In Vivo Tyrosine Phosphorylation Sites of Activated Ephrin-B1 and EphB2 from Neural Tissue*

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EphB2 is a receptor tyrosine kinase of the Eph family and ephrin-B1 is one of its transmembrane ligands. In the embryo, EphB2 and ephrin-B1 participate in neuronal axon guidance, neural crest cell migration, the formation of blood vessels, and the development of facial structures and the inner ear. Interestingly, EphB2 and ephrin-B1 can both signal through their cytoplasmic domains and become tyrosine-phosphorylated when bound to each other. Tyrosine phosphorylation regulates EphB2 signaling and likely also ephrin-B1 signaling. Embryonic retina is a tissue that highly expresses both ephrin-B1 and EphB2. Although the expression patterns of EphB2 and ephrin-B1 in the retina are different, they partially overlap, and both proteins are substantially tyrosine-phosphorylated. To understand the role of ephrin-B1 phosphorylation, we have identified three tyrosines of ephrin-B1 as in vivo phosphorylation sites in transfected 293 cells stimulated with soluble EphB2 by using mass spectrometry and site-directed mutagenesis. These tyrosines are also physiologically phosphorylated in the embryonic retina, although the extent of phosphorylation at each site may differ. Furthermore, many of the tyrosines of EphB2 previously identified as phosphorylation sites in 293 cells (Kalo, M. S., and Pasquale, E. B. (1999) Biochemistry 38, 14396–14408) are also phosphorylated in retinal tissue. Our data underline the complexity of ephrin-B bidirectional signaling by implicating many tyrosine phosphorylation sites of the ligand-receptor complex.

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Both the GPI-linked ephrin-A ligands and the transmembrane ephrin-B ligands have dual functions. As classical ligands do, the ephrins stimulate the kinase activity of the Eph receptors; the ephrin-A ligands preferentially activate EphA receptors, and the ephrin-B ligands preferentially activate EphB receptors (1). The ephrin extracellular domain is sufficient for this function as long as it is clustered (2, 3). The ephrins also have a second function, which has been described as “reverse signaling,” whereby they activate signaling pathways in the cells in which they are expressed (4–6).

Several lines of evidence indicate that reverse signaling is important for transducing signals from ephrin-Eph receptor complexes. For example, the ephrin-B cytoplasmic domain is required to restrict intermingling between populations of Eph receptor-bearing cells and B-ephrin-bearing cells (7). This implies that activation of ephrin-B signaling drives cell sorting in the developing hindbrain, similar to activation of Eph receptor signaling (8). Furthermore, ectopic overexpression of ephrin-B1 in Xenopus embryos causes cell dissociation (9, 10), an effect that requires the 19 carboxyl-terminal amino acids of the ephrin-B1 cytoplasmic domain (9). The cytoplasmic domain of ephrin-B1 is also required to inhibit oncogenic signaling pathways activated by tyrosine kinases (11, 12).

Knock out studies in the mouse support the idea that signals mediated by ephrin-B1 and ephrin-B3 control axon pathfinding in the embryonic brain anterior commissure and the developing retina (13, 14), whereas signals mediated by EphB2 control the formation of the vascular system (15, 16).

Consistent with the ephrin-B ligands having receptor-like intrinsic signaling properties, a soluble form of the EphB4 receptor ectodomain induces angiogenic sprouting of endothelial cells in vitro, presumably by “activating” signaling through the ephrin-B2 ligand expressed in these cells (16). In addition, in vitro experiments the EphB2 ectodomain inhibits chemotraction of cerebellar granule cells to the SDF-1 chemokine, presumably by activating signaling through the ephrin-B1 ligand expressed in these cells (17).

