A positional marker for the dorsal embryonic retina is homologous to the high-affinity laminin receptor

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Summary

In a search for determinants of positional information in the embryonic eye, we isolated two monoclonal antibodies that label strongly the dorsal part of the undifferentiated embryonic retina in mammals, bird and cold-blooded vertebrates. In the chick, the optic tectum is labeled in a corresponding fashion, the ventral tectum more heavily than the dorsal tectum. Through biochemical and molecular analysis both antibodies were found to recognize a protein that has been cloned repeatedly, first in a screen with antibodies to the '68K-laminin receptor' (Wewer \textit{et al.} (1986) \textit{Cancer Res.} 47, 5691–5698), a name that may not exhaustively describe its function. Western blots show the protein to be present in most or all tissues, and Western and Southern blots reveal a high degree of conservation in the detected signals up to invertebrates and bacteria. Despite the very strong and selective labeling of the dorsal retina in conventional immunohistochemical preparations, the protein and its mRNA are present in even amounts throughout the embryonic retina, as demonstrated by Western and Northern blots of bisected retinas, and immunohistochemically in retinas fixed with ethylene glycole bisuccinimide (EGS), an $\text{NH}_2$-group crosslinker with very long spacer arm. This indicates that the dorsoventral asymmetry in the embryonic retina is not in the amount but in the configuration of this protein; whether this difference relates to laminin binding is not known.

Key words: eye, antigen, retina, laminin receptor, positional information.

Introduction

Establishment of topographical order in retinotectal projections is resistant to a wide range of experimental manipulations, which points to a chemotactic mechanism underlying the initial map formation, but other mechanisms, including axon–axon interactions, timing and activity, are thought to play a role in the refinement and stabilization of the map (Bonhoeffer and Gierer, 1984). Sperry's chemoaffinity hypothesis postulates that the specificity of retinotopic projections is established through graded chemical differences between cells in the retina, which match up with corresponding graded differences in the optic targets (Sperry, 1963). He suggested that a system of two qualitatively distinct graded properties, one in the anterior–posterior and the other in the dorsoventral dimension, would endow each retinal point with a unique local address; and that a third system, graded in the radial dimension, might increase the precision of the map when superimposed on a combination of the first two (Sperry, 1951).

Over the years this hypothesis has stimulated an extensive search for asymmetrically distributed properties in the retina, as a first step to the identification of the biochemical nature of the determination process for positional information. (1) Dissociated cells from ventral and dorsal retinal halves were shown to adhere preferentially to their target halves on the tectal surface (Barbera \textit{et al.} 1973), and dorsal retinal cells adhere preferentially to ventral retinal cells \textit{in vitro} (Gottlieb \textit{et al.} 1976). (2) Axons from temporal retina show stronger fasciculation than nasal axons, and growth cones from temporal but not nasal retina respond to a repulsive property in the caudal tectum. The transition for the two retinal properties from nasal to temporal is an abrupt step function, whereas the repulsive property in the tectum has a graded distribution (Bonhoeffer and Huf, 1982; Bonhoeffer and Huf, 1985; Halfter \textit{et al.} 1981; Walter \textit{et al.} 1987). (3) The TOP antigen, a 47K ($K=10^3M_r$) cell-surface glycoprotein defined by a monoclonal antibody, is distributed in a dorsoventral gradient in the retina and a ventrodorsal gradient in the tectum of the embryonic chick. TOP's exact biochemical elucidation has proven difficult, e.g. as antisera to the immune-precipitated protein fail to show a prefer-
ence for dorsal retina (Trisler and Collins, 1987; Trisler et al. 1981). (4) Finally, the JONES antigen, an acetylated GD3 ganglioside, is restricted to the dorsal retina of the embryonic rat (Constantine-Paton et al. 1986).

Here we describe an antigen in the dorsal part of the undifferentiated embryonic eye, which very likely reflects a particular configuration of the high-affinity laminin receptor, a molecule whose exact structure and function is much under debate at present. A link of a potential positional marker for the dorsoventral axis of the retina to the laminin system is intriguing in view of the known role of laminin in retinal outgrowth (Cohen et al. 1987; Cohen et al. 1986; Smalheiser et al. 1984), but this laminin receptor may well in addition serve other functions that are not related to laminin.

