Bi-directional Regulation of Ser-985 Phosphorylation of c-Met via Protein Kinase C and Protein Phosphatase 2A Involves c-Met Activation and Cellular Responsiveness to Hepatocyte Growth Factor*

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Previous studies indicated that treatment of cells with 12-O-tetradecanoylphorbol-13-acetate induced phosphorylation of Ser-985 at the juxtamembrane of c-Met, the receptor tyrosine kinase for hepatocyte growth factor (HGF), and this was associated with decreased tyrosine phosphorylation of c-Met. However, the regulatory mechanisms and the biological significance of the Ser-985 phosphorylation in c-Met remain unknown. When A549 human lung cancer cells were exposed to oxidative stress with H$_2$O$_2$, H$_2$O$_2$ treatment induced phosphorylation of Ser-985, but this was abrogated by an inhibitor for protein kinase C (PKC). Likewise, treatment of cells with NaF (an inhibitor of protein phosphatases) allowed dephosphorylation of Ser-985, and a protein phosphatase responsible for dephosphorylation of Ser-985 was identified to be protein phosphatase 2A (PP2A). The effects of PKC inhibitors revealed that PKC$\delta$ and $\epsilon$ were responsible for the Ser-985 phosphorylation of c-Met, and pull-down analysis indicated that associations of PKC$\delta$ and $\epsilon$ with c-Met may be involved in the regulation of Ser-985 phosphorylation of c-Met. Instead, PP2A was constitutively associated with c-Met, whereas its activity to dephosphorylate Ser-985 of c-Met was decreased when cells were exposed to H$_2$O$_2$. Addition of HGF to A549 cells in culture induced c-Met tyrosine phosphorylation, the result being mitogenic response and cell scattering. In contrast, in the presence of H$_2$O$_2$ stress, HGF-dependent tyrosine phosphorylation of c-Met was largely suppressed with a reciprocal relationship to Ser-985 phosphorylation, and this event was associated with abrogation of cellular responsiveness to HGF. These results indicate that Ser-985 phosphorylation of c-Met is bi-directionally regulated through PKC and PP2A, and the Ser-985 phosphorylation status may provide a unique mechanism that confers cellular responsiveness/unresponsiveness to HGF, depending on extracellular conditions.

Hepatocyte growth factor (HGF),* originally identified and cloned as a mitogenic protein for hepatocytes (1–3), evokes multiple cellular responses, including mitogenesis, morphogenesis, migration, and anti-apoptosis (4–6). These biological activities of HGF are triggered by tyrosine phosphorylation of c-Met, a specific receptor tyrosine kinase for HGF (7). Biological activities of HGF support tissue organization during development and regeneration of organs, including the liver, kidney, placenta, and skeletal muscle (4–6), but unregulated and/or constitutive activation of the c-Met receptor endows tumor cells with invasive and metastatic characteristics (8, 9).

The c-Met receptor is a heterodimeric protein composed of extracellular $\alpha$-chain and membrane-spanning $\beta$-chain, which contains an intracellular tyrosine kinase domain. Specific binding of HGF to the c-Met receptor activates tyrosine kinase activity, thereby facilitating phosphorylation of C-terminal tyrosine residues, so-called multifunctional docking sites (10, 11). In addition to the catalytic tyrosine kinase domain, the $\beta$-chain contains a juxtamembrane domain of 47 amino acid residues, which is highly conserved in distinct species (12). Previous studies indicated that a serine residue at position 985, which resides in the juxtamembrane domain of c-Met, is phosphorylated by treatment of cells with 12-O-tetradecanoylphorbol-13-acetate (TPA), an activator for protein kinase C (PKC). Most interesting, Ser-985 phosphorylation was associated with decreased tyrosine phosphorylation of the c-Met receptor (13); however, neither regulatory mechanisms nor the biological significance of the Ser-985 phosphorylation of c-Met has been elucidated.

