

# Phosphorylation of Serine 985 Negatively Regulates the Hepatocyte Growth Factor Receptor Kinase\*

(Received for publication, August 5, 1993)

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The receptor for hepatocyte growth factor/scatter factor (HGF/SF) is an  $\alpha\beta$  tyrosine kinase of 190 kDa which mediates growth and motility in several cell types. We have previously shown that tyrosine autophosphorylation enhances the receptor kinase activity, while serine phosphorylation by protein kinase C or other  $\text{Ca}^{2+}$ -dependent kinase(s) is inhibitory. We now identify Ser<sup>985</sup> as the major phosphorylation site for the protein kinases responsible for such inhibition. Both phorbol esters or  $\text{Ca}^{2+}$  ionophore treatment induces phosphorylation of the same tryptic phosphopeptide corresponding to the sequence Leu<sup>983</sup>-Arg<sup>987</sup> located in the juxtamembrane domain of the receptor  $\beta$  chain. Purified protein kinase C phosphorylates *in vitro* a synthetic peptide (V14S) including Ser<sup>985</sup>. Trypsin digestion of the phosphorylated V14S generates a single phosphopeptide comigrating in reverse-phase high performance liquid chromatography with the tryptic peptide phosphorylated *in vivo*. Phorbol ester treatment of cultured cells inhibits the ligand-induced tyrosine autophosphorylation of the receptor. *In vitro*, Ser<sup>985</sup> phosphorylation inhibits the receptor tyrosine kinase activity on exogenous substrates. Substitution of Ser<sup>985</sup> by site-directed mutagenesis results in increased tyrosine phosphorylation of the receptor and abolishes down-modulation by protein kinase C. These data show that phosphorylation of Ser<sup>985</sup> is a key mechanism for the negative regulation of HGF/SF receptor.

The receptor for hepatocyte growth factor (HGF),<sup>1</sup> a powerful mitogen for several epithelial cells, is a transmembrane tyrosine kinase encoded by the *MET* proto-oncogene (Bottaro *et al.*, 1991; Naldini *et al.*, 1991a; Giordano *et al.*, 1993). The tyrosine kinase is also known as the receptor for "scatter factor" (SF), a molecule identical to HGF, that triggers cell motility and promotes invasiveness (Weidner *et al.*, 1991; Naldini *et al.*, 1991b). HGF/SF is secreted as an inactive single chain precursor and processed to the active two chain form by extracellular proteolytic cleavage mediated by urokinase (Naldini *et al.*, 1992). The receptor protein is an heterodimer of 190 kDa (p190MET) composed of an

$\alpha$ -chain of 50 kDa, exposed at the cell surface, linked by disulfide bonds to a  $\beta$ -chain of 145 kDa (p145  $\beta$ ; Giordano *et al.*, 1989a, 1989b). The latter spans the plasma membrane and is endowed with tyrosine kinase activity (Tempest *et al.*, 1986; Park *et al.*, 1987; Giordano *et al.*, 1989a). Two C-terminal truncated HGF/SF receptor forms have been identified: a 140-kDa heterodimer bound to the cell surface and a 130-kDa heterodimer released in the extracellular environment. (Prat *et al.*, 1991b). In humans, the HGF/SF receptor is widely expressed in cells of epithelial origin and in a variety of epithelial tumors (Prat *et al.*, 1991a; Di Renzo *et al.*, 1991, 1992). Recently, the receptor has been found in the microglia (Di Renzo *et al.*, 1993) and on endothelial cells (Bussolino *et al.*, 1992).

Both the mitogenic and the motogenic HGF/SF signals are mediated by the receptor tyrosine kinase activity which results in autophosphorylation at specific tyrosine sites recruiting intracellular signal transducers (Graziani *et al.*, 1991; Bardelli *et al.*, 1992). HGF/SF stimulation enhances the activity of PI 3-kinase (Ponzetto *et al.*, 1993) of a RAS guanine nucleotide exchanger (Graziani *et al.*, 1993) and of a tyrosine phosphatase (Villa-Moruzzi *et al.*, 1993).

We have also shown that tyrosine autophosphorylation of the receptor enhances its own intrinsic kinase activity (Naldini *et al.*, 1991c; Ferracini *et al.*, 1991). Our previous work has demonstrated as well that the HGF/SF receptor can be phosphorylated on serine by two independent mechanisms involving activation of protein kinase C or activation of a protein kinase sensitive to the concentration of intracellular  $\text{Ca}^{2+}$  (Gandino *et al.*, 1990, 1991).

In this work we have identified Ser<sup>985</sup> as the major phosphate acceptor site for the phosphorylations triggered by both protein kinase C and the  $\text{Ca}^{2+}$ -dependent kinase. We show that phosphorylation of this residue acts as a negative modulator of the tyrosine kinase activity of the receptor *in vitro* and in transfected cells. We suggest that phosphorylation of Ser<sup>985</sup> is a mechanism for the negative regulation of HGF/SF receptor.

