Retinoic acid regulates the expression of dorsoventral topographic guidance molecules in the chick retina

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Summary

Asymmetric expression of several genes in the early eye anlagen is required for the dorsoventral (DV) and anteroposterior (AP) patterning of the retina. Some of these early patterning genes play a role in determining the graded expression of molecules that are needed to form the retinotectal map. The polarized expression of retinoic acid (RA) activity is asymmetrically distributed along the DV axis in the retina, suggesting that it may play a patterning role. RA signaling was found to play a role in regulating the expression of EphB2, EphB3 and ephrin B2, three molecules whose graded expression in the retina along the DV axis is important for establishing the correct retinotectal map. Blocking RA signaling by misexpression of a RA degrading enzyme, Cyp26A1 recapitulated some but not all the effects of DNhRARα. It also was found that Vax, a ventrally expressed transcription factor that regulates the expression of the EphB and ephrin B molecules, functions upstream of, or in parallel to, RA. Expression of DNhRARα led to increased levels of RA-synthesizing enzymes and loss of RA-degrading enzymes. Activation of such compensatory mechanisms when RA activity is blocked suggests that RA homeostasis is very strictly regulated in the retina.

Key words: Retinoic acid, Dorsoventral, EphB, Ephrin B, Patterning, Retinotectal map

Introduction

Establishment of the DV and AP axes constitutes a fundamental step in eye development. Several transcription factors and signaling molecules expressed asymmetrically in the early retina are key to establishing the AP and DV axes of the retina: e.g. Vax (Barbieri et al., 2002; Mui et al., 2002; Schulte et al., 1999) and Ventroptin (Sakuta et al., 2001) in the ventral retina, and Tbx5 and BMP4 (Koshiba-Takeuchi et al., 2000) in the dorsal retina. In addition to these early DV molecules, retinoic acid (RA) activity is asymmetrically distributed along the DV axis in the retina, suggesting that it may play a patterning role. The presence of a dorsal and a ventral zone of RA activity in the retina, separated by a middle zone that is free of RA, is conserved among vertebrate species such as zebrafish, chick and mice (Marsh-Armstrong et al., 1994; McCaffery et al., 1999; Mey et al., 1997). The two zones of RA activity are determined by the expression of two RA-synthesizing enzymes, RALDH1 in the dorsal retina (McCaffery et al., 1992; McCaffery et al., 1991) and RALDH3, formerly known as RALDH6, in the ventral retina (Li et al., 2000; Mic et al., 2000; Suzuki et al., 2000). The mid-zone is free of RA owing to the expression of multiple members of the Cyp26 family of RA-degrading enzymes, including Cyp26A1, Cyp26B1 and Cyp26C1 (Fujii et al., 1997; MacLean et al., 2001; Ray et al., 1997; Reijntjes et al., 2003; Reijntjes et al., 2004; Swindell et al., 1999; Tahayato et al., 2003). Retinoic acid signal transduction is initiated by the binding of RA to the heterodimer of retinoic acid receptor (RAR) and retinoid X receptor (RXR) (Kastner et al., 1997). This is followed by binding of the receptor-ligand complex to the DNA response elements of RA-responsive genes (Umesono et al., 1991). Retinoic acid has been demonstrated to play a crucial role in the development of many tissues in the embryo, including the eye. Embryos that develop under conditions of vitamin A deficiency are often born with microphthalmia or anopthalmia (Kalter and Warkany, 1959). In zebrafish, RA activity is important for formation of the ventral eye (Hyatt et al., 1996b; Marsh-Armstrong et al., 1994), and later in the patterning/differentiation of photoreceptors (Hyatt and Dowling, 1997; Hyatt et al., 1996a). Knockout mice generated for either of the two RA-synthesizing enzymes in the retina, RALDH1 or RALDH3, did not produce any changes in expression of early DV patterning genes in the retina (Dupe et al., 2003; Fan et al., 2003). In the RALDH1 knockout mice, compensation by other RA-synthesizing enzymes, such as RALDH3, expressed in the dorsal RPE and dorsal lens at E10.5 (Li et al., 2000; Mic et al., 2000), may account for the lack of an eye-patterning phenotype. Several of the retinoic acid receptors in mice have been mutated either singly or in some combinations. In mice where multiple retinoic acid receptors have been knocked out,
there were severe defects in the eye, e.g. in the lens, cornea, conjunctiva and sclera (reviewed by Mark and Chambon, 2003). In the compound mutants for Rarb2 and Rarg2, there was retinal dysplasia and degeneration (Grondona et al., 1996), which may be due to underlying patterning defects. Analysis of patterning defects per se has not been carried out in these mutants.

In the neural retina, the early and patterned expression of genes such as Vax, Tbx5, Bmp4 and Ventroptin provides spatial cues to the retinal ganglion cells such that their axons project in an orderly fashion to higher brain centers, e.g. the superior colliculus (SC) in mammals and the optic tectum (OT) in birds (Crossland et al., 1974). Ganglion cells next to each other in the retina send projections to neighboring regions in the SC/OT, giving rise to a topographic map. Retinal ganglion cells originating in the dorsal retina project to the lateral tectum and those from the ventral retina send projections to the medial tectum. Similar order is maintained along the AP axis to give rise to the retinotectal map (reviewed by McLaughlin et al., 2003a).