Interestingly, treatment of cultured cells expressing ephrin-B ligands with soluble EphB receptor ectodomains induces tyrosine phosphorylation of the B-ephrins (12, 18). The cytoplasmic domains of the three B-ephrins consist of ~80 amino acids, including five conserved tyrosine residues. Ephrin-B1, in addition, contains a sixth cytoplasmic tyrosine. Tyrosine phosphorylation likely plays an important role in ephrin-B signaling, but the specific tyrosines involved have remained elusive. Here we identify tyrosine phosphorylation sites in the cytoplasmic domain of ephrin-B1 from 293 cells stimulated with a soluble form of the EphB2 ectodomain. Importantly, these ephrin-B1 phosphorylation sites were also detected in the embryonic neural retina, indicating that the in vivo phosphorylation sites in retinal tissue are the same as in cultured cells. In addition, we identify phosphorylated tyrosines of EphB2 from embryonic retinal tissue that confirm that phosphorylation sites previously identified in 293 cells (19) have physiological significance in vivo. These phosphorylated tyrosines likely regulate EphB2 kinase activity and interactions with signaling proteins in response to ligand binding. Thus, ephrin-B ligands and EphB receptors form bidirectional signaling complexes in which both the receptor and the ligand become tyrosine-phosphorylated in their cytoplasmic domains and have increased signaling capability. An important step in the characterization of ephrin-B-EphB bidirectional signaling is to identify the tyrosines that become phosphorylated in the complex under physiological conditions. The approaches that we have used can
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be generally applied to the determination of protein-tyrosine phosphorylation sites in animal tissues.

EXPERIMENTAL PROCEDURES

Materials—Protease inhibitors, anti-phosphotyrosine antibodies coupled to agarose (clone PT-66), and protein A-peroxidase were obtained from Sigma. Endoproteinases used in mapping phosphorylated tyrosines by mass spectrometry were of sequencing grade from Promega or Roche Molecular Biochemicals. 1-Octyl-β-D-glucopyranoside and α-cyano-4-hydroxycinnamic acid were obtained from Aldrich. Chemicals used for mass spectrometry experiments were of high pressure liquid chromatography grade or the best grade available. The Voyager MALDI-TOF mass spectrometer was from PerSeptive Biosystems. Chromatography grade or the best grade available. The Voyager MALDI-TOF mass spectrometer was from PerSeptive Biosystems.

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trometer equipped with a nitrogen laser, delayed extraction, and a reflector. Spectra were externally calibrated with angiotensin I (MH+ = 1296.69) and adrenocorticotropic hormone fragments (1–17, MH+ = 2093.09; 18–39, MH+ = 2465.20; 7–38, MH+ = 3657.93). For interpretation of the mass spectra, a list of predicted molecular masses was generated by theoretical cleavage with specific endoproteinases using the MS-Digest program (prospector.ucsf.edu). 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 with an accuracy of ~0.1% or better.

**Electrospray Mass Spectrometry**—For samples infused into an electrospray mass spectrometer, the zip tip containing bound phosphopeptides from ephrin-B1 was pre-equilibrated appropriately, washed in 5% formic acid, and eluted in 50% methanol, 5% formic acid directly into a nanospray capillary. Electrospray mass spectra were recorded at a nL/min flow rate on an API 3000 triple quadrupole mass spectrometer in positive ion mode. Q1 scans were acquired with a mass step of 0.1 atomic mass units and a dwell time of 1 ms. For product ion scans of selected doubly charged phosphopeptides, nitrogen was used as the collision gas, and a mass step of 0.2 atomic mass units and a dwell time of 2 ms were used. The collision energy for product ion scans was adjusted individually for each experiment. Ion series in tandem mass spectra were identified with the assistance of the MS-Product program (prospector.ucsf.edu) and BioMultiView (PE-Sciex).

**RESULTS**

**Tyrosine Phosphorylation Sites of Ephrin-B1 Expressed in 293 Cells**—To identify which of the six tyrosines in the cytoplasmic domain of ephrin-B1 become phosphorylated upon engagement with EphB receptors, we transfected ephrin-B1 in 293 cells. The transfected cells were stimulated with cross-linked EphB2 Fc, a soluble fusion protein consisting of the ectodomain of EphB2 fused to the Fc portion of human IgG1 and preincubated with anti-Fc antibodies. EphB2 Fc increased the level of ephrin-B1 tyrosine phosphorylation (Fig. 1A), as expected (12). For MALDI mass spectrometric analysis, ephrin-B1 immunoprecipitated from EphB2 Fc-treated cells was digested with trypsin and endoproteinase Glu-C. The ephrin-B1 tyrosine-phosphorylated peptides were isolated from the mixture of digested peptides using anti-tyrosynophosphoryl antibodies conjugated to agarose beads. This level of purification, which permits ionization of peptides directly from beads, had previously been sufficient to identify by mass spectrometry the tyrosine-phosphorylated peptides generated from EphB2 and EphB5 expressed in transfected 293 cells (19). However, additional acidic elution and reverse phase purification steps with microcolumns were required for ephrin-B1 to reduce background noise associated with the previous protocol and detect clearly the lower level of phosphorylation of ephrin-B1.