In the present study, we found that the phosphorylation status of juxtamembrane Ser-985 of the c-Met receptor is bi-directionally regulated through reverse activities of PKC$\delta$/$\epsilon$ and protein phosphatase 2A (PP2A), a serine/threonine protein phosphatase. Likewise, oxidative stress in cells induced PKC-mediated Ser-985 phosphorylation, and this was associated with inhibition of c-Met tyrosine phosphorylation and subsequent biological responses upon HGF stimulus. Our observations mean that the Ser-985 phosphorylation of c-Met mediated via PKCs and PP2A provides a unique mechanism, which confers cellular responsiveness/unresponsiveness to HGF, depending on the extracellular environment and conditions.

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**EXPERIMENTAL PROCEDURES**

**Materials**—A polyclonal anti-human c-Met antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-phospho-c-Met (pY1234/1235) antibody, monoclonal anti-phosphotyrosine (4G10) and anti-P2PA antibodies, recombinant PKC isoforms, recombinant protein phosphatase 1 (PP1), and protein phosphatase 2A (PP2A) proteins were purchased from Upstate Biotechnology, Inc. A polyclonal anti-phospho-c-Met (pY1365) antibody was purchased from BIO SOURCE International (Camarillo, CA). A monoclonal anti-bromodeoxyuridine (BrdUrd) antibody and monoclonal anti-PKCK antibodies were purchased from BD Biosciences. Bisindolylmaleimide (BIM), KN-62, G6976, G6983, calyculin A, and cyclosporin A were from Calbiochem. TO-PRO-3 was from Molecular Probes (Eugene, OR). A polyclonal anti-phospho-Ser-985 c-Met antibody against synthetic peptide corresponding to the 11 amino acid sequence at the juxtamembrane domain of c-Met (amino acids 978–988) was raised in rabbit wherein Ser-985 was phosphorylated. The antibody was purified using an affinity column immobilized with the antigenic peptide.

**Cell Culture**—A549 cells obtained from the Japanese Cancer Research Resources Bank were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO2.

**Immunoprecipitation and Western Blotting**—Cells were lysed in lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 25 mM β-glycerophosphate, 50 mM NaF, 1 mM Na3VO4, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml antipain, pepstatin A, leupeptin, and 1% aprotinin and then incubated on ice for 30 min. After centrifugation, the supernatant was incubated with antibodies and precipitated by protein G-Sepharose at 4°C overnight. These immunoprecipitates were separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad). The membrane was incubated with 5% nonfat dry milk in phosphate-buffered saline at 4°C overnight, blotted with antibodies at room temperature for 2 h, and subsequently labeled with horseradish peroxidase-conjugated antibody against mouse or rabbit immunoglobulin at room temperature for 1 h. The membranes were washed, incubated with ECL immunoblotting detection reagents (Amersham Biosciences). 5-Bromo-2′-deoxyuridine (BrdUrd) Labeling—Cells were stimulated 10 ng/ml HGF for 12 h with or without 0.5 mM H2O2 and labeled with 10 μM BrdUrd during the last 2 h of the culture period. The cells were then fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 3 min. Cells were further treated with 2 M HCl for 15 min and neutralized with 0.1 M sodium tetraborate for 30 min. After washing with phosphate-buffered saline, these cells were incubated with anti-BrdUrd antibody for 2 h and subsequently with fluorescence labeled secondary antibody for 1 h. The cells were washed with phosphate-buffered saline, and the nuclei were then stained with TO-PRO-3. The number of cells stained positively for BrdUrd and nuclei was counted using fluorescence microscopy.

**Protein Kinase C Assay**—Immunoprecipitated c-Met from nonstimulated A549 cells was incubated with recombinant PKC isoforms in the reaction buffer (20 mM Tris-HCl, 10 mM MgCl2, 20 μM ATP, 25 mM β-glycerophosphate, 10 mM NaF, 1 mM Na3VO4, 1 mM dithiothreitol, 1 mM CaCl2, 0.1 mg/ml phosphatidylyserine, and 0.01 mg/ml diacylglycerol) for 30 min at 30°C. The reaction mixture was separated by SDS-PAGE, and the phosphorylated Ser-985 of c-Met was detected by Western blotting with anti-phospho-Ser-985 c-Met antibody.

