Reciprocal Expression of the Eph Receptor Cek5 and Its Ligand(s) in the Early Retina

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Recent evidence suggests that Eph receptor tyrosine kinases and their ligands provide positional information in the developing visual system. We previously found that the Eph receptor Cek5 is more highly expressed in the ventral than dorsal chicken embryonic retina. We now report the identification of a chicken ligand for Cek5 (cCek5-L) that is 75% identical to the ligand LERK2. In situ hybridization experiments do not reveal a dorsoventral gradient of cCek5-L transcripts in the optic tectum at Embryonic Day 8, suggesting that this ligand is not involved in guiding Cek5-expressing axons in the tectum. Surprisingly, it is in the retina that high levels of cCek5-L mRNA are present. In the early retina, cCek5-L is more highly expressed in the dorsal than the ventral aspect. Similarly, a Cek5 Ig chimera labels dorsal but not ventral retina, indicating that even if several Cek5 ligands are present, their overall distribution is complementary to that of Cek5. Hence, Cek5 and cCek5-L may both contribute to define anatomical compartments within the early retina. In contrast, in the 11-day embryonic retina the distributions of Cek5 and its ligand(s) show considerable overlap, suggesting changing functions as development progresses. In dissociated cultures of dorsal or ventral retinal cells seeded on plates coated with either receptor or ligand Ig chimeras, the interaction between Cek5 and its ligand(s) or cCek5-L and its receptor(s) is sufficient to mediate cell adhesion and allows neurite outgrowth.

INTRODUCTION

Numerous studies have established the importance of receptor protein–tyrosine kinases in the development of the embryonic nervous system. Members of the Eph family, the largest known family of receptor tyrosine kinases, are highly expressed in developing neural tissue (van der Geer et al., 1994), as are some of their ligands (Cheng and Flanagan, 1994; Bergemann et al., 1995; Carpenter et al., 1995; Fletcher et al., 1994a). Interestingly, some Eph receptors are distributed in manners that suggest their involvement in defining cell position both in peripheral tissues (Patel et al., 1996) and in tissues of the nervous system. For example, several Eph receptors are expressed in specific rhombomeres of the developing hindbrain, suggesting that they may help cells establish and/or maintain the segmental boundaries of rhombomeres (Nieto et al., 1992; Becker et al., 1994; Ganju et al., 1994; Ruiz and Robertson, 1994; Xu et al., 1995). Eph receptors are also likely to define subsets of neurons, as they are expressed in specific subpopulations of embryonic spinal cord motor neurons (Kilpatrick et al., 1996; Ohta et al., 1996) and retinal ganglion cells (Cheng et al., 1995; Holash and Pasquale, 1995).

The development of the visual pathway is a complex process that ultimately results in point to point retinotectal topographic connections in which axons from the ventral retina project to the dorsal optic tectum and axons from the dorsal retina project to the ventral tectum. Likewise, axons from the anterior retina project to the posterior tectum and vice versa (reviewed by Holt and Harris, 1993). The mechanisms that underlie topographic specificity are poorly understood, but recent evidence strongly supports the possibility that Eph receptors are involved. The Eph receptor Cek4 is present in an increasing anterior to posterior gradient in the retina, while in the optic tectum the anterior to posterior gradient of Cek4, which is expressed on the axons of the ingrowing retinal ganglion cells, is reversed (Cheng et al., 1995). Two ligands for Cek4, ELF-1 and RAGS, are more highly expressed in the posterior than the anterior portion of the retina.
of the optic tectum (Cheng et al., 1995; Drescher et al., 1995), and thus the distributions of these ligands are complementary to that of their cognate receptor Cek4. These expression patterns are consistent with a model in which a retinal axon recognizes its position in the tectum because it expresses a specific quantity of a given Eph receptor on its growth cone, and therefore it responds to the concentration of the cognate ligand in its environment. The interaction between receptor and ligand then regulates axon pathfinding within the optic tectum. Support for this hypothesis is provided by in vitro experiments, which demonstrate that RAGS and ELF-1 direct the growth of retinal ganglion cell axons in stripe assays (Drescher et al., 1995; Nakamoto et al., 1996). Furthermore, ELF-1 ectopically expressed in the tectum causes abnormal targeting of Cek4 expressing axons (Nakamoto et al., 1996).