Materials and methods

Generation of hybridomas

Balb-c mice were injected intraperitoneally with doses of about 50 formalin-fixed eyes from E11-13 mice, followed over one week by multiple injections of cyclophosphamide (100mg/kg-1). Two weeks later the mice received a second injection of eyes, and after four days the spleen cells were fused to NS1 cells following standard procedures (Köhler and Milstein, 1975). The hybridomas were screened on fixed embryo sections, and the two antibodies described here were cloned at least three times by limiting dilutions. The Dolce clone was initially unstable; only by very extensive cloning and testing of several hundred clones did we eventually establish a stable cell line. Neither the Dolce, nor the Jula cell line express antibody on the cell surface. Both the Dolce and Jula antibodies, as well as all other antibodies that we generated with the same immunization protocol, are of the IgM type. For morphological work in embryonic mice, this is desirable, because detection with light-chain-specific secondary antisera gives no background at all, as only IgG but not IgM cross the placental barrier. While the Dolce antibody works well and reliably in immunohistochemistry and Western blots, the Jula antibody can be difficult: its titer obtainable in supernatants is highly variable; when grown as ascites it seems to stimulate autoantibodies; it is unstable upon storage; and its signal in Western blots is usually rather weak.

Histology

Mouse embryos from an outbred colony were staged according to Theiler (Theiler, 1972) and fixed with periodatelysine-paraformaldehyde (Mc Lean and Nakane, 1974), or with 3.6% paraformaldehyde/0.13% picric acid, or (for Fig. 2F) with a 95% methanol/0.5% acetic acid mixture. The tissue was cryo-protected in 25% sucrose, frozen and cut at 12 μm on a cryostat. The sections were preincubated with 0.1-0.4% Triton X-100, exposed to antibody on a shaker and processed for immunofluorescence. In some cases, we cut sections on a vibratome and visualized antibody binding by immunofluorescence or with a horseradish-peroxidase- or alkaline-phosphatase-based detection system.

For fixation of the crosslinker ethylene glycol bissuccinimide (EGS; Pierce), retinas from E12-14 mouse embryos were dissected free, which was necessary due to the large size and poor tissue penetration of EGS as compared to formaldehyde. The retinas were washed thoroughly in Hanks balanced salt solution; EGS dissolved in DMSO was added to final concentrations of 1-10 mm, and in some samples 0.1-0.01% of Triton X-100 was included; after 30 min the reactions were terminated with glycine; the retinas were embedded in one block alternating with conventionally fixed retinas and processed for immunohistochemistry.

Preliminary tests for the biochemical nature of the antibody epitopes were done on cryostat sections of fixed embryos. Prior to incubation with the antibodies the slides were exposed to a wide range of treatments: high temperature, low and high pH, high salt concentrations, alcohols, lipid solvents, denaturing reagents, reducing reagents, sodium periodate, and a series of enzymes.

To label cells in culture, cos-1 cells (= large-T transformed green monkey kidney cells) and NIH-3T3 cells were grown on gelatin-coated slides for 3-4 days. For surface labeling, the cells were either exposed to antibody supernatant at room temperature for 30 min, fixed with p-formaldehyde/picric acid and processed for immunofluorescence, or they were fixed in the presence of 5-15% sucrose before being exposed to antibody. Both protocols gave similar results but the alive-labeling procedure avoids artefacts, which are only partially eliminated by the sucrose and which are probably due to damage of membranes at fine processes by the fixative. For the cytoplasmic labeling, the cells were fixed and lipid-extracted with 0.1-0.2% Triton X-100 prior to antibody exposure.

Western blots

The tissue was homogenized on ice in 0.05 M Tris buffer at pH 6.8 with 0.1% Triton X-100, 2 mM PMSF, 5 mM EDTA, and in some cases aprotinin and leupeptin; the homogenate was boiled in SDS-sample buffer with or without β-mercaptoethanol for 5 min, and separated by SDS-PAGE according to Laemmli (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose paper with Towbin buffer (Towbin et al. 1979). The blot was stained for proteins with Compete S (Sigma), photographed, destained and blocked overnight at 4°C with or without 10% hemoglobin, 0.05% Tween-20 in 0.1 M phosphate buffer at pH 7.4, or by replacing the hemoglobin with 20% newborn calf serum, polyvinylpyrrolidone, or 5% dry milk. Blots were incubated with Dolce supernatant containing 10% hemoglobin or 20% newborn calf serum for 1 h at room temperature and thoroughly washed. Antibody binding was made visible either with sheep anti-mouse labeled with Iodine (Amersham) or with goat anti-mouse-IgM coupled to alkaline phosphatase. The rabbit antisera were used in the following dilutions: 0.9 and 0.9a p. at 1:600, 380 at 1:500, Kiri at 1:700, and the monoclonal antibody supernatants were either used straight or diluted 1:2.

