Identification of Tyrosine 489 in the Carboxy Terminus of the Tpr-Met Oncoprotein as a Major Site of Autophosphorylation†

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ABSTRACT: The Met receptor tyrosine kinase is the receptor for hepatocyte growth factor/scatter factor. HGF/SF is a multifunctional cytokine that can stimulate proliferation, motility, and morphogenesis in epithelial and endothelial cells. Oncogenic activation of the Met receptor occurs through a genomic rearrangement that generates a hybrid protein in which *tpr* sequences are directly fused amino terminal to the *met* receptor kinase domain. The resultant Tpr-Met hybrid protein posseses tyrosine kinase activity, is constitutively phosphorylated on tyrosine residues *in vivo*, and transforms fibroblasts in culture. We have identified two tyrosine residues within the catalytic domain of the Tpr-Met oncoprotein (Y365, Y366) and Met receptor (Y1234, Y1235) that are phosphorylated and essential for both the catalytic and biological activity of the oncoprotein and receptor. However, a detailed analysis of phosphorylation in these proteins has not been undertaken. In order to determine the sites of tyrosine phosphorylation in the Tpr-Met oncoprotein, *in vitro* mutagenesis, phosphopeptide mapping, and dephosphorylation protection assays were performed. Here we identify that a single tyrosine (Y489) in the carboxy terminus of the Tpr-Met oncoprotein is highly phosphorylated and is essential for biological activity. In contrast, additional tyrosines (Y482, Y498) located in the carboxy terminus are not phosphorylated at detectable levels and are not essential for the biological activity of the oncoprotein.

Binding of growth factors to cell surface receptors initiates a series of events culminating in a variety of cellular responses including cell division, motility, and morphogenesis. Many peptide growth factors bind a class of transmembrane receptors having intrinsic tyrosine kinase activity. Ligand binding to receptor tyrosine kinases (RTKs)[1] induces dimerization of adjacent receptor molecules and autophosphorylation. Autophosphorylation is believed to occur by a trans mechanism facilitated by dimerization and is critical for intracellular signaling by the receptor (Ullrich & Schlessinger, 1990; Cantley et al., 1991).

The *met* proto-oncogene is a RTK that was identified as the receptor for hepatocyte growth factor/scatter factor (HGF/SF) (Bottaro et al., 1991; Naldini et al., 1991; Rosen et al., 1989). HGF/SF is a multifunctional cytokine that stimulates proliferation, motility, or morphogenesis in a variety of epithelial and endothelial cells (Matsumoto & Nakamura, 1992; Stoker & Gherardi, 1991; Rosen et al., 1989). The Met receptor is a 190 kDa heterodimer consisting of a 45 kDa extracellular α subunit and a 140 kDa β subunit. The β subunit spans the membrane and contains the catalytic kinase domain as well as a number of potential tyrosine phosphorylation sites (Gonzatti-Haces et al., 1988; Giordano et al., 1989; Rodrigues et al., 1991). Activation of the Met receptor is sufficient to transduce both motogenic and morphogenic signals in epithelial cells (Yarden & Schlessinger, 1987; Zhu et al., 1994a,b) and confer tumorigenic potential (Iyer et al., 1990; Rong et al., 1992) and increased motility to fibroblasts (Giordano et al., 1993).

Oncogenic activation of the Met receptor occurs through a genomic rearrangement that places sequences from a locus on chromosome 1 (*tpr*) into the *met* gene on chromosome 7 (Park et al., 1986). This rearrangement results in the generation of a chimeric protein containing.142 novel amino acids from Tpr fused directly to the Met kinase domain (Park et al., 1987). The Tpr domain contains a leucine zipper motif that mediates constitutive dimerization of the Met kinase and is essential for activation of the kinase and cellular transformation by the Tpr-Met oncoprotein (Rodrigues & Park, 1993). We have proposed that the leucine zipper-mediated dimerization of the Tpr-Met oncoprotein mimics dimerization and activation of the Met RTK following ligation with HGF/SF and, thus, may represent a general model for oncogenic activation of RTKs (Rodrigues & Park, 1993, 1994a; Sawyers & Denny, 1994).

Tyrosine phosphorylation is crucial for full biological activity of receptor tyrosine kinases. Mutations that impair phosphotransferase activity of the receptor abolish ligand-induced biological responses (Honegger et al., 1988; Chen et al., 1987; Chou et al., 1987; McClain et al., 1987; Escobedo et al., 1988). In many receptor tyrosine kinases, autophosphorylation of a tyrosine residue(s), that corresponds to Y416 in the kinase domain of c-Src, has been shown to correlate with increased enzymatic activity (Kmiecik & Shalloway, 1987; Piwnica-Worms et al., 1987; Rosen et al., 1983; Yu & Czech, 1984; Sasaki et al., 1985; Fantl et al.,

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[1] Abbreviations: HGF, hepatocyte growth factor; SF, scatter factor; SH2, Src homology 2; PI3′K, phosphatidyl inositol 3′-kinase; PLC, phospholipase C; RTK, receptor tyrosine kinase; CNBr, cyanogen bromide.

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1989; Kazlauskas et al., 1991; Van Der Geer & Hunter, 1991; Rodrigues & Park, 1994b). We have identified two autophosphorylation sites within the catalytic domain of the Tpr-Met oncoprotein (Tyr-365 and Tyr-366) that are essential for enzymatic activity and transforming activity of the oncoprotein (Rodrigues & Park, 1994b). Furthermore, mutation of the corresponding tyrosine residues in the Met receptor generates a receptor with reduced kinase activity (Longati et al., 1994; Zhu et al., 1994a) that is biologically inactive (Zhu et al., 1994a).

Phosphorylated tyrosine residues located outside the catalytic domain of receptor tyrosine kinases have been identified as representing the primary interaction sites with the SH2 domains of several substrates (Songyang et al., 1993; Pawson & Gish, 1992). Tryptic phosphopeptide mapping of the Tpr-Met oncoprotein revealed that multiple tyrosine residues are phosphorylated (Rodrigues & Park, 1994b), but no comprehensive study of tyrosine phosphorylation sites has been undertaken for either the Tpr-Met oncoprotein or the Met receptor. To identify sites of tyrosine phosphorylation in the Tpr-Met oncoprotein, *in vitro* mutagenesis, tryptic phosphopeptide mapping, and dephosphorylation protection assays were performed. Here we show that a single tyrosine (Y489) in the carboxy terminus of the Tpr-Met oncoprotein is highly phosphorylated and is essential for biological activity of the oncoprotein. Furthermore, we demonstrate using a dephosphorylation protection assay that Y489 is a direct binding site for the Grb2 adaptor protein. In contrast, additional tyrosine residues located in the carboxy terminus (Y482, Y498) are not phosphorylated at detectable levels and are not essential for the biological activity of the oncoprotein.