The resulting improved mass spectra contained only three major peaks corresponding to three ephrin-B1 monophosphorylated peptides: two containing tyrosines 312 and 317 and another containing the carboxyl-terminal tyrosines 331 and 332 (Fig. 1B and Table I). This result indicates that one or both of the tyrosines in each peptide are sites of phosphorylation. Unfortunately, it was not possible to more precisely identify the tyrosine phosphorylation sites of ephrin-B1 using this mass spectrometric approach because shorter peptides containing only one of these tyrosines could not be readily obtained with the sequence-specific proteases available.

To directly identify the phosphorylated tyrosines, electrospray tandem mass spectrometry was used to sequence the doubly charged ion of the carboxyl-terminal peptide with a molecular weight of 1488.90 (m/z = 744.8), which contains tyrosines 331 and 332 (Fig. 2). Upon fragmentation, a series of expected daughter “a,” “b,” and “y” ions were detected in the spectra, allowing the sequence to be determined from the mass differences between the fragment ions. Most of the sequence was verified by a set of singly and doubly charged carboxy-terminal y ions, where the mass difference between y3 and y2 indicates phosphorylation at tyrosine 331. Phosphorylation at tyrosine 332, however, was not detected in the y ion series, because the mass difference between y2 and y1 only indicates the presence of tyrosine and not phosphotyrosine. As expected, all larger carboxyl-terminal peptides (y1/y2/y3), which contain both tyrosines, were detected as monophosphorylated ions. In addition, the amino-terminal b10 fragment, containing tyrosine 331 but not tyrosine 332, was detected as a monophosphorylated ion. Based on this analysis, tyrosine 331 is the phosphorylation site in the ephrin-B1 peptide with a weight of 1488.90. Because the size of a peak in a MALDI spectrum is not an accurate indication of the abundance of the corresponding peptide, we used site-directed mutagenesis to determine whether…
Tyrosines 312, 317, and 331 of ephrin-B1 are phospho-
ylation sites. Ephrin-B1 mutants were transfected in 293 cells, and the cells were treated with EphB2 Fc to induce ephrin-B1 tyrosine phosphorylation. The cell extracts were then immunoprecipitated (IP) with anti-ephrin-B1 antibodies. The immunoprecipitates were probed by immunoblotting (IB) with anti-phosphotyrosine antibodies (PTyr) and reprobed with anti-ephrin-B1 antibodies. Tyrosines 301, 305, 312, 317, and 331 were mutated to phenylalanine singly or in combination, as indicated. wt, wild type ephrin-B1.

**FIG. 3. Tyrosines 312, 317, and 331 of ephrin-B1 are phosphorylation sites.** Ephrin-B1 mutants were transfected in 293 cells, and the cells were treated with EphB2 Fc to induce ephrin-B1 tyrosine phosphorylation. The cell extracts were then immunoprecipitated (IP) with anti-ephrin-B1 antibodies. The immunoprecipitates were probed by immunoblotting (IB) with anti-phosphotyrosine antibodies (PTyr) and reprobed with anti-ephrin-B1 antibodies. Tyrosines 301, 305, 312, 317, and 331 were mutated to phenylalanine singly or in combination, as indicated. wt, wild type ephrin-B1.

Tyrosine 331 is highly phosphorylated. Tyrosine phosphorylation of an ephrin-B1 mutant with phenylalanine at position 331 (ephrin-B1 Y331F) was greatly reduced compared with wild type ephrin-B1 (Fig. 3). This indicates that tyrosine 331 is a major phosphorylation site of ephrin-B1 in 293 cells stimulated with EphB2 Fc.

To determine whether tyrosine 312 and 317 are phosphorylated, we mutated these tyrosines to phenylalanine. Mutation of either tyrosine slightly reduced tyrosine phosphorylation of wild type ephrin-B1 and the Y331F mutant in 293 cells stimulated with EphB2 Fc (Fig. 3). This indicates that both tyrosines are phosphorylated but to a lesser degree than tyrosine 331. Thus, the ephrin-B1 peaks detected at 1643 m/z and 1771 m/z (Fig. 1B) likely represent mixtures of two peptides, one phosphorylated at tyrosine 312 and the other phosphorylated at tyrosine 317. This may explain the unclear results obtained with these peptides in tandem mass spectrometry experiments (data not shown).

