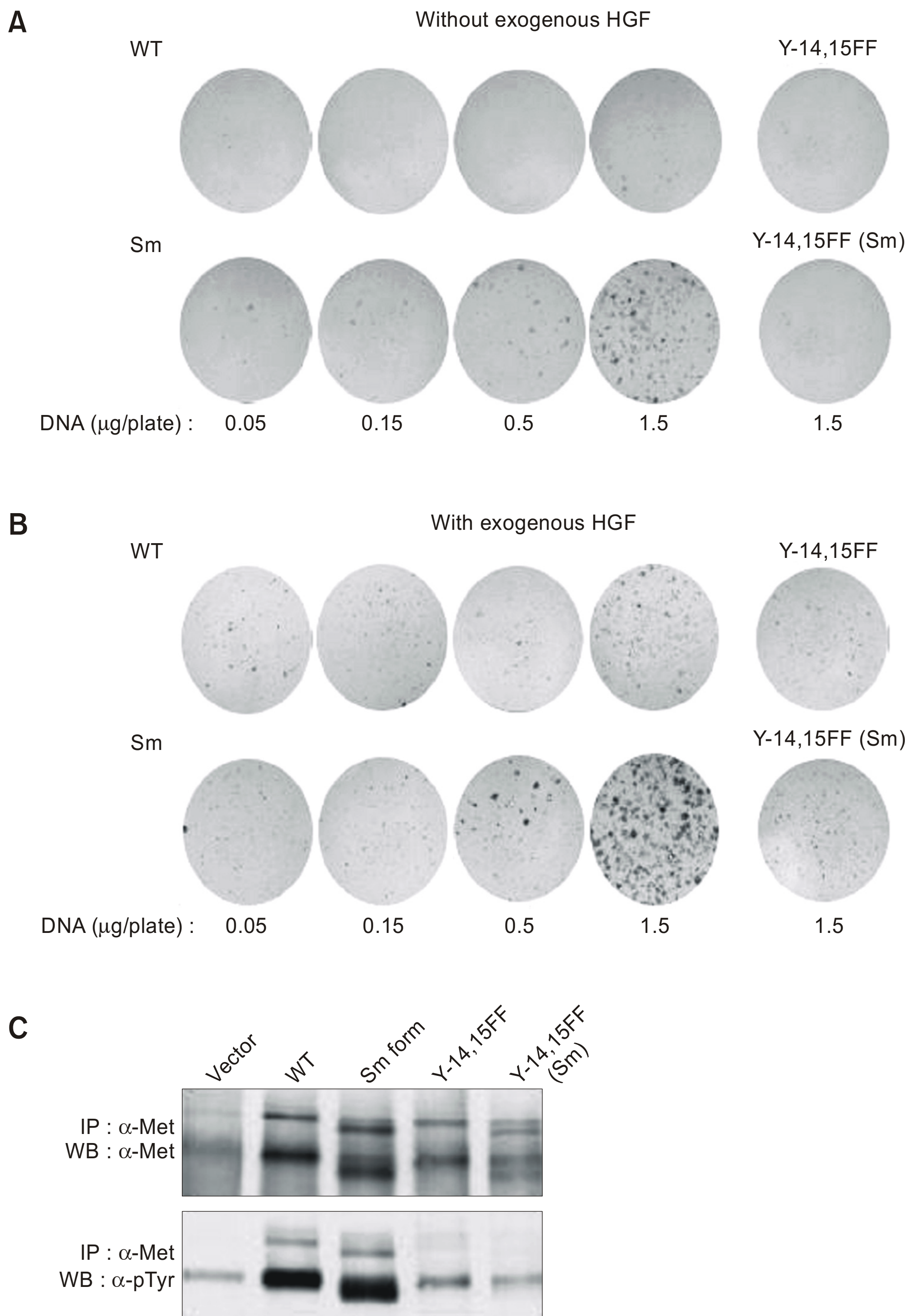


**Figure 1.** Differential foci induction by Met-WT and Met-SM. Cells transfected with indicated amounts of Met-WT (WT) or Met-SM (Sm) cDNA were assessed for focus-forming ability in the absence (A) or presence (B) of HGF/SF (200 scatter units/ml). Cells transfected with 1.5 μg DNA/plate of Y14,15-FF mutant of Met-WT (Y14,15-FF) or Y14,15-FF mutant of Met-SM (Y14,15-FF (Sm)) were also assessed for focus-forming ability. (C) Immunoprecipitation followed by Western blot analysis was performed to assess the protein amount and tyrosine phosphorylation of Met-WT and Met-SM transfected cells. Representative results are shown from independent experiments performed three times (A, B).

