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c-Met Function Requires N-Linked Glycosylation Modification of Pro-Met

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ABSTRACT

c-Met, the receptor for hepatocyte growth factor (HGF), is cell surface tyrosine kinase that controls cancer cell growth, survival, invasion, and metastasis. Post-translational modification, such as glycosylation, plays an essential role in regulating the function of cell surface molecules. Whether glycosylation modification regulates the enzymatic properties of c-Met is unknown. In this study, we investigated the effect of glycosylation on the function of c-Met. We found that c-Met is an N-linked glycosylated protein. Both pro-Met and p145Met (the β subunit of mature c-Met) have N-linked glycosylation. Glycosylation inhibitor studies revealed that the *N*-glycosylation modification of p145Met is from pro-Met, but not due to the further modification of pro-Met. Importantly, blocking the *N*-glycosylation targets pro-Met to cytoplasm and initiates its phosphorylation independent of HGF engagement. Nonglycosylated pro-Met activates c-Met downstream pathways to a certain extent to compensate for the degradation of p145Met induced by glycosylation blocking-mediated endoplasmic reticulum (ER) stress. J. Cell. Biochem. 114: 816–822, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: c-Met; GLYCOSYLATION; PHOSPHORYLATION; pro-Met

Hepatocyte growth factor (HGF) receptor, c-Met, is a transmembrane tyrosine kinase. Phosphorylation on Tyr-1234/1235 residues are crucial for c-Met activation, whereas phosphorylation on Tyr-1349/1356 residues are essential for c-Met to form the docking site and activate its downstream signaling pathways [Ferracini et al., 1991; Dai et al., 2012]. Upon HGF binding, Tyr-1234/1235 residues in the catalytic domain of c-Met are phosphorylated and the receptor is activated. Once activated, c-Met activates multiple downstream signaling pathways, including the PI3K/Akt and MEK/ERK pathways [Boccaccio and Comoglio, 2006; You et al., 2011; Dai et al., 2012]. Through these intermediary pathways, HGF/c-Met governs a diversity of important cellular responses, including proliferation, differentiation, and migration [Gay et al., 1999; Boccaccio and Comoglio, 2006; Forte et al., 2006]. Deregulation of HGF/c-Met signaling is implicated in the development and metastasis of many types of human tumor [Ueki et al., 1997; Wang et al., 2001; Boccaccio and Comoglio, 2006; Benvenuti and Comoglio, 2007; Bellon et al., 2008; You et al., 2011].

Both transcriptional and post-translational mechanisms control the expression of c-Met and its roles in extracellular signaling transduction. It has been reported that aberrant activation of c-Met is strongly associated with overexpression of the receptor without HGF engagement [Desiderio, 2007; You et al., 2011]. c-Met undergoes post-translational modification, including glycosylation, disulfide bonds formation and endoproteolytic cleavage. c-Met is initially synthesized as a partially glycosylated single-chain precursor (pro-Met) in the endoplasmic reticulum (ER). Pro-Met undergoes disulfide bonds formation, posttranslational glycosyla-

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Run Chen, Juan Li, and Chunhong Feng contributed equally to this work.

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tion, and endoproteolytic cleavage to produce the mature heterodimeric form of c-Met (p190Met$^{\alpha\beta}$) [Giordano et al., 1989]. p190Met$^{\alpha\beta}$ consists of a 50-kDa α subunit (p50Met) and a 145-kDa β subunit (p145Met) which are joined by disulfide bonds. The α subunit is entirely extracellular, while the β subunit spans the membrane and contains the tyrosine kinase catalytic domain [Giordano et al., 1989].

In this study, we investigated the roles of glycosylation in c-Met function. We found that c-Met undergoes N-linked but not O-linked glycosylation. Interestingly, N-linked glycosylation (*N*-glycosylation) blocking targets pro-Met to cytoplasm and initiates its phosphorylation. Moreover, cytoplasm located pro-Met cannot activate c-Met downstream pathways as effectively as mature c-Met. These data indicate that *N*-glycosylation modification is required for the effective function of c-Met.