For the tests of dorsal and ventral retina halves about 15 E12-14 retinas per sample were bisected in tissue culture medium and processed for Western blots in neighboring lanes. Variations in the methods included different homogenization buffers, reducing versus non-reducing sample buffers, native gels, fixation of the embryos as for immunohistochemistry prior to gel analysis and tests for proteolysis.

Molecular biology

The cDNA library was constructed in the bacteriophage expression vector Agt11 using RNA obtained from E16 rat brains and following the protocol described in Gubler and Hoffman (1983) and Neve et al. (1986a). The library, consisting of 3x10^6 independent recombinant phage, was screened with the Dolce antibody following Huynh et al. (1987). Seven positive clones were isolated, and the largest one, termed RD4A, was subcloned into the plasmid vector pGEM3. Two EcoRI-Pst restriction fragments from the RD4A clone, of
400 and 800 basepairs, were subcloned into Mpi8 and Mpi9 to sequence both strands. Each insert was sequenced by the chain termination method (Sanger et al. 1977) using Sequenase (US Biochemical). All subsequent probings were done with the fragment of RD4A that represents the 3’-fragment of the full-length clone published by Yow et al. (1988). For the RNA blots, tissue from E13 embryos was dissected under sterile conditions in tissue culture medium and quick-frozen in liquid nitrogen. RNA was isolated and probed with the radiolabeled clone according to Neve et al. (1986a), and by RNA slot blot analysis following Mobley et al. (1988). For the in situ hybridization, E14 to E16 rat embryos were sectioned on a cryostat and probed with the radiolabeled clone according to Neve et al. (1988). For Southern blot analysis, DNA was prepared following Neve et al. (1986b), digested with EcoRI and probed with the partial clone.

Results and Discussion

We generated monoclonal antibodies (Köhler and Milstein, 1975) to eyes from embryonic day 11 to 13 (E11–13) mice, following a protocol that involves immune-suppression (Matthews and Patterson, 1983) and fusion of the primary response. The two antibodies described here, Jula and Dolce, were obtained in separate fusions. They probably recognize the same antigen (see below), but their epitopes differ in response to several treatments: the Jula epitope is relatively more sensitive to denaturation and fixatives, and it is affected both by alcohol and by very high periodate concentrations, treatments that do not harm the Dolce epitope. Although the labeling patterns of the two antibodies are similar overall, there are differences in particular in the brain; here only the similarities will be described.

A series of coronal sections through mouse eyes from E9 to newborn, labeled with Dolce, is shown in Fig. 1. The ‘dorsal antigen’ appears at the late eye-vesicle stage at E9.5 (Fig. 1B). At E11 (Fig. 1E), before the onset of ganglion-cell differentiation, the labeling extends over its maximal extent, more than a third of retinal area; at this stage the border of the labeled region is quite sharp. With the wave of ganglion-cell differentiation expanding from central to peripheral retina, the antigen is gradually pushed towards the dorsal margin, and the labeling becomes more graded, as seen for E14.5 in Fig. 1G. Neighboring sections labeled with Dolce and Jula; in this case, the cytoplasmic pool co-localizes with Jula; in this case, the cytoplasmic pool co-localizes.