Positional values must be assigned to ganglion cells in order for them to form an accurate topographic map. In his chemoaffinity model, Sperry suggested that this process is guided by AP and DV expression gradients of molecules (Sperry, 1963). These were later found to be the Eph family of receptor tyrosine kinases in the retina, and similar rostrocaudal and mediolateral expression gradients of their ligands, the ephrins, have been found in the SC/OT (Simon and O’Leary, 1992a; Simon and O’Leary, 1992b; Simon and O’Leary, 1992c; Wilkinson, 2001). Members of the EphA family of receptor tyrosine kinases in the retina and the ephrin A family members expressed in the tectum (Cheng et al., 1995; Drescher et al., 1995) determine retinotectal projections along the AP axis (Feldheim et al., 2000; Sakurai et al., 2002; Yates et al., 2001). The receptors of EphB family, EphB2 and EphB3, are expressed asymmetrically in a ventral-to-dorsal gradient in the retina (Braisted et al., 1997; Connor et al., 1998; Holash and Pasquale, 1995) and the ligand ephrin B1 is expressed in a mediolateral gradient in the optic tectum (Braisted et al., 1997). Recent experiments using misexpression studies with ephrin B1 in the chick optic tectum (McLaughlin et al., 2003b), and the study of Ephb2 and Ephb3 double knockout mice (Hindges et al., 2002), have demonstrated the importance of these molecules for correct DV mapping of the ganglion cells onto the SC/OT.

Very little is known about how the graded expression of the Eph receptors and ephrin molecules are established within the retina. The early DV patterning genes such as Vax and Tbx5 have been demonstrated to play a role in regulating the DV expression gradient of the EphB receptors and the ephrin B ligands (Barbieri et al., 2002; Mui et al., 2002; Schulte et al., 1999). The asymmetric distribution of RA activity along the DV axis and the timeline of expression of the RA-synthesizing and -degrading enzymes suggest that RA might also play a role in the regulation of the expression gradient of the EphB receptors and the ephrin B ligands. The onset of expression of patterning genes such as Vax and Tbx5 is at HH stage 14 (Koshiba-Takauuchi et al., 2000; Schulte et al., 1999). The expression of ephrin B1 and ephrin B2 is detected at HH stage 18-19 in the retina (Braisted et al., 1997; Peters and Cepko, 2002), and the expression of EphB2 and EphB3 can be detected in the ventral retina by HH stage 24 (Holash and Pasquale, 1995; Peters and Cepko, 2002). The timeline of expression of the RA-synthesizing enzymes RALDH1 and RALDH3, and Cyp26A1, the RA-degrading enzyme, is as follows. RALDH1 is faintly detected in the dorsal eye region at HH stage 14 and subsequently expression becomes stronger by HH stage 16 (Suzuki et al., 2000). RALDH3 is first detected in the ventral eye region at HH stage 12, but only in the surface ectoderm. By HH stage 14, strong expression could be seen in the ventral eye in the presumptive neural retina, pigment epithelium and surface ectoderm (Blentic et al., 2003; Suzuki et al., 2000). Cyp26A1 expression is restricted to dorsal lens alone at HH stage 18 and then is detected in a horizontal stripe across the retina at E6 (Blentic et al., 2003). However, another member of the family, Cyp26B1, is detected in a horizontal stripe across the middle of the retina as early as HH stage 18 (Reijntjes et al., 2003). Thus, RA-synthesizing and -degrading enzymes are expressed at a time and place that is consistent with RA signaling playing a role in regulating the DV topographic guidance molecules.

Redundancy among the various receptor subtypes and the RA-synthesizing enzymes has made it difficult to study the effects of loss of RA activity on patterning in the retina by conventional loss of function studies. In this study, some of these difficulties were surmounted by using a replication-competent avian virus vector, RCAS, to express a dominant-negative form of the human retinoic acid receptor α (DNhRARα) in the chick eye. This method of blocking RA activity in the retina allowed initial eye development to progress to a certain degree, providing an opportunity to study the role of RA in early eye development. It was observed that expression of DNhRARα led to loss of expression of the EphB2 and EphB3 receptor tyrosine kinases in the ventral retina, and of ephrin B2, one of the dorsally expressed EphB molecules within the retina. In addition to this, it was found that expression of Vax was unchanged by loss of RA activity. However, misexpression of Vax in the retina led to loss of expression of the dorsal RA-synthesizing enzyme, RALDH1, and ectopic expression of the ventral RA-synthesizing enzyme, RALDH3, in the dorsal retina. This indicates that Vax may regulate expression of EphB2/B3 and ephrin B2 by acting in parallel to, or upstream of, RA activity in the retina.

