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A dorso–ventral asymmetry in the embryonic retina defined by protein conformation

(laminin receptor/retinotopic specification/ribosomes)

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ABSTRACT **In a search for determinants of retinotopic specification we previously identified an antigen in the dorsal embryonic retina as a protein called the 68-kDa laminin receptor. A dorso–ventral asymmetry in a laminin receptor seemed consistent with the known responsiveness of embryonic optic axons to laminin, but there were three peculiar points. (*i*) The molecular mass of this presumed laminin receptor in immunoblots is not 68 kDa but 43 kDa, and the molecular mass of the protein deduced from the mRNA is only 33 kDa. (*ii*) The antigen does not have the localization expected of a receptor for the extracellular matrix: the antibodies label mainly a granular cytoplasmic antigen in dorsal retina; an additional sparse cell-surface antigen present on a few cells does not show a dorso–ventral asymmetry. (*iii*) Despite the pronounced dorso–ventral difference seen immunohistochemically, in immunoblots the 43-kDa protein (p40) is evenly distributed throughout the retina. Here we show that (*i*) native p40 and *in vitro*-translated gene product are indistinguishable and their anomalous migration in denaturing gels probably is due to low pI; (*ii*) p40 is bound in a Mg^{2+}-dependent manner to large cytoplasmic complexes that appear to include ribosomes; and (*iii*) there is a labile conformational difference in p40 between dorsal and ventral retina: dorsally it is more accessible to proteolysis, suggesting a more open conformation. In conjunction with the recent hypothesis that p40 constitutes a translation initiation factor (D. Auth and G. Brawerman, personal communication), these observations point to a dorso–ventral asymmetry in some aspect of protein translation, which in turn may set up differences in recognition factors on retinal growth cones.**

The retina projects onto its central target regions in retinotopic maps that are similar in all vertebrates. Most evidence suggests a biochemical basis for the initial steps of the map formation (1). Sperry's chemoaffinity hypothesis postulates a system of two graded chemical determinants, which are orthogonally arranged in the antero–posterior and dorso–ventral axes of the retina (2). A long-ongoing search for the molecular mechanism of this process has proven difficult (3), which is not surprising if it turns out to resemble in complexity the axial determination of the early *Drosophila* embryo. Here the determination of the dorso–ventral axis alone involves the concerted action of proteins encoded by 12 genes, of which the dorsal protein functions as the morphogen, whose nuclear localization is spatially controlled by other factors, and which, in turn, influences the transcription from four genes (4–6).

In the developing vertebrate retina one has to assume the existence of at least two separate components: an early one that sets up an asymmetry in the retina as an embryonic field and a later one, expressed on growth-cone surfaces, that executes this asymmetry in the guidance of axons to their targets. In a search for factors involved in retinal determination, we generated two monoclonal antibodies that label very strongly the dorsal part of the embryonic retina in cold-blooded vertebrates, birds, and mammals (7). This dorsal antigen seemed to belong to the early component of factors acting in an embryonic field, as the antigen has a cytoplasmic localization and appears very early in eye development—at the late eye-vesicle stage—and disappears with differentiation of the retina. Upon isolating the cDNA encoding this antigen, however, we were surprised to learn that it is identical to a cell-surface component: a receptor for the extracellular matrix known as the high-affinity or 68-kDa laminin receptor (7–14). Here we present a short biochemical characterization of the protein, and we provide evidence for its interaction with large cytoplasmic complexes that include ribosomes, in which conformation may determine an asymmetrical function in the dorso–ventral axis. It seems now likely that the protein belongs to the early component of factors, possibly playing a role in differential control of protein synthesis.

METHODS

The monoclonal antibodies were generated by following protocols described previously, as were the protocols for immunoblotting and for immunohistochemistry (7). The immunofluorescent double-labelings were done on COS cells, large tumor antigen-transformed monkey kidney cells. Dolce, a monoclonal IgM antibody (7), was used for all immunoblot analyses of the 43-kDa protein (p40). The nondenaturing gel analysis was done by omitting $NaDodSO_4$ from the protocol of Laemmli (15). For the two-dimensional gels, which involved isoelectric focusing in the first dimension and $NaDodSO_4$ separation in the presence of 2-mercaptoethanol in the second dimension, we followed the procedure described by Strahler *et al.* (16).