As the presence of cell-surface labeling cannot be diagnosed in fixed tissue sections, we applied the antibodies to live, non-permeabilized cells. For Fig. 3F a culture of COS cells was incubated alive with Dolce: the surface of a small fraction of the cells is labeled in a speckled pattern, while in two pairs which might be interpreted to be postmitotic sisters because of their mirror-symmetrical morphology (Albrecht-Buehler, 1977; Solomon, 1979). There were more such surface-labeled cells, when the cultures were healthy and in vigorous growth phase. Comparisons with Nomarski optics showed the labeling clearly associated with the cytoplasmic membrane, and there was no sign of damage to the cells. Not shown here is bright surface labeling on rounded cells that are apparently in mitosis, on cells in telophase, but also on cells with no obvious link to mitosis. These observations tentatively suggest that one of the functions of the surface antigen in the cell lines is a role in postmitotic spreading. When similar cultures were fixed and permeabilized, the much more abundant cytoplasmic antigen present in all cells in the form of perinuclear granules became visible. Fig. 3E shows fixed and permeabilized 3T3 cells labeled with Jula; in this case, the cytoplasmic pool co-localizes partially with stress fibres. When embryonic retinas were gently isolated and labeled alive in culture, the surface of a few cells was brightly labeled in a speckled pattern, similar to the surface labeling in the cell lines.
Fig. 1. Coronal sections through mouse embryos in chronological order, from embryonic day 9 (E9) to postnatal day 1 (P1), labeled with Dolce; in the photographs dorsal is up and ventral down. Neighboring sections labeled with Jula (not shown) gave identical patterns in the eye. (A–C) E9–10; (D) E10.5; (E) E11; (F) E12.5; (G) E14.5; (H) E16.5; (I) P1. Scales A–H: 200 μm; I: 500 μm.
The labeled cells were arranged in a circular fashion in the retina, a pattern whose significance is not clear. We have no direct evidence that the surface antigen is related to the cytoplasmic antigen rather than representing a chance cross-reactivity with an unrelated cell surface component, but as both antibodies described here, in addition to antisera to the same antigen (Wewer et al. 1987), and several other monoclonal antibodies (in preparation), show surface labeling in a few cells, there may well be a relationship.

In order to determine the nature of the antigen(s) seen by the two antibodies, we tested immunohistochemically the vulnerability of the antibody epitopes to a series of reagents: both epitopes were selectively destroyed by several proteases, suggesting that the antigen is a protein. In Western blots of extracts from embryonic eyes or other embryonic and adult tissues, Dolce recognizes strongly and specifically a band at 44K (Fig. 4A). We did not find any tissue lacking this immunoreactive band (except for red blood cells), even
Fig. 3. (A,B) Coronal sections through eye and tectum of E4 chick labeled with Dolce; note the preferential labeling of the dorsal eye and the ventral tectum. (C) Coronal section through mouse E15 hindbrain labeled with Dolce; note the labeling of the midline and the inner ear. (D) Coronal section through striatum and nucleus accumbens of a P4 mouse labeled with Dolce. (E) 3T3 cells grown for 3 days on gelatin-coated slide, fixed with p-formaldehyde/picric acid, permeabilized with 0.1% Triton X-100 and labeled with Jula. Note the cytoplasmic localization of the antigen which partially follows the pattern of stress fibers. In addition, even a subpopulation of the nuclei are labeled here, possibly in relation to some functional state of the cells; both monoclonal antibodies showed this variable nuclear labeling. (F) Culture of cos cell exposed alive at room temperature to Dolce supernatant for 30 min, fixed and treated with secondary antibody. Although this field is covered by cells, only a small subpopulation contains surface antigen, here two pairs with symmetrical morphology which might represent postmitotic sisters. (G) Section through muscle in a late mouse embryo labeled with Jula; at high power the thick labeled stripes can be seen interrupted by a thin central gap, a pattern that resembles the location of myosin heads. Scales. A, 200 \mu m; B,C, 500 \mu m; D, 1 \mu m; E,F, 50 \mu m; G, 40 \mu m.

the Dolce hybridoma cells give a strong signal when blotted with their own antibody (Fig. 4A). No such band was seen with a large number of control antibodies. The 44K band ran in our system slightly higher than actin, as visualized by several actin antibodies, and our antibodies did not react with purified actin (not shown). A similar strong band is seen in blots from a diverse set of species, either at the same molecular weight or slightly higher (Fig. 4A); e.g. in the chick the molecular weight is about 46K. The Jula antibody recognizes the same 44K band but more weakly (Fig. 4C). In addition to the strong 44K band, much weaker bands at 130K and 68K became visible after longer exposures of Western blots with Dolce, which one would tend to ignore were it not for the results described below. In subcellular fractionations, probed with Dolce and with the rabbit antisera described below, the 44K band was totally in the soluble pool and the 68K band both in the membrane and soluble fractions (not shown).

