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A Novel Recognition Motif for Phosphatidylinositol 3-Kinase Binding Mediates Its Association with the Hepatocyte Growth Factor/Scatter Factor Receptor

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The pleiotropic effects (mitogenesis, motogenesis, and morphogenesis) elicited by hepatocyte growth factor/scatter factor (HGF/SF) are mediated by the activation of the tyrosine kinase receptor encoded by the *MET* proto-oncogene. Following autophosphorylation, the receptor associates with the p85/110 phosphatidylinositol (PI) 3-kinase complex in vivo and in vitro. By a combination of two complementary approaches, competition with synthetic phosphopeptides and association with Tyr-Phe receptor mutants, we have identified Y-1349 and Y-1356 in the HGF/SF receptor as the binding sites for PI 3-kinase. Y-1349VHV and Y-1356VNV do not conform to the canonical consensus sequence YXXM for PI 3-kinase binding and thus define YVXV as a novel recognition motif. Y-1349 and Y-1356 are located within the C-terminal portion of the HGF/SF receptor and are phosphorylation sites. The affinity of the N- and C-terminal *src* homology region 2 (SH2) domains of p85 for the phosphopeptides including Y-1349 and Y-1356 is 2 orders of magnitude lower than that measured for Y-751 in the platelet-derived growth factor receptor binding site. However, the closely spaced duplication of the novel recognition motif in the native HGF/SF receptor may allow binding with both SH2 domains of p85, thus generating an efficient docking site for PI 3-kinase. In agreement with this model, we have observed that a phosphopeptide including both Y-1349 and Y-1356 activates PI 3-kinase in vitro.

A new concept in the field of signal transduction which links tyrosine kinase receptors to downstream signalling events has emerged in the past 2 years. A number of cytoplasmic molecules that mediate cellular responses to growth factors have been shown to interact with activated receptors through their *src* homology region 2 (SH2) domains (22). Each SH2 domain appears to be capable of binding to a distinct phosphotyrosine embedded in a given amino acid sequence of the receptor (3). This model has been confirmed in the last year by the work of several laboratories which have identified autophosphorylation sites in epidermal growth factor receptor, platelet-derived growth factor (PDGF) receptor, colony-stimulating factor 1 receptor, and fibroblast growth factor receptor acting as specific docking sites for phosphatidylinositol (PI) 3-kinase, phospholipase Cγ, and *ras*-GTPase activating protein (*ras*GAP) (3, 18, 23, 30, 32).

Hepatocyte growth factor/scatter factor (HGF/SF) elicits mitogenesis and/or motogenesis in epithelial cells (16, 24, 33, 37, 39). It should be noted that while its biological effects vary depending on the target cell, the HGF/SF signal is mediated by a single receptor, the tyrosine kinase encoded by the *MET* proto-oncogene (for a review, see reference 6). Thus, it is likely that the basis for these distinct biological responses will be found in a bifurcation in the pathways lying downstream to the receptor. We have previously shown that the HGF/SF receptor associates in vitro with a number of cytoplasmic signal transducers (2). We have also shown that addition of HGF/SF to responsive cells (A549 lung carcinoma cells) induces association of PI 3-kinase with the *Met* receptor in vivo (11) and stimulates the *ras*-guanine nucleotide exchanger (12). Identification of the transducer binding sites is a prerequisite to develop model systems aimed at correlating biological response with a specific signalling pathway.

In this work we sought to identify the binding site for PI 3-kinase in the HGF/SF receptor. PI 3-kinase catalyzes the production of phospholipids which may act as second messengers without need for hydrolysis (5, 28). This enzyme is known to interact with a number of tyrosine kinase receptors by means of the SH2 domains of its regulatory subunit, p85 (8, 25, 34). Its function has been shown to be indispensable for mitogenesis in some cell types (9, 31). In the HGF/SF receptor there is a potential recognition motif (Y-1313EVM), which could represent a binding site. Our results, obtained with both synthetic phosphopeptides and receptor Tyr-Phe mutants, show that although the synthetic phosphopeptide containing the consensus Y-1313EVM is capable of binding to PI 3-kinase, tyrosine 1313 can be eliminated from the receptor without affecting PI 3-kinase binding. The true binding sites in the native receptor are phosphotyrosine 1349 (Y-1349VHV) and phosphotyrosine 1356 (Y-1356VNV). These residues thus identify YV(N or H)V as a novel recognition motif for PI 3-kinase.

MATERIALS AND METHODS

Reagents, cells, and antibodies. All reagents, unless otherwise specified, were purchased from Sigma Chemical Co. Protein A covalently coupled to Sepharose was purchased from Pharmacia LKB Biotechnology Inc. All radioactive

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isotopes were purchased from Amersham Corp. A549 lung carcinoma cells and COS-7 cells, purchased from American Type Culture Collection, were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Flow Laboratories, Inc.) in a 5% CO_2-water-saturated atmosphere. *Spodoptera frugiperda* (Sf9) cells, from American Type Culture Collection, were grown in monolayer cultures with SF-900 medium (GIBCO-BRL). Antisera and monoclonal anti-*Met* antibodies (kindly provided by M. Prat) were raised against a synthetic peptide corresponding to the 19 C-terminal amino acids of the human *Met* protein. Antibodies against p85 are described by Otsu et al. (25). Synthetic phosphopeptides were a generous gift of C. Turck (Howard Hughes Medical Institute, San Francisco, Calif.) and were synthesized with an amino-terminal phosphotyrosine (7, 38). Recombinant HGF/SF was kindly provided by G. Gaudino.

Expression of HGF/SF receptor and p85 cDNAs in insect cells, using baculovirus vectors. Recombinant HGF/SF receptor and p85 baculoviruses were constructed as previously described (2, 25) and used to infect Sf9 cells.