cells were pulsed in 0.2 mCi $^{35}$S-labeled methionine and cysteine (0.2 mCi/ml; ICN) in the presence or absence of exogenous HGF/SF (100 scatter units/ml). After 1 h, the cells were washed with DMEM and cultured in DMEM containing 3 mg/ml methionine. At different time points, the cells were lysed and used for immunoprecipitation with anti-Met antibody. Precipitated proteins were resolved by 8% Tris-glycine gel electrophoresis, and the resulting gels were used for autoradiography.

### *In vivo* tumorigenecity assay

Pools of G418-resistant NIH 3T3 cells expressing the indicated Met proteins were generated as described above and were shown to express nearly equal amount of exogenous Met before use. The cells were plated as described, and 5 × $10^5$ cells were inoculated subcutaneously into 4-week-old female athymic nude mice.

**Table 1.** Nude mouse tumorigenesis

| Met construct | No. mice with tumors/ No. mice injected[a] | Mean tumor size, $mm^2$ (mean ± S.D.)[b] |
|---|---|---|
| WT | 0/6[c] | 0 |
| SM | 6/6 (10) | 57.8 ± 9.1 |
| Y-14,15 FF (WT) | 0/6 | 0 |
| Y-14,15 FF (SM) | 0/6 | 0 |

[a]Number of mice with tumors 18 day after inoculation of 5 × $10^5$ cells; (days post-inoculation when tumors were first detected in each animal). [b]Size at 18 day after injection (subsequently, all mice with tumor burden were sacrificed). [c]At 25 day, the first mouse developed a palpable tumor.

