Ephrin-A6, a New Ligand for EphA Receprors in the Developing Visual System

Patricia Menzel, Fatima Valencia, Pierre Godement, Vincent C. Dodelet, and Elena B. Pasquale
The Burnham Institute, 10901 N. Torrey Pines Road, La Jolla, California 92037

In the embryonic visual system, EphA receptors are expressed on both temporal and nasal retinal ganglion cell axons. Only the temporal axons, however, are sensitive to the low concentrations of ephrin-A ligands found in the anterior optic tectum. The poor responsiveness of nasal axons to ephrin-A ligands, which allows them to traverse the anterior tectum and reach their targets in the posterior tectum, has been attributed to constitutive activation of the EphA4 receptor expressed in these axons. EphA4 is highly expressed throughout the retina, but is preferentially phosphorylated on tyrosine (activated) in nasal retina. In a screen for EphA4 ligands expressed in chicken embryonic retina, we have identified a novel ephrin, ephrin-A6. Like ephrin-A5, ephrin-A6 has high affinity for EphA4 and activates this receptor in cultured retinal cells. In the embryonic day 8 (E8) chicken visual system, ephrin-A6 is predominantly expressed in the nasal retina and ephrin-A5 in the posterior tectum. Thus, ephrin-A6 has the properties of a ligand that activates the EphA4 receptor in nasal retinal cells. Ephrin-A6 binds with high affinity to several other EphA receptors as well and causes growth cone collapse in retinal explants, demonstrating that it can elicit biological responses in retinal neurons. Ephrin-A6 expression is high at E6 and E8, when retinal axons grow to their tectal targets, and gradually declines at later developmental stages. The asymmetric distribution of ephrin-A6 in retinal cells, and the time course of its expression, suggest that this new ephrin plays a role in the establishment of visual system topography. © 2001 Academic Press

Key Words: protein tyrosine phosphorylation; retinotectal topography; axon guidance; growth cone collapse; patterning.

INTRODUCTION

Eph receptor tyrosine kinases and their ephrin ligands are required for the topographic organization of the embryonic visual system (Feldheim et al., 2000, 1998; Frisen et al., 1998). The EphA3 receptor is expressed in an increasing nasal to temporal gradient in the developing axons of chicken retinal ganglion cells (Cheng and Flanagan, 1994) and the ligands ephrin-A2 and ephrin-A5 are expressed in increasing anterior to posterior gradients in the target of retinal ganglion cell axons, the optic tectum (superior colliculus in the mouse) (Cheng and Flanagan, 1994; Drescher et al., 1995; Feldheim et al., 1998; Frisen et al., 1998; Nakamoto et al., 1996). According to one model, repulsion by gradually increasing levels of ephrin-A ligands prevents the growth of temporal axons to the posterior tectum (Drescher et al., 1995; Nakamoto et al., 1996). According to another model, temporal axons expressing EphA receptors initially extend into the posterior tectum, where interaction with ephrin-A2 and ephrin-A5 subsequently causes their withdrawal or degeneration (Feldheim et al., 1998; Frisen et al., 1998; Gao et al., 1999; Roskies et al., 1995). A topographic map is generated because the concentration of ephrin required for axon repulsion, or elimination, is inversely proportional to the EphA receptor concentration on the temporal axons (Nakamoto et al., 1996). Thus, tectal regions with high ephrin-A2 and ephrin-A5 expression are ultimately devoid of axons with high EphA receptor expression.

The regulation of retinotectal topography by EphA receptors is, however, more complex. Several EphA receptors in addition to EphA3 are present in retinal cells with different spatial distributions (Cheng et al., 1995; Connor et al., 1998; Feldheim et al., 2000; Marcus et al., 1996; Monschau et al., 1997; Sefton et al., 1997; Zhang et al., 1996). We have previously reported that the EphA4 receptor, for example, is highly expressed in the embryonic retina and optic nerve (Holash and Pasquale, 1995). Although uniformly expressed in different sectors of the retina at embryonic day 8 (E8),
EphA4 is asymmetrically phosphorylated on tyrosine, with higher phosphorylation levels in the nasal than the temporal retina (Connor et al., 1998). This tyrosine phosphorylation of EphA4 may have functional significance for the topographic organization of the retinotectal projection by attenuating the repulsive effects of ephrin-A ligands encountered by nasal retinal axons (Hornberger et al., 1999; Rosentreter et al., 1998). This effect could explain the ability of nasal axons to reach regions of the optic tectum that contain substantial levels of ephrin-A2 and ephrin-A5 even though these axons express EphA receptors (Dutting et al., 1999; Feldheim et al., 2000; Hornberger et al., 1999).

The uneven phosphorylation pattern of EphA4 suggests that this receptor is activated by a ligand present in the E8 nasal retina (Connor et al., 1999). We initially thought that this ligand may be ephrin-A5 (Connor et al., 1998) because ephrin-A5 binds EphA4 with high affinity (Monschau et al., 1997) and is present at higher levels in the nasal than the temporal retina (Connor et al., 1998; Dutting et al., 1999; Hornberger et al., 1999). We have found, however, that ephrin-A5 protein is expressed at low levels in the chicken embryonic retina, suggesting that another ephrin activates EphA4 in the nasal retina. We have, therefore, used RT-PCR with degenerate primers to identify the ephrins expressed in the chicken embryonic retina. One of the amplified products encodes a novel ephrin, which we have designated ephrin-A6. We demonstrate here that ephrin-A6 has the properties expected for the main ligand that activates EphA4 in nasal retinal cells.

