Multiple in Vivo Tyrosine Phosphorylation Sites in EphB Receptors†

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ABSTRACT: Autophosphorylation regulates the function of receptor tyrosine kinases. To dissect the mechanism by which Eph receptors transmit signals, we have developed an approach using matrix-assisted laser desorption-ionization (MALDI) mass spectrometry to map systematically their in vivo tyrosine phosphorylation sites. With this approach, phosphorylated peptides from receptors digested with various endoproteinases were selectively isolated on immobilized anti-phosphotyrosine antibodies and analyzed directly by MALDI mass spectrometry. Multiple in vivo tyrosine phosphorylation sites were identified in the juxtamembrane region, kinase domain, and carboxy-terminal tail of EphB2 and EphB5, and found to be remarkably conserved between these EphB receptors. A number of these sites were also identified as in vitro autophosphorylation sites of EphB5 by phosphopeptide mapping using two-dimensional chromatography. Only two in vitro tyrosine phosphorylation sites had previously been directly identified for Eph receptors. Our data further indicate that in vivo EphB2 and EphB5 are also extensively phosphorylated on serine and threonine residues. Because phosphorylation at each site can affect receptor signaling properties, the multiple phosphorylation sites identified here for the EphB receptors suggest a complex regulation of their functions, presumably achieved by autophosphorylation as well as phosphorylation by other kinases. In addition, we show that MALDI mass spectrometry can be used to determine the binding sites for Src homology 2 (SH2) domains by identifying the EphB2 phosphopeptides that bind to the SH2 domain of the Src kinase.

The Eph receptors are a large family of receptor tyrosine kinases that regulate a variety of key processes during embryonic development, including axon pathfinding, arborization and pruning, the organization of cellular boundaries, and the formation of the circulatory system (*1*−*7*). Abnormally expressed Eph receptors have also been suggested to contribute to carcinogenesis (*8*−*11*).

On the basis of sequence homology, the 14 known Eph receptors are divided into two groups (*12*). Receptors of the EphA group bind preferentially the glycosylphosphatidylinositol (GPI[1])-linked ephrin-A ligands, and receptors of the EphB group bind preferentially the transmembrane ephrin B ligands. The binding of ephrin-B ligands to EphB receptors presumably requires a cell−cell interaction, because both are transmembrane proteins, and results in the phosphorylation of tyrosine residues in the intracellular domains of both the receptor and the ligand (*13*, *14*). Thus, formation of a complex of an ephrin-B ligand and an EphB receptor has been proposed to initiate bidirectional signaling cascades, which propagate in both the receptor-bearing cells and the ligand-bearing cells. Tyrosine phosphorylation of EphB receptors likely occurs at least in part by autophosphorylation, whereas the ephrin-B ligands, which lack a kinase domain, are presumably phosphorylated by an associated tyrosine kinase. Because each phosphorylation site may differentially affect the signaling properties of an ephrin-B ligand−EphB receptor complex, it is important to identify the specific tyrosines that become phosphorylated in order to understand the regulatory mechanisms of this complex. We have focused on identifying the tyrosine phosphorylation sites of EphB receptors, which contain a kinase domain.

Tyrosine-phosphorylated sequences in the cytoplasmic domain of receptor tyrosine kinases serve as docking sites for signaling proteins and regulate kinase activity (*15*). Tyrosine phosphorylation of Eph receptors is required for their association with a number of signaling proteins (*13*, *16*, *17*), which in turn may contribute to propagation of some Eph receptor signals. Signal transduction may also occur through Eph receptor kinase activity. Activated EphB2, for example, causes an increased level of tyrosine phosphorylation of proteins such as the neuronal cell adhesion molecule, L1 (*18*), the Ras binding protein, AF6 (*19*), the low-molecular weight phosphotyrosine phosphatase, LMW-PTP (*20*), and the docking protein p62dok (*21*). Tyrosine phosphorylation of these proteins by activated Eph receptors

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[1] Abbreviations: SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TPCK, *N*-(*p*-tolylsulfonyl)-L-phenylalanyl chloromethyl ketone; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; MALDI-TOF, matrix-assisted laser desorption-ionization time-of-flight; TBS, Tris-buffered saline; TFA, trifluoroacetic acid; PVDF, polyvinyl difluoride; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PCR, polymerase chain reaction; SH2, Src homology 2; PTB, phosphotyrosine binding; SAM, sterile α motif; LMW-PTP, low-molecular weight phosphotyrosine phosphatase; HPLC, high-pressure liquid chromatography; GPI, glycosylphosphatidylinositol; EGF, epidermal growth factor.

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may influence their function, thereby causing changes in cell behavior.

On the basis of phosphopeptide mapping of the receptors, EphA4 and EphB2, two conserved tyrosines in the juxtamembrane region of Eph receptors were identified as autophosphorylation sites. In both EphA4 and EphB2, one tyrosine (Y611 in EphB2) appeared to be more highly phosphorylated than the other (Y605 in EphB2) (*22*, *23*). The existence of additional autophosphorylation sites in Eph receptors has been suspected, however, because some phosphopeptides remained unassigned in the phosphopeptide maps of EphB2 (*23*). Furthermore, a conserved tyrosine in the carboxy-terminal tail of the EphB receptors, EphB1, has been indirectly identified as a phosphorylation site because mutation of this tyrosine disrupted the binding of the adaptor protein, Grb10, which contains an SH2 domain (*24*). The presence of another tyrosine phosphorylation site in the kinase domain of EphB1 was inferred on the basis of the phosphorylation-dependent association with the adaptor protein, Grb2, which also contains an SH2 domain (*24*).

Previous studies did not determine whether the two tyrosines identified as in vitro phosphorylation sites of Eph receptors are actually phosphorylated in vivo. Indeed, no direct efforts to identify in vivo phosphorylation sites of Eph receptors have been reported so far. We have used a two-dimensional phosphopeptide mapping approach to identify the in vitro autophosphorylation sites of the EphB receptor, EphB5, and a mass spectrometry approach to identify the in vivo phosphorylation sites of EphB5 and the related receptor, EphB2. We also report an approach for identifying the binding sites for SH2 domains using matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry.

EXPERIMENTAL PROCEDURES

Materials. Cellulose plates and the Hunter HTLE-7000 Thin Layer Electrophoresis System were purchased from C. B. S. Scientific. Protease inhibitors, TPCK-trypsin, anti-phosphotyrosine antibodies coupled to agarose (clone PT-66), and protein A−peroxidase were obtained from Sigma Chemical Co. Endoproteinases used in mapping by mass spectrometry were sequencing grade from Promega. 1-*O*-Octyl β-D-glucopyranoside (98%), α-cyano-4-hydroxycinnamic acid, and 2,5-dihydroxybenzoic acid were obtained from Aldrich Chemical Co. Chemicals used for mass spectrometry experiments were HPLC grade or the best grade available. The Voyager MALDI-TOF mass spectrometer was from PerSeptive Biosystems. Anti-phosphotyrosine antibodies conjugated to horseradish peroxidase were from Transduction Laboratories. The enhanced chemiluminescence system was from Amersham Life Sciences. GammaBind Plus Sepharose was from Pharmacia Biotech.

Plasmids Encoding Eph Receptors. The pcDNA3-EphB2 plasmid has been described (*18*). The cDNA encoding chicken EphB5 (*25*) was cloned into pcDNA3 as follows. Nucleotides 107−556 of EphB5 were obtained by PCR amplification from an 11-day-old chicken embryonic retina cDNA library (*26*) with the sense primer ATTGGTACCGGTGGAGTCTACAGAGAATC, containing an *Asp*718 site (underlined), and the antisense primer CTGGCAGTTCCTGAGAGGCT, and cloned into the TA vector pCRII. Nucleotides 1890−3147 of EphB5 were obtained by PCR from a partial EphB5 cDNA (*27*) with the sense primer CCGGATCCTTCAAGAGTAAAAGGCGAG, containing a *Bam*HI site (underlined), and the antisense primer ACTTCTCGAGTCACACTTCAACTGGTTCA, containing a *Xho*I site (underlined), and cloned into expression vector pGEX-4T-1. An EphB5 fragment obtained by digestion of the PCR product in pCRII at the introduced *Asp*718 site and at an endogenous *Sal*I site at position 529, together with a *Sal*I to *Eco*RI fragment from EphB5 cDNA (nucleotides 530−2284) (*25*), and a *Eco*RI to *Xho*I fragment from the PCR product in pGEX-4T-1 (nucleotides 2285−3147) were ligated into *Asp*718- and *Xho*I-digested pcDNA3.

Site-Directed Mutagenesis. To mutate tyrosines in the cytoplasmic domain of EphB5, site-directed mutagenesis was performed by sequential PCR steps using appropriate EphB5 cDNAs in pBluescript as templates and overlapping primers encoding the mutation (*28*). The PCR product encoding the mutation was then inserted into pcDNA3-EphB5 via unique restriction sites and sequenced to confirm the desired construct.

To generate the Y610F mutant, the overlapping sense primer AGTAT**TTC**ATTGATCCTTCC and antisense primer TCAAT**GAA**ATACTTCACTCC (mutated codons are indicated in bold) were used together with the T7 and M13 reverse primers to amplify the EphB5 cDNA in pBluescript. The final PCR product was digested with *Blp*I and *Eco*RI and ligated into the *Blp*I- and *Eco*RI-digested pcDNA3-EphB5 plasmid. The Y592F, Y599F, Y609F, and Y616F mutants were generated in the same manner by using the following overlapping primers: CTCCA**TTC**ACAGACCGCCTG and TCTGT**GAA**TGGAGTCTCTCG (Y592F), AGCAG**TTT**ATCAGTACACGAG and CTGAT**AAA**CTGCTGCAGGCG (Y599F), TGAAG**TTT**TACATTGATCCTTC and ATGTA**AAA**CTTCACTCCAAG (Y609F), and CCACG**TTT**GAAGATCCCAATG and TCTTC**AAA**CGTGGAAGGATC (Y616F). The Y610F/Y616F mutant was produced similarly by using the final Y616F PCR product as a template and the overlapping primers containing the Y610F mutation. The Y609F/Y610F mutant was constructed by using the overlapping primers TGAAG**TTTTTC**ATTGATCCTTCC and TCAAT**GAAAAA**CTTCACTCCAAG, which contained both mutant codons. The Y609F/Y610F/Y616F mutant was produced by using the final Y616F PCR product as a template and the overlapping primers containing the Y610F/Y609F mutation.