Materials and methods

Chick embryos

Fertilized White Leghorn eggs (SPAFAS, Norwich, CT) were incubated at 38°C for the times indicated. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Retroviral constructs and the lacZ reporter construct

The insert from pRS-hRARα, a gift from Ronald M. Evans (Damm et al., 1993) was PCR-amplified using GCAGAAGACCCCATGGC-CAGCAACAGCAGCTCTCT as the 5’ primer and CCGAAATTCCACATTTCCTCGGATGAGAGGCGG as the 3’ primer. The PCR product was digested with BbsI and EcoRI, and subcloned into the NcoI/EcoRI sites of pSlax21 to generate pSlax21-DNhRARα. The CdiI fragment of pSlax21-DNhRARα was further subcloned into the RCASBp(A) vector (Morgan and Fekete, 1996; Petopoulos and
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Development

RNA in situ hybridization

Flat-mount in situ hybridization was performed as described previously (Bruhn and Cepko, 1996) with some modifications (Chen and Cepko, 2002). Double in situ hybridization was carried out with digoxigenin-labeled RNA probes for specific markers and the fluorescein-labeled probe specific for RCAS to detect virally infected cells. The probes labeled with digoxigenin were first detected with NBT/BCIP until the desired purple signal developed. This was followed by incubation with AP-conjugated anti-fluorescein antibody. The second in situ hybridization signal was detected with BCIP alone until the desired blue color developed. In some cases the second probe for RCAS was not used; instead, immunostaining was performed with 3c2 mAb recognizing the viral Gag protein, based on the protocol described previously (Chen and Cepko, 2002) to identify virus-infected regions of the retinae.

Results

Expression of DNHRARα in chick embryonic DF-1 cells blocks RA signaling

A dominant-negative allele of the human RARs was created by Damm et al. (Damm et al., 1989). It is truncated at the level of amino acid 403 and thus lacks a region of the C-terminal domain, which is proposed to be involved in transcriptional activation (Damm et al., 1989; Zenke et al., 1990). This truncated receptor is capable of binding to its heterodimeric partner, RXR, and can bind to the retinoic acid response element (RARE) present in the upstream regions of genes regulated by RA signaling (Damm et al., 1993). A replication competent avian retrovirus, RCAS, was used to transduce this gene (RCAS-DNHRARα). Prior to injecting this virus in the chick eye, it was necessary to determine if this human gene was capable of blocking RA signaling in chick cells. Cells from the chick embryonic cell line DF-1 were co-transfected with RCAS-DNHRARα and RARE-lacZ, a reporter for RA activity (Wagner et al., 1992). This reporter consists of the β-galactosidase gene under the control of the RA-binding site (RARE) and the basal promoter from RARβ. When an assay for β-galactosidase was performed with DF-1 cells transfected with the RARE- lacZ reporter alone, they turned blue (Fig. 1A). By contrast, those co-transfected with the RARE-lacZ reporter and DNA for RCAS-DNHRARα did not turn blue (Fig. 1B).

Expression of DNHRARα in the chick retina leads to reduction in eye size

Microphthalmia has been observed in mammalian embryos born from females fed with a diet deficient in vitamin A (Hale, 1937; Wilson, 1953). Thus, a reduction in eye size might occur when RA activity is blocked by expression of DNHRARα in the chick retina. Such a phenotype would be an additional means of confirming that the DNHRARα functions in the chick retina. When the eyes of chick embryos injected with RCAS-DNHRARα at HH stage 17 were compared at E7, the injected right eye was observed to be significantly smaller than the
uninjected left eye. This phenotype was observed in >20 injected embryos of which one is shown in Fig. 2A. In some animals, the injected eye size was as small as one half of the control eye size. In addition, the ventral fissure (white arrowhead) appeared to be wider and more distinct in the injected eye than in the contralateral uninjected eye (Fig. 2A). This smaller eye phenotype was apparent from E5 and the severity of the phenotype correlated with the extent of viral infection in the retina as assayed by staining with the 3c2 antibody against viral Gag protein. The small eye phenotype as well as all other phenotypes observed when injecting the RCAS-DNhRAR\(\alpha\)/H9251 virus were the same regardless of whether the stage of injection was HH stage 10 or HH stage 17. The only difference was in the total area of the retina that was infected, with a greater extent of infection in those infected at HH stage 10. Thus, mostly retinae injected at HH stage 17 are shown in the figures in order to allow for a comparison of infected and uninfected regions within the same retina.

A reduction in eye size could be caused either by increased cell death or decreased rate of cell proliferation. A TUNEL assay was performed on sections of embryonic day 7 (E7) chick retinae from eyes injected with RCAS-DNhRAR\(\alpha\). TUNEL-positive cells were not found in the infected or uninfected regions of the retina (data not shown). This suggests that the size reduction in the retina is not primarily due to increased cell death. In order to discover if changes in cell proliferation might account for the smaller eyes, explant cultures from nine different E7 retinae, R1 to R9, injected in vivo at HH stage 10 with RCAS-DNhRAR\(\alpha\), were labeled with \(^{3}\)H thymidine for 4 hours. Retinal explants were then dissociated into single cells and immunohistochemistry was performed with 3c2 antibody against the viral Gag protein to identify infected cells (Fig. 2B,D). The number of \(^{3}\)H thymidine-labeled cells (cells containing at least 10 grains per cell) among the infected, 3c2+ population (green bar and dark blue bars) as well as the uninfected, 3c2− population (red bars and light blue bars). The data for RCAS-DNhRAR\(\alpha\) are from nine infected retinae and the data for RCAS-GFP are from six infected retinae.