**MATERIALS AND METHODS**

Reverse Transcription and Polymerase Chain Reaction

For polymerase chain reaction (PCR) with degenerate primers, mRNA was prepared from E8 chicken retina using the MicroFastTrack Kit (Invitrogen) and reverse transcription was performed using the cDNA cycle kit (Invitrogen) with random primers according to the instructions of the manufacturer. PCR was then carried out with degenerate primers corresponding to sequences conserved in the A-ephrins or the B-ephrins. A-ephrins were amplified using two sense degenerate primers, GTCT(A/T)CT-AG(T/C)AA(T/C)CC or GTCT(A/T)CTGGAACTAG(C/T)TCG(A/T)C(A/C)TCC, and one anti-sense degenerate primer, GGA(T/G)GT(G/A)AGAG(A/C)G(T/G)AA(A/T)TC(T/T)(C/T). B-ephrins were amplified using the sense primer CGGATCCAC(G/A)T(C/A)AT(A/T)(C/A)AA(G/A)TT(T/C)CA(G/A)GA-G(A/T) (introduced BamHI site in italics) and the antisense primer GGAATTC(G/A)T(C/A)AC(T/C)TT(G)GAT(G/A)TA(G/A)TAT-(G/A)TT (introduced EcoRI site in italics). PCR amplifications were performed using five cycles with annealing at 37°C followed by 30 cycles with annealing at 45°C, and Taq polymerase was added only after the initial cycle of denaturation at 95°C. Bands of the appropriate size were gel purified and reamplified either with the same primers (B-ephrin primers) or with longer primers that included an EcoRI site (A-ephrin primers), subcloned into pGEX-4T-1 vector (Pharmacia) or pT7Blue vector (Novagen), and sequenced.

PCR with ephrin-A6 specific primers, reverse transcription was performed either with E8 dorsal retina mRNA and random primers or with E8 ventral retina mRNA and the ephrin-A6 specific anti-sense primer GGATCTCCTACTACCGGGGTTAACC (Barn A6, containing an introduced BamHI site, in italics). A first PCR amplification was carried out with the sense primer CGTACGCGCAGCGTCTACTGG (Nhe A6, containing an Nhel site, in italics) and the Barn A6 antisense primer. Two bands of approximately 400 and 1000 bp were obtained from amplification of the dorsal retina cDNA, with the larger band being more prominent. Only the 400-bp band was detectable in the amplification of the ventral retina mRNA. The two PCR fragments were reamplified using (1) the Nhe A6 sense primer and the antisense primer GAAGGTTTATTCACCTCCC (3′ A6–2), which yielded the same fragment (comprising nucleotides 123–285, Fig. 2B) from amplification of both bands; (2) the sense primer GAAGGTTTATTCACCTCCC (5′ A6–2) and the 3′ A6–2 anti-sense primer, which yielded the same fragment (comprising nucleotides 21–285) from amplification of both bands; (3) the 5′ A6–2 sense primer and the Barn A6 antisense primer, which yielded a fragment comprising nucleotides 123–956 of ephrin-A6 from amplification of the larger band and a fragment comprising nucleotides 123–524 of ephrin-A6 (or the corresponding regions of ephrin-A6γ, δ, or ε) from amplification of the smaller band.

**Rapid Amplification of cDNA Ends (RACE)**

E8 chicken retina mRNA was isolated using the MicroFastTrack kit and used for 5′ and 3′ RACE using the 5′ RACE System Kit (GibcoBRL) according to the instructions of the manufacturer. For 5′ RACE, cDNA synthesis was primed using the antisense primer GAACGGGGCACGACGAGGC (5′ A6–1). Terminal deoxynucleotidyl transferase was used to add a homopolymeric dC tail to the 3′ end of the purified single-stranded cDNA. The dC-tailed cDNA was then amplified by PCR with the 5′ RACE Abridged Anchor Primer (GibcoBRL) containing a 3′ sequence complementary to the polyC tail and the 3′ A6–1 primer and reamplified using the Abridged Universal Amplification Primer (AUAP) (GibcoBRL) and the nested antisense primer 3′ A6–2. PCR products were digested with SalI (in the AUAP) and PstI (in the amplified A6 product), cloned into SalI and PstI digested pBluescript SK vector, and sequenced. For 3′ RACE, cDNA synthesis was primed using the oligo-dT 3′ RACE Adapter Primer (GibcoBRL), which contains the sequence of the AUAP. The cDNA was then amplified using the sense primer CACGTTCTCGAGGACGT (5′ A6–3) and the AUAP and reamplified using the nested sense primer GAGACGTCCAGCCTGTTCAT (5′ A6–4) and the AUAP. PCR products were digested with SpeI (in the AUAP) and Nari (in the amplified A6 product), cloned into SpeI and Bsp106 digested pBluescript SK vector, and sequenced.

**Fc and AP Chimeras**

The sequences encoding the extracellular domains of chicken ephrin-A5 (amino acids 21–208) (Drescher et al., 1995), ephrin-A6 (amino acids 21–184), EphA3 (amino acids 22–540) (Sajjadi et al., 1991), and EphA4 (amino acids 19–547) (Ohla et al., 1996) were amplified by PCR with specific primers containing a Nhel restriction site (sense primer) and a BamHI restriction site (antisense primer). The amplified products were cloned into a Nhel–BamHI digested pcDNA3 vector containing the sequences encoding the signal peptide of CDS (to the 5′ end of the Nhel site) and the Fc portion of human IgG1 (to the 3′ end of the BamHI site), as
previously described (Shao et al., 1994), and verified by sequencing. The EphA3 Fc and EphA4 Fc expression plasmids were transiently transfected into COS cells using Superfect transfection reagent (Qiagen). Cell culture supernatants containing the fusion protein were centrifuged at 4000 g to eliminate cell debris and stored frozen at −20°C. The ephrin-A5 Fc and ephrin-A6 Fc expression plasmids were stably transfected into 293T cells. Recombinant ephrin-A5 and ephrin-A6 Fc fusion proteins were purified from cell-culture supernatants using Affi-Gel protein A (Bio-Rad) as previously described (Shao et al., 1994) and stored frozen in aliquots in PBS containing 1 mM CaCl$_2$.

