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## Function and spatial distribution in developing chick retina of the laminin receptor $\alpha_6\beta_1$ and its isoforms

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### Abstract

We have recently shown that the laminin-binding integrin receptor,  $\alpha_6\beta_1$ , is prominently expressed in the developing chick retina, and its expression and activity are regulated during development on both retinal ganglion cells and other neural retinal cells. In the present study, we show that antibodies specific for the extracellular portion of the chick  $\alpha_6$  subunit dramatically inhibit interactions in vitro between embryonic day 6 neural retinal cells and laminin, showing that  $\alpha_6\beta_1$  functions as an important laminin receptor on developing retinal neurons. In previous work, we showed that  $\alpha_6$  mRNA levels on retinal ganglion cells decrease dramatically after E6 during the period that RGC axons innervate the optic tectum. In the present study, we show decreases in  $\alpha_6$  mRNA are not prevented by ablation of the optic tectum, indicating that tectal contact is not the major cause of this decrease. Within the embryonic retina, the  $\alpha_6$  subunit is codistributed, in part, with laminin, suggesting that it functions as a laminin receptor during retina development in vivo. Furthermore, two isoforms of the  $\alpha_6$  protein with distinct cytoplasmic domains generated by differential splicing have quite different distribution patterns in the retina, suggesting that these two isoforms may have different functions during retinal development.

### Keywords

laminin; integrin; retina; chick retina

## INTRODUCTION

During the development of the vertebrate nervous system, each neuron has to find its way specifically to a final target where synapse formation occurs. In this process, the growth cones of developing neurons must recognize correct pathways reliably. The complex structure of the embryo makes it necessary for growth cones to respond to a diverse variety of cellular and extracellular substrata which contain the information needed to orient axonal outgrowth. Several classes of molecules have now been identified in the extracellular environment in vivo, which seem to be involved in promoting and guiding axons (reviewed by Dodd and Jessell, 1988; Jessell, 1988). Among these, several components of the extracellular matrix (ECM) seem likely to play important roles (reviewed by Sanes, 1989; Reichardt and Tomaselli, 1991). The best characterized receptors for ECM constituents are integrins, and several members of this family have been shown to be expressed on neurons and to promote neurite outgrowth on ECM-coated substrata in vitro (reviewed by de Curtis, 1991). Laminin (LN) is considered one of the most potent neurite outgrowth-promoting ECM molecules for several neuronal types in culture.

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Perhaps as many as 27 distinct isoforms of laminin exist as trimeric complexes of three subunits: an A homologue plus a B1 homologue plus a B2 homologue. The rapidly expanding family of identified laminin subunits consists at present of 8 polypeptides, three of which are homologues of the A subunit; two or three of which are B1 subunit homologues; and two of which are B2 chain homologues (cf. Sanes et al., 1990; Kallunki et al., 1992; O'Rear, 1992). While a few preliminary studies have been done characterizing neuronal interactions with partially purified preparations of other laminin isoforms, the most definitive studies to date have been done only with the first identified laminin isoform, which contains the A, B1 and B2 subunits (reviewed in de Curtis, 1991). In particular, studies *in vitro* have shown that embryonic day 6 (E6) neural retinal cells attach and spread on laminin, extending long neurites within 24 hours (Cohen et al., 1986; Hall et al., 1987). The effect of laminin on neurite outgrowth from E6 neural retinal cells and retinal ganglion cells can be completely abolished by the presence of monoclonal antibodies to the integrin  $\beta_1$  subunit (Cohen et al., 1986; Hall et al., 1987), implicating one or more  $\beta_1$ -class integrin receptors in interactions of these cells with laminin. The presence of laminin along the vitreal surface of the embryonic retina and in the developing optic pathway suggests possible roles for this extracellular matrix protein during the development of the retina and primary visual projection (McLoon, 1984; Adler et al., 1985; Cohen et al., 1987; Halfter and Fua, 1987; McLoon et al., 1988).

Recently, we have shown that at least two potential LN-binding integrins,  $\alpha_3\beta_1$  and  $\alpha_6\beta_1$ , are present in embryonic retina. The  $\alpha_6\beta_1$  heterodimer is expressed in E6 neural retinal cells and in a highly enriched preparation of retinal ganglion cells (de Curtis et al., 1991). By using antibodies and cDNA clones specific for the chick  $\alpha_6$  subunit, we showed that the expression of this protein is down regulated between embryonic days 6 and 12 during the development of the chick neural retina, correlating with loss of ability of these neurons to interact with laminin. In particular, dramatic decreases in the levels of mRNA and protein were observed in retinal ganglion cells over this time span. The present study presents direct evidence, using inhibitory antibodies, that the  $\alpha_6\beta_1$  integrin functions as an important laminin receptor for retinal neurons. Colocalization of  $\alpha_6$  with laminin *in vivo* suggests that the receptor is functional *in vivo*.

## MATERIALS AND METHODS

### Reagents and solutions

White Leghorn chicken eggs were purchased from Feather Hill Farm (Petaluma, CA). Restriction enzymes and Klenow fragment of DNA polymerase were from New England Biolabs (Beverly, MA) and Boehringer Mannheim Diagnostics, Inc. (Houston, TX). Sequence enzyme, reagents for sequencing and random hexanucleotide primers were from kits supplied by U. S. Biochemical Corp. (Cleveland, OH). [ $\alpha$ - $^{35}\text{S}$ ]dATP was from Amersham Corp. (Arlington Heights, IL). D-[6- $^3\text{H}$ (N)]-glucosamine hydrochloride and EN $^3$ HANCE were from NEN, DuPont Co. (Wilmington, DE). Protein A-sepharose CL-4B, CNBr-Sepharose CL-4B, and thiopropyl-Sepharose CL-4B were from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Oligonucleotide primers and synthetic peptides were provided by facilities in the Howard Hughes Medical Institute at University of California at San Francisco. Laminin (LN) was purified from Engelbreth-Holm Swarm sarcoma as published (Timpl et al., 1979). Collagen IV was from Collaborative Research Inc. (Bedford, MA). Vectastain ABC kits for immunohistochemical staining were from Vector Laboratories (Burlingame, CA).

### Antibodies

The polyclonal antibody against the chicken integrin  $\beta_1$  subunit was raised against the last 23 carboxy-terminal residues of this protein by Dr Kevin Tomaselli in this laboratory and is described by Tomaselli et al. (1988). Two peptides corresponding to carboxyl terminal portions

of the two alternative cytoplasmic portions of the human  $\alpha_6$  protein (Tamura et al., 1991) were synthesized at the Howard Hughes Medical Institute at University of California, San Francisco. The 35 amino acid-long cytoplasmic A peptide (CGFFKRNKDHYDATYHKAEIHAQPSDKERLTSDA) and the 35 amino acid-long cytoplasmic B peptide, which contained an added amino-terminal cysteine not present in the  $\alpha_6$ -B sequence, (CRIRKEEREIKDEKYIDNLEKKQWITKWNRNESYS) were coupled to KLH through N-terminal cysteines (Calbiochem-Behring Corp., San Diego, CA). The conjugates were used to immunize rabbits, as described previously (de Curtis et al., 1991). In this paper, the antibodies raised against these two peptides were referred to as  $\alpha_6$ -cytoA and  $\alpha_6$ -cyto B antibodies, respectively. The purification and use of the  $\alpha_6$ -cytoA antibody has been previously described (de Curtis et al., 1991). The polyclonal antibody  $\alpha_6$ -EX was raised against a fusion protein containing the amino-terminal portion of the extracellular domain of the chicken  $\alpha_6$  integrin subunit. A portion of the  $\alpha_6$  protein corresponding to amino acids -16 to +514 (de Curtis et al., 1991) was cloned into the pAR3040 plasmid vector, at the *Bam*HI site (Studier and Moffatt, 1986; Hoey and Levine, 1988). The final fusion protein expressed in BL21 (DE3)pLysS cells, contained an extra 14 amino acids at the amino-terminus and 16 amino acids at the carboxy-terminus derived from the plasmid. The fusion protein was used for immunization of rabbits after purification by SDS-PAGE and electroelution. IgG fractions from the serum were used for affinity purification of the antibody on a fusion protein-CNBr-Sepharose CL-4B column. A rabbit polyclonal antibody to mouse LN, named JW2, was prepared in this laboratory by Dr Janet Winter and was purified by affinity chromatography on LN as described (Lander et al., 1985). The monoclonal antibody CSAT was purified from ascites made by using hybridoma cells generously provided by Dr A. F. Horwitz, University of Pennsylvania, Philadelphia, PA. The monoclonal antibody against chick G4 was a kind gift of Dr F. Rathjen, Zentrum für Molekulare Neurobiologie, Hamburg, Federal Republic of Germany.