**Protein Phosphatase Assay**—Ser-985 of the c-Met receptor was phosphorylated by treatment of the cells with 25 mM NaF for 30 min. Immunoprecipitated PKC isoforms from HGF- or H2O2-stimulated A549 cells were incubated with the substrate peptide in the reaction buffer containing 1 μCi of the [γ-32P]ATP for 10 min at 30°C. The reaction mixture was transferred onto a P-81 phosphocellulose paper, and this radioactivity on the paper was measured in a scintillation counter.

**RESULTS**

**Inhibition of Cellular Response to HGF by H2O2—Oxidative stress induces growth arrest in proliferating cells (14). We first asked whether the mitogenic stimulus of HGF was influenced under oxidative stress. DNA synthesis was measured by BrdUrd incorporation into A549 cells cultured in the absence or presence of HGF and/or H2O2 (Fig. 1). In the control culture, the number of cells labeled with BrdUrd reached 32%. When the cells were cultured in the presence of 0.5 mM H2O2 for 12 h, numbers of BrdUrd-positive cells decreased to 4%, indicating that serum-dependent proliferation of cells was strongly inhibited by H2O2. On the other hand, addition of HGF (10 ng/ml) stimulated BrdUrd labeling to 60%, indicating that HGF exerts mitogenic activity for A549 cells even in the presence of fetal bovine serum. However, exposure of the cells to H2O2 in the presence of HGF inhibited BrdUrd uptake to a level similar to that seen in cells treated with H2O2. These results demonstrate that the mitogenic stimulus of HGF, as well as serum components, was down-regulated by H2O2.

Because cell scattering is a unique biological response through activation of the c-Met receptor and serum components do not induce cell scattering for A549 cells (see below), we also asked if the cell scattering induced by HGF is influenced under the oxidative stress. A549 cells were cultured under the absence or presence of HGF and/or H2O2 for 12 h (Fig. 1B). HGF (10 ng/ml) significantly induced scattering of cells, although it was inhibited in the presence of 0.5 mM H2O2. Addition of H2O2 alone did not induce cell scattering. Taken together, in addition to the mitogenic response, H2O2 treatment diminished biological responses to HGF in A549 cells.

PKC-mediated Ser-985 Phosphorylation Under H2O2 Stress—To search for mechanisms by which H2O2 treatment suppressed biological responses to HGF in A549 cells, we focused on the Ser-985 phosphorylation of c-Met, as well as tyrosine phosphorylation of the c-Met receptor, because phosphorylation of Ser-985 induced by TPA treatment was associated with suppression of tyrosine phosphorylation of the c-Met receptor (13). To specifically detect phosphorylated Ser-985, we prepared a polyclonal antibody against a synthetic peptide (amino acids 978–988 in the c-Met), which contains phosphorylated Ser-985. A549 cells were treated with or without TPA, and the cell lysate was subjected to immunoprecipitation for c-Met and subsequent Western blot with anti-phosphorylated Ser-985 antibody. The positive signal was detectable only in TPA-treated cells (Fig. 2A, left panel), and reprobing with anti-c-Met antibody indicated that the positive signal for phosphorylated Ser-985 coincided with the size of the c-Met β-chain (Fig. 2A, right panel). The c-Met receptor was then immunoprecipitated, incubated with or without λ protein phosphatase, and analyzed by Western blots using anti-phosphorylated Ser-985 antibody (Fig. 2B). The immunoreactive band for phosphorylated Ser-985 was diminished when the immunoprecipitate was treated with λ protein phosphatase. Moreover, when the antibody was preadsorbed with phosphorylated Ser-985 antigenic peptide, the specific band corresponding to the phosphorylated Ser-985 of c-Met seen in TPA-treated cells was not detectable (Fig. 2C, middle panel). In contrast, the antibody preadsorbed with nonphosphorylated antigenic peptide was reactive to phosphorylated Ser-985 of c-Met from TPA-treated cells (Fig. 2C, right panel). Thus the antibody is highly specific for the phosphorylated Ser-985 of the c-Met receptor.