To produce the alkaline phosphatase (AP) fusion constructs, the sequences of the extracellular domains of ephrin-A5 (amino acids 21–208) and ephrin-A6 (amino acids 21–184) were amplified by PCR from the corresponding Fc vectors together with the sequence encoding the CDS signal peptide. Primers used for amplification were T7 (sense, to pCDNA3 sequences) and either the ephrin-A5 antisense primer TGGGAGATCTCGGTCTGTG CCG (introduced Bgl II site in italics) or the ephrin-A6 antisense primer GCTAGATTCGGCGCCG (introduced Bgl II site in italics). Amplified products were digested with HindIII (in pCDNA3) and BglII and cloned into similarly digested APtag2 vector (Cheng et al., 1995). Partial restriction digests were used for ephrin-A5, which contains an internal HindIII site. After sequence verification, the expression plasmids were transiently transfected into 293T cells. Cell culture supernatants containing the AP fusion proteins were centrifuged at 4000 g to eliminate cell debris, supplemented with Hepes buffer pH 7.5 to 20 mM and NaN$_3$ to 0.02%, and stored frozen at −20°C.

**Antibodies**

Anti-ephrin-A5 and anti-ephrin-A6 antibodies were prepared by injecting into rabbits the corresponding Fc fusion proteins. The immune serum was affinity-purified on columns prepared by coupling ephrin-A5 Fc or ephrin-A6 Fc to AffiGel-10 (Bio-Rad). Anti-Fc antibodies were then absorbed out by passing the affinity-purified antibodies on a human Fc column (Cappel). The anti-EphA3, EphA4, EphB2, and EphB5 antibodies have been described previously (Holash and Pasquale, 1995; Soans et al., 1994, 1996). The PY20 monoclonal antibody to phosphotyrosine, conjugated to peroxidase, was from Transduction Laboratories. Affinity-purified anti-human Fc antibody was from Cappel and antialkaline phosphatase polyclonal antibody from GenHunter Corporation.

**Immunohistochemistry and Whole Mounts**

For immunohistochemistry, E8 chicken heads were fixed overnight in 4% paraformaldehyde in PBS and cryoprotected in 25% sucrose in PBS. Vertical frozen sections of E8 chicken heads were labeled with anti-ephrin-A5 or anti-ephrin-A6 antibodies as indicated. Immunoperoxidase labeling was with a Vectastain Elite ABC kit (Vector Laboratories). For whole mounts, E3 embryos were fixed in 4% paraformaldehyde in PBS for 2 h at room temperature, rinsed in HBS/Triton/BSA (150 mM NaCl, 20 mM Hepes buffer, pH 7.4, 0.1% Triton X-100, 0.5 mg/ml BSA), incubated for 2 h with 0.5 ml cell culture supernatant containing ephrin-A6 AP and 0.1% Triton X-100, washed 6 × 10 min in HBS/Triton/BSA, heat inactivated at 65°C for 20 min, equilibrated in AP buffer (100 mM NaCl, 100 mM Tris, pH 9.5, 5 mM MgCl$_2$, 0.1% Triton X-100) for 20 min at room temperature, and finally incubated with substrate (0.17 mg/ml BCIP, 0.33 mM NBT in AP buffer). When the desired labeling intensity was achieved, the embryos were rinsed in PBS to stop color development.

**Retinal Cultures**

Dissociated retinal cells were prepared as described in Holash et al. (1997) from E7 chicken retinas. After 1 h in cell culture medium containing 10% serum, the cells were cultured overnight in medium with 0.5% serum. Cultures were then treated with 10 μg/ml ephrin-A5 Fc or ephrin-A6 Fc for 15 min, before collecting in RIPA buffer for immunoprecipitation with anti-Eph receptor antibodies.

**Immunoprecipitation and Immunoblotting**

Immunoprecipitations were performed as described (Holash et al., 1997). For pull-down experiments with Fc fusion proteins, 10 μg of the Fc portion of human IgG1 (Fc), ephrin-B2 Fc, ephrin-A5 Fc, and ephrin-A6 Fc were bound to StaphA (Boehringer Mannheim) and then incubated with E8 chicken retina extracts in 1% Brij 96 buffer as described (Zisch et al., 1997). The proteins that remained bound to the StaphA-Fc complexes were separated by SDS-PAGE and probed by immunoblotting.

For immunoblotting with anti-Fc receptor or anti-ephrin antibodies, filters were blocked for 1 to 16 h in 3% BSA in TBS (Tris-hydroxyethyl aminoethane-buffered saline) or 0.2% nonfat dry milk in PBS and then incubated for approximately 4 h at room temperature, or overnight at 4°C, with 2–3 μg/ml antibody. After several washes, the filters were incubated with 0.2 μg/ml protein-A peroxidase (Sigma) in TBS containing 3% BSA for 1 h. Peroxidase-conjugated anti-phosphotyrosine antibody (0.05 μg/ml in 3% BSA) was used to detect tyrosine phosphorylation. The filters were then washed and developed using enhanced chemiluminescence reagents (Amersham).