To obtain the Y944F mutant, the overlapping primers GTCGT**TTC**AAGGAGAATTTTG and TCCTT**GAA**ACGACCCATCTTG were used together with the flanking primers ATGAACTATGTGCATCG and ACTTCTCGAGTCACACTTCAACTGGTTCA (containing a *Xho*I site, underlined) to amplify the EphB5 cDNA fragment in pGEX-4T-1 described above. The final PCR product containing the mutation was digested with *Pflm*I at an endogenous site and *Xho*I and ligated with an *Eco*RI to *Pflm*I EphB5 cDNA fragment (nucleotides 2285−2552) into the *Eco*RI- and *Xho*I-digested pcDNA3-EphB5 plasmid. To produce the Y794E mutant, overlapping primers CCACT**GAG**ACTGGAGCTCTGGG and CCAGT**CTC**AGTGGGATTTGAAG were used together with the flanking primers ATCCACCTGGAGGGCGTGGTC and ACTTCTCGAGTCACACTTCAACTGGTTCA (containing a *Xho*I site, underlined) to

amplify the EphB5 cDNA fragment in pGEX-4T-1. The final PCR product containing the mutation was digested with *Eco*RI and *Pflm*I at endogenous sites and ligated with a *Pflm*I to *Xho*I EphB5 fragment (nucleotides 2285−3147) into an *Eco*RI- and *Xho*I-digested pcDNA3-EphB5 plasmid. To generate the Y841F and Y857F mutants, overlapping primers GACCT**TTC**TGGGACATGTCC and TCCCA**GAA**AGGTCTCTCACC for Y841F and AGGAC**TTT**CGCCTGCCACCAC and AGGCG**AAA**GTCCTGGTCAATG for Y857F were used together with flanking primers to amplify the EphB5 cDNA in pGEX-4T-1. The final PCR products were digested with *Eco*RI and *Xho*I and ligated into an *Eco*RI- and *Xho*I-digested EphB5-pcDNA3 plasmid.

Ephrin-B2 Fc Chimera. The sequence encoding the extracellular domain of chicken ephrin-B2 (nucleotides 452−1046) (GenBank accession number AF180729) was amplified by PCR with the sense primer ACTCGCTAGCCAAGTCCATCGTTTTAGAC containing an *Nhe*I site (underlined) and the antisense primer CGGGATCCACTTCTGAACCCAGTATAC containing a *Bam*HI site (underlined). The PCR product was digested with *Nhe*I and *Bam*HI and cloned into pcDNA3 containing the CD5 signal peptide and the Fc portion of human IgG1 as described previously (*26*, *29*). The Fc chimera was purified from cell culture supernatants of stably transfected 293 cells as described previously (*26*, *29*).

Cell Culture and Transfections. The 293 human embryonal kidney cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg of streptomycin, and 1 mM sodium pyruvate. Transient transfections were performed by the calcium phosphate method as previously described (*23*).

Phosphopeptide Mapping by Two-Dimensional Chromatography. Phosphopeptide mapping was performed as previously described (*23*, *30*) using wild-type and mutant forms of EphB5 immunoprecipitated from transiently transfected 293 cells. After elution of the appropriate band from gel slices, γ-^{32}P-autophosphorylated receptor was digested with 0.4 μg/μL TPCK-trypsin. Three thousand counts per minute of each digest were resolved in two dimensions on 100 μm × 20 cm × 20 cm thin-layer cellulose plates by electrophoresis followed by ascending chromatography. Electrophoresis was performed at pH 1.9 in 50:156:1794 88% formic acid/glacial acetic acid/water for 40 min at 1000 V on a Hunter HTLE-7000 Thin Layer Electrophoresis System, and ascending chromatography was carried out in 750:500:150:600 *n*-butanol/pyridine/glacial acetic acid/water. The plates were exposed to X-ray film for 3 days at −70 °C with an intensifying screen.

Preparation of Receptors for Mass Spectrometry. The 293 cells transiently transfected with EphB2 or EphB5 were lysed in 1% Brij 97 in Tris-buffered saline (TBS) containing protease inhibitors and 5 mM EDTA. Untransfected 293 cells were used as a control. Lysates were incubated with 100 μL of GST−Src SH2 bound to glutathione agarose (~1 μg/μL protein) for 30 min at 4 °C. GST−Src SH2 and GST−Grb10 SH2 (nucleotides 1968−2276) were prepared as described previously (*23*). Alternatively, lysates were incubated with 50 μg of ephrin-B2 Fc bound to 10 μL of GammaBind Plus Sepharose for 30 min at 4 °C. The sample was washed three times in 1% Brij in TBS containing 1 mM sodium orthovanadate and 10 mM NaF, and three times in 0.1% 1-*O*-octyl β-D-glucopyranoside (98%) and 100 mM NH_4HCO_3 (pH 7.5). Samples were boiled for 3 min, cooled on ice, and centrifuged. Proteolytic digestions of the receptors were performed with sequence grade proteases according to one of the following methods. Trypsin digests (50 ng/μL) were carried out in 100 μL of 100 mM NH_4HCO_3 (pH 8) at 37 °C overnight, followed by a second 5 h digestion. Endoproteinase Glu-C/trypsin digests were carried out in 100 μL of 100 mM NH_4HCO_3 (pH 8) at 37 °C overnight with endoproteinase Glu-C (50 ng/μL) first, followed by a 6 h tryptic digestion at 37 °C (50 ng/μL). Endoproteinase Glu-C/Arg-C digests were carried out by digesting first with endoproteinase Glu-C (50 ng/μL) overnight at 37 °C in 100 μL of 100 mM NH_4HCO_3 (pH 8), drying the sample in a speed vac, dissolving the sample in 10 mM Tris (pH 7.5), 2.5 mM dithiothreitol, and 1 mM $CaCl_2$, and digesting with endoproteinase Arg-C (50 ng/μL) for 6 h at 37 °C. Endoproteinase Glu-C/Lys-C digests were carried out by digesting first with endoproteinase Glu-C (50 ng/μL) overnight at 37 °C in 100 μL of 100 mM NH_4HCO_3 (pH 8), drying the sample in a speed vac, dissolving the sample in 25 mM Tris (pH 7.7) and 1 mM EDTA, and digesting with endoproteinase Lys-C (50 ng/μL) for 6 h at 37 °C. Endoproteinases were denatured by boiling for 5 min and further inhibited with PMSF (100 μg/mL) or, in the case of endoproteinase Lys-C, with 1 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone. The digested samples were centrifuged, and the supernatants were removed. The sedimented beads were washed once with 100 μL of 100 mM NH_4HCO_3 (pH 8), and the supernatant and wash were combined with 10 μL of anti-phosphotyrosine antibodies conjugated to agarose (*31*) or to GST−Src SH2 bound to glutathione agarose for incubation at 4 °C for 6 h. The beads were washed four times with 100 mM NH_4HCO_3 (pH 8) and once with water and dried in a speed vac.

MALDI-TOF Mass Spectrometry. Mass spectra were collected on a Voyager MALDI-TOF mass spectrometer using the delayed extraction mode. The bound phosphopeptide mixtures were analyzed in linear and reflector mode. Sixty-four to 128 shots from a nitrogen laser (337 nm) were averaged to yield each recorded mass spectrum. Spectra were externally calibrated with angiotensin I ($MH^+ = 1296.6853$) and adrenocorticotropic hormone fragment (7−38; $MH^+ = 3657.93$). The matrix solution was prepared immediately before use by mixing 20 mg of α-cyano-4-hydroxycinnamic acid and 4 mg of 2,5-dihydroxybenzoic acid in 500 μL of 0.1% trifluoroacetic acid (TFA) in 50% CH_3CN/dH_2O, vortexing for 1 min, centrifuging for 1 min, and subsequently diluting the clear supernatant 1:4 with 0.1% TFA in 50% CH_3CN/dH_2O. Five microliters of the diluted matrix solution was mixed with the dried, tyrosine-phosphorylated peptides bound to anti-phosphotyrosine agarose (*31*), and a 1 μL suspension was spotted on the target and allowed to air-dry completely before mass analysis.

For interpretation of the mass spectra, a list of predicted molecular weights was generated by theoretical cleavage with specific endoproteinases using the MS-Digest program available at the University of California, San Francisco (http://prospector.ucsf.edu/ucsfhtml3.2/msdigest.htm). Each peptide was assumed to contain at least one phosphate group. The masses of the peaks recorded in the mass spectra were matched to the calculated masses within ~0.1% or better.