### Cell attachment assay and neurite outgrowth assay

E6 chick retinae were dissected and incubated for 6 minutes at 37°C in 0.1% trypsin (Worthington Biochemical Corp., Freehold, NJ) in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS (CMF-PBS). Digestion was stopped by adding 0.2×volume of heat-inactivated fetal calf serum. Pellets were washed once in F12 nutrient mixture, and triturated in F12 containing 0.002% DNase I. For cell attachment, Linbro/Titer plates (Flow laboratories, Inc., Mc Lean, VA) were coated overnight with 20 µg/ml of LN or collagen IV in CMF-PBS. Coated and uncoated wells were incubated for 2 hours at room temperature with 1% BSA in CMF-PBS. Wells were washed twice with CMF-PBS and about 100,000 retinal cells were added to each well, after preincubation in a sterile tube for 20 minutes at room temperature in F12 medium with additives (5 µg/ml insulin, 30 nM selenium, 25 µg/ml human transferrin, 100 U/ml penicillin and streptomycin, according to Bottenstein et al., 1980). When indicated, preincubation occurred in the presence of  $\alpha_6$ -EX or NR IgG. Cells were sedimented at the bottom of the wells by centrifugation, and incubated for 1 hour at 37°C in 5% CO<sub>2</sub> atmosphere in the same medium used during the preincubation. Unattached cells were removed by the brisk addition of warm medium followed by gentle vacuum suction. The cells were fixed with 3% paraformaldehyde, stained with Crystal Violet (0.5% in 20% methanol) and washed with water (Bodary et al., 1989). After solubilization with 1% SDS, A540 was measured in each well. The percentage of inhibition of cell attachment on LN or collagen IV was calculated as follows:

$$\% \text{ inhibition} = 1 - \frac{A540(\alpha_6\text{-EX}) - A540(\text{BSA})}{A540(\text{NR}) - A540(\text{BSA})} \times 100$$

In each experiment every sample was in triplicate.

For neurite outgrowth assays, trypsinized retinal cells were prepared and preincubated with IgG as described above. About 30,000 cells were added to each well. Cells were cultured overnight at 37°C in a 5% CO<sub>2</sub> atmosphere, and cultures were examined in the microscope for neurite outgrowth.

### Metabolic labeling and immunoprecipitation

Aliquots of cell lysate prepared from dissociated E6 retinal cells metabolically labeled with [<sup>3</sup>H]glucosamine were used for immunoprecipitation with different anti-integrin antibodies. Preparation of cells, metabolic labeling, lysate preparation, and immunoprecipitation procedures were the same as described by de Curtis et al. (1991).

### Immunohistochemical staining

Paraformaldehyde-fixed E6 and E12 chick retinae were embedded in O.C.T. (Miles Inc., Elkhart, IN) and frozen for sectioning. 16 μm cryosections were blocked for 1 hour at room temperature with 10% goat serum, 0.2% Triton X-100 in PBS. The sections were incubated for 1 hour at room temperature with 2.5 μg/ml preimmune or affinity purified immune IgG ( $\alpha_6$ -cytoA and  $\alpha_6$ -EX antibodies), preimmune or immune serum diluted 1:500 ( $\alpha_6$ -cytoB antibody), affinity-purified anti-LN IgG (JW2 polyclonal antibody, Lander et al., 1985), anti-chick LN IgG (a gift of Dr Charles Little) or 5 μg/ml IgG (monoclonal antibody G4). The sections were successively incubated with Vectastain ABC reagents (Vector Laboratories, Inc., Burlingame, CA) and developed to visualize HRP-catalyzed reaction product according to the supplier.

### Tectal ablation and purification of retinal ganglion cells

Eggs were windowed at embryonic day 3 (E3) and allowed to continue to develop until E6. The optic tecta of E6 embryos were removed completely and embryos were allowed to develop until day E9–E11. In each experiment, a number of unoperated embryos were allowed to develop until E9–E11 in windowed eggs as controls. Retinal cells from operated and unoperated embryos between E9 and E11 were dissociated by incubation in hyaluronidase and trypsin and fractionated in Percoll gradients. 2–5 embryos were used for each sample. This procedure and characterization of retinal ganglion cell-enriched and depleted fractions are described in de Curtis et al. (1991). These two fractions, referred to as Fractions I and II, respectively in that paper, were used for RNA analysis.

### RNA analysis

Total RNA was prepared as described in Chomczynski and Sacchi (1987). RNA electrophoresis, transfer to nitrocellulose, and detection with <sup>32</sup>P-labeled  $\alpha_6$  cDNA and actin cDNA probes was done as described in de Curtis et al. (1991). For photography, blots were exposed to Kodak XAR-5 film. Bands were quantitated on a Molecular Dynamics Computing Densitometer. Each value for  $\alpha_6$  transcript was normalized to the corresponding value for  $\beta$ -actin transcript.

## RESULTS

### Characterization of three polyclonal antibodies raised against different portions of the $\alpha_6$ polypeptide

To study the possible functions of the chick  $\alpha_6\beta_1$  integrin in mediating interactions of retinal neurons with laminin in vitro and to characterize its distribution during retinal development in vivo, three polyclonal antibodies have been prepared to different portions of the  $\alpha_6$  polypeptide. The  $\alpha_6$ -EX antibody was prepared to the external domain of  $\alpha_6$ , using a fusion protein. The  $\alpha_6$ -cytoA and  $\alpha_6$ -cytoB antibodies were prepared using as antigens the carboxy-terminal

peptide sequences present in the cytoplasmic domains of two distinct variants of the human  $\alpha_6$  subunit, which are generated by alternative RNA processing (Tamura et al., 1991). Fig. 1 shows the high degree of homology between the chick and human  $\alpha_6A$  cytoplasmic domains, where 34 of the 35 amino acid residues are conserved. Furthermore, analysis of the most 3' portions of chick  $\alpha_6$  cDNAs, sequenced by us previously (de Curtis et al., 1991), reveals the presence of nucleic acid residues which share 88% identity with the corresponding nucleic acid human sequence (Fig. 1B). The 3' portion of this sequence contains a portion of an open reading frame encoding 19 amino acid residues that appears to correspond to a chick equivalent of the first portion of the 53-residue long human  $\alpha_6B$  cytoplasmic domain. 18 of these 19 residues are identical in the human and chick amino acid sequences, including several residues that are highly conserved in all integrin  $\alpha$  subunits. These data suggested to us that two isoforms of the integrin  $\alpha_6$  subunit are also present in the chick.

To obtain direct evidence for this possibility, the  $\alpha_6$ -EX,  $\alpha_6$ -cytoA and  $\alpha_6$ -cytoB antibodies were used to immuno-precipitate proteins from a lysate prepared from [ $^3$ H]glucosamine-labeled E6 retinal neurons. SDS-PAGE analysis under non-reducing conditions of immunoprecipitates (Fig. 2) showed that all three antibodies recognize a band of  $140 \times 10^3 M_r$ , corresponding to the chick  $\alpha_6$  integrin subunit (de Curtis et al., 1991). With all three antibodies the  $140 \times 10^3 M_r$  band coprecipitated with a polypeptide with a smaller apparent molecular mass, which comigrated with the integrin  $\beta_1$  subunit (Fig. 2, compare lanes 1–3 with lane 4). In addition, small amounts of a  $180 \times 10^3 M_r$  band were also coprecipitated by the  $\alpha_6$ -cytoB antibody (Fig. 2, lane 3). This band was not observed in immunoprecipitates using anti- $\beta_1$  antibodies (Fig. 2, lane 4) and seems likely to be the integrin  $\beta_4$  subunit which has been shown to associate with  $\alpha_6$  in other cells (e.g. Sonnenberg et al., 1990).

#### $\alpha_6$ is involved in the interactions of retinal cells with LN in vitro

When E6 retinal neurons were plated onto LN-coated wells, cell attachment to the substratum was inhibited by the  $\alpha_6$ -EX antibody in a dose-dependent manner (Fig. 3). 66% of cell attachment was inhibited when 1 mg/ml of  $\alpha_6$ -EX IgG were present during a 1 hour incubation at 37°C. No significant inhibition was observed when the incubation was performed on collagen IV (70+/-6% inhibition) or when 1 mg/ml of normal rabbit (NR) IgG was present (not shown). An antibody to the integrin  $\beta_1$  subunit (CSAT) virtually eliminated attachment of E6 retinal neurons to LN during a 1 hour incubation, consistent with previous results (Hall et al., 1987; de Curtis et al., 1991).

To test the effect of  $\alpha_6$ -EX antibody on neurite outgrowth, E6 retinal neurons were plated on LN or collagen IV in the presence of 0.5 mg/ml  $\alpha_6$ -EX IgG and cultured for 24 hours at 37°C. Results are quantitated in Table 1 and are illustrated in Fig. 4. Results in Table 1 show that fewer than half as many neurons developed neurites on laminin in the presence of  $\alpha_{6EX}$  IgG compared to control IgG or no IgG. As expected,  $\alpha_{6EX}$  IgG did not inhibit neurite formation on collagen IV. Previous results have shown that antibodies to the integrin  $\beta_1$  subunit almost completely eliminate retinal neurite formation on laminin and collagen IV (Hall et al., 1987). Although fewer cells extended neurites, data in Table 1 show that those neurons able to form neurites grew comparatively long neurites even in the presence of  $\alpha_{6EX}$  IgG. Taken together, the effects of  $\alpha_{6EX}$  IgG in cell adhesion and neurite outgrowth assays suggest that  $\alpha_6\beta_1$  is a prominent, but is not the only laminin receptor present on these neurons. The failure of  $\alpha_{6EX}$  IgG to reduce neurite length can be interpreted in many ways. For example, cell heterogeneity could explain these observations.