By using specific antibodies, phosphorylation of tyrosines and Ser-985 of c-Met was analyzed by immunoprecipitation and subsequent Western blots (Fig. 3A). Tyrosine phosphorylation of c-Met was induced within 5 min with HGF stimulus; however, the phosphorylation level strongly decreased when...
the cells were pretreated with H2O2 for 60 min prior to HGF stimulus (Fig. 3A, upper panel). In contrast to tyrosine phosphorylation of c-Met, Ser-985 was not phosphorylated by HGF but was strongly phosphorylated in the presence of H2O2 (Fig. 3A, middle panel). Therefore, H2O2 stimulus induces Ser-985 phosphorylation, and Ser-985 phosphorylation is reciprocally associated with the tyrosine phosphorylation status of the c-Met receptor.

When cells were treated with 0.5 mM H2O2 alone, phosphorylation of Ser-985 was within 10 min and was decreased but evidently sustained until 120 min after exposure to H2O2 (Fig. 3B). Ser-985 phosphorylation was induced by 0.1 mM H2O2 and increased in a dose-dependent manner (Fig. 3C). On the other hand, Ser-985 phosphorylation was almost completely inhibited by BIM (an inhibitor of PKC), indicating that PKC is involved in H2O2-induced Ser-985 phosphorylation (Fig. 3D). Reprobing of Western blots with anti-c-Met antibody revealed that the c-Met level was mostly equal at each immunoprecipitation procedure. These results indicate that changes in phospho-
phorylation levels of Ser-985 and tyrosine residues in c-Met by H$_2$O$_2$ and HGF could be attributed to phosphorylation/dephosphorylation events and that PKC is responsible for H$_2$O$_2$-induced Ser-985 phosphorylation.

**Transient Phosphorylation of Ser-985 by HGF Stimulus**—Because PKCs are downstream signal transducers of c-Met following HGF stimulus, we speculated that the HGF stimulus would induce Ser-985 phosphorylation to some extent even without H$_2$O$_2$ treatment. When cells were treated with HGF for 10 min, Ser-985 was weakly phosphorylated in a concentration-dependent manner (Fig. 4A). In the time course experiment (Fig. 4B), Ser-985 phosphorylation was detected within 10 min by stimulus with 10 ng/ml HGF and sustained for up to 60 min; however, it disappeared 120 min after the stimulus. When the same immunoblots were reprobed using anti-phosphotyrosine antibody (Fig. 4B, middle panel), c-Met was tyrosine-phosphorylated within 1 min and then gradually increased until 60 min after the stimulus. Thereafter, the tyrosine phosphorylation level slightly declined in parallel to a slight decrease in the expression level of c-Met but remained at a high level until 120 min after the stimulus. Taken together with the finding that HGF stimulus allows for a mitogenic response in A549 cells in the absence of H$_2$O$_2$, these results indicate that HGF weakly induces Ser-985 phosphorylation at least to some extent, but it is transient and allows for HGF-dependent tyrosine phosphorylation of c-Met, which is capable of inducing a mitogenic stimulus.

When phosphorylation levels of Ser-985 induced by HGF and TPA were compared, Ser-985 phosphorylation induced by HGF was much weaker than that induced by TPA (Fig. 4C). Tyrosine phosphorylation of c-Met was induced by HGF, whereas when cells were simultaneously treated with HGF and TPA, HGF-dependent tyrosine phosphorylation was much weaker than that seen with HGF alone (Fig. 4C). Thus, the level of HGF-induced tyrosine phosphorylation of c-Met was again in a re-
ciprocal relationship to the Ser-985 phosphorylation level when Ser-985 phosphorylation was induced by TPA.