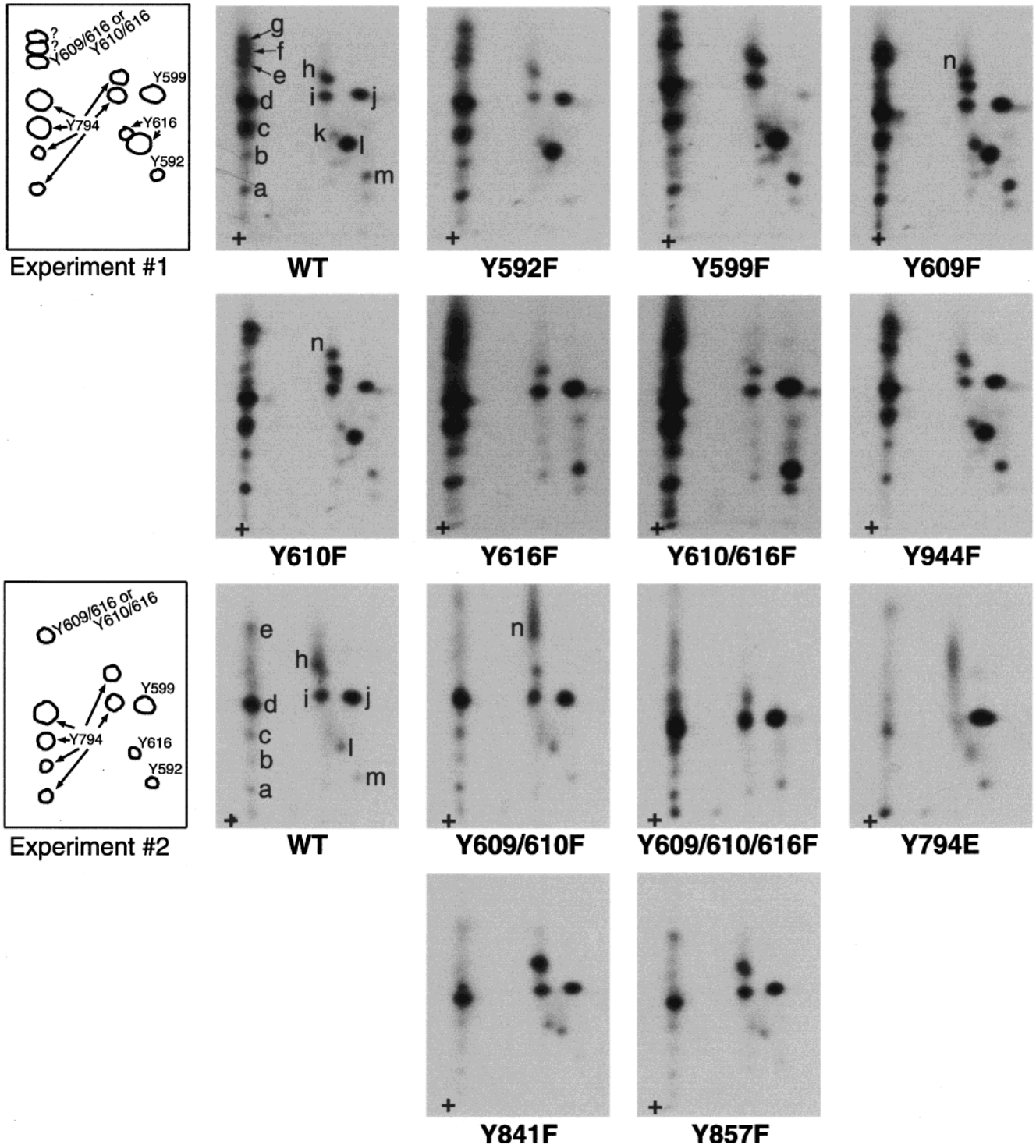

FIGURE 1: Tryptic phosphopeptide maps of wild-type and mutant forms of EphB5. Wild-type and mutant receptors expressed in 293 cells were immunoprecipitated, autophosphorylated with [γ-^{32}P]ATP, and resolved by SDS−PAGE. Radioactive bands representing phosphorylated forms of EphB5 were excised, digested with TPCK-trypsin, and subjected to two-dimensional chromatography. Maps were exposed to film for 3 days at −70 °C with an intensifying screen. Starting at the origin (+), electrophoretic separation is from left to right, and ascending chromatography is from bottom to top. Thirteen phosphopeptides are labeled **a**−**m** in both wild-type panels. An additional phosphopeptide (labeled **n**) appeared only in certain mutants. Peptides containing identified phosphorylated tyrosines are indicated on the left in two schematic representations, one for each set of experiments.

Immunoblots. Samples separated by SDS−PAGE were electrotransferred to PVDF membranes (Millipore) and incubated with primary antibodies for 1−2 h in 3% bovine serum albumin (BSA) and 0.1% polyoxyethylenesorbitan monolaurate (Tween 20) in TBS. Tyrosine phosphorylation was detected with anti-phosphotyrosine antibodies conjugated to horseradish peroxidase (1:10000 dilution). EphB5 was detected with affinity-purified polyclonal antibodies recognizing the carboxy terminus (1 μg/mL) (*25*), and EphB2 was detected with affinity-purified polyclonal antibodies recognizing the carboxy terminus (1 μg/mL) (*32*) or the extracellular domain (1 μg/mL), followed by protein A−peroxidase (1:10000 dilution). An enhanced chemiluminescence system was used for detection. Reprobing was performed after

stripping bound antibodies with a 1 h incubation in 2% SDS, 100 mM mercaptoethanol, and 62.5 mM Tris (pH 7.5).

RESULTS

Mapping in Vitro Autophosphorylation Sites of the EphB5 Receptor. To determine which of the 16 tyrosines in the cytoplasmic domain of the EphB receptor, EphB5 (*25*), are sites of autophosphorylation, we used two-dimensional phosphopeptide mapping in conjunction with site-directed mutagenesis (*23*, *30*). EphB5 was immunoprecipitated from transiently transfected 293 human embryonal kidney cells and autophosphorylated in vitro in a kinase buffer containing [γ-^{32}P]ATP. Following gel electrophoresis, the radiolabeled EphB5 was extracted from the gel and digested with trypsin. After the resulting tryptic peptides had been resolved by two-dimensional chromatography, 13 distinct phosphopeptides (**a**−**m**) were detected (Figure 1).

To identify the phosphorylated tyrosines within the tryptic peptides, the tyrosines in the juxtamembrane region (Y592F, Y599F, Y609F, Y610F, and Y616F) and carboxy-terminal tail (Y944F) were mutated to phenylalanine because in other receptor tyrosine kinases many autophosphorylation sites are located in these regions (*15*, *33*). Because Y609, Y610, and Y616 cannot be separated into different tryptic peptides, we also generated Y609F/Y610F, Y610F/Y616F, and Y609F/Y610F/Y616F (double and triple EphB5 mutants) to better detect autophosphorylation at these sites. Tyrosine 794 in the kinase domain was mutated to phenylalanine because it corresponds to a conserved tyrosine in the activation loop that is phosphorylated in other tyrosine kinases (*15*, *33*, *34*). The Y794F mutant, however, could not be analyzed because of insufficient γ-^{32}P incorporation. We, therefore, generated a glutamic acid mutant (Y794E), which exhibited sufficient γ-^{32}P incorporation for mapping analysis. Finally, Y841 and Y857 in the kinase domain were mutated because they were predicted to be possible binding sites for the SH2 domains phosphatidylinositol 3-kinase (Y841) and Shc (Y857) (*35*, *36*), which interact with EphB5 (unpublished data).

Tryptic phosphopeptide maps of the EphB5 mutants were similar to those of wild-type EphB5, except for the absence of certain γ-^{32}P-labeled phosphopeptides in some mutants (Figure 1). The most dramatic case was the Y794E mutant, which lacked a number of major phosphopeptides (**a**−**d**, **h**, and **i**), consistent with Y794 being a major autophosphorylation site.

Phosphopeptide **m** was absent in the Y592F mutant, and phosphopeptide **j** was absent in the Y599F mutant, indicating that Y592 and Y599 are both phosphorylated. Phosphopeptides **e**, **k**, **l**, and **n** all appear to contain Y609, Y610, and Y616, because they are absent in the Y609F/Y610F/Y616F mutant. The absence of phosphopeptides **k** and **l** in the mutant Y616F suggests that they represent peptides monophosphorylated at Y616. Consistent with this assignment, peptides **k** and **l** are also absent in maps of the Y610F/Y616F mutant. Phosphopeptides **e** and **n** appear to represent alternative forms of the same peptide that probably differ in their degree of phosphorylation. Phosphopeptide **e** migrates vertically with the same mobility as phosphopeptide **n**, but resolves differently on the basis of charge, and is absent in the maps containing phosphopeptide **n**. Moreover, the presence of phosphopeptide **e** in the Y616F mutant suggests that either Y609, Y610, or both are phosphorylated.

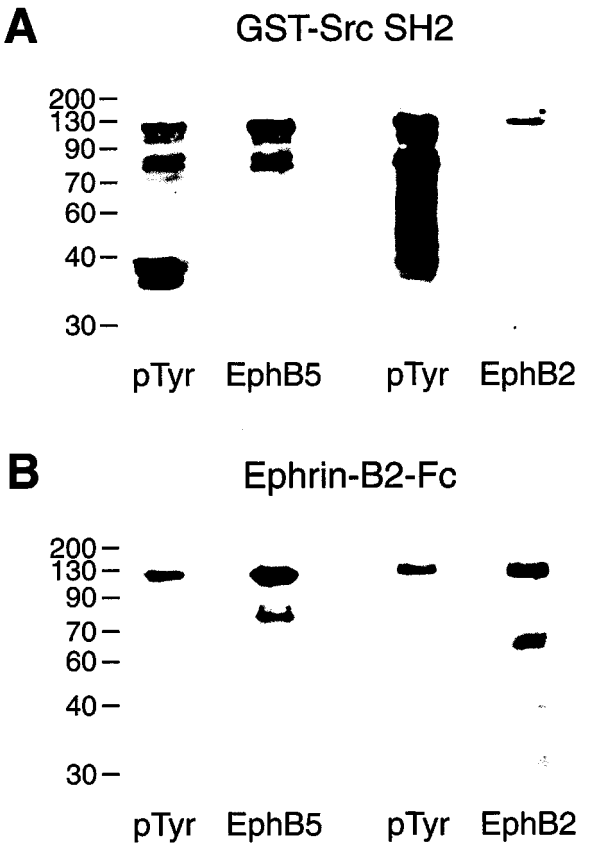

FIGURE 2: Tyrosine-phosphorylated EphB5 and EphB2 isolated using the SH2 domain or the ephrin-B2 ligand. (A) A GST fusion protein of the SH2 domain immobilized on glutathione sepharose and (B) an Fc fusion protein of the extracellular domain of ephrin-B2 immobilized on GammaBind Plus Sepharose were incubated with lysates of 293 cells expressing EphB5 and EphB2. Bound proteins were resolved by SDS−PAGE and labeled by immunoblotting with antibodies specific for phosphotyrosine (pTyr), EphB5, or EphB2, as indicated.