### Table 1. Percentage of infected cells (3c2+) and uninfected cells (3c2−) that were labelled with \(^{3}\)H thymidine in retinae infected with RCAS-DNhRAR\(\alpha\) and RCAS-GFP

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Mean=15.23 Mean=9.42 Mean=18.1 Mean=18.8
s.d.=2.37 s.d.=2.245 s.d.=0.748 s.d.=1.15

Student’s t-test (type 2, tail 1): \(P=1.98378 \times 10^{-7}\).
of the infected cells were labeled (Table 1 and Fig. 2C, light-blue and dark-blue bars). Thus, RCAS-DNhRARα-infected cells were significantly reduced in proliferation \((P<0.0001)\). When several rounds of cell division are taken into consideration, this difference is likely to be sufficient for the observed reduction in eye size.

**Blocking RA signaling in the retina alters expression of molecules that direct retinotectal projections along the DV axis**

In several vertebrate species, there is a zone of RA signaling in the dorsal and ventral retina. These zones are separated in the middle by a zone which is free of RA. Infection with RCAS-DNhRARα in the chick retina was carried out to determine if the expression of genes that were asymmetrically expressed along the DV axis might be RA dependent. Some of the candidate genes examined for expression changes were the EphB family of tyrosine kinase receptors and their ligands, the ephrin B molecules. The EphB family members, EphB2 and EphB3, are expressed in a ventral high to dorsal low gradient (Fig. 3A,E) in the retina in both chick and mouse (Braisted et al., 1997; Hindges et al., 2002; Holash and Pasquale, 1995). Two ephrin B family members, ephrin B1 and ephrin B2, which serve as ligands for the EphB receptors, also are expressed, but in reciprocal dorsal high to ventral low gradients in the retina (Fig. 3I,M) (Braisted et al., 1997). Chick embryos at HH stage 17 were injected in the right eye alone with RCAS-DNhRARα, and allowed to develop to E7. The retinae from the right eyes of injected animals were harvested, flat-mounted and in situ hybridization was performed to detect expression changes in ephrin B1, ephrin B2, EphB2 and EphB3. Expression of DNhRARα led to an absence of expression of EphB2, EphB3, ephrin B2 and ephrin B1 in uninfected and RCAS-DNhRARα infected retinae. In situ hybridization carried out on flat-mounted E7 retina either uninjectected or injected with RCAS-DNhRARα. Retinae shown with dorsal towards the top, ventral towards the bottom, anterior (nasal) towards the left and posterior (temporal) towards the right. In situ hybridization of uninjected E7 retina, with EphB2 (A), EphB3 (E), ephrin B2 (I) and ephrin B1 (M). Double in situ hybridization of RCAS-DNhRARα injected E7 retina with EphB2 and RCAS probes (B-D). Magnified view of the boxed area from B showing EphB2 expression (purple signal, C) and RCAS infection (blue signal, D). RCAS-DNhRARα injected E7 retina with double in situ hybridization for EphB3 and RCAS probes (F-H). Magnified view of the boxed area in F showing EphB3 expression (purple signal, G) and RCAS-infected area (blue signal, H). RCAS-DNhRARα-injected E7 retina with double in situ hybridization for ephrin B2 and RCAS probes (J-L). Magnified view of boxed area in J showing ephrin B2 expression (purple signal, K) and RCAS-infected area (blue signal) (L). RCAS-DNhRARα injected E7 retina with double in situ hybridization for ephrin B1 (purple signal, N) and RCAS (blue signal, O). The white arrows indicate infected areas with subtle downregulation of ephrin B1 expression.
Development phenotype caused by DNhRAR/H9252 (DNcTR2) was chosen as a control to determine if the signal.

was observed within the infected areas (Fig. 3D,H, blue expression of both EphB2 (Fig. 3B-D) and EphB3 (Fig. 3F-H) not that of ephrin B1. In the ventral retina, absence of a significant role in regulating the expression of ephrin B2, but not that of ephrin B1. In the ventral retina, absence of expression of both EphB2 (Fig. 3B-D) and EphB3 (Fig. 3F-H) was observed within the infected areas (Fig. 3D,H, blue signal).

A dominant-negative form of thyroid hormone receptor β2 (DNCTRβ2) was chosen as a control to determine if the phenotype caused by DNhRARα might be produced by indirect effects on thyroid hormone (TH) signaling. DNCTRβ2 was constructed by deleting a single amino acid residue within the ligand-binding domain. A similar mutation in the human thyroid hormone receptor has been demonstrated to give rise to a dominant-negative form (Banaihmad et al., 1992; Usala et al., 1991). The ability of this construct to function as a TH-reporter construct, DR4- lacZ, whereas almost no cells with blue nuclei were observed among the cells transfected with DR4- lacZ+RCAS- DNCTRβ2 (Fig. 4I).

RNA-mediated signaling is mediated through heterodimers of RA receptor (RAR) and retinoid X receptor (RXR), whereas TH signaling is mediated through heterodimers of the thyroid hormone receptor (TR) and RXR (Kliwer et al., 1992). Thus, there is a possibility that the DNhRARα could block TH signaling by sequestering all available RXR molecules in a cell. For example, when DF-1 cells were co-transfected with either RARE-lacZ (RA-reporter) + RCAS- DNCTRβ2 or with DR4-lacZ (TH-reporter) + RCAS-DNhRARα, it was observed that very few to none of the cells turned blue (data not shown), indicating that both RA signaling and TH signaling can be blocked by each construct.