## EXPERIMENTAL PROCEDURES

**Reagents and Cells**—Phorbol 12-myristate 13-acetate (TPA), A23187 ionophore, myelin basic protein (MBP), sodium deoxycholate, and ATP were from Sigma, Triton X-100 was from Pierce, and okadaic acid was from Moana Bioproducts, Inc. [ $\gamma$ -<sup>32</sup>P]ATP (specific activity 7000 Ci/mmol), [<sup>32</sup>P]orthophosphate (10 mCi/ml), and <sup>125</sup>I-protein A were obtained from Amersham. Nitrocellulose filters for Western blots were from Bio-Rad. The peptide V14S corresponding to amino acid residues 975–988 (VHTPHLDRLVSARS) of the *MET* sequence derived from cDNA cloning of the major transcript (Ponzetto *et al.*, 1991; EMBL/GenBank accession number X54559), was obtained from Neo System. Purified protein kinase C was kindly provided by Dr. Peter J. Parker (Imperial Cancer Research Fund, London), calcium/calmodulin-dependent protein kinase-II by Angus C. Nairn (Rockefeller University, New York). Anti-phosphotyrosine antibodies (anti-Tyr(P)) were raised in rabbits as described (Comoglio *et al.*, 1984). Rabbit and mouse monoclonal anti-Met antibodies, directed against the  $\beta$ -chain of p190MET receptor, were produced as described previously (Prat *et al.*, 1991b). Human HGF/SF was purified to homogeneity from tissue culture medium con-

\* This work was supported by funds from the Associazione Italiana Ricerche sul Cancro and from Consiglio Nazionale delle Ricerche Special Project ACRO, 92.02169.PF 39 (to P. M. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by an Associazione Italiana Ricerche sul Cancro fellowship.

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<sup>1</sup> The abbreviations used are: HGF, hepatocyte growth factor; SF, scatter factor; TPA, 12-O-tetradecanolyphorbol-13-acetate; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

ditioned by the human fibroblast line MRC5 as described previously (Bussolino *et al.*, 1992). GTL-16 cells are a clonal cell line derived from a poorly differentiated gastric carcinoma featuring a constitutively tyrosine-phosphorylated HGF/SF receptor due to overexpression of an amplified, structurally normal *MET* oncogene (Giordano *et al.*, 1989a; Ponzetto *et al.*, 1991). COS-7 cells were obtained from the American Type Culture Collection. The cells were cultured at 37 °C, under a humidified atmosphere of 95% air, 5% CO<sub>2</sub>, in RPMI-1640 medium supplemented with 10% fetal calf serum (Seromed).

**[<sup>32</sup>P]Orthophosphate Labeling and Immunoprecipitation**—Subconfluent cultures of GTL-16 cells were incubated for 4 h at 37 °C with 1 mCi/ml of [<sup>32</sup>P]orthophosphate in phosphate-free RPMI medium in the absence of serum. Cells were stimulated with 160 nM TPA, or dimethyl sulfoxide alone (Me<sub>2</sub>SO), for 30 min at 37 °C. The cultures were placed on ice, washed twice with cold phosphate buffer saline and lysed in RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 100 μM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 10 mM okadaic acid) and a mixture of protease inhibitors. Cell lysates were clarified at 30,000 revolutions/min for 30 min at 4 °C, and immunoprecipitated for 2 h at 4 °C with anti-*Met* antibodies coupled to Sepharose-protein A. Bound proteins were washed several times and eluted in boiling Laemmli buffer. Eluted proteins were subjected to 8% SDS-PAGE followed by autoradiography at -70 °C using intensifying screens.

**HPLC Phosphopeptide Mapping and Phosphoamino Acid Analysis**—For phosphopeptide mapping the <sup>32</sup>P-labeled bands were excised from SDS-PAGE and further processed as described elsewhere (Ferracini *et al.*, 1991). The phosphopeptides were analyzed on a reverse-phase C<sub>2</sub>-C<sub>18</sub> Superpack Pep-S column (Pharmacia LKB), resolved on a gradient of acetonitrile in buffer A of 0.46%/min (from 0 to 32% acetonitrile in 82 min) with a flow rate of 1 ml/min. The eluted radioactivity was monitored by a Radiomatic A-100 radioactive flow detector (Packard). The fractions corresponding to the largest radioactive peaks were pooled, lyophilized, and subjected to phosphoamino acid analysis. Phosphoamino acid analysis was performed as described by Cooper *et al.* (1983).

**In Vitro Phosphorylation of the Synthetic Peptide V14S**—The peptide V14S was phosphorylated *in vitro* by protein kinase C as follows: 50 μg of peptide were incubated at 30 °C for 10 min in 40 μl of a buffer containing 50 mM HEPES, pH 7.5, 0.5 mM EGTA, 12.5 mM MgCl<sub>2</sub>, 0.75 mM CaCl<sub>2</sub>, 50 μg of phosphatidylserine, 50 ng of TPA, together with 30 μCi of [<sup>32</sup>P]ATP and 3 units of purified kinase. Phosphorylation by calcium/calmodulin-dependent kinase-II was performed in a buffer containing 120 mM NaCl, 16 mM Tris-HCl pH 8.00, 10 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>, 0.07 mM EGTA, 0.1 mM β-mercaptoethanol, 1 μM calmodulin, 50 μM [<sup>32</sup>P]ATP, and 300 ng of purified kinase. The reaction was terminated by adding trichloroacetic acid at a final concentration of 10%. After 1 hr on ice the reaction mixture was centrifuged at 15,000 revolutions/min at 4 °C for 15 min. The pellet was dissolved in Laemmli buffer and analyzed in an 8% SDS-PAGE followed by autoradiography. The supernatant was subjected to Seppak™ chromatography and analyzed in reverse-phase HPLC as described above. The peak was collected, lyophilized, resuspended in 50 mM NH<sub>4</sub>CO<sub>3</sub>, pH 7.8, and incubated with 50 μg of L-1-tosyl-amido-2-phenylethyl chloromethyl ketone-treated trypsin for 2 h at 37 °C. The desiccated sample was then resuspended in buffer A and analyzed on HPLC. The phosphorylated peak was recovered from HPLC, hydrolyzed, and analyzed in thin layer chromatography using phosphorylated amino acids as standards.