Because maps of the EphB5 mutants Y841F, Y857F, or Y944F contained the same phosphopeptides as the wild type, we concluded that either these tyrosines are not autophosphorylation sites or the peptides containing these tyrosines are not detected in our peptide maps. Taken together, the results of these mapping experiments indicate that Y592, Y599, Y609 and/or Y610, and Y616 in the juxtamembrane region and Y794 in the kinase domain are in vitro autophosphorylation sites of EphB5. Additional autophosphorylation sites may exist in EphB5, since phosphopeptides **g** and **f** remain unidentified.

Mapping in Vivo Tyrosine Phosphorylation Sites in the EphB5 Receptor. To determine whether the autophosphorylation sites that we identified for EphB5 in vitro are also phosphorylated in the cellular environment, as well as to identify possible additional tyrosine phosphorylation sites, we used a mass spectrometric approach. EphB5 was isolated from transiently transfected 293 cells, where this receptor is expressed in its tyrosine-phosphorylated form (Figure 2A), by using two alternative approaches. In the first approach, EphB5 was isolated by binding to immobilized glutathione *S*-transferase (GST)−Src SH2 domain (Figure 2). As expected, a number of tyrosine-phosphorylated proteins bound

to GST−Src SH2, although the major phosphorylated band was EphB5. Immobilized ephrin-B2 Fc was also successfully used to isolate EphB5 (Figure 2), although ephrin-B2 has not been reported to be a high-affinity ligand for EphB5. The proteins bound to GST−Src SH2 or ephrin-B2 Fc were digested with various combinations of endoproteinases. Tyrosine-phosphorylated peptides were selectively isolated from the resulting complex mixture of peptides by using anti-phosphotyrosine antibodies conjugated to agarose, and directly analyzed by MALDI-TOF mass spectrometry.

Representative examples of the mass spectra for EphB5 that was purified with either GST−Src SH2 or ephrin-B2 Fc are shown in Figure 3. The observed masses were compared to a database of masses predicted for appropriate proteolytic digests of EphB5 (top halves of Tables 1−3). Because the peptides were purified with anti-phosphotyrosine antibodies, at least one phosphate group was included in calculating the masses of the predicted fragments. Peak assignments indicate that EphB5 peptides were often phosphorylated at multiple sites. Many of the peaks in the spectra could be mapped to phosphorylated sequences in EphB5, and the corresponding observed masses are denoted in the spectra shown in Figure 3. Peaks observed in spectra obtained from the material purified from untransfected cells were denoted as "background" (B) peaks.

Only a small number of peaks remained unassigned, which are presumably due to nonspecific cleavages, unknown modifications, and damage that may occur to the receptor during the experiment. Some of the unidentified peaks in the GST−Src SH2 domain-purified samples may be phosphopeptides from cellular proteins phosphorylated by EphB5. The spectra from GST−Src SH2-purified samples indeed contained more unassigned peaks than those from ephrin-B2 Fc-purified samples. Furthermore, although fewer experiments were carried out with ephrin-B2 Fc, the patterns of tyrosine-phosphorylated peptides were similar to those obtained with GST−Src SH2. Nine of the 17 peptides identified in the ephrin-B2 Fc experiments were identical to those identified in the GST−Src SH2 experiments (footnotes e and f in Tables 1 and 2), and all the phosphorylated tyrosines, except for Y944, were represented in the peptides from ephrin-B2 Fc-isolated EphB5. In a few instances, peak assignments were ambiguous, because two different EphB5 phosphorylated peptides could account for an observed peak ("?" in Tables 1−3). The peak at *m*/*z* 2153.84 in Figure 3A, for example, could represent two isobaric phosphopeptides, one from the juxtamembrane region (Table 1) and the other from the kinase domain (Table 2).

Many phosphorylated peptides were from the juxtamembrane region of EphB5, which contains five tyrosines (Table 1). Several of these phosphopeptides contain only one tyrosine (either Y592 or Y599), indicating, in agreement with our in vitro mapping experiments, that Y592 and Y599 are phosphorylated in vivo. Because some short peptides containing Y592 are phosphorylated at multiple sites, threonines 590 and 593 were also identified as phosphorylation sites.

Some of the largest observed peaks, such as that at *m*/*z* 2025.96 in Figure 3B, correspond to peptides containing Y609, Y610, and Y616. Assignment of the tyrosine phosphorylation sites in this region of EphB5 is ambiguous, however, because there are no suitable proteolytic sites between the three tyrosines. Our data suggest that at most only two of these tyrosines are phosphorylated in the same peptide. On the basis of the mapping studies described in the previous section, Y616 is likely to be one of the phosphorylated tyrosines.

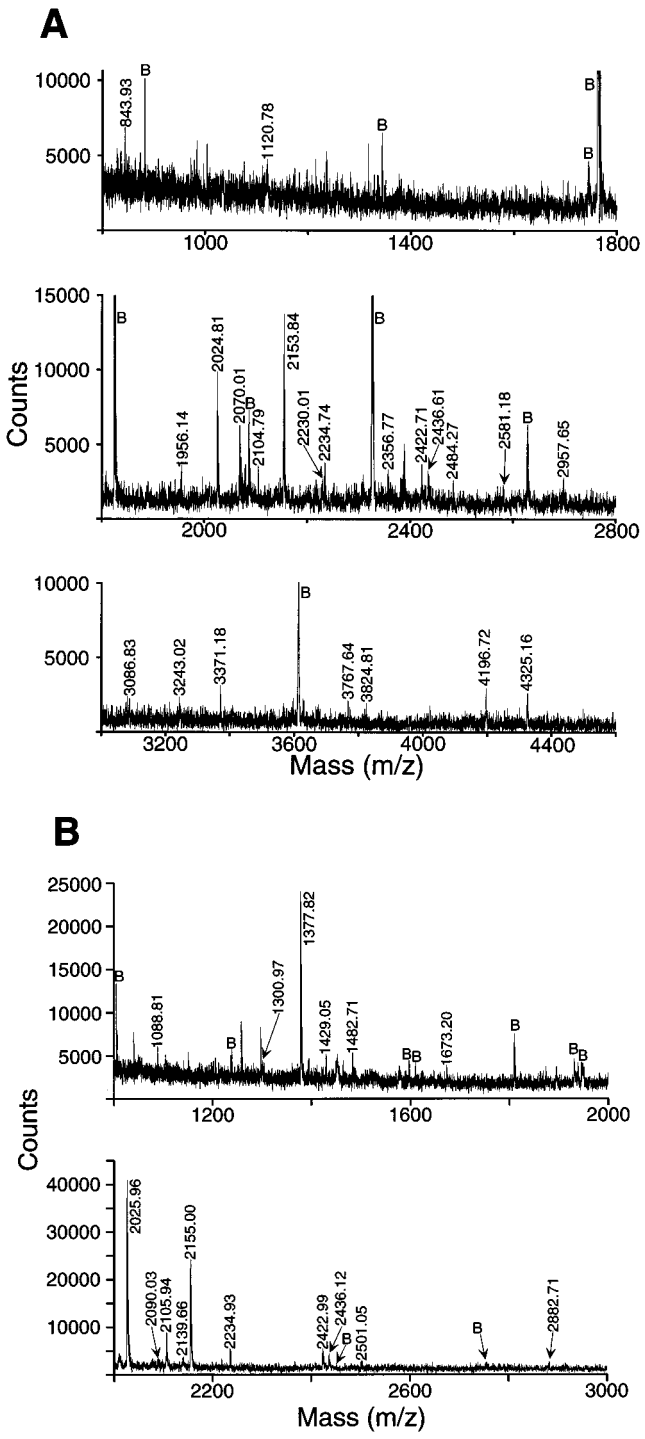

FIGURE 3: MALDI mass spectra of EphB5 phosphopeptides. EphB5 bound to (A) the GST−Src SH2 domain or (B) ephrin-B2 Fc was digested with endoproteinase Glu-C and trypsin. Tyrosine-phosphorylated peptides were isolated from the resulting peptide mixture by binding to anti-phosphotyrosine antibodies conjugated to agarose, and analyzed by MALDI mass spectrometry in the reflector mode. Peaks corresponding to tyrosine-phosphorylated peptides of EphB5 are denoted by their masses and identified in Tables 1−3. Peaks marked with a B are from background ions.

