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Identification of the Major Autophosphorylation Site of the *Met*/ Hepatocyte Growth Factor Receptor Tyrosine Kinase*

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The *MET* proto-oncogene encodes a transmembrane tyrosine kinase receptor for HGF ($p190^{MET}$). In this work, $p190^{MET}$ was immunoprecipitated, allowed to phosphorylate in the presence of [γ-^{32}P]ATP, and digested with trypsin. A major phosphopeptide was purified by reverse phase chromatography. The phosphorylated tyrosine was identified as residue 1235 (Tyr^{1235}) by Edman covalent radiosequencing. A synthetic peptide derived from the corresponding *MET* sequence was phosphorylated by $p190^{MET}$ in an *in vitro* assay and coeluted in reverse phase chromatography. Tyr^{1235} lies within the tyrosine kinase domain of $p190^{MET}$, within a canonical tyrosine autophosphorylation site that shares homology with the corresponding region of the insulin, CSF-1 and platelet-derived growth factor receptors, and of $p60^{src}$ and $p130^{gag\text{-}fps}$. The $p190^{MET}$ kinase is constitutively phosphorylated on tryosine in a gastric carcinoma cell line (GTL16), due to the amplification and overexpression of the *MET* gene. Metabolic labeling of GTL-16 cells with [^{32}P]orthophosphate followed by immunoprecipitation and tryptic phosphopeptide mapping of $p190^{MET}$ showed that Tyr^{1235} is a major site of tyrosine phosphorylation *in vivo* as well. Since phosphorylation activates $p190^{MET}$ kinase, we propose a regulatory role for Tyr^{1235}.

Tyrosine kinase activity is a critical transducing mechanism for the mitogenic signal. The role of autophosphorylation has been described for different receptor and nonreceptor protein tyrosine kinases. Several examples are known in which tyrosine phosphorylation is an early event in signal transduction, acting as a regulatory step of enzymatic activity (Hunter and Cooper, 1985; Hunter, 1987; Ullrich and Schlessinger, 1990). Phosphorylation takes place at specific sites and, in different receptors, may enhance (Honegger *et al.*, 1988; Bertics and Gill, 1985) or inhibit (Downward *et al.*, 1985) the subsequent phosphorylation of relevant cellular targets. Several protein tyrosine kinases catalyze their phosphorylation on multiple sites (Hunter and Cooper, 1985; Tornqvist *et al.*, 1987; Hazan *et al.*, 1990). Many oncogene products derived from cellular tyrosine kinases reveal altered autophosphorylation sites compared with their normal counterparts (Yarden and Ullrich, 1988).

$p190^{MET}$ is a 190-kDa transmembrane protein, encoded by the *MET* oncogene, with structural and functional properties of a tyrosine kinase receptor (Giordano *et al.*, 1988, 1989a; Gonzatti-Haces *et al.*, 1988). The molecule features a heterodimeric ($\alpha\beta$) structure. A 145-kDa β chain that spans the membrane is disulfide-linked with a 50-kDa α chain exposed to the cell surface (Giordano *et al.*, 1989a, 1989b). Recent works by others and by us (Bottaro *et al.*, 1991; Naldini *et al.*, 1991b) suggest that $p190^{MET}$ is the receptor for the hepatocyte growth factor (Miyazawa *et al.*, 1989; Nakamura *et al.*, 1989; Zarnegar *et al.*, 1990). We previously showed that $p190^{MET}$ tyrosine kinase is activated by *in vitro* autophosphorylation (Naldini *et al.*, 1991a). To identify the residue involved in activation, we have analyzed tyrosine phosphorylation sites of the β subunit in the conditions used for *in vitro* kinase activation. The major phosphorylation site *in vitro* was found to be tyrosine 1235[1] (Tyr^{1235}). The presence of the same tyrosine-phosphorylated peptide *in vivo* was confirmed by [^{32}P]orthophosphate labeling experiments. Given the amino acid homology in the catalytic domain of protein tyrosine kinases (Hanks *et al.*, 1988), Tyr^{1235} of the *MET* sequence corresponds to a major *in vitro* tyrosine autophosphorylation site of the CSF-1 receptor (Roussel *et al.*, 1990; Tapley *et al.*, 1990; Van Der Geer and Hunter, 1990), platelet-derived growth factor receptor (Kazlauskas and Cooper, 1989), $p60^{src}$ (Cooper, 1989; Veillette and Bolen, 1989) and $p130^{gag\text{-}fps}$ (Weinmaster *et al.*, 1984). Tyr^{1235} of $p190^{MET}$ is also homologous to one of the three *in vitro* phosphorylated tyrosines of the insulin receptor (Herrera and Rosen, 1986; Tornqvist *et al.*, 1987). In some of the above receptors, autophosphorylation on tyrosine residues homologous to Tyr^{1235} represents an activation step. Since the $p190^{MET}$ protein is activated by autophosphorylation *in vitro*, we propose a regulatory role for Tyr^{1235}.

MATERIALS AND METHODS

Reagents and Cells—Staphylococcus aureus protein A covalently coupled to Sepharose was purchased from Pharmacia LKB Biotechnology Inc. [γ-^{32}P]ATP was obtained from Amersham Corp. Protease inhibitors were from Sigma. Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[2] were from Bio-Rad.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X54559.

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[1] The $p190^{MET}$ sequence numbering refers to the cDNA sequence revised by Ponzetto *et al.* (1991).

[2] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; CHAPS, 3-[(3-cholamido-propyl)dimethylammonio]-1-propanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; MES, 4-morpholineethanesulfonic acid; RP, reverse phase; HGF, hepatocyte growth factor.