We previously reported that the Eph receptor Cek5 is expressed in a polarized fashion in the developing chick retina, with higher expression in the ventral portion (Holash and Pasquale, 1995). Similar results have been reported for the mouse and quail homologs of Cek5, NuK and Qek5, respectively (Kenney et al., 1995; Henkemeyer et al., 1996). However, we had not detected dorsoventral differences of Cek5 protein expression in the optic tectum (Holash and Pasquale, 1995), leading us to suspect that the interaction between Cek5 and its ligands may not play a role in guiding retinal ganglion cell axons to their final tectal targets. To investigate the role of Cek5 and its ligands in the visual system, we have identified a chicken ligand closely related to the mammalian Cek5 ligand Cek5-L, also known as LERK2 (Beckmann et al., 1994; Shao et al., 1994) and compared its expression with that of Cek5 by using in situ hybridization. Because at least three distinct ligands have been shown to interact with Cek5, Cek5-L, HTK ligand (HTK-L, also known as LERK2) (Beckmann et al., 1994; Shao et al., 1994) and compared its expression with that of Cek5 by using in situ hybridization. Because at least three distinct ligands have been shown to interact with Cek5, Cek5-L, HTK ligand (HTK-L, also known as LERK2) (Bennett et al., 1995; Cerretti et al., 1995), and NLERK2 or Elk-L3 (Gale et al., 1996b; Nicola et al., 1996), and additional Cek5 ligands may exist, we also labeled sections of retina with a Cek5 extracellular domain Ig chimera to determine the collective protein distribution of Cek5 ligands. In addition we have examined the effects of a persistent interaction between Cek5 and cCek5-L in cultures of retinal cells.

MATERIALS AND METHODS

Cloning of the Chicken Ligand cCek5-L

An 11-day chicken embryonic retina cDNA library constructed in the vector λEX1lox (custom cDNA library, Novagen), was screened to isolate cCek5-L clones. A digoxigenin-labeled probe corresponding to nucleotides 58 to 1038 of mouse Cek5-L was prepared by PCR amplification. The mouse Cek5-L cDNA (Shao et al., 1994) was used as the template. The library was screened according to standard procedures for low stringency hybridization: probe hybridization was carried out at 37°C in a buffer containing 35% formamide, 0.75 M NaCl, 0.075 M sodium citrate, pH 7.0, 0.06% Ficoll, 0.06% polyvinylpyrrolidone, 0.06% BSA, 0.5% SDS, and 200 μg/ml sheared salmon sperm DNA. Five plaques among 20,000 plaques screened were identified as positive and subjected to several rounds of purification. Recombinant phage clones were then converted to plasmid subclones by cre-mediated excision according to the instruction of the manufacturer (Novagen). Sequencing and restriction mapping showed that all five clones represent fragments of the same cDNA. Three of the clones contain distinct but mostly overlapping inserts of approximately 1.8 kb, while two contained distinct inserts of approximately 0.4 kb corresponding to the 3’ end of the cDNA sequence. The longest clone was completely sequenced on both strands to obtain the sequence of the chicken Cek5-L shown in Fig. 1A.

Preparation of Recombinant Ig Chimeras

The sequences encoding the extracellular domains of Cek5 (amino acids 19–541) and chicken Cek5-L (amino acids 22–226) were amplified by PCR with specific primers containing a NheI restriction site (sense primer) and a BamHI restriction site (antisense primer). After sequencing the amplified products were cloned into a NheI- and BamHI-digested pcDNA3 vector containing the sequences encoding the signal peptide of CD5 (to the 5’ end of the NheI site) and the Fc portion of human Ig G1 (to the 3’ end of the BamHI site), as previously described (Shao et al., 1994). The expression plasmids were then stably transfected into 293T cells by the calcium phosphate method, using a transfection kit from Gibco BRL and according to the instructions of the manufacturer. The recombinant Ig chimeras were purified from the cell culture supernatants using Affi-Gel protein A (Bio-Rad) as described previously (Shao et al., 1994) and stored frozen in aliquots in PBS containing 1 mM CaCl2. The preparation of an Ig chimera containing the N-terminal 25 amino acids of mature B61, which was used as a control, was as described (Shao et al., 1994).

Retinal Cultures

To examine the behavior of retinal cells in culture, the retinae were removed from embryos at 8 days in ovo (for substrate studies) or 7 days in ovo (for ligand activation studies). After removal, eyes were separated into dorsal and ventral halves and the neural retina was separated from the retinal pigmented epithelium and washed in PBS. For ligand activation studies, only the ventral retina was used. The harvested retinae were incubated in trypsin/EDTA for 5 min at 37°C, after which culture medium (Medium 199 containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM pyruvic acid) was added and the suspensions were triturated with a fire-polished Pasteur pipet. Following dissociation, cells were pelleted and resuspended in culture medium. This resulted in cell suspensions which contained single cells as well as small clumps of cells.