From the kinase domain, we isolated many phosphorylated peptides that contained Y794 in the activation loop, con-

Table 1: Juxtamembrane Peptides from in Vivo Phosphorylated EphB5 and EphB2 Identified by Mass Spectrometry

tyrosine	measured *m*/*z*	M_{calcd}	%Δ[a]	peptide	protease[b]
EphB5 peptide					
592	1120.78	1121.30[c]	0.046	ETPYTDR+3P	E, T[e]
592	1197.71	1197.43[c]	0.023	RETPYTDR+2P	T[e]
592	1569.99	1568.66[c]	0.085	SKRRETPYTDR+2P	E, R[e]
592	4328.96	4330.10[d]	0.026	LPLIVGSALGGLAFLVIAAIAILAIIFKSKRRETPYTDR+2P	T[e]
599	1088.81	1088.51[c]	0.028	LQQYISTR+1P	E, T[f]
592/599 ?[g] (2)	2107.00	2107.00[c]	0.000	RETPYTDRLQQYISTR+1P	T[e]
592/599 ? (5)	2356.77	2356.11[c]	0.028	TPYTDRLQQYISTRGLGVK+2P	E, T[e]
592/599	2422.71	2423.03[c]	0.013	RRETPYTDRLQQYISTR+3P	E, T[e]
592/599 ? (9)	2422.99	2423.03[c]	0.002	RRETPYTDRLQQYISTR+3P	E, T[f]
592/599	2434.61	2436.08[c]	0.060	TPYTDRLQQYISTRGLGVK+3P	E, T[e]
592/599	2436.12	2436.08[c]	0.002	TPYTDRLQQYISTRGLGVK+3P	E, T[f]
592/599	2484.27	2485.15[c]	0.035	ETPYTDRLQQYISTRGLGVK+2P	E, T[e]
592/599	2882.09	2882.71[c]	0.022	RETPYTDRLQQYISTRGLGVK+5P	E, T[f]
592/599 ? (6)	2957.65	2958.92[c]	0.043	RRETPYTDRLQQYISTRGLGVK+4P	E, T[e]
609/610/616	2024.81	2025.85[c]	0.051	YYIDPSTYEDPNEAIR+1P	E, T[e]
609/610/616	2025.96	2025.85[c]	0.005	YYIDPSTYEDPNEAIR+1P	E, T[f]
609/610/616	2026.07	2025.85[c]	0.011	YYIDPSTYEDPNEAIR+1P	T[e]
609/610/616	2104.79	2105.81[c]	0.048	YYIDPSTYEDPNEAIR+2P	E, T[e]
609/610/616	2105.94	2105.81[c]	0.006	YYIDPSTYEDPNEAIR+2P	E, T[f]
609/610/616 ? (2)	2106.25	2105.81[c]	0.021	YYIDPSTYEDPNEAIR+2P	T[e]
609/610/616	2139.66	2139.92[c]	0.012	GLGVKYYIDPSTYEDPNE+1P	E, T[f]
609/610/616 ? (4)	2153.84	2154.89[c]	0.049	YYIDPSTYEDPNEAIRE+1P	E, T[e]
609/610/616	2155.00	2154.89[c]	0.005	YYIDPSTYEDPNEAIRE+1P	E, T[f]
609/610/616	2234.74	2234.86[c]	0.005	YYIDPSTYEDPNEAIRE+2P	E, T[e]
609/610/616	2234.93	2234.86[c]	0.003	YYIDPSTYEDPNEAIRE+2P	E, T[f]
609/610/616	2501.05	2501.09[c]	0.002	YYIDPSTYEDPNEAIREFAK+1P	E, T[f]
609/610/616	2581.18	2581.06[c]	0.005	YYIDPSTYEDPNEAIREFAK+2P	E, T[e]
609/610/616	2610.28	2609.18[c]	0.042	GLGVKYYIDPSTYEDPNEAIRE+1P	E, R[e]
609/610/616 ? (6)	2957.65	2957.17[c]	0.016	GLGVKYYIDPSTYEDPNEAIREFAK+1P	E, T[e]
599/609/610/616	3371.18	3371.27[d]	0.003	LQQYISTRGLGVKYYIDPSTYEDPNE+4P	E, T[e]
599/609/610/616	3373.19	3371.27[d]	0.057	LQQYISTRGLGVKYYIDPSTYEDPNE+4P	E, R[e]
592/599/609/610/616	3615.34	3616.63[c]	0.036	ETPYTDRLQQYISTRGLGVKYYIDPSTYE+2P	E, R[e]
EphB2 peptide					
586	1167.54	1168.29[c]	0.064	ADSEYTDK+3P	T[e]
586	1245.46	1244.42[c]	0.084	RADSEYTDK+2P	E, T[e]
586	1325.52	1324.39[c]	0.085	RADSEYTDK+3P	E, T[e]
586	1325.66	1324.39[c]	0.096	RADSEYTDK+3P	E, K[e]
586 ? (11)	1808.70	1809.79[c]	0.060	RRGFERADSEYTDK+1P	E, T[f]
593	1667.72	1667.71[c]	0.000	LQHYTSGHMTPGMK+1P	T[e]
593	1667.79	1667.71[c]	0.005	LQHYTSGHMTPGMK+1P	E, T[e]
586/593	2733.75	2733.18[c]	0.021	RADSEYTDKLQHYTSGHMTPGMK+1P	E, K[e]
605/611	1775.86	1775.65[c]	0.012	IYIDPFTYEDPNE+2P	E, T[e]
605/611	2021.55	2021.89[c]	0.017	IYIDPFTYEDPNEAVR+1P	E, T[f]
605/611	2021.89	2021.89[c]	0.000	IYIDPFTYEDPNEAVR+1P	E, T[e]
605/611	2101.48	2101.86[c]	0.018	IYIDPFTYEDPNEAVR+2P	E, T[f]
605/611	2101.88	2101.86[c]	0.001	IYIDPFTYEDPNEAVR+2P	T[e]
605/611	2102.02	2101.86[c]	0.008	IYIDPFTYEDPNEAVR+2P	E, T[e]
605/611	2150.43	2150.93[c]	0.023	IYIDPFTYEDPNEAVRE+1P	E, T[f]
605/611	2151.07	2150.93[c]	0.007	IYIDPFTYEDPNEAVRE+1P	E, T[e]
605/611	2151.17	2150.93[c]	0.011	IYIDPFTYEDPNEAVRE+1P	E, K[e]
605/611	2180.84	2181.82[c]	0.045	IYIDPFTYEDPNEAVR+3P	E, T[e]
605/611	2230.50	2230.90[c]	0.018	IYIDPFTYEDPNEAVRE+2P	E, T[f]
605/611	2231.02	2230.90[c]	0.005	IYIDPFTYEDPNEAVRE+2P	E, T[e]
605/611	2231.05	2230.90[c]	0.007	IYIDPFTYEDPNEAVRE+2P	E, K[e]
605/611	2628.48	2627.77[c]	0.027	IYIDPFTYEDPNEAVREFAKE+1P	E, T[e]
586/593/605/611	4390.91	4389.56[d]	0.031	YTDKLQHYTSGHMTPGMKIYIDPFTYEDPNEAVRE+3P	E, K[e]

[a] Percent difference between measured mass and calculated mass. [b] Trypsin (T) and endoproteinases Glu-C (E), Arg-C (R), and Lys-C (K). [c] Monoisotopic mass. [d] Average mass. [e] Receptor purified by GST−SH2 domain. [f] Receptor purified by ephrin-B2 Fc. [g] ? indicates pairs of candidate peptides corresponding to the same peak, identified by the same number in parentheses.

sistent with the in vitro mapping experiments, and Y662, Y673, Y750, Y756, and Y814 (Table 2). Y662, Y673, and Y814 were determined to be phosphorylated because some phosphopeptides included only one of these tyrosines. Y750 and Y756, however, could not be separated into different peptides, and it is not known whether one or both are phosphorylated. Some of the peptides from the kinase domain have multiple phosphorylation sites, indicating the presence of several serine/threonine phosphorylation sites.

Several of the phosphopeptides that were isolated contain Y944, the only tyrosine in the carboxy-terminal tail of EphB5 (Table 3), consistent with this tyrosine being phosphorylated. These peptides were phosphorylated at multiple sites, indicating that the carboxy-terminal tail of EphB5 is phosphorylated on serine/threonine residues as well.

Mapping in Vivo Tyrosine Phosphorylation Sites in the EphB2 Receptor. To determine whether tyrosine phosphorylation sites are conserved in different EphB receptors,

Table 2: Kinase Domain Peptides from in Vivo Phosphorylated EphB5 and EphB2 Identified by Mass Spectrometry