MATERIALS AND METHODS

Cells and DNA Transfection. Fischer rat 3T3 (Fr3T3) and COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Flow Laboratories). Transient transfections were carried out with 6 μg of DNA per 100 mm plate using the DEAE-dextran method (McCutchan & Pagano, 1968). At 12−16 h post-transfection, cells were treated with 100 μM chloroquine for 3−4 h (Luthman & Magnusson, 1983). Cells were harvested ~72 h following transfection. Infections of Fr3T3 cells to generate stable cell lines were carried out using the method of Kreigler (1990). Helper-free retrovirus was generated by transient overexpression in COS-1 cells as described previously (Muller et al., 1991). Briefly, mutant cDNAs were subcloned into the retrovirus vector pLXSN (Miller & Rosman, 1989) and cotransfected with pSV-Ψ^--E-MLV (O'Shea et al., 1991) into COS-1 cells using the DEAE-dextran method. Virus was harvested 3−5 days post-transfection, filtered through 0.45 μm filters, and used to infect Fr3T3 cells plated at a density of (3−5) × 10^5 cells per 60 mm plate 24 h prior to infection. The medium was removed 16 h later and replaced with DMEM containing 10% heat inactivated fetal bovine serum retrovirus and 4 μg of Polybrene per milliliter. The cells were split the following day into 4 × 100 mm plates, and 2 days after addition of the virus, G418 was added to two of these plates. Two to three weeks post-infection, foci were counted from each infection, as a percentage of total G418 resistant colonies.

Polymerase Chain Reaction (PCR). Reactions were carried out in a Perkin Elmer Cetus thermal cycler. Twenty five cycles, each consisting of a 1 min denaturation step at 94 °C, followed by an annealing step of 2 min at the appropriate T_m and a final extension step carried out at 72 °C for 3min, were conducted. Reaction mixtures contained 50 pmol of each primer, 200 μM of each of the four deoxynucleoside triphosphates, and 5 units of Taq polymerase (Pharmacia) in 100 μL of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.75 mM $MgCl_2$, and 0.01% (w/v) gelatin.

Plasmid Construction. The isolation of the Tpr-Met cDNA and the cloning of pTpr-Met-1 and K241A have been previously described (Rodrigues et al., 1991; Rodrigues & Park, 1993). Mutagenesis was carried out either by using a PCR based protocol, site-directed mutagenesis by overlap extension (SOE) (Ho et al., 1989), or as described (Deng & Nickoloff, 1992). The Y489F mutant was generated using the following set of complementary oligonucleotides containing the appropriate nucleotide substitutions (substitutions are underlined): Y489F; 5′-CATTGGGGAGCACTTTGTC-CATGTG-3′. This primer was used in conjunction with 5′ and 3′ primers in the vector to generate two DNA fragments with overlapping ends. Fragments were then used as templates for a second round of PCR. The final products were substituted back into pTpr-Met-1 following digestion with *Spe*I. The other mutations were created with the Deng and Nickloff protocol, using the following sets of primers (substitutions are underlined): Y482F, 5′-GGAG-CACTTTGTCCATGTTAACGCTAC-3′; Y498F, 5′- GT-GTCGCTCCGTTTCCTTCTCTGTTG-3′. All DNAs were sequenced by the dideoxy chain termination method (Sanger et al., 1977) using the Sequenase system (United States Biochemical Corp.).

Immunoprecipitation. Cells were lysed with CSK buffer (100 mM KCl, 10 mM PIPES, pH 7.0, 0.5% Nonidet P-40, 300 mM sucrose, and 3 mM $MgCl_2$). Immunoprecipitations were conducted using the rabbit polyclonal antibody, Ab144 (Rodrigues et al., 1991). Immunocomplexes were collected on protein A−Sepharose (Pharmacia), washed alternatively with CSK buffer and CSK high salt buffer (CSK + 500 mM NaCl), resuspended in Laemmli sample buffer, boiled for 5 min, and subjected to SDS−polyacrylamide gel electrophoresis (PAGE).

In Vitro Protein Kinase Assays. Cells were lysed with CSK buffer, and immunoprecipitations were carried out as described. The immunocomplexes were washed twice with kinase buffer (20 mM PIPES, pH 7.4, 10 mM $MnCl_2$) and resuspended in 15 μL of kinase buffer. Following the addition of 20 μCi of [γ-^{32}P]ATP, samples were incubated at 25 °C for 10 min. Immune complexes were washed alternatively with CSK buffer and high salt buffer, resuspended in Laemmli sample buffer, and resolved by SDS−PAGE. For exogenous substrate assays $MgCl_2$ was substituted for $MnCl_2$ in the kinase buffer and reactions were preincubated for 5 min at 25 °C with 25 μCi of [γ-^{32}P]ATP prior to the addition of myelin basic protein to a final concentration of 0.5 mM and an additional 25 μCi of [γ-^{32}P]-ATP. Reactions were incubated at 25 °C for 10 min, then terminated by the addition of Laemmli sample buffer, and resolved by SDS−PAGE. Relative amounts of phosphorylation of both Tpr-Met and myelin basic protein (MBP) were quantified using the FUJI BAS 1000 phosphorimager.

Phosphopeptide Mapping. Phosphopeptide mapping was carried out according to the method of Boyle *et al.* (1991). Briefly, proteins were phosphorylated with 25 μCi of [γ-^{32}P]-

ATP, as above, and resolved by SDS−PAGE and the band of interest was excised. The Tpr-Met proteins were then eluted from the gel, precipitated with tricholoroacetic acid, oxidized with performic acid, and then digested with 50 μg of trypsin (Worthington) for 24 h at 37 °C. Following the addition of a second aliquot of trypsin and incubation for an additional 12−24 h, samples were lyophilized, washed extensively, resuspended in water, and spotted on cellulose TLC plates (Machery-Nagel). Electrophoresis was conducted at pH 8.9 for 40 min at 1 kV, followed by ascending chromatography in buffer containing 1-butanol, pyridine, acetic acid, and water (15:10:3:12). Phosphopeptides were visualized by autoradiography.

CNBr Cleavage. Tpr-Met protein was gel isolated and TCA precipitated as described. The precipitated pellet was resuspended in 75 μL of 70% (v/v) formic acid, and >100-fold excess of crysatalline CNBr (Sigma) was added in a 1.5 mL microcentrifuge tube. The tube was saturated with nitrogen gas, capped, and placed in the dark overnight, at 25 °C.

Dephosphorylation Protection Assay. Dephosphorylation protection assays were performed essentially as described previously (Rotin et al., 1992; Batzer et al., 1994). Briefly, Tpr-Met protein was immunoprecipitated from $^1/_5$ of a transiently transfected COS-1 cell lysate and phosphorylated *in vitro* with 20 μCi of [γ-^{32}P]ATP as above. The phosphorylated proteins were washed 3 times in TNE (50 mM Tris base, pH 8.0, 1% NP-40, 2 mM EDTA) and preincubated for 30 min at 25 °C with 1 μM Grb2-GST fusion protein with agitation, prior to dephosphorylation with 5 units of alkaline phosphatase (Boehringer Mannheim), or lysate from half of a 100 mm plate of confluent wild-type-9 cells (FR3T3 cells stably expressing Tpr-Met (Fixman et al., 1995)) for 30 min at 37 °C. Proteins were resolved by 8% SDS−PAGE, and tryptic phosphopeptide mapping was performed as above.