Because the amino acid sequence encompassing Ser-985 (Ser-Ala-Arg) coincides with the consensus sequences in substrates of PKC ((S/T/R)(R/K)) and CaMK II (CaMK II) ((S/T/X)(R/K)), involvement of PKC and CaMK II in Ser-985 phosphorylation was analyzed by using specific inhibitors (Fig. 4D). BIM almost completely inhibited Ser-985 phosphorylation induced by TPA and HGF. In contrast, KN-62 (CaMK II inhibitor) could not reduce Ser-985 phosphorylation induced by HGF and TPA. These results suggest that PKC is responsible for HGF-induced Ser-985 phosphorylation.

PKC Isoforms Responsible for Ser-985 Phosphorylation—Because TPA targets both conventional and novel types of PKCs, we addressed PKC isoforms responsible for Ser-985 phosphorylation of c-Met. Western blot analysis using cell lysate revealed that PKC isoforms, α (conventional), δ, ε, and θ (novel) but not β, γ, and ϖ were expressed in A549 cell (Fig. 5A). We then tested whether these PKC isoforms directly phosphorylate Ser-985 of c-Met, using recombinant PKC isoforms (α, δ, ε, and θ). In vitro kinase assay for immunoprecipitated c-Met indicated that each PKC isoform phosphorylated Ser-985 of c-Met to a comparable extent, at least in vitro (Fig. 5B). To identify which PKC isoforms are responsible for Ser-985 phosphorylation in the cells, we next assessed the effects of isoform-specific inhibitors for PKC on H2O2-induced Ser-985 phosphorylation (Fig. 5C). Pretreatment of cells with BIM (inhibitor for α, δ, and ε) almost completely inhibited Ser-985 phosphorylation induced by H2O2, indicating PKC θ is not involved in H2O2-induced Ser-985 phosphorylation. G69876 (an inhibitor for α) had no effect on Ser-985 phosphorylation, whereas G69893 (an inhibitor for α and δ) partially inhibited Ser-985 phosphorylation. Collectively, these results indicate that PKCδ and -ε are responsible for H2O2-induced Ser-985 phosphorylation of c-Met.

We addressed mechanisms coordinating these activities for the regulation of Ser-985 phosphorylation. A potential mechanism would be association of PKC and c-Met. To determine whether PKCs are associated with c-Met, c-Met was immunoprecipitated from H2O2-treated cells, and the immunoprecipitates were subjected to Western blotting with anti-PKC antibodies. Association of PKCδ and -ε with c-Met was marginal in unstimulated cells, whereas it was detectable 10 min after H2O2 stimulus and was sustained for up to 60 min (Fig. 5D). These results suggested that the association of PKCδ and -ε with c-Met may be involved in the regulation of Ser-985 phosphorylation of c-Met.

To address further the PKC isoform-dependent Ser-985 phosphorylation, we measured changes in activity of PKCδ and -ε. PKC isoforms were immunoprecipitated from HGF- or H2O2-stimulated cells, respectively, and subjected to in vitro kinase assay by using synthetic peptide corresponding to the 11 amino acid residues of c-Met (amino acids 978–988), including Ser-985 as a substrate. H2O2 increased activity of PKCδ and -ε within 10 min, and the activities thereby declined to lower levels than those seen at 10 min (Fig. 5E). HGF stimulus also increased PKCδ and -ε activity with a similar time dependence to that seen in the case of H2O2 stimulus; however, the level of PKC activity during HGF stimulus was less than that enhanced by H2O2.