High performance liquid chromatography (HPLC) apparatus was from Pharmacia, and HPLC-grade solvents were from Baker Co. Inc. The molecular weight markers used in SDS-PAGE were from GIBCO/BRL. Trypsin was from Worthington. *S. aureus* V8 protease was purchased from Sigma. Tyrosine containing synthetic peptides was synthetized by Neosystem Laboratories and further purified on a C_{18} Vydac RP HPLC column (The Separations Group).

GTL16 cell line is a clonal cell line derived from a poorly differentiated gastric carcinoma line (Giordano *et al.*, 1988). In this line, the *MET* gene is amplified, overexpressed, and $p190^{MET}$ is phosphorylated (Giordano *et al.*, 1989a). The GTL-16 *MET* gene has been cloned and sequenced. No mutations were detected when compared with the nonamplified *MET* genes sequenced in different cell lines (Ponzetto *et al.*, 1991). Cells were grown in RPMI 1640 medium containing 10% fetal bovine serum and maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Anti-Met antibodies were raised in rabbits immunized against the synthetic peptide VDTRPASFWETS corresponding to the amino acid sequence at the carboxyl-terminal end of the predicted c-*MET* gene product as described (Tempest *et al.*, 1986). Monoclonal anti-Met antibodies were used as culture supernatant of hybrid cells screened from fusions of the spleen cells of mice immunized with GTL-16 cells.

Immunoprecipitation and Phosphorylation Reaction—Cells were washed twice with cold phosphate-buffered saline and scraped with a rubber policeman in cold phosphate-buffered saline containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. Cells were then pelleted at low speed at 4 °C and extracted with 1% CHAPS in HEPS buffer (25 mM HEPES-NaOH, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 100 mM NacL, 10% glycerol, and a mixture of protease inhibitors including 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 100 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, and 10 μg/ml pepstatin for 30 min at 4 °C with stirring. The cell lysates were cleared by centrifugation at 15,000 × *g* for 15 min at 4 °C. After these treatments, in the absence of phosphatase inhibitors, the $p190^{MET}$ is completely dephosphorylated (Naldini *et al.*, 1991a). Protein A-Sepharose (30 μl of packed beads/100 μl of lysate) was preincubated with anti-Met peptide antiserum (3 μl/100 μl of lysate) or monoclonal antibodies (1 μl of ascitic fluid/100 μl of lysate), washed twice with buffer, and incubated with the lysates for 3 h at 4 °C with stirring. Bound proteins were then washed three times with HEPS buffer and twice with KB buffer (containing 20 mM HEPES-NaOH, pH 7.1, 5 mM $MgCl_2$, 100 mM NaCl). Then beads were washed once with KB buffer, and the phosphorylation reaction was performed in the presence of [γ-^{32}P]ATP, 10 mM unlabeled ATP for 15 min at 37 °C. The reaction was stopped by adding concentrated boiling Laemmli buffer (Laemmli, 1970), and then the sample was subjected to 8% SDS-PAGE in reducing conditions. Gel was dried and subjected to autoradiography. Phosphoamino acid analysis of ^{32}P-*in vitro*-labeled $p190^{MET}$ was done as previously described (Cooper *et al.*, 1983; Naldini *et al.*, 1991b).

Immunoprecipitation of in Vivo [^{32}P]Orthophosphate-labeled $p190^{MET}$—After an overnight incubation in serum-free RPMI media, GTL-16 cells were washed twice with RPMI minus phosphate (Irvine Scientific), starved for 15 min in RPMI minus phosphate, and radiolabeled with [^{32}P]orthophosphate by incubation for 4 h in RPMI minus phosphate containing 37 MBq/ml [^{32}P]orthophosphate. Cells were then lysated with HEDS buffer containing protease and phosphatase inhibitors (50 mM sodium pyrophosphate, 100 mM NaF, 2 mM Na_3VO_4, 30 mM phenyl phosphate, 1 mM $ZnCl_2$, and protease inhibitors as described above) and 2% SDS, sonicated and diluted to 0.2% SDS with the same buffer and centrifuged at 12,000 rpm to remove insoluble material. Lysates were then precipitated with anti-p145 carboxyl-terminal peptide antiserum for 2 h. Beads were washed five times with HEDS. Laemmli buffer was added, and the immunoprecipitated labeled protein was separated on an 8% SDS-PAGE in reducing conditions. The protein was further digested with trypsin as previously described.

Phosphopeptide Mapping—For phosphopeptide mapping, the [γ-^{32}P]ATP-labeled bands were excised from the polyacrylamide gel, washed twice with 10% methanol to remove SDS, minced, and dried in a lyophilizer. The gel slices were rehydrated with 50 mM NH_4CO_3, pH 7.8, containing 50 μg/band of Trypsin-TPCK and incubated for 2 h at 37 °C. The tryptic digestion was repeated once, and the gel slices were further eluted with 50 mM NH_4CO_3, pH 7.8 (Honegger *et al.*, 1988). Eluates were then lyophilized and further incubated with 100 μg/ml trypsin for 2 h at 37 °C. The dessicated sample was then resuspended in Buffer A (100% water containing 0.1% trifluoroacetic acid) and filtered on a 0.2-μm Acrodisc filter (Gelman Instrument Co.). The phosphopeptides were analyzed on a reverse phase C_2/C_{18} Superpack Pep-S column (Pharmacia), resolved on a gradient of acetonitrile in Buffer A of 0.46%/min (from 0–32% acetonitrile in 70 min) with a flow rate of 1 ml/min. The eluted radioactivity was monitored by a Radiomatic A-100 radioactive flow detector (Packard Instrument Co.).