**Myelin Basic Protein Phosphorylation Assay**—GTL-16 cells treated or not with TPA were washed twice with ice-cold phosphate-buffered saline and lysed in RIPA buffer in the presence of serine phosphatase inhibitors (see above) to prevent serine dephosphorylation. Tyrosine phosphatase inhibitors were deliberately omitted to allow *in vitro* dephosphorylation of tyrosyl residues. Immunoprecipitation was performed as described above with anti-*Met* antibodies. Immunocomplexes were washed in KB buffer (25 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, and 0.1 mM dithiothreitol) in the presence of different concentrations of MBP or [<sup>32</sup>P]ATP. After 10 min at 37 °C, the reaction was stopped by addition of equal volume of 2-fold concentrated Laemmli buffer (Laemmli, 1970). Samples were separated in 15% SDS-PAGE and autoradiographed. The relative amount of phosphate incorporated in MBP was estimated by measuring the optical density of the corresponding autoradiographic band with a laser densitometer (LKB 2202 Ultrascan). Alternatively, phosphorylation of MBP was directly quantified by liquid scintillation counting of the <sup>32</sup>P-labeled protein bound to phosphocellulose filters as described previously (Naldini *et al.*, 1991c).

**HGF/SF Stimulation and Detection of Tyrosine Phosphorylation in Vivo**—Subconfluent cultures, control or treated with TPA as above, were

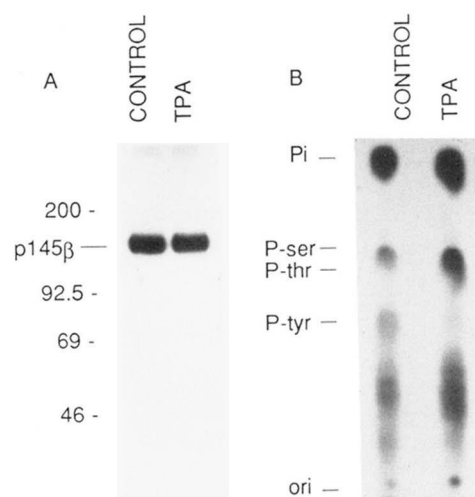


FIG. 1. Analysis of amino acids phosphorylated in HGF/SF receptor in response to TPA treatment. A, SDS-PAGE of proteins solubilized from [<sup>32</sup>P]orthophosphate-labeled GTL-16 cells and immunoprecipitated with anti-*Met* antibodies. CONTROL, unstimulated cells; TPA, cells treated with TPA for 30 min. B, high voltage electrophoresis of phosphoamino acids hydrolyzed from the 145-kDa β-chains, eluted from the SDS-PAGE bands shown in A. P-ser, phosphoserine; P-thr, phosphothreonine; P-tyr, phosphotyrosine.

washed and incubated in the presence of HGF/SF (50 ng/ml) for 10 min at 37 °C. Monolayers were washed with ice-cold phosphate-buffered saline and lysed in RIPA buffer. Immunoprecipitation was performed as described above with anti-*Met* antibodies. Eluted proteins were subjected to 8% SDS-PAGE, transferred to nitrocellulose sheets, and probed with 10 μg/ml of purified anti-Tyr(P) antibodies followed by <sup>125</sup>I-labeled protein A as described elsewhere (Di Renzo *et al.*, 1986). To quantify the amount of HGF/SF receptor, anti-*Met* antibodies were used to reprobe the blots. Filters were subjected to autoradiography as above.

**Site-directed Mutagenesis and Expression in COS-7 Cells**—The cloning of the *MET* c-DNA has been reported previously (Ponzetto *et al.*, 1991; Giordano *et al.*, 1992). The nucleotide tract comprised between the *Eco*RI restriction site and the 3' end of the *MET* cDNA sequence was subcloned in the pSELECT™-1 vector (Promega). Mutagenesis was performed using an *in vitro* oligonucleotide site-directed mutagenesis system (Promega), as specified by the manufacturer. Oligonucleotides were synthesized with an Applied Biosystem 391 apparatus. Bacterial colonies containing the mutated *MET* cDNA were identified by sequencing (T7 sequencing kit from Pharmacia). Full-size *MET* cDNA carrying the Ser<sup>985</sup>-Ala<sup>985</sup> mutation was reconstructed in the PMT2 vector. The PMT2 vector, containing the major late adenovirus promoter, was transiently transfected in COS-7 cells by the lipofection procedure (GIBCO-BRL). Protein experiments were performed 48 h after transfection.