In order to ascertain whether the effects on expression of EphB2, EphB3 and Ephrin B2 could be mediated by blocking thyroid hormone signaling, RCAS-DNCTRβ2 was introduced into the chick retina. Infection with RCAS-DNCTRβ2 did not alter the expression patterns of EphB2 (Fig. 4A,B), EphB3 (Fig. 4C,D), ephrin B1 (Fig. 4E,F) or EphB2 (Fig. 4G,H). Infection with RCAS-DNCTRβ2 also did not lead to alteration of expression of the two RA-synthesizing enzymes RALDH1 and RALDH3 (data not shown), indicating that there is no interaction between the RA signaling and TH pathway at this level.

Expression of a RA-degrading enzyme, CYP26A1, can reproduce some, but not all, of the effects of DNhRARα

In order to investigate the role of RA signaling in regulating the expression of EphB2, EphB3 and ephrin B2 by an independent approach, RA signaling was reduced by expression of a RA-degrading enzyme, Cyp26A1. Retinas were harvested at E7 from chick embryos injected with RCAS-Cyp26A1 at HH stage 10. Infection with RCAS-Cyp26A1 in the ventral retina produced no change in expression of EphB2 (Fig. 5C,G, white arrowhead) or EphB3 (Fig. 5D,H, white arrowhead). However, in the dorsal retina loss of expression of ephrin B2 (Fig. 5A, black arrow) was observed within RCAS-Cyp26A1-infected regions (Fig. 5E, black arrow), but no change in expression of ephrin B1 was observed (Fig. 5B,F, white arrowhead). The changes observed in the dorsal retina were very similar to the effects of expression of DNhRARα. The normal expression pattern of Cyp26A1 in the chick retina is a midline stripe along the DV border (Fig. 5K, between the white arrows) with very low level...
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The differential effects of RCAS-Cyp26A1 on ephrin B1 and ephrin B2 may be due to the fact that the ventral border of expression of ephrin B1 extends further ventrally than that of ephrin B2 in the wild-type retina (Fig. 5I,J, white arrows) such that it overlaps with the expression domain of CYP26A1. In the ventral retina, EphB2 and EphB3 expression was not affected by RCAS-Cyp26A1. When DF-1 cells were co-transfected with either RARE-lacZ alone, RARE-lacZ + RCAS-DNhRARα or RARE-lacZ + RCAS-Cyp26A1 (Fig. 5L,M,N), DNhRARα was able to completely block the reporter, although there were a few blue cells (Fig. 5N) with Cyp26A1. As this experiment was carried out in vitro, in the presence of RA levels in excess of that in the retina, it suggests that Cyp26A1 may not be as efficient at blocking RA signaling as DNhRARα.

The expression of RALDH1, RALDH3 and Cyp26A1 is altered by expression of DNhRARα

There are four known retinaldehyde dehydrogenase enzymes that synthesize RA (Grun et al., 2000; Lee et al., 1991; Li et al., 2000; Lin et al., 2003; Suzuki et al., 2000; Zhao et al., 1996) and three members of the cytochrome P450-linked oxidase family (CYP26) that degrade RA (Abu-Abed et al., 2002; Fuji et al., 1997; MacLean et al., 2001; Ray et al., 1997; Tahayato et al., 2003; Taimi et al., 2004; White et al., 2000). Of the synthesizing enzymes, RALDH1 and RALDH3 are expressed in the retina in spatially restricted domains in the dorsal and ventral retina, respectively. The reduction of RA signaling by RCAS-DNhRARα may lead to activation of homeostatic mechanisms to restore RA levels. Thus, the expression of the two RA-synthesizing enzymes and Cyp26A1 were assayed following infection with RCAS-DNhRARα. Upregulation of RALDH1 within virus-infected areas in the dorsal retina (Fig. 6A,D,G,J) and upregulation of RALDH3 within virus-infected areas in the ventral retina (Fig. 6B,E,H,K) were observed. The upregulation of RALDH1 was restricted to its normal domain of expression in the dorsal retina (Fig. 6A,D, arrowhead), even if there was viral infection in the ventral retina (Fig. 6D, arrow). The upregulation of RALDH3 was similarly restricted to its normal ventral domain (Fig. 6B,E, arrowhead; Fig. 6E, arrow). Cyp26A1 is expressed at high levels in an equatorial stripe running across the DV border in the retina with very low levels of expression outside this central domain. Cyp26A1 expression was downregulated in virus-infected areas (Fig. 6C,F,I,L) both within its normal central domain of expression as well as in other regions of the retina where there was virus infection.

Vax regulates the expression of RA-synthesizing enzymes, but RA activity is not required for expression of Vax

In the chick retina, the homeodomain-containing transcription factor Vax is expressed at HH stage 14 in the ventral retina. Misexpression of Vax in the chick retina has been demonstrated to cause loss of expression of both ephrin B1 and
ephrin B2 in the dorsal retina, and lead to ectopic expression of EphB2 and EphB3 in the dorsal retina (Schulte et al., 1999). In order to understand the epistatic relationship between Vax and RA with respect to regulation of EphB and ephrin B, the expression of Vax and another early patterning gene, Tbx5, was examined following expression of DNhRARα. The ventral retinal expression of Vax (Fig. 7A,B), as well as the dorsal retinal expression of Tbx5 (Fig. 7C,D) appeared to be unchanged within regions of virus infection.