MATERIALS AND METHODS

MATERIALS

Tunicamycin (Tun, inhibitor of *N*-glycosylation), *N*-glycanase, *O*-glycanase, MG132 (proteasome inhibitor), and cycloheximide (CHX, protein synthesis inhibitor) were purchased from Sigma Chemical Company. The c-Met inhibitor PF-2341066 was purchased from Selleck Chemicals. Antibodies against c-Met, GRP78, and β-actin were purchased from Santa Cruz Biotechnology. Antibodies against phospho-Akt (Ser-473), phospho-ERK (Thr-202/Tyr204), phospho-Met (Tyr-1234/1235/1349), Akt, ERK, and were purchased from Cell Signaling Technology.

CELL CULTURE AND TREATMENTS

Human HCC cell line MHCC-97H and human cholangiocarcinoma cell line RBE were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator containing 5% CO_2 and 95% ambient air at 37°C.

WESTERN BLOT

Cells were lysed in Triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 10 mM NaF, 5 mg/ml aprotinin, 20 mM leupeptin, and 1 mM sodium orthovanadate) and centrifuged at 12,000*g* for 15 min. Protein concentrations were measured using the BCA assay. Protein samples were denatured with 4× SDS-loading buffer (200 mM Tris, pH 6.8, 8% SDS, 400 mM DTT, 0.4% bromophenol blue, 40% glycerol) at 100°C for 5 min and subjected to standard SDS–PAGE and Western blot analysis as previously described [Dai et al., 2009].

DEGLYCOSYLATION

Deglycosylation was performed according to the manufacturer's manual. A denaturation process was required for the deglycosylation of glucose oxidase. For treatment with *N*-glycanase/*O*-glycanase, 20 μl of G7 buffer, 20 μl of 10% Nonidet P-40, and 5 μl of *N*-glycanase/*O*-glycanase were added, and the mixture was incubated at 37°C for 3 h. The deglycosylated protein samples were subjected to standard SDS–PAGE and Western blot analysis.

IMMUNOFLUORESCENCE STAINING AND CONFOCAL MICROSCOPY ANALYSIS

Cells were re-plated on chamber slides. When cultured to 60% confluence, cells that were incubated with anti-phospho-Met and anti-Met antibodies conjugated to CY3 (Invitrogen) for immunofluorescence and confocal microscopy assay.

HUMAN HGF ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

A total of 5×10^6 cells were cultured in 2 ml of RPMI-1640 medium in 60-mm culture dishes for 24 h and medium was collected. The HGF concentration was measured using human HGF ELISA kit (R&D systems) according to manufacturer's protocol.

MUTATION ANALYSIS

Total RNA was isolated with TRIzol reagent (Invitrogen) according to manufacturer's protocol. Complementary single strand DNA was

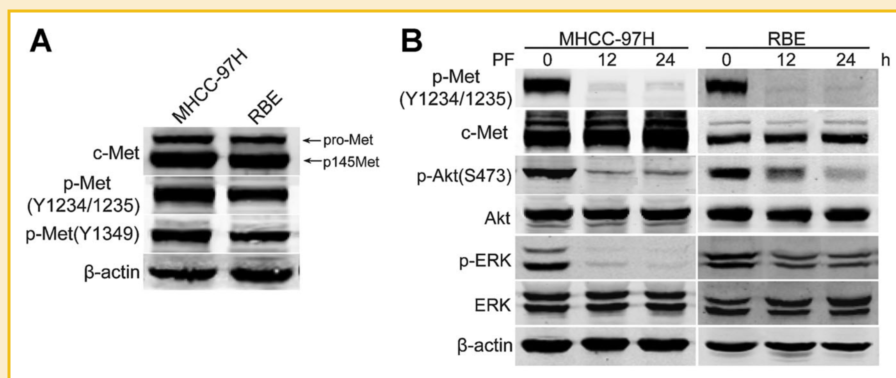

Fig. 1. c-Met expression and its downstream pathways. A: Western blot analysis of phosphorylation and protein levels of c-Met in MHCC-97H and RBE cells. B: c-Met inhibition inhibits PI3K/Akt and MEK/ERK activation. After treated with PF-2341066 (PF, 100 nM) for indicated time periods, MHCC-97H and RBE cells were subjected to Western blot analysis.