## RESULTS

**Activation of Protein Kinase C with TPA Induces Serine Phosphorylation in a Single Tryptic Peptide of the HGF/SF Receptor β-Chain**—Due to amplification and overexpression of the *MET* oncogene in GTL-16 cells the HGF/SF receptor is constitutively phosphorylated on tyrosine even in the absence of the ligand (Giordano *et al.*, 1989a; Ponzetto *et al.*, 1991). After metabolic labeling with [<sup>32</sup>P]orthophosphate, the 145-kDa β-chain immunoprecipitated with anti-*Met* antibodies was heavily phosphorylated both in tyrosine and in serine. After TPA treatment, the overall amount of phosphate detected in the β-chain was unaffected (Fig. 1A). Phosphoamino acid analysis showed that TPA treatment decreased the phosphotyrosine and increased the phosphoserine content (Fig. 1B).

<sup>32</sup>P-Labeled 145-kDa β-chains, immunoprecipitated from control or TPA-treated cells, were digested with trypsin and analyzed by reverse-phase HPLC (Fig. 2). In the β-chain from control cells, the peptide eluting at 35 min contained the phosphorylated Tyr<sup>1235</sup>, which has been previously identified as the major autophosphorylation site of the HGF/SF receptor (Fer-

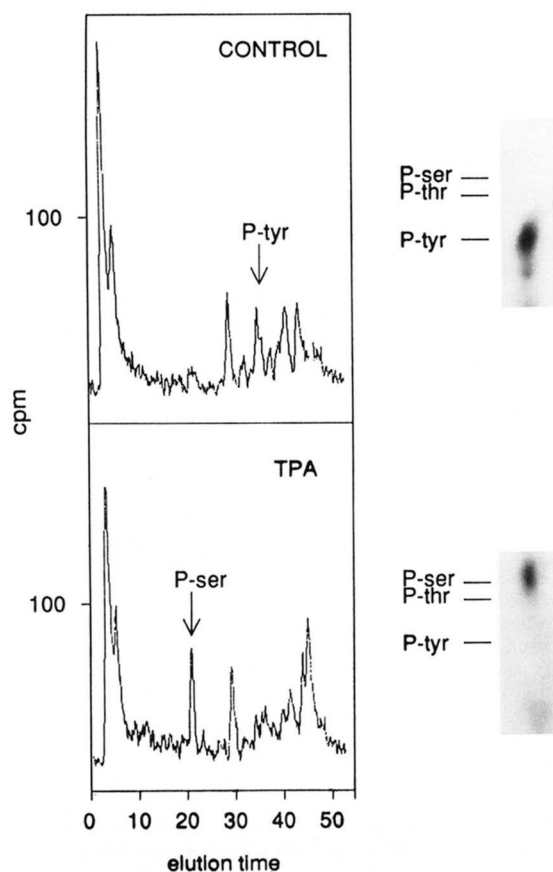


FIG. 2. Analysis of tryptic peptides phosphorylated in HGF/SF receptor in response to TPA treatment. Elution profiles of [ $^{32}$ P]orthophosphate-labeled HGF/SF receptor  $\beta$ -chains trypsin digested and analyzed on reverse-phase HPLC as described under "Experimental Procedures." Upper panel, untreated cells. Lower panel, cells treated with TPA. The phosphoamino acid analysis of the peaks pointed by arrows are shown on the right. P-ser, phosphoserine; P-thr, phosphothreonine; P-tyr, phosphotyrosine.

racini *et al.*, 1991). The amount of  $^{32}$ P incorporated in this peptide was reduced in the  $\beta$ -chain immunoprecipitated from TPA-treated cells. On the other hand, a significant amount of radioactivity was incorporated in a peak, barely detectable in control cells, eluting at 20 min. Phosphoamino acid analysis of this peak showed that phosphoserine was the only labeled amino acid.

**Activation of a  $Ca^{2+}$ -dependent Kinase or Protein Kinase C Induces Serine Phosphorylation of the HGF/SF Receptor  $\beta$ -Chains in the Same Tryptic Peptide**—[ $^{32}$ P]orthophosphate-labeled GTL-16 cells were treated for 5 min with the calcium ionophore A23187 (10  $\mu$ M). As described previously, under these conditions a  $Ca^{2+}$ -dependent protein kinase phosphorylates the HGF/SF receptor in a single major phosphopeptide (Gandino *et al.*, 1991). After A23187 or TPA treatment, the receptors were immunoprecipitated with anti-Met antibodies, the  $\beta$ -chains were trypsin digested, and compared in HPLC. In both conditions an identical major tryptic phosphopeptide was eluted at approximately 20 min (Fig. 3). In both cases, serine was the only phosphorylated amino acid (not shown).

**The Major Serine Phosphorylation Site of the HGF/SF Receptor  $\beta$ -Chain Maps at Residue 985**—A number of peptides derived from the sequence of the cytoplasmic domain of the HGF/SF receptor  $\beta$ -chain were synthesized and incubated *in vitro* with purified protein kinase C in the presence of [ $\gamma$ - $^{32}$ P]ATP. These included the sequence between Val<sup>976</sup>-Ser<sup>989</sup> (V14S). The peptide V14S was phosphorylated *in vitro* by protein kinase C (Fig. 4A) and, at lower efficiency, by calcium/