Phosphopeptide Edman Radiosequencing—The fractions collected from HPLC separations were counted in a β-counter (Packard), and the fractions corresponding to the largest radioactive peak were pooled and lyophilized. After having been dissolved in 30% acetonitrile in water, samples were applied to sequelon-arylamine membrane disks at 55 °C. The membrane was allowed to dry, removed from the heating block, and rewet with a minimal amount of water-soluble ethyl carbodiimide (5 ml of 10 mg/ml) in MES buffer, pH 5.0. The reaction was allowed to proceed for 20 min at room temperature. The membranes were then thoroughly washed with 100% trifluoroacetyl acid and applied to a MilliGen/Biosearch 6600 liquid phase Pro-Sequencer protein sequencing system for Edman degradation. After each cycle, the eluted amino acid was collected and counted on a β-counter (for a method of phosphotyrosine Edman radiosequencing, see also Meyer *et al.*, 1991).

Phosphorylation of Synthetic Peptides—Phosphorylation reaction on the immunoprecipitated $p190^{MET}$ was done in the presence of different concentrations of three synthetic peptides: D5K: N′-DMYDK-C′ amino acids 1228–1232, E8K: N′-EYYSVHNK-C′ amino acids 1233–1240, and G21R: N′-GAPYPDVNTFDIT-VYLLQGRR-C′ amino acids 1280–1300. Amino acid numbering refers to the $p190^{MET}$ predicted sequence. For the kinetic analysis, the reaction mixture was incubated for 5 min at 4 °C. The reaction was stopped by adding EDTA to a final concentration of 10 mM. The supernatant was collected and cleared by precipitation with 5% trichloroacetic acid for 1 h at 4 °C. Equal volumes were spotted on a 3.5-cm square phosphocellulose paper (P-81 ion exchange chromatography paper from Whatman), washed once in 30% acetic acid for 15 min, twice in 15% acetic acid for 15 min, and once in acetone for 5 min, air dried, and counted in a β-counter (Packard) with scintillation fluid, as described (Glass *et al.*, 1978).

D13K peptide was generated by exhaustive tryptic cleavage of V21K synthetic peptide (V21K: N′-VADFGLARDMYDKEY-YSVHNK-C′ amino acids 1229–1240 of $p190^{MET}$). The digestion was performed incubating 500 μg of V21K peptide with 50 μg of trypsin for 2 h at 37 °C, and the treatment was repeated twice. The obtained D13K peptide (N′-DMYDKEYYSVHNK-C′ amino acids 1228–1240 of $p190^{MET}$) was purified on a C_{18} Vydac RP HPLC column.

50 μg of E8K, D13K, and V21K were subjected to phosphorylation by the immunoprecipitated kinase as previously described. The reaction was incubated for 15 min at 37 °C and stopped by addition of EDTA to a final concentration of 10 mM. The immunoprecipitate was pelleted, and the supernatants were cleared by centrifugation after addition of trichloroacetic acid to a final concentration of 5%. The supernatants were vacuum dried, resuspended in water, and loaded on a Seppack light C_{18} cartridge (Waters Associates) to separate the phosphorylated peptide from free phosphate and labeled ATP. The peptides were then eluted with 80% acetonitrile, 0.1% trichloroacetic acid in water, vacuum dried, and subjected to HPLC analysis. The peptides were digested when indicated. Trypsin digestion was done on the phosphorylated samples with a double incubation of 20 μg of trypsin for 2 h at 37 °C. *S. aureus* V8 protease digestion of the phosphorylated peptides was accomplished with an incubation of 50 μg of enzyme for 2 h.

Peptide phosphorylation by v-*abl* tyrosine kinase domain was done as follows. 350 ng of purified enzyme were incubated with 50 μg of E8K peptide in the presence of 10 μCi [γ-^{32}P]ATP, 10 μM cold ATP, 25 mM Tris, pH 8, 10 mM $MgCl_2$, and 100 μM dithiothreitol in a final volume of 50 μl. The reaction was incubated for 15 min at 37 °C. The reaction was blocked by adding 50 μl of 5% trichloroacetic acid in water. Samples were then incubated for 30 min at 4 °C and pelleted for 5 min at 4 °C at 12,000 rpm. Supernatants were vacuum dried and resuspended in water. Phosphopeptides were bound to a Seppack light C_{18} cartridge (Waters), eluted as previously described, and subjected to HPLC analysis. Bacterial purified v-*abl* tyrosine kinase domain produced in *Escherichia coli* (Ferguson *et al.*, 1985) was kindly provided by P. Dalla Zonca.

RESULTS

p190^{MET} was immunoprecipitated from GTL-16 cells, dephosphorylated, and incubated *in vitro* with [γ-^{32}P]ATP under conditions activating the tyrosine kinase. The immunoprecipitation was performed with antibodies directed either toward the amino or the carboxyl terminus of the molecule with equivalent results (Naldini *et al.*, 1991).

The phosphorylated β subunit (p145^{MET}) was separated by SDS-polyacrylamide gel electrophoresis in reducing conditions and identified by autoradiography (Fig. 1*a*). Phosphoamino acid analysis, performed on the protein eluted from the excised band, proved that radiolabeled phosphate was incorporated exclusively on tyrosine residues (Fig. 1*b*).