tyrosine	measured *m/z*	M_{calcd}	%Δ[a]	peptide	protease[b]
EphB5 peptide					
662	1139.65	1139.49[c]	0.014	REYTVAIK+2P	T[e]
662	1404.94	1405.58[c]	0.046	EYTVAIKTLK+3P	T[e]
662	1482.71	1481.72[c]	0.067	REYTVAIKTLK+2P	E, T[f]
673 (1) ?[g]	1377.76	1377.61[c]	0.011	TLKSGYTDEQR+1P	T[e]
673 (8) ?	1377.82	1377.61[c]	0.016	TLKSGYTDEQR+1P	E, T[f]
673	1956.14	1956.68[c]	0.028	SGYTDEQRREFLSE+3P	E, T[e]
662/673	2090.03	2088.71[c]	0.063	YTVAIKTLKSGYTDE+5P	E, T[f]
662/673 (9) ?	2422.99	2421.94[c]	0.043	EYTVAIKTLKSGYTDEQR+4P	E, T[f]
662/673	2919.93	2917.31[c]	0.090	HPGKREYTVAIKTLKSGYTDEQR+2P	T[e]
750/756 (1) ?	1377.76	1377.57[c]	0.014	YLSDMNYVHR+1P	T[e]
750/756 (8) ?	1377.82	1377.57[c]	0.018	YLSDMNYVHR+1P	E, T[f]
750/756	4196.72	4200.70[d]	0.095	EGQFSVLQLVGMLRGIAAGMRYLSDMNYVHRDLAAR+2P	E, T[e]
750/756	4199.21	4200.70[d]	0.036	EGQFSVLQLVGMLRGIAAGMRYLSDMNYVHRDLAAR+2P	T[e]
750/756	4325.16	4328.88[d]	0.086	KEGQFSVLQLVGMLRGIAAGMRYLSDMNYVHRDLAAR+2P	E, T[e]
750/756 (3) ?	5094.72	5091.70[d]	0.059	GIAAGMRYLSDMNYVHRDLAARNILVNSNLVCKVSDFGLSRFLE+2P	E, R[e]
794	1673.20	1672.60[c]	0.036	DDASNPTYTGALGCK+2P	E, T[f]
794	2070.01	2071.95[c]	0.094	DDASNPTYTGALGCKIPIR+1P	E, T[e]
794 (4) ?	2153.84	2151.92[c]	0.089	DDASNPTYTGALGCKIPIR+2P	E, T[e]
794	2230.01	2231.89[c]	0.084	DDASNPTYTGALGCKIPIR+3P	E, T[e]
794	2461.20	2462.69[d]	0.060	FLEDDASNPTYTGALGCKIPIR+1P	T[e]
794	3086.83	3084.96[d]	0.061	VSDFGLSRFLEDDASNPTYTGALGCK+4P	E, T[e]
794	3243.02	3244.92[d]	0.059	VSDFGLSRFLEDDASNPTYTGALGCK+6P	E, T[e]
794 (7) ?	3824.81	3824.99[d]	0.005	FLEDDASNPTYTGALGCKIPIRWTAPEAVQYR+3P	E, T[e]
794	5209.60	5209.65[d]	0.000	DLAARNILVNSNLVCKVSDFGLSRFLEDDASNPTYTGALGCKIPIR+3P	E, R[e]
814	715.40	716.31[c]	0.128	AVQYR+1P	E, R[e]
814	843.93	844.41[c]	0.057	AVQYRK+1P	E, T[e]
814	1300.97	1300.57[c]	0.031	WTAPEAVQYR+1P	E, T[f]
814	1429.05	1428.67[c]	0.027	WTAPEAVQYRK+1P	E, T[f]
814	1429.74	1428.67[c]	0.075	WTAPEAVQYRK+1P	T[e]
814	1509.09	1508.63[c]	0.030	WTAPEAVQYRK+2P	E, R[e]
EphB2 peptide					
668	1392.46	1391.47[c]	0.071	TLKSGYTEKQR+1P	E, T[e]
668	1649.31	1649.86[c]	0.033	IFVAIKTLKSGYTE+1P	E, K[e]
668 (11) ?	1808.70	1809.79[c]	0.060	IFVAIKTLKSGYTE+3P	E, T[f]
668	1956.15	1955.73[c]	0.021	SGYTEKQRRDFLSE+3P	E, T[e]
668	2222.78	2223.03[c]	0.011	REIFVAIKTLKSGYTEK+3P	T[e]
668	2269.82	2271.12[c]	0.057	EIFVAIKTLKSGYTEKQR+2P	E, T[f]
668	4151.46	4151.45[d]	0.000	SGYTEKQRRDFLSEASIMGQFDHPNVIHLEGVVTK+2P	E, K[e]
745/751	1361.45	1361.57[c]	0.009	YLADMNYVHR+1P	E, T[f]
745/751	1361.52	1361.57[c]	0.004	YLADMNYVHR+1P	E, T[e]
745/751 (10) ?	1441.74	1441.54[c]	0.014	YLADMNYVHR+2P	E, T[e]
745/751	2070.27	2069.87[c]	0.019	GIAAGMKYLADMNYVHR+2P	E, T[e]
745/751	3087.89	3087.50[d]	0.013	YLADMNYVHRDLAARNILVNSNLVCK+1P	E, K[e]
789	1609.45	1607.65[c]	0.112	DDTSDPTYTSALGGK+1P	E, T[f]
789	1996.48	1996.84[c]	0.018	FLEDDTSDPTYTSALGGK+1P	E, T[f]
789	2156.98	2156.78[c]	0.010	FLEDDTSDPTYTSALGGK+3P	T[e]
789	2087.32	2086.97[c]	0.017	DDTSDPTYTSALGGKIPIR+1P	E, T[e]
809	858.28	858.42[c]	0.017	AIQYRK+1P	E, K[e]
809 (10) ?	1441.74	1442.68[c]	0.065	WTAPEAIQYRK+1P	E, T[e]
809	1875.79	1873.88[c]	0.102	IPIRWTAPEAIQYR+2P	T[e]
809	1920.67	1922.01[c]	0.070	IPIRWTAPEAIQYRK+1P	T[e]
809/821	2876.79	2877.18[c]	0.014	AIQYRKFTSASDVWSYGIVMWE+3P	E, K[e]

[a] Percent difference between measured mass and calculated mass. [b] Trypsin (T) and endoproteinases Glu-C (E), Arg-C (R), and Lys-C (K). [c] Monoisotopic mass. [d] Average mass. [e] Receptor purified by GST−SH2 domain. [f] Receptor purified by ephrin-B2 Fc. [g] ? indicates pairs of candidate peptides corresponding to the same peak, identified by the same number in parentheses.

we investigated the in vivo tyrosine phosphorylation sites of EphB2 (*37*), a receptor closely related to EphB5. Tyrosine-phosphorylated peptides from EphB2 were isolated as described for EphB5, since both the SH2 domain and ephrin-B2 bind phosphorylated EphB2 (Figure 2). In addition, a GST fusion protein of the SH2 domain of the adaptor Grb10 was used for some of the experiments. Similar mass spectra were obtained by purifying EphB2 with GST−Src SH2 or GST−Grb10 SH2. Representative examples of the mass spectra for EphB2 that was purified with either GST−Src SH2 or ephrin-B2 Fc are shown in Figure 4. Most of the peaks in these spectra were identified as phosphopeptides predicted from proteolytic digestion of EphB2 or background ions, and their peptide assignments are summarized in the bottom halves of Tables 1−3.

Many phosphopeptides were from the juxtamembrane region of EphB2 (Table 1). Of the four tyrosines in the juxtamembrane region of EphB2, all appear to be phosphorylated. Peptides including tyrosines 605 and 611, such as that at *m/z* 2101.48 in Figure 4, correspond to some of the major peaks observed for EphB2. Serine 584 and threonine 587 in EphB2, like the corresponding threonines 590 and 593 in EphB5, also appear to be phosphorylated. On the basis of one peptide (at *m/z* 2180.84), threonine 610 was also determined to be phosphorylated.

Table 3: Carboxy-Terminal Tail Peptides from in Vivo Phosphorylated EphB5 and EphB2 Identified by Mass Spectrometry

tyrosine	measured *m/z*	M_{calcd}	%Δ[a]	peptide	protease[b]
EphB5 peptide					
944	1587.96	1589.76[c]	0.113	WLDAIKMGRYKE+1P	E, R[e]
944	2197.55	2196.04[c]	0.069	YKENFDQAGLITFDVISR+1P	T[e]
944 (5) ?[g]	2356.77	2355.97[c]	0.034	YKENFDQAGLITFDVISR+3P	E, T[e]
944	2698.11	2700.13[c]	0.075	MGRYKENFDQAGLITFDVISR+3P	E, T[e]
944	3767.64	3768.88[d]	0.033	MGRYKENFDQAGLITFDVISRMTLEDLQR+4P	E, T[e]
944 (7) ?	3824.81	3823.23[d]	0.041	WLDAIKMGRYKENFDQAGLITFDVISRMTLE+2P	E, T[e]
944 (3) ?	5096.54	5100.57[d]	0.079	KPSALKATGTGSSRPSQPLLSNSPPDFPSLSNAHEWLDAIKMGRYK+2P	E, R[e]
944	5227.47	5229.69[d]	0.042	KPSALKATGTGSSRPSQPLLSNSPPDFPSLSNAHEWLDAIKMGRYKE+2P	E, R[e]
EphB2 peptide					
939	816.39	816.24[c]	0.018	MSQYK+2P	E, T[f]
939	864.27	865.32[c]	0.121	MSQYKE+1P	E, T[e]
939	864.45	865.32[c]	0.101	MSQYKE+1P	E, T[f]
939	1591.59	1591.72[c]	0.008	WLDAIKMSQYKE+1P	E, R[e]
939	3614.19	3613.01[d]	0.033	WLDAIKMSQYKESFASAGFTTFDIVSQMTVE+1P	E, R[e]
939	3610.50	3613.01[d]	0.069	WLDAIKMSQYKESFASAGFTTFDIVSQMTVE+1P	E, K[e]
939	5096.55	5096.56[d]	0.000	TIPDYTSFNTVDEWLDAIKMSQYKESFASAGFTTFDIVSQMTVE+1P	E, R[e]

[a] Percent difference between measured mass and calculated mass. [b] Trypsin (T) and endoproteinases Glu-C (E), Arg-C (R), and Lys-C (K). [c] Monoisotopic mass. [d] Average mass. [e] Receptor purified by GST−SH2 domain. [f] Receptor purified by ephrin-B2 Fc. [g] ? indicates pairs of candidate peptides corresponding to the same peak, identified by the same number in parentheses.

In the kinase domain, several different phosphopeptides contained the conserved Y789 in the activation loop (Table 2). Other phosphopeptides indicate that Y668, Y745, Y751, and Y809 in the kinase domain of EphB2 are also phosphorylated. The kinase domain of EphB2 appears to be phosphorylated on multiple serine/threonine residues as well.

In the carboxy-terminal tail, there are two tyrosines in EphB2. On the basis of the phosphorylated peptides that were isolated (Table 3), we concluded that Y939 is phosphorylated and Y921 is not phosphorylated. Carboxy-terminal phosphopeptides from EphB2 appear to be less heavily phosphorylated on serine/threonine residues than those from EphB5.

Mapping the Binding Site(s) for the SH2 Domain by Mass Spectrometry. To determine whether a modification of the procedure described above for identifying protein phosphorylation sites can be used to identify binding sites for protein interaction domains, we mapped the binding site(s) in EphB2 for the SH2 domain of the Src kinase. Tyrosine-phosphorylated EphB2 was initially purified with GST−Src SH2. In contrast to the original method, the protease-digested fragments were purified with GST−Src SH2 immobilized on glutathione agarose, rather than anti-phosphotyrosine antibodies. The mass spectrum obtained from the peptides bound to the SH2 domain exhibited a few peaks (Figure 5). These peaks correspond to phosphorylated peptides containing Y605 and Y611 in the juxtamembrane domain, except for a minor peak corresponding to a peptide containing Y668 in the kinase domain (Table 4).