Immunoblotting. Immunoprecipitates prepared as described above were resolved by SDS−PAGE and transferred to nitrocellulose membrane (Schleicher and Schuell). The membranes were blocked in TBST (10 mM Tris, pH 7.5, 50 mM NaCl, and 0.1% Tween 20) containing 3% bovine serum albumin and 2.5 mM EDTA for 1 h. Membranes were then incubated for 1.5 h with Ab144. Membranes were washed extensively with TBST + 2.5 mM EDTA, and immunoreactive proteins were detected by incubation with protein A conjugated to horseradish peroxidase followed by enhanced chemiluminescence (ECL) detection reagents (Amersham).

RESULTS

Characterization of Phosphorylated Tyrosine Residues in Tpr-Met. From structure/function analyses, we have determined that a truncated Tpr-Met oncoprotein, lacking the three carboxy terminal tyrosine residues (Y482, Y489, and Y498), is nontransforming although it remains an active kinase.[2] To determine if any of these tyrosine residues in the constitutively active Tpr-Met oncoprotein are sites of autophosphorylation, mutant proteins were generated in which each tyrosine was substituted with a phenylalanine residue (Y482F, Y489F, Y498F; Figure 1). From tryptic phosphopeptide maps, the sites of tyrosine phosphorylation of the Tpr-Met oncoprotein are identical when the protein is labeled with ^{32}P either *in vitro* or *in vivo* (Rodrigues & Park, 1994b). When assayed *in vitro*, each of the mutant proteins retained comparable levels of autokinase activity and phosphorylated the exogenous substrate myelin basic protein to similar levels (data not shown) (Ponzetto et al., 1995). Therefore, because the phosphorylation events which occur *in vitro* accurately reflect those which occur *in vivo*, we chose to examine the phosphorylation sites when labeled *in vitro*. COS-1 cells were transiently transfected with an expression vector encoding the wild-type Tpr-Met product (Wt), or for each mutant (Y482F, Y489F, Y498F). The Tpr-Met proteins were immunoprecipitated and incubated with kinase buffer and [γ-^{32}P]ATP, resolved by SDS−PAGE, and isolated as described (Boyle et al., 1991). Although there are 16 potential sites of tyrosine phosphorylation in the Tpr-Met oncoprotein, tryptic digestion of the Tpr-Met oncoprotein theoretically yields 12 peptides each containing one or more tyrosine residues. The two tyrosine residues that are essential for catalytic activity (Y365 and Y366) (Rodrigues & Park, 1994b) are localized to a single tryptic fragment. Similarly, from analysis of putative trypsin cleavage sites in the amino acid sequence of the Tpr-Met oncoprotein, Y482 and Y489 are predicted to be located on a single tryptic peptide, whereas Y498 is located on a separate tryptic peptide corresponding to the carboxy terminus of the oncoprotein (Figure 3a).

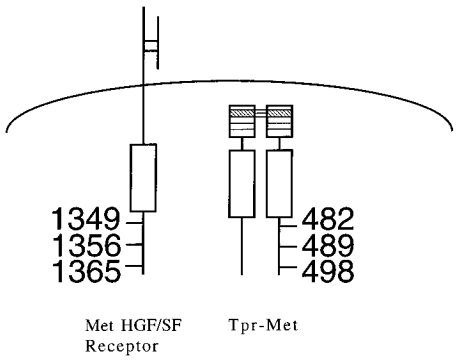

FIGURE 1: Carboxy terminal tyrosines in Met and Tpr-Met. Schematic representation of the Met HGF/SF receptor (left) and the Tpr-Met oncoprotein (right). The three carboxy terminal tyrosines are indicated at their corresponding positions in both the Met receptor and Tpr-Met. Open boxes represent the kinase domain, and small arrows indicate autophosphorylation in Tpr-Met. Hatched boxes in Tpr-Met indicate the leucine zipper dimerization domain, encoded by Tpr, which is essential for constitutive activation of Tpr-Met.

A tryptic phosphopeptide map of the Wt Tpr-Met oncoprotein yields 13 major phosphopeptides (Rodrigues & Park, 1994b), labeled A−M, as well as several minor phosphopeptides (Figure 2a,e). Peptides A and B correspond to the mono- and bis-phosphorylated peptide containing the major autophosphorylation sites in Tpr-Met (Y365/366) (Rodrigues & Park, 1994b), whereas peptide C is a partial digestion product containing these same residues. The trypsin cleavage site (K365) downstream of Y365/366 is surrounded by acidic amino acids (Asp and Glu) and as a consequence is inefficiently digested by trypsin (Ferracini et al., 1991).

Phosphopeptide maps of the Y482 and Y498F mutant proteins were essentially identical to that of Wt Tpr-Met (Figure 2), indicating that these tyrosines were not highly phosphorylated *in vitro*. The tryptic phosphopeptide maps of the Y482F and Y498F mutants are similar to that of the

[2] G. A. Rodrigues, J. Lin, and M. Park, unpublished.