In the 293 cells, phosphorylation was essentially undetectable for the ephrin-B1 Y312F/Y317F/Y331F mutant, which lacks all three tyrosines (Fig. 3). Only very low exposures of the film revealed a faint band (data not shown). This suggests that the other three tyrosines present in the cytoplasmic domain of ephrin-B1 are not significantly phosphorylated when ephrin-B1 is stimulated with EphB2 Fc in 293 cells. This conclusion is consistent with the data obtained by mass spectrometry. Fragmentation of the carboxyl-terminal monophosphorylated peptide did not show any evidence of phosphorylation at tyrosine 332 in the tandem mass spectra (Fig. 2). Furthermore, in the MALDI mass spectra (Fig. 1B) we did not detect peaks corresponding to a doubly phosphorylated peptide containing tyrosines 331 and 332, nor peaks corresponding to phosphorylated peptides containing tyrosines 301 and 305. In addition, mutation of tyrosine 301 and 305 did not detectably reduce the level of ephrin-B1 tyrosine phosphorylation (Fig. 3).

**Ephrin-B1 and EphB2 Are Phosphorylated on Tyrosine in Embryonic Neural Retina—**We have previously shown that the EphB2 receptor is phosphorylated on tyrosine in vivo in the developing chicken neural retina (26). This phosphorylation is presumably caused by ephrin-B ligands that bind to EphB2, causing its activation. Indeed ephrin-B1 and ephrin-B2, two ligands for EphB2, are expressed in the embryonic neural retina (Fig. 4A) (20, 24). According to the reverse signaling model, ephrin-B ligands in the retina should be phosphorylated on tyrosine upon interaction with EphB2. Consistent with this possibility, we detected ephrin-B1 tyrosine phosphorylation in embryonic retina (Fig. 4B). The apparent molecular weight of tyrosine-phosphorylated ephrin-B1 is different at different developmental stages, consistent with the presence of several immunoreactive ephrin-B1 bands that are developmentally regulated (Fig. 4A). The different bands may represent alternatively spliced forms of ephrin-B1, represent proteolytic fragments, or be the result of post-translational modifications. They do not represent other ephrin-B ligands because the antibodies are highly specific for chicken ephrin-B1 and do not cross-react with other ephrin-B ligands.2

The phosphorylation of ephrin-B1 and EphB2 in the embry-
Ephrin-B1 and EphB2 in Vivo Tyrosine Phosphorylation Sites

**Fig. 4.** Ephrin-B1 is phosphorylated on tyrosine in vivo in the embryonic retina. A, ephrin-B1 protein is present in the chicken retina between days 8 and 15 of embryonic development. Equal amounts of retinal extracts from the indicated days of development were resolved by SDS-polyacrylamide gel electrophoresis, stained for total protein with Amido Black, and probed by immunoblotting (IB) with anti-ephrin-B1 antibodies. The arrows indicate the positions of several ephrin-B1 immunoreactive bands, which appear to be differentially regulated at different developmental stages. B, ephrin-B1 is phosphorylated on tyrosine in the chicken retina between days 8 and 15 of embryonic development. Ephrin-B1 was immunoprecipitated from chicken retinal tissue at the days of development indicated at the bottom. The lane marked (12) is a control immunoprecipitate from E12 retina with nonimmune rabbit IgG. Immunoprecipitates were probed by immunoblotting (IB) with anti-phosphotyrosine antibodies and reprobed with anti-ephrin-B1 antibodies.

Ephrin-B1 is expressed in the ventral retina (Fig. 5, A and B) but using only the halves of the dorsal or ventral retina that are distal from the dorsal-ventral midline. Phosphorylation in these regions confirms that the interaction between EphB2 and ephrin-B1 is not restricted to the border of their expression domains in the region between dorsal and ventral retina. IB, immunoblot.

