An ephrin-A-dependent Signaling Pathway Controls Integrin Function and Is Linked to the Tyrosine Phosphorylation of a 120-kDa Protein*

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The Eph family of receptor tyrosine kinases and their ligands, the ephrins, have been implicated in the development of the retinotectal projection. Here, glycosphosphatidylinositol-anchored A-ephrins are not only expressed in the tectum but also on retinal axons, raising the possibility that they function in this context as receptors. We now show that activation of ephrin-A2 or ephrin-A5 by one of their receptors, ephA5, results in a β1-integrin-dependent increased adhesion of ephrin-A-expressing cells to laminin. In the search for an ephrin-A-dependent signaling pathway controlling integrin activation, we identified a 120-kDa raft membrane protein that is tyrosine-phosphorylated specifically after ephrin-A activation. Tyrosine phosphorylation of this protein is not seen after stimulating ephrin-A2-expressing cells with basic fibroblast growth factor, epidermal growth factor, insulin growth factor, or fetal calf serum containing a large set of different growth factors. The role of p120 as a mediator of an ephrin-A-integrin coupling is supported by the finding that inhibiting tyrosine phosphorylation of p120 correlates with an abolishment of the β1-dependent cell adhesion.

During development of the retinotectal projection, members of the Eph family of receptor tyrosine kinases and their “ligands”, the ephrins, are strongly involved in guiding retinal axons to, and in, the tectum (for review see Refs. 1–3). Besides the graded expression of Eph receptors on retinal axons and of ephrins in the tectum, the ephrin-As are also differentially expressed on retinal axons themselves (4, 5). Gain of function and loss of function analyses suggest that here the ephrin-As modulate the function of the coexpressed receptors in that coexpression of ligands and receptors runs in parallel to a decrease in sensitivity for the repellent activity of the tactally expressed ephrins (4, 5). These findings fit to subsequent results of in vivo analyses of ephrin-A5 single and ephrin-A5; ephrin-A2 double knockout mice (6).

A hint of how ephrin-A functions on retinal axons may be provided by the concept that these ligands are localized, because of their membrane attachment by a glycosphosphatidylinositol (GPI) anchor, to so-called rafts (7–12). rafts are small dynamic microdomains in the membrane with a special glycosphingolipid and cholesterol composition, which have been proposed, besides having other functions, to serve as localized platforms for signal transduction (13).

GPI-anchored proteins are bound to the outer leaflet of the raft membranes (with no direct contact to the cytosol), whereas signaling molecules such as members of the src family are localized to the inner leaflet of rafts. In fact, “activated” GPI-anchored proteins can transduce signals to the cytosol, leading, for example, to changes in intracellular Ca2+ concentrations and activation of specific signaling pathways. This signaling might occur through formation of larger raft domains triggered by clustering of GPI-anchored proteins, which allows an interaction of signaling molecules normally separated through direct interactions between raft lipids or through (regulated) association of GPI-anchored molecules with appropriate transmembrane (co) receptors (9, 14). For example, the GPI-anchored cell adhesion molecule contactin/F11 associates with the transmembrane protein CASPR (15), representing a coreceptor involved in signal transduction after contactin/F11 stimulation. Other examples of GPI-anchored proteins capable of mediating signals include Thy-1 (16), CNTF (17), GDNF (18), and neurturin (19).

Simons and coworkers (7) have put forward the idea that a fundamental principle guiding the way raft microdomains exert their function in signal transduction is the (regulated) separation of different membrane proteins (7, 20). According to this concept, GPI-anchored molecules and other specific sets of membrane proteins have a strong tendency to associate with these domains, whereas others, such as molecules with a transmembrane domain, rarely appear in these areas.

Recently, it was shown that activation of ephrin-A5 results in an increase in adhesion of the ligand-expressing cells and in the level of tyrosine phosphorylation of molecules with molecular masses of 75–80 and 60 kDa (21). These changes, however, were not specific for an ephrin-A activation and were also seen after incubation of these cells with, for example, basic fibroblast growth factor (bFGF) (22).