For subcellular fractionations we applied the protocol worked out by Maeda *et al.* (17), in which the tissue is homogenized in a Mg^{2+}-containing buffer and spun at 100,000 $\times$ *g* through 41% sucrose. The titrations aimed to determine the binding conditions of p40 to sedimentable complexes were first worked out on retinas from postnatal day 1 (P1) mice and later applied to embryonic day 12.5–14 (E12.5–14) retinas; binding conditions did not differ between postnatal and embryonic retinas. The tissue was homogenized on ice in a pH 7.2 solution containing 10 mM sodium phosphate buffer (or 10 mM Tris buffer for Ca^{2+} titrations), 30 mM NaCl, 0.02% sodium azide, 0.5 mM phenylmethylsulfonyl fluoride and aprotinin at 10 μg/ml, and with various concentrations and combinations

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Abbreviations: SLE, systemic lupus erythematosus; p40, 43-kDa protein.

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of Mg^{2+}, Ca^{2+}, EDTA, and EGTA. By spinning the homogenate at 4°C in a microcentrifuge at 12,000 × *g* for 20 sec, a low-speed pellet was generated. The supernatant was then spun in an Airfuge at 100,000 × *g* for 1 hr at 4°C, resulting in the soluble fraction and a high-speed pellet.

For the protease assays we dissected retinas from E12.5–14 mouse embryos into dorsal and ventral halves in tissue culture medium, using 5 to 10 retina halves per sample point. For Fig. 6*A* the retinas were washed in Hanks' solution and homogenized in a buffer volume of ≈20 times the tissue volume containing 10 mM Tris (pH 7.4), 30 mM NaCl, 0.02% sodium azide, 3 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and aprotinin at 10 μg/ml. The homogenate was digested for 45 min at 30°C with various concentrations of trypsin (GIBCO), and the remaining p40 was assayed by immunoblot. For experiments as in Fig. 6*B* the retina halves were washed and incubated intact in ≈4 times the tissue volume of Hanks' solution at pH 8.5 containing 4 mM Mg^{2+}, 2 mM Ca^{2+}, 5% sucrose, 0.05% Triton X-100, and 500 μg of trypsin. The Triton X-100 was necessary to allow trypsin to enter the cells. By testing the tissue versus solution we found only small amounts of p40 leaking out of the cells, presumably because most of it was bound to the cytoplasmic complexes. Incubations were done at room temperature and were terminated after various lengths of time.

A p40 partial clone previously isolated from a rat library with the Dolce antibody (7) was used to isolate a full-length clone from a human library. Its identity to the published human clone (13) was verified through restriction mapping and sequencing from both ends. *In vitro* synthesis of 5′ capped RNA from the clone was done with Promega reagents. *In vitro* translations were done in a reticulocyte–lysate system (Promega).

RESULTS

We used gel electrophoresis to compare p40 derived from embryonic mouse tissue to *in vitro*-translated protein from the full-length human clone (Fig. 1). Both forms migrated in denaturing gels as a band around 43 kDa, which was split into a doublet under nonreducing conditions. In nondenaturing gels both proteins migrated as two widely separated bands, between which several faint bands appeared in heavily loaded preparations. In two-dimensional gels both proteins accumulated at identical locations with a major spot around an apparent pI of 5.2 and a tail and minor spot toward less acidic pI levels.

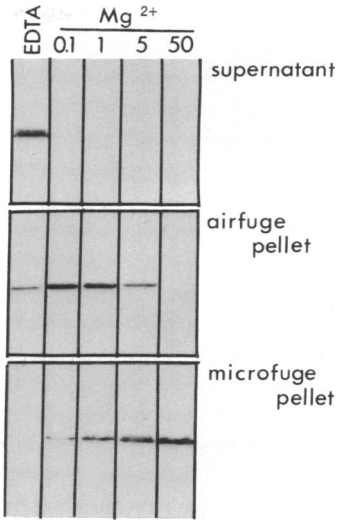

FIG. 2. Fractionation of p40 from P1 retinas into a soluble pool and low- and high-speed pellets, detected by immunoblots. Mg^{2+} concentrations are in mM.

In subcellular fractionations of tissues homogenized in EDTA-containing buffer we previously found all p40 in the soluble fraction (7). When we used the protocol of Maeda *et al.* (17), which includes Mg^{2+} rather than EDTA, p40 was not detectable by immunoblot in the soluble or the membrane fractions, but all of it was in the particulate fraction (data not shown). To determine the binding characteristics of p40 to the particulate fraction, we did titrations with EDTA or EGTA and/or Mg^{2+} or Ca^{2+} and tested the supernatant, low-speed pellets and high-speed pellets for p40. With increasing Mg^{2+} concentrations p40 moved from the soluble fraction into complexes of apparently increasing size (Fig. 2). EGTA was very ineffective in solubilizing p40. Under conditions that corresponded to ≈80 μM free Mg^{2+} or 1 mM free Ca^{2+} (18) about one-third of p40 was in the soluble fraction (data not shown). These titrations and the poor effect of EGTA indicate that physiologically most p40 is bound in a Mg^{2+}-dependent manner to the complexes.