**Fig. 5.** EphB2 and ephrin-B1 have partially overlapping distributions and are substantially phosphorylated on tyrosine in the developing chicken retina. A, immunoperoxidase labeling (dark) shows that EphB2 protein is concentrated in the ventral retina, whereas ephrin-B1 protein is concentrated in the embryonic dorsal retina. Paraffin sections cut vertically from the same region of an E6 chicken head were stained with antibodies to ephrin-B1 and EphB2. The arrows mark the position of the optic fissure (of), located in the middle of the ventral retina. B, immunoblotting shows that ephrin-B1 is also expressed in the ventral retina, although at a low level, and EphB2 is also expressed in the dorsal retina, although at a low level. Retinas were cut in four parts, perpendicular to the optic fissure. Dt, top half of the dorsal retina; Db, bottom half of the dorsal retina; Vt, top half of the ventral retina; Vb, bottom half of the ventral retina. The left panel is an Amido Black-stained filter, which shows that equal amounts of protein were present in the lanes. In the right panel, the top portion of the same filter was probed with antibodies to EphB2, and the bottom portion was probed with antibodies to ephrin-B1. C, EphB2 and ephrin-B1 are both phosphorylated on tyrosine in the dorsal and the ventral retina. Ephrin-B1 and EphB2 were immunoprecipitated (IP) from dorsal (D) and ventral (V) retina and probed by immunoblotting with anti-phosphotyrosine (PTyr) antibodies followed by anti-ephrin-B1 or anti-EphB2 antibodies, respectively. Note that the phosphotyrosine signal for EphB2 is higher in E12 dorsal than ventral retina even though less EphB2 was immunoprecipitated. In contrast, the phosphotyrosine signal for ephrin-B1 is higher in ventral than dorsal retina, even though less ephrin-B1 was immunoprecipitated. D, EphB2 and ephrin-B1 are both phosphorylated on tyrosine in the top half of the dorsal retina and the bottom half of the ventral retina. This experiment was carried out as in C but using only the halves of the dorsal or ventral retina that are distal from the dorsal-ventral midline. Phosphorylation in these regions confirms that the interaction between EphB2 and ephrin-B1 is not restricted to the border of their expression domains in the region between dorsal and ventral retina. IB, immunoblot.
ferences in the extent of ephrin-B1 phosphorylation at different tyrosines in the retina and 293 cells. Alternatively the samples may have been digested differently by the proteases, or there may have been different components in the retina and the 293 cell samples, which differentially affected the amount of each peptide that was ionized. An additional peak of 1851 m/z was also observed in the ephrin-B1 sample from retina, which corresponds to a peptide containing tyrosines 312 and 317 and two phosphates. In this peptide, both tyrosines may be phosphorylated, or serine 309 may be phosphorylated in addition to one of the tyrosines.

**Tyrosine Phosphorylation Sites of EphB2 in Neural Retina and 293 Cells**—We used the same MALDI mass spectrometry approach to identify EphB2 tyrosine-phosphorylated peptides in retinal tissue. Many peaks were present in the spectra of EphB2 immunoprecipitated from E12 retina and digested with trypsin and endoproteinase Glu-C, and most of the major peaks have masses corresponding to EphB2-phosphorylated peptides (Fig. 7A and Table II). These peptides contain many of the same tyrosines previously identified as phosphorylation sites of EphB2 expressed in 293 cells (19). Thus, many tyrosines in the cytoplasmic domain of EphB2 appear to be phosphorylated in retina tissue as they are in the 293 cells. In particular, four prominent peaks from retinal EphB2 correspond to peptides containing the juxtamembrane tyrosines 605 and 611. Differences were noted, however, in the relative representation of peptides containing different tyrosines. In the spectra of EphB2 from retina, for example, three of the twelve EphB2 peaks correspond to peptides containing tyrosine 668, whereas peptides containing this tyrosine had been poorly represented in spectra of EphB2 from 293 cells (19). In addition, one of the peaks corresponds to a peptide that contains tyrosines 821 and 831, two residues that had not been previously identified as phosphorylation sites.