## Differential localizations of two alternatively spliced forms of $\alpha_6$ protein in the developing chick retina

Cryosections of E6 retinæ were stained with each of the three antibodies against the chick  $\alpha_6$  subunit to study the localization of this LN receptor and its isoforms in the neural retina. At low magnification the  $\alpha_6$ -EX antibody showed a relatively uniform distribution of this integrin subunit through the width of the retina at E6 (Fig. 5A). Staining with the  $\alpha_6$ -cytoA and  $\alpha_6$ -cytoB antibodies revealed two dramatically different patterns of distribution of their respective antigens. The  $\alpha_6$ -cytoB antibody showed a reasonably uniform staining of cell surfaces throughout the E6 retina, which was weaker but similar to that observed when the  $\alpha_6$ -EX antibody was used (Fig. 5C). The  $\alpha_6$ -cytoA antibody stained an antigen restricted to a small region of the retina adjacent to the optic nerve and to the optic nerve itself (Fig. 5B). Sections incubated with each of the three preimmune sera showed very low backgrounds (Fig. 5D–F). At higher magnification the antigens recognized by the  $\alpha_6$ -EX and the  $\alpha_6$ -cytoB antibodies were detected throughout the retina (Fig. 5H,I). A discontinuous layer of cells characterized by spherical cell bodies located near the vitreal surface, developing retinal ganglion cells, showed more intense staining. At higher magnification, staining with the  $\alpha_6$ -cytoA antibody (Fig. 5K) was comparable to that obtained by using the corresponding preimmune serum (Fig. 5L), with the exception of the areas of the retina adjacent to the optic nerve (see diagram Fig. 5G, box 2), where an intense stain was detected throughout the thickness of the retina (Fig. 5J).

Similar patterns of staining were seen in sections of E12 retinæ. In this case, both the  $\alpha_6$ -EX and the  $\alpha_6$ -cytoB antibodies strongly stained a small fraction of the cell bodies and axons present in the now recognizable ganglion cell layer (Fig. 6A,B). The majority of the spherical cell bodies in this layer showed no or weak staining. The intensities of the staining of most areas in the E12 chick retinæ by the  $\alpha_6$ -cytoA antibody (Fig. 6C) were comparable to the staining obtained with the corresponding preimmune serum (Fig. 6D). In Fig. 7, the distribution of the  $\alpha_6$  cyto A antigen is illustrated in low magnification photomicrographs of the retina and surrounding tissues adjacent to the optic nerve. Similar to the expression pattern in E6 retinæ, specific staining with this antibody appeared to be restricted to areas of the neural retina around the optic nerve, and to the nearby vasculature protruding into the vitreous at this developmental stage.

## Codistribution of the $\alpha_6$ polypeptide and LN in the developing chick retina

To determine whether the distribution of the  $\alpha_6$  integrin subunit is consistent with this protein functioning as one subunit of a LN receptor ( $\alpha_6\beta_1$ ) in the developing chick retina, we compared the distribution of  $\alpha_6$  with that of LN in E6 retinæ. Parallel sections from E6 retinæ were stained with the polyclonal anti-LN antibody JW2 (Fig. 8B), with  $\alpha_6$ -EX (Fig. 8C), or with the monoclonal antibody G4 which is specific for the cell adhesion molecule NgCAM (Fig. 8D). The G4 antibody has been shown to recognize specifically retinal ganglion cells in the developing chick retina where it is concentrated on their axons (Lemmon and Mc Loon, 1986). As expected, this antibody stained the layer of retinal ganglion cell axons running between the retina and the vitreous (Fig. 8D, arrowheads). When adjacent sections were incubated with the  $\alpha_6$ -EX antibody, a layer of round cell bodies colocalizing with Ng-CAM-expressing retinal ganglion cells was heavily stained in E6 retinæ. Within the layer of axons projecting from retinal ganglion cells,  $\alpha_6$  appeared concentrated on axons in close proximity to the basal lamina at the vitreal surface (Fig. 8C). A similar pattern of distribution was observed for LN, which was also concentrated in the basal lamina at the interface between the retina and the vitreous (Fig. 8B).

## Effects of target ablation on levels of $\alpha_6$ mRNA in retinal ganglion cells

Between E6 and E10, retinal ganglion cells lose responsiveness to laminin at a time correlating with innervation by their axons of the optic tectum (Cohen et al., 1989). These neurons also reduce by at least 4-fold  $\alpha_6$  mRNA levels during this period of development (de Curtis et al., 1991). Tectal ablations appear to reverse, at least partially, the loss of laminin responsiveness (Cohen et al., 1989), making it seem possible that tectal innervation down regulates  $\alpha_6$  mRNA levels.

To determine whether  $\alpha_6$  mRNA levels in retinal ganglion cells are regulated by innervation of the optic tectum, the optic tecta of E5-6 chicks were removed and mRNA levels examined in retinal neurons at E9-E11. RNA blots of total RNA from retinal ganglion cell-enriched and depleted fractions were assayed, using cells from unoperated chicks of the same ages as controls. Results in Fig. 9 show that a single  $\alpha_6$  transcript of 5.3 kb was detected in control and tectal-ablated embryos, in agreement with previous observations (de Curtis et al., 1991). Ablation of the tectum at E5-6 did not appear to increase  $\alpha_6$  mRNA levels dramatically in either retinal ganglion cells or other neuroretinal cells at E9-10. To quantitate these results more carefully, values for  $\alpha_6$  mRNA levels were normalized to the corresponding values for  $\beta$ -actin mRNA, used as a standard in the RNA blots. The ratio of  $\alpha_6$  mRNA levels in the experimental versus control animals is presented in Table 2. No significant change in  $\alpha_6$  mRNA was seen in the fraction of retinal cells depleted of retinal ganglion cells. Individual experiments varied between a maximum 29% increase and minimum 16% decrease with an average of 0% change and a standard deviation of 20%. In contrast, a small average increase of approx. 20% was seen in retinal ganglion cells from operated animals. Individual experiments varied between a maximum 47% increase and minimum 24% decrease with a standard deviation of 33%. The levels in operated animals were not statistically significantly different from levels in controls. Since the level of  $\alpha_6$  mRNA in E6 retinal ganglion cells is at least 300% higher than that in E12 retinal ganglion cells (de Curtis, 1991), one might have expected a similar magnitude increase in  $\alpha_6$  mRNA in operated animals if the tectum provided the major signal down-regulating  $\alpha_6$  mRNA in retinal ganglion cells. This thus seems unlikely.

## DISCUSSION

Four major conclusions can be made from the results of this paper. First, the integrin  $\alpha_6\beta_1$  is a prominent, functional receptor for LN utilized by embryonic retinal neurons in vitro. Second, the presence of  $\alpha_6$  antigen at contact sites between these neurons and LN in vivo suggests that this receptor is utilized in vivo. Third, striking differences in the spatial distributions of alternative variants of  $\alpha_6$  suggest that there may be different functions for the two LN receptors,  $\alpha_6A\beta_1$  and  $\alpha_6B\beta_1$ , in the developing retina. Finally, tectal ablation does not significantly affect  $\alpha_6$  mRNA levels in retinal ganglion cells. Thus, previously observed effects of contact with optic tectum on laminin-responsiveness in retinal ganglion cells seem unlikely to reflect transcriptional regulation of  $\alpha_6$  expression.

In previous work, survival and differentiation of neurons has been shown to be dramatically influenced by LN (reviewed in Sanes, 1989; Reichardt and Tomaselli, 1991). In particular, E6 retinal neurons have been shown to respond to LN-coated substrata by extending long neurites. In contrast, most E12 neural retinal cells in culture can neither adhere to nor extend neurites on LN (Cohen et al., 1986; Hall et al., 1987). The same authors have shown that  $\beta_1$  integrins are prominent receptors utilized by these neurons to interact with LN in vitro.

In a recent paper, we showed that the  $\alpha_6\beta_1$  integrin is one of the candidate LN receptors that is expressed in E6 retinal neurons, suggesting that it may be an important LN receptor for these cells (de Curtis et al., 1991). In the present study, we have provided direct evidence for this. An antibody,  $\alpha_6$ -EX, was prepared to a large fragment of the extra-cellular domain of the  $\alpha_6$

subunit. This antibody was shown to recognize  $\alpha_6$  specifically. In functional cell adhesion and neurite outgrowth assays, the  $\alpha_6$ -EX antibody strongly inhibits in a dose-dependent manner interactions of embryonic retinal neurons with LN, but not collagen IV. In agreement with previous work, antibodies to the  $\beta_1$  subunit were also shown to inhibit interactions of these neurons with LN. Since  $\alpha_6$  and  $\beta_1$  coassociate in the embryonic neuroretina (de Curtis et al., 1991), the results imply that  $\alpha_6\beta_1$  is a functional receptor on these neurons for LN, but not collagen, consistent with its properties in other cells (Hall et al., 1990; Sonnenberg et al., 1990).