In order to examine the possibility that Vax regulates the expression of the ephrin B and EphB molecules in the chick retina through RA, the effects of misexpression of Vax on the expression of RALDH1 and RALDH3 were determined. E6 retinae were harvested from chick embryos injected with RCAS-Vax at HH stage 10, and in situ hybridization was performed for RALDH1 and RALDH3. Vax misexpression led to downregulation of the dorsal RA-synthesizing enzyme RALDH1, (Fig. 7I,J) and ectopic expression of the RALDH3 enzyme in the dorsal retina within the virus-infected areas (Fig. 7E-H).

**Discussion**

**Retinoic acid signaling is essential for expression of EphB2, EphB3 and ephrin B2 in the chick retina**

The fact that RA plays a fundamental role in eye development has been appreciated for many decades. However, previous studies have only identified an early function of RA activity in development of the DV axis in the eye. This has been demonstrated in zebrafish, where altering RA levels has a profound effect on development of the ventral retina (Hyatt et al., 1996b; Marsh-Armstrong et al., 1994). The extent to which RA influences the formation of the ventral retina is variable in other vertebrate species. In mice, some of the strains with RA receptor mutations (Mark and Chambon, 2003) and loss of RALDH3 (Dupe et al., 2003) have a shortened ventral retina. In chick, perturbing RA activity had no effect on the formation of the ventral retina (Mey et al., 2001). However, the division of the retinal DV axis into several zones of RA activity suggests that, in addition to being required very early for development of the ventral retina in some species, RA activity plays a role in other aspects of development along the DV axis. In this study, the RCAS viral vector delivered DNhRARα primarily to the retina and presumably blocked RA signaling by any RAR, providing a means to overcome lethality and/or redundancy problems encountered by previous gene targeting studies in mice. This approach permitted successful identification of later functions of RA in the retina.

Signaling through RA receptors was shown to be required for the expression of the EphB2 and EphB3 receptor tyrosine kinases in the ventral retina.

**Fig. 6.** Expression of RALDH1, RALDH3 and Cyp26A1 on retinae infected with RCAS-DNhRARα. Flat-mounted E7 retina oriented with dorsal towards the top, ventral towards the bottom, anterior (nasal) towards the left and posterior (temporal) towards the right, injected with RCAS-DNhRARα, on which double in situ hybridization was carried out with the following probes: RALDH1 (purple signal, A,D,G) and RCAS (blue signal, D), RALDH3 (purple signal, B,E,H) and RCAS (blue signal, E) and Cyp26A1 (purple signal, C,F,L) and RCAS (blue signal, F,L). White arrowhead (A) indicates area of upregulation of RALDH1 within its normal domain of expression. Black arrowhead (B) indicates upregulation of RALDH3 within its normal domain of expression. Black arrowheads indicate RCAS-infected regions within the normal domain of expression. Black arrows indicate RCAS-infected regions outside the normal domain of expression of RALDH1 (D) and of RALDH3 (E). (G,J) Magnified views of boxed region in A showing upregulated RALDH1 expression (G) and RCAS infection (J); (H,K) magnified views of boxed region in B showing upregulated RALDH3 expression (H) and RCAS infection (K); (I,L) magnified views of C and F showing loss of Cyp26A1 expression (I) and RCAS infection (L). Uninjected E7 retinae showing RALDH1 (M) and RALDH3 (N) expression domains.
ratina, and also for ephrin-B2 in the dorsal retina. The fact that EphB-ephrin B signaling specifies retinotopic mapping along the DV axis has been demonstrated by several studies. Recent studies with mice carrying mutations in both Ephb2 and Ephb3 receptors have shown that EphB forward signaling controls topographic mapping of the retinal DV axis along the lateromedial axis of the superior colliculus (Hindges et al., 2002). Other studies in Xenopus have also shown that EphB-ephrin B interactions are responsible for retinotopic mapping. However, in this case, ephrin B reverse signaling is dominant (Mann et al., 2002). It is postulated that such signaling may also contribute to retinotopic mapping in higher vertebrates, especially for the dorsal RGC axons. A retina completely infected with DNhRARα has almost no, or extremely low, expression of EphB2, EphB3 and ephrin B2 (data not shown). This is not unlike the double mutant mice for EphB2 and EphB3, and therefore would be expected to have a similar phenotype with respect to mapping DV retinal projections along the lateromedial axis of the tectum. Thus, RA signaling is important for establishment of the DV retinotopic map through regulating the expression of the EphB2, EphB3 and ephrin B2.