GST-SH2 domain fusion proteins. The N- and C-terminal SH2 domains of the bovine PI 3-kinase p85 subunit (amino acids 314 to 431 and 612 to 722) were obtained by polymerase chain reaction and cloned into the pGEX-2 bacterial expression vector (35). Glutathione *S*-transferase (GST)–SH2 fusion proteins were purified from bacterial lysates by glutathione affinity chromatography (26). Amino acid analysis on an Applied Biosystems 420A analyzer was used to determine the concentrations of the recombinant proteins.

Site-directed mutagenesis and expression in COS 7 cells of the *MET* cDNA. The cloning of the *MET* cDNA has been reported previously (29) (EMBL data bank accession number X54559). The 3′ end fragment from nucleotide 2355 to the end was subcloned in pSELECT-1. Site-directed mutagenesis was performed by using an in vitro oligonucleotide site-directed mutagenesis system (Altered Sites in vitro mutagenesis system; Promega). Oligonucleotides were synthesized with an Applied Biosystem 391 apparatus. Mutant clones were identified by sequencing (T7 sequencing kit from Pharmacia). Full-size *MET* cDNAs carrying the appropriate Tyr-Phe mutation were reconstructed in the PMT2 vector, which contains the major late adenovirus promoter. All plasmids were transfected by Lipofectin (GIBCO-BRL) in COS 7 cells.

In vitro association experiments. Sf9 cells expressing the recombinant HGF/SF receptor (approximately 4×10^6 cells per point) were lysed 36 h after infection in buffer A (10 mM Tris-HCl buffer [pH 7.5], 10% glycerol, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA) supplemented with 0.2 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 0.1 TIU of aprotinin per ml, and 1 μg of pepstatin per ml. Lysates were clarified at 15,000 × *g* at 4°C for 15 min, and the supernatants were immunoprecipitated after 2 h of incubation with anti-*Met* antibodies coupled to protein A-Sepharose. Immunocomplexes were washed three times with buffer A, once with buffer B (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA), and once with buffer C [25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) (pH 7.2), 100 mM NaCl, 5 mM $MgCl_2$]. Samples were prephosphorylated by incubation for 15 min at 25°C in buffer C with 10 μM unlabelled ATP and then washed three times with cold buffer A supplemented with 1 mM sodium orthovanadate. Association between the immobilized receptor and the baculovirus-expressed p85 was carried out as previously described (2). For the association experiments with the PI 3-kinase holoenzyme, A549 cells (approximately 2×10^6 cells per point) serum starved for 3 days were used as a source of PI 3-kinase. A549 cells were Dounce homogenized in MOPS buffer [20 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.5), 1 mM $MgCl_2$, 0.1 mM EDTA, 200 mM sucrose, 1 mM sodium orthovanadate], supplemented with 0.2 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 0.1 TIU of aprotinin per ml, and 1 μg of pepstatin per ml. Homogenates were centrifuged at 100,000 × *g* for 20 min at 4°C. When the ability of phosphopeptides to block the association with the receptor was checked, cell lysates were preincubated with the phosphopeptides for 1 h at 4°C prior to incubation with the immobilized recombinant HGF/SF receptor. Following association, the complexes were washed three times with buffer A, twice with buffer D (0.5 M LiCl, 100 mM Tris-HCl [pH 7.6]), and twice with buffer B. The presence of the p85 subunit of the PI 3-kinase in the receptor immunoprecipitate was determined by (i) labelling of the receptor and associated proteins with [γ-^{32}P]ATP by in vitro kinase assay and (ii) Western immunoblotting. The presence of PI 3-kinase holoenzyme was assessed by activity assay.

In vivo association experiments. Transfected COS 7 cells expressing HGF/SF receptor mutants were stimulated for 10 min at 37°C with HGF/SF (12 ng/ml) and lysed in buffer A in the presence of 1 mM sodium orthovanadate. Lysates were clarified at 15,000 × *g* at 4°C for 15 min, and the supernatants were immunoprecipitated after 2 h of incubation with anti-*Met* antibodies (specific for the human protein) coupled to protein A-Sepharose. Complexes were washed twice with buffer A, twice with buffer D, and twice with buffer B. The presence of the receptor-associated PI 3-kinase in the complex was determined by PI 3-kinase assay as described by Whitman et al. (40).

In vitro kinase assay. Receptor-associated proteins were labelled in 20 μl of buffer C in the presence of 10 μCi of [γ-^{32}P]ATP (specific activity, 7,000 Ci/mmol; Amersham) at 25°C for 15 min. The reaction was stopped by adding 1 ml of ice-cold buffer A without protease inhibitors. Samples were washed three times with cold buffer A. The labelled immunocomplexes were eluted from protein A-Sepharose in boiling Laemmli buffer. Supernatants were then subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western immunoblotting. Immunoprecipitates after the association were solubilized in boiling Laemmli buffer, separated by SDS–8% PAGE, and electrotransferred into nitrocellulose filters (Hybond; Amersham). Filters were then incubated with the indicated antibodies, and specific binding was detected by the enhanced chemiluminescence system (Amersham).

Tryptic phosphopeptide mapping. ^{32}P-labelled bands corresponding to in vitro-phosphorylated wild-type and mutant HGF/SF receptors were excised from polyacrylamide gels and treated as previously described (10). Tryptic peptide digests were dissolved in 100% dimethylformamide, diluted 1:1 with the high-performance liquid chromatography (HPLC) loading buffer (0.1% trifluoroacetic acid in water), and separated by HPLC on a reverse-phase C_2/C_{18} Superpack Pep-S column (Pharmacia) with a gradient of acetonitrile (0 to 32% in 70 min) in the presence of 0.1% trifluoroacetic acid, with a flow of 1 ml/min. The eluted radioactivity was monitored by a Radiomatic A-100 radioactive flow detector (Packard Instrument Co.). As a control, a synthetic peptide (I24K, Neosystem Laboratories) was separated by HPLC as described above and analyzed at 214 nm. I24K encompasses 24 amino acids from isoleucine 1337 to lysine 1360 of the *Met* protein sequence and thus corresponds to the predicted tryptic phosphopeptide of interest, except for the fact that it is not phosphorylated.