synthesized using an omniscript RT kit (Qiagen). The primers used in this study were as follows: sense primer, 5′-CTTTGTGAGCAGATGCGGAG-3′ and anti-sense primer, 5′-CAGGTATAGGCAGTGACAAG-3′. PCR product was purified using a QIAquick Gel Extraction kit (Qiagen) and sequenced at Beijing Genomics Institute (BGI). Sequencing results were compared to human c-Met gene sequence using blast alignment analysis.

RESULTS

c-Met EXPRESSION AND ITS DOWNSTREAM PATHWAYS

First, we examined the expression of phosphorylated and non-phosphorylated c-Met in MHCC-97H and RBE cells. As shown in Figure 1A, MHCC-97H and RBE cells showed strong expression and constitutive phosphorylation of c-Met. As PI3K/Akt and MEK/ERK are two typical downstream pathways of c-Met [Boccaccio and Comoglio, 2006; You et al., 2011; Dai et al., 2012], we tested whether the phosphorylation of Akt and ERK are c-Met-dependent in MHCC-97H and RBE cells. The results showed that c-Met inhibitor PF-2341066 obviously decreased the basal activation of Akt and ERK (Fig. 1B), indicating that PI3K/Akt and MEK/ERK are downstream pathways of c-Met in MHCC-97H and RBE cells.

Since HGF is the natural ligand for c-Met activation, we investigated whether the basal activation of c-Met was due to autocrine secretion of HGF. The enzyme-linked immunosorbent assay (ELISA) analysis failed to demonstrate any HGF secreted by MHCC-97H and RBE cells (data not shown). As the mutations in c-Met gene correlate with the auto-activation of this receptor in multiple different cancers [Jeffers et al., 1997, 1998; Schmidt et al., 1999], we investigated whether the activation of c-Met in MHCC-97H and RBE cells was due to a mutation. Sequencing of the c-Met gene demonstrated none of the reported mutations was found in MHCC-97H and RBE cells (data not shown). Taken together, these results suggest that sustained high basal activation of c-Met in MHCC-97H and RBE cells independent of HGF production and gene mutation.

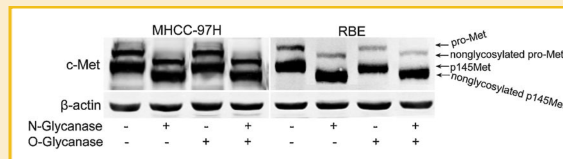

Fig. 2. c-Met is an N-linked glycosylated protein. Following digested with *N*-glycanase and *O*-glycanase, samples of MHCC-97H and RBE cells were analyzed via Western blot.