The independent approach of using RCAS-Cyp26A1 to lower the levels of RA signaling in the retina produced effects on EphB2 and EphB3 that were different from those obtained with RCAS-DNhRARαs. With RCAS-Cyp26A1, there was no change in the expression of EphB2 and EphB3. This difference might be explained by the existence of lower levels of RA in the dorsal retina when compared with the ventral retina, as determined by HPLC (Mey et al., 1997). This is due to differences in substrate range and enzyme kinetics between the dorsal RA-synthesizing enzyme RALDH1 and the ventral RA-synthesizing enzyme RALDH3 (McCaffery et al., 1992). Lowering of RA levels by Cyp26A1 in the ventral retina might be compensated for by the higher RA-synthesizing activity of RALDH3, perhaps leading to a minor reduction in RA signaling, and thus no effect on the expression of EphB2 and EphB3. The fact that Cyp26A1 maybe less efficient at blocking RA signaling compared with DNhRARαs is demonstrated by the inability of RCAS-Cyp26A1 to completely turn off the RA-reporter in DF-1 cells in the presence of excess RA.

**Perturbations of RA activity in the retina lead to activation of homeostatic mechanisms to maintain RA levels**

Retinoic acid is required for the morphogenesis, organogenesis and differentiation of many tissues during development (Kalter and Warkany, 1959). In order to achieve this, it is likely that appropriate levels of RA need to be strictly maintained, with adjustments in RA levels made according to local requirements. Given the fact that both deficiency of, and excess of, RA have profound effects on development, it might be predicted that compensatory mechanisms are active in the developing embryo to regulate RA levels. We found that when there is a significant block in RA signaling by expression of the DNhRARα receptor, upregulation of the RA-synthesizing enzymes RALDH1 and RALDH3 occurred. The domains of upregulation of each enzyme were within their normal field of expression, suggesting that there are other spatially restricted factors that regulate the expression of RALDH1 and RALDH3. In addition, expression of DNhRARα led to downregulation of Cyp26A1, the RA-degrading enzyme. This is not surprising because the studies that first identified Cyp26A1 showed that its expression was increased by RA (Ray et al., 1997; White et al., 2000). The expression of Cyp26B1, another member of this family, is also regulated by RA (Reijntjes et al., 2003).

Expression of DNhRARα leads to conditions that are somewhat different when compared with deficiency of vitamin A. In the case of RA deficiency, the ligand is missing. By contrast, DNhRARα blocks transcription through formation of
inactive RAR-RXR heterodimers that cannot activate target genes. Thus, even if compensatory mechanisms are recruited to increase the levels of RA, the block in transcription of target genes will not be overcome. Yet, compensatory mechanisms appear to be activated to try to maintain optimal RA signaling. This underscores the importance of RA homeostasis in developing and mature tissues.

**Relationship of RA activity to other DV patterning genes in the retina**

The patterning of the AP and DV axes of the retina and retinotectal topographic mapping are intrinsically linked to each other. Some of the transcription factors expressed asymmetrically in the retina to define its axial polarity, such as Vax and Tbx5, have been implicated in the regulation of expression of topographic guidance molecules, e.g. EphB and ephrin B family members. In the Vax2 knockout mouse, the expression of ephrin B1 and ephrin B2 are expanded into the ventral retina, while there is loss of expression of EphB2 and EphB3 in the ventral retina (Barbieri et al., 2002; Mui et al., 2002).

Observations made after blocking RA signaling in the retina indicate that RA also plays an important role in regulating the expression of some of these guidance molecules. Thus, Vax may function either upstream of or in parallel to RA signaling to regulate the expression of ephrin B2, EphB2 and EphB3. The epistatic relationship between RA activity and some of the early DV axis determining genes in the retina was investigated in this study. Blocking RA activity in the retina did not alter the dorsal expression of Tbx5 or the ventral expression of Vax. This might have been predicted given that Vax and Tbx5 are expressed prior to RALDH1, RALDH3 and Cyp26 in the retina. However, misexpression of Vax in the retina alters the expression of the RA-synthesizing enzymes RALDH1 and RALDH3. Vax misexpression downregulates the expression of RALDH1 in the dorsal retina and leads to ectopic expression of the ventral enzyme RALDH3 in the dorsal retina. These observations could be consistent with the two models described below.

**Model 1**

The transcription factors Vax and Tbx5 function through RA to regulate the expression of EphB2, EphB3 and ephrin B2 in the retina. According to this model, Vax regulates the expression of EphB3 and EphB2 in the ventral retina by turning on the expression of RALDH3, which produces RA. Vax misexpression downregulates the expression of RALDH1 in the dorsal retina and leads to loss of expression of RA. Ephrin B2. Misexpression of Vax in the dorsal retina leads to loss of expression of RALDH1. This could be due to a direct inhibition of RALDH1 by Vax or it could act through downregulation of Tbx5, which has previously been reported (Schulte et al., 1999). Thus, Vax misexpression leads to loss of ephrin B2 expression because there is no RALDH1 in the dorsal retina.

**Model 2**

Vax regulates the expression of EphB2 and EphB3 in the ventral retina and Tbx5 regulates ephrin B2 in the dorsal retina, by acting in parallel to RA. According to this model, both Vax and RA activity are required in the ventral retina for expression of EphB2 and EphB3. Thus, in the absence of either Vax or RA activity, EphB2 and EphB3 are not expressed. This would be possible because both Vax and RA independently exerted positive effects on the enhancers for these genes (Fig. 8B). In the dorsal retina, RA activity and Tbx5 would act independently to positively regulate the expression of ephrin B2. Missing either factor would lead to loss of ephrin B2 expression. When Vax is misexpressed in the dorsal retina, it downregulates ephrin B2 expression through downregulating Tbx5 and/or RALDH1.