We constructed an E16 rat brain cDNA library in the expression vector Agt11 (Young and Davis, 1983) and
Fig. 4. (A) Western blot autoradiography of extracts of tissues from several species probed with Dolce: human embryonic brain; newborn gerbil retinas and brain; eyes from embryonic mice, rats and chicks; heads of *Xenopus* and zebrafish larvae; lobster larvae; heads of adult *Drosophila*; and the Dolce hybridoma cell line. An immunoreactive band around 44K was detected in all tested samples shown here, and in addition in retinas, brain, muscle and liver from adult mice (not shown); only red blood cells lacked the band. (B) Protein stain (left) and Western blot (right) of a gel loaded with the protein encoded by the full-length human laminin-receptor clone fused into the β-galactosidase gene (Segui-Real et al. 1988) and β-galactosidase as control. The blot was reacted with Jula, Dolce and a control antibody, α-SSEA1; the detection system was alkaline phosphatase. Jula and Dolce specifically recognized the fusion protein but not the β-galactosidase portion of it; α-SSEA1 was negative. (C) Western blots of extracts from mouse embryonic eyes (E13–14) incubated with the monoclonal antibodies Dolce and Jula and with a series of rabbit antisera to different forms of the high affinity laminin receptor: the antiserum 0.9 against the human laminin-receptor fusion protein and affinity purified 0.9 (=0.9a.p.) (Segui-Real et al. 1988), the antiserum 3801 against a synthetic peptide deduced from the C-terminal portion of the cloned sequence, and the antiserum Kiri against the native receptor (Wewer et al. 1987). The polyclonal antibodies 0.9 a.p. and 3801, as well as Dolce and Jula reacted here only with the 44K protein; Kiri labeled only the 67K region, and 0.9 labeled both the 44K and the 67K proteins. The detection system was alkaline phosphatase. Note the molecular weight heterogeneity in the 67K regions recognized by the Kiri and 0.9 antisera in these blots which are from neighboring lanes in the gel. This heterogeneity, as well as the observation that the affinity purified 0.9 antisera lacks 67K-reactive antibodies, raises the possibility that some of the multiple bands at 67K are artefacts.

screened 10^6 clones with the Dolce antibody. Seven independent positively reacting clones were isolated through successive screens. Of these clones, six contained cDNA inserts less than 0.5 kilobases (kb) in size, while one contained an insert of about 1.2 kb. This clone, termed RD4A, was subcloned into the plasmid vector pGEM3 for further analysis. A Northern blot, in which radiolabeled RD4A was used to probe RNA from E13 mouse eye region, revealed a single abundant RNA species of about 1.3 kb. In several brain tumor samples, but not in normal embryonic tissue, we detected an additional band around 5.5 kb. We sequenced RD4A with the dideoxy-chain termination method (Sanger et al. 1977) and found that it contained two independent cDNA inserts. The insert conferring both antibody and Northern blot specificity codes for the C-terminal portion of a protein whose deduced amino acid sequence is part of the published sequence of the '68K-laminin receptor' (Wewer et al. 1986; Yow et al. 1988), a protein believed to play a role in invasive processes such as in metastasis of malignant tumors and in bacterial infections (Lopez et al. 1985). The six other clones contained inserts indistinguishable from this one by restriction mapping. Southern blots probed with RD4A showed a high degree of conservation: we saw strong signals in all species tested in the Western blot (Fig. 4A), and in addition in yeast, nematode and *E. coli*.

In order to test the authenticity of RD4A, we compared our monoclonal antibodies to reagents prepared by two groups working on this laminin receptor. First, we did Western blots of the protein encoded by the full-length human laminin-receptor clone fused into the β-galactosidase gene (Segui-Real et al. 1988): both monoclonal antibodies recognize specifically the fusion protein but not β-galactosidase (Fig. 4B). Second, we compared by Western blots the monoclonal antibodies to several rabbit antisera against the laminin receptor: an antiserum to purified receptor (Kiri), to a deduced peptide sequence (3801) (Wewer et al. 1987), to the
fusion protein (0.9), and affinity-purified 0.9 (0.9a.p.) (Segui-Real et al. 1988). The rabbit antisera label either the 44K band, or multiple bands around 68K, or both regions (Fig. 4C). Third, we did immunohistochemistry with the rabbit antisera. Although they work less well on fixed tissue sections than on Western blots, they do resemble our monoclonal antibodies in many details: they label the midline of the embryonic brain, the inner ear and a striped pattern in muscle; and one of them, the antiserum to the purified laminin receptor, labels preferentially the dorsal retina although rather weakly (Fig. 2F). In addition, the localization of the antigen seen with our antibodies in cultured cells – an abundant cytoplasmic pool, in some cases a partial co-localization of the cytoplasmic antigen with stress fibres, and a cell-surface component – is identical to the published labeling pattern with several laminin-receptor antibodies (Wewer et al. 1987; Yannariello-Brown et al. 1988).