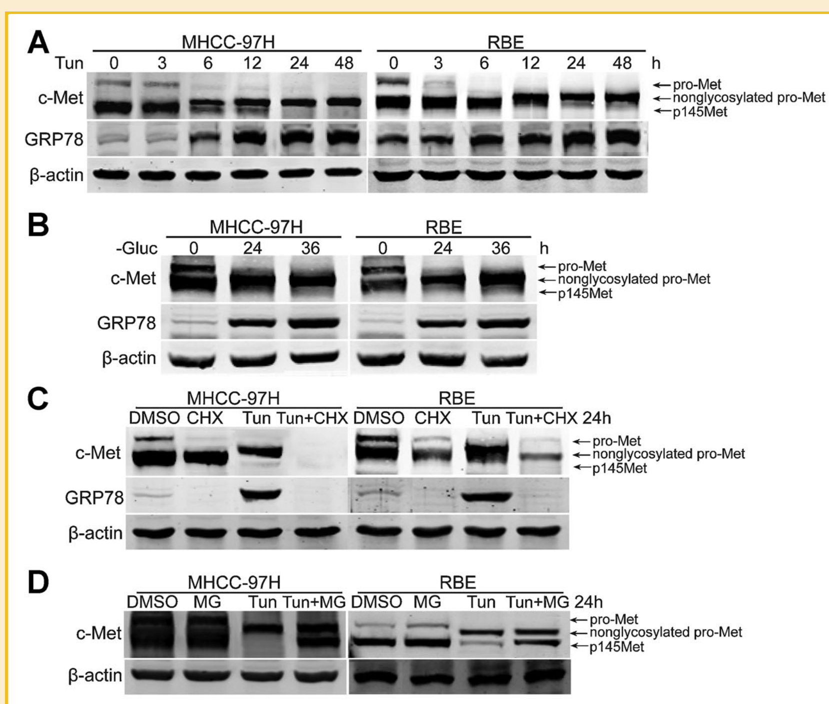

Fig. 3. N-linked glycosylation of Pro-Met responsible for the glycosylation modification of c-Met. A: Tunicamycin inhibits pro-Met glycosylation. After treated with tunicamycin (Tun, 2.5 μg/ml) for indicated time periods, MHCC-97H and RBE cells were subjected to Western blot analysis. B: Glucose deprivation inhibits pro-Met glycosylation. After incubated in glucose-deficient culture media for indicated time periods, MHCC-97H and RBE cells were subjected to Western blot analysis. C: Cycloheximide inhibits the expression of nonglycosylated pro-Met upon tunicamycin treatment. After treated with tunicamycin (Tun, 2.5 μg/ml) for indicated time periods with or without cycloheximide (CHX, 10 μM) pre-incubation for 1 h, MHCC-97H and RBE cells were subjected to Western blot analysis. D: MG132 inhibits tunicamycin-induced p145Met degradation. After treated with tunicamycin (Tun, 2.5 μg/ml) for 24 h with or without MG132 (MG, 20 μM) pre-incubation for 1 h, MHCC-97H and RBE cells were subjected to Western blot analysis.

c-Met IS AN N-LINKED GLYCOSYLATED PROTEIN

To investigate the effect of glycosylation on the function of c-Met, we analyzed the *N*-glycosylation and *O*-glycosylation modification of c-Met in MHCC-97H and RBE cells. NetNGlyc, a glycosylation analysis program (http://www.cbs.dtu.dk/services/NetNGlyc/), predicts that c-Met has multiple N-linked but not O-linked glycosylation sites. Consistent with this prediction, we observed a discrepancy in the molecular mass of pro-Met and p145Met and their mobility on SDS gels upon *N*-glycanase treatment (Fig. 2). However, *O*-glycanase treatment had no effect on this molecular mass shift of pro-Met and p145Met (Fig. 2), indicating that c-Met is an N-linked but not O-linked glycosylated protein.

PRO-MET RESPONSIBLE FOR N-LINKED GLYCOSYLATION OF c-Met

Since both pro-Met and p145Met are glycosylated, it is interest to analyze the more detailed mechanism of c-Met glycosylation. To investigate whether pro-Met undergoes further glycosylation to yield p145Met, tunicamycin, an inhibitor of N-linked glycosylation, was used in our study. The results showed that tunicamycin treatment caused a progressive shift in pro-Met molecular mass over a period of 3–48 h (Fig. 3A), confirming that pro-Met is an *N*-glycosylated protein. It is notable that tunicamycin did not cause a shift in p145Met molecular mass (Fig. 3A), indicating that pro-Met does not undergo further *N*-glycosylation after its translation. The results that glycosylated pro-Met responsible for N-linked glycosylation modification of c-Met was confirmed by our data which showed that glucose deprivation resulted in a progressive shift in the molecular mass of pro-Met, but not in p145Met (Fig. 3B). Furthermore, protein synthesis inhibitor CHX pre-treatment blocked tunicamycin-mediated nonglycosylated pro-Met induction (Fig. 3C), confirming that pro-Met synthesis responsible for the post-translational *N*-glycosylation of c-met.