DISCUSSION

By systematically mapping the tyrosine-phosphorylated, proteolytic fragments of two EphB receptors, we found that these receptors are extensively phosphorylated in vivo. Ten of the 15 tyrosines in EphB2 and 10−12 of the 16 tyrosines in EphB5 were identified as phosphorylation sites (Figure 6). Phosphorylation of serine and threonine residues was also detected in these receptors. Often, a peptide presented several phosphorylation states, suggesting complex in vivo patterns of EphB receptor phosphorylation in which different sites are differentially phosphorylated.

Many phosphopeptides corresponding to major peaks in the mass spectra were from the juxtamembrane region of EphB2 and EphB5, consistent with juxtamembrane tyrosines being major in vivo phosphorylation sites. We previously identified Y605 and Y611 of EphB2 as in vitro autophosphorylation sites by using two-dimensional phosphopeptide mapping (*23*). Our mass spectrometry data indicate that either Y605, Y611, or both are also phosphorylated in vivo. Of the corresponding tyrosines in EphB5, we have identified Y616 and possibly Y610 as phosphorylation sites by two-dimensional phosphopeptide mapping and by mass spectrometry. These two juxtamembrane tyrosines are highly conserved in the Eph receptors, and evidence suggests that their phosphorylation has functional significance. First, it is required for Eph receptor association with the SH2 domains of different signaling proteins, including Src, as we show here, and other Src family kinases, the Ras GTPase activating protein (RasGAP), and the adaptors Crk and Nck (*21*, *23*, *38*, *39*). Second, phosphorylation of Y605 and Y611 of EphB2 has been proposed to regulate kinase activity, because preventing phosphorylation by mutating them to phenylalanine severely impaired receptor kinase activity (*23*). Mutation to glutamic acid, which like phosphotyrosine is negatively charged, instead resulted in an active receptor (unpublished data).

The two other tyrosines present in the juxtamembrane region of both EphB2 (Y586 and Y593) and EphB5 (Y592 and Y599), which are conserved in all other EphB receptors but not in the EphA receptors, were also identified as in vivo phosphorylation sites. Their significance is not known, however, and their sequence context does not suggest association with specific SH2 or phosphotyrosine-binding (PTB) domains (*35*, *36*).

The tyrosine in the activation loop of EphB2 (Y789) and EphB5 (Y794) appears to be a major phosphorylation site both in vitro and in vivo, because many phosphopeptides from both in vitro autophosphorylated EphB5 and in vivo phosphorylated EphB2 and EphB5 contained this tyrosine. Phosphorylation at this site has not been previously reported for Eph receptors. The corresponding tyrosine, however, is a major phosphorylation site in other receptor tyrosine

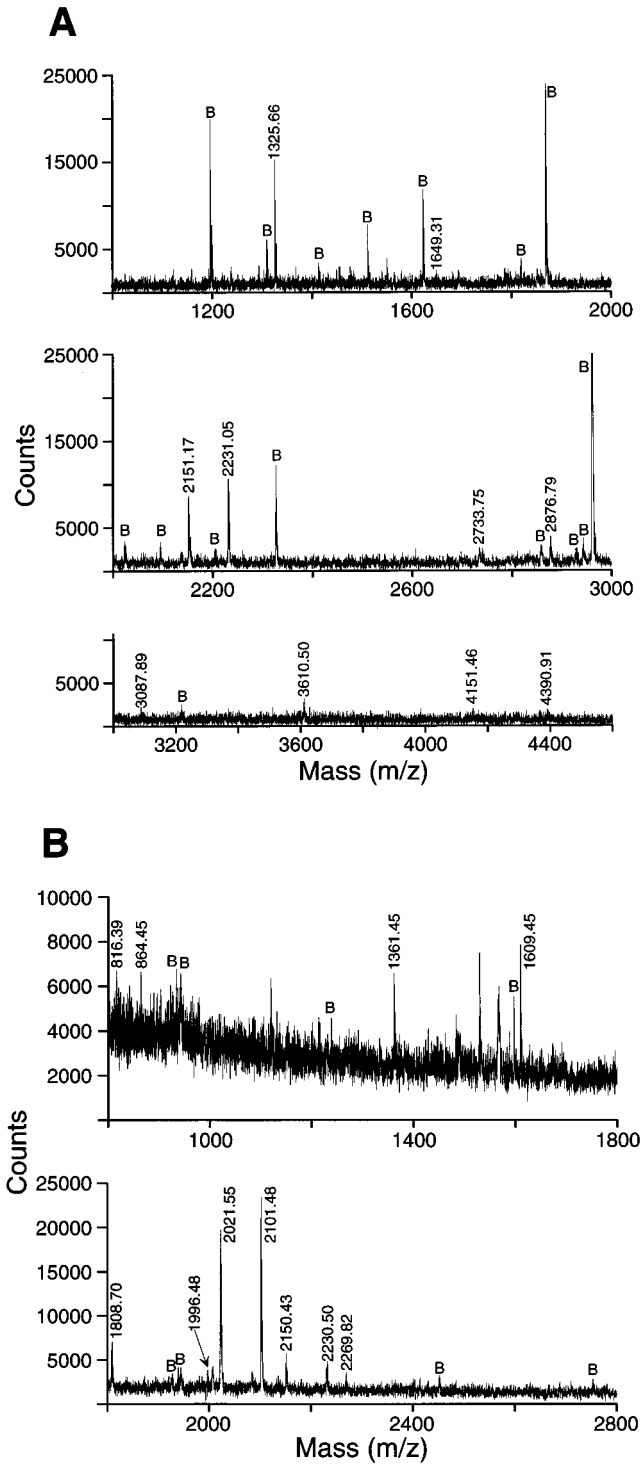

FIGURE 4: MALDI mass spectra of EphB2 phosphopeptides. (A) EphB2 bound to GST−Src SH2 was digested with endoproteinase Glu-C and endoproteinase Lys-C, and (B) EphB2 bound to ephrin-B2 Fc was digested with endoproteinase Glu-C and trypsin. Tyrosine-phosphorylated peptides were isolated from the resulting peptide mixture by binding to anti-phosphotyrosine antibodies conjugated to agarose, and analyzed by MALDI mass spectrometry in the reflector mode. Peaks corresponding to tyrosine-phosphorylated peptides of EphB2 are denoted by their masses and identified in Tables 1−3. Peaks marked with a B are from background ions.

kinases (*15*, *33*). Phosphorylation of this tyrosine has been proposed to regulate kinase activity, because its mutation to phenylalanine has inactivating effects in a number of tyrosine kinases (*15*, *40*−*44*). Structural studies on the insulin receptor

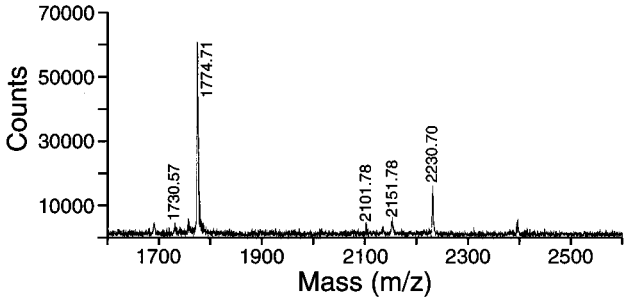

FIGURE 5: Mapping the SH2 domain binding site(s) in EphB2 by mass spectrometry. EphB2 bound to GST−Src SH2 was digested with endoproteinase Glu-C and trypsin. SH2 domain-interacting peptides were isolated from the resulting peptide mixture by binding to GST−Src SH2 bound to glutathione agarose, and analyzed by MALDI mass spectrometry in the reflector mode. Peaks corresponding to tyrosine-phosphorylated peptides of EphB2 are denoted by their masses and identified in Table 4.

Table 4: Peptides Bound to the SH2 Domain of Src from in Vivo Phosphorylated EphB2

tyrosine	measured *m/z*	M_{calcd}	%Δ[a]	peptide[b]
668	1730.57	1729.82[c]	0.043	IFVAIKTLKSGYTE+2P
605/611	1774.71	1775.65[c]	0.053	IYIDPFTYEDPNE+2P
605/611	2101.78	2101.86[c]	0.004	IYIDPFTYEDPNEAVR+2P
605/611	2151.78	2150.93[c]	0.040	IYIDPFTYEDPNEAVRE+1P
605/611	2230.70	2230.90[c]	0.020	IYIDPFTYEDPNEAVRE+2P

[a] Percent difference between measured mass and calculated mass. [b] EphB2 was isolated on the GST−Src SH2 domain and digested with endoproteinase Glu-C and trypsin. The resulting peptide mixture was purified on the SH2 domain of Src. [c] Monoisotopic mass.

and Src family kinases have indeed shown that tyrosine phosphorylation in the activation loop stabilizes an active conformation of the kinase domain (*45*, *46*). Our data suggest a regulatory role for phosphorylation of Y794 in the activation loop of EphB5, because its mutation to phenylalanine essentially abolished in vitro autophosphorylation of EphB5.

The tyrosines in the amino-terminal portion of the kinase domain were also identified as in vivo phosphorylation sites of EphB receptors. In the amino-terminal, ATP-binding lobe of the kinase domain (*33*, *47*), we detected phosphorylation of Y668 of EphB2, the corresponding Y673 of EphB5, and the nonconserved Y662 of EphB5. In the carboxy-terminal, larger lobe of the kinase domain, we detected phosphorylation of Y745 and Y751 of EphB2 and the corresponding Y750 and Y756 of EphB5, which are located near the catalytic loop (*33*, *47*), and Y809 of EphB2 and the corresponding Y814 of EphB5, which are located near the activation loop. We did not obtain evidence, by mass spectrometry or two-dimensional phosphopeptide mapping, for phosphorylation of the other four tyrosines in the kinase domain of EphB2 or EphB5.