Analysis of the interaction of Y-1349 and Y-1356 with the p85 N- and C-terminal SH2 domains with the BIAcore. Details of the construction and principle of operation of the BIAcore biosensor have been described (14, 15, 17). The SH2 domains used in these experiments were desalted through a Pharmacia column on a SMART chromatography system in order to achieve buffer exchange to the BIAcore running buffer, consisting of 20 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, and 4 mM dithiothreitol. Avidin (Boehringer) at 50 μg/ml in 20 mM sodium acetate buffer, pH 4.0, was immobilized on the sensor chip surface after activation with a 1:1 mixture of *N*-hydroxysuccinimide and *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (Pharmacia). Excess reactive groups were blocked with ethanolamine (1.0 M). Biotinylated phosphopeptide Y-751 (DMSKDESVDY*VPMLDMK) was injected over the avidin at a flow rate of 5 μl/s for 50 s. Nonspecifically bound material was removed with a short pulse (4 s) of 0.1% SDS. GST-SH2 domain fusion proteins were mixed with a range of concentrations of HGF/SF receptor phosphopeptides and injected over the surface at 5 μl/min for 40 s at a constant temperature of 25°C. The material bound to the surface was removed with a 4-s pulse of 0.1% SDS, which brought the signal to background level.

In vitro activation of PI 3-kinase. Cytosolic extracts of A549 cells serum starved for 3 days were prepared as described above. PI 3-kinase holoenzyme was immunoprecipitated for 2 h at 4°C by anti-p85 monoclonal antibodies coupled to protein-A Sepharose. Immunocomplexes were washed three times with buffer A (see above) and incubated for 30 min at 4°C in the same buffer with the synthetic peptides (either phosphorylated or unphosphorylated as a control) at a concentration of 100 μM. Following incubation, the immunoprecipitates were assayed for PI 3-kinase activity as previously described (40).

RESULTS

Competition experiments with synthetic phosphopeptides. In our initial studies, we made use of synthetic phosphopeptides to inhibit binding of p85 or PI 3-kinase in in vitro association experiments with the recombinant HGF/SF receptor. The phosphopeptides were designed to cover all the possible tyrosines present in the cytoplasmic portion of the HGF/SF receptor. The list of the phosphopeptides is shown in Table 1. Sixteen phosphopeptides were eight amino acids long and began with a phosphotyrosine residue at the N terminus. One phosphopeptide was 12 amino acids long and included two phosphotyrosines. Previous work carried out with the PDGF receptor had shown that the four amino acids located immediately downstream to the phosphotyrosine are important for defining the SH2 recognition site (9).

In the experiment shown in Fig. 1, we used lysates of insect cells (Sf9) infected with a recombinant baculovirus as a source of p85 protein (25). Such lysates were diluted appropriately (see Materials and Methods) and preincubated with each of the phosphopeptides (10 μM) before incubation with the HGF/SF recombinant receptor. The receptor was immunoprecipitated from lysates of Sf9 cells infected with a recombinant baculovirus carrying the full-size human *MET* cDNA (2). In these cells, the receptor is synthesized largely in the form of the uncleaved precursor (MET_{Bac} in the figures), which is, however, fully functional (2). The receptor was immobilized on protein-A Sepharose beads and prephosphorylated with cold ATP. After association, the beads were washed and the complexes were phosphorylated with

TABLE 1. Tyrosine-phosphorylated peptides derived from cytoplasmic sequences within the HGF/SF receptor[a]

Position of tyrosine in HGF/SF receptor sequence	Receptor phosphopeptide
971	Y*DARVHTP
1003	Y*RATFPED
1026	Y*PLTDMSP
1093	Y*HGTLLDN
1159	Y*MKHGDLR
1192	Y*LASKKFV
1230	Y*DKEYYSV
1234	Y*YSVHNKT
1235	Y*SVHNKTG
1284	Y*PDVNTFD
1295	Y*LLQGRRL
1307	Y*CPDPLYE
1313	Y*EVMLKCW
1349	Y*VHVNATY
1349–1356	Y*VHVNATY*VNVK
1356	Y*VNVKCVA
1365	Y*PSLLSSE

[a] Octameric peptides were synthesized with an amino-terminal phosphotyrosine (7, 38). In the left column, the amino acid position of the N-terminal tyrosine of each peptide is indicated. *, phosphorylated tyrosine residue. Peptide 1349-1356 is phosphorylated on both Y-1349 and Y-1356.

[γ-^{32}P]ATP. During the phosphorylation reaction, both the receptor and p85 become labelled and thus detectable by SDS-PAGE. Figure 1 shows that only three of the phosphopeptides efficiently inhibited p85 binding: Y-1313EVM LKCW, Y-1349VHVNATY, and Y-1356VNVKCVA. The phosphopeptide Y-1349VHVNATY-1356VNVK also completely prevented p85 binding, while phosphopeptide Y-1307CPDPLYE was only partially effective. In the experiment shown in Fig. 2, we sought to roughly define the relative affinities of these phosphopeptides for p85. The experiment was carried out in the same manner as the previous one, but the phosphopeptides were used at various concentrations between 10 nM and 1 μM. The efficiency at inhibiting p85 binding was highest for Y-1313EVMLKCW and was followed by Y-1356VNVKCVA, Y-1349VHVNATY, and Y-1307CPDPLYE. The phosphopeptide Y-1349VHVN

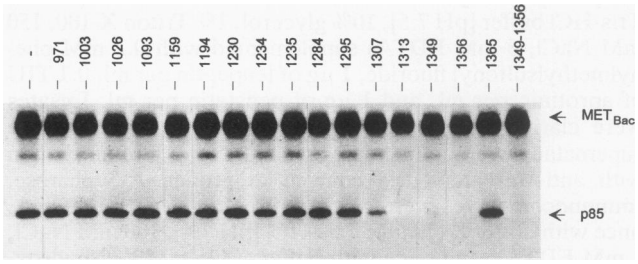


FIG. 1. Inhibition of binding of p85 to HGF/SF receptor by tyrosine-phosphorylated peptides. Recombinant HGF/SF receptor was purified by immunoprecipitation from baculovirus-infected Sf9 cells by using a rabbit polyclonal antiserum and phosphorylated with cold ATP. Lysates of Sf9 cells expressing p85 were preincubated with each of the phosphopeptides (10 μM). The lysates were then allowed to associate with immobilized recombinant HGF/SF receptors. Following association, the complex was washed, and receptor-bound p85 was detected by an in vitro kinase assay, as described in Materials and Methods. The phosphopeptides are identified by the number of the amino-terminal tyrosine (see list of peptide sequences in Table 1).