Since the inhibitory effects of  $\alpha_6$ -EX are strong, but incomplete, results in the present paper also suggest that an additional integrin functions as a LN receptor on retinal neurons. This receptor must contain the  $\beta_1$  subunit, since antibodies to  $\beta_1$  prevent virtually all interactions of these neurons with LN (this paper and Hall et al., 1987). Several other integrin  $\alpha$  subunits -  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  and  $\alpha_7$  - have now been shown to associate with  $\beta_1$  to form functional LN receptors. In previous work, we detected  $\alpha_3\beta_1$ , but were not able to detect either  $\alpha_1\beta_1$  or  $\alpha_2\beta_2$  in the retina (de Curtis et al., 1991). By immunocytochemistry, though,  $\alpha_1$  is clearly present in E7.5 retina and is reported to be expressed by retinal ganglion cells (Duband et al., 1992). Neither  $\alpha_1\beta_1$  nor  $\alpha_2\beta_1$  functions as laminin receptors in all cells in which they are expressed (cf. Chan and Hemler, 1993; Ignatius and Reichardt, unpublished).  $\alpha_1\beta_1$  also appears to recognize a site in laminin not recognized by retinal neurons (de Curtis et al., 1991). Thus, it may function as a retinal laminin receptor, but this is uncertain. In more recent work, we have not detected the  $\alpha_7$  subunit in the embryonic neuroretina of the mouse, using immunocytochemistry (D. Sretavan and L. F. Reichardt, unpublished results), suggesting it is not likely to be present in the chick retina. Thus, the most likely candidate to be an additional LN receptor(s) in the embryonic chick retina is  $\alpha_3\beta_1$  (and possibly  $\alpha_1\beta_1$ ). If so, the activity of  $\alpha_3\beta_1$  must be regulated by post-translational mechanisms, since it is expressed by neurons at E12 that don't interact strongly with LN (de Curtis et al., 1991).

To study the possible role of  $\alpha_6\beta_1$  as a LN receptor in vivo, we examined its expression during retinal development. As assessed using the  $\alpha_6$ -EX antibody, the  $\alpha_6$  protein is broadly distributed in the retina at E6, with particularly high expression in cells close to the vitreal surface, where the retinal ganglion cells are developing at this stage (Thanos and Bonhoeffer, 1983; Rager, 1980). At this stage, retinal ganglion cell axons are growing along the vitreal surface, and many of them are entering the optic nerve, where they extend towards their target, the optic tectum (Rager, 1980). We found that the  $\alpha_6$  polypeptide was most strongly localized to a subset of axons or perhaps domains of axons, those portions in contact with the vitreal surface, where it colocalized with the sites of highest levels of expression of LN. In contrast, NgCAM, a marker for the axons of mammalian and chick retinal ganglion cells (Lemmon and McLoon, 1986; Pigott and Davies, 1987), was expressed at high levels throughout the entire width of the retinal ganglion cell axon tract consistent with its previously demonstrated role in axon-axon interactions (cf. Chang et al., 1987).

In E12 retinas, the overall distribution of the  $\alpha_6$ -EX antigen was more restricted. Fewer cells still expressed high levels of this protein (Fig. 6). At this stage of development, virtually all retinal ganglion cells have reached the optic tectum. The layer of retinal ganglion cell axons between the retina and the laminin-rich vitreous is thicker at this stage, but only domains of axons in proximity to the laminin-rich vitreal surface showed a high level of expression of the  $\alpha_6$  subunit (Fig. 6). The correlation between the patterns of distribution of  $\alpha_6$  and LN in vivo, together with the demonstrated role of  $\alpha_6\beta_1$  as LN receptor in retinal cells in vitro strongly suggest that this integrin is an important LN receptor in the developing chick retina. Numerous previous studies on non-neuronal cells have documented many examples where integrins are colocalized on domains of plasmalemma in contact with their ligands (cf. Carter et al., 1990a,b).



Possible functions of laminin mediated through this receptor include regulation of migration or morphogenesis of neurons in early retinal development at times when both laminin and  $\alpha_6\beta_1$  are expressed at moderate levels throughout the width of the retina. Isoforms of laminin have also been detected along virtually the entire embryonic retinal-tectal pathway (McLoon, 1984; McLoon et al., 1988; Adler et al., 1985; Cohen et al., 1987; Halfter and Fua, 1987). Expression of laminin is transient in the optic stalk and correlates with the ability of retinal ganglion cells to utilize laminin as a substratum (cf. Cohen et al., 1987; 1989). These observations suggest that laminin and the  $\alpha_6\beta_1$  integrin will prove to be important in regulating development and axon outgrowth of retinal ganglion cells. It should now be possible to test these possible functions in vivo using  $\alpha_6$ -specific inhibitory antibodies and other appropriate reagents.

The present paper presents evidence that  $\alpha_6$  antibodies fail to stain the majority of retinal ganglion cells in E12 neuroretina. Previously, analysis of the expression of the  $\alpha_6$  integrin subunit at the mRNA and polypeptide levels has shown a dramatic decrease in the expression of this mRNA and protein between E6 and E12 in retinal ganglion cells (de Curtis et al., 1991). This suggests that transcriptional regulation of the expression of  $\alpha_6$  accounts, at least in part, for the decreased responsiveness of older retinal ganglion cells to LN (de Curtis et al., 1991). Since significant  $\alpha_6$  protein is seen on a proportion of these neurons, though, transcriptional regulation does not appear to be sufficient to account for the loss of responsiveness in neurite outgrowth assays of essentially all retinal ganglion cells neurons to laminin. As will be discussed below, the ligand-binding activity of integrins has been shown to be regulated on other neurons in the retina (Neugebauer and Reichardt, 1991) and may potentially be reduced also in older retinal ganglion cells. Alternatively, other developmental changes may reduce the signals transmitted by laminin binding to  $\alpha_6\beta_1$  which result in neurite outgrowth.

Tectal ablation has been reported to prevent partially the decrease in responsiveness of older retinal ganglion cells to laminin (Cohen et al., 1989), suggesting that target contact may regulate integrin expression in these cells. In the present paper, we have attempted to determine whether contact with the optic tectum regulates  $\alpha_6$  expression at the mRNA level by examining effects of tectal ablation at E5-6 on  $\alpha_6$  mRNA levels at E9-11. In normal animals during approximately this interval,  $\alpha_6$  mRNA levels in retinal ganglion cells are reduced to less than one-fourth of the E6 levels (de Curtis et al., 1991). This is not prevented by ablation of the tectum (Fig. 9 and Table 2). Thus, tectal contact does not appear necessary for down-regulation of  $\alpha_6$  mRNA. The report that tectal ablation partially prevents the decrease in laminin-responsiveness of retinal ganglion cells (Cohen et al., 1989) could be explained by effects on expression of other laminin receptors, such as  $\alpha_1\beta_1$  or  $\alpha_3\beta_1$ . Alternatively, the ligand-binding activity of residual  $\alpha_6\beta_1$  receptors on retinal ganglion cells may be activated by tectal ablation.

Results in the present paper demonstrate changes in distribution and reductions in apparent levels of  $\alpha_6$  subunit expression in most areas of the developing retina (see Fig 6, Fig 7, Fig 8). In previous work, using protein and RNA blots, dramatic decreases in expression in non-retinal ganglion cell populations were not seen (de Curtis et al., 1991), even though these cells at E12 have lost the abilities to adhere to or extend neurites on laminin (containing the A, B1 and B2 subunits). Our previous work also demonstrated that substantial levels of the  $\alpha_6\beta_1$  receptor were present on the surfaces of older retinal cells fractionated to remove retinal ganglion cells. Taken together, the results suggest that  $\alpha_6\beta_1$  is present in a comparatively inactive state on the surfaces of these cells. Consistent with this possibility, binding assays indicate that the affinity of laminin-binding sites for laminin is reduced approx. 100-fold on the neurites of E9 compared to E6 retinal cells (Cohen et al., 1989). In addition, a monoclonal antibody, TASC, that binds the  $\beta_1$  subunit and activates ligand-binding by  $\beta_1$ -integrins restores the ability of E12 retinal neurons to bind to laminin (Neugebauer and Reichardt, 1991). Posttranslational activation of

the surface  $\alpha_6\beta_1$  receptor has been shown previously in macrophages (Shaw et al., 1990). These results suggest that a large fraction of the  $\alpha_6\beta_1$  receptor present in E12 neuroretina is not active, but can be activated by appropriate physiological stimuli, which remain to be identified.

Alternative RNA processing, including in some cases alternative exon splicing, has been described for some integrin subunits, and more recently variants with alternative cytoplasmic domains generated by differential RNA processing have been described for the mammalian  $\alpha_3$  and  $\alpha_6$  subunits (Tamura et al., 1991). By using antibodies specific for the two alternative cytoplasmic domains of the  $\alpha_6$  subunit, we found that both forms were present in the developing chick neural retina, and that the patterns of distribution of the two  $\alpha_6$  isoforms were quite different. The  $\alpha_6$ -cytoA subunit was detected in a very restricted distribution, with high levels only near the optic nerve, while the distribution of the  $\alpha_6$ -cytoB isoform was virtually identical to that of the  $\alpha_6$  subunit in toto, as detected using the  $\alpha_6$ -EX antibody. In previous work, the two different  $\alpha_6$  isoforms have been shown to be expressed differentially in different tissues and during differentiation using PCR analysis (Tamura et al., 1991; Cooper et al., 1991). The present paper presents the first evidence for differential distribution of these isoforms within an organ or tissue. Since the sequence of each of the alternatively expressed cytoplasmic tails is conserved in mammalian and avian species, each seems likely to have a distinct function. As one possibility, different cytoplasmic domains on the  $\alpha_6$  subunit might allow a single ECM protein, laminin, to transmit different signals to different cells.