For ligand activation studies, cells from the retinae of one embryo (two eyes) were divided into 12–16 wells of a 24-well tissue culture plate and cultured at 37°C. Several hours after plating, medium was removed and replaced with medium containing only 0.5% serum. After serum starvation for about 16 hr, cells were stimulated with the Cek5-L Ig chimera for various periods of time. Following ligand treatment, wells were rinsed with PBS and then the cells were collected in 0.025 M NaCl, 0.01 M sodium phosphate, pH 7.2, 1% deoxycholate, 1% Triton X-100, and 0.1% SDS with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.2 ttryp-
A sense probe was prepared from pBluescript KS. Immunoprecipitation

Antibodies

Diluted 1:50 in 3% BSA. The Ig chimeras were Embryos were fixed in 4% paraformaldehyde and embedded in 30 min at room temperature with a biotinylated goat anti-human Ig chimera or the cCek5-L Ig chimera at a concentration of 0.25 μg/ml was added to the wells. After incubating for 4 hr at 4°C, wells were washed with PBS and retinal cell suspensions were added. The cells from dorsal or ventral retinae from one embryo (two eyes) were distributed into 8–16 wells and cultured at 37°C. The behavior of the cells was observed 1 hr after plating and again the next day. The cultures were then fixed with 4% paraformaldehyde. Both living and fixed cells were photographed using phase-contrast microscopy.

**Ig Chimera Immunohistochemistry**

White leghorn chicken embryos were incubated to the appropriate stage of development, and embryos were embedded in OCT compound and frozen unfixed in 2-methylbutane chilled with dry ice. Sections that were 10–20 μm thick were collected on poly-L-lysine-coated slides and then dipped in 70% ethanol and air-dried. Sections were blocked with 1.5% normal goat serum in PBS and incubated for 60 min at 4°C. Sections were washed several times, sections were fixed in 4% paraformaldehyde, washed, and incubated for 30 min at room temperature with a biotinylated goat anti-human antibody (Vector) diluted 1:50 in 3% BSA. The Ig chimeras were visualized by using the Vectastain avidin–biotin technique (Vector). After reacting in diaminobenzidine for approximately 2–3 min, the slides were washed, air-dried, and mounted in Permount for photography.

**Antibodies**

Two different anti-Cek5 antibodies were used, which have previously been described (Pasquale, 1991; Holash and Pasquale, 1995). Anti-Cek5(897–995) antibodies, which were used for immunoblotting, were prepared using a glutathione S-transferase fusion protein comprising amino acids 897–995 of Cek5. These antibodies were affinity purified with the same fusion protein coupled to N-hydroxy-succinimide-activated agarose (Bio-Rad). Anti-Cek5(163–995) antibodies, which were used for immunoprecipitation, were prepared using a β-galactosidase–Cek5 fusion protein comprising amino acids 163–995 of Cek5 (Pasquale, 1991). These antibodies were affinity-purified using the β-galactosidase–Cek5 fusion protein and absorbed on a β-galactosidase affinity column. The anti-phosphotyrosine antibodies used have been described previously (Pasquale et al., 1994).

**Immunoprecipitation**

Lysates from cultured cells were precleared with Staph A (Boehringer-Mannheim) and incubated for 40 min with 10–20 μg of anti-Cek5(163–995) antibodies absorbed on Staph A. The immunoprecipitated material was washed three times with RIPA buffer and once with PBS. SDS-containing sample buffer was added and the immunoprecipitates were boiled for 5 min.

To assess Cek5 binding to the CcCek5-L Ig chimera, 11-day retinæ were sonicated in PBS containing protease inhibitors (see above). Sonicated material corresponding to one retina was incubated for 1 hr at 4°C with either 5 μg of chicken CcCek5-L Ig chimera or 10 μg of nonimmune rabbit IgG complexed with Staph A. After washing, the immunoprecipitated material was eluted from the Staph A pellet after boiling for 5 min in 0.1% SDS and diluted 1:10 with RIPA buffer. Since direct probing of Ig chimera immunoprecipitates produced high background in immunoblots, a second immunoprecipitation with anti-Cek5(163–995) antibodies was performed as described above for the cell culture extracts.