To determine whether these differences were caused by the modifications introduced in the protocols used, we obtained a MALDI mass spectrum of phosphopeptides from EphB2 overexpressed in 293 cells using the same protocol used for retinal EphB2 (Fig. 7B and Table II). Modifications of the previous protocol involve isolating EphB2 by immunoprecipitation (rather than using immobilized ephrin-B1 Fc or glutathione S-transferase fusion proteins of SH2 domains) (19) and eluting the peptides from the anti-phosphotyrosine antibody coupled to beads followed by a reverse phase chromatography step. These modifications improve sensitivity and selectivity, because they reduce the number of background peaks that do not correspond to EphB2 phosphopeptides. This analysis identified an additional tyrosine (tyrosine 821) of EphB2 from 293 cells as a
Ephrin-B1 and EphB2 in Vivo Tyrosine Phosphorylation Sites

**Table II**

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<tr>
<th>Tyrosine residue</th>
<th>Measured mass</th>
<th>Calculated mass</th>
<th>Mono or Avg</th>
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\( ^a \) m, monoisotopic mass; a, average mass.

\( ^b \) PO4, phosphate group.

phosphorylation site, consistent with the identification of peptides containing this tyrosine in the EphB2 retinal sample. Thus, tyrosine 821 is one of the in vivo phosphorylation sites of EphB2. In addition, peptides containing tyrosines 605, 611, 668, 789, and 939 were detected in EphB2 from both retina and 293 cells, consistent with previous results (19). In the 293 cell sample, all five most prominent peaks correspond to peptides containing tyrosines 605 and 611. Hence, many in vivo tyrosine phosphorylation sites are similar in EphB2 ectopically expressed in 293 cells or endogenously expressed in embryonic retinal tissue.

Interestingly, the majority of the EphB2 phosphopeptides obtained from retina and 293 cells were different, even though they contained the same tyrosines. This suggests differential protease digestion and/or differential detection of EphB2 peptides from retina and 293 cell samples. These factors may also explain some of the discrepancies in the phosphorylation sites identified in 293 cells and retina. Peptides containing tyrosines 745, 751, and 921 were detected in EphB2 from 293 cells and not retina, whereas peptides containing tyrosine 586 were detected only in retina (although tyrosine 586 had been previously identified as a phosphorylation site of EphB2 in 293 cells (19)). The differences observed in the spectra may also reflect some differences in the phosphorylation sites of EphB2 in embryonic retina and 293 cells. Further experiments digesting EphB2 with different combinations of proteases may help resolve these issues.

**DISCUSSION**

**Ephrin-B1 and EphB2 Tyrosine Phosphorylation in Embryonic Retina**—We previously reported that the EphB2 receptor is substantially phosphorylated on tyrosine in the developing neural retina (26). This finding suggested that ligands that stimulate EphB2 phosphorylation must also be expressed in this tissue. Indeed, ephrin-B1 and ephrin-B2 are expressed in the embryonic retina (20, 24). Consistent with binding of ephrin-B1 to EphB2 in the retina, we show here that not only EphB2 but also ephrin-B1 is phosphorylated on tyrosine in retinal tissue.

At E8 and E12, ephrin-B1 is more highly expressed in the dorsal half of the chicken retina, and EphB2 is more highly expressed in the ventral half (20, 24). Interaction and tyrosine phosphorylation of ephrin-B1 and EphB2 could occur at the boundary between their regions of expression. In the hindbrain, for example, Eph receptors and ephrins are present in adjacent rhombomeres, and their activation at the boundaries prevents intermingling of cells between rhombomeres (7, 8). However, our data indicate that phosphorylation is not restricted to the boundary between dorsal and ventral retina. Tyrosine-phosphorylated EphB2 and ephrin-B1 were detected not only in the central portions of the retina but also in the distal dorsal and ventral portions. This is consistent with their overlapping expression in the top half of the dorsal retina (where ephrin-B1 expression is high and EphB2 expression is low) and the bottom half of the ventral retina (where EphB2 expression is high and ephrin-B1 expression is low). Interestingly, at earlier developmental stages EphB2 and ephrin-B1 overlap even more extensively, at least in the mouse retina (13). Additional EphB receptors and ephrins expressed in the retina (28) likely also contribute to the spatial patterns of ephrin-B1 and EphB2 tyrosine phosphorylation. For example, the presence of another EphB receptor competing with EphB2 for binding to ephrin-B1 in the E8 retina could explain the low
levels of EphB2 phosphorylation observed at this stage. In addition, growth factor receptor tyrosine kinases may also cause phosphorylation of ephrin-B1 in the retina (10, 12, 29). Notably, fibroblast growth factor can up-regulate tyrosine phosphorylation of endogenous ephrin-B1 in embryonic retinal tissue (10).