In summary, the results presented in this paper show that the  $\alpha_6\beta_1$  receptor is a prominent LN receptor for retinal neurons, and suggest roles for the  $\alpha_6\beta_1$  integrin during retinal development and retinal ganglion cell axonogenesis. It will be important to analyze in the future the exact role of this and other integrins in mediating retinal development in vivo, and to see whether its different isoforms have more specific roles in regulating neuronal differentiation.

## Abbreviations

CMF-PBS,  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS; ECM, extracellular matrix; E6, embryonic day 6; E12, embryonic day 12; LN, laminin; NR, normal rabbit.

## Acknowledgments

We are grateful to Kristine Venstrom for her help with the preparation and purification of the  $\alpha_6$ -cytoA and  $\alpha_6$ -cytoB antibodies, and to Dr Vito Quaranta (Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037) for providing us with the sequence of the human  $\alpha_6$ -cytoB peptide. We also would like to acknowledge the generous gift of the G4 monoclonal antibody (anti-NgCAM) from Dr F. Rathjen (Zentrum für Molekulare Neurobiologie, Hamburg, Federal Republic of Germany), the CSAT monoclonal antibody (anti- $\alpha_1$ ) from Dr A. F. Horwitz (University of Pennsylvania, Philadelphia, PA), anti-EHS-laminin antibodies (JW2) from Janet Winter, and anti-chick laminin antibodies from Dr Charles Little (University of Virginia, Charlottesville, VA). We thank Dr Frances Lefcort for helpful comments on the manuscript.

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A

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B

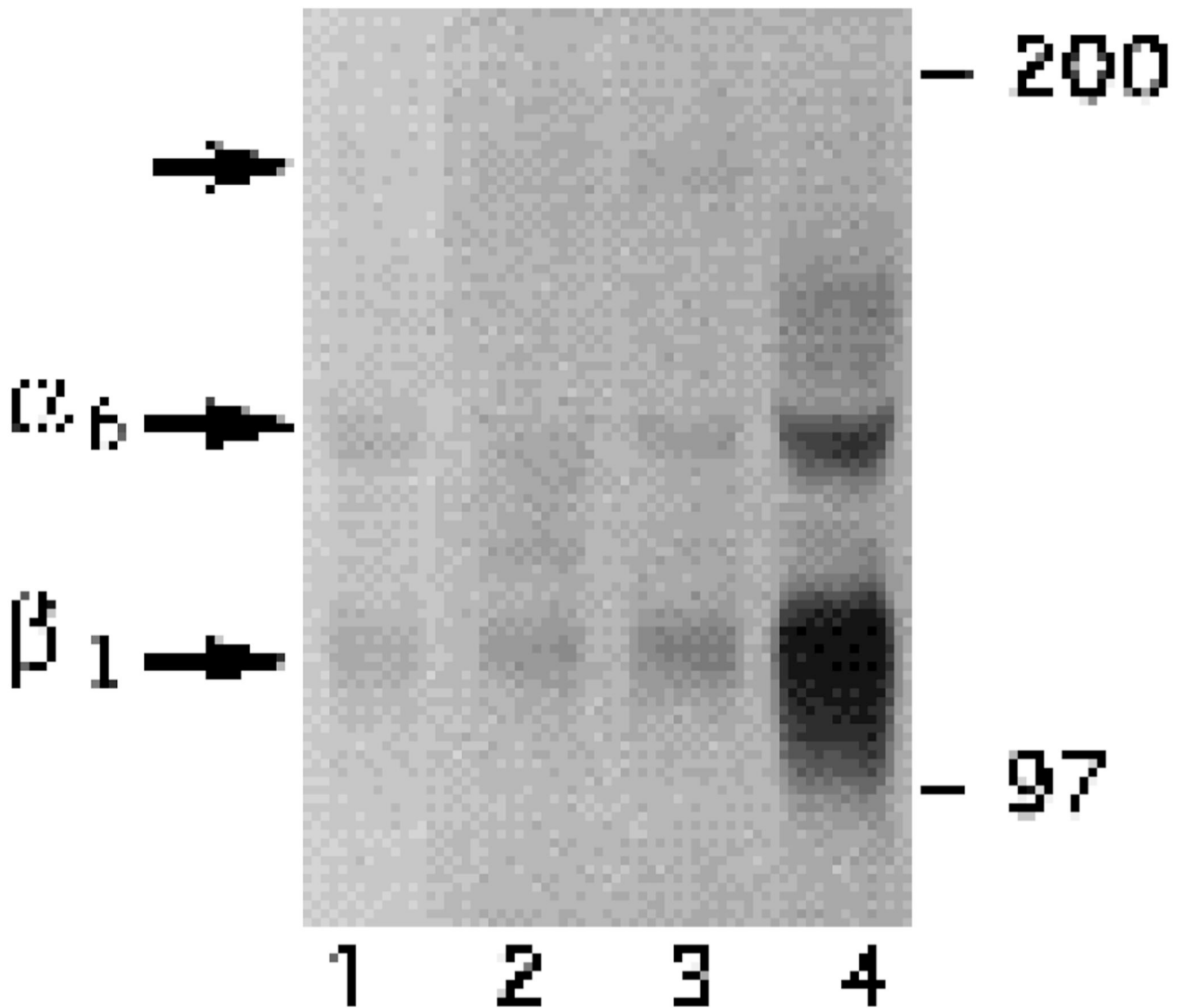
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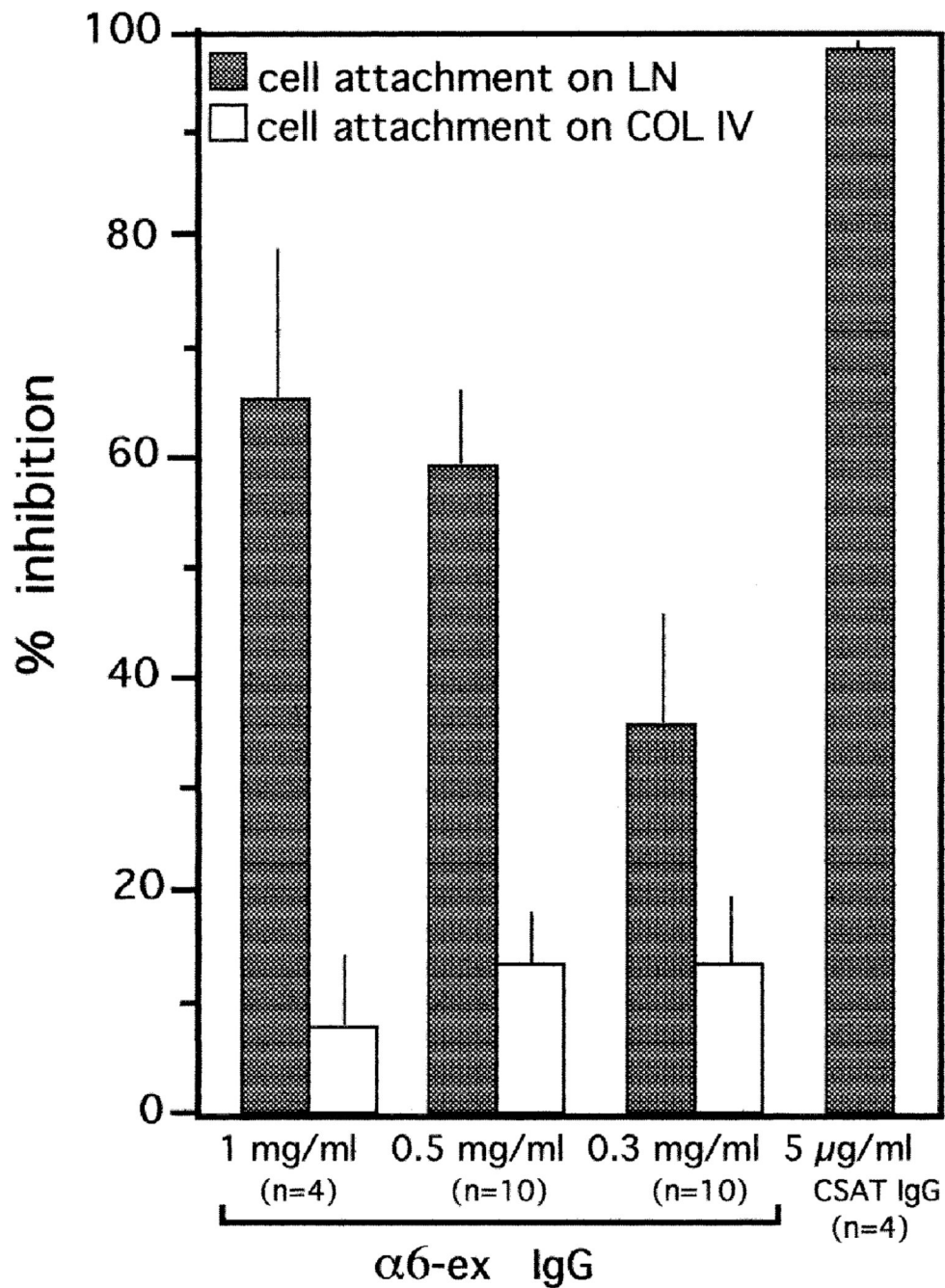
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**Fig. 1.**