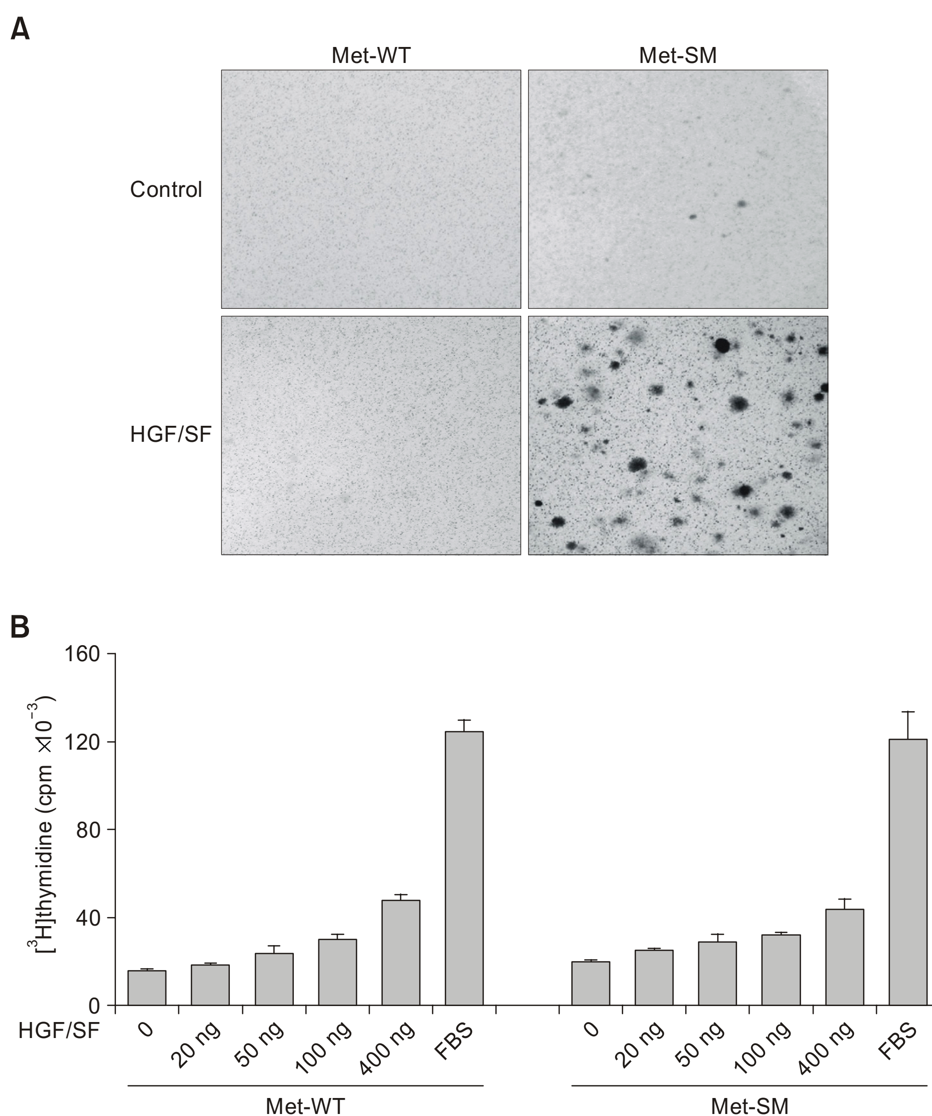


**Figure 2.** Anchorage-independent growth of NIH 3T3 cells transfected with Met-SM. (A) NIH 3T3 cells transfected with indicated vectors were diluted in 0.3% Noble agar solution (0.3% Noble agar in DMEM with 10% FBS) and added into each well (5000 cells in 1 ml) with pre-formed 0.7% agar layer containing DMEM with 10% FBS. Plates were incubated at 37°C, with 5% $CO_2$ condition for three weeks. Twice a week, DMEM containing 10% FBS with (HGF/SF) or without HGF/SF (Control) were added (1 ml/well) to provide required nutrition and growth factors. The pictures of colonies were taken after crystal violet staining. Representative fields of view from three independent experiments are shown. (B) HGF/SF-induced cell proliferation was assessed by thymidine incorporation assay as described under Materials and Methods.

## Results

### Enhanced tumorigenecity by Met-SM

To address the biological activity of Met-SM, we transfected NIH 3T3 cells with different amounts of wild type Met (Met-WT) or Met-SM expressing vector. The cells were maintained in DMEM containing 800 μg/ml G-418 for 12 d and stained with crystal violet. Whereas the wild type produced few foci even at a concentration of 1.5 μg DNA per plate, Met-SM produced more foci with less DNA in a dose-dependent manner (Figure 1A), showing the Met-SM was truly transforming. To address the effect of exogenous HGF/SF in the transforming activity of Met-SM, the ligand was added to the culture at a concentration of 200 scatter units/ml every 2-3 d. Exogenous HGF/SF clearly increased the number and size of foci both with Met-WT and Met-SM, and the extent of the increase was far more significant with Met-SM (Figure 1B). Both the expression level and the phosphotyrosine level were comparable between Met-WT and Met-SM (Figure 1C). As another *in vitro* method to test the transforming activity, a soft agar colony generation assay was performed. The results showed that only Met-SM transfected NIH 3T3 cells formed large colonies in soft agar, although HGF/SF-induced proliferation rate was comparable (Figure 2A and B). More importantly, when these cells were used for *in vivo* tumorigenesis assay in nude mice, Met-SM was highly tumorigenic based on both the number of mice with tumors and the size of the tumors developed (Table 1).

Y14, 15 (the $14^{th}$ and $15^{th}$ Tyr residues in cytosolic portion of Met) form a common docking site for several different signaling molecules downstream of Met. The Y14, 15 mutants of Met-WT are known to block Met signaling and were used as controls. Met-WT and Met-SM mutants Y14, 15-FF molecules were transfected into NIH 3T3 cells and compared to Met-WT and Met-SM. The transforming capacity of Met-SM observed in a focus-formation assay and an *in vivo* nude mice assay were abolished by the introduction of Y14, 15-FF mutation (Figrue 1 and Table 1). These data indicate that the transforming signaling from Met-SM is mediated through the Y14, 15 docking site as Met-WT.