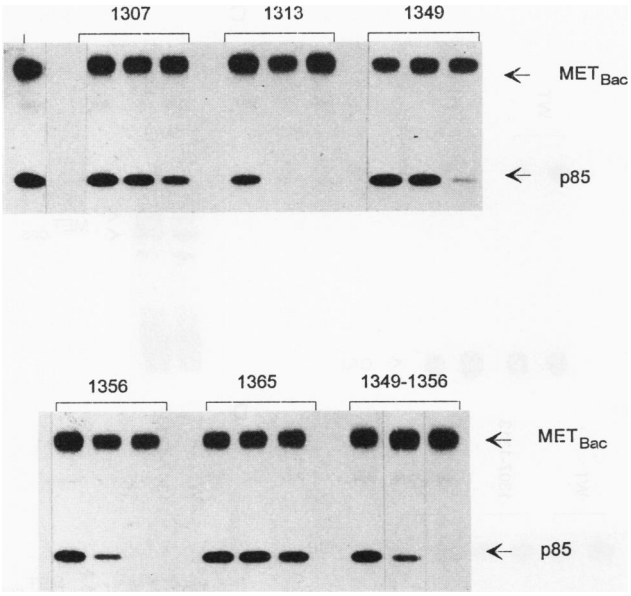


FIG. 2. Inhibition of binding of p85 to HGF/SF receptor with different concentrations of tyrosine-phosphorylated peptides. The phosphopeptides which efficiently inhibited p85 binding to the HGF/SF receptor (Fig. 1) were used at increasing concentrations (10 nM, 100 nM, and 1 μM from left to right) to define their relative affinities for p85. Conditions of the experiment were as described in the legend to Fig. 1. Phosphopeptide 1365 was used as a negative control.

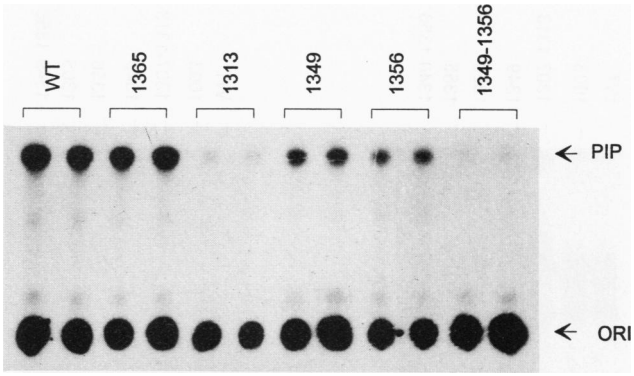


FIG. 3. Inhibition of binding of PI 3-kinase holoenzyme to HGF/SF receptor by tyrosine-phosphorylated peptides. The phosphopeptides which efficiently inhibited p85 (Fig. 1) were assayed for the ability to interfere with binding of the PI 3-kinase holoenzyme to the HGF/SF receptor. Cytosolic extracts from 3-day-starved A549 cells were preincubated with each of the phosphopeptides (10 μM) prior to incubation with the immobilized recombinant HGF/SF receptor. The presence of receptor-associated PI 3-kinase in the immunocomplexes was determined by PI 3-kinase activity assay, as described in Materials and Methods. The position of the PI 3-phosphate (PIP) product of the PI 3-kinase reaction is indicated.

ATY-1356VNVK seemed to be comparable to Y-1356VNVKCVA in its affinity for p85.

Since the interaction with the p110 catalytic subunit could affect the conformation of p85, these results might not reflect the true properties of the SH2 domains of p85 when present in the complex. To exclude this possibility, we carried out the same kind of competition experiments with lysates of A549 cells as a source of PI 3-kinase holoenzyme. The association between the holoenzyme and the recombinant receptor was visualized by measuring the PI 3-kinase activity recovered in the receptor immunoprecipitates. Figure 3 shows that the phosphopeptides which had proven capable of inhibiting p85 binding also interfere with binding of the PI 3-kinase holoenzyme to the recombinant HGF/SF receptor. In particular it should be noted that the phosphopeptide including both phosphotyrosine 1349 and phosphotyrosine 1356 seems to be more efficient at displacing the PI 3-kinase holoenzyme from the HGF/SF receptor than those including just one of these residues.

The results of this first set of experiments suggested the possible existence of a double binding site for PI 3-kinase in the HGF/SF receptor, consisting of the phosphotyrosine pairs 1307-1313 and 1349-1356. That a pair of phosphotyrosine residues could be involved had been previously shown for the PDGF receptor, where Y-740 and Y-751 are known to form the PI 3-kinase binding site (9, 18).

In vitro association of p85 with HGF/SF receptor Tyr-Phe mutants. A series of constructs were made by site-directed mutagenesis of the wild-type *Met* receptor cDNA. These constructs were transiently expressed in COS 7 cells, to obtain the corresponding Tyr-Phe *Met* mutants Phe-1003, Phe-1307, Phe-1313, Phe-1349, Phe-1356, and Phe-1365. In addition to single-amino-acid substitutions, some multiple substitutions were also made. In particular, we produced the two double-site mutants necessary to further elucidate the results of the competition experiments: the *Met* mutants Phe-1307-1313 and Phe-1349-1356.