**Immunoblotting**

Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). After transfer, the filters were blocked for 1 to 16 hr in 3% BSA in TBS (Tris-hydroxymethyl aminomethane-buffered saline) and then incubated for approximately 4 hr at room temperature, or overnight at 4°C, with 3 μg/ml of anti-phosphotyrosine antibodies. After several washes, the filters were incubated with 0.2 μg/ml protein–A peroxidase (Sigma) in TBS containing 3% BSA for 1 hr. The filters were then washed and developed using enhanced chemiluminescence reagents (Amersham). The filters were then air-dried, incubated in 3% BSA containing 0.2% sodium azide, and reprobed with anti-Cek5(897–995) antibodies at a concentration of 3 μg/ml.

**In Situ Hybridization**

Embryos were fixed in 4% paraformaldehyde and embedded in paraffin. Five to 2-μm sections were cut with a microtome and mounted on Fisher Superfrost Plus slides. In situ hybridization was performed according to standard procedures (Simmons et al., 1989). Briefly, deparaffinized sections were deproteinized in 0.2 M HCl and treated with 2.5 μg/ml protease K. After acetylation and dehydration in ethanol, the sections were air-dried. The sections were then incubated overnight with the [33P]labeled riboprobes (approximately 105 dpm/μl) in hybridization buffer (300 mM NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1× Denhardt's, 0.2% SDS, 10 mM DTT, 0.25 mg/ml tRNA, 50% formamide, 10% dextran sulfate) at 50°C. Following hybridization, the slides were immersed in 4× SSC (150 mM NaCl, 15 mM Na2 citrate, 2 H2O), followed by two 15-min washes in 2× SSC at 61–63°C, RNase A digestion, one 30-min wash in 0.1× SSC at 63°C, and one 10-min wash at room temperature. After dehydration the sections were air-dried, exposed to X-ray film for 2 to 4 days, and then dipped in photographic emulsion and exposed for 2 to 4 weeks prior to development. The developed slides were then counterstained with cresyl violet and photographed.

The sense and antisense riboprobes for Cek5 were prepared by using a template to Cek5 fragment (nucleotides 302 to 572) amplified by PCR and cloned in both orientations in the pCRII vector (Invitrogen). The antisense chicken Cek5-L probe was prepared by using a Cek5-L fragment (nucleotides 301–602) amplified by PCR and cloned in the pCRII vector as a template. The corresponding sense probe was prepared from the cDNA of Cek5(163–995) containing a BamHI to EcoRV fragment from the pCRII cCek5-L vector. After linearization of the plasmids with BamHI (Cek5) or HindIII (cCek5-L), the riboprobes were synthesized with T7 RNA polymerase using a Promega in vitro transcription system and [32P]UTP, according to the specifications of the manufacturer for the preparation of high-specific activity riboprobes, with some modifications. The modifi-
cations were as follows: the reaction volume was reduced from 20 to 11.5 μl, and spin columns (CHROMA SPIN-10, Clontech) were used to remove unincorporated nucleotides.

RESULTS

Cloning of Chicken Cek5-L

To identify the chicken homolog of mammalian Cek5-L, which would then allow us to prepare RNA probes for in situ hybridization experiments, we screened an 11-day chicken embryo cDNA retina library at low stringency with a mouse Cek5-L DNA probe. Although the probe used includes the sequences encoding the cytoplasmic domain, which is highly conserved in Cek5-L and two other transmembrane ligands, HTK-L and NLERK2 (Nicola et al., 1996), all the clones isolated correspond to the same gene. We have designated this gene cCek5-L (chicken Cek5 ligand). Since five distinct positive clones were identified after screening only 20,000 plaques, cCek5-L is presumably abundantly expressed in the 11-day chicken embryo retina. Three of the clones isolated contain the entire open reading frame, and the sequence of the longest clone, with the corresponding deduced amino acid sequence, is shown in Fig. 1A. cCek5-L contains two hydrophobic stretches of amino acids, presumably representing a signal peptide and a membrane spanning domain. Four cysteines and a single consensus site for N-linked glycosylation are present in the extracellular domain. cCek5-L exhibits a high degree of similarity with mouse Cek5-L (75% overall amino acid identity), human LERK2 (76% amino acid identity), human HTK-L (55% amino acid identity), and human NLERK2 (36% amino acid identity) (Fig. 1B). The phylogenetic relationships, deduced on the basis of sequence similarities, between Cek5-L and the two other known transmembrane ligands for Eph receptors are shown in Fig. 1C. cCek5-L belongs to the same subgroup as the mammalian species homologs of LERK2/Cek5-L, which is distinct from the LERK5/HTK-L and NLERK2 subgroups.

cCek5-L Binds to and Activates Cek5

A chimeric molecule composed of the extracellular domain of the newly identified cCek5-L fused to a human Ig domain was prepared for receptor binding and activation studies. To confirm that the newly identified ligand binds to Cek5, the cCek5-L Ig chimera was used to immunoprecipitate Cek5 from extracts of 11-day embryonic retina. As shown in Fig. 2A, the recombinant ligand efficiently bound Cek5 present in the retinal extract.