These comparisons demonstrate that our antibodies recognize the same protein that has been previously cloned as the ‘68K-laminin receptor’; however, this protein seems to occur in several molecular weight forms (see below). Immunohistochemically all six reagents show similarities, e.g. in labeling muscle and midline, but they differ in other places. In particular, only one of the antisera resembles the monoclonal antibodies in preferentially labeling the dorsal eye. In order to test biochemically for a dorsoventral difference, we ran Western blots on embryonic retinas bisected into dorsal and ventral halves. There was no difference detectable: the levels of the 44K protein in dorsal and ventral retina were perfectly identical, and we never found a band unique to dorsal retina, although we tried several different extraction and gel electrophoresis protocols. In addition, we did not detect any difference in mRNA levels between dorsal and ventral embryonic retinas either by in situ hybridization or by RNA slot blots prepared from bisected retinas. These experiments indicate that both the mRNA and the 44K protein are present throughout the embryonic eye in similar high amounts, but the sites recognized by the two antibodies appear masked in ventral retina in immunohistochemical specimens prepared under a range of different conditions: fixation with formaldehyde, picric acid, periodate-lysine-paraformaldehyde or methanol/acetic acid; on cryostat or vibratome sections; with or without detergents; and to some extent even in unfixed tissue (although most antigen will wash out under such conditions). This presumed masking cannot directly involve a covalent modification, as it appears to be broken by the detergent sodium dodecyl sulfate (SDS) used in Western blotting. To test this explanation morphologically, we fixed embryonic retinas with ethylene glycole bissuccinimide (EGS), a homobifunctional reagent that crosslinks NH₂-groups via a very long (1.6 nm) spacer arm, thus creating a very wide meshwork. Under these conditions, both monoclonal antibodies label the entire retina homogeneously (Fig. 5A); no difference in intracellular antigen distribution and appearance is detectable between dorsal and ventral retina. This is not due to general stickiness of the tissue, as the distribution of several other antigens, such as the HNK-1 antigen (Fig. 5B), is not affected by EGS-fixation. These observations make it likely that the ‘dorsal antigen’ represents a particular configuration of the laminin receptor rather than a spurious cross-reactivity with an unknown antigen that is immunologically closely related to the laminin receptor, has the same subcellular distribution, but escapes all attempts of biochemical detection. The assumption of a dorsoventral difference in the configuration of the laminin receptor is consistent with the labeling patterns of three additional monoclonal antibodies to the same laminin receptor which we isolated recently: one of them labels the embryonic retina with a slight dorsal

Fig. 5. Pairs of embryonic retinas fixed with ethylene glycole bissuccinimide (upper specimens) or periodate-lysine-paraformaldehyde (lower specimens). The right pair (A) was labeled with Dolce, the left pair (B) with HNK-1 (Abo and Bale, 1981; Noronha et al. 1986).
preference, the second shows no preference at all, and the third labels preferentially the ventral retina (in preparation). A factor in the epitope accessibility by the antibodies may be their large size, as all are of the IgM type.

A point at issue in the published work on the '68K-laminin receptor' is that its mRNA size of 1.3 kb is too small to code for a 68K protein. The calculated relative molecular mass of the deduced protein is only 33K, and primer extension shows this to be the full-length mRNA (Rao 