Figure 4 shows the results of an association experiment similar to that represented in Fig. 1, carried out by using the same source of p85 (expressing Sf9 cells) and using lysates of transfected COS 7 cells as a source of wild-type and mutant HGF/SF receptors. Transfected COS 7 cells express the single-chain receptor precursor as well as the proteolytically processed mature form in a 1:1 ratio. After the association reaction, the samples were split in two and processed differently to yield the results shown in Fig. 4A and B. In Fig. 4A, both the receptor and p85 are visualized by phosphorylation after a kinase assay. This panel shows that the mutant receptors are all active and are present in comparable amounts. While all the other mutants (and in particular Phe-1307-1313) still bind and phosphorylate p85, only the double mutant Phe-1349-1356 does not. In Fig. 4B, after the association, the kinase assay was omitted, and the samples were run in SDS-PAGE and transferred to nitrocellulose. The Western blot was then decorated with monoclonal antibodies specific for p85. This experiment confirms that only the mutant Phe-1349-1356 has lost the ability to bind p85. It should be noted that by comparing Fig. 4A and B, the mutant Phe-1356 appears to be still capable of binding but not of phosphorylating p85. This suggests that the p85 associated with this receptor mutant may not be positioned correctly for efficient phosphorylation.

In vivo association of PI 3-kinase with HGF/SF receptor Tyr-Phe mutants. Figure 5 shows that these results are essentially reproducible also in vivo. In these experiments, the *Met*/PI 3-kinase complex was immunoprecipitated with anti-*Met* antibodies from lysates of COS 7 cells (expressing the wild-type and mutant *Met* receptors) after HGF/SF stimulation. Lysis and immunoprecipitation were carried out in the presence of sodium orthovanadate to prevent receptor dephosphorylation. After extensive washing, the PI 3-kinase assay was carried out on the immunoprecipitates, equalized

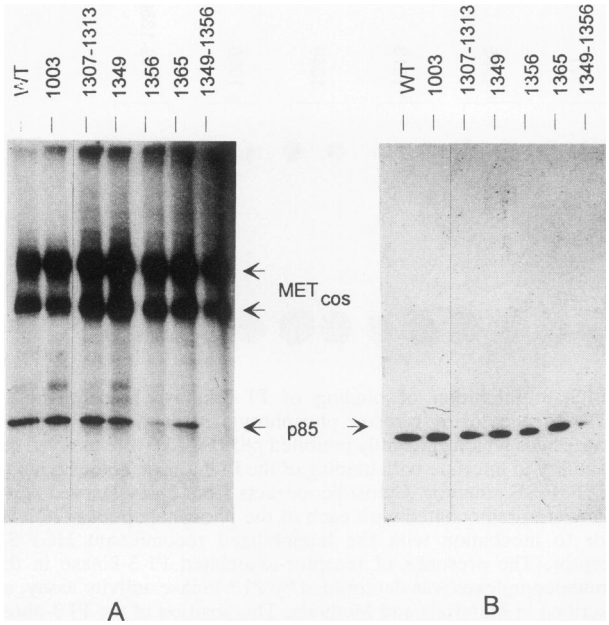


FIG. 4. Effects of Tyr-Phe mutations on the interaction of HGF/SF receptor with p85. COS 7 cells were transfected with plasmids encoding wild-type HGF/SF receptor (WT) or receptors in which the tyrosine codon at the indicated position was converted to a phenylalanine codon individually, or in combination. COS 7 cells express endogenous HGF/SF receptor. However, the simian protein is not recognized by a monoclonal antibody directed against the carboxyl-terminal human-specific peptide. These antibodies were used to selectively immunoprecipitate the human HGF/SF receptor from transfected COS 7 cells. Immobilized prephosphorylated receptors were incubated with lysates of Sf9 cells expressing p85. (A) Both the receptor and p85 were labelled by an in vitro kinase assay as described in Materials and Methods. (B) The presence of p85 in the receptor immune complex was determined by immunoblotting with anti-p85 monoclonal antibodies.

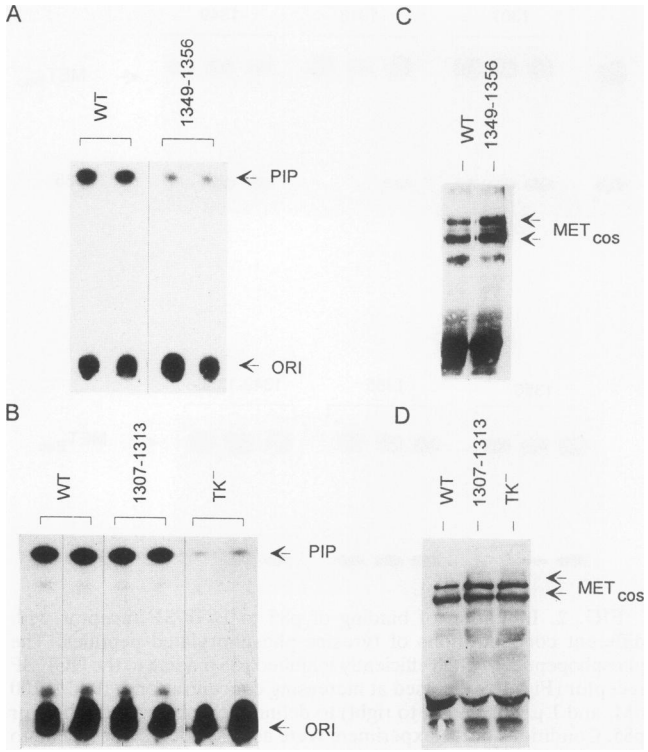


FIG. 5. In vivo effects of Tyr-Phe mutations on the interaction of the HGF/SF receptor with the PI 3-kinase holoenzyme. COS 7 cells expressing wild-type or mutated receptors were stimulated with HGF/SF and lysed. Receptors were immunoprecipitated with human-specific monoclonal antibodies. (A and B) The presence of the receptor-associated PI 3-kinase was determined by PI 3-kinase activity assay, as described in Materials and Methods. The position of the PI-3-phosphate (PIP) product of the PI 3-kinase reaction is indicated. (C and D) Immunoblots (with human-specific monoclonal antibodies) show that the samples tested for PI 3-kinase activity contained equivalent amounts of recombinant receptor. The thymidine kinase-negative (TK^-) receptor mutant was obtained by converting the aspartic acid at position 1204 to an asparagine residue. This resulted in a kinase-inactive (not shown) HGF/SF receptor.