Involvement of Protein Phosphatase 2A in Ser-985 Dephosphorylation—Because the HGF-induced Ser-985 phosphorylation was transient and the level was weak compared with that induced by H2O2 (Fig. 3) and TPA (Fig. 4), we speculated that Ser/Thr protein phosphatases might attenuate the level of phosphorylation of Ser-985 during HGF stimulation. To address this possibility, the effect of NaF was analyzed, which is a broad inhibitor for protein phosphatases on phosphorylation levels of Ser-985 and tyrosine of c-Met receptor (Fig. 6A). In this experiment, phosphorylation of tyrosine residues at 1234/1235 and 1365 of c-Met, which are critical autophosphorylation sites to evoke intracellular signals of HGF, was analyzed by using specific antibodies. In control culture without NaF, HGF induced transient Ser-985 phosphorylation and phosphorylation of Tyr-1234/1235 and Tyr-1365. On the other hand, pretreatment with NaF for 10 min allowed for Ser-985 phosphorylation, and the level was higher than that induced by HGF stimulus, suggesting that protein phosphatase(s) is involved in the regulation of Ser-985 phosphorylation. HGF stimulation in the presence of NaF further increased Ser-985 phosphorylation to higher levels than that seen with NaF alone. Most impor-
Regulation of Ser-985 Phosphorylation of c-Met via PKC and PP2A

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Fig. 6. Dephosphorylation of Ser-985 by PP2A and change in PP2A activity by HGF and H2O2 stimulus. A, effects of NaF on the phosphorylation of Ser-985, Tyr-1234/1235, and Tyr-1365 during HGF stimulus. A549 cells were pretreated in the presence or absence of 25 mM NaF for 10 min and stimulated with 10 ng/ml HGF, c-Met was immunoprecipitated from the cells with anti-c-Met antibody and subjected to Western blotting with anti-phospho-Ser-985 antibody. The blot was successively reprobed with anti-phospho-c-Met (pY1234/1235) (upper middle panel), anti-phospho-c-Met (pY1365) (lower middle panel), and anti-c-Met antibodies (lower panel). B, enhancement of HGF-dependent Ser-985 phosphorylation by calyculin A. Cells were pretreated with or without 2 μM calyculin A (CalA) or 2 μM cyclosporin A (CysA) for 10 min and stimulated with 10 ng/ml HGF for 10 min. c-Met was immunoprecipitated from the cells with anti-c-Met antibody and subjected to Western blotting with anti-phospho-Ser-985 antibody. The blot was reprobed with anti-c-Met antibody. C, in vitro dephosphorylation of Ser-985 by recombinant PP2A. The cells were untreated or treated with 25 mM NaF for 10 min, and c-Met was immunoprecipitated from cell lysate with anti-c-Met antibody. Ser-985-phosphorylated c-Met residues immunoprecipitated from NaF-treated cells were incubated with or without recombinant PP1 or PP2A. Samples were subjected to SDS-PAGE and subsequent immunoblotting with anti-phospho-Ser-985 antibody (upper panel). The blot was reprobed with anti-c-Met antibody (lower panel). D, association of PP2A with c-Met. Cells were stimulated with 10 ng/ml HGF or 0.5 mM H2O2, and PP2A was then immunoprecipitated from cell lysates. PP2A activity to dephosphorylate phosphorylated Ser-985 was analyzed by Western blots, using Ser-985-phosphorylated c-Met prepared from cells untreated or treated with HGF or H2O2 as a substrate.

We then attempted to identify the phosphoserine phosphatase responsible for the attenuation of Ser-985 phosphorylation. Because NaF broadly inhibits the activities of PP1, PP2A, and PP2B, the effects of selective inhibitors, calyculin A (specific to PP1/2A) and cyclosporin A (specific to PP2B), were evaluated (Fig. 6B). Treatment with calyculin A further enhanced the Ser-985 phosphorylation induced by HGF, but cyclosporin A did not influence the Ser-985 phosphorylation induced by HGF, thereby suggesting the involvement of PP1 and/or PP2A in Ser-985 dephosphorylation. To address this, the c-Met receptor possessing phosphorylated Ser-985 was immunoprecipitated from A549 cells pretreated with NaF, and the catalytic activity of purified recombinant PP1 and PP2A to dephosphorylate Ser-985 was analyzed, respectively (Fig. 6C). Treatment with PP2A but not PP1 almost completely dephosphorylated Ser-985 of c-Met; hence, PP2A is presumed to be responsible for dephosphorylation of phospho-Ser-985 of c-Met.