To further determine whether the chicken ligand that we cloned exhibits the properties expected of a bona fide ligand for Cek5, the cCek5-L Ig chimera was used to treat cultures of dissociated cells from ventral retina. Receptor tyrosine kinase ligands typically cause rapid autophosphorylation on tyrosine of their cognate receptors. This is followed at later times by receptor internalization and degradation, which likely serve to attenuate the signals generated (van der Geer et al., 1994; Ullrich and Schlessinger, 1990). As expected, phosphorylation on tyrosine of Cek5 was already apparent 5 min after addition of ligand at a concentration of 2.5 μg/ml and reached maximal levels after 10 to 15 min (Fig. 2B). Cek5 remained phosphorylated on tyrosine for as long as 24 hr after addition of the recombinant ligand (Fig. 2B). A marked downregulation of the level of Cek5 receptor was also apparent, beginning at 1 hr after addition of Cek5-L.

Reciprocal Compartmentalization of cCek5-L and Cek5 in the Retina but Not in the Optic Tectum at Embryonic Day 8

To determine whether cCek5-L exhibits a polarized distribution in the optic tectum that may influence target recognition of Cek5 expressing retinal axons, we have used in situ hybridization to compare its expression levels in dorsal and ventral tectum at Embryonic Day 8. At this stage in development retinal ganglion cell axons have begun to reach the optic tectum. The intensity of the cCek5-L signal that we detected was low and similar in regions of the dorsal and ventral tectum which, based on cresyl violet staining, are apparent, beginning at 1 hr after addition of Cek5-L.

In the retina at Embryonic Day 8, cCek5-L mRNA is concentrated in the dorsal aspect (Figs. 4A, 4C, 4D, and 4E), and thus its distribution is complementary to that of the Cek5 mRNA (Figs. 4B, 4G, 4H, and 4I). This distribution is apparent in both sagittal (Fig. 4A) and frontal (Fig. 4C) sections through the chicken embryo head. Outside the visual system, a striking concentration of cCek5-L transcripts was noticed in a subregion of the extreme anterior part of the brain (Fig. 4C).

Since the possibility exists that Cek5 ligands other than cCek5-L are expressed in the developing retina, sections of retina obtained from 4- to 8-day-old embryos were labeled with a chimeric molecule consisting of the extracellular portion of Cek5 fused to a human Ig domain. This chimera is expected to bind to all of the Cek5 ligands, whether known or not yet identified, and therefore was used to deter-
FIG. 1. Chicken Cek5 ligand (cCek5-L) sequence and homologies with the other transmembrane ligands of the B61 family. (A) The putative signal peptide sequence and transmembrane domain are underlined, and the possible site of N-glycosylation is marked by a dotted overline. Amino acids and nucleotides are numbered at right. (B) The ligand sequences were aligned using the program CLUSTAL. The signal peptides and transmembrane domains are underlined, conserved cysteines in the extracellular receptor-binding domain are indicated by the symbol *, and the position of introns in the mouse Cek5 ligand (Eplg2; Fletcher et al., 1994b) is indicated by large black dots above the sequences. Small dots replace residues that are identical to the corresponding residue in cCek5-L, and hyphens represent gaps introduced in the sequences to aid the alignment. Amino acids are numbered at left. cCek5-L, chicken Cek5 ligand; mCek5-L, mouse Cek5 ligand; hLERK2, human LERK2; hHTK-L, human HTK ligand; hNLERK2, human NLERK2. (C) Phylogenetic tree for the transmembrane ligands of the B61 family. The tree was constructed using the program CLUSTAL. The GenBank accession number for the cCek5-L sequence is U72394.

mine the overall distribution of ligands for Cek5. Consistent with our in situ hybridization results, the Cek5 Ig chimera preferentially labeled the dorsal portion of the retina both at Embryonic Day 4 (Fig. 5A) and at Embryonic Day 8 (Figs. 5C and 5D), presumably reflecting a high concentration of the ligands in the dorsal portion of the retina.