for *Met* protein content, to quantify the amount of endogenous PI 3-kinase coprecipitated in complex with the wild-type receptor and its mutant forms. Only the Phe-1349-1356 double mutant coprecipitated with an amount of PI 3-kinase activity significantly lower than that associated with the wild-type receptor. The low residual activity bound to the Phe-1349-1356 double mutant is probably due to the formation of receptor dimers with the endogenous *Met* protein from COS 7 cells. This interpretation is supported by the fact that the same amount of residual binding is also present on immunoprecipitates obtained from COS 7 cells transfected with a kinase-inactive mutant (Fig. 5, TK^-).

The results of this second set of experiments indicate that residues Y-1349 and Y-1356 mediate binding of PI 3-kinase to the HGF/SF receptor, while residues Y-1307 and Y-1313 do not.

Phosphopeptide mapping of wild-type and mutant receptors. The results obtained with the mutant receptors imply that tyrosines 1349 and 1356 are phosphorylated in vivo. A synthetic peptide (I24K) was constructed to correspond to the tryptic peptide including these two residues. This peptide required a combination of aqueous and organic solvents for best recovery and eluted at a very late time from the HPLC column used for the separation (Fig. 6A). When the same procedure was used to run a tryptic digest obtained from a wild-type receptor that had been phosphorylated in vitro, a peak eluting at a time very close to that of the unphosphorylated I24K peptide was recovered (Fig. 6B). This novel peak is absent in the double-site mutant (Fig. 6C) and is reduced in the single-site mutants (not shown). All receptors were expressed in COS 7 cells and phosphorylated in vitro prior to tryptic digestion.

These results indicate that tyrosines 1349 and 1356 are indeed in vitro phosphorylation sites and, considered in combination with the results showing the loss of in vivo association of *Met* with PI 3-kinase in COS cells transfected with the corresponding double-site mutant, imply that the same tyrosine residues are also in vivo phosphorylation sites.

Evaluation of the relative affinities of phosphotyrosines 1349 and 1356 for the N- and C-terminal SH2 domains of p85. The presence of two SH2 domains in the p85 molecule and the need to eliminate two phosphotyrosines in the HGF/SF receptor to abolish PI 3-kinase binding suggest a model in which each SH2 domain interacts with one of the two tyrosines (4, 18, 19, 36). It is therefore interesting to measure the relative affinities of the two phosphopeptides for the N- and C-terminal SH2 domains of p85.

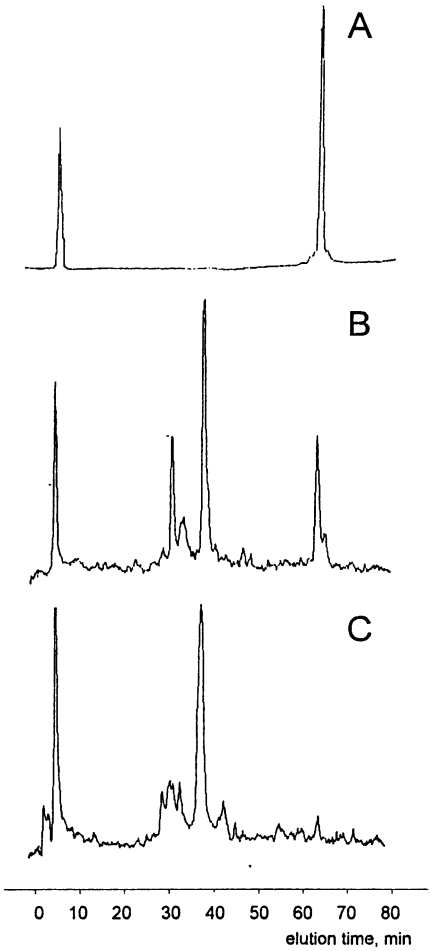


FIG. 6. Identification of Y-1349 and Y-1356 as in vitro phosphorylation sites in the HGF/SF receptor by tryptic phosphopeptide mapping. (A) HPLC analysis at 214 nm of a synthetic nonphosphorylated peptide (I24K), which corresponds to the tryptic peptide containing tyrosines Y-1349 and Y-1356 in the HGF/SF receptor. I24K elutes from the HPLC column after 65 min. (B and C) Radio-HPLC elution profiles of tryptic phosphopeptides derived from in vitro [γ-^{32}P]ATP-phosphorylated wild-type receptor (B) and the Phe-1349-1356 receptor mutant (C).