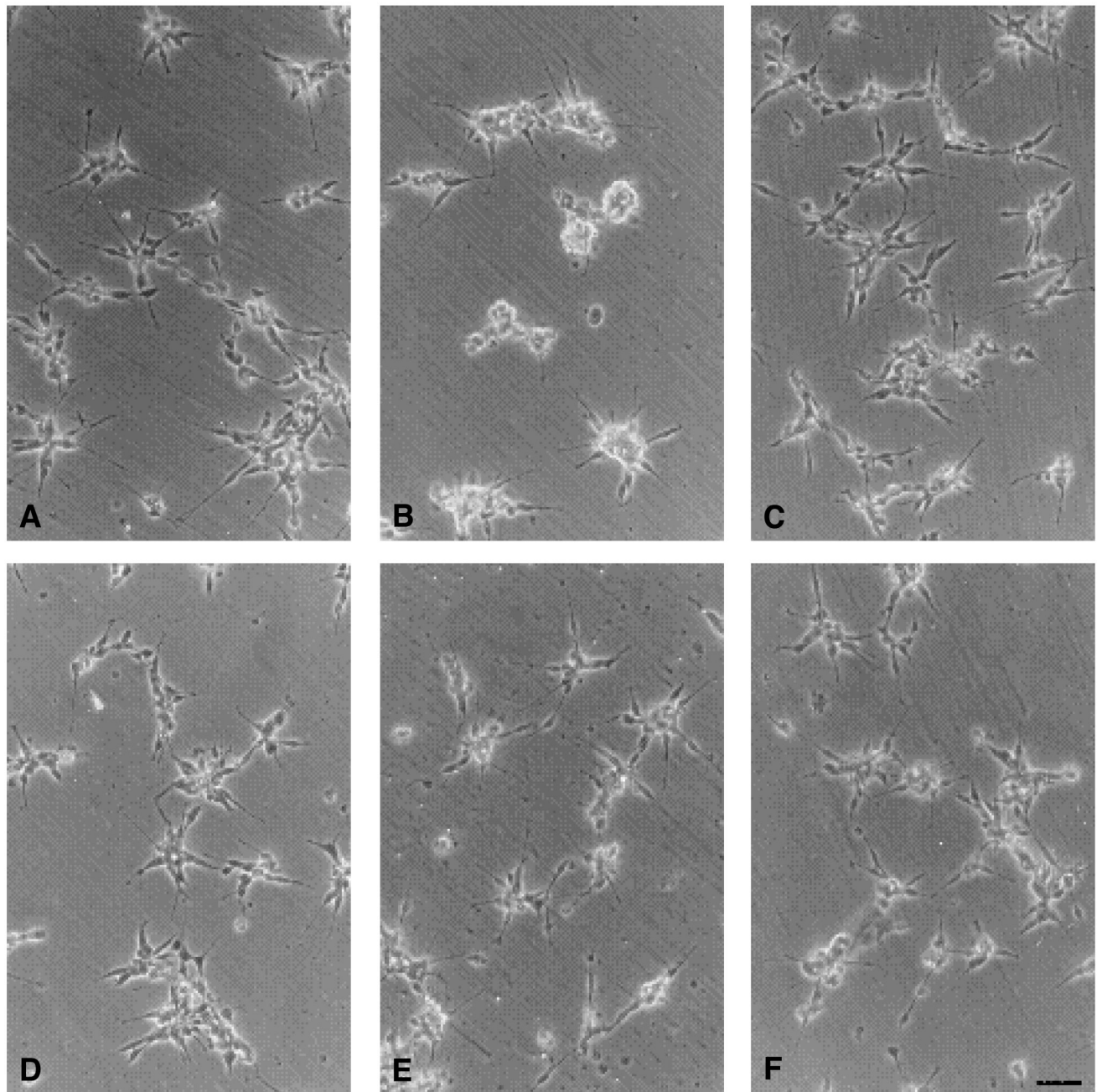
Sequence comparisons between human and chick cytoplasmic domains of integrin  $\alpha_6$  proteins. In A, the entire amino acid sequences of the chick (top) and human (bottom) A class cytoplasmic domains are aligned. Note that 34 of 35 amino acid residues are identical. In B, the most 3' sequenced portion of the chick  $\alpha_6$  mRNA is aligned with the same region in the human  $\alpha_6$  mRNA. Note the strong homology at the nucleic acid level. Note also that an open reading frame in the chick sequence (top) is very similar to an open reading frame in the human sequence (bottom), which has been shown to constitute the cytoplasmic domain of the B-isoform of the human  $\alpha_6$  subunit (Tamura et al., 1991). Only the amino terminal portion of this reading frame is present in sequenced chick cDNAs and is aligned in this figure. Vertical lines indicate that identical amino acids that are present in each sequence.



**Fig. 2.** Immunoprecipitations from a cell lysate of metabolically labeled E6 retinal neurons. Cell lysates were prepared as described in the Experimental Procedures. For each lane, the same amount of TCA-precipitable radioactivity was used for immunoprecipitation with  $\alpha_6$ -EX (lane 1),  $\alpha_6$ -cytoA (lane 2),  $\alpha_6$ -cytoB (lane 3), or chick  $\beta_1$  (lane 4) polyclonal antibodies.

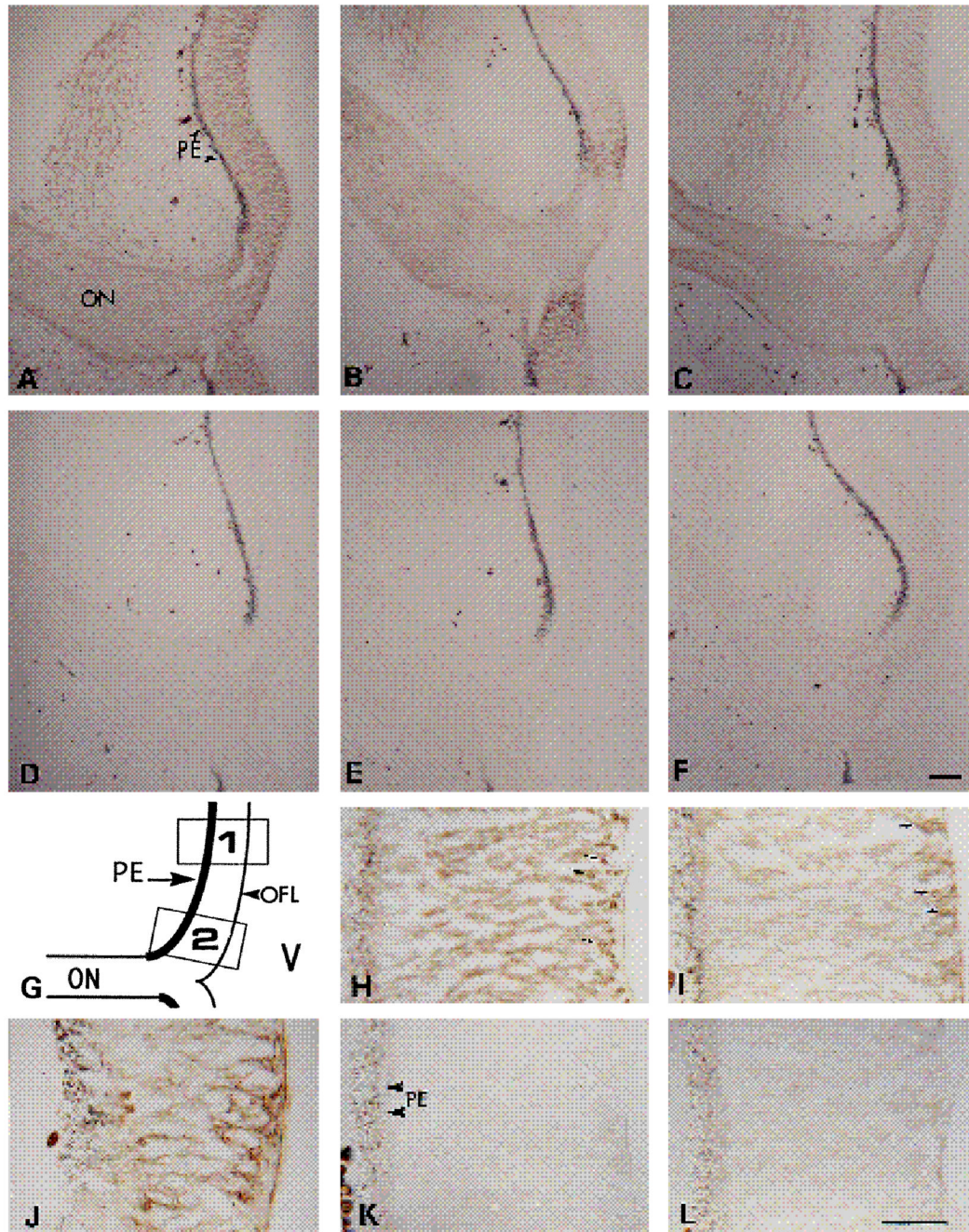


**Fig. 3.** Cell attachment assay of E6 retinal neurons on laminin (LN) or collagen IV (COL IV). Cells were prepared by trypsinization of E6 retinae and the cell attachment assay was performed for 1 hour at 37°C as described in the Experimental Procedures. Results of experiments with a function blocking  $\beta_1$  subunit-specific mAb (CSAT) and  $\alpha 6$ EX IgG are shown. In every experiment, each sample was tested in triplicate. Bars indicate the standard error; *n* represents the number of experiments.