Association of PP2A with c-Met and PP2A Activity Modulated by HGF- and H2O2 Stimulation—The above results indicate that the phosphorylation status of Ser-985 is bi-directionally regulated by phosphorylation and dephosphorylation mediated by PKC and PP2A, respectively. We therefore addressed mechanisms coordinating these activities for the regulation of Ser-985 phosphorylation. A potential mechanism would be association of PKC and/or PP2A to the c-Met receptor. To determine whether PP2A is associated with c-Met, c-Met was immunoprecipitated, and the immunoprecipitates were subjected to Western blotting with anti-PP2A antibody. PP2A was clearly associated with the c-Met receptor even in unstimulated cells, and HGF stimulus did not significantly alter the association of PP2A with c-Met (Fig. 6D). Hence, we suggest a constitutive association of PP2A with the c-Met receptor. When changes in the association of PP2A were analyzed in cells treated with H2O2, H2O2 stimulation did not change the association of PP2A with c-Met following H2O2 stimulus (Fig. 6D), again suggesting a constitutive association of PP2A with c-Met regardless of any extracellular stimuli with HGF and H2O2.

We then considered possible changes in PP2A activity even though PP2A is constitutively associated with c-Met. To address this possibility, the catalytic activity of PP2A required to dephosphorylate phosphorylated Ser-985 of c-Met was analyzed. PP2A was immunoprecipitated from A549 cells untreated or treated with HGF or H2O2, and PP2A activity was analyzed by Western blots, using phosphorylated Ser-985 of c-Met from NaF-treated cells as a substrate (Fig. 6E). Ser-985 was equally dephosphorylated by PP2A prepared from cells untreated or treated with HGF for up to 60 min, indicating that PP2A associated with c-Met was active in unstimulated cells.
Regulation of Ser-985 Phosphorylation of c-Met via PKC and PP2A

A previous study demonstrated that Ser-985 phosphorylation of c-Met was induced by TPA stimuli, and this event resulted in a decrease of c-Met tyrosine phosphorylation (13). In our work, strong Ser-985 phosphorylation induced by H$_2$O$_2$ stimulus and inhibition of protein phosphatase, as well as TPA, was associated with a decreased c-Met tyrosine phosphorylation upon HGF stimulus. Thus, the phosphorylation status of Ser-985 seems to play a functional regulatory role in activation of the c-Met receptor, thereby involved in distinct cellular responsiveness to HGF, which depends on distinct extracellular conditions. However, the regulation of Ser-985 phosphorylation by extracellular stimuli, potential molecules responsible for Ser-985 phosphorylation/dephosphorylation, and involvement of Ser-985 phosphorylation in regulation of cellular responsiveness mediated by c-Met remain to be addressed.

In the present study, we found that Ser-985 was phosphorylated and dephosphorylated, respectively, by PKC (PKC$_\alpha$ and -$\epsilon$) and PP2A, and that Ser-985 phosphorylation was bi-directionally regulated by the differential activation of PKC and PP2A depending on cellular conditions. Likewise, we found that Ser-985 was phosphorylated by oxidative stress and that the stress-induced Ser-985 phosphorylation was associated with inhibition of tyrosine phosphorylation of c-Met and subsequent cellular responsiveness to HGF. Possible regulatory mechanisms of Ser-985 phosphorylation and its involvement in c-Met receptor activation and cellular responsiveness are schematically described in Fig. 7. Various intracellular signaling molecules are associated with the c-Met receptor (8, 9); however, the association of PKC$_\alpha$, PKC$_\epsilon$, and PP2A with the c-Met receptor has not been demonstrated. Because activation of PKC is a downstream event triggered by HGF and H$_2$O$_2$ stimuli (15, 16), we speculated that the association of PKC$_\alpha$ and -$\epsilon$ with c-Met is likely to be related to the activation of these isoforms and/or their association may facilitate preferentially Ser-985 phosphorylation of c-Met. On the other hand, PP2A seems to constitutively associate with c-Met, yet its enzymatic activity is regulated by extracellular stimuli.