![Fig. 8. Models describing the two possible modes of regulation of expression of EphB2, EphB3 and ephrin B2 by RA, Vax and Tbx5. Model 1: Vax and Tbx5 function in the dorsal and ventral retina to regulate the expression of ephrin B2, EphB2 and EphB3 exclusively through the RA synthesizing enzymes, RALDH1 and RALDH3. Model 2: Vax and Tbx5 function independently of RA to regulate expression of EphB2, EphB3 and ephrin B2. According to this model, both Tbx5-dependent and RA-dependent positive inputs are required to express ephrin B2 in the dorsal retina. The enhancer regions of EphB2 and EphB3 would require positive inputs from both Vax and RA for their expression in the ventral retina.](image-url)
If the first model is true, then the effects of Vax on the expression of EphB2, EphB3 and ephrin B2 have to be mediated only through regulation of RA activity. In that case, there would have to be some intrinsic difference between the RA activity in the dorsal retina and the ventral retina to account for the expression of the ephrin B molecules in the dorsal retina versus the EphB molecules in the ventral retina. The only known difference in RA activity between the dorsal and the ventral retina is in the source of RA synthesis, which is RALDH1 in one case and RALDH3 in the other. There are two differences between RALDH1 and RALDH3, one is in their ability to synthesize the different forms of RA and the second one is in their efficiency at synthesizing RA. RALDH1 has the ability to synthesize both 9-cis RA and all-trans RA (Labrecque et al., 1995), whereas RALDH3 can only synthesize all-trans RA (Yoshida et al., 1998). The retinoid X receptor (RXR), which is the heterodimeric partner for the retinoic acid receptor (RAR) can also signal by forming RXR-RXR homodimers that exclusively uses 9-cis RA as a substrate (Mangelsdorf et al., 1995). This raises the possibility that the regulation of ephrin B2 expression is mediated through 9-cis RA binding to RXR-RXR homodimers, while EphB2 and EphB3 expression, might be regulated by all-trans RA binding to RAR-RXR heterodimers.

Two facts argue against the use of 9-cis RA binding to RXR-RXR homodimer to regulate the expression of ephrin B2: (1) when the levels of the various forms of RA, all-trans RA, 9-cis RA and 13-cis RA, were measured by HPLC at various stages of development in the chick retina, no significant levels of 9-cis RA were detected at any stage (Mey et al., 1997); and (2) if the first model were to be true then mis-expression of RALDH1 in the ventral retina may be sufficient to turn on the expression of ephrin B2 ectopically. Experiments carried out to misexpress RALDH1 in the ventral retina failed to turn on ephrin B2 ectopically in that location (see Fig. S1 in the supplementary material). The other major difference between RALDH1 and RALDH3 is that RALDH3 is much more efficient than RALDH1 and therefore can act faster and at lower concentrations of the substrate. This difference could account for a large difference in gene expression.

Although we cannot completely discount Model 1, Model 2 most probably describes the mechanism by which EphB2, EphB3 and ephrin B2 are regulated. The enhancer regions of EphB2 and EphB3 may have sites that are regulated positively by Vax and also possibly have other sites positively regulated by RA either directly or indirectly. The presence of Vax, as well as RA activity, is required to activate transcription of EphB2 and EphB3, which does not happen in the absence of either factor. In the case of ephrin B2, the enhancer region may contain sites that are positively regulated by Tbx5 and other sites positively regulated by RA; the presence of both would be necessary to express ephrin B2. Further characterization of the enhancer regions of these guidance molecules to identify factors that directly bind to their enhancer regions and regulate their expression should shed more light on this. This could possibly resolve the intricate web of interactions between the early patternting genes such as Vax, Tbx5 and RA and any downstream factors that may mediate their function by regulating the expression of the topographic guidance molecules.

Potential role of RA in other aspects of retinal patterning and development

The functional significance of the different ventral borders of expression of the two ephrin B molecules expressed in the dorsal retina is completely unknown. Our study reveals the strong dependence of expression of ephrin B2 on RA activity. The regulation of expression of ephrin B1 in the dorsal retina is either independent of RA signaling or only mildly dependent on it. Its expression is primarily determined by as yet unidentified upstream factors whose ventral border of expression must extend beyond the ventral border of Tbx5/RALDH1. Misexpression of Cyp26A1 led to loss of ephrin B2 without affecting the expression of ephrin B1, EphB2 or EphB3. This provides a unique situation to study the function of ephrin B2 in the developing retina. Reverse signaling through the ephrin B molecules may be involved in intra-retinal targeting of RGC axons to the optic disc (Birgbauer et al., 2000). One could glean more information about the function of the ephrin B molecules in the retina using RCAS-DNhRARα and RCAS-Cyp26A1 as tools.

We thank Ronald M. Evans for the DNhRARα construct. Eric Swindell for Cyp26A1 cdNA and B. Vennström for providing cTRβ2 cDNA. We are grateful to the members of the Cepko and Tabin laboratories for critical discussion of the manuscript. This work was supported by Howard Hughes Medical Institute to C.L.C.; C.L.C. and J.S. are supported by HHMI.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/23/5147/DC1

References


Retinoic acid regulates DV topographic molecules