The eluted ^{32}P-radiolabeled-p145^{MET} β subunit was subjected to trypsin digestion. Phosphopeptides were separated on a C_2/C_{18} RP HPLC column and eluted with a gradient of acetonitrile/water at pH 2.0. In these conditions, six *peaks* (*A–F*) were resolved (Fig. 2). Inconsistency of the *F peak* with a late elution suggested it to be a large partial tryptic peptide. Since its presence was not reproducible in all the profiles, it was not further characterized. DEAE-anion exchange HPLC showed that *peaks A, B,* and *D* contained a single phosphopeptide, while *peaks C* and *E* contained a mixture of different peptides (not shown). It was thus concluded that the peptide eluted in *peak A* at 35′ in RP (peptide A) contained the major tyrosine phosphorylation site *in vitro*.

Fractions corresponding to the peptide A were collected, pooled, and subjected to Edman covalent radiosequencing. Yield of the covalent coupling to the arylamine membrane was about 20%. The amount of ^{32}P bound to the phenylthiohydantoin amino acid eluted after each cycle was measured. A peak of radioactivity was detected at the eighth cycle (Fig. 3). This showed that a phosphotyrosine residue was present in peptide A at position number eight from the amino terminus. According to the published amended sequence of the *MET* protein (Ponzetto *et al.*, 1991), no tyrosine is present at the eighth position from the amino terminus of the peptides resulting from an exhaustive tryptic digestion. To find a tyrosine residue at the eighth position of a tryptic peptide, we have to assume that Lys^{1232}, a potential tryptic site surrounded by two acidic residues (*DKE*), remains uncleaved (Fig. 4). In

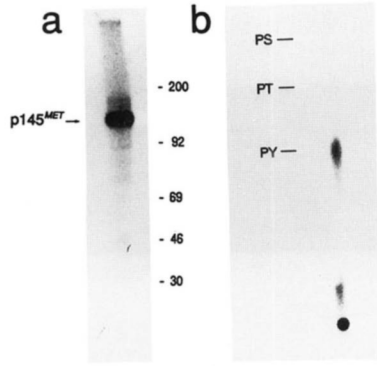

FIG. 1. ***In vitro* phosphorylation of p190^{MET} immunoprecipitated from GTL-16 cells.** Met protein was precipitated and phosphorylated with [γ-^{32}P]ATP as described under "Materials and Methods." A band of 145 kDa corresponding to the phosphorylated β subunit was visualized by autoradiography of an SDS-PAGE gel (*panel a*). The migration of the molecular weight standards in kDa is indicated on the *right*. Phosphoamino acid analysis was performed by thin layer electrophoresis of the eluted labeled phosphoprotein (*panel b*). The migration of standard phosphoamino acids is shown on the *left*. Phosphotyrosine was the only detectable phosphoamino acid. *PS*, phosphoserine; *PT*, phosphothreonine; *PY*, phosphotyrosine.

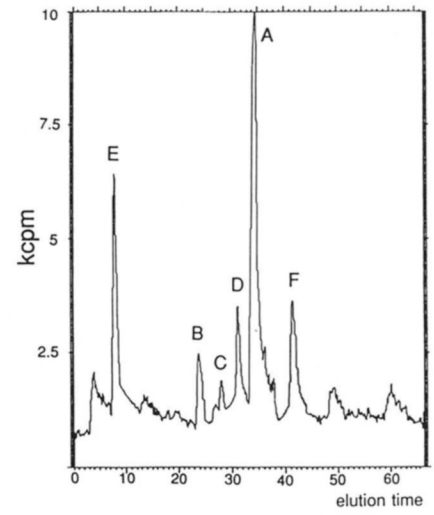

FIG. 2. **Tryptic phosphopeptide analysis of p145^{MET} β subunit labeled *in vitro* with [γ-^{32}P]ATP.** Labeled SDS-PAGE bands of p145^{MET} were subjected to TPCK trypsin digestion in 50 mM ammonium carbonate, pH 7.8, for 4 h at 37 °C. The cleavage products were analyzed on an RP HPLC PepS column developed with a 0–32% acetonitrile gradient in 82 min. Six major peaks designated *A–F* were resolved.

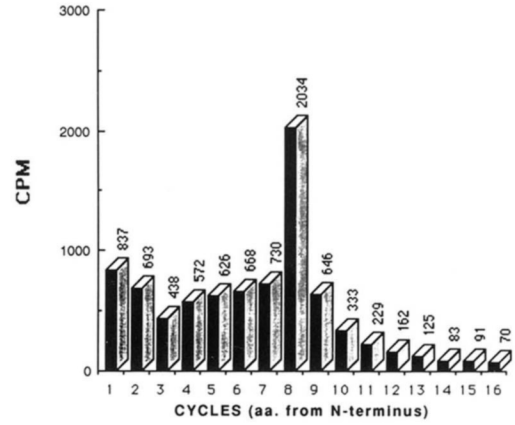

FIG. 3. **Edman radiosequencing of HPLC-purified peptide A.** Radioactive *peak A* was bound to sequelon-arylamine membrane disks. Membranes were then processed with a MilliGen/Biosearch 6600 liquid phase ProSequencer protein sequencing system for Edman degradation. After each cycle, the eluted phenylthiohydantoin-derivative was collected and counted on a β counter. Of the 40,000 cpm recovered from the *peak A*, about 20% of the counts were covalently linked to the membrane disks. A peak of radioactivity was detected in correspondence to the eighth cycle.