**Equilibrium Binding Experiments**

High-binding 96-well ELISA plates (Corning) were coated overnight at 4°C with 100 μl of a 30 μg/ml anti-human Fc antibody solution in PBS. After rinsing and blocking with 3% BSA for 1 h, the wells were incubated on a moving platform at room temperature for 2 h with 100 μl PBS, 1 mM CaCl$_2$ containing 0.25–1 μg/ml Eph receptor Fc (R&D Systems) or 100–1000 μl cell culture supernatants containing chicken EphA3 Fc or EphA4 Fc. The wells were then rinsed and incubated for 3 h at room temperature with 200–μl cell culture supernatants containing ephrin-A5 or ephrin-A6 AP (at the lower concentrations, the AP solution was replaced with a second aliquot after 1.5 h to avoid possible ligand depletion). After washing, 225 μl of 0.012 M para-nitrophenylphosphate was added and incubated for 20 min at 37°C. OD$_{405}$ was measured using an ELISA plate reader. Measurements of bound ligand AP were carried out in duplicate. The concentration of the ephrin-A5 and ephrin-A6 AP fusion proteins, and their integrity, were determined by immunoblotting the ephrin AP supernatants with anti-alkaline phosphatase antibody and comparing with a purified alkaline phosphatase standard (GenHunter) by densitometric analysis. The activities of the ephrin-A5 AP and ephrin-A6 AP supernatants were also determined after heating for 10 min at 65°C (Cheng and Flanagan, 1994; Flanagan and Leder, 1990). The specific activity of the ephrin-AP fusion proteins was approximately 125 U/mg protein (Berger et al., 1988). $K_d$ values were calculated directly from the binding curves by nonlinear regression analysis using an hyperbolic one site binding equation (GraphPad Prism). Final $K_d$ values are averages of the values obtained in several experiments.
Growth Cone Collapse Experiments

Explants dissected from E6 chicken retinas were cultured on polylysine (200 μg/ml) and laminin (20 μg/ml)-coated glass coverslips for 24 h in 1 ml of F12-DMEM medium containing 10% FBS and 0.4% methylcellulose. Three hours before adding Fc proteins, the medium was exchanged to 0.5 ml medium without methylcellulose. Ephrin-A6 Fc and human Fc were cross-linked by a 30 min incubation with a 1/10 concentration of anti-Fc antibodies and added to the explant cultures (0.5 ml/well, in culture medium equilibrated with CO₂) at 1 μg/ml final concentration. After 30 min the cultures were fixed by adding 2% cold glutaraldehyde and then mounted in PBS–glycerol. For quantitative analysis about 80 growth cones were scored for each explant, and experiments were repeated three times. Growth cones were observed under phase contrast optics and pictures were taken with a SPOT CCD camera. Growth cones with no lamellipodia or filopodia remaining at the tip of the neurite were scored as collapsed.

RESULTS

Ephrin-A5 Protein Expression Is High in the Posterior Optic Tectum and Low in the Retina at Embryonic Day 8

We previously detected higher levels of ephrin-A5 mRNA in the chicken nasal retina compared to the temporal retina at embryonic day 8 (E8) (Connor et al., 1998). To determine whether ephrin-A5 is the ligand that causes EphA4 tyrosine phosphorylation in the E8 nasal retina, we examined the expression of ephrin-A5 at the protein level. While both immunohistochemistry (Fig. 1A) and immunoblotting (Fig. 1B) experiments revealed the expected high expression of ephrin-A5 in the posterior optic tectum (Drescher et al., 1995; Monschau et al., 1997), ephrin-A5 protein expression in the nasal retina appeared to be very low. This pattern of ephrin-A5 protein expression suggested that another ligand for EphA4 must be highly expressed in the E8 nasal retina.

RT-PCR Approach to Identify Candidate EphA4 Ligands Expressed in the Nasal Retina

EphA4, like other EphA receptors, can be activated by several A-ephrins (Eph Nomenclature Committee, 1997). In addition, EphA4 has substantial affinity for some B-ephrins. We used an RT-PCR approach to search for candidate EphA4 ligands expressed in the E8 chicken retina (Fig. 2A) because this approach can identify known as well as unknown ephrins. Degenerate primers designed to amplify B-ephrins yielded ephrin-B1 and ephrin-B2. These B-ephrins are concentrated in the dorsal rather than the nasal retina (Holash et al., 1997; Marcus et al., 1996) and therefore are not good candidates as ligands that activate EphA4 in the nasal retina. Degenerate primers designed to amplify A-ephrins yielded ephrin-A2, ephrin-A5, and a cDNA encoding an ephrin fragment not closely related to previously known ones. Of the A-ephrins amplified, ephrin-A2 does not appear to be a good candidate as the main ligand activating EphA4 in the nasal retina because it binds poorly to EphA4 (Monschau et al., 1997). Ephrin-A5, as shown above, is poorly expressed in the E8 retina and is therefore unlikely to cause substantial activation of EphA4. Thus, we focused our efforts on the further characterization of the putative new ephrin identified.