In addition to inhibiting pro-Met glycosylation, tunicamycin treatment also led to a marked reduction in protein level of p145Met in a time-dependent manner (Fig. 3A,B). Incubation of MHCC-97H and RBE cells with tunicamycin induced the induction of ER stress biomarker glucose-regulated protein 78 (GRP78; Fig. 3A,C), indicating the activation of ER stress response. As tunicamycin induces ER stress through inhibiting N-linked glycosylation, it is reasonable that tunicamycin promotes p145Met degradation through ER stress associated degradation. This notion is supported by the data that proteasome inhibitor MG132 inhibited tunicamycin-induced p145Met degradation (Fig. 3D). Furthermore, MG132 inhibited tunicamycin-induced p145Met degradation without

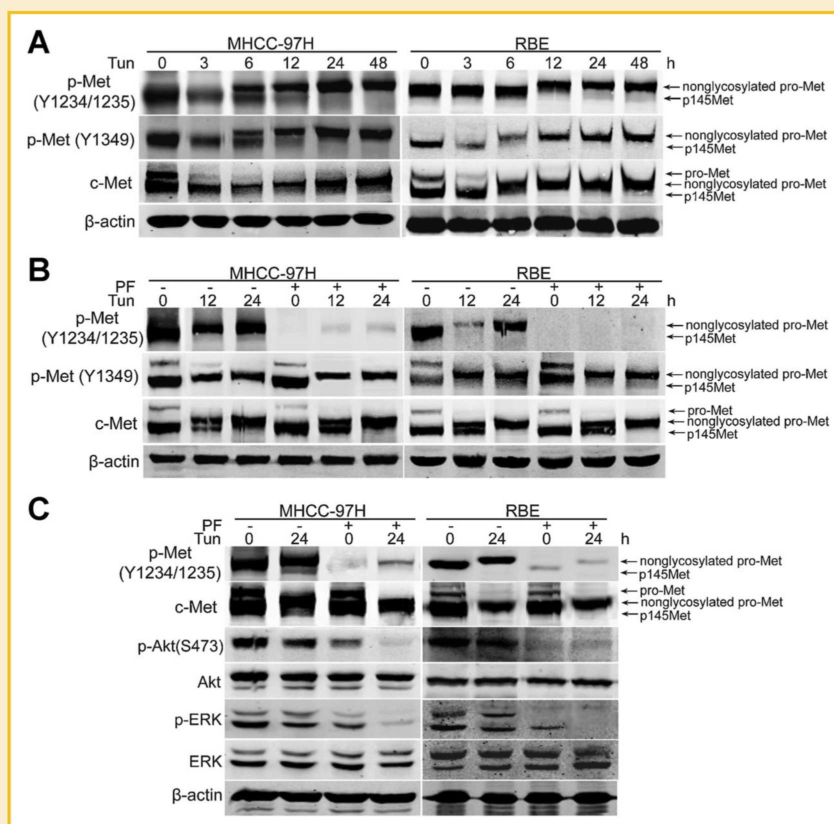

Fig. 4. Glycosylation blocking initiates the phosphorylation of pro-Met. A: Tunicamycin initiates pro-Met phosphorylation. After treated with tunicamycin (Tun, 2.5 μg/ml) for indicated time periods, MHCC-97H and RBE cells were subjected to Western blot analysis. B: PF-2341066 inhibits tunicamycin-induced pro-Met phosphorylation. After treated with tunicamycin (Tun, 2.5 μg/ml) for indicated time periods with or without PF-2341066 (PF, 100 nM) pre-incubation for 1 h, MHCC-97H and RBE cells were subjected to Western blot analysis. C: PF-2341066 blocks the activity of nonglycosylated pro-Met and inhibits PI3K/Akt and MEK/ERK phosphorylation. After treated with tunicamycin (Tun, 2.5 μg/ml) for 24 h with or without PF-2341066 (PF, 100 nM) pre-incubation for 1 h, MHCC-97H and RBE cells were subjected to Western blot analysis.

nonglycosylated p145Met induction (Fig. 3D) confirmed the notion that pro-Met does not undergo further glycosylation to yield p145Met.