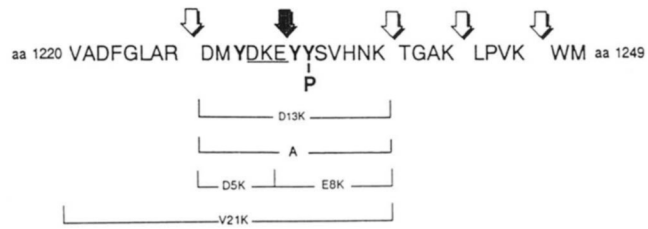

FIG. 4. **The amino acid sequence of p190^{MET} surrounding the major phosphorylation site.** *Open arrows* show the trypsin-specific digestion sites (Arg-*X*, Lys-*X*). The *solid arrow* points at *S. aureus* V8 protease site. The residues (*underlined*) correspond to a putative tryptic site that is inefficiently cleaved in our conditions. Tyr^{1235} is 8 residues after the amino-terminal tryptic cleavage site Arg^{1227}. Note the presence of a canonical consensus sequence for tyrosine phosphorylation, Arg^{1227}-3*X*-Asp^{1231}-3*X*-Tyr^{1235}.

this case, Tyr^{1235}, included in the peptide starting from Asp^{1228} to Lys^{1240}, corresponds to the expected tyrosine. The impairment of trypsin cleavage by acidic residues surrounding arginine or lysine has been previously described (Allen, 1990). To prove that the described tryptic digestion site is not cleaved in the above stated conditions, a trypsin digestion of a synthetic peptide D13K designed on the predicted $p190^{MET}$ sequence (Fig. 4) and containing the DKE residues (*underlined*) was performed. The D13K peptide was phosphorylated *in vitro* by $p190^{MET}$, and the elution time of the D13K peptide was unaffected by tryptic digestion. In addition, the phosphorylated peptide coeluted in RP HPLC with phosphopeptide A (Fig. 5), strongly suggesting the identity of the tryptic phosphopeptide D13K with the peptide A.

Peptide A contains three tyrosines, Tyr^{1230}, Tyr^{1234}, and Tyr^{1235}. To show that Tyr^{1235} is indeed the only autophosphorylated site, two peptides reproducing the sequence indicated in Fig. 4 (*E8K* and *D5K*) were synthetized and tested as exogenous substrates for $p190^{MET}$ kinase *in vitro*. Phosphorylation occurred on E8K including Tyr^{1234} and Tyr^{1235} but not on D5K including Tyr^{1230}. The K_m of phosphorylation was 1.3 mM (Fig. 6). To prove that Tyr^{1230} did not skip phosphorylation due to the shortness of the peptide and/or to the lack of a complete amino-terminal consensus sequence for the kinase, we analyzed the phosphorylation of the longer peptide V21K, which includes the D5K sequence and possesses a long amino-terminal stretch upstream of Tyr^{1230}. In V21K, Tyr^{1230} is opposite to Tyr^{1234}-Tyr^{1235} with respect to a V8 site (Glu^{1233}-Tyr^{1234}) (see Fig. 4). Digestion with V8 protease of V21K showed that the only tyrosine phosphorylated was in the fragment containing Tyr^{1234} and Tyr^{1235} (Fig. 7*a*). In fact, this fragment comigrated with E8K digested with V8 protease (Fig. 7*b*). The E8K peptide contains the two tyrosines Tyr^{1234} and Tyr^{1235}. Only one tyrosine was phosphorylated *in vitro* by $p190^{MET}$. In fact, phosphorylated E8K eluted as a single peak in RP HPLC (Fig. 8*a*) and in anion exchange (not shown), suggesting that E8K is indeed phosphorylated only at 1 residue. On the contrary, when E8K was phosphorylated by a tyrosine kinase with a relaxed substrate specificity, as the v-*abl*-encoded *E. coli*-expressed kinase domain, both tyrosines were phosphorylated, and two peaks were observed in RP HPLC (Fig. 8*b*). A further evidence for phosphorylation of Tyr^{1235} but not of Tyr^{1234} in synthetic peptides by $p190^{MET}$ was given by chymotryptic digestion experiments. Chymotrypsin cleaves, among others, Tyr-*X* bonds, when the tyrosine residue is not phosphorylated (Allen, 1990). A chymotryptic treatment of E8K or V21K after phosphorylation by $p190^{MET}$ originated a unique phosphopeptide that eluted at 22 min in RP HPLC (not shown). The only possible phosphopeptide common to E8K and V21K corresponds to the sequence Tyr^{1235}-Lys^{1240} resulting from the cleavage of the Tyr^{1234}-Tyr^{1235} bond (see Fig. 4).

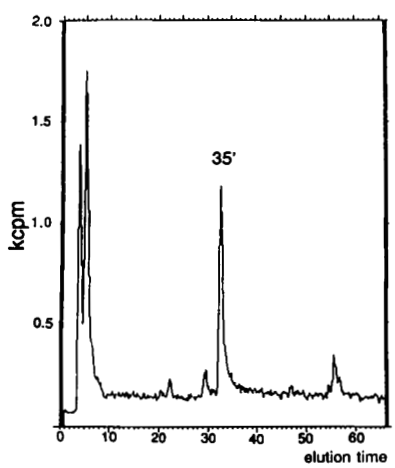

FIG. 5. **RP HPLC elution profile of D13K phosphorylated peptide.** D13K after $p190^{MET}$ phosphorylation was resolved in a single major phosphopeptide peak eluting at 35 min. The peak comigrates with phosphopeptide A as shown in Fig. 2. The phosphorylated peptide was not cleaved by trypsin, determined by an identical elution after trypsin incubation (not shown).