et al. 1989; Segui-Real et al. 1988; Yow et al. 1988). Although it was thought to be an integral membrane receptor, the deduced protein does not contain a signal sequence or a proper membrane spanning region. As the deduced 33K protein is rather acidic, it is likely to migrate anomalously slowly in SDS gels, which makes it plausible that the 44K band in Western blots represents the 33K protein. The relationship between the 44K and 68K proteins, however, remains a puzzle. There are three possible explanations. (1) The two proteins may be merely immunologically related. The degree of similarity must be extensive, however, given the diversity of observations linking the proteins (Douvile et al. 1988; Kleinman et al. 1988; Rao et al. 1989; von der Mark and Risse, 1987; Wewer et al. 1987). As similar structure could imply similar function, this first possibility raises the question of a functional homolog to laminin binding for the cytoplasmic 44K protein. (2) The 68K protein may represent a posttranslational modification of the 44K protein, an explanation favored by Rao et al. (1989). If one assumes that the 68K form somehow enables the association with the membrane, there remains the difficult problem of how the protein can be transposed to the outside face of the plasma membrane. (3) The two proteins may derive from alternatively spliced mRNAs. This possibility is supported by the observation of an mRNA around 5.5 kb in addition to the 1.3 kb mRNA (Segui-Real et al. 1988; Yow et al. 1988). However, if the multiple bands recognized by the laminin antisera in Western blots of embryonic eyes (see Fig. 4C) do indeed represent the 68K protein rather than an artefact, this third explanation is unlikely, because we saw the large mRNA only in several tumors and not in normal embryonic tissue.

Two questions regarding the retino-tectal system have to remain open at present. The first is whether our antigen is related to the TOP antigen, a cell-surface glycoprotein of unknown biochemical nature which, like our antigen, is distributed in a dorsoventral pattern in the retina and a ventrodorsal pattern in the tectum of the embryonic chick (Trisler and Collins, 1987; Trisler et al. 1981). The second question concerns the role, if any, the protein might play in the establishment of retino-tectal topography. While in the embryonic chick eye the labeling appears quite graded, in the mouse the distribution resembles more a step function than a gradient. One could argue that the radially expanding wave of N-CAM expression which indicates ganglion cell differentiation (Fig. 2A−D), in combination with the receding border of the 'dorsal antigen', create a spatio-temporal gradient, reminiscent of Sperry's radial dimension in retinal specification (Sperry, 1951). Alternatively, a step-like function may give rise to secondary monotonic gradients (Bonhoeffer and Gierer, 1984).

The dorso-ventral difference seen immunohistochromically by the antibodies appears to be in the configuration of the laminin receptor rather than its amount. As the difference is conserved in species as far apart as chick and mouse, it is probably of functional significance. However, so far we have no hint of what it might represent at the molecular level, as it has eluded all attempts to demonstrate it biochemically. The notion of an asymmetrical distribution in the attribute of a high-affinity laminin receptor seems attractive, since laminin is the most potent outgrowth factor for embryonic retinal ganglion cells identified so far (Mc Loon et al. 1988; Kleinman et al. 1988; Smalheiser et al. 1984). Laminin itself is distributed homogeneously and at high levels in the course of optic axons in the embryo (Cohen et al. 1987), but disappears later, as does the ganglion-cell response to laminin. A dorso-ventral asymmetry in the receptor could introduce the asymmetry in adhesion of growth cones postulated by the chemoaﬃnity hypothesis (Sperry, 1963). In this case, the high cytoplasmic pool might function to allow the dynamic expression of the receptor on the growth cone surface (Wewer et al. 1987). In view of the high affinity of the '68K-laminin receptor' − Kd of 2 nm − (Liotta et al. 1985; Malinoff and Wicha, 1983), it can be argued that a rapid turnover at the surface is necessary to prevent irreversible sticking of the growth cone on the abundant laminin in its course. The apparent dynamic expression of the antigen on the surface of mitotic cells observed in cell lines (Fig. 3F) may serve as a model for the growth cone. Observations made incidental to other studies are indeed consistent with this possibility: in retinal explants from mouse embryos, dorsal axons responded stronger to the laminin substratum than ventral axons (Godemont and Bonhoeffer, 1989).

At present it may be prudent to wait, however, before formulating any models on dorsoventral determination, until the relationship of the 44 and 68K proteins and the question of their function are solved: even if the presumed membrane-associated 68K protein acts as a laminin receptor, the functional relationship of the cytoplasmic 44K protein to laminin cannot be taken for granted. In addition the proteins may serve other not yet identified functions, as suggested by the wide distribution and high conservation of the 44K band. Nevertheless it is provocative that two other examples of dorsoventral determining factors appear thematically related: the unc-6 and unc-5 genes in nematode, which are involved in dorsoventral axonal guidance, code for the homolog of the laminin-B2 chain and a protein that may function as a laminin receptor (Culotti et al. 1988), and markers for the dorsal and ventral compartments of Drosophila wing disc are related to the integrins which include the low-affinity laminin receptor (Leptin et al. 1987).

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