### Impaired down-regulation of Met-SM by HGF/SF

To study the mechanism responsible for the biological activity of Met-SM, we pooled colonies of either Met-WT or Met-SM-transfected NIH 3T3 cells for biochemical analysis. We first detected Met protein tyrosine phosphorylation in response to HGF/SF. In the absence of exogenous HGF/SF, Met phosphotyrosine was not significantly different between Met-WT and Met-SM (Figure 1C and 3, top panel). However, in the presence of exogenous HGF/SF, Met-WT phosphotyrosine was the highest at one hour, then decreased. By contrast, Met-SM phosphorylation was greater than Met-WT at each time and dramatically increased even to 24 hours. When the same blot was stripped and probed for Met, it turned out that the striking difference in phosphotyrosine at least partly resulted from the difference in the amounts of Met protein (Figure 3, second panel from the top). After HGF/SF treatment, the amount of Met-WT decreased, while that of Met-SM increased and was maximum at 24 h. The difference in phosphotyrosine and c-Met protein was also observed in whole cell lysates by Western blot analysis (Figure 3, the third and fourth panel from the top). In both panels, the level of Met-SM phosphorylation and Met protein p140 increased dramatically over 24 hours. Since the cDNAs of the two forms of Met are

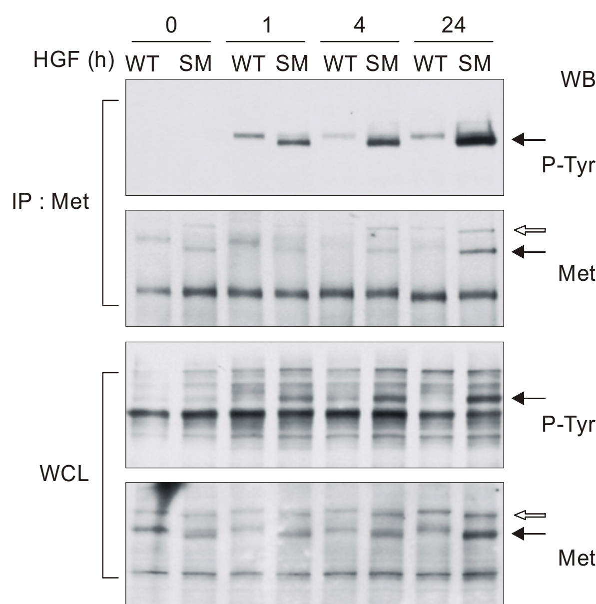


**Figure 3.** Accumulation of the protein amount and phosphorylation of Met-SM upon HGF/SF treatment. Cells stably transfected with vectors expressing Met-WT (WT) or Met-SM (SM) were cultured in DMEM/10% CS to confluency of 40-50%. HGF/SF (200 scatter units/ml) was added to each dish at various time points before the harvest as indicated (0, 1, 4, and 24 h). Four hundred μg of cell lysate was immunoprecipitated with anti-Met antibody (SP260). The precipitate (IP: α–Met) was resolved on an 8% gel, and analyzed by Western blot analysis using anti-phosphotyrosine antibody (α-P-Tyr, top panel) or anti-Met antibody (α–Met, second panel from the top). Forty μg of whole cell lysate (WCL) was resolved on an 8% gel and examined by Western blot analysis using anti-phosphotyrosine antibody (α-P-Tyr, third panel from the top). The filters were stripped and reprobed with anti-Met antibody (α–Met, bottom panel). Blank and filled arrows denote the 170 kDa single chain precursor of Met and the 140 kDa β-chain of the mature Met heterodimer, respectively.