Here we show that binding of the eph3 receptor to ephrin-A2 and ephrin-A5 leads to an activation of the integrin system, as seen in a β1-integrin-dependent increase in adhesion of the ephrin-A-expressing cells. Activation of ephrin-As and changes in adhesion correlate well with the tyrosine phosphorylation of a protein with a molecular mass of 120 kDa.

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† The abbreviations used are: GPI, glycosylphosphatidylinositol; PBS, phosphate-buffered saline; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; IGF, insulin growth factor; FCS, fetal calf serum; AP, alkaline phosphatase.
which is observed only after ephrin-A activation. p120 thus might represent a component of a new ephrin-A-dependent signaling pathway functionally linking ephrin-A to integrins.

**EXPERIMENTAL PROCEDURES**

**Establishment of ephrin-A-expressing Cell Lines—HEK293 cell clones stably expressing chick ephrin-A2 or the long or the short form of mouse ephrin-A5 were established according to standard protocols. In brief, about 2 × 10^6 HEK293 cells were transfected with 25 μg of the respective pCMNeo-based expression plasmids and were selected for about 2 weeks in standard medium (Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and antibiotics) containing 400 μg/ml G418 (PAA Laboratories). Then individual clones were isolated, expanded, and analyzed for ephrin-A expression using ephA3-AP staining and later, Western blots. Those cell clones most strongly expressing ephrin-A were further expanded and used in this study.

**Monoclonal Antibodies and Reagents—** ephA3-AP and AP were produced as described previously (23, 24); protein G-agarose is from Roche Molecular Biochemicals. Antibodies against chicken ephrin-A2 were described by Hornberger et al. (4), monoclonal antibodies to phosphotyrosine (clone 4G10), the β1-integrin-neutralizing antibody (clone DE9), and anti-human fyn (rabbit whole serum, used for immunoprecipitation) were from Upstate Biotechnologies. Monoclonal antibodies to fyn and caveolin-2 were from Transduction Laboratories, and the horseradish peroxidase-conjugated secondary antibodies were from Dianova.

**Activation of ephrin-A by ephA3—** About 75% confluent HEK293 cell clones expressing the indicated ephrin-A were starved for 36 h in serum-free RPMI medium, detached from the dishes with prewarmed FUCS-EDTA (5 mM KCl, 130 mM NaCl, 3 mM NaHCO3, 5 mM d-glucose, 10 mM HEPES, pH 7.0, 0.1 mM EDTA), pelleted at 1000 rpm for 5 min, washed once with serum-free RPMI medium, pelleted again, and resuspended in serum-free RPMI medium containing either 10 nM ephA3-AP or 10 nM AP. Then the cells were incubated at 37 °C for the indicated times, placed immediately on ice, and washed once with ice-cold PBS containing 1 mM Na3VO4. Subsequently the cells were collected as caveolin fraction, and the last fraction was collected as the soluble fraction (see also Ref. 21). The cell lysate was used for Optiprep density gradient ultracentrifugation.

**Purification of Detergent-insoluble Glycolipid-enriched Domains by Optiprep Density Gradient Ultracentrifugation—** The cell lysate was diluted to 35% (v/v) in Optiprep (Nycomed, Oslo, Norway). 0.96 ml of this solution was overlaid with 3 ml of 30% Optiprep in lysis buffer, 0.5 ml of 10% AP Triton X-100, 20 μM aprotinin, 50 micromolars of leupeptin, 2 μM pepstatin, 1 mM Na3VO4. This lysate was used for Optiprep density gradient ultracentrifugation.

**Immuno precipitation and Immunoblotting—** The “caveolin” fraction was diluted 1:3 with 0.5% Triton X-100 lysis buffer containing protease inhibitors and was then precleared with appropriate amounts of protein G-agarose for 2 h at 4 °C under rotating conditions. Immunoprecipitations were done by incubating the precleared lysate with antibody-conjugated protein G-agarose for at least 4 h at 4 °C under rotating conditions. Subsequently the immunoprecipitate was centrifuged and washed three times with lysis buffer. SDS polyacrylamide gel electrophoresis and immunoblotting were done according to standard protocols.