The complexes presumably represent the cytoplasmic granules seen immunohistochemically (7). To test the possibility that the granules are aggregates of ribosomes, we double-labeled COS cells with the Dolce antibody and a

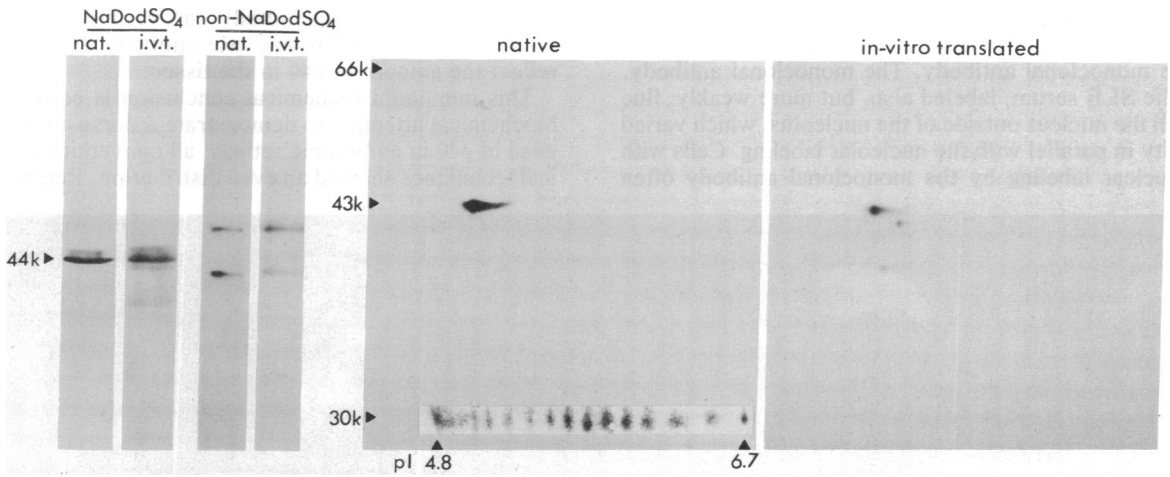

FIG. 1. Comparisons of native p40 (nat.) from embryonic mouse eyes and *in vitro*-translated p40 (i.v.t.) in denaturing (left lanes), nondenaturing (middle lanes), and isoelectric focusing (*Right*) gels. Native p40 was detected by immunoblot and *in vitro*-translated p40 through [^{35}S]methionine autoradiography. The pI markers are carbonic anhydrase Carbamylytes (Pharmacia) detected by Ponceau S (Sigma). Note that the signals for the two proteins are identical except for two minor proteins in the *in vitro*-translated p40 preparation, which probably represent degradation of the mRNA; p40 protein is very resistant to endogenous proteolysis (7). k, kDa.