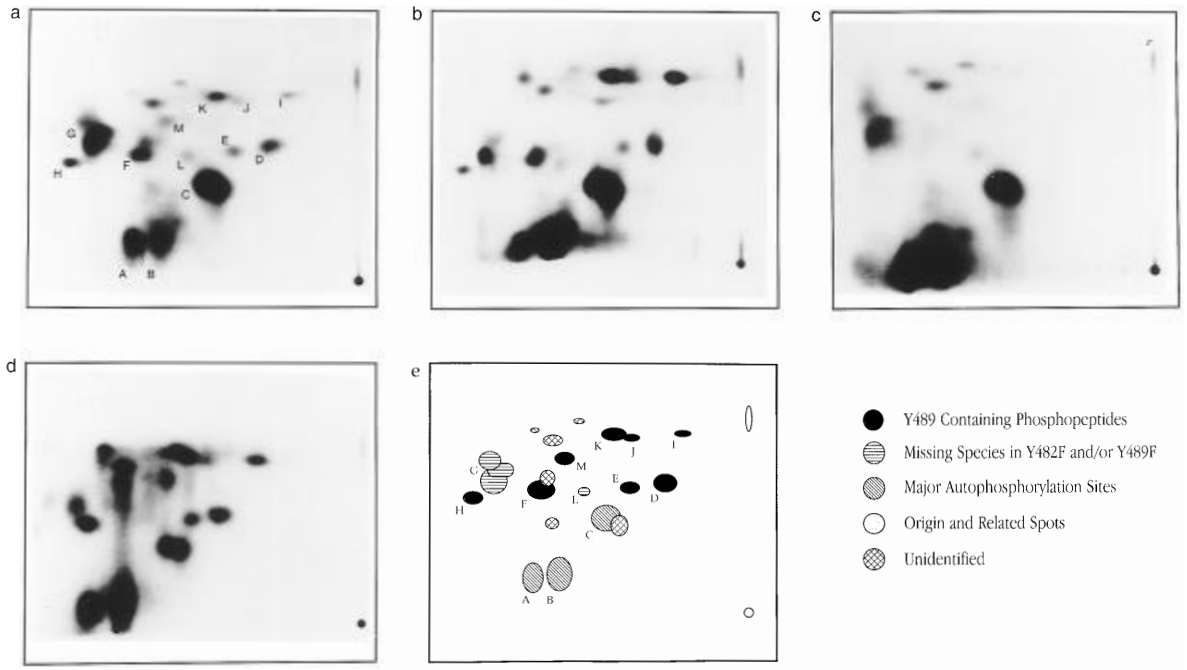

FIGURE 2: Y489 is the most highly phosphorylated tyrosine residue in the carboxy terminus of Tpr-Met. Wild-type and mutant forms of Tpr-Met from transiently transfected COS-1 cells were immunoprecipitated and labeled in an *in vitro* kinase reaction with [γ-^{32}P]ATP. Tpr-Met proteins were then isolated from 10% polyacrylamide−SDS gels and digested with trypsin for 24−36 h and the phosphopeptides separated by electrophoresis in the horizontal direction followed by ascending thin layer chromatography in the vertical direction. (a) Wild-type Tpr-Met, (b) Y482F, (c) Y489F, (d) Y498F, (e) schematic representation of phosphopeptides.

Wt Tpr-Met protein product, with the exception of the loss of part of a complex of phosphopeptides designated G (Figure 2d). However, the phosphopeptide map of the Y489F mutant was dramatically different from that of the Wt phosphopeptide map, which showed the loss of eight phosphorylated species (D, E, F, H, I, J, K, and M (Figure 2c)). A schematic representation of all phosphorylated species which are missing upon mutation of each tyrosine residue is shown in Figure 2e. Together, these data suggest that Y489 is a major site of autophosphorylation in the carboxy terminus of the Tpr-Met oncoprotein.

Tyrosine 489 Is the Only Highly Phosphorylated Residue in the Carboxy Terminus of the Tpr-Met Oncoprotein. The loss of many phosphorylated species (8; Figure 2c) upon mutation of a single tyrosine (Y489) residue indicates one of two possibilities: (i) partial digestion of the Tpr-Met oncoprotein by trypsin, or (ii) that phosphorylation of Y489 is required for phosphorylation of other tyrosine residues, thus acting as part of a phosphorylation cascade. To investigate which of the two possibilities was occurring, gel purified Wt Tpr-Met oncoprotein was digested with CNBr, prior to trypsinization. CNBr, which cleaves after methionine residues, is predicted to release a carboxy terminal fragment which contains all three of the carboxy terminal tyrosines (Figure 3a). The CNBr cleavage site at M459 is amino terminal to the trypsin cleavage sites at R469 and K493. Thus, digestion of the CNBr carboxy terminal fragment with trypsin is expected to generate two authentic tryptic phosphopeptides, one containing Y482 and Y489, and another containing Y498. The Wt Tpr-Met oncoprotein was transiently expressed in COS-1 cells, immunoprecipitated, labeled with [γ-^{32}P]ATP, and resolved by 10% SDS−PAGE. The labeled Tpr-Met oncoprotein was then eluted from the gel and subjected to cleavage with CNBr. The CNBr digested Tpr-Met fragments were immunoprecipitated with Ab144, directed against the extreme carboxy terminus of Tpr-Met. Resolution of the immunoprecipitates by 15% SDS−PAGE revealed a ladder of bands, which is consistent with partial cleavage by CNBr (Figure 3b). For example, CNBr cleavage at M459 generates a carboxy terminal fragment of 7.2 kDa (fragment I; Figure 3a), whereas cleavage by CNBr at M449, but not M459, would generate an 8.4 kDa peptide (fragment II; Figure 3a). Similarly, cleavage at M410, but not M449 or M459, would generate a peptide of 12.9 kDa (fragment III; Figure 3a). The electrophoretic mobility of the lower band (I) is consistent with that of the fully CNBr digested carboxy terminal product (fragment I; Figure 3a), and the mobility of the upper band is consistent with that of a partially digested fragment III (Figure 3a). Fragment II, which would be intermediate in mobility to fragments I and III, was not detected (Figure 3a,b).

Tryptic digestion followed by two-dimensional phosphopeptide mapping of the 7.2 kDa peptide (fragment I) showed recovery of spots D and I, which are present in the Wt Tpr-Met map, but missing in the Y489F map. Fragment I contains all three tyrosines (Y482, Y489, Y498) which are present in the carboxy terminus of Tpr-Met. Therefore, any phosphopeptides recovered from fragment I arise directly from the carboxy terminus of Tpr-Met. This is consistent with phosphopeptides D and I (Figure 3e) representing a fully cleaved tryptic peptide containing Y482 and Y489 and a partial digestion product containing Y482, Y489, and Y498. Because neither of these tryptic fragments was altered in the Y482F or Y498F mutants, we conclude that phosphopeptides D and I represent a *mono*phosphorylated pY489 tryptic phosphopeptide containing Y482 and Y489, or a *mono*phosphorylated pY489 tryptic phosphopeptide containing Y482, Y489, and Y498. Fragment III contains the three tyrosines in the carboxy terminus of Tpr-Met (Y482, Y489, Y498), as well as four tyrosines within the kinase domain (Y417,