A variety of cellular damages and stresses such as oxidative stress, UV irradiation, and heat induce cell cycle arrest (14, 17, 18). Cell cycle arrest is a critical event, which allows cells to be restored from the damages (e.g., DNA-repair and refolding of denatured enzymes) or eliminated by apoptotic pathways; however, precise mechanisms of cell cycle arrest have yet to be elucidated. We found that the growth-promoting effects of HGF were abolished by suppression of the tyrosine phosphorylation event of c-Met when cells were exposed to oxidant stress, and the suppression was mediated by phosphorylation of Ser-985. To our knowledge, this is the first finding that H$_2$O$_2$-induced growth arrest is primarily regulated by receptor inactivation even when cells are exposed to mitogens. We propose that attenuation of the cellular responsiveness to growth factors may be an initial step to avoid disadvantages associated with cell cycle transition in damaged cells.

Previous studies indicated that Ser/Thr phosphorylation in receptor tyrosine kinases is involved in inhibition of tyrosine phosphorylation events following ligand stimulus, suggesting that a specific Ser/Thr phosphorylation provides an autoinhibitory mechanism in distinct receptor ligand stimulus. Tyrosine phosphorylation events in epidermal growth factor receptor (19), insulin receptor (20), c-Kit (21), and RET (22) are reciprocally inhibited by PKC-mediated Ser/Thr phosphorylation. Although related mechanisms remain to be addressed, the inhibition of receptor dimerization and enhancement of internalization in the epidermal growth factor receptor have been noted (23, 24). As for c-Met, pretreatment of cells with TPA and NaF induced extensive Ser-985 phosphorylation, and this was associated with suppression of tyrosine phosphorylation of c-Met upon HGF stimulus without any accompanying c-Met receptor degradation (Figs. 4C and 5A). Furthermore, TPA and NaF treatment after HGF stimulus also induced extensive Ser-985 phosphorylation and resulted in tyrosine dephosphorylation without c-Met degradation. Thus, we speculate that phosphorylation of Ser-985 may mediate the inhibition of receptor dimerization and/or association of protein-tyrosine phosphatases.

c-Met has a structural variant that lacks a cytoplasmic juxtamembrane region (\Delta Jx-Met) including Ser-985, and it is generated through the alternative splicing of exon 14 (12). The juxtamembrane domain of c-Met has been considered to play a negative regulatory function, based on findings that addition of the juxtamembrane domain to oncogenic TPR-Met fusion protein abolishes its transforming activity (25), and phosphorylation of Tyr-1003 in this domain facilitates proteasome-mediated degradation of c-Met through Cbl association (26). Furthermore, mutations in the juxtamembrane domain were identified in human gastric cancer and small cell lung cancer, and these mutant c-Met are more susceptible to tyrosine phosphorylation upon HGF stimulus (27, 28), again indicating that the domain contributes to negative regulation of c-Met. Most interesting, \Delta Jx-Met is expressed in various tissues (12), suggesting that the \Delta Jx-Met plays some c-Met related roles in biological events. \Delta Jx-Met expression may provide a condition

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wherein the activation of ΔJxt-Met simply depends on the ligand binding regardless of the extracellular environment and stimuli in a cell and/or tissue type-specific manner.

PKC is activated by diverse extracellular stimuli such as cellular stress, cell-cell adhesion, cell-extracellular matrix interaction, and cellular (tissue) injury (16, 29–31). Likewise, c-Met-mediated cellular responsiveness is modified depending on extracellular conditions, including cellular stress, cell-cell adhesion, and cell extracellular matrix interaction (32–34). Taken together, Ser-985 phosphorylation mediated by activation of PKCα, PKCζ, and PP2A is likely to play a role in the regulation of c-Met receptor activation and cellular responsiveness to HGF, which depend on the extracellular environment and stimuli. Further studies on regulatory mechanisms responsible for c-Met receptor activation through juxtamembrane domain and Ser-985 phosphorylation will provide even further understanding of the HGF-c-Met system in development, tissue regeneration, and tumorigenesis.

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