Cloning of Ephrin-A6

Using 5’ RACE we obtained the sequence encoding the amino-terminal part of the new ephrin, except for the
FIG. 2. Ephrin-A6 is a new ephrin ligand. (A) RT-PCR with degenerate primers to identify ephrins present in E8 chicken retina. The amino acid sequences corresponding to the degenerate primers are indicated. (B) DNA and amino acid sequence of ephrin-A6 and its variant forms. Ephrin-A6 has 12 hydrophobic amino acids at the carboxy terminus, consistent with a signal for GPI linkage; ephrin-A6a, b, d, and e are predicted to be secreted ligands. The putative signal peptide sequence is underlined and the possible site of N-glycosylation is marked by a dotted overline. Amino acids and nucleotides are numbered at right. Amino acids that are different in the five ephrin-A6 variant forms are boxed. Nucleotides that are present only in some forms are shaded in gray, and 3' sequence common to all forms is underlined. GenBank accession number AF317286. (C) PCR amplification is consistent with the presence of different ephrin-A6 transcripts in E8 retina. mRNA from E8 retina was reverse transcribed and primers corresponding to 5' and 3' sequences of ephrin-A6 were used for amplification. Two fragments were obtained (larger fragment and smaller fragment) and portions of each fragment were reamplified with three sets of primers. Amplification products corresponding to the 5' end (lanes 2 and 5) and the middle portion (lanes 1 and 4) were the same, whereas amplification products corresponding to the 3' end (lanes 3 and 6) were different, indicating that the two bands contain the same 5' region and differ in their 3' regions. (D) Alignment of ephrin-A6 with the other ephrin-A ligands. The program CLUSTAL W was used for the alignment. Amino acids that are identical in ephrin-A6 and in at least one of the other ligands are shaded in gray; cysteines are shaded in black. Arrows indicate the positions where an intron has been identified in at least one of the ephrins; the amino acids corresponding to the position of identified introns are underlined. An arrow with a question mark indicates the position where the four variant forms of ephrin-A6 diverge. (E) Tree showing the relationship of ephrin-A6 with the other known ephrins. Chicken ephrin-A2, ephrin-A5, ephrin-B1, and ephrin-B2 are compared to the corresponding mouse ephrins, to show that the chicken and mouse counterparts of the same ephrin are much more closely related than ephrin-A6 is to any of the other ligands. Ephrin-A6, therefore, does not appear to be the chicken homolog of a previously identified mammalian ephrin.
several amino acids at the beginning of the signal peptide (which are not part of the mature protein) (Fig. 2B). By 3’ RACE we obtained several 3’ sequences that differ in the number of nucleotides comprising a poly-C stretch. These sequences are predicted to encode secreted proteins that differ in their most carboxy-terminal amino acids (ephrin-A6, A New Ligand for EphA Receptors).
A6, b, g, d, and e in Fig. 2B). To obtain a cDNA containing both 5' and 3' sequences, we performed RT-PCR using E8 retina mRNA as a template and primers corresponding to 5' and 3' sequences obtained by RACE. The PCR reaction yielded two bands: a smaller one of the expected size of approximately 400 bp, and a larger one of approximately 1000 bp (Fig. 2C). Additional PCR amplifications with internal primers and sequence analysis showed that the two bands contain the same 5' region but different 3' regions. The 1000-bp band encodes a longer form that contains a putative hydrophobic signal for GPI linkage at the C-terminus (ephrin-A6a in Fig. 2B), consistent with the sequence of an ephrin of the A subclass. Interestingly, all four variant forms diverge in the same region, suggesting that they originate by alternative splicing. In agreement with this hypothesis, an intron has been identified in the corresponding region of ephrin-A2, ephrin-A3, and ephrin-A5 (arrows in Fig. 2D). An alignment of the new sequence with those of the five known A-ephrins (Fig. 2D) and a phylogenetic tree (Fig. 2E) do not show a particularly close similarity between the new sequence and any of the other ephrins. This is consistent with the sequence encoding a novel ephrin and not the chicken homolog of a previously known one. We designated the new ephrin, ephrin-A6, according to the guidelines of the Eph Nomenclature Committee (Eph Nomenclature Committee, 1997).

Ephrin-A6 Expression Is High in the Nasal Retina and Low in the Optic Tectum, a Distribution Distinct from That of Ephrin-A5

To examine the distribution of ephrin-A6 in the chicken visual system, we prepared anti-ephrin-A6 polyclonal antibodies. These antibodies do not recognize ephrin-A5 in immunoblotting and immunoprecipitation experiments (data not shown). Conversely, our anti-ephrin-A5 polyclonal antibodies do not recognize ephrin-A6. In sagittal sections of E8 chicken heads, ephrin-A6 immunoreactivity is most prominent in the nasal retina (Fig. 3A). Ephrin-A6 is evenly distributed throughout the retinal layers including the nerve fiber layer, which contains the axons of retinal ganglion cells (Fig. 3B). In contrast, in the optic tectum ephrin-A6 immunoreactivity is very faint (Fig. 3A). Thus, at E8 ephrin-A5 and ephrin-A6 have a complementary pattern of expression, with ephrin-A5 highly expressed in the posterior tectum and ephrin-A6 highly expressed in the topmost layer, which is mostly brown because it is devoid of cell bodies. The pigmented epithelium (black) is at the bottom. (C) Developmental regulation of ephrin-A5 and ephrin-A6 in embryonic chicken retina and tectum. Anti-ephrin-A6 and anti-ephrin-A5 antibodies were used to immunoprecipitate the proteins from equal amounts of protein extracts from the retina (R) and the tectum (T) at the days of embryonic development indicated. The positions of molecular weight standards, in Kd, are indicated at left.
nasal retina. Indeed, at all developmental stages examined by immunoblotting, ephrin-A5 is predominantly expressed in the optic tectum rather than the retina, whereas ephrin-A6 is predominantly expressed in the retina (Fig. 3C). Interestingly, the developmental regulation of ephrin-A6 protein expression in the visual system follows a time course similar to that of ephrin-A5. Both ligands are present at high levels at E6 and E8, when retinal axons are growing toward their tectal targets (Goldberg, 1974). Ephrin-A5 and ephrin-A6 expression gradually decreases at later developmental stages and by E18, when retinotectal topography has been established, their expression is barely detectable.