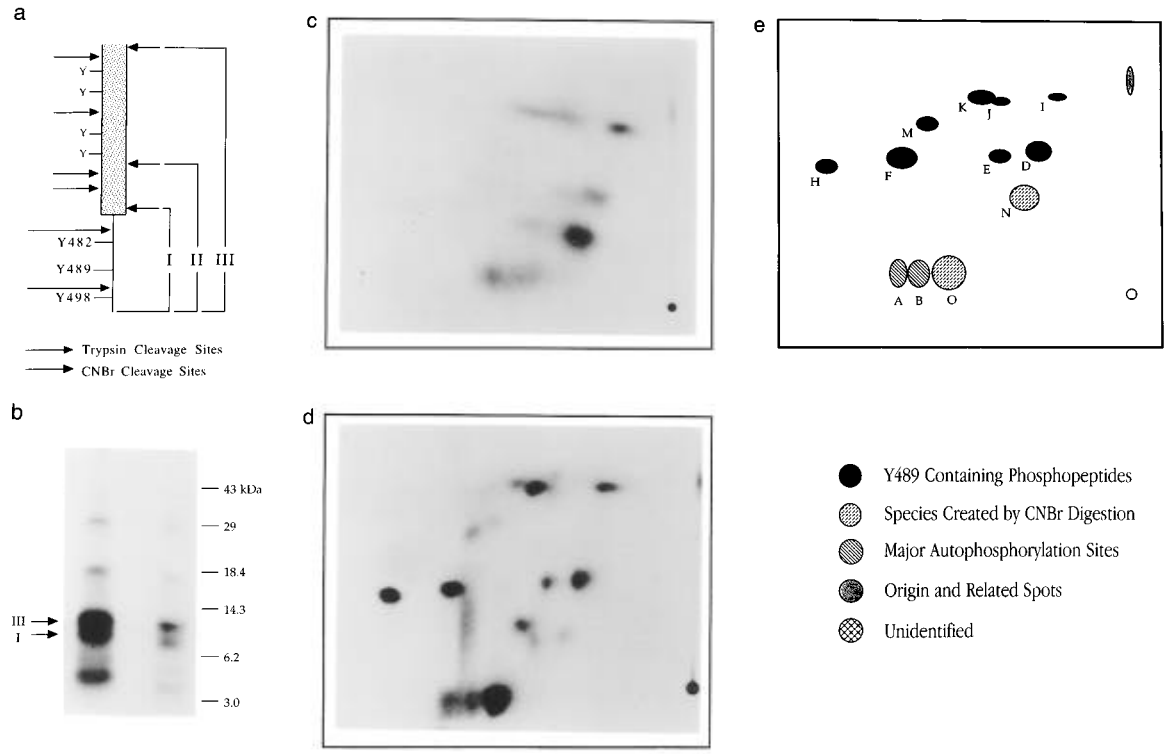

FIGURE 3: Partial digestion.by trypsin gives multiple Y489 containing species. Wild-type Tpr-Met from transiently trasnsfected COS-1 cells was immunoprecipitated and labeled in an *in vitro* kinase reaction with [γ-^{32}P]ATP, and isolated by SDS−PAGE as in Figure 2. (a) Schematic representation of potential CNBr cleavage sites in the carboxy terminal region of the Tpr-Met oncoprotein. (b) The isolated Tpr-Met protein was then digested with CNBr in darkness for 24 h at 25 °C, and carboxy terminal fragments were isolated by immunoprecipitation and resolved by SDS−PAGE on 15% polyacrylamide gels (left). The same sample was reboiled in Laemmli sample buffer for 5 min and loaded in an adjacent lane (right). Phosphopeptides corresponding to bands I and III were isolated (indicated by arrows), trypsinized, and separated in two dimensions as in Figure 2. (c) Phosphopeptide map of fragment I. (d) Phosphopeptide map of fragment III. (e) Schematic representation of phosphopeptides recovered from trypsinization of fragments I and III.

Y428, Y440, and Y446). Spots D, E, F, H, I, J, K, and M were recovered when fragment III was subjected to tryptic phosphopeptide mapping. All of the labeled peptides that are recovered from phosphopeptide mapping of fragment III are also present in Wt and missing in the map of the Y489F mutant, which is consistent with the presence of partial trypsin digestion products, each containing a phosphorylated Y489 residue. In each of the maps of both CNBr cleaved fragments I and III, there is a single phosphopeptide, which is not present in the Wt map (N and O, respectively; Figure 3c−e). These phosphopeptides represent new species which do not correspond to any of the tryptic digestion products seen in the Wt map. We therefore conclude that these fragments correspond to CNBr fragments I and III, respectively, which were not fully cleaved by trypsin.

Analysis of Y489 Containing Tryptic Phosphopeptides Using a Dephosphorylation Protection Assay. To further examine if the the tryptic phosphopeptides missing in Y489F mutant represent partial digestion products, each containing a phosphorylated Y489, we used a dephosphorylation protection assay (Rotin et al., 1992; Batzer et al., 1994). In the Tpr-Met oncoprotein, the downstream amino acid sequence of Y489 is a consensus sequence for the binding of the SH2 domain of the Grb2 adaptor protein (pYVNV) (Songyang et al., 1994). A GST-Grb2 fusion protein binds to a Wt or Y482F mutant Tpr-Met *in vitro* (Fixman et al., submitted; Ponzetto et al., 1994), but fails to bind to the Y489F mutant (Fixman et al., 1995; Ponzetto et al. 1994). Furthermore, the association between Wt Tpr-Met and GST-Grb2 was abolished by preincubation of GST-Grb2 with a phosphopeptide based on the sequence surrounding Y489 of Tpr-Met (VHVNATpYVNVK), whereas a phosphopeptide containing the sequences surrounding Y482 (TFIGEH-pYVHVN) could not compete for binding of Tpr-Met to GST-Grb2 (Fixman et al., 1995). Thus, in order to identify pY489 containing tryptic phosphopeptides, a GST-Grb2 fusion protein was used to block the dephosphorylation of pY489.

Wt Tpr-Met proteins were immunoprecipitated from transiently transfected COS-1 cells, phosphorylated with [γ-^{32}P]ATP, and then subjected to a dephosphorylation reaction with alkaline phosphatase in the presence of purified GST-Grb2 fusion protein. Proteins were resolved by SDS−PAGE, and tryptic phosphopeptide mapping was performed on the pY489 protected Tpr-Met proteins. Figure 4 shows that 7 of the 8 tryptic phosphopeptides lost when Y489 is substituted with phenylalanine are protected (D, E, F, H, I, J, and K) from dephosphorylation by alkaline phosphatase. Phosphopeptide M (Figures 2 and 3) was not protected in this assay. The low stoichiometry of this species (M) in tryptic phosphopeptide maps of Wt Tpr-Met (Figure 2a), however, may result in the under-representation of this species in this assay. Phosphopetides B and C which represent the major sites of autophosphorylation in Tpr-Met (Y365, Y366) (Rodrigues & Park, 1994b) are not completely dephosphorylated by alkaline phosphatase, or by a lysate from a Tpr-Met expressing cell line (wild-type-9). Since Tpr-Met in the absence of protection by a GST-Grb2 fusion protein failed to be completely dephosphorylated (data not shown), the high stoichiometry of phosphorylation at these