DEGLYCOSYLATION INITIATES THE PHOSPHORYLATION OF PRO-MET

As *N*-glycosylation plays an important role in the function of cell surface molecules, we addressed whether *N*-glycosylation regulates the phosphorylation of pro-Met. As shown in Figure 4A, pro-Met cannot be detected by antibodies against phosphorylated form of c-Met, indicating that pro-Met is not phosphorylated in MHCC-97H and RBE cells. Interestingly, tunicamycin-induced nonglycosylated pro-Met was detected by antibodies against phosphorylated form of c-Met, suggesting that the nonglycosylated pro-Met is phosphorylated (Fig. 4A). Furthermore, the phosphorylation of nonglycosylated pro-Met was blocked by c-Met inhibitor PF-2341066 (Fig. 4B).

To investigate the function of nonglycosylated pro-Met, we analyzed the effect of *N*-glycosylation blocking on c-Met downstream pathways. As shown in Fig. 4C, tunicamycin treatment decreased the phosphorylation levels of Akt and ERK in MHCC-97H and RBE cells. More importantly, c-Met inhibitor PF-2341066 not only blocked the phosphorylation of nonglycosylated pro-Met but also decreased the phosphorylated Akt and ERK in tunicamycin-treated MHCC-97H and RBE cells (Fig. 4C). Together, these data suggest that nonglycosylated pro-Met can activate, at least in part, c-Met downstream pathways.

GLYCOSYLATION IS REQUIRED FOR c-Met MEMBRANE TARGETING

To test the role of *N*-glycosylation in the cellular location of c-Met, we investigated the cellular localization of c-Met in tunicamycin-treated MHCC-97H cells. First, we monitored the location of c-Met in MHCC-97H cells. As shown in Figure 5A,B, both phosphorylated and non-phosphorylated c-Met were membrane targeting in DMSO-treated MHCC-97H cells. After treated with tunicamycin for 24 h, both phosphorylated and non-phosphorylated pro-Met accumulated in the cytoplasm in MHCC-97H cells (Fig. 5A,B). Hence, *N*-glycosylation is required for c-Met membrane targeting.

DISCUSSION

Glycosylation is a critical step in the maturation of the majority of the proteins that traffic through the ER [Hebert and Molinari, 2007]. N-linked glycosylation is often essential for the folding, intracellular transport, secretion, and function of glycoproteins [Branza-Nichita et al., 2004; Ambasta et al., 2007]. In this study, we investigated the effect of glycosylation on c-Met function. Our data demonstrated that c-Met is an *N*-glycosylated protein, and *N*-glycosylation is an essential post-translational modification mechanism for the function of c-Met.

Glycosylation of c-Met was removed by *N*-glycanase but not by *O*-glycanase, indicating that c-Met is an *N*-glycosylated protein. Considering that both pro-Met and p145Met, which is processed