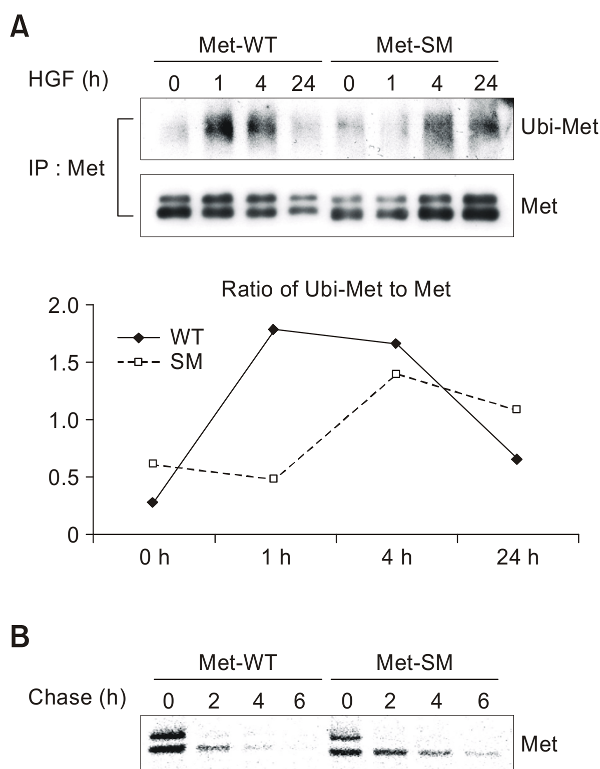


**Figure 4.** Enhanced stability of Met-SM. (A) Delayed ubiquitination of Met-SM protein. NIH 3T3 cells stably expressing Met-WT or Met-SM were serum-starved for 12 h, and then treated with HGF/SF (100 scatter unit/ml) for indicated times. Cells were washed with ice-cold PBS and lysed in ice-cold RIPA buffer. Five hundred microgram of each lysate was immunoprecipitated with anti-Met antibody. Precipitated proteins were resolved on an 8% gel and probed with anti-ubiquitin antibody. The ratio of ubiquitinated Met to total precipitated Met based on the band intensity in (A) was measured by Sion image analysis software. (B). HGF/SF-induced receptor down-regulation of Met-SM is impaired. NIH 3T3 cells stably expressing Met-WT or Met-SM were cultured in serum free medium containing HGF/SF (100 scatter unit/ml) for 24 h. Then the cells were pulse-labeled with [$^{35}$S]Trans label mix (0.2 mCi/ml) and chased for the indicated hours under the continuing presence of exogenous HGF/SF (100 scatter unit/ml).

in the same construct and since there was no difference in the amount of Met proteins in the absence of HGF/SF, it is highly unlikely that this difference in the amount of protein resulted from the difference in the rate of *de novo* protein synthesis.

Ubiquitination is required for ligand-induced degradation of Met (Jeffers *et al*., 1997b). We therefore, examined the ubiquitination of Met-WT and Met-SM upon HGF/SF treatment. The NIH 3T3 clones expressing Met-WT and Met-SM were treated with HGF/SF for different times. Met protein was immunoprecipitated from cell lysates and the ubiquitinated Met was detected by Western blot analysis with anti-ubiquitin antibody. HGF/SF induced rapid and strong ubiquitination of Met-WT, whereas the ubiquitination of Met-SM was significantly delayed (Figure 4A).

To examine whether the delayed ubiquitination of Met-SM was associated with increased stability, we performed pulse-chase analysis. After 1 h of pulse labeling, two bands with molecular weights of 170 kDa and 140 kDa respectively were detected. The 140 kDa band is the mature form of Met derived from the 170 kDa precursor form. Following pulse-chase analysis, in the presence of HGF/SF, Met-SM showed decreased degradation when compared with Met-WT (Figure 4B). Taken together, the accumulation of SM-Met resulted at least partially from decreased degradation possibly caused by the delay in the ligand-induced ubiquitination of Met.

## Discussion

One of the authors previously identified an HGF/SF receptor isoform (Met-SM form) that has a deletion of 47 amino acids in the juxtamembrane domain, apparently by RNA splicing (Lee *et al*., 1994). The differential splicing and its resultant protein occur in a variety of mouse tissues, suggesting the biological significance of this isoform. We have tried to find Met-SM in rat tissues and human tissues by RT-PCR. While the Met-SM was present in various tissues from rat, it was not observable from any human normal tissues tested (unpublished observation by J.H. Lee). Multiple reasons may contribute to the different expression of Met-SM in normal tissue between murine and human (reviewed by Lopez *et al*., 1998). In spite of the difference in the expression in normal tissue, its strong oncogenic activity shown in this study suggests its potential role in human cancer. Recently, some researchers claimed to find Met-SM from a primary human lung cancer sample (Ma *et al*., 2005), strongly suggesting possible involvement of this alternative splicing form in human tumorigenesis.