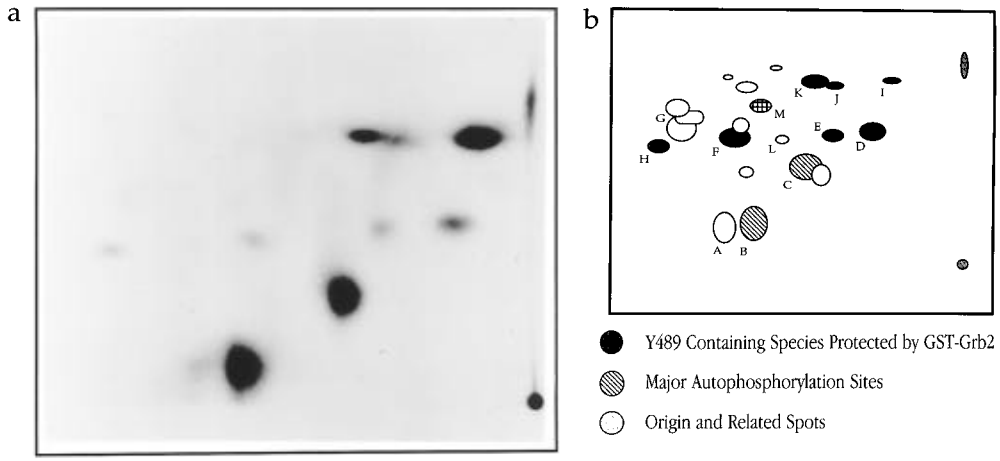

FIGURE 4: Dephosphorylation assay using the GST-Grb2 fusion protein. Wild-type Tpr-Met from transiently transfected COS-1 cells was immunoprecipitated and labeled in an *in vitro* kinase reaction with [γ-^{32}P]ATP as in Figure 2. Immunoprecipitates were then incubated with 500 nM purified GST-Grb2 fusion protein for 30 min at 25 °C prior to dephosphorylation with 5 units of alkaline phosphatase. Proteins were resolved by SDS−PAGE, and tryptic phosphopeptide mapping was performed on Tpr-Met as in Figure 2. (a) Tryptic phosphopeptide map of Tpr-Met protein, with pY489 containing phosphopeptides protected from dephosphorylation. (b) Schematic representation of species protected from dephosphorylation by GST-Grb2.

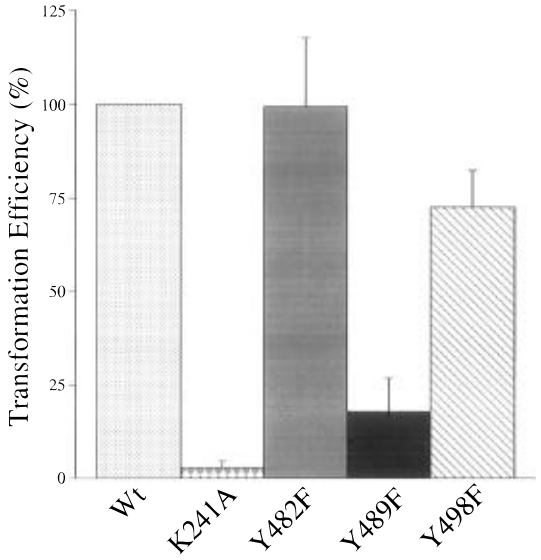

FIGURE 5: Transformation efficiency of carboxy terminal Tpr-Met mutants. Transforming efficiency represents a given number of foci from a single experiment expressed as a percentage of G418 resistant colonies from the same experiment and normalized to Wt. The standard deviation represents the results from a minimum of 4 independent experiments, each performed in duplicate.

sites may render them refractory to complete dephosphosphorylation. However, these data demonstrate that the tryptic phosphopeptides missing in the maps of Y489F mutants each contain a pY489 and support our interpretation that these phosphopeptides arise as products of partial digestion by trypsin.

Transforming Activity of Carboxy Terminal Tyrosine−Phenylalanine Mutants. To investigate the importance of the phosphorylation state of the carboxy terminal tyrosines in Tpr-Met *in vivo*, focus forming assays were performed. Fr3T3 cells were infected with retrovirus containing the various mutants of Tpr-Met and tested in parallel for both resistance to G418, and their ability to overgrow a confluent monolayer. The transforming activity of the Wt Tpr-Met oncoprotein was normalized to 100% (foci counted as a percentage of G418 resistant colonies (Figure 5)). As shown previously when compared with Wt, the Y489F mutant showed a dramatic reduction in the transforming activity (17.8 ± 0.83%; Figure 5) (Ponzetto et al., 1994; Fixman et al., 1995). In contrast, the Y482F mutant showed essentially Wt levels of focus forming activity (99.3 ± 18.47%), whereas the Y498F mutant oncoprotein consistently had a reduced focus forming activity (72.6 ± 9.71%; Figure 5).

DISCUSSION

Tyrosine phosphorylation is crucial for full biological activity of both receptor and nonreceptor tyrosine kinases and their derived oncoproteins. We have identified two tyrosine residues within the catalytic domain of the hepatocyte growth factor receptor (Met) (Y1234, Y1235) (Zhu et al., 1994b; Longati et al., 1994) and its oncogenic counterpart, Tpr-Met (Y365, Y366), that are phosphorylated (Rodrigues & Park, 1994b) and are essential for full catalytic and biological activity in both the Tpr-Met oncoprotein (Rodrigues & Park, 1994b) and the Met receptor (Zhu et al., 1994b). Both the Tpr-Met oncoprotein (which lacks any missense mutations) and the Met receptor each contain a total of 16 tyrosine residues. However, a detailed analysis of phosphorylation in these proteins had not been undertaken. In order to determine the state of phosphorylation of the tyrosines in Tpr-Met, tryptic phosphopeptide mapping was performed. It was previously shown that the sites of tyrosine phosphorylation are identical in Tpr-Met when radiolabeled with [γ-^{32}P]ATP *in vitro* or *in vivo* (Rodrigues & Park, 1994b) and that these sites are identical in the Met receptor (Zhu et al., 1994a). Because the tryptic phosphopeptide maps of both the receptor and oncoprotein are identical, the sites of tyrosine phosphorylation on both the receptor and the oncoprotein are likely to be the same.

Peptide mapping and *in vitro* mutagenesis experiments revealed that Y489 in the carboxy terminus of the Tpr-Met oncoprotein was highly phosphorylated, whereas Y482 and Y498 were not (Figure 2). Since Y489 is phosphorylated in immunoprecipitates *in vitro*, we conclude that phosphorylation at this site is due to autophosphorylation. A tryptic phosphopeptide map of a mutant Tpr-Met oncoprotein containing a substitution of phenylalanine for tyrosine at residue 489 (Y489F) results in the loss of eight phosphory-

lated species (Figure 2). The loss of many phosphorylated peptides upon mutation of a single tyrosine residue may suggest partial digestion by trypsin or that phosphorylation of Y489 is required for phosphorylation of other tyrosine residues. From tryptic digestion of a CNBr released fragment containing the Tpr-Met carboxy terminus, we believe that the loss of many species is the result of generation of many Y489 containing phosphopeptides due to partial trypsin digestion (Figure 3). This is demonstrated by the ability of a GST-Grb2 fusion protein to protect all putative pY489 containing tryptic phosphopeptides from dephosphorylation by alkaline phosphatase (Figure 4). The potential trypsin cleavage sites in the carboxy terminus of Tpr-Met are surrounded by both acidic amino acids and proline residues, which are known to be refractory to trypsin cleavage (Boyle et al., 1991), and similar partial digestion by trypsin has been observed in the PDGF (Kashishian et al., 1992; Valius et al., 1993) and CSF-1 receptors (Van Der Geer & Hunter, 1993). In contrast, Y482 and Y489 mutant Tpr-Met proteins displayed tryptic phosphopeptide maps essentially identical to that of the Wt oncoprotein. Tyrosines 482 and 489 in Tpr-Met, which are conserved between the three members of the Met family, Met, Sea, and Ron (Huff et al., 1993; Ronsin et al., 1993), are located on the same tryptic phosphopetide in Met, and it has been suggested that both tyrosines are phosphorylated in the Met receptor (Y1349, Y1356, corresponding to Y482 and Y489, respectively). Indeed, both Y482 and Y489, when present in a synthetic peptide, can be phosphorylated by the Met receptor *in vitro* (Ponzetto et al., 1994). However, our data suggest that both tyrosines are not phosphorylated in the intact Tpr-Met molecule. Our data support the interpretation that Y489 is a highly phosphorylated residue whereas Y482 or 498 are not phosphorylated to detectable levels. Mutation of Y482 does not affect the mobility of any tryptic phosphopetides lost in the Y489F mutant (Figure 2b,c), suggesting that both tyrosines (482 and 489) are not detectably phosphorylated on the same tryptic peptide. Moreover, the eight phosphopeptides that are lost in the Y489F mutant are unaffected in the Y482F mutant (Figure 2), further demonstrating that Tpr-Met molecules containing a phosphorylated Y482 are rare.