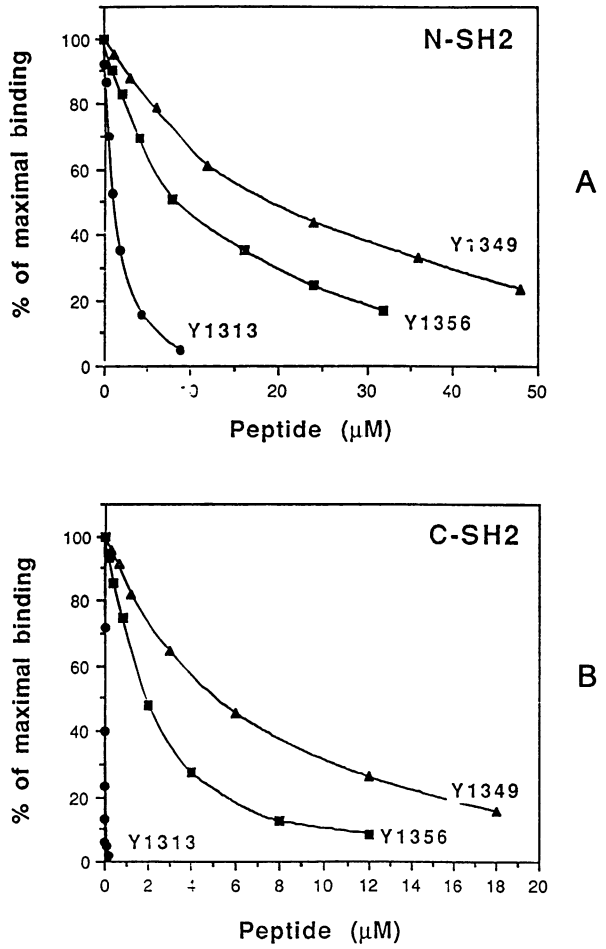
We initially attempted to do this by biospecific interaction analysis with the BIAcore instrument (14, 15, 17). However, the coupling of the phosphopeptides to the matrix either directly or, after biotinylation, by binding to matrix-immobilized avidin resulted in no significant response. This was presumably because the phosphotyrosine in these peptides is at the N terminus, and immobilization perturbs its binding capacity. We therefore did the affinity measurements in an indirect way, by measuring the ability of the phosphopeptides to inhibit the interaction of the SH2 domains with an immobilized phosphopeptide which includes phosphotyrosine 751 (Y-751) in the human PDGF receptor and has been shown to have a high affinity for the two SH2 domains (27). The N- and C-terminal SH2 domains of p85 were mixed with a range of concentrations of the *Met* phosphopeptides and injected over immobilized Y-751 phosphopeptide. Figure 7 shows the results of these measurements, expressed as percent inhibition of binding to phosphopeptide Y-751. Although it was not possible to derive the absolute affinities for these interactions, comparison of the values at which half-maximal inhibition is observed provided useful information about the relative affinities. A summary of the data is shown in Fig. 7C. The highest apparent affinity is displayed by the Y-1313 phosphopeptide, which contains the canonical YXXM motif. The phosphopeptides Y-1349 and Y-1356, which include the unconventional binding site YVXV, also inhibit binding of p85 N- and C-terminal SH2 domains to phosphopeptide Y-751, but at higher concentrations. All phosphopeptides, but more evidently Y-1313, show a higher affinity for the C-terminal SH2 than for the N-terminal SH2. These data are in agreement with those obtained from the experiment shown in Fig. 2 and indicate that, at least in vitro and under our experimental conditions, the novel binding motif YVXV has an affinity for p85 2 orders of magnitude lower than the canonical consensus.

Activation of PI 3-kinase in vitro with HGF/SF receptor phosphopeptides. It has been previously shown that incubation with tyrosine-phosphorylated insulin receptor substrate 1 causes a four- to fivefold increase in PI 3-kinase activity and that this activation is mimicked by the synthetic tyrosine-phosphorylated peptide containing the sequence Y-628MPM of the insulin receptor substrate 1 protein (1). We tested the phosphorylated peptides Y-1313EVMLKCW, Y-1349VHVNATY, Y-1356VNVKCVA, and Y-1349VHV NATY-1356VNVK for their abilities to activate the PI 3-kinase holoenzyme in vitro. Figure 8 shows that at 100 μM, the peptide containing phosphotyrosine 1313 activates slightly. Similar results were obtained with the single peptides containing either phosphotyrosine 1349 or phosphotyrosine 1356 (not shown). The peptide containing phosphotyrosines 1349 and 1356 shows a clearer activation effect (approximately fivefold), comparable to that which has been observed with the insulin receptor substrate 1 protein (1).

DISCUSSION

On the basis of work carried out in a number of laboratories over the last 3 years, it has become an accepted notion that following ligand binding, tyrosine kinase receptors physically associate with a unique subset of cytoplasmic signalling proteins containing SH2 domains (22). The affinity and selectivity of the various SH2 domains depend on the amino acid sequence surrounding critical phosphotyrosine residues in the receptors (3, 36). The striking specificity of these interactions has been underscored in a series of recent papers reporting the mapping of distinct binding sites for PI 3-kinase, phospholipase Cγ, and *ras*-GTPase activating protein on several tyrosine kinase receptors (9, 18, 23, 30, 32). These results have opened the possibility, once the location of a specific recognition sequence in the receptor is known, to selectively eliminate by site-directed mutagenesis a specific binding site from a receptor without interfering with the others. This powerful new approach should facilitate elucidation of the role of the various transductional pathways in eliciting the biological response to a ligand.

From these studies it has also emerged that the four amino acids located immediately downstream of a phosphotyrosine residue are sufficient to specify a SH2 binding site (9). The first of such consensus sequences to be identified was the YXXM motif, specific for PI 3-kinase binding (3, 9, 18, 20, 40). Two copies of this sequence are present in the KI region of the PDGF receptor, one copy is in the KI region of the colony-stimulating factor 1 receptor and c-Kit, and nine copies are found in the insulin receptor substrate 1 protein.



C

Phosphopeptide	N-SH2	C-SH2
Y1313	0.9μM	0.004μM
Y1349	19μM	5μM
Y1356	8μM	2μM

FIG. 7. Evaluation of the relative affinities of phosphotyrosine 1313, 1349, and 1356 for the N- and C-terminal SH2 domains of p85. Affinities were determined by biospecific interaction analysis with the BIAcore instrument (14, 15, 17). Relative affinities were determined by measuring the ability of the phosphopeptides to inhibit the interaction of the SH2 domains with an immobilized phosphopeptide (DMSKDESVDY*VPMLDMK) which includes Y-751 in the human PDGF receptor. (A and B) Results of these measurements, expressed as percent inhibition of binding to phosphopeptide Y-751. (C) Phosphopeptide concentrations necessary to reach half-maximal inhibition of binding.