**Adhesion Assay—** 96-Well microtiter plates were coated with serially diluted purified chick ephrin-A2 (0.625–20 μg/ml) in PBS/diluent or poly-l-lysine over night at 4 °C and washed once with PBS. Non specific binding sites were blocked at room temperature for 1 h using 1% bovine serum albumin/ PBS. The starved cells (36 h) were detached from the dishes with prewarmed FUCS-EDTA, washed once, and resuspended in serum-free RPMI medium. ephA3-AP or AP was added to a final concentration of 10 nM. Subsequently, about 2 × 10^6 cells in 100 μl were plated per well (in triplicates) and incubated at 37 °C for 30 min. Then the medium was discarded, and remaining cells were washed once with PBS. Subsequently the cells were fixed with 0.5% paraformaldehyde/0.5% glutaraldehyde at room temperature for 30 min and stained with 0.5% crystal violet in 20% methanol at room temperature for 10 min. The cells were washed three times with H2O and were extracted with 50% ethanol/50 mM sodium citrate, pH 4.5 (50 μl/well). Absorbance was measured at 550 nm. Data shown in Figs. 1 and 6 were calculated on the basis of two to three independently performed experiments.

**RESULTS**

**Increased Adhesion of ephrin-A2-expressing Cells after Activation by Its Receptor—** To characterize a possible receptor or signaling function of the GPI-anchored ephrin-A ligands, we established a HEK293 cell line stably expressing ephrin-A2. As the ephA system has been shown to be involved for example in the regulation of cell migration (25), we investigated whether treatment of these cells with their receptor changes the adhesive properties of these cells to laminin. For an activation we used ephA3-AP or AP (the latter serving as a negative control).

**FIG. 1. Increased adhesion to laminin after stimulating HEK293-ephrin-A2 cells with ephA3. ephrin-A2-expressing cells were starved for 36 h, detached from the culture dishes, and plated in the presence of 10 mM ephA3-AP or 10 mM AP on dishes coated with increasing concentrations of laminin (0.625–20 μg/ml) or poly-l-lysine (PLL) (410–20 μg/ml). 30 min later unbound cells were washed away. The number of adherent cells was determined using a colorimetric assay (see “Experimental Procedures”), y axis, number of adherent cells in arbitrary units. A, activation of ephrin-A2 with ephA3-AP results in a specific increase in adhesion to laminin, but not to poly-l-lysine (PLL), when compared with cells incubated with AP. B, the increase in ephA3-AP-mediated adhesiveness can be blocked by incubation with a neutralizing antibody to β1-integrin. Here ephrin-A2-expressing cells were incubated in the presence of ephA3-AP either with an anti-β1-integrin antibody (10 μg/ml) or with a control antibody (anti-human Fc; 10 μg/ml).
adhesion to laminin but not to poly-L-lysine, which indicates an activation of the integrin system. To confirm that the increase in adhesion is indeed dependent on the presence of ephrin-A ligands, we performed this assay also using the parental cell line HEK293. Here we did not observe any increase in cell adhesion after ephA3-AP treatment (data not shown). To investigate the role of integrins directly, we performed the adhesion assay in the presence of a function-blocking monoclonal antibody to β1-integrin, used at a concentration of 10 μg/ml (see “Experimental Procedures”). Here we observed a strong reduction in the ephA3-induced increase in adhesion, whereas a control antibody used at the same concentration did not affect this increase (Fig. 1B). Thus, activation of ephrin-A by its receptors leads to an activation of the integrin system.

Ephrin-A2 Is Associated with Raft Microdomains—To understand the signaling function of ephrin-A ligands more closely, we were interested in identifying those proteins that link stimulated ephrin-A ligands to the integrin system. As ephrin-A molecules are GPI-anchored, they possess no direct link to the cytosol and thus might require a “coreceptor” for exerting such a signaling function. GPI-anchored molecules are found in so-called rafts, which are believed to represent platforms in which molecules involved in signal transduction are concentrated, thus a coreceptor might colocalize here with ephrin-A.