The role of the juxtamembrane region of Met in cell transformation is relatively unknown. It was reported that the addition of the juxtamembrane domain to TPR-Met fusion protein abolished the transforming activity of TPR-Met (Vigna *et al*., 1999), suggesting this domain has inhibitory effect on the transforming activity of activated Met. Actually, the part of juxtamembrane domain deleted in Met-SM was believed to act as regulatory sites of the enzymatic activity of Met. Specifically, two sites have been described as regulatory sites. Ser985 (Ser983 in mouse Met, accession number: NP032617, NCBI) was described as a substrate of PKC, and its phosphorylation by PKC results in the downregulation of tyrosine kinase activity of Met (Gandino *et al*., 1994). In addition, it was reported that a specific protein tyrosine phosphatase, PTP-S, binds to this region

and acts as a down-regulation mechanism after the activation of Met receptor by its ligand (Villa-Moruzzi *et al*., 1998). The remarkable difference in tyrosine phosphorylation level between two forms after adding HGF/SF strongly suggests the difference in down-regulation mechanism.

There were some reports showing the involvement of a RING-finger protein c-Cbl in the degradation of c-Met protein by ubiquitination (Peschard *et al*., 2001; Taher *et al*., 2002). They showed that the tyrosine residue Y1003 (Y1001 in mouse Met, accession number: NP032617, NCBI) in the juxtamembrane domain is essential for the binding of c-Cbl to Met. Since Y1003 resides in the exon-14 coding region, it was highly probable that Met-SM is less ubiquitinated than Met-WT. Consistent with these reports, we detected a significantly delayed ubiquitination of Met-SM in response to HGF/SF treatment relative to that of Met-WT. The delayed ubiquitination of Met-SM correlated with its enhanced stability as revealed by pulse-chase analysis, suggesting that differences in the protein degradation pathway may responsible for the enhanced oncogenic activity.

Although the NIH 3T3 cells used in this study secrete two scatter units/ml of HGF/SF (data not shown), this secretion didn't affect much in terms of the amount of tyrosine phosphorylation (see Figure 1). However, the difference in the focus-forming assay was significant between the two Met forms even in the absence of exogenous HGF, suggesting possible qualitative differences in the downstream signaling. One of the authors showed the differential induction of three tyrosine-phosphorylated proteins by the receptor isoforms in COS-7 cells, suggesting that the juxtamembrane domain of Met plays a role in selective signal transduction (Lee *et al*., 1995). Furthermore, the p85 subunit of phosphatidylinositol-3-OH kinase (PI3 kinase) co-precipitated with the small isoform of the HGF/SF receptor, suggesting that the juxtamembrane region plays a role in negative regulation of the binding of PI3 kinase to the HGF/SF receptor (Lee *et al*., 1995). Multiple cellular responses are regulated by PI3K, including cell growth, inhibition of apoptosis, actin cytoskeleton reorganization, vesicle transport, and cellular transformation (reviewed by Cantley *et al*., 2002). Moreover, PI3K activity is reported to be critical for Met-mediated cell migration, *in vitro* tubulogenesis (Derman *et al*., 1995), and resistance to apoptotic agents in MDA-MB-453 cells and in glioma cell lines (Bowers *et al*., 2000; Fan *et al*., 2000). The majority of activated PI3K downstream from the Met receptor is associated with the multisubstrate adaptor protein Gab-1 (Furge *et al*., 2000), whose recruitment and phosphorylation requires Y15 and, to a lesser extent, Y14 (Nguyen *et al*., 1997). Consistent with this, the Y14, 15FF mutation abolished the transforming activity of Met-SM in both the absence and presence of HGF/SF. However, since Y14, 15 is known to be a docking site for several downstream signaling molecules such as PLC-r, pp60-Src, and Grb-2 (Lee *et al*., 1995), we can not exclude the possible involvement of other signaling molecules in the transforming activity of Met-SM.

In this paper, we have shown the transforming activity of Met-SM, a natural isoform of Met receptor produced by alternative splicing. This strongly suggests the possibility of the role of Met-SM in tumorigenesis by HGF/SF-Met signaling. It would be very interesting to explore the role of Met-SM in different human cancers reported to show enhanced HGF-Met signaling. Moreover, since this alternative splicing form presents in normal tissues from as early as the day-9 fetus of mice (Lee *et al*., 1994), it may also play a role during normal mouse development.

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