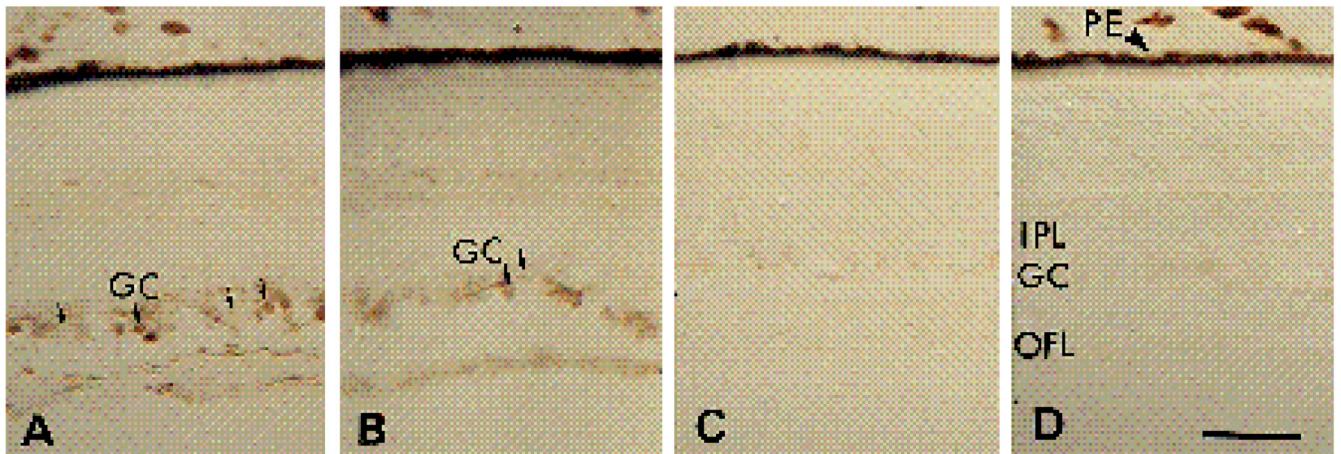


**Fig. 4.** Neurite outgrowth of E6 retinal neurons on LN (A–C) or COL IV (D–F). Retinal neurons were prepared from E6 retinae and used in the neurite outgrowth assay as described in the Materials and methods. The cells were cultured overnight without IgG (A,D), with 0.5 mg/ml  $\alpha_6$ -EX IgG (B,E), or with 0.5 mg/ml normal rabbit IgG (C,F). Bar, 40  $\mu$ m.

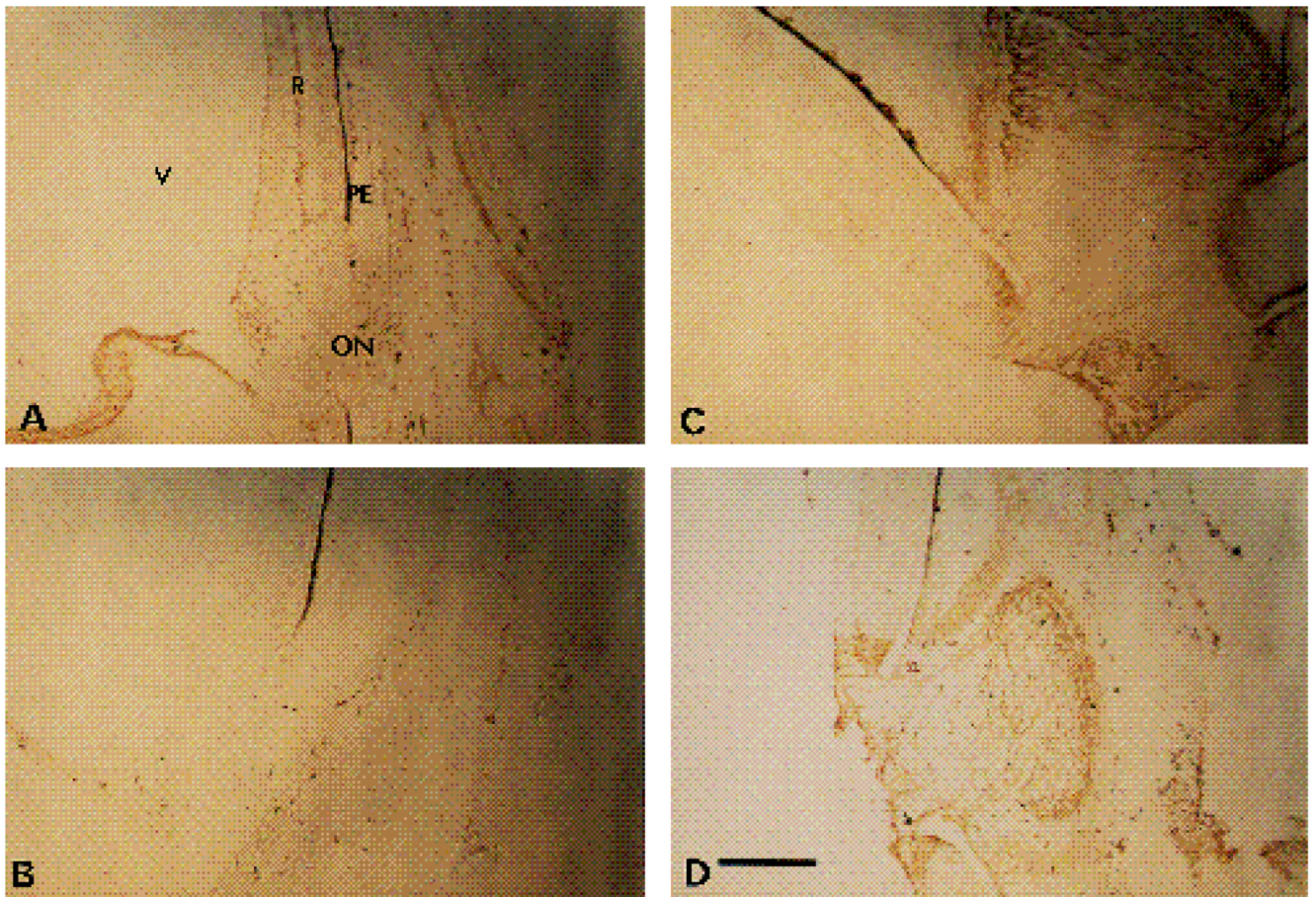




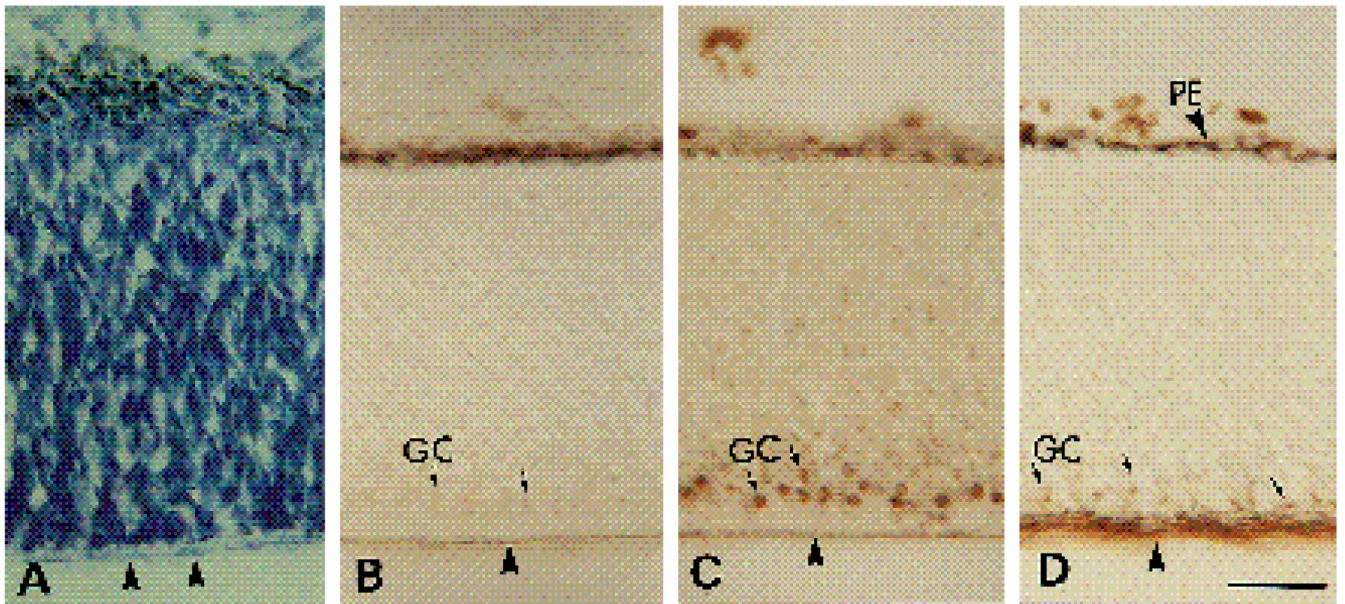
**Fig. 5.** Micrographs of immunoperoxidase staining on E6 chick retina. 16  $\mu\text{m}$  sections of E6 retina were incubated with  $\alpha_6$ -EX (A,H),  $\alpha_6$ -cytoA (B,J,K), or  $\alpha_6$ -cytoB (C,I) antibodies. D,E and F are control stainings with preimmune IgG, for A,B and C, respectively. L is the control staining with preimmune IgG for J and K. G shows a diagram of the low power magnification of the retina as shown in panels A–F. Box 1 indicates the area of the retina shown in panels H,I,K and L. Box 2 indicates the area of the retina shown in J. ON, optic nerve; PE, pigmented epithelium; OFL, optic fiber layer; V, vitreous. Bars are 20  $\mu\text{m}$  in A–F, 10  $\mu\text{m}$  in H–L.