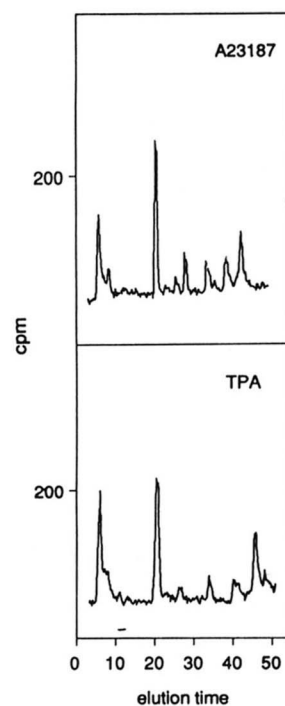


FIG. 3. Analysis of tryptic peptides phosphorylated in HGF/SF receptor in response to TPA or A23187 treatment. Elution profiles of [ $^{32}$ P]orthophosphate-labeled HGF/SF receptor  $\beta$ -chains trypsin digested and analyzed as in Fig. 2. Upper panel, cells treated with the ionophore for 5 min. Lower panel, cells treated with TPA.

calmodulin-dependent kinase-II (data not shown). The phosphorylated peptide V14S was subjected to trypsin digestion and compared with the tryptic phosphopeptide generated from HGF/SF receptor  $\beta$ -chains phosphorylated *in vivo* after TPA treatment. Identity was proven by the same retention time in HPLC and by co-elution when mixed (Fig. 4, B and C). The only phosphorylated amino acid was serine, showing that protein kinase C and the  $Ca^{2+}$ -activated kinase trigger the phosphorylation of serine 985.

**Phosphorylation of Ser<sup>985</sup> Is Associated with the Inhibition of Ligand-induced Tyrosine Autophosphorylation of the HGF/SF Receptor *in Vivo***—GTL-16 cells, due to overexpression of an amplified MET gene, display a basal level of constitutive tyrosine phosphorylation of the HGF/SF receptor. As previously described, the level of tyrosine phosphorylation can be further increased upon binding of HGF/SF to the receptor (Naldini *et al.*, 1991a). After treatment of GTL-16 cells with TPA for 30 min, the level of constitutive tyrosine phosphorylation of the receptor  $\beta$ -chain was reduced (Fig. 5A). Successive treatment with HGF/SF, at concentrations effective in control cells, failed to induce autophosphorylation on tyrosine of the receptor. TPA treatment did not apparently change the amount of HGF/SF receptor protein, as detected in Western blots probed with anti-Met antibodies (Fig. 5B).

**Phosphorylation of Ser<sup>985</sup> Inhibits the HGF/SF Receptor Tyrosine Kinase Activity *in Vitro***—The effect of Ser<sup>985</sup> phosphorylation on the HGF/SF receptor kinase activity was analyzed *in vitro* by studying tyrosine phosphorylation of an exogenous substrate. Identical amounts of HGF/SF receptor, immunoprecipitated with anti-Met antibodies from GTL-16 cells stimulated or not with TPA, were analyzed. Immunoprecipitation was performed in a buffer supplemented with EDTA and Ser/Thr phosphatase inhibitors, but lacking  $Na_3VO_4$  in order to allow dephosphorylation of tyrosine residues. The amount of protein precipitated and the content in phosphotyrosine of samples solubilized from control and treated cells was checked

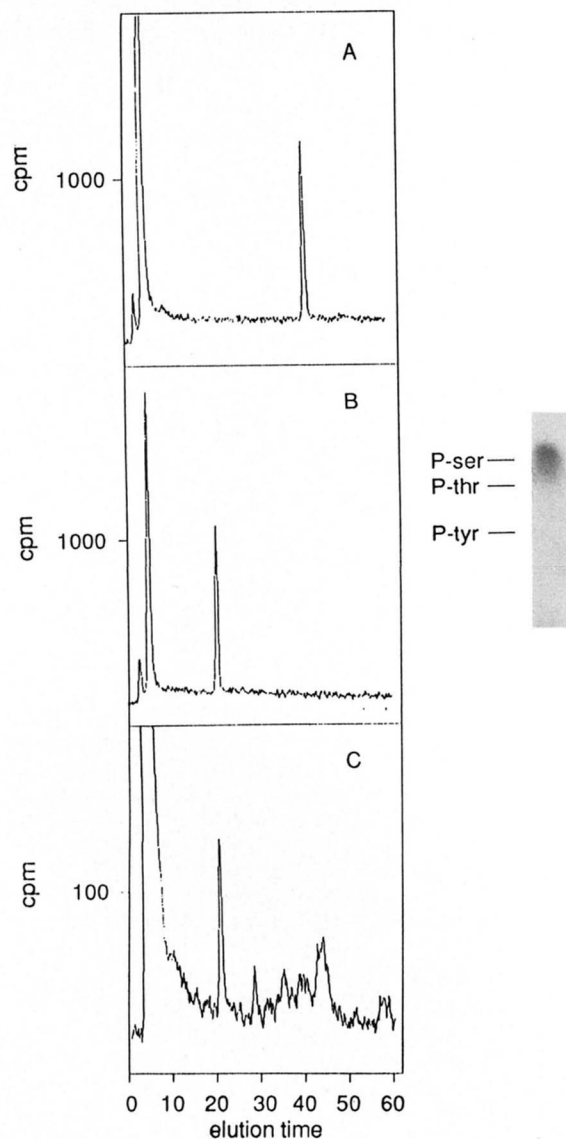


FIG. 4. Analysis of the V14S synthetic peptide phosphorylated *in vitro* by protein kinase C. A, elution profile of the phosphorylated intact V14S synthetic peptide (VHTPHLDRLVSARS). B, elution profile of the tryptic fragment (LVSAR) derived from the phosphorylated V14S; the phosphoamino acid analysis is shown on the right. C, elution profile of *in vivo* [ $^{32}$ P]orthophosphate-labeled HGF/SF receptor  $\beta$ -chains trypsin digested and mixed with the tryptic fragment derived from V14S phosphorylated *in vitro*. P-ser, phosphoserine; P-thr, phosphothreonine; P-tyr, phosphoryrosine.