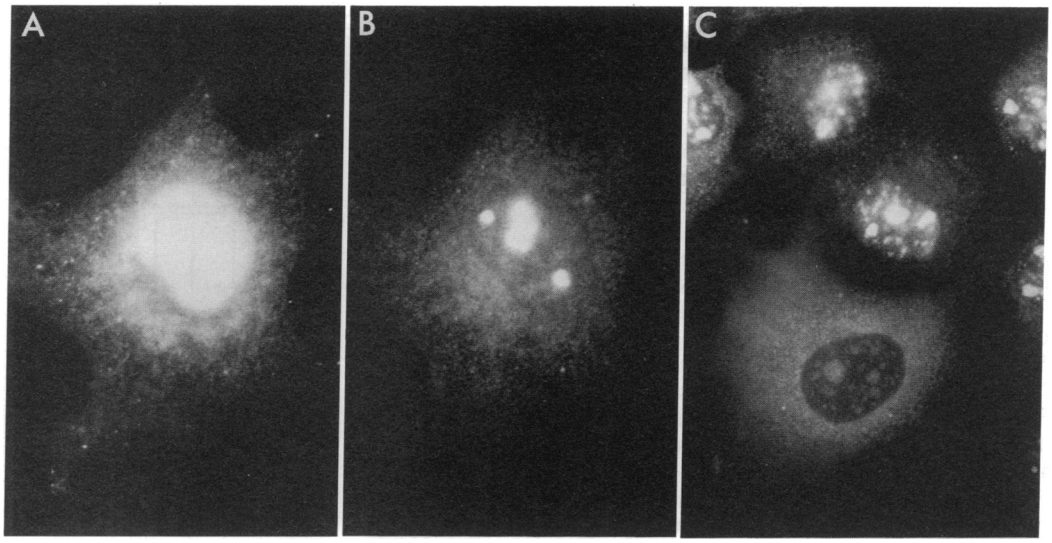

FIG. 3. Immunofluorescence of COS cells double-labeled with the SLE serum (*A*) and Dolce antibody (*B*). (*C*) Groups of COS cells to illustrate the variable nuclear labeling with Dolce antibody.

serum from patients with systemic lupus erythematosus (SLE), which recognizes a common epitope present on three P proteins of the large ribosomal subunit (19); none of the proteins labeled in immunoblots by the SLE serum lined up with the p40 band (data not shown). Both antibodies showed similar granular cytoplasmic labeling patterns (Fig. 3 *A* and *B*). While at most locations the granules were too small and densely packed for a detailed comparison at the light-microscopic level, at some places, such as thin cellular processes or veils, some, but not all, of the granules could be identified under both fluorescence filters. In addition, we confirmed D. Auth and G. Brawerman's unpublished observation that p40 is probably associated with the small ribosomal subunit (personal communication): in sucrose gradients of the high-speed pellet from liver homogenate, the p40 signal coincided with a protein and RNA peak in the 40S region (data not shown).

Both the SLE serum and the monoclonal antibody labeled, in addition to the cytoplasmic granules, some of the nuclei. The SLE serum showed weak general nuclear labeling and in some nuclei strong labeling of a lobulated compartment that most likely represents the nucleolar compartment involved in ribosomal assembly. The monoclonal antibody labeled the identical nucleolar compartment in some of the cells; in others this compartment was labeled only by the SLE serum or by the monoclonal antibody. The monoclonal antibody, but not the SLE serum, labeled also, but more weakly, fine patches in the nucleus outside of the nucleolus, which varied in intensity in parallel with the nucleolar labeling. Cells with strong nuclear labeling by the monoclonal antibody often appeared to have weaker cytoplasmic labeling and vice versa, a morphological observation consistent with the possibility that the antigen shifts in and out of the nucleus (Fig. 3*C*). The criterion for the variability in nuclear labeling detected by the monoclonal antibody may be linked to growth behavior, as many of the cells that were apparently actively growing had strong nuclear antigen, but cells packed in dense clumps often had little or none.

Embryonic retinas labeled by the two monoclonal antibodies described (7) and by three additional monoclonal antibodies are illustrated in Fig. 4: the Dolce and Jula antibodies showed a very strong preference for the dorsal retina, the Sandy antibody had a much weaker dorsal preference, the Leez antibody labeled the retina homogeneously, and the Counter antibody had a very weak preference for the ventral retina. All five monoclonal antibodies recognized in immunoblots of nondenaturing gels a similar doublet, and in blots of denaturing gels they recognized the same 43-kDa band that had been shown (7) for the Dolce and Jula antibodies to correspond to the p40 clone (Fig. 5). More extensive proof for the reaction of all five antibodies with p40 will be given elsewhere. The main point illustrated here is that the immunohistochemical pattern with different monoclonal antibodies to p40 can vary substantially; this variation suggests that the pattern revealed by any one antibody does not simply reflect the amount of p40 in the tissue.

This immunohistochemical conclusion is consistent with biochemical attempts to demonstrate a dorso–ventral difference in p40 in embryonic retinas: all conventional biochemical techniques showed an even distribution. Retinas cut into