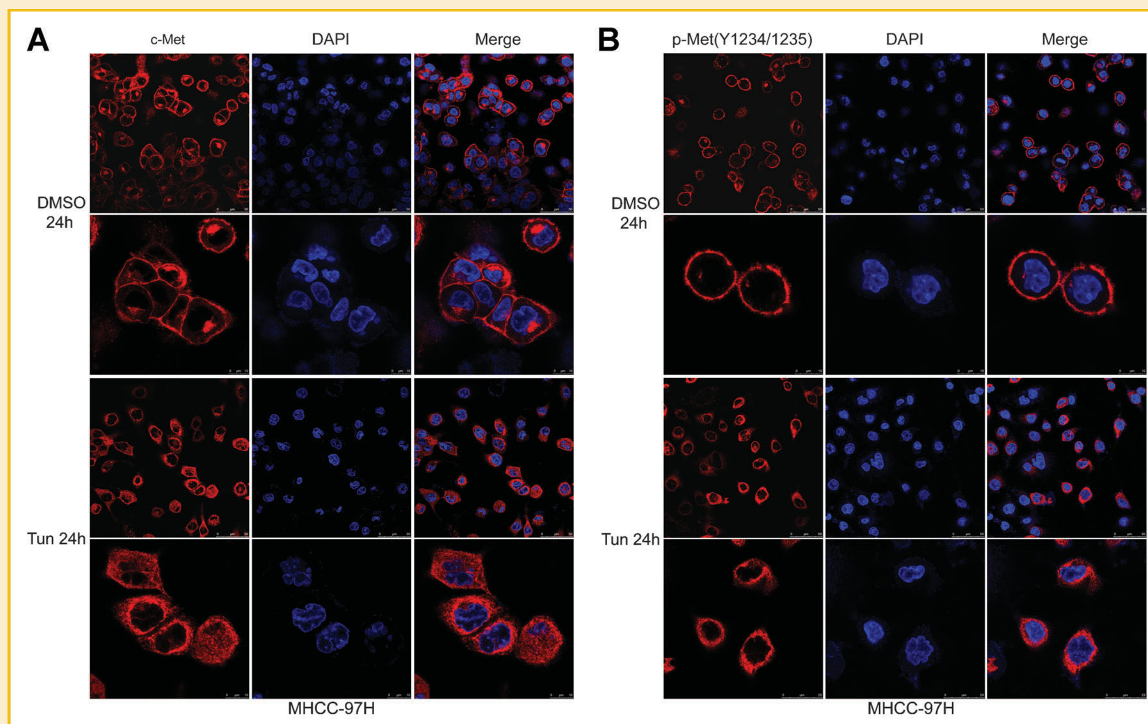

Fig. 5. Glycosylation is required for c-Met membrane targeting. A: Localization of c-Met in MHCC-97H cells. After treated with dimethyl sulfoxide (DMSO) or tunicamycin (Tun, 2.5 μg/ml) for 24 h, MHCC-97H cells were subjected to immunofluorescence staining and confocal microscopy analysis. B: Localization of phosphorylated c-Met in MHCC-97H cells. After treated with dimethyl sulfoxide (DMSO) or tunicamycin (Tun, 2.5 μg/ml) for 24 h, MHCC-97H cells were subjected to immunofluorescence staining and confocal microscopy analysis.

from pro-Met, were *N*-glycosylated proteins, we asked whether pro-Met is further glycosylated to yield p145Met. Based on the data that *N*-glycosylation inhibitor tunicamycin inhibited *N*-glycosylation of pro-Met without p145Met *N*-glycosylation inhibition, we suggest that c-Met is initially synthesized as an *N*-glycosylated pro-Met, and pro-Met does not undergo further glycosylation to yield p145Met. Thus, the c-Met mRNA is translated into an *N*-glycosylated pro-Met in ER under physiological conditions, and the pro-Met folds into a structure stabilized by intra-chain disulfide bonds without further glycosylation. Then, the pro-Met is cleaved by a protease to originate mature c-Met.

MHCC-97H and RBE cells express high levels of phosphorylated c-Met without HGF autocrine and gene mutation. Interestingly, nonglycosylated pro-Met was detected by antibodies against phosphorylated form of c-Met in MHCC-97H and RBE cells. As pro-Met was not phosphorylated in MHCC-97H and RBE cells, it is reasonable to suggest that removal of *N*-glycosylation initiates the phosphorylation of pro-Met. As nonglycosylated pro-Met is phosphorylated on Tyr-1234/1235/1349 independent of HGF engagement, it is interesting to investigate the function of nonglycosylated pro-Met. Since the activation of c-Met downstream pathways PI3K/Akt and MEK/ERK were c-Met-dependent in MHCC-97H and RBE cells, the function of nonglycosylated pro-Met can be analyzed through investigating the effect of nonglycosylated pro-Met on PI3K/Akt and MEK/ERK activation. As blocking nonglycosylated pro-Met by c-Met inhibitor PF-2341066 inhibited the phosphorylation of PI3K/Akt and MEK/ERK in tunicamycin-treated MHCC-97H and RBE cells, it is reasonable to suggest that nonglycosylated pro-Met sustains the activation of c-Met downstream pathways, such as PI3K/Akt and MEK/ERK, to a certain extent.