Tyrosine 489 but not tyrosine 482 in the Tpr-Met oncoprotein has been shown to be essential *in vivo* for association with or activation of multiple SH2 domain containing substrates, including PLCγ, the Grb2 adaptor protein, and PI3′K (Fixman et al., 1995). However, a comparison of the sequence YVHV (Y482) and YVNV (Y489) with the optimal binding sequences for SH2 domain containing proteins (Songyang et al., 1993, 1994) indicates that both tyrosines represent a possible binding site for a number of SH2 domain containing proteins. The Grb2 adaptor protein has an absolute requirement for an asparagine at the +2 position downstream from the tyrosine and has been demonstrated to bind exclusively to Y489 in the oncoprotein (Fixman et al., 1995) and to the corresponding tyrosine (Y1356) in the Met receptor (Ponzetto et al., 1994; Zhu et al., 1994a). However, other SH2 domain containing substrates such as PLCγ and PI3K would be predicted to bind to either tyrosine (482 or 489) if phosphorylated (Songyang et al., 1993). The data presented here suggest that Y489, as a highly phosphorylated residue, acts as a magnet for SH2 domain containing substrates. In contrast, Y482, although a potential SH2 binding site for many substrates, is not detectably phosphorylated and, as anticipated from these studies, when substituted by phenylalanine, has no affect on the ability of substrates to associate with the Tpr-Met oncoprotein *in vivo* (Fixman et al., 1995). This is in contrast to the Met receptor, where it has been suggested that both Y1349 and Y1356 are essential for interactions with these substrates *in vitro* (Ponzetto et al., 1994). This may reflect a difference between the Tpr-Met oncoprotein which has a constitutively active kinase and the Met receptor whose kinase activity is regulated by ligand binding. However, we have shown that the tryptic phosphopeptide maps of the receptor are essentially identical to that of the oncoprotein (Rodrigues & Park, 1994b; Zhu et al., 1994a), and we have demonstrated that Y1356 is essential for the biological activity of the receptor (Zhu et al., 1994a), whereas Y1349 is not (Weidner et al. 1995, Fournier et al., in preparation), suggesting that Y1356 but not Y1349 is a critical residue.

As shown for the Met receptor (Zhu et al., 1994a), replacement of Y489 in the Tpr-Met oncoprotein with phenylalanine has a profound effect on the ability of the Tpr-Met oncoprotein to transform fibroblasts in culture (Fixman et al., 1995; Ponzetto et al., 1994). Substitution of Y489 with phenylalanine resulted in decreased focus forming ability (17.8 ± 9%) when compared with the Wt oncoprotein, whereas substitution of Y482 with phenylalanine had no affect on the transforming activity (Figure 5). This is consistent with our phosphopeptide mapping data (Figure 2c) and other studies that have identified Y489 as a crucial residue for the biological activity of the Tpr-Met oncoprotein or receptor (Fixman et al., 1995; Zhu et al., 1994a; Ponzetto et al., 1994). Our data differ from those of a previous study, where a Y482F Tpr-Met mutant was reported to transform fibroblasts with 50% the efficiency of the Wt oncoprotein (Ponzetto et al., 1994). This may reflect the variability of cellular transfections versus retroviral infection, whereas in our study, focus forming activity was internally controlled by comparing the number of G418 resistant colonies obtained from the same infection.

Interestingly, in both the Y482F and Y498F mutants the phosphorylation or mobility of components of complex G was altered. From the tryptic phosphopeptide maps of the carboxy terminus generated following CNBr cleavage (Figure 3c,d), peptide G does not appear to correspond to a C-terminal phosphorylated site, suggesting that the presence of Y482 or Y498 may be required for phosphorylation of a tyrosine residue(s) located outside the carboxy terminus. Although the transforming activity of the Y482F mutant was not altered when compared with Wt, the transforming activity of the Y498F mutant is consistantly decreased (72.6 ± 9.71%; Figure 5). Significantly, a Met receptor containing a Y498F substitution is also impaired in its ability to stimulate branching morphogenesis (Weidner et al., 1995).

We have identified that Y489 in the carboxy terminus of the Tpr-Met oncoprotein is a highly phosphorylated tyrosine residue that is essential for full biological activity of the oncoprotein. However, there are several other phosphorylated species which are generated through tryptic phosphopeptide mapping of the Tpr-Met oncoprotein that are as yet unidentified (Figure 2a,e). This indicates that there are one or more unidentified sites of tyrosine phosphorylation in the Tpr-Met oncoprotein which may be important for biological activity.