Colocalization of cCek5-L and Cek5 in the 11-Day Embryonic Retina

In the more differentiated retina of the 11-day chicken embryo, ligands for Cek5 become concentrated in the forming inner plexiform layer, as determined by using the Cek5

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Cek5 Ligands Are Polarized in the Retina

FIG. 1—Continued

Ig chimera (Fig. 5E). Immunostaining of the Embryonic Day 11 retina with anti-Cek5 antibodies demonstrated that at this stage of development Cek5 is expressed in the nerve fiber layer and inner plexiform layer throughout the 11-day retina (Pasquale et al., 1994), albeit at somewhat lower levels in the dorsal retina than in the ventral retina (not shown). Thus, in contrast to the earlier developmental stages, during retina differentiation Cek5 and its ligand(s) have overlapping patterns of expression, particularly in the inner plexiform layer. In agreement with this, Cek5 is highly phosphorylated on tyrosine (activated) at Embryonic Day 11 (Pasquale et al., 1994).

Cek5-L and Cek5 transcripts are also similarly distributed in the Embryonic Day 11 dorsal retina (Figs. 6D and 6E and Pasquale et al., 1994). Like Cek5, Cek5-L mRNA is expressed in neurons, particularly those whose cell bodies...
are in the inner portion of the inner nuclear layer (amacrine cells) and in the retinal ganglion cell layer. However, we have not determined whether Cek5 and cCek5-L are coexpressed in the same cells or present in different cells of the same type. The absence of cCek5-L transcripts in the optic nerve (Fig. 6B) indicates that cCek5-L is not expressed in those glial cells whose cell bodies are within the optic nerve.

**Effects of cCek5-L and Cek5 as Culture Substrates for Retinal Cells**

We examined the effects of the interaction between Cek5 and cCek5-L in cultures of retinal cells by plating dissociated Embryonic Day 8 dorsal or ventral retinal cells on surfaces coated with either the cCek5-L Ig chimera or the Cek5 Ig-coated surfaces (Shao et al., 1994). When observed the following day, ventral retinal cells appeared more spread in the wells that had been coated with ligand (Fig. 7E) than in those that had been coated with receptor (Fig. 7F), whereas dorsal retinal cells showed a higher degree of attachment and spreading on receptor-coated plates (Figs. 7G and 7H). Over time cell-cell contacts also developed, causing the formation of large clumps of cells, particularly in the absence of interaction with the substrate (Figs. 7F and 7G). Neurites were detectable in 24-hr cultures of ventral retinal cells plated on ligand and dorsal retinal cells plated on receptor, suggesting that prolonged interaction between receptor and ligand allows neurite extension. This is important with regard to the 11-day embryonic retina, where the colocalization of ligand and receptor in the inner plexiform layer suggests that they may interact in vivo for extended periods during development.

**DISCUSSION**

A notable feature of visual system development is the formation of both short-range neuronal projections within
FIG. 4. cCek5-L mRNA expression in the chicken retina at Embryonic Day 8. Sagittal (A and B) or frontal (C–J) sections through chicken embryo heads were hybridized with antisense RNA probes to cCek5-L (A, C–E) or Cek5 (B, G–I). cCek5-L is more highly expressed in the dorsal than the ventral retina. A region of dorsal retina (F) hybridized with a cCek5-L sense probe demonstrates that the labeling in (D) is specific. Unlike cCek5-L, Cek5 message is more highly concentrated in the ventral than the dorsal retina. Labeling of ventral retina with Cek5 sense probe (J) demonstrates the specificity of the labeling. (C) and (G) show autoradiographs of hybridized sections, while the remaining panels are dark-field photographs of retina developed in photographic emulsion. Arrows in (A) and (B) and arrowheads in (C) and (G) mark the ventral region of the eye. The bright line marking the outer portion of the retina in the dark-field photographs (A, B, D–F, and H–J) is the retinal pigmented epithelium, which is nonspecifically bright; re, retina; t, tectum. In the panels showing dorsal retina (D, F, and H) the pigmented epithelium is at the top, while in the panels showing ventral retina (E, I, and J) the pigmented epithelium is at the bottom. The intense labeling above the eyes in (C) corresponds to the anterior portion of the forebrain. Scale bars: (B) = 500 μm, (C) = 2 mm, (H) = 100 μm.