by Western blots probed with anti-*Met* and anti-*Tyr(P)* antibodies (Fig. 6A). Under these conditions the difference in basal phosphorylation on tyrosine of the HGF/SF receptor samples from control and TPA-treated cells was abolished while the difference in Ser<sup>985</sup> phosphorylation was maintained as judged from phosphoamino acid analysis (not shown). The kinase assay was performed by incubating equal amounts of the receptor kinase with 50  $\mu$ M MBP in the presence of increasing concentrations of [ $\gamma$ - $^{32}$ P]ATP. Assays were also performed by varying the concentration of MBP at fixed concentrations of ATP. After 10 min at 0  $^{\circ}$ C, the reaction was stopped and the phosphorylated MBP was quantified either in 15% SDS-PAGE (Fig. 6B) or by binding to phosphocellulose paper (data not shown). Ser<sup>985</sup> phosphorylation reduced the kinase activity of the HGF/SF receptor toward the exogenous substrate. The reduction affected mostly the  $V_{max}$  (~50%) leaving unaffected the  $K_M$  for both substrates. The  $K_M$  for MBP was in the range of 3–15  $\mu$ M; the  $K_M$  for ATP was ~50  $\mu$ M.

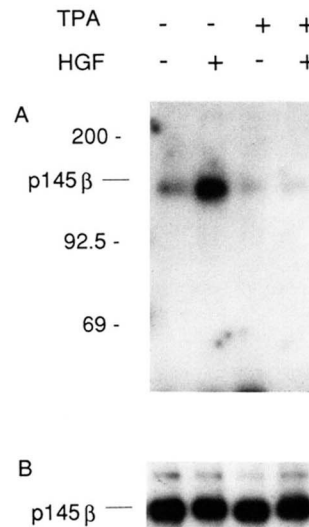


FIG. 5. Serine-985 phosphorylation inhibits the ligand-induced tyrosine autophosphorylation of HGF/SF receptor *in vivo*. Western blot analysis of HGF/SF receptors immunoprecipitated with anti-*Met* antibodies from cells exposed to TPA and to HGF/SF. A, Western blot probed with anti-*Tyr(P)* antibodies; B, Western blot probed with anti-*Met* antibodies.

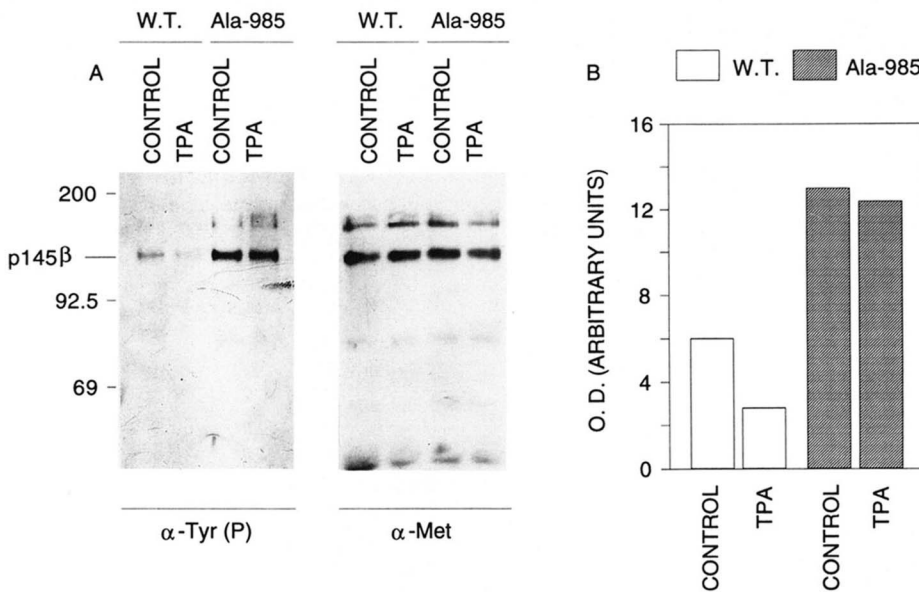
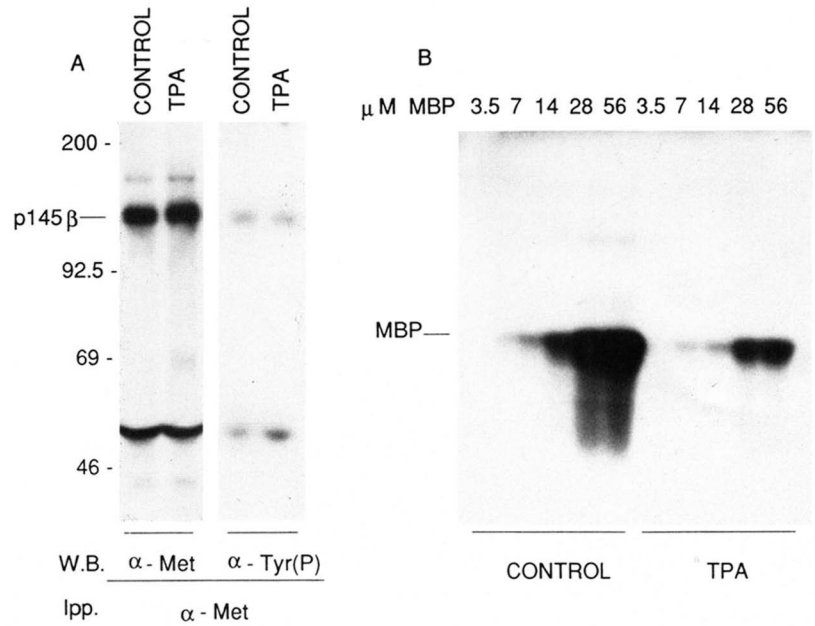
**Mutation of Ser<sup>985</sup> Abolishes Negative Regulation of HGF/SF Receptor Kinase Activity by Protein Kinase C**—To further confirm the regulatory role of Ser<sup>985</sup> on the HGF/SF receptor tyrosine kinase activity, we used *in vitro* site-directed mutagenesis to replace Ser<sup>985</sup> with alanine. The Ala<sup>985</sup> HGF/SF receptor cDNA construct was cloned into PMT2 vector, as described under "Experimental Procedures" and transfected into COS-7 cells. Wild-type receptor cDNA was transfected as control. In transfected cells, due to over-expression (Bardelli *et al.*, 1992), the recombinant wild-type HGF/SF receptor is tyrosine phosphorylated in the absence of the ligand. As in GTL-16 cells, the level of autophosphorylation can be further increased in the presence of HGF/SF (not shown). In both cases, tyrosine phosphorylation was reduced in response to TPA treatment (Fig. 7). Tyrosine phosphorylation of the Ala<sup>985</sup> mutant was significantly higher than phosphorylation of the wild-type receptor, both in the presence or in the absence of the ligand. Moreover, negative regulation by TPA treatment was abolished (Fig. 7).