In initial experiments we investigated whether ephrin-A2 is indeed associated with rafts. A criterion for the association of a particular protein with rafts is the presence of that protein in detergent-insoluble glycolipid-enriched domains, which can be isolated because of their high lipid content by sucrose gradient centrifugation (26). Thus ephrin-A2-expressing cells were subjected to an Optiprep (sucrose) step gradient centrifugation, and individual fractions were subjected to Western blot analysis using monoclonal antibodies to ephrin-A2 and caveolin-2. Caveolins (27) are proteins that are often found associated with these rafts and might function in stabilizing the raft domains (13). These analyses showed that ephrin-A2 is contained in the same fractions as caveolin-2, indicating that ephrin-A2 is indeed localized to rafts. Fractions containing soluble proteins neither contained ephrin-A2 nor caveolin-2 (Fig. 2, A and C). This purification step considerably increased the sensitivity of our analyses, as the majority of cell proteins was contained in the nonraft fractions (data not shown).

Activation of Ephrin-A2 Leads to Changes in the Tyrosine Phosphorylation Pattern of Raft Proteins—To identify proteins that might be components of an ephrin-A2 signaling pathway, we compared the raft fractions from HEK293-ephrin-A2 cells after incubation with either ephA3-AP or with AP. Using Western blot analysis we focused in particular on changes in tyrosine phosphorylation levels using phosphotyrosine-specific monoclonal antibodies. Before activation, cells were serum-starved for 36 h, followed by an incubation with 10 nM ephA3-AP or 10 nM AP for the indicated times (0, 30, and 60 min). Subsequently raft fractions (C), as well as fractions containing soluble proteins (S), were isolated by Optiprep centrifugation and subjected to Western blot analysis using the anti-phosphotyrosine-specific antibody 4G10 (A). Then the filter was stripped and reprobed with antibodies specific for (B) fyn and (C) caveolin-2 (cav-2). α-Cav-2 antibodies were used to show a separation of raft-associated proteins versus soluble proteins and also to verify that similar amounts of the raft fractions had been loaded. Molecular mass markers (in kDa) are indicated to the left. Labelings to the right highlight the tyrosine phosphorylation patterns of p120, p80, and Src family kinases at roughly 60 kDa. Whereas the tyrosine phosphorylation of p120 is transiently up-regulated, the phosphorylation of p80 is strongly reduced after ephrin-A activation. Apparently neither the concentration nor the phosphorylation level of fyn is changed (A and B). D, to confirm the identity of fyn, the raft fraction was immunoprecipitated (IP) using an anti-fyn antibody and was then probed with the anti-phosphotyrosine antibody 4G10 (left) and, after stripping, with a different fyn antibody (right). The left lanes in each figure show the raft fraction before immunoprecipitation.

![Image](https://www.jbc.org/)