**Ephrin-B1 Phosphorylation Sites**—Various stimuli can induce tyrosine phosphorylation of ephrin-B1, including binding to EphB receptors, activation of Src kinase, platelet-derived growth factor stimulation and, as mentioned above, fibroblast growth factor stimulation (10, 12, 18). The Tie-2 receptor and fibroblast growth factor receptor 1 can also phosphorylate the cytoplasmic domain of ephrin-B1 in vitro (10, 29). Ephrin-B1 is tyrosine-phosphorylated in mouse embryonic tissues (18) and, as we show here, in chick embryonic retina. The precise physiological consequences of ephrin-B1 phosphorylation, however, remain to be determined. One report shows that tyrosine phosphorylation of the cytoplasmic domain of ephrin-B1 inhibits its ability to cause cell dissociation in *Xenopus* embryos (10). In addition, EphB-dependent activation of ephrin-B signaling has been implicated in pathfinding of anterior commissure axons (30) and dorsal retinal ganglion cell axons (13). The molecular mechanisms involved in pathfinding of ephrin-B1-bearing axons remain to be determined, but presumably ephrin-B1 tyrosine phosphorylation plays a crucial role.

We have developed approaches that for the first time identify the *in vivo* tyrosine phosphorylation sites of ephrin-B1. By MALDI mass spectrometry, we have detected the same tyrosine-phosphorylated peptides containing tyrosines 312/317 and 331/332 in both embryonic retinal tissue and 293 cells stimulated with multimeric EphB2 Fc. If phosphorylation of ephrin-B1 is influenced by its oligomeric state or the nature of the stimulus (interaction with EphB receptors or activation of other receptor tyrosine kinases), then the transfected 293 cells closely mimic the *in vivo* situation in neural tissue. Electrospray tandem mass spectrometry and site-directed mutagenesis more precisely identified tyrosines 312, 317, and 331 of ephrin-B1 as phosphorylation sites. These sites are likely to have physiological significance because they are present in *in vivo*

We have determined that tyrosine 331 is the major in *in vivo* phosphorylation site of ephrin-B1 bound to EphB2. Tyrosine 331 is the residue at the −3 position from the carboxyl terminus of ephrin-B1 and part of the PDZ domain-binding site. Several PDZ domain-containing proteins bind to ephrin-B1. They include GRIP1, GRIP2, and syntenin, which are adaptor proteins that consist solely of PDZ domains; the protein kinase C-interacting protein Pick1, the phosphotyrosine phosphatase FAP-1; PHIP, which is related to a *Caenorhabditis elegans* protein involved in regulating polarity in the early embryo; and PDZ-RGS3, which functionally connects B-ephrins and trimeric G proteins (17, 31–33). Interaction of ephrin-B1 with these PDZ proteins may contribute to the localization and clustering of ephrin-B1 and mediate downstream signaling events. Structural studies have suggested that the residue at the −3 position confers binding specificity for the PDZ domain (34). Thus, phosphorylation at tyrosine 331 could differentially affect the affinity of ephrin-B1 for different PDZ domains. Ephrin-B1 phosphorylation, however, has not been found to drastically affect most PDZ domain interactions examined so far (33).

The residue at position −2 from the carboxyl terminus is also important for regulating PDZ domain binding (35) and is also a tyrosine in ephrin-B1 (tyrosine 332). Phosphorylation at the −2 position affects binding of an ephrin-B1 synthetic peptide to syntenin (33). However, we have found no evidence that tyrosine 332 is phosphorylated. MALDI mass spectra identified only the monophosphorylated form of the peptide containing tyrosines 331 and 332, and electrospray mass spectra only detected tyrosine 331 as a phosphorylated amino acid in this peptide. Consistent with the mass spectrometry data, an ephrin-B1 mutant containing tyrosine 332, but not tyrosines 312, 317, and 331, was not significantly phosphorylated in 293 cells treated with EphB2 Fc.