In all these proteins, this motif mediates the interaction with PI 3-kinase (1, 3, 9, 18, 30). The same consensus is also present in the HGF/SF receptor (Y-1313EVM), which also responds to ligand stimulation, becoming associated with PI 3-kinase (11). Given the distinct effects of HGF/SF, depending on the target cell, and given the possible involvement of PI 3-kinase in diverse signalling processes, it seemed to us of particular interest to identify its binding site in the HGF/SF receptor. Although (Y-1313EVM) fulfills the sequence requirement for a potential PI 3-kinase recognition sequence, it is located toward the end of, but still within, the tyrosine kinase domain. This tyrosine residue has not been identified as a phosphorylation site either in vivo or in vitro (10). The tyrosine and methionine residues in this position are conserved in many tyrosine kinases (13), suggesting that they may be important for proper folding of the catalytic domain. Moreover, all of the transducer's binding sites so far identified are located either in the kinase insert or in the C-terminal portion of the receptor molecule.

We mapped the actual PI 3-kinase binding site through the combination of two complementary experimental approaches; competition with synthetic phosphopeptides and association experiments with Tyr-Phe mutants. The results of the competition experiments narrowed the number of the candidate phosphotyrosine residues to four. The association with the corresponding receptor mutants unequivocally identified the binding site in a pair of tyrosine residues located eight amino acids apart in the C-terminal region of the receptor molecule: Y-1349VHV and Y-1356VNVK. These two phosphotyrosines are both located in a single tryptic peptide which has been identified by comparing HPLC phosphopeptide profiles obtained from phosphorylated wild-type and mutant receptors. Tyrosines 1349 and 1356 thus represent two newly described phosphorylation sites.

The presence of a double docking site for PI 3-kinase in the HGF receptor is reminiscent of the PDGF receptor's primary structure, in which the recognition sequence YXXM is also repeated twice (3, 7). Several models have been proposed to explain the reason for the need of two binding sites for PI 3-kinase (4, 19, 21). Some of these models take into account the fact that p85 has two SH2 domains which could both bind to the receptor. In the case of the PDGF receptor, the affinities of p85 for the two sites seem to be different. It has been proposed that the p85 C-terminal SH2 binds one of the two tyrosines with high affinity and the N-terminal SH2 mediates a lower-affinity interaction with

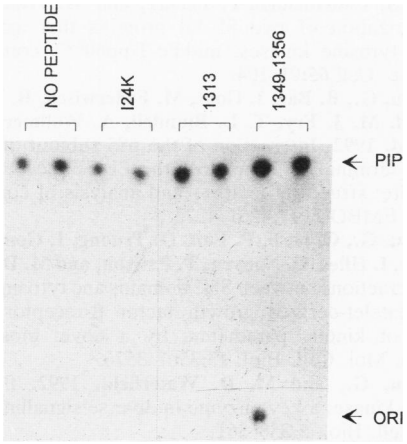


FIG. 8. Activation of PI 3-kinase in vitro by HGF/SF receptor phosphopeptides. PI 3-kinase holoenzyme was immunoprecipitated from cytosolic extracts of 3-day-starved A549 cells by using anti-p85 monoclonal antibodies. Immunoprecipitates were incubated either with a nonphosphorylated peptide (I24K) containing tyrosines 1349 and 1356 or with the indicated phosphopeptides. All peptides were tested at 100 μM. After incubation, the immunoprecipitates were assayed for PI 3-kinase activity.

the remaining tyrosine (18, 19). The low-affinity interaction may be affected by phosphorylation of p85 following receptor binding (19).

A semiquantitative measurement of the affinities of the N- and C-terminal SH2 domains of p85 for phosphotyrosines 1349 and 1356 was obtained by using the BIAcore biosensor. Similar to what has been observed for the PDGF receptor, the C-terminal SH2 domain of p85 seems to display higher affinity for both phosphotyrosines than the N-terminal SH2. However, from these measurements, the interaction of the SH2 domains of p85 with the phosphotyrosines in the novel consensus YVXV seems to be 2 orders of magnitude lower than that determined for the canonical recognition sequence YXXM. This large difference in affinity is consistent with the predictions of Songyang et al. (36), derived by using a degenerate phosphopeptide library to determine the sequence specificity of the peptide binding sites for 14 different SH2 domains. In fact, the relative affinity of the C-terminal SH2 domain of p85 for the sequence YVXV compared with that for YEXM is expected to be quite low, since the preference for M at the +3 position is very strong. E and V have similar affinities at the +1 position, but they confer a small fraction of the total binding energy. The N-terminal SH2 domain, on the other hand, is expected to show less preference for YEXM over YVXV, since this protein selects more on the basis of the residue at the +1 position (E and V are both strongly selected at +1). Although M is preferred at +3, it is not as strongly selected as it is in the case of the C-terminal SH2 domain.

It should be noted that since these experiments were carried out with single phosphopeptides and isolated SH2 domains instead of, respectively, the intact receptor and p85, these in vitro results may not reflect the physiological affinity. The closely spaced duplication of the novel recognition motif in the HGF/SF receptor may in fact allow both SH2 domains of p85 to bind simultaneously in vivo (36), thus conferring to the receptor a much greater affinity for PI 3-kinase than that displayed by a single binding site. This hypothesis is strengthened by two observations. First, the phosphopeptide which includes both phosphotyrosine residues is more efficient than those including just one of the two at displacing the PI 3-kinase holoenzyme from the HGF/SF receptor in an in vitro association experiment (Fig. 3). Second, the phosphopeptide which includes the two phosphotyrosine residues activates PI 3-kinase in vitro to a higher extent than that containing the canonical consensus sequence or the singly phosphorylated peptides. This is in agreement with the finding by Carpenter et al. (4) that PI 3-kinase is activated by a phosphopeptide containing both tyrosine 740 and tyrosine 751 of the PDGF receptor binding site to a much greater extent than by the singly phosphorylated peptides, although these still bound quite tightly. The authors argue that binding to both SH2 domains simultaneously causes a conformational change that activates PI 3-kinase. This seems to be happening with the two sites on the carboxy-terminal region of the HGF/SF receptor.

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