**Fig. 2.** Analysis of the tyrosine phosphorylation pattern of raft fractions after activating ephrin-A2 by ephA3. A fibroblast cell line stably expressing ephrin-A2 (HEK293-ephrin-A2) was serum-starved for 36 h, followed by an incubation with 10 nM ephA3-AP or 10 nM AP for the indicated times (0, 30, and 60 min). Subsequently raft fractions (C), as well as fractions containing soluble proteins (S), were isolated by Optiprep centrifugation and subjected to Western blot analysis using the anti-phosphotyrosine-specific antibody 4G10 (A). Then the filter was stripped and reprobed with antibodies specific for (B) fyn and (C) caveolin-2 (cav-2). α-Cav-2 antibodies were used to show a separation of raft-associated proteins versus soluble proteins and also to verify that similar amounts of the raft fractions had been loaded. Molecular mass markers (in kDa) are indicated to the left. Labelings to the right highlight the tyrosine phosphorylation patterns of p120, p80, and Src family kinases at roughly 60 kDa. Whereas the tyrosine phosphorylation of p120 is transiently up-regulated, the phosphorylation of p80 is strongly reduced after ephrin-A activation. Apparently neither the concentration nor the phosphorylation level of fyn is changed (A and B). D, to confirm the identity of fyn, the raft fraction was immunoprecipitated (IP) using an anti-fyn antibody and was then probed with the anti-phosphotyrosine antibody 4G10 (left) and, after stripping, with a different fyn antibody (right). The left lanes in each figure show the raft fraction before immunoprecipitation.
Fig. 3. Temporal changes in the tyrosine phosphorylation of proteins localized in raft fractions after activation of ephrin-A2 by ephA3. Serum-starved HEK293-ephrin-A2 cells were incubated for the indicated times with ephA3-AP. Subsequently raft fractions were isolated and subjected to Western blot analysis. A, tyrosine phosphorylation patterns using the anti-phosphotyrosine-specific monoclonal antibody 4G10, (B) fyn, (C) cav-2, and (D) ephrin-A2. 15 min after start of activation with ephA3, there is a marked increase in the tyrosine phosphorylation of p120, which, however, is again reduced after 60 min. The tyrosine phosphorylation of p80 is significantly decreased already after 5 min. The decrease in the tyrosine phosphorylation of a protein of roughly 45 kDa is observed also after incubation with AP alone (see Fig. 2). E, cell adhesion of HEK293-ephrin-A2 cells measured at different times of activation by ephA3-AP (right bars) or AP (left bars).

In an attempt to determine the identity of p120, we have probed the cells using ephrin-A5-AP or ephrin-A2-AP, and we have analyzed the raft fraction with selected antibodies. However, results from these experiments ruled out the possibility that p120 corresponds to an ephA receptor or focal adhesion kinase (data not shown).

Specificity of the Activated Signaling Pathway—Next we asked whether the changes in phosphorylation observed were specific for ephrin/Eph interactions or whether other proteins such as growth factors also induce these or similar changes in tyrosine phosphorylation. For this purpose, we analyzed a number of different signaling molecules known to affect the tyrosine phosphorylation patterns of cellular proteins, such as epidermal growth factor (EGF), insulin growth factor (IGF), and bFGF. We also included fetal calf serum (FCS) in our analysis, which contains a large variety of different growth factors. We observed a decrease in the phosphorylation of p80 after FCS treatment; however, none of these factors led to changes in the tyrosine phosphorylation pattern of p120 comparable with those caused by ephA3-AP (Fig. 4).

Activation of ephrin-A5 Leads to an Activation of the Same Signaling Pathway—We then wanted to know whether the observed changes in tyrosine phosphorylation can be observed also for other ephrin-A ligands. We established HEK293 cells stably expressing either the long or the short splice form of ephrin-A5 (28) and performed the same experiments as described above for ephrin-A2. Indeed, after activation by ephA3-AP, both ligands induced the down-regulation of p80 phosphorylation and the transient up-regulation of p120 phosphorylation (Fig. 5).

Blocking Tyrosine Phosphorylation of p120 Abolishes the Increase in Cell Adhesion—Our next step was to investigate more closely the inter-relationship between ephrin-As and integrins. A number of molecules are known to be involved in the regulation of integrin signaling, and we were interested in whether signaling pathways used in ephrin-A and integrin signaling share common members. We concentrated on src family kinases, which are known to be involved in integrin signaling and are required for an optimal adhesion efficiency but appear not to be essential for these events (29–33). We used the pyrazolopyrimidine PP2, which selectively inhibits src family kinases (34). This inhibitor and a closely related molecule, PP1, have both been used in a number of investigations to show an involvement of src family kinases in different developmental...
with respect to tyrosine phosphorylation of raft proteins. The long and short splice forms of ephrin-A5 induce the same changes in tyrosine phosphorylation of raft proteins as ephrin-A2. HEK293 cell lines stably expressing either the long or the short splice form of ephrin-A5 were serum-starved for 36 h and were subsequently treated with 10 nM ephA3-AP for the indicated times. Raft fractions were isolated and subjected to Western blot analysis using the phosphotyrosine-specific antibody 4G10. Similar to the results obtained with the HEK293-ephrin-A2 cell line (see Figs. 2 and 3), there is a transient increase in the amount of tyrosine-phosphorylated p80. There is no apparent difference between the two different ephrin-A5 splice forms with respect to tyrosine phosphorylation of raft proteins.