Motifs of ephrin-B1 containing phosphorylated tyrosines could represent binding sites for Src homology 2 (SH2) domains or phosphotyrosine-binding domains. Interestingly, the ephrin-B1 interacting proteins identified so far contain PDZ domains and not SH2 or phosphotyrosine-binding domains. Indeed, pull-down assays could not detect interactions between tyrosine-phosphorylated ephrin-B1 and a number of known SH2 domains fused to glutathione-S-transferase, including those of Src, Abl, Arg, phosphatidylinositol 3-kinase, SHEP1, RasGAP, Crk, Grb10, and ShcB. Additional experiments will determine whether other SH2 or phosphotyrosine-binding domains could bind to phosphorylated ephrin-B1 and mediate ephrin-B1 signals. Unfortunately, the sequences near tyrosines 312, 317, and 331 do not provide strong clues as to which other SH2 domains should be examined (36, 37). On the other hand, tyrosines 312 and 317 have been shown to be required for association with activated fibroblast growth factor receptor 1 (10).

The identity of the kinase(s) that phosphorylate the B-ephrins remains a mystery. Interestingly, however, tyrosine 317 and 331 are in sequence contexts that may be preferentially phosphorylated by a receptor tyrosine kinase (38). In contrast, a cytoplasmic tyrosine kinase such as Abl is more likely to phosphorylate tyrosine 312. Perhaps, different tyrosine kinases phosphorylate different tyrosines of ephrin-B1.

**EphB2 Phosphorylation Sites**—We have also identified the tyrosine residues of EphB2 that are phosphorylated *in vivo* in retinal tissue. Many of the tyrosines in the EphB2 cytoplasmic domain are phosphorylated in the E12 retina. The multiple phosphorylation sites detected in 293 cells (39) (Fig. 7B), therefore, are not artifacts caused by overexpression of transfected EphB2. The juxtamembrane tyrosines 605 and 611, in particular, appear to be prominent phosphorylation sites in both retinal tissue and cultured cells. Phosphorylation of these tyrosines regulates both binding of many SH2 domain-containing proteins and kinase activity (39, 40). Thus, tyrosines 605 and 611 could play a particularly important functional role in EphB2 signaling in *in vivo*. Phosphorylation of tyrosine 779, the conserved tyrosine in the activation loop of the kinase domain, likely also regulates kinase activity. Other phosphorylation sites were identified within the kinase domain, including tyrosines 668, 741/751, and 821. Phosphorylation of these tyrosines may also affect EphB2 kinase activity as well as represent protein-binding sites that are regulated by phosphorylation. Although mass spectrometry does not provide precise quantitative information, tyrosine 668 in the kinase domain was represented by several large peaks in the spectra from retinal tissue. This may imply that this tyrosine is a prominent phosphorylation site in the retina. Interestingly, tyrosine 668 in the kinase domain and tyrosine 586 in the juxtamembrane domain are predicted to have similar binding specificity for SH2 domains because both are in a similar sequence context (586**Y**DK and 586**Y**TEK) (36, 37). Experiments are in progress to determine whether tyrosines 586 and 668 mediate the phosphorylation-dependent protein interactions that do not depend on tyrosines 605 and 611 of EphB2 (40, 41). Whether the identified phosphorylation sites of EphB2 are the result of autophosphorylation or are phosphorylated by other tyrosine kinases remains to be
Ephrin-B1 and EphB2 in Vivo Tyrosine Phosphorylation Sites

determined, but they are likely to be physiologically relevant because they are phosphorylated in vivo in neural tissue.

Identification of tyrosine phosphorylation sites is an important step in understanding tyrosine kinase signaling pathways. As more sensitive methods are used, it becomes increasingly clear that many tyrosines in proteins have the potential to be phosphorylated. Thus, regulation of proteins by tyrosine phosphorylation may be a much more prevalent form of regulation than previously thought. Some of the phosphorylation sites may occur with low stoichiometry or be phosphorylated only very transiently. Nevertheless, these phosphorylation sites could have important functions. Mass spectrometry has made possible the identification of in vivo phosphorylation sites in live cells grown under physiological phosphate concentrations and in tissues (19, 27, 42, 43). We have identified the tyrosine phosphorylation sites in ephrin-B1 and EphB2 in a tissue where these proteins are normally expressed and tyrosine-phosphorylated. Identification of the sites that are phosphorylated in tissues will help elucidate the physiological mechanisms underlying the activities of ephrin-B ligands and EphB receptors. For example, it will be possible to genetically engineer mice expressing tyrosine mutants of ephrin-B1 and EphB2 to identify the in vivo function of each phosphorylation site. Antibodies specific for each phosphorylation site may also yield important insights as to the spatial distribution of the tyrosine-phosphorylated forms of these proteins, which are the forms activated to signal.

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