the retina or optic tectum and long-range projections connecting the retina to the optic tectum. Many molecules with polarized expression along the axes of the retina and/or the optic tectum have been identified (reviewed in Kaplanian and Patterson, 1994). The characterization of these molecules will help elucidate the molecular mechanisms governing pattern formation in the visual system. Recently, members of the Eph family of receptor tyrosine kinases and their ligands have been proposed to define functional compartments within the embryonic nervous system, thus contributing to the establishment of anatomical boundaries. In addition, they appear to regulate axon fasciculation and
Fig. 5. Cek5 Ig chimera labeling of the retina. An Ig chimera containing the extracellular domain of Cek5 was used to label vertical sections of Embryonic Day 4 (A), Day 8 (C and D), and Day 11 (E) retina. A control chimera was used to label the section of retina shown in (B). The Cek5 Ig chimera preferentially labeled the dorsal retina at Embryonic Days 4 (A, top) and 8 (C), whereas labeling in the ventral retina was very low (A, bottom) or undetectable (D). At Embryonic Day 11 (E), the chimera preferentially labeled the inner plexiform layer in the dorsal retina. The pigmented epithelium is at the top in the dorsal retina shown in (C and E) and at the bottom in the ventral retina shown in (D). Scale bars: (A) (for panels A and B) = 150 µm; (D) for panels (C–E) = 100 µm.

Cek5 Ligands Are Polarized in the Retina

It is in the early retina that Cek5 and its ligand(s) display complementary restricted distributions. These distributions are consistent with the compartmentalization that others have recently observed for Eph receptors and their ligands (Gale et al., 1996a,b) and suggest that both Cek5 and cCek5-L provide cells in the retina with information regarding their dorsal or ventral location and contribute to maintaining their compartmentalization. In addition, our previous work suggests that Cek5 provides positional information in the optic nerve as well, where it is highly expressed in only about half of the retinal ganglion cell axons (Holash and Pasquale, 1995). This restricted expression of Cek5 in the optic nerve indicates that this receptor may define boundaries between groups of axons leaving the retina and therefore operates in the organization of axons in the optic nerve. The importance of ordering of the ganglion cell axons in the retina and optic nerve is poorly understood; however, axons in the optic nerve of the chicken are clearly organized according to a stereotyped pattern (Rager et al., 1988; Thanos and Bonhoeffer, 1983).

The results of Cheng et al. (1995), Drescher et al. (1995), and Nakamoto et al. (1996) suggest that some Eph receptors and their ligands are involved in establishing anterior–posterior topography in the tectum, but expression of the ligands in the retina or optic nerve has not been reported until now. It is possible that the mechanisms that govern anterior–posterior topography in the visual system are distinct from those that govern dorsoventral topography. Alternatively, the function of Cek5 and its ligand(s) may be restricted to the retina and possibly the optic nerve, while additional receptor–ligand pairs may be involved in establishing dorsoventral topography in the tectum. Because the Eph family is so large, and redundancy exists in receptor–ligand interactions (Davis et al., 1994; Brambilla et al., 1995; Bergemann et al., 1995), it is plausible that a complex set of interactions involving several receptor–ligand pairs governs the formation of topographic mapping along both tectal axes as well as pattern formation within the retina and optic tectum. In support of this hypothesis, mRNA encoding an Eph receptor, Ebk, is more highly concentrated in the anterior than in the posterior mouse tectum (Ellis et al., 1995).

Although a number of Eph receptors bind the ligand Cek5-L and additional ligands for Cek5 exist (Bergemann et al., 1995), the adhesive properties that we observed in dissociated cells from the dorsal ventral retina plated on Cek5 or cCek5-L Ig chimeras are consistent with a compartmentalized expression of functional Cek5 ligands in the dorsal retina and of functional cCek5-L receptors in the ventral retina. Furthermore, the interaction of Cek5 with dorsally expressed ligand(s) or of cCek5-L with ventrally expressed receptor(s) is sufficient to affect the behavior of retinal cells. Interestingly, the identification of Cek5-L was based on a cellular adhesion assay (Shao et al., 1994), and a recent report indicates that stable cell–cell contacts are mediated by the interaction of cells expressing the ligand LERK2 with cells expressing the Eph receptor Hek2 (Böhme...
FIG. 6. cCek5-L is expressed in neurons in the visual pathway at Embryonic Day 11. Sections of retina and optic nerve obtained from 11-day chicken embryos were hybridized with cCek5-L antisense (B and D) or sense (C) probes and with Cek5 antisense (E) or sense (F) probes. (A) is a bright-field view of (B), stained with cresyl violet. In the retina, both cCek5-L and Cek5 antisense probes strongly label the ganglion cell layer (arrow) and the inner portion of the inner nuclear layer, which contains amacrine cells and is located more towards the retinal pigmented epithelium. (D, E, and F) represent portions of dorsal retina. The optic nerve (on) in (B) is not significantly labeled above control levels (C), suggesting that cCek5-L is not expressed in the glial cells that are located in the optic nerve. Labeling in the retinal pigmented epithelium, which appears as a black line in (A) and as a white line in (B, D, and E) is nonspecific, as can be seen in (C). Scale bar in (A) = 150 μm.