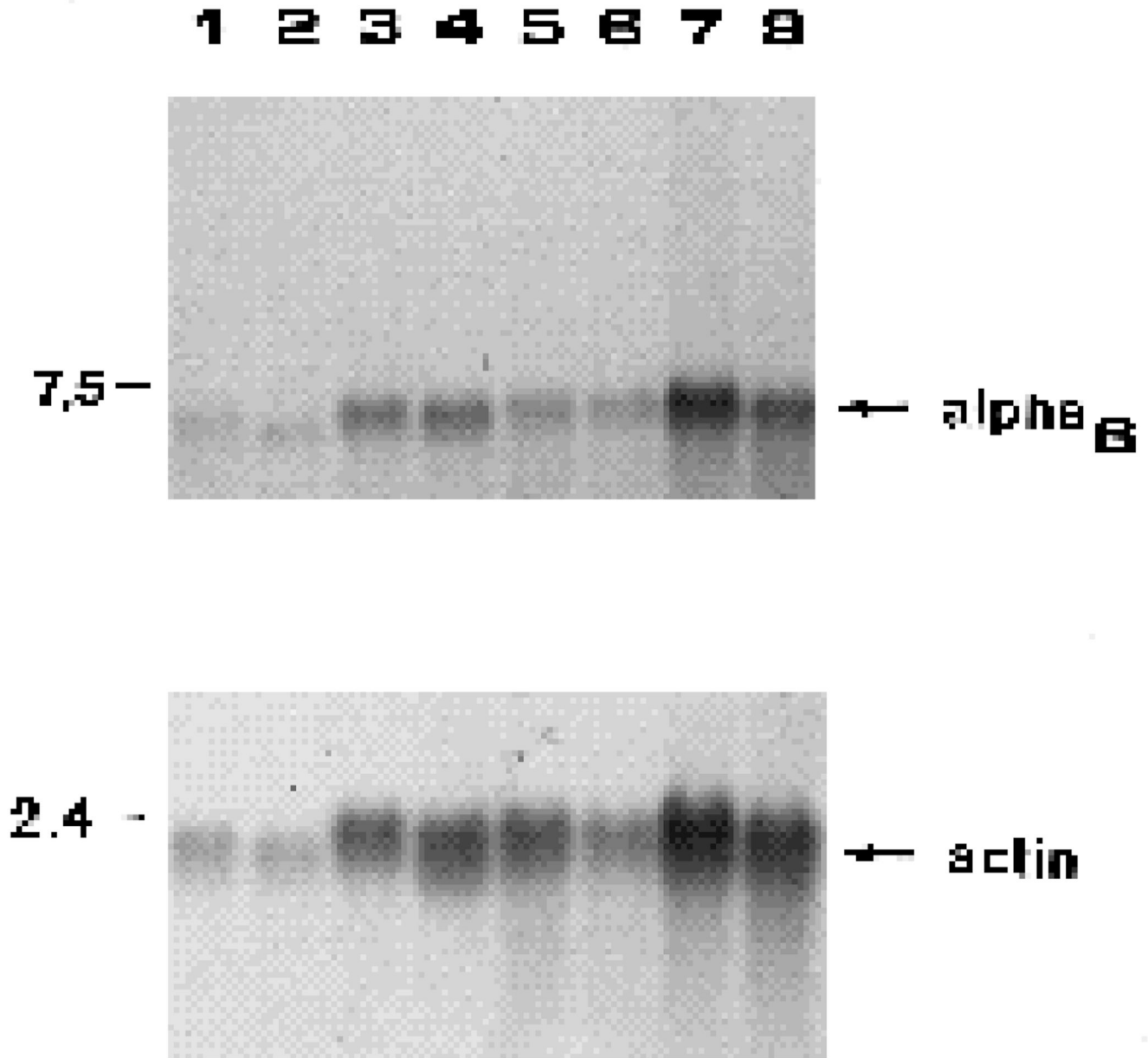
**Fig. 6.** Micrographs of immunoperoxidase staining on E12 chick retina. 16  $\mu\text{m}$  sections were stained with  $\alpha_6\text{-ex}$  (A),  $\alpha_6\text{-cytoB}$  (B) and  $\alpha_6\text{-cytoA}$  (C) antibodies. D is a section stained with preimmune IgG for the  $\alpha_6\text{-cytoA}$  antibody. IPL, inner plexiform layer; OFL, optic fiber layer; PE, pigmented epithelium. Arrows point to examples of cells with strong or faint immunoperoxidase reaction product in the ganglion cell layer. Bar, 10  $\mu\text{m}$ .



**Fig. 7.** Low power micrographs of  $\alpha_6$  and  $\alpha_{6A}$  expression patterns in E12 chick retina. 16  $\mu\text{m}$  sections were stained with  $\alpha_{6EX}$  IgG (A), preimmune IgG for  $\alpha_{6EX}$  (B), or  $\alpha_{6cytoA}$  (C and D). The region surrounding the optic nerve is visible in each panel. ON, optic nerve; PE, pigment epithelium; V, vitreous. Reaction product is brown. Note localized distribution of  $\alpha_{6A}$  in retina adjacent to the optic nerve, within the optic nerve, and in tissues surrounding the retina. Bar, 10  $\mu\text{m}$ .



**Fig. 8.** Micrographs of E6 (A–D) 16  $\mu\text{m}$  thick retina sections stained with thionin (A) or with immunoperoxidase after incubation with anti-LN (B),  $\alpha_6$ -EX (C), or G4 (D) IgG. GC, ganglion cells (arrows); PE, pigmented epithelium. Arrowheads indicate the border between the optic fiber layer and the vitreous. Bar, 10  $\mu\text{m}$



**Fig. 9.** Effects of tectal ablation on integrin  $\alpha_6$  mRNA levels in retinal ganglion cells and other retinal cells. Retinal ganglion cells (RGC) and retinal ganglion cell depleted fractions (non-RGC) were used to prepare total RNA samples. Lanes 1–4 are RNA samples from one experiment; lanes 5–8 are RNA samples from a second experiment. RGC, controls (lanes 1,5); RGC, tectum-ablated (lanes 2,6); non-RGC, controls (lanes 3,7); non-RGC, tectum-ablated (lanes 4,8). RNA samples were fractionated by agarose gel electrophoresis, transferred to nitrocellulose and incubated with  $^{32}\text{P}$ - $\alpha_6$  probe. The upper section of this blot was incubated with the  $^{32}\text{P}$ - $\alpha_6$  probe, the lower section with the  $^{32}\text{P}$ actin probe. The positions of DNA standards are indicated in kb on the left. When quantitated, tectal ablation resulted in 15% and 47% increases in  $\alpha_6$  mRNA in the RGC fractions, and 11% and 16% decreases in the non-RGC fractions respectively in the two experiments depicted here.

**Table 1**Effects of  $\alpha_6\text{EX}$  antibody on neurite formation and neurite outgrowth by retinal neurons

Substrate	IgG	% cells with neurites	Average neurite length
LN	–	70	30.1±2.2
LN	$\alpha_6\text{EX}$	32	25.6±2.1
LN	NR	71	31.4±1.6
col IV	–	55	26.6±1.9
col IV	$\alpha_6\text{EX}$	55	26.8±1.9
col IV	NR	52	24.7±1.9

Dissociated E6 retinal neurons were plated on laminin (LN)- or collagen IV (col IV)-coated substrata and incubated for 24 hours at 37°C in culture medium plus no IgG (–), 0.5 mg/ml of  $\alpha_6\text{EX}$  IgG ( $\alpha_6\text{EX}$ ) or 0.5 mg/ml of normal rabbit IgG (NR), as described in Materials and Methods. For quantitation of neurite formation by attached cells, 200 neurons in each culture condition were scored for the presence or absence of neurites. Results are presented as % of cells with neurites. For quantitation of neurite length, 50 neurons with neurites were examined in each culture condition. Neurite length is indicated as average neurite length  $\pm$  standard error.

**Table 2**

Effect of tectal ablations on  $\alpha_6$  mRNA levels in retinal ganglion cells (RGC) and cells depleted of retinal ganglion cells (non-RGC)

Experiment	$\alpha_6$ mRNA ablation	
	$\alpha_6$ mRNA control	
	RGC	Non-RGC
1	1.45	0.99
2	0.76	1.29
3	1.15	0.89
4	1.47	0.84
average	1.21±0.33	1.00±0.20

Retinal ganglion cells were separated from other cells on Percoll gradients as described in Materials and Methods. Each experiment used 3–5 embryos for each sample. The complete optic tecta were removed from embryos in windowed eggs at E5-6. Embryos were sacrificed between E9-11; total RNA was prepared and specific transcripts were quantitated by RNA blot as described in Materials and Methods. Values were normalized using  $\beta$ -actin mRNA as a control. Values in the table are ratios of normalized  $\alpha_6$  mRNA levels in ablated versus control animals. Average values  $\pm$  standard deviations are indicated.