#### DISCUSSION

Hepatocyte growth factor, also known as scatter factor, exerts pleiotropic cellular responses mediating regulation of cell division, motility, and differentiation (for a review, see Gherardi and Stoker, 1991). Such critical functions are mediated by binding and activation of a cell surface tyrosine kinase receptor whose structure, biosynthesis, and biochemical properties have been elucidated in a series of previous reports from this and other laboratories (for a review, see Cooper, 1992; Comoglio, 1993).

The response to growth factors can be modulated both positively and negatively by covalent modifications of the corresponding receptors. It is widely accepted that phosphorylation plays an important role in regulation of signal transduction (Yarden and Ullrich, 1988; Nishizuka, 1988; Ullrich and Schlessinger, 1990). Positive regulation by tyrosine phosphorylation has been shown for the *v-fps* oncogene product (Weinmaster *et al.*, 1986) and the insulin receptor (Rosen *et al.*, 1983; Yu and Czech, 1984, 1986; Klein *et al.*, 1986). Furthermore, the kinase activity of pp60<sup>c-src</sup> (Cartwright *et al.*, 1987; Kmiecik and Shalloway, 1987; Pivnicka-Worms *et al.*, 1987), of pp120gag-fps (Meckling-Hansen *et al.*, 1987) and of the insulin receptor

**FIG. 6. Effect of serine-985 phosphorylation on the kinase activity of the HGF/SF receptor *in vitro*.** *A*: HGF/SF receptors were immunoprecipitated (*ipp.*) with anti-*Met* antibodies and transferred to nitrocellulose filters. The Western blots (*W.B.*) were probed either with anti-*Met* antibodies, to normalize the amount of protein, or with anti-*Tyr(P)* antibodies, to assess the level of tyrosine phosphorylation. *CONTROL*, unstimulated cells. *TPA*, cells treated with TPA for 30 min. *B*, SDS-PAGE analysis of myelin basic protein (*MBP*) phosphorylated *in vitro* by the immunoprecipitated HGF/SF receptors analyzed in *A*.



**FIG. 7. Effect of TPA on the tyrosine phosphorylation of wild-type or Ala<sup>985</sup> HGF/SF receptor.** *A*, Western blot analysis of recombinant HGF/SF receptors immunoprecipitated with anti-*Met* antibodies from COS-7 cells transfected with wild-type (*W.T.*) or Ala<sup>985</sup>. The same blot was probed with anti-*Tyr(P)* antibodies, stripped, and with anti-*Met* antibodies. *CONTROL*, unstimulated cells. *TPA*, cells treated with 160 nM TPA for 30 min. *B*, relative amount of phosphotyrosine present in HGF/SF receptor  $\beta$ -chains, estimated by measuring the optical density (*O.D.*) of the corresponding band in the radiogram of the blot probed with anti-*Tyr(P)* antibodies shown in *A*.

(Ellis *et al.*, 1986) was shown to be greatly diminished upon substitution of a critical tyrosine residue with a phenylalanine.