Ephrin-A6 Is a High-Affinity Ligand for EphA4

To gather clues on the receptor binding properties of ephrin-A6, we stained whole E3 chicken embryos with an ephrin-A6 alkaline phosphatase fusion protein (ephrin-A6 AP). Rhombomeres 3 and 5 were prominently labeled (Fig. 4A). This result suggests that ephrin-A6 is a high-affinity ligand for EphA4, the Eph receptor known to be concentrated in rhombomeres 3 and 5 (Nieto et al., 1992). This initial observation was confirmed in pull-down experiments with an ephrin-A6 Fc fusion protein (Fig. 4B). Among the different Eph receptors present in E8 chicken retinal extracts, ephrin-A6 Fc bound EphA4 and also EphA3, but not EphB5. In comparison with ephrin-A6, ephrin-A5 Fc bound similar amounts of EphA4 and higher amounts of EphA3. To determine the affinity of ephrin-A6 for different EphA receptors, we performed equilibrium binding assays (Fig. 5). In these assays, different concentrations of ephrin-A6 AP were incubated with EphA receptor Fc fusion proteins immobilized on ELISA plates coated with anti-Fc antibodies. The apparent dissociation constants ($K_D$) measured in these experiments for the binding of ephrin-A6 to chicken and mouse EphA4 were $9.5 \times 10^{-10}$ and $7.9 \times 10^{-10}$ M, as expected for high-affinity interactions. In comparison, the $K_D$ for the binding of ephrin-A5 to EphA4 was $11 \times 10^{-10}$ M. Ephrin-A6 also exhibited high binding affinities (low $K_D$ values) for other EphA receptors that are expressed in the visual system, including EphA3, EphA5, EphA7, and EphA8, suggesting that ephrin-A6 may be a physiological ligand for some of these receptors. Ephrin-A6 exhibited a lower affinity for EphA6 ($K_D = 46 \times 10^{-9}$ M) and substantially lower but appreciable affinity for EphB2 ($K_D = 146 \times 10^{-8}$ M). Overall, the binding affinities of ephrin-A6 for different Eph receptors appeared similar to those of ephrin-A5 (Fig. 5).

In agreement with the affinity measurements, ephrin-A6 Fc caused increased phosphorylation on tyrosine of both EphA3 and EphA4 in cultures of retinal neurons (Fig. 6). This result confirmed that ephrin-A6 is an activating ligand for these EphA receptors. At the same concentration, ephrin-A6 Fc did not detectably activate EphB2 and EphB5. In an in vitro functional assay with retinal explants, ephrin-A6 Fc added to the cultures caused collapse of both temporal and nasal growth cones (Fig. 7). The percentage of collapsed growth cones was much higher in temporal (78%) than nasal (19%) growth cones, in agreement with the reported lower sensitivity of nasal axons for ephrin-A ligands.
FIG. 5. Ephrin-A6 binds with high affinity to EphA receptors expressed in the visual system. (A) Representative binding curves and corresponding Scatchard plots for the binding of ephrin-A6 AP to EphA receptors and to EphB2. (B) Summary of apparent $K_D$ values for the binding of ephrin-A6 and ephrin-A5 to EphA receptors and to EphB2. The values shown are averages of $K_D$ values calculated by nonlinear regression analysis of binding curves similar to those shown in (A). Apparent $K_D$ values are indicated in the table below, the number of binding experiments ($n$) used to calculate each $K_D$ is indicated in parentheses, and error bars represent standard errors.
**DISCUSSION**

Ephrin-A ligands have been implicated in the formation of various axonal projections, including the retinotectal (Cheng and Flanagan, 1994; Drescher et al., 1995; Feldheim et al., 2000, 1998; Frisen et al., 1998; Nakamoto et al., 1996), hippocamposeptal (Gao et al., 1996; Zhang et al., 1996), enthorinohippocampal (Stein et al., 1999), and thalamocortical (Gao et al., 1998) projections. They have also been proposed to regulate boundary formation between hindbrain segments (Xu et al., 1995); demarcation of boundaries and Purkinje cell compartments in the developing cerebellum (Karam et al., 2000; Rogers et al., 1999); formation of functional domains, layers, and circuits in the developing cortex (Castellani et al., 1998; Donohue and Rakic, 1999); and pathfinding of motor axons (Eberhart et al., 2000; Kury et al., 2000).

We have identified a new ephrin, ephrin-A6, which is highly expressed in the chicken embryonic nasal retina. Consistent with playing a role in the developing visual system, ephrin-A6 activates EphA receptors and causes growth cone collapse in cultured retinal neurons. Among the other five ephrins of the A subgroup, only ephrin-A2 and ephrin-A5 have been detected in the visual system (Cheng and Flanagan, 1994; Drescher et al., 1995; Feldheim et al., 2000). Ephrin-A2 and ephrin-A5 are the two most closely related ephrins (Fig. 2D), have partially overlapping distributions in several neural structures (Cheng and Flanagan, 1994; Drescher et al., 1995; Onschau et al., 1997; Zhang et al., 1996), and have redundant functions (Feldheim et al., 1998). Ephrin-A6, on the other hand, does not have especially high homology with ephrin-A2 or ephrin-A5 and has a distinctive expression pattern in the embryonic visual system. While ephrin-A2 and ephrin-A5 are predominantly expressed in the optic tectum, ephrin-A6 is predominantly expressed in retinal cells, including the axons of retinal ganglion cells. The differences between ephrin-A6 and ephrin-A2/A5 in amino acid sequence, expression patterns, receptor binding properties, and secreted versus cell surface-associated forms, suggest that ephrin-A6 has a role quite distinct from those proposed for ephrin-A2 and ephrin-A5.