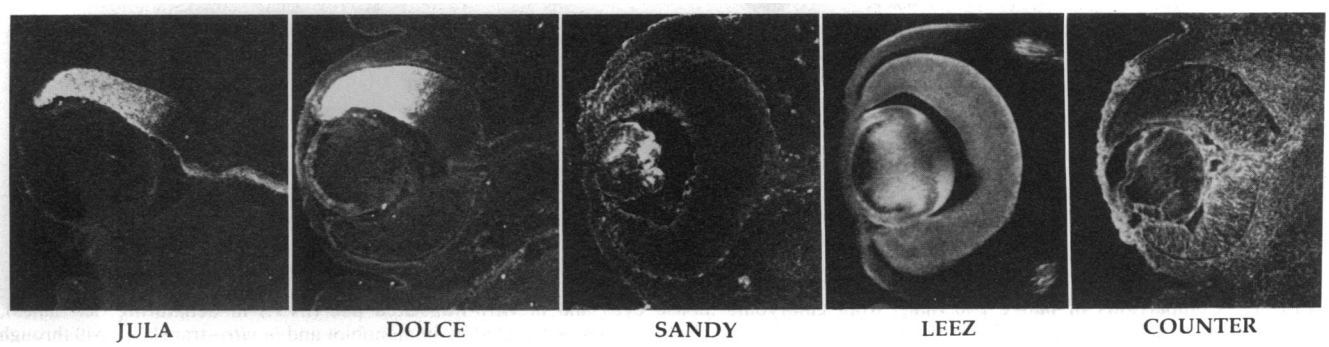

FIG. 4. Retinas from E11.5–13.5 mouse embryos labeled with five monoclonal antibodies to p40. Heads were sectioned in the coronal plane; dorsal retina is up, and ventral retina is down.

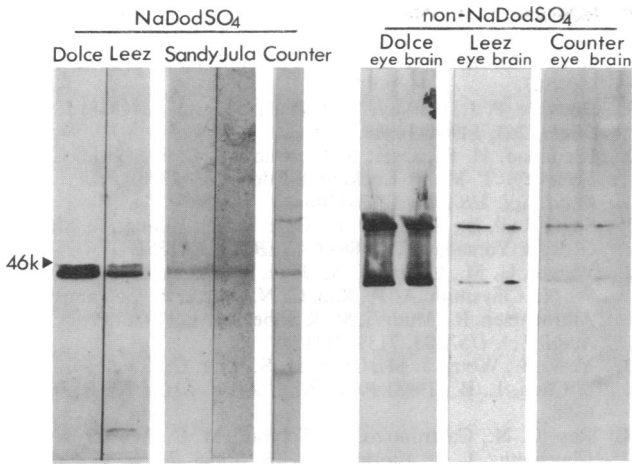

FIG. 5. Immunoblots of the five monoclonal antibodies on embryonic eyes and brains under denaturing (*Left*) and nondenaturing (*Right*) conditions. Samples for the tests with Sandy, Jula, and Counter antibodies under denaturing conditions (*Left*) and for the nondenaturing gel (*Right*) were treated with 2-mercaptoethanol. Samples for Dolce and Leez antibodies (*Left*) were prepared under nonreducing conditions, which caused the p40 band to migrate as a doublet. k, kDa.

dorsal and ventral halves had identical amounts and appearance of p40, as detected by the Dolce antibody in denaturing and native gels, in immunoprecipitations and by isoelectric focusing (data not shown). There was no difference in susceptibility to trypsin digestion of tissue extracts (Fig. 6*A*) and in the Mg^{2+}-dependent binding of p40 to the complexes: the conditions for shifting p40 into the soluble fraction were identical for dorsal and ventral retinas (data not shown). However, when the trypsin digestions were done in live

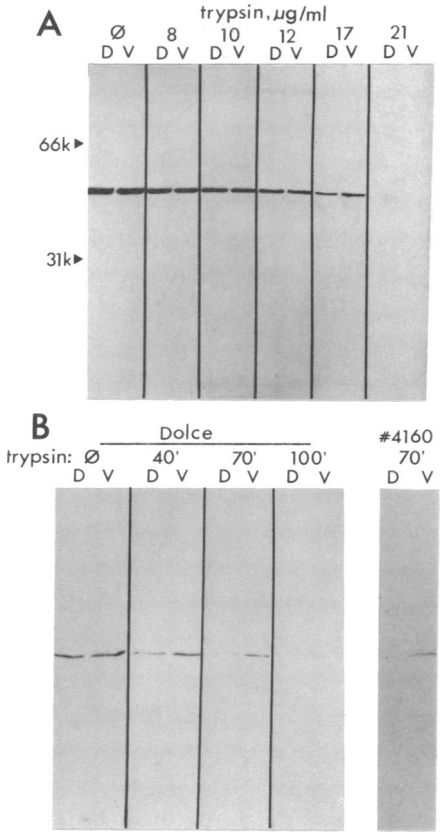

FIG. 6. Immunoblot of trypsin digestions of dorsal (D) and ventral (V) retina halves done in extract (*A*) and *in situ* (*B*).

retinas *in situ*, p40 turned out to be more accessible to proteolysis in dorsal than in ventral retina (Fig. 6*B*). Asymmetrical proteolysis under these conditions was also seen with the p40 antiserum 4160 (14), which does not show a dorso–ventral difference immunohistochemically. This result points to a conformational difference that is apparently labile and possibly dependent on a factor lost during extraction.