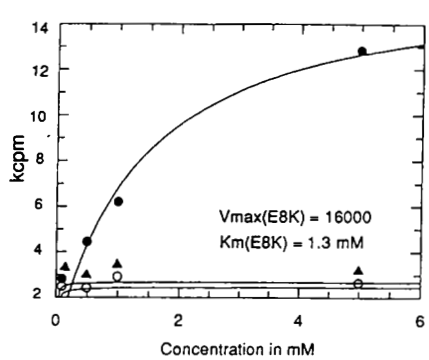

FIG. 6. **Tyrosine phosphorylation of synthetic peptides by $p190^{MET}$.** The phosphorylation reaction with the immunoprecipitated $p190^{MET}$ was done as described under the "Materials and Methods." The peptide containing Tyr^{1235} is the only one phosphorylated. D5K: N′-DMYDK-C′ corresponds to amino acids 1228–1232 of MET (○); E8K: N′-EYYSVHNK-C′ corresponds to amino acids 1233–1240 of MET (●); G21R: N′-GAPYPDVNTFDITVYLLQGRR-C′ corresponds to amino acids 1280–1300 of MET (▲).

To identify the tyrosine phosphorylated peptides *in vivo*, we did a [^{32}P]orthophosphate labeling of GTL-16 cells in culture. In this cell line, $p190^{MET}$ is constitutively phosphorylated on tyrosine due to the amplification and overexpression of a normal *MET* gene (Giordano *et al.*, 1988; Ponzetto *et al.*, 1991). The ^{32}P-metabolically labeled $p190^{MET}$ was immunopurified and subjected to trypsin digestion. RP HPLC analysis of the labeled tryptic phosphopeptides was performed as previously described. Seven labeled peaks were resolved, one of which (*peak A*) was eluted with identical retention time to the major peptide phosphorylated on tyrosine *in vitro* (Fig. 9). Phosphoamino acid analysis of *peak A* (35 min) and of the other major peak resolved at 4 min, 21 min (*K*), 30 min (*H*), and 44 min (*G*) were performed (Fig. 10). The peak eluting at 4 min contained free phosphate only. *Peak A* was the only one containing exclusively phosphotyrosine. *Peak G* contained phosphoserine and a low amount of phosphotyrosine. *Peaks K* and *H* contained exclusively phosphoserine. These data show that Tyr^{1235} of peptide A is a major tyrosine-phosphorylated site of $p190^{MET}$ both *in vitro* and *in vivo*. The *in vivo* serine phosphorylated peptides *G*, *K*, and *H* were expected, since a serine phosphorylation of $p190^{MET}$ *in vivo* was shown in previous work (Gandino *et al.*, 1990, 1991).

DISCUSSION

It has been demonstrated that autophosphorylation positively regulates the tyrosine kinase activity of insulin receptor increasing the V_{max} of the enzyme and modulates the biological responses to insulin (Rosen *et al.*, 1983; Tornqvist and Avruch, 1988; Ellis *et al.*, 1986). Tyrosine residue 1146 (Tyr^{1146}) represents the major *in vitro* autophosphorylation site of the receptor with Tyr^{1150} and Tyr^{1151} that are phosphorylated alternatively and to a lesser extent (Tornqvist *et al.*, 1987, 1988). Phosphorylation on these sites has an activation role on the tyrosine kinase, and it is required *in vivo* for biological response to insulin (White *et al.*, 1988; Yu and Czech, 1984; Tornqvist and Avruch, 1988; Ellis *et al.*, 1986).

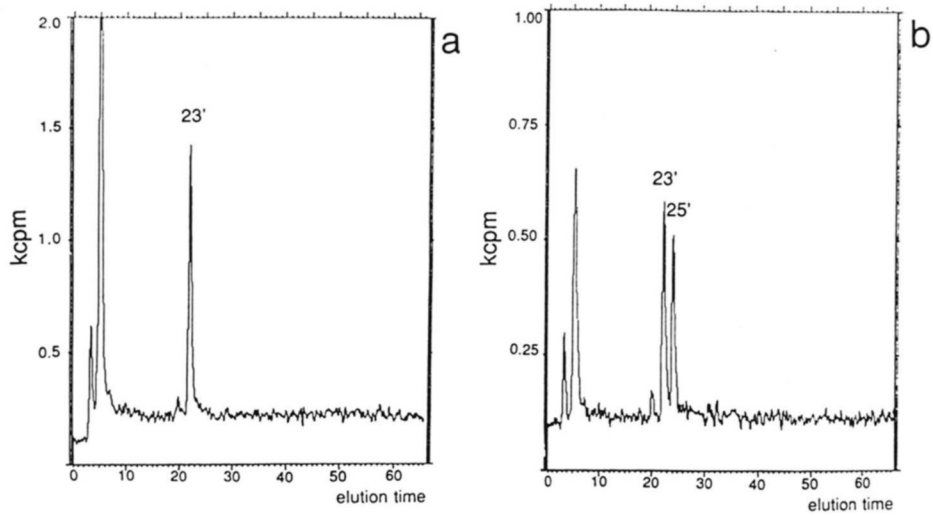

FIG. 7. **RP HPLC elution profile of V21K and E8K phosphorylated by p190^{MET} and digested with *S. aureus* V8 protease.** After selective cleavage of Glu-*X* bonds, V21K phosphopeptide shifts to a retention time (*profile a*) identical with the one obtained after cleavage of E8K (*profile b*). In *profile b*, the undigested form of E8K phosphopeptide is detectable at 25 min. The *early peaks* correspond to residual-free [γ-^{32}P]ATP and free [^{32}P]phosphate.