Extensive autophosphorylation in the amino-terminal portion of the kinase domain has not been previously reported for other receptor tyrosine kinases (*15*, *33*). A tyrosine corresponding to Y668 of EphB2 is present in most Eph receptors, but not in other families of tyrosine kinases, and Y662 of EphB5 is not conserved within the Eph family itself. These, therefore, may be unique Eph receptor phosphorylation sites. It should be noted that previous mutagenesis studies on tyrosine kinases focused on tyrosines outside the

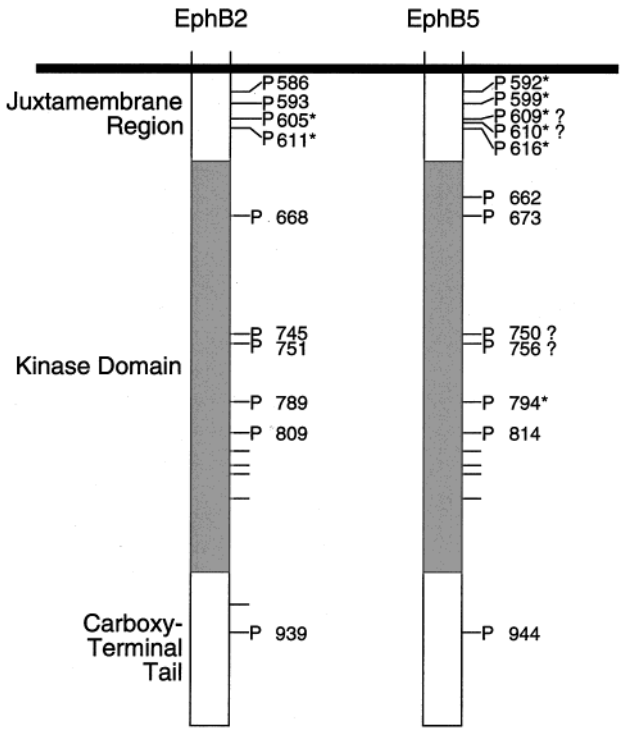

FIGURE 6: Tyrosine phosphorylation sites in the cytoplasmic region of EphB2 and EphB5. Tyrosine residues are represented by a horizontal line; P indicates a phosphate group. In vivo tyrosine phosphorylation sites identified by mass spectrometry are indicated by their residue number. In vitro autophosphorylation sites of EphB2 (*23*) and EphB5 identified by two-dimensional phosphopeptide mapping are marked with asterisks. Tyrosines whose levels of phosphorylation could not be conclusively demonstrated are denoted with ?.

kinase domain as well as the conserved tyrosine in the activation loop, because these tyrosines were thought likely to be phosphorylated. At least one other example of tyrosine phosphorylation in the amino-terminal lobe of the kinase domain has been reported, however. This is Y703 in the epidermal growth factor (EGF) receptor (*48*). Interestingly, this is not an autophosphorylation site but rather a site that can be phosphorylated by the Src kinase both in vitro and in vivo. Src family kinases can interact with Eph receptors (*16*), and perhaps some of the phosphorylation sites that we have detected in vivo are phosphorylated by these kinases.

Tyrosines corresponding to Y745, Y751, and Y809 of EphB2 are present in other families of tyrosine kinases, where they may be phosphorylated. Indeed, Y803 of the EGF receptor, which corresponds to Y745 of EphB2, has been shown to be a site of autophosphorylation that can also be phosphorylated by Src (*49*, *50*).

Tyrosine phosphorylation sites in the kinase domain of Eph receptors may affect catalytic activity. For example, phosphorylation of tyrosines in the ATP-binding lobe may influence the affinity for ATP, and phosphorylation of tyrosines near the catalytic and activation loops may influence the activation state of Eph receptors. Phosphotyrosine residues in the activation loop of the insulin receptor have also been implicated in binding signaling proteins (*51*−*53*). Similarly, the $NPXY_{794}$ sequence of EphB5 may represent a PTB domain binding site when it is phosphorylated (*54*, *55*). The sequence motifs surrounding other phosphorylated tyrosines in the kinase domain of EphB2 and EphB5, however, do not conform to predicted binding sites for SH2 or PTB domains (*35*, *36*).

The single conserved tyrosine in the carboxy-terminal tail of Eph receptors, Y939 in EphB2 and Y944 in EphB5, was identified as an in vivo phosphorylation site by mass spectrometry. This tyrosine, however, was not detected as an in vitro autophosphorylation site of EphB5 by two-dimensional phosphopeptide mapping. This discrepancy suggests that this tyrosine may be phosphorylated by a kinase present in vivo and not in the EphB5 immunoprecipitates. Tyrosine phosphorylation in the carboxy-terminal tail of Eph receptors has been proposed to regulate binding of effector proteins, such as the adaptor Grb10 and LMW-PTP (*20*, *24*). In addition, tyrosine phosphorylation in the carboxy-terminal tail may regulate Eph receptor dimerization (*56*, *57*).

EphB2 contains a second tyrosine (Y921) in its carboxy-terminal tail, which does not appear to be phosphorylated. Notably, the crystal structure of the carboxy-terminal tail of EphB2 indicated that Y921 anchors interfaces in the dimerized structure and is, therefore, unlikely to be phosphorylated (*56*, *57*). The hydroxyl group of Y939 is instead exposed to the solvent and therefore accessible for phosphorylation.

Although serine/threonine phosphorylation has not been previously reported for Eph receptors, our data indicate that in vivo EphB2 and EphB5 are substantially phosphorylated on serine/threonine residues throughout the cytoplasmic region. Serine/threonine phosphorylation was observed in the juxtamembrane region of both EphB2 and EphB5. In other receptor tyrosine kinases, such as the receptors for insulin, EGF, and hepatocyte growth factor, phosphorylation of juxtamembrane serine and/or threonine residues regulates kinase activity (*15*, *58*−*60*).

The phosphorylation of threonine 802 in the kinase domain of EphB2, and the corresponding threonine 807 in EphB5, is intriguing. This threonine is conserved in the Eph receptors and the non-receptor tyrosine kinases, while it is a methionine in the other families of receptor tyrosine kinases (*34*). The nature of this residue influences substrate specificity (*61*, *62*), consistent with its location in the P+1 loop, which contributes a binding surface for the substrate (*46*, *47*). Phosphorylation of this threonine may, therefore, disrupt substrate binding or result in a shift in substrate specificity, and thus provide a novel mechanism for regulating which downstream signaling pathways are activated.

MALDI mass spectrometry has proven to be a fast and sensitive method for determining the complicated in vivo phosphorylation patterns of EphB receptors and has allowed us to identify many Eph receptor phosphorylation sites that were previously unknown. It should be noted that our approach could be sensitive enough to allow identification of sites that are phosphorylated at low levels. Because peak size in the mass spectra does not necessarily correlate with the amount of the corresponding peptide, alternative approaches will be needed to accurately compare the extent of phosphorylation at different sites and/or determine their physiological relevance. Similar patterns of phosphorylation sites were determined for two EphB receptors on the basis of the masses of their different sets of peptides, which is consistent with an accurate identification of the sites. Furthermore, many of the same tyrosine phosphorylation sites were identified by comparing radiolabeled peptides from wild-type and mutated EphB5.

Unlike other approaches used to identify phosphorylation sites, mass spectrometry does not require mutagenesis of selected amino acid residues. This is preferable because the results are not biased by preconceived ideas regarding which residues are likely to be phosphorylated nor affected by possible changes in protein folding caused by the mutation. Furthermore, mutation of an amino acid can influence the phosphorylation of other sites, as we have observed after mutating Y605 and Y611 of EphB2 (*23*) and Y794 of EphB5 to phenylalanine. MALDI mass spectrometry also does not require radioactive labeling of the phosphorylated residues. ^{32}P labeling may cause artifactual patterns of phosphorylation because of the low ATP or phosphate concentrations and possible lack of equilibration between the radioactive and nonradioactive phosphate pools.

We were able to separate the tyrosine-phosphorylated peptides from a complex mixture of peptides by using a final purification step with anti-phosphotyrosine antibodies conjugated to agarose beads (*31*), or GST−Src SH2 domain bound to glutathione agarose. The peptides bound to the agarose beads were mixed with matrix and directly subjected to MALDI analysis (*63*). This approach did not require HPLC separation of individual tyrosine-phosphorylated peptides prior to mass spectrometric analysis, as previously described for mapping phosphorylation sites of other large proteins (*31*, *64*, *65*). Remarkably, the GST−Src SH2 domain, which was not covalently attached to agarose, did not suppress ionization of the bound phosphopeptides. The approach we have used, therefore, appears to be suitable for identifying the tyrosine-phosphorylated binding sites for the SH2 domains of other signaling proteins, provided that the binding site is not cleaved by the endoproteinases that are used.

In this study, we have identified the in vivo phosphorylation sites of EphB2 and EphB5 from transiently transfected 293 human embryonal kidney cells as a first step toward elucidating the molecular mechanisms underlying Eph receptor function. Untransfected 293 cells do not express detectable levels of endogenous EphB2 or EphB5, but they express at least one EphB receptor, EphB4. They, therefore, represent a suitable cell line for analyzing EphB receptor signaling properties. By immunoblotting extracts of transfected 293 cells, we found that the expression levels of EphB2 and EphB5 appeared to be comparable (unpublished data). Transfected EphB2 and EphB5 were constitutively tyrosine-phosphorylated, presumably because of either their high levels of expression or the presence of endogenous ephrin-B ligands in 293 cells. In vivo, in some embryonic tissues, EphB2 and EphB5 are also highly expressed and constitutively tyrosine-phosphorylated (*25*, *66*). Refinements of the mass spectrometry approach that we describe here should allow characterization of the phosphorylation patterns of Eph receptors isolated from embryonic tissues. This will determine whether the complicated pattern of EphB receptor tyrosine phosphorylation that we have observed in cultured cells is also present in tissues, consistent with a complex physiological regulation of Eph receptor function during developmental processes.

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