The HGF/SF receptor tyrosine kinase is activated by tyrosine autophosphorylation. The raise in phosphorylation rate is due to an increase in the  $V_{max}$  of the enzyme-catalyzed phosphotransfer reaction (Naldini *et al.*, 1991c). The major phosphorylation site of the HGF/SF receptor has been mapped to Tyr<sup>1235</sup> (Ferracini *et al.*, 1991). Tyr<sup>1235</sup> is located within the kinase domain in a segment homologous to the major autophosphorylation sites of other receptor and non-receptor kinases (Hanks *et al.*, 1988). We recently demonstrated that this site is directly involved in the kinase activation of the receptor.<sup>2</sup>

Negative regulation by serine or threonine phosphorylation has been shown to operate in a number of membrane receptor kinases including the receptors for insulin (Jacobs *et al.*, 1983; Takayama, *et al.*, 1988), insulin-like growth factor-1 (Jacobs *et al.*, 1983), and epidermal growth factor (Davis and Czech, 1984). Epidermal growth factor receptor is phosphorylated by protein kinase C at a specific site, Thr<sup>654</sup> (Hunter *et al.*, 1984).

Phosphorylation of this residue inhibits the receptor tyrosine kinase activity (Downward *et al.*, 1985; Friedman *et al.*, 1984; Cochet *et al.*, 1984, Hunter *et al.*, 1984; Countaway *et al.*, 1990) and, as a consequence, the ability to trigger the mitogenic response (Livneh *et al.*, 1988). Other reports indicate that the epidermal growth factor receptor may also be negatively modulated upon threonine/serine phosphorylation mediated by a yet unidentified protein kinase activated by the increased concentration of intracellular Ca<sup>2+</sup> (Fearns and King, 1985; Friedman *et al.*, 1989; Verheijden *et al.*, 1990).

Previous work has shown that serine phosphorylation induced by TPA treatment reduces tyrosine autophosphorylation of the HGF/SF receptor (Gandino *et al.*, 1990). A similar effect was also observed after serine phosphorylation induced by a Ca<sup>2+</sup>-sensitive kinase, acting through a protein kinase C-independent mechanism (Gandino, *et al.*, 1991). We now report that in both cases a single residue, Ser<sup>985</sup>, is phosphorylated. Substitution of this residue with alanine resulted in complete loss of negative regulation.

Ser<sup>985</sup> is located within the juxtamembrane domain of the HGF/SF receptor, a position similar to that of the regulatory

<sup>2</sup> P. Longati, A. Bardelli, C. Ponzetto, L. Naldini, and P. M. Comoglio, submitted for publication.

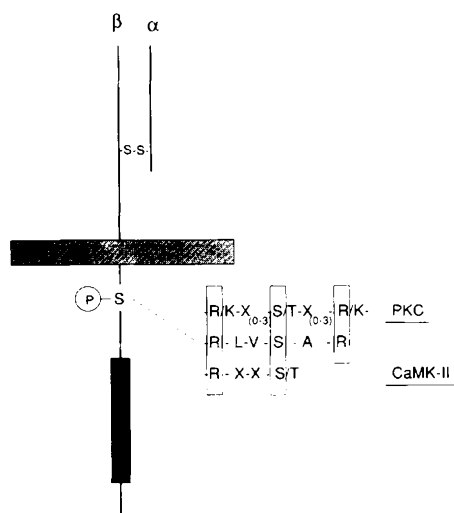


FIG. 8. Consensus sequences for phosphorylation surrounding Ser<sup>985</sup> in the HGF/SF receptor. Ser<sup>985</sup> is located within the juxta-membrane domain of the HGF/SF receptor  $\beta$ -chain. On the right the sequence flanking Ser<sup>985</sup> is compared with the consensus sequences for phosphorylation by protein kinase C (PKC) or by calcium/calmodulin-dependent protein kinase-II (CaMK-II).

Thr<sup>654</sup> of epidermal growth factor receptor. Ser<sup>985</sup> is surrounded by a stretch of amino acids that fits the consensus sequence for protein kinase C phosphorylation. Interestingly, Ser<sup>985</sup> and the 3 residues upstream generate a second motif that is the canonical consensus sequence for phosphorylation by calcium/calmodulin-dependent kinase II (Fig. 8; Kennelly and Krebs, 1991). The synthetic peptide V14S, corresponding to the sequence Val<sup>975</sup>-Ser<sup>988</sup> of the HGF/SF receptor and including Ser<sup>985</sup>, was phosphorylated *in vitro* by purified protein kinase C and by calcium/calmodulin-dependent kinase-II, suggesting that these enzymes might directly phosphorylate the HGF/SF receptor  $\beta$ -chain *in vivo*. However, attempts to phosphorylate the immunoprecipitated receptor have been so far unsuccessful (data not shown).

Phosphorylation of Ser<sup>985</sup> causes inhibition of the HGF/SF receptor kinase as shown by reduced phosphorylation of an exogenous substrate *in vitro* and inhibition of autophosphorylation *in vivo*. Kinase activation and subsequent tyrosine phosphorylation of growth factor receptors are critical to recruit and to activate cytoplasmic signal transducers which ultimately generates the biological response (for a review, see Cantley *et al.*, 1991). In this context phosphorylation of Ser<sup>985</sup> is a negative regulatory event interfering with the signal transduction pathways triggered by HGF/SF.

**Acknowledgments**—We thank Dr P. J. Parker for generously supplying purified protein kinase C and Dr. A. Nairn for calcium/calmodulin-dependent kinase-II and for performing some peptide phosphorylation experiments. The technical assistance of R. Callipo and L. Palmas and the excellent secretarial help of A. Cignetto are gratefully acknowledged.

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