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REFERENCES

Batzer, A. G., Rotin, D., Urena, J. M., Skolnik, E. Y., & Schlessinger, J. (1994) *Mol. Cell. Biol. 14*, 5192.
Bottaro, D. P., Rubin, J. S., Faletto, D. L., Chan, A. M.-L., Kmiecik, T. E., Vande Woude, G. F., & Aaronson, S. A. (1991) *Science 251*, 802.
Boyle, W. J., Van Der Geer, P., & Hunter, T. (1991) *Methods Enzymol. 201*, 110.
Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., & Soltoff, S. (1991) *Cell 64*, 281.
Chen, W. S., Lazar, C. S., Poenie, M., Tsien, R. Y., Gill, G. N., & Rosenfeld, M. G. (1987) *Nature 328*, 820.
Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebwhol, D., Ullrich, A., & Rosen, O. M. (1987) *J. Biol. Chem. 262*, 1842.
Deng, W. P., & Nickoloff, J. A. (1992) *Anal. Biochem. 200*, 81.
Escobedo, J. A., Barr, P. J., & Williams, L. T. (1988) *Mol. Cell. Biol. 8*, 5126.
Fantl, W. J., Escobedo, J. A., & Williams, L. T. (1989) *Mol. Cell. Biol. 9*, 4473.
Ferracini, R., Longati, P., Naldini, L., Vigna, E., & Comoglio, P. M. (1991) *J. Biol. Chem. 266*, 19558.
Fixman, E. D., Naujokas, M. A., Rodrigues, G. A., Moran, M. F., & Park, M. (1995) *Oncogene 10*, 237.
Giordano, S., Ponzetto, C., Di Renzo, M. F., Cooper, C. S., & Comoglio, P. M. (1989) *Nature 339*, 155.
Giordano, S., Zhen, Z., Medico, E., Gaudino, G., Galimi, F., & Comoglio, P. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 649.
Gonzatti-Haces, M., Seth, A., Park, M., Copeland, T., Oroszlan, S., & Vande Woude, G. F. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 21.
Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene 77*, 51.
Honegger, A., Dull, T. J., Szapary, D., Komoriya, A., Kris, R., Ullrich, A., & Schlessinger, J. (1988) *EMBO J. 7*, 3053.
Huff, J. L., Jelinek, M. A., Borman, C. A., Lansing, T. J., & Parsons, J. T. (1993) *Proc. Natl. Acad. Sci. U.S.A 90*, 6140.
Iyer, A., Kmiecik, T. E., Park, M., Daar, I., Blair, D. G., Dunn, K. J., Sutrave, P., Ihle, J. N., Bodescot, M., & Vande Woude, G. F. (1990) *Cell Growth Differ. 1*, 87.
Kashishian, A., Kazlauskas, A., & Cooper, J. A. (1992) *EMBO J. 11*, 1373.
Kazlauskas, A., Durden, D. L., & Cooper, J. A. (1991) *Cell Regul. 2*, 413.
Keating, M. T., Escobedo, J. A., & Williams, L. T. (1988) *J. Biol. Chem. 263*, 12805.
Kmiecik, T. E., & Shalloway, D. (1987) *Cell 49*, 65.
Kriegler, M. (1990) in *Gene Transfer and Expression*, Stockton Press, New York, London, Tokyo, Melbourne.
Longati, P., Bardelli, A., Ponzetto, C., Naldini, L., & Comoglio, P. M. (1994) *Oncogene 9* (1), 49.
Luthman, H., & Magnusson, G. (1983) *Nucleic Acids Res. 11*, 1295.
Matsumoto, K., & Nakamura, T. (1992) *Crit. Rev. Oncog. 3*, 27.
McClain, D. A., Maegwa, H., Lee, J., Dull, T. J., Ullrich, A., & Olefsky, J. M. (1987) *J. Biol. Chem. 262*, 14663.
McCutchan, J. H., & Pagano, J. S. (1968) *J. Natl. Cancer Inst. 41*, 351.
Miller, D. A., & Rosman, G. J. (1989) *BioTechniques 7*, 980.
Muller, A. J., Young, J. C., Pendergast, A.-M., Pondel, M., Landau, N. R., Littman, D. R., & Witte, O. N. (1991) *Mol. Cell. Biol. 11*, 1785.
Naldini, L., Weidner, K. M., Vigna, E., Gaudino, G., Bardelli, A., Ponzetto, C., Narsimhan, R. P., Hartmann, G., Zarnegar, R., Michalopoulos, G. A., Birchmeier, W., & Comoglio, P. M. (1991) *EMBO J. 10*, 2867.
O'Shea, E. K., Klemm, J. D., Kim, P. S., & Alber, T. (1991) *Science 254*, 539.
Park, M., Dean, M., Cooper, C. S., Schmidt, M., O'Brien, S. J., Blair, D. G., & Vande Woude, G. (1986) *Cell 45*, 895.
Park, M., Dean, M., Kaul, K., Braun, M. J., Gonda, M. A., & Vande Woude, G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6379.
Pawson, T., & Gish, G. D. (1992) *Cell 71*, 359.
Piwnica-Worms, H., Saunders, K. B., Roberts, T. M., Smith, A. E., & Cheng, S. H. (1987) *Cell 49*, 75.
Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G., & Comoglio, P. M. (1994) *Cell 77*, 261.
Rodrigues, G., & Park, M. (1993) *Mol. Cell. Biol. 13*, 6711.
Rodrigues, G. A., & Park, M. (1994a) *Curr. Opin. Genet. Dev. 4*, 15.
Rodrigues, G. A., & Park, M. (1994b) *Oncogene 9*, 2019.
Rodrigues, G. A., Naujokas, M. A., & Park, M. (1991) *Mol. Cell. Biol. 11*, 2962.
Rong, S., Bodescot, M., Blair, D., Dunn, J., Nakamura, T., Mizuno, K., Park, M., Chan, A., Aaronson, S., & Vande Woude, G. F. (1992) *Mol. Cell. Biol. 12*, 5152.
Ronsin, C., Muscatelli, F., Mattei, M.-G., & Breathnach, R. (1993) *Oncogene 8*, 1195.
Rosen, E. M., Goldberg, I. D., Kacinski, B. M., Buckholz, T., & Vinter, D. W. (1989) *Cell Dev. Biol. 25*, 163.
Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M., & Cobb, M. H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3237.
Rotin, D., Margolis, B., Mohamadi, M., Daly, R. J., Daum, G., Li, N., Fischer, E.H., Burgess, W. H., Ullrich, A., & Schlessinger, J. (1992) *EMBO J. 11*, 559.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.
Sasaki, N., Rees-Jones, R. W., Zick, Y., Nissley, S. P., & Rechler, M. M. (1985) *J. Biol. Chem. 260*, 9793.
Sawyers, C. L., & Denny, C. T. (1994) *Cell 77*, 171.
Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechlelder, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., & Cantley, L. C. (1993) *Cell 72*, 767.
Songyang, Z., Shoelson, S. E., McGlade, J., Olivier, P., Pawson, T., Bustelo, X. R., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T., Ren, R., Baltimore, D., Ratnofsky, S., Feldman, R. A., & Cantley, L. C. (1994) *Mol. Cell. Biol. 14*, 2777.
Stoker, M., & Gherardi, E. (1991) *B. B. A. Cancer Rev. 1072*, 81.
Ullrich, A., & Schlessinger, J. (1990) *Cell 61*, 203.
Valius, M., Bazenet, C., & Kazlauskas, A. (1993) *Mol. Cell. Biol. 13*, 133.
Van Der Geer, P., & Hunter, T. (1991) *Mol. Cell. Biol. 11*, 4698.
Van Der Geer, P., & Hunter, T. (1993) *EMBO J. 12*, 5161.
Weidner, K. M., Sachs, M., Riethmacher, D., & Birchmeier, W. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2597.
Yarden, Y., & Schlessinger, J. (1987) *Biochem. J. 26*, 1443.
Yu, K. T., & Czech, M. P. (1984) *J. Biol. Chem. 259*, 5277.
Zhu, H., Naujokas, M. A., Fixman, E. D., Torossian, K., & Park, M. (1994a) *J. Biol. Chem. 269*, 29943.
Zhu, H., Naujokas, M. A., & Park, M. (1994b) *Cell Growth Differ. 5*, 359.

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