DISCUSSION

The protein characterized here is being studied in several laboratories as the 68-kDa laminin receptor because a clone encoding it was originally identified in a screen of an expression library with antibodies to the 68-kDa laminin-binding protein (12). There were questions about its identity, however, because its deduced size is only 33 kDa (13, 14), because its only consistent signal in immunoblot with a range of antibodies is ≈43 kDa (the 68-kDa signal seen with some antisera represents contaminating antibodies to skin keratins, "finger proteins"; P.McC., U.C.D., unpublished observations) (7), and because immunohistochemically the antigen is mainly located in the cytoplasm (7). Recently this protein was found identical to a protein of unknown function whose mRNA is in the ribonucleoprotein fraction and under strong translational control (20–22). This result cast further doubt on the assumption that its main function is in laminin binding because a receptor for the extracellular matrix does not possess the requirement for rapid and tight control of synthesis, which is a characteristic of proteins whose mRNA is in the ribonucleoprotein fraction in mammals.

Here we show that the native and *in vitro*-translated proteins behave identically in tests using denaturing, nondenaturing, and isoelectric-focusing gel analyses, indicating that the p40 signal in immunoblots corresponds to the primary or only minimally modified gene product. Its anomalous migration in $NaDodSO_4$ gels at 43 kDa, despite its calculated molecular mass of 33 kDa, is presumably explained by its low pI, as acidic proteins are known to migrate at apparently higher mass. Further, we show that p40 is bound in a Mg^{2+}-dependent manner to precipitable complexes that appear to include ribosomes. The morphological correlate of these complexes is a cytoplasmic granular labeling pattern with the p40 antibody, similar to the pattern with an SLE serum specific for ribosomal proteins (19). In addition p40 immunoreactivity appears in the nucleolar compartment of the nucleus, which is known as a transient location of ribosomal proteins (23). We find p40 in all cells and tissues of the body including reticulocytes, but it is lost from mature erythrocytes, the only cell type lacking ribosomes (7). Evolutionarily it is very conserved: similar proteins or genes can be detected in a large number of species including animals, plants, and bacteria (7, 24). Our results are consistent with a role of p40 in protein translation, perhaps in initiation of translation as hypothesized by D. Auth and G. Brawerman (personal communication).

The nature of the relationship of p40 to the 68-kDa laminin receptor is not known. Rao *et al.* postulate that p40 is posttranslationally processed to the 68-kDa protein (14). Alternatively the two proteins may only be very similar but coded for by different genes, as p40, which is present in unicellular organisms, may represent the evolutionary precursor to the laminin receptor: when for multicellular organisms the need of interaction with the environment arose, a mechanism for protein–protein interaction may have been duplicated from the ribosome system. So far we have found no evidence for laminin binding to p40[§]. The p40 mRNA seems to be abundant, and it has been identified repeatedly.

[§]McCaffery, P., Roth, H. & Dräger, U. C., in Proceedings of the 20th Annual Meeting of the Society for Neuroscience, Oct. 28–Nov. 2, 1990, Saint Louis, p. 302.

A presumably much rarer mRNA for the 68-kDa receptor may not yet have been detected, but a candidate is the 5.5 kilobase mRNA seen with the p40 cDNA probe (7, 13); its immunohistochemical mark could be the sparse cell-surface component labeled by our monoclonal antibodies (7).

The search for a biochemical correlate of the dorso–ventral difference in the embryonic eye, seen immunohistochemically with some p40 antibodies, proved very difficult. None was detectable with a wide range of assays. The only difference found was in trypsin susceptibility, as long as the digestions were done under *in situ* conditions. In conjunction with the immunohistochemistry this indicates a more accessible, open conformation of the protein in the dorsal retina. The lability of this conformational difference suggests that a soluble factor maintains the open conformation (25). A dorso–ventral difference in the conformation of p40, a protein that appears to associate with ribosomes, could convey a dorso–ventral difference in some aspect of regulation of protein synthesis, which in turn could result in differences in effector molecules expressed on growth-cone surfaces.

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