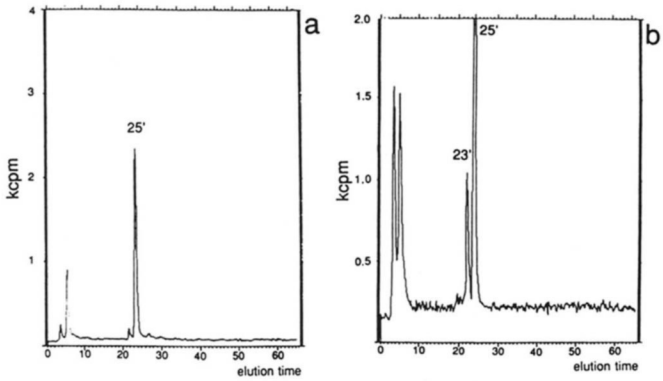

FIG. 8. **Phosporylation of E8K by p190^{MET} and recombinant v-*abl* kinase.** RP HPLC elution profile of E8K peptide phosphorylated by p190^{MET} kinase (*profile a*) and by a truncated tyrosine kinase domain from bacterial v-*abl* (*profile b*). Phosphorylation by p190^{MET} originates mainly one phosphorylated form of E8K eluting at 25 min. E8K phosphorylated by p190^{MET} was resolved as a single phosphopeptide also on the anion exchange HPLC column (data not shown). The v-*abl* enzymatic activity produces two different phosphorylated forms of the peptide (23 min and 25 min of elution time). The *early peaks* present in *both profiles* correspond to residual-free [γ-^{32}P]ATP and free [^{32}P]phosphate.

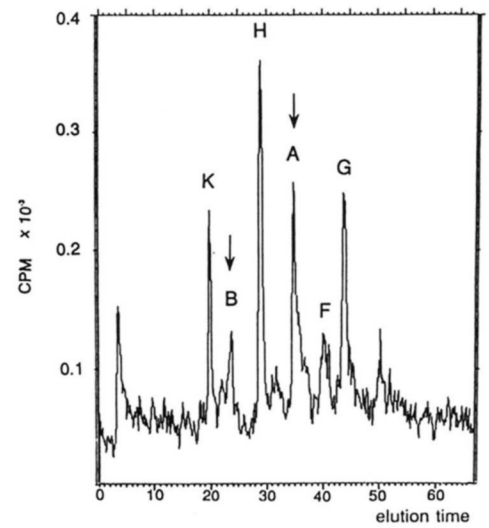

FIG. 9. **Tryptic phosphopeptide analysis of p145^{MET} protein labeled *in vivo* with [^{32}P]orthophosphate.** Five major *peaks* designated *A, B, G, H* and *K* were resolved. *Arrows* point to the elution times of the corresponding phosphopeptides phosphorylated *in vitro* (see Fig. 3).

Tyrosine phosphorylation on residue 807, together with residues 697 and 706, is detectable on the CSF-1 receptor both *in vitro* and *in vivo* after CSF-1 binding (Tapley *et al.*, 1990). Tyr^{807} phosphorylation is critical for CSF-1-induced mitogenesis (Roussel *et al.*, 1990). Kinase activation by tyrosine autophosphorylation has also been described for the v-*fps* kinase p130$^{gag\text{-}fps}$ (Weinmaster *et al.*, 1984; Meckling-Hansen *et al.*, 1987) and for p60^{src} kinase (Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987). The major *in vitro* tyrosine autophosphorylation sites are located in the catalytic domain (Tornqvist *et al.*, 1987, 1988; Weinmaster *et al.*, 1984). Tyr^{416} is the major autophosphorylation site of p60^{src} *in vitro*. It is constitutively phosphorylated *in vivo* in all transforming mutants of the molecule (Smart *et al.*, 1981; Hanafusa, 1987; Iba *et al.*, 1984; Cartwright *et al.*, 1986). Its phosphorylation increases the V_{max} of the p60^{src} kinase for exogenous substrates (Veillette and Bolen, 1989; Cooper, 1988). Substitution of the Tyr^{416} residue with phenylalanine by site-directed mutagenesis impairs kinase activity *in vitro* (Kmiecik *et al.*, 1988) and provides a mutant with a decreased transforming potential (Ferracini and Brugge, 1990; Piwnica-Worms *et al.*, 1987; Kmiecik and Shalloway, 1987). Tyr^{1073} of p130$^{gag\text{-}fps}$, the homologous residue of p60^{src} Tyr^{416}, has a similar behavior (Weinmaster *et al.*, 1984; Meckling-Hansen *et al.*, 1987). In the epidermal growth factor receptor, the tyrosine homologous to p60^{src} Tyr^{416} in the kinase domain is not phosphorylated.

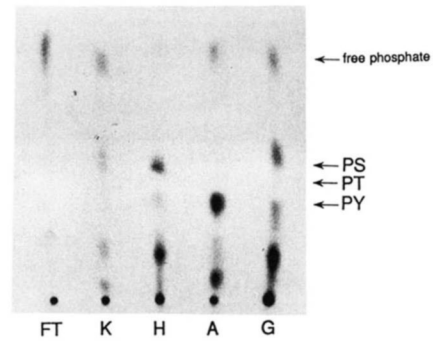

FIG. 10. **Phosphoamino acid analysis of tryptic peptides phosphorylated *in vivo*.** Thin layer electrophoresis was done on the acid-hydrolized peptides resolved on Fig. 11. The migration of standard phosphoamino acids is shown on the *left*. *PS*, phosphoserine; *PT*, phosphothreonine; *PY*, phosphotyrosine.