**Ephrins in the Retina Regulate Retinotectal Topography**

Ephrin-A2 and ephrin-A5 expressed in the chicken optic tectum and in a region ventrocaudal to the optic chiasm control the topographic mapping of incoming retinal ganglion cell axons, presumably by activating a combination of EphA receptors present on the surface of these axons (Drescher, 1997; Flanagan and Vanderhaeghen, 1998; Marcus et al., 2000; O'Leary and Wilkinson, 1999). EphA receptors expressed in nasal retinal cells, however, appear to be also regulated by ephrin-A ligands that are coexpressed in the retinal cells (Dutting et al., 1999; Feldheim et al., 2000; Hornberger et al., 1999). It has been suggested that by causing constitutive EphA receptor activation, ephrin-A ligands present on the axons of nasal retinal ganglion cells render these axons less sensitive to the ephrin repellent...
Ephrin-A6 induces collapse of retinal growth cones. (A)
Percentages of collapsed growth cones in cultures of temporal and nasal retinal explants, following addition of 1 μg/ml clustered ephrin-A6-Fc or human Fc. Values represent the mean ± SEM from 7 explants (ephrin-A6 Fc, temporal); 7 explants (Fc, temporal); 6 explants (ephrin-A6 Fc, nasal); and 4 explants (Fc, nasal). (B) Growth cones from temporal retinal explants following addition of 1 μg/ml ephrin-A6-Fc or 1 μg/ml human Fc.

FIG. 7. Ephrin A6 induces collapse of retinal growth cones. (A) Percentages of collapsed growth cones in cultures of temporal and nasal retinal explants, following addition of 1 μg/ml clustered ephrin-A6-Fc or human Fc. Values represent the mean ± SEM from 7 explants (ephrin-A6 Fc, temporal); 7 explants (Fc, temporal); 6 explants (ephrin-A6 Fc, nasal); and 4 explants (Fc, nasal). (B) Growth cones from temporal retinal explants following addition of 1 μg/ml ephrin-A6-Fc or 1 μg/ml human Fc.

Several properties of ephrin-A6 point to a likely role as a mainly retinal expressed molecule that could, however, influence the growth of retinal fibers in the optic tectum. First, ephrin-A6 is preferentially expressed in the retina and its binding affinities for different Eph receptors are high and similar to those of ephrin-A5. Ephrin-A2 and ephrin-A5 have also been detected in the nasal retina and in the axons of nasal retinal ganglion cells (Brennan et al., 1997; Connor et al., 1998; Dutting et al., 1999; Hornberger et al., 1999), but these ephrins are expressed at much lower levels in the chicken embryonic retina than in the tectum (Fig. 3C) (Monschau et al., 1997). In fact, the expression level of ephrin-A5 in the chicken retina declines to low levels before the retinotectal projection is established (Fig. 3) [(Dutting et al., 1999) and our unpublished data].

Second, we have detected ephrin-A6 in the nerve fiber layer of the E8 chicken retina, where the axons of retinal ganglion cells are located. Hence, ephrin-A6 could influence the behavior of nasal retinal axons. The time course of ephrin-A6 expression in the retina parallels that of ephrin-A5 expression in the tectum: both ligands are highly expressed at E6, when retinal axons begin to grow on the tectal surface. Their expression remains substantial during the establishment of the retinotectal projection (E8 to E12) and drastically declines at the later developmental stages. This expression pattern is consistent with ephrin-A6 playing a role in the regulation of retinotectal topography. Third, in contrast to ephrin-A5, ephrin-A6 is strongly expressed throughout nasal retina and the boundary between high and low expression of ephrin-A6 in the retina coincides with the location of the optic fissure. Interestingly, in stripe assays there is a step transition in correspondence of the optic fissure between the responses of nasal and temporal retinal axons to membrane-bound tectal guidance cues (Rosentreter et al., 1998). Axons from areas just posterior to the optic fissure avoid stripes of posterior tectal membranes while axons just anterior to it are essentially insensitive to such cues even though they express EphA4 receptor. Hence, the pattern of expression of ephrin-A6 is
consistent with a role in modifying the guidance behaviors of nasal versus temporal retinal axons in the stripe assays and in vivo (Feldheim et al., 2000). In addition, our growth cone collapse experiments demonstrate that ephrin-A6 can indeed influence the behavior of retinal axons.

It is not known if ephrin-A6 has similar high expression in the mouse retina as in the chicken retina, but this appears likely. Labeling with EphA3 Fc or EphA5 Fc probes suggested that ephrin-A ligands are present in the mouse nasal retina (Feldheim et al., 2000; Marcus et al., 1996). Ephrin-A5 mRNA was detected in the embryonic mouse retina, but the level of expression appeared to be low and confined to the extreme nasal retina. Interestingly, nasal retinal axons from ephrin-A2/ephrin-A5 double knock out mice remain less sensitive than temporal axons to wild-type striped posterior tectal membranes (Feldheim et al., 2000), unlike axons of chicken nasal retina when deprived of GPI-linked molecules (Hornberger et al., 1999). Retinal axons of these knock out mice also retain some degree of anterior–posterior topographic organization in vivo (Feldheim et al., 2000). An explanation for both these observations could be that a third ephrin-A ligand, such as ephrin-A6, is still present in the visual system of the ephrin-A2/ephrin-A5 double knock out mice. As described above, ephrin-A6 may play a role in nasal retinal cells by increasing the basal levels of constitutive activation of the coexpressed Eph receptors. Ephrin-A6, however, may also act as a receptor-like molecule that transduces signals. Although this role of the A-ephrins is not yet well understood, signals mediated by GPI-linked ephrins have been shown to affect cell adhesion and movement (Chin-Sang et al., 1999; Davy et al., 1999; Wang et al., 1999).