In parallel to these binding assays, we performed Western blot analyses of raft fractions from ephA3-AP-treated cells, which showed that PP2 pretreatment of these cells almost completely abolished the increase in tyrosine phosphorylation of p120, whereas PP3 did not affect this pattern (Fig. 7). The tyrosine phosphorylation of p120 is abolished after ephrin-A stimulation. It has been proposed that raft microdomains serve as platforms in the membrane, within which molecules involved in signaling to the cytosol are organized (7, 12, 13). Both GPI-anchored ephrin-A ligands and B-class ephrins, which are transmembrane-anchored, are found in these structures. Whereas A-ligands appear to be constitutively associated with these rafts, the incorporation of B-ligands in these structures might be regulated, as ephrin-Bs without their cytoplasmic tail no longer associate with rafts (37), suggesting an active (regulated) transport of B-ephrins into and out of rafts. Interaction of ephB receptors with their ligands results in the phosphorylation of highly conserved tyrosines in the intracellular domain of the ligands, which then might serve as binding sites for other signaling molecules (37–41).

After stimulation of ephrin-A2-expressing cells with ephA3-AP, we observed an increase in cell adhesion, which could be abolished by using a neutralizing monoclonal antibody to β1-integrin, indicating the existence of an ephrin-A-dependent signaling pathway controlling integrin function. The mechanism by which ephrin-As exert this function, e.g. signal into the cytosol, is poorly understood. It is quite possible that a communication between ephrin-As (bound to the outer leaflet of the membrane) and intracellular signaling molecules such as lipidated src family kinases (bound to the inner leaflet of the membranes) is mediated by (conformational changes of) transmembrane proteins. The proteins we have identified on the basis of their presence in raft membranes and their differential tyrosine phosphorylation pattern in response to ephrin/Eph interactions represent candidates for such a role. p120 is only slightly phosphorylated in the unstimulated state but becomes strongly tyrosine-phosphorylated after ephrin-A activation. We cannot exclude the possibility that the increase in tyrosine phosphorylation of p120 is because of a recruitment of already phosphorylated proteins having a molecular mass of about 80 kDa, whose tyrosine phosphorylation was down-regulated after ephrin-A stimulation. However, the strong increase in adhesion typically seen after stimulating ephrin-A2-expressing cells leads to an activation of the integrin system and an increased tyrosine phosphorylation of a 120-kDa raft protein. Both the increase in adhesion and in tyrosine phosphorylation of p120 can be abolished by inhibiting the function of src family kinases. Besides p120, we identified a set of proteins having a molecular mass of about 80 kDa, whose tyrosine phosphorylation was down-regulated after ephrin-A stimulation.

**DISCUSSION**

In this report we have shown that binding of ephA3 to ephrin-A-expressing cells leads to an activation of the integrin system and an increased tyrosine phosphorylation of a 120-kDa raft protein. The long and short splice forms of ephrin-A5 induce the same changes in tyrosine phosphorylation of raft proteins as ephrin-A2. HEK293 cell lines stably expressing either the long or the short splice form of ephrin-A5 were serum-starved for 36 h and were subsequently treated with 10 nM ephA3-AP for the indicated times. Raft fractions were isolated and subjected to Western blot analysis using the phosphotyrosine-specific antibody 4G10. Similar to the results obtained with the HEK293-ephrin-A2 cell line (see Figs. 2 and 3), there is a transient increase in the amount of tyrosine-phosphorylated p80. There is no apparent difference between the two different ephrin-A5 splice forms with respect to tyrosine phosphorylation of raft proteins.