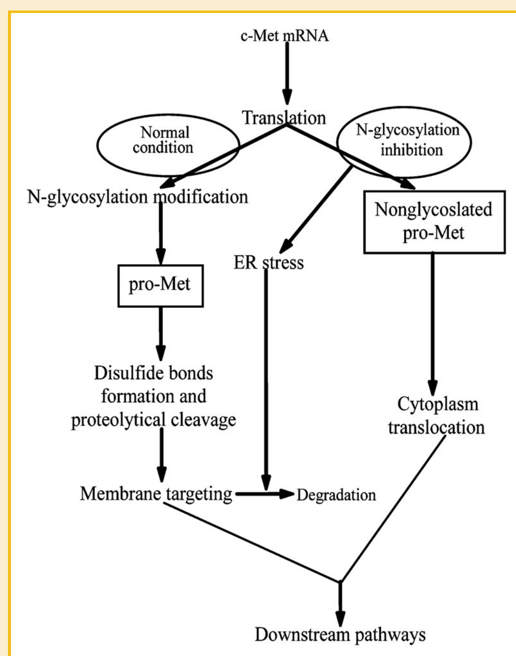

Fig. 6. Schematic presentation of the effect of *N*-glycosylation blocking on c-Met function. Under physiological conditions, c-Met mRNA is translated into *N*-glycosylated pro-Met in ER. Within a few minutes, *N*-glycosylated pro-Met folds into a structure stabilized by intra-chain disulfide bonds. Then, the pro-Met is cleaved by a protease to originate membrane targeting c-Met. Once activated, membrane targeting c-Met activates its downstream pathways effectively. Blocking N-linked glycosylation promotes the translocation of pro-Met to cytoplasm without further procession. Cytoplasm location initiates the phosphorylation of nonglycosylated pro-Met to activate c-Met downstream pathways to a certain extent, which compensating for the degradation of p145Met induced by glycosylation blocking-mediated ER stress.

An important question now before us is why nonglycosylated pro-Met but not glycosylated pro-Met can be phosphorylated. Under physiological conditions, the c-Met mRNA is translated into *N*-glycosylated pro-Met in ER. Within a few minutes, *N*-glycosylated pro-Met folds into a structure stabilized by intra-chain disulfide bonds in ER. Then, the pro-Met is cleaved by a protease to originate membrane targeting c-Met. Thus, pro-Met is located in the ER in the process of c-Met maturation, and it is reasonable to suppose that pro-Met cannot be phosphorylated in the ER. However, removing the *N*-glycosylation of pro-Met inhibits its further procession and maturation. It has been accepted that unfolded protein will be exported to the cytosol and subsequently degradated by ubiquitin proteasome system [Eisele and Wolf, 2008]. As we demonstrated that nonglycosylated pro-Met is translocated to cytoplasm. Thus, it is reasonable to suggest that cytoplasm location results in the phosphorylation of nonglycosylated pro-Met. Considering that membrane targeting is critical for c-Met to activate its downstream pathways, we speculate that nonglycosylated pro-Met cannot effectively activate c-Met downstream pathways duo to its cytoplasm location.

In brief, the present work reveals that *N*-glycosylation is required for c-Met function. *N*-glycosylation blocking attenuates c-Met function, at least in part, through inhibiting its cell membrane targeting (Fig. 6). Future studies of the role of specific *N*-glycosylation sites may provide further insights into the regulatory functions of glycosylation in c-Met trafficking and signaling functions.

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