et al., 1996). Our adhesion assay also indicates that neither Cek5 nor its ligand are able to mediate strong homophilic interactions, unlike some other receptor tyrosine kinases containing Ig and fibronectin type III repeats in their extracellular domains (Bellosta et al., 1995).

The relative distributions of Cek5 and its ligand(s) change as the retina differentiates, suggesting that they have different functions during retinal development. Early in retinal development, Cek5 and cCek5-L have complementary distributions and therefore may function primarily to establish boundaries between different anatomical regions, as previously reported for the Eph receptor Sek1 in the hindbrain (Xu et al., 1995). Our in vitro experiments suggest that interactions between cCek5-L-expressing cells and Cek5-expressing cells, which presumably occur at the boundary between dorsal and ventral retina, result in intracellular signaling as well as cell adhesion. Increased tyrosine phosphorylation of Cek5 and, as recently shown, cCek5-L (Holland et al., 1996) may generate signals that inhibit cell migration across the dorsal–ventral boundary, whereas the formation of stable cell–cell contacts may prevent cells that have crossed the boundary from migrating further into the opposite compartment. Hence, it is conceivable that both cell adhesion and repulsive signaling effects cooperate to maintain cell position along the dorsal–ventral axis of the early retina. As development progresses, Cek5 and cCek5-L are colocalized in the inner plexiform layer and therefore may regulate the interactions and/or targeting of neuronal processes growing within the inner plexiform layer. The persistent enhanced phosphorylation on tyrosine of Cek5 that is observed in the retina both in vivo after Embryonic Day 10 (Pasquale et al., 1994) and in cultured retinal cells treated with ligand (Fig. 2) is consistent with the colocalization of receptor and ligand that we observed in the 11-day retina.

Unlike cCek5-L, which in the 11-day chicken embryonic retina is expressed in neurons, the ligand AL-1 (which is the mammalian homolog of chicken RAGS), is thought to be expressed in glial cells (Drescher et al., 1995; Winslow et al., 1995). The interaction of AL-1 with one of its receptors, Rek7, appears to cause axons to leave the glial surface and fasciculate, perhaps as a result of repulsive effects (Winslow et al., 1995). Additionally, the ligands RAGS and ELF-1 have a repulsive effect on retinal ganglion cell axons (Drescher...
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FIG. 7. Effects of the interaction between Cek5 and cCek5-L in cultured retina cells. After approximately 1 hr in culture, dissociated cells from the ventral retina show greater attachment to surfaces coated with a cCek5-L Ig chimera (A) than they do to surfaces coated with a Cek5 extracellular domain Ig chimera (B). In contrast, cells isolated from the dorsal retina attach preferentially to receptor-coated surfaces (D) than to ligand-coated surfaces (C). After 24 hr in culture, cells from the ventral retina are attached and spread on the ligand-coated plates (E), but not on the receptor-coated plates (F), and cells from the dorsal retina are attached and spread on the receptor-coated plates (H), but not on the ligand-coated plates (G). Cells in (F) and (G) preferentially adhere to each other than to the substrate, forming large clumps. Cells in (E) and (H) extend neurites, some of which are indicated by arrows. Cells in (C) are out of focus because they float in the cell culture medium. Scale bar in (H) = 60 μm.

et al., 1995; Nakamoto et al., 1996). Biological effects mediated by repulsion or adhesion may prove to be fundamental differences between the mechanisms of action of the two groups of ligands for Eph receptors, membrane-bound such as Cek5-L and GPI-linked such as AL-1/RAGS and ELF-1. However, our in vitro assay did not address the short-term effects of ligand receptor interaction on growth cone behavior, nor did it provide axons with a choice between substrates. Therefore, an alternative possibility is that the short-term effects of cCek5-L on growth cones may be similar to those of RAGS and ELF-1 and result in transient growth cone collapse (Drescher et al., 1995), while a prolonged interaction between ligands and Eph receptors, such as that presumably occurring between Cek5 and cCek5-L in the inner plexiform layer of the retina, may regulate cell adhesion and axon fasciculation rather than causing growth cone repulsion.

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