Treating the ephrin-A2-expressing cells with 10 μM PP2 alone, thus without stimulation by ephA3-AP, led to only a small reduction in the adhesiveness of these cells (Fig. 6). However, the strong increase in adhesion typically seen after ephA3-stimulation (see Figs. 1 and 6) was prevented by PP2, i.e. by blocking src family kinases. The incubation in the presence of 10 μM PP3, which is a structurally closely related but functionally inactive compound to PP2, had no effect on the increase in cell adhesion (Fig. 6).

In parallel to these binding assays, we performed Western blot analyses of raft fractions from ephA3-AP-treated cells, which showed that PP2 pretreatment of these cells almost completely abolished the increase in tyrosine phosphorylation of p120, whereas PP3 did not affect this pattern (Fig. 7). The tyrosine phosphorylation of p120 is abolished after ephrin-A stimulation. It has been proposed that raft microdomains serve as platforms in the membrane, within which molecules involved in signaling to the cytosol are organized (7, 12, 13). Both GPI-anchored ephrin-A ligands and B-class ephrins, which are transmembrane-anchored, are found in these structures. Whereas A-ligands appear to be constitutively associated with these rafts, the incorporation of B-ligands in these structures might be regulated, as ephrin-Bs without their cytoplasmic tail no longer associate with rafts (37), suggesting an active (regulated) transport of B-ephrins into and out of rafts. Interaction of ephB receptors with their ligands results in the phosphorylation of highly conserved tyrosines in the intracellular domain of the ligands, which then might serve as binding sites for other signaling molecules (37–41).

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 Activation of Integrins by ephrin-As

we think this is unlikely, because the increase in p120 tyrosine phosphorylation can be blocked by inhibiting src family kinases. It remains to be investigated whether a similar modification of p120 can be observed also after clustering of other GPI-anchored proteins expressed at similar levels in HEK293 cells. It is noteworthy that the strong increase in p120 tyrosine phosphorylation occurs only after a lag phase of about 10 min, suggesting the involvement of other signaling components. Possibly the lag phase is correlated with changes in the structure or composition of ephrin-A-containing raft microdomains. Thus treatment with oligomeric ephA3-AP might lead to a clustering of ephrin-A2 and a concomitant fusion of initially smaller raft microdomains, which results, by an unknown mechanism, in an activation of src family kinases, p120 tyrosine phosphorylation, and integrin activation. In good agreement with p120 playing a role in coupling ephrin-A activation to integrin activation, we have shown here (using a specific src family kinase inhibitor) that blocking p120 tyrosine phosphorylation correlates with an abolishment of the increase in cell adhesion. In further support, the increase in cell adhesion occurs subsequent to the increase in p120 tyrosine phosphorylation, which thus supports the concept that p120 phosphorylation is involved in the control of cell adhesion rather than its being a consequence. The p80 protein might not be involved in this process, as its dephosphorylation was observed for example also after FCS treatment, which on the other hand does not affect cell adhesion.

Interestingly, activation of ephA receptors leads to a suppression of integrin function and to an inhibition of cell spreading and migration (of fibroblast cells) (42–45). Therefore, the unexpected picture arises that activation of ephA and ephrin-A might contribute to an understanding of the development of the retinotectal projection, where on subpopulations of retinal axons ephrin-A and ephA-A are coexpressed.

Specificity of the Tyrosine Phosphorylation of p120 and p80—Recently, in an approach similar to the one used here, Davy et al. (21) identified a number of proteins whose tyrosine phosphorylation level increased after binding of ephA5-Fc to an ephrin-A5-expressing cell line. They found most prominently a 75–80-kDa protein, which became highly phosphorylated after binding of ephrin-A5-expressing cell line. They found most prominently a 75–80-kDa protein, which became highly phosphorylated after binding of ephrin-A5-expressing cell line. They found most prominently a 75–80-kDa protein, which became highly phosphorylated after binding of ephrin-A5-expressing cell line. They found most prominently a 75–80-kDa protein, which became highly phosphorylated after binding of ephrin-A5-expressing cell line. They found most prominently a 75–80-kDa protein, which became highly phosphorylated after binding of ephrin-A5-expressing cell line. 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Jisen Huai and Uwe Drescher

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