L A R L I E D N E **Y** T A R	Y 416 RSV p60$^{v\text{-}src}$
L A R L I E D N E **Y** T A R	Y 701 Y73 p90$^{gag\text{-}yes}$
L A R E E A D G V **Y** A A S	Y1073 FSV p130$^{gag\text{-}fps}$
L A R D I **Y** E T D **Y** **Y** R K	Y1146-Y1150-Y1151 IR
L A R D M Y D K E Y **Y** S V	Y1235 MET
L A R D I M N D S N **Y** I V	Y 807 CSF-1 R
L A R D I M R D S N **Y** I V	Y 857 PDGF R

FIG. 11. **Sequence homologies surrounding the major *in vitro* phosphorylated sites of different tyrosine kinases.** These sequences are located within the philogenetically conserved tyrosine kinase domain. The major phosphorylation sites are in *boldface letters*. These include Tyr^{1146}, Tyr^{1150}, and Tyr^{1151} of the insulin receptor (Tapley *et al.*, 1990), Tyr^{807} of the CSF-1 receptor (Tornqvist *et al.*, 1988), Tyr^{857} of the platelet-derived growth factor β receptor (Kazlauskas and Cooper, 1989), Tyr^{416} of p60^{src} (Patchinsky *et al.*, 1982; Cross and Hanafusa, 1983), Tyr^{1073} of p130$^{gag\text{-}fps}$ (Meckling-Hansen *et al.*, 1987; Van Der Geer and Hunter, 1990), and Tyr^{701} of p90$^{gag\text{-}yes}$ (Kitamura *et al.*, 1982). Modified from Hanks *et al.* (1988).

The autophosphorylation sites were identified on the long carboxyl-terminal tail of the molecule (Downward *et al.*, 1985; Margolis *et al.*, 1989). Their role in modulating the kinase activity by phosphorylation has been debated (Bertics and Gill, 1985; Bertics *et al.*, 1988; Downward *et al.*, 1985; Honegger *et al.*, 1989). The V_{max} of exogenous substrates phosphorylation is not affected, while the K_m is decreased (Honegger *et al.*, 1988; Downward *et al.*, 1985). As in the epidermal growth factor receptor, all the autophosphorylation sites of the putative receptor encoded by HER2 are located on the carboxyl terminus (Hazan *et al.*, 1990). The platelet-derived growth factor receptor appears to be phosphorylated on a site *in vitro* after stimulation with the specific ligand. The site is located in the tyrosine kinase domain and shares homology with the Tyr^{416} phosphorylation site on p60^{src}. Its role on regulation of tyrosine kinase in the molecule is still controversial (Fantl *et al.*, 1989; Kazlauskas and Cooper, 1989).

This paper shows that Tyr^{1235} is a major site of p190^{MET} tyrosine autophosphorylation both *in vitro* and *in vivo*. Other peptides appear to be phosphorylated on tyrosine, albeit to a lesser extent. It must also be realized that in GTL-16 cells, p190^{MET} is constitutively phosphorylated due to overexpression of the *MET* gene (Giordano *et al.*, 1989a; Ponzetto *et al.*, 1991) and thus analyzed *in vivo* under steady-state conditions. However, a number of considerations point to this residue as the critical autophosphorylation site of the receptor kinase activated in physiological conditions. The Tyr^{1235} residue is embedded in a typical consensus pattern for tyrosine phosphorylation (Pinna, 1990; Patchinsky *et al.*, 1982; Hunter, 1982; Cooper *et al.*, 1984; Kemp and Pearson, 1990; Gealen and Harrison, 1990). It is located 8 residues downstream an arginine residue, and 2 acidic amino acids (aspartic and glutamic) are present 2 and 5 residues upstream. Tyr^{1235} is located within the tyrosine kinase domain in a segment homologous to the major autophosphorylation sites of other receptor and nonreceptor kinases (Hanks *et al.*, 1988) (see Fig. 11). The *MET* sequence, including Tyr^{1235}, shares the highest homology with the corresponding sequence of the insulin receptor as expected from the phylogenetic relatedness (Hanks *et al.*, 1988; Yousuke *et al.*, 1985; Park *et al.*, 1987; Ponzetto *et al.*, 1991). In the insulin receptor, phosphorylation of Tyr^{1146}, Tyr^{1150}, and Tyr^{1151}, homologous to Tyr^{1235} of p190^{MET}, is required for full catalytic activity and biological response to insulin (Weinmaster *et al.*, 1984; Ellis *et al.*, 1986). In the human CSF-1 receptor Tyr^{807}, which is again the homologous of Tyr^{1235}, is a site of ligand-dependent receptor phosphorylation *in vivo* (Tapley *et al.*, 1990), and its deletion impairs the mitogenic response (Roussel *et al.*, 1990). The *src* homologue of p190^{MET} Tyr^{1235} is the previously mentioned Tyr^{416}. All oncogenic variants of the *src* gene are constitutively phosphorylated at this site (Hanafusa, 1987; Iba *et al.*, 1984; Cartwright *et al.*, 1986). Phosphorylation of p60^{src} at Tyr^{416} causes *in vitro* elevation of kinase activity (Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987).

Previous work has shown that serine phosphorylation plays a negative role in the regulation of the kinase activity of p190^{MET}. Serine phosphorylation of the molecule is enhanced by triggering the activity of protein kinase C or by increasing the intracellular concentration of Ca^{2+} ions (Gandino *et al.*, 1990, 1991). Conditions stimulating the serine phosphorylation of p190^{MET} decrease correspondingly the tyrosine phosphorylation only on Tyr^{1235} *in vivo*, as described (Gandino *et al.*, 1991). This suggests that phosphorylation of this site is involved in the full activation of the kinase in physiological conditions. We have also shown that phosphorylation on tyrosine enhances the kinase activity of p190^{MET} *in vitro* toward synthetic substrates (Naldini *et al.*, 1991a). Taken together, these data suggest that autophosphorylation on Tyr^{1235} plays a positive regulatory role in the kinase activity of the receptor encoded by the *MET* proto-oncogene.

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