Ephrin-A6 and Patterning of the Retina

The distribution of ephrin-A6 in the chicken retina is also consistent with a role for this new ligand in the establishment and/or stabilization of intraretinal organization along the anterior–posterior axis. The complementary expression of ephrin-A6 and EphA3, a receptor that is activated by ephrin-A6, could contribute to keep nasal and temporal retinal ganglion cells spatially separate. In fact, recent work has shown that in rhombomeres of the hindbrain adjacent populations of Eph receptor and ephrin expressing cells are prevented from intermingling by the repulsive signals generated upon receptor-ligand interaction (Mellitzer et al., 1999; Xu et al., 1999). In addition, unlike ephrin-A2 and ephrin-A5, which are confined to the nerve fiber layer (Fig. 3B and Hornberger et al., 1999), ephrin-A6 is expressed in all layers of the E8 nasal retina and, therefore, can influence the behaviors of other types of cells in the retina in addition to retinal ganglion cells. Because ephrin-A6 exhibits extensive overlap with EphA4, which is also expressed throughout the layers of the nasal retina at E6–E8 (Holash and Pasquale, 1995), ephrin-A6 expression in the retina produces two populations of retinal cells: a nasal population containing activated EphA4 and a temporal population containing inactive EphA4. The functional consequences of this pattern of EphA4 tyrosine phosphorylation in retina organization, however, remain to be determined.

Soluble Ephrin-A6

We have detected mRNAs encoding putative secreted forms of ephrin-A6 in the E8 chicken retina. Hence, in addition to GPI-linked ephrin-A6, secreted forms of this ligand may be produced as a result of gene splicing. Soluble forms of other ephrins have been previously detected in cell culture but presumably they are released from the cell surface through cleavage by proteases or phospholipases (Bartley et al., 1994; Bohme et al., 1996). Recent data have indeed shown that ephrin-A2 is cleaved from the cell surface by a metalloprotease activated following Eph receptor–ephrin interaction (Hattori et al., 2000). Secreted forms of ephrin-A6 could contribute to make retinal cells less responsive to ephrin guidance cues. In vitro stripe assays, for example, monomeric ephrin-A2 added to the culture medium inhibited retinal axon guidance without activating Eph receptors (Ciossek et al., 1998). Monomeric ephrin-A6 may also decrease the degree of receptor clustering induced by GPI-linked ephrins, which may change the effects of Eph receptor activation on integrin-mediated cell adhesion (Stein et al., 1998). Antibodies that distinguish GPI-linked and soluble ephrin-A6 will be required to determine the relative levels of expression, distribution, and developmental regulation of these different forms.

Three Ephrin-A Genes Are Differentially Expressed in the Visual System

As discussed above, recent studies have shown that Ephrin-A ligands have important functions in both the retina and the optic tectum. Here we show that at least three different ephrin-A genes are preferentially expressed in either the chicken retina or the tectum. Why is ephrin-A6 preferentially expressed in the retina and ephrin-A2/ephrin-A5 in the tectum, rather than the same genes being expressed in both structures? There are several possible reasons. First, the three ephrins may interact differently with the Eph receptors expressed in the visual system. Ephrin-A2 has been reported to bind to several EphA receptors with lower affinity than ephrin-A5 (Flanagan and Vanderhaeghen, 1998). In binding assays ephrin-A6 appears to have overall Eph receptor binding properties similar to those of ephrin-A5 but subtle differences between the Eph receptor binding specificities of ephrin-A5 and ephrin-A6 may exist and result in significantly different biological activities. Interestingly, differences in Eph receptor binding were observed when ephrin-A5 Fc and ephrin-A6 Fc were incubated with chicken retinal extracts. When competing for binding with the other Eph receptors present in retinal extracts, EphA3 associated more efficiently with ephrin-A5 than ephrin-A6. Second, secreted forms of ephrin-A ligands, such as the secreted forms of ephrin-A6, may be required in
the retina but not the tectum. Third, differentially regulating the spatial and temporal expression of several ephrin-A genes can yield complex ephrin-A expression patterns, which may be required for visual system patterning. Several pieces of evidence point to such complex spatial and temporal regulation of ephrins in the visual system (Cheng et al., 1995; Connor et al., 1998; Drescher et al., 1995; Dutting et al., 1999; Feldheim et al., 2000; Marcus et al., 1996, 2000). Fourth, even though A-ephrins expressed both on retinal axons and on tectal cells regulate formation of topographic maps, it may nevertheless be necessary to have different ephrin genes that are placed under different genetic control mechanisms in the retina versus the tectum. The existence of several A-ephrins that are differentially expressed in the visual system might have the advantage that they could be independently regulated by different transcription factors, affording more flexibility in the control of their expression in projecting (e.g., retina) versus target (e.g., tectum) regions.

In conclusion, ephrin-A6 is the newest member of the ephrin family, and our data suggest that it plays a distinctive role in the topographic organization of the visual system. As the families of molecules expressed in retinal and tectal cells, and along the visual pathway, continue to be identified, it becomes clear that complex signaling and adhesive interactions between nerve fibers and cells as well as between nerve fibers themselves are required to provide the sophisticated regulation necessary for visual structures to assemble. It will be important to determine the contribution of ephrin-A6 to this process. In addition, future studies will reveal whether ephrin-A6 is expressed outside the visual system and is required for the development of certain neural structures.

ACKNOWLEDGMENTS

The authors thank Robbin Newlin for immunohistochemical staining of sections, Rick Lindberg for the degenerate primers to ephrin-A ligands, and John Flanagan for the APTag-2 vector. This work was supported by NIH Grant EY10576 (E.B.P.).

REFERENCES


Xu, Q., All dus, G., Holder, N., and Wilkinson, D. G. (1995). Expression of truncated Sek-1 receptor tyrosine kinase disrupts...
the segmental restriction of gene expression in the Xenopus and zebrafish hindbrain. Development 121, 4005–4016.

Received September 14, 2000
Accepted October 31, 2000
Published online January 4, 2001