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Widespread Expression of β1 Integrins in the Developing Chick Retina: Evidence for a Role in Migration of Retinal Ganglion Cells

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During extension of axons, critical neuronal interactions with extracellular matrix (ECM) and other cells are thought to be mediated in part by heterodimeric β1 integrin receptors. In this report, we examine the expression and function of β1 integrins in the developing chick retina. Expression of the β1 subunit, assayed by *in situ* hybridization and antibody staining of dissociated cells, was widespread in undifferentiated neuroepithelial cells, before the initiation of axons. Expression persisted in most retinal cell layers throughout embryonic development, during and after axon extension. The repertoire of β1-associated α subunits was examined using reverse transcription–polymerase chain reaction. In addition to the α6 and α8 subunits previously reported, chick homologues of the α2 and α4 subunits were detected. Developmental Northern blots revealed varying patterns of integrin subunit expression and showed that expression of β1 and the mRNAs of its associated α subunits are not always coregulated during retinal development. The timing and distribution of expression suggested that β1 integrins may be involved in other developmental events in addition to axon extension. To address functions carried out by β1 integrins in the early retina, explanted eye cups were incubated in the presence of function blocking anti-β1 antibody and migration of newly born retinal ganglion cells (RGCs) was assessed. RGC migration from the ventricular zone to the vitreal border was significantly inhibited, suggesting that β1 integrins play a role in neuroblast migration in the retina.

INTRODUCTION

During development of the vertebrate nervous system, contacts with other cells and with extracellular matrix (ECM) are essential in events such as cell fate determination, neuroblast migration, axon outgrowth, and synapse formation (Venstrom and Reichardt, 1993; Reichardt and Tomaselli, 1991; Sanes, 1989). Integrins, a widely expressed family of heterodimeric, transmembrane receptors, have been shown to mediate adhesive and migratory interactions between neural cells and ECM and cell surface ligands and may play roles in multiple phases of neural development, particularly axon outgrowth (Schwartz *et al.*, 1995; Hynes and Lander, 1992). At least 8 β and 16 α integrin subunits have been identified thus far, giving rise to over 20 different heterodimers. As many as 10 of these have been documented on cells of neural origin (Venstrom and Reichardt, 1993).

Specific combinations of integrin α and β subunits give rise to receptors with different ligand binding properties. However, some heterodimers are multifunctional, and many appear to have overlapping functions. For example, α2β1 functions as a dual collagen and laminin receptor, but at least seven other heterodimers have also been reported to bind laminin (Schwartz *et al.*, 1995). Mechanically, integrins link the actin cytoskeleton with extracellular ligands. In addition, integrins serve as bidirectional signal transducing receptors capable of transmitting signals that are extracellular and intracellular in origin (Sastry and Horwitz, 1993; Schwartz *et al.*, 1995).

Several members of the β1 subfamily, the largest integrin subfamily, have been shown to be expressed in developing chick and mouse retina (Hall *et al.*, 1987; de Curtis and Reichardt, 1993; de Curtis *et al.*, 1991; Bossy *et al.*, 1991; Cohen *et al.*, 1987; Sheppard *et al.*, 1994; Duband *et al.*, 1992). In the embryonic chick retina, retinal ganglion cells

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(RGCs), the only retinal cell type to project an axon out of the retina, are strongly immunoreactive with antibodies against the β1 subunit in both tissue sections and in cultured cells (Cohen *et al.*, 1987). Specific β1-associated α subunits that have been detected during chick retinal development include α3, α8, and isoforms of α6. The α3 subunit has been detected by Western blot analysis of Embryonic Day 6 (E6) retinal tissue but little is known about its spatial distribution, ligand binding, or function during retinal development. Isoforms of α6 have been detected on E6 and E12 RGCs and elsewhere in the developing chick retina (de Curtis and Reichardt, 1993). Immunoprecipitation suggests that α6 associates with β1 in the chick retina (de Curtis *et al.*, 1991) and polyclonal antibodies raised against the α6 extracellular domain decrease the ability of retinal cells to adhere and extend neurites on laminin-1 (Ln-1). The α8 subunit is expressed strongly on the axons of E6 and E12 RGCs and selected cells of the inner nuclear layer (INL) in the E12 chick retina (Bossy *et al.*, 1991). Although recent evidence suggests that α8β1 mediates neurite outgrowth of chick embryonic sensory and motor neurons on tenascin and fibronectin (Varnum-Finney *et al.*, 1995; Muller *et al.*, 1995), the function and ligand binding specificity in the embryonic chick retina have not been reported. The expression of αv has also been detected in the developing chick retina, where it associates with β3 and probably β5 (but not β1) and acts as a vitronectin receptor (Neugebauer *et al.*, 1991; Gervin *et al.*, 1996).

β1 Integrins appear to play major roles in axon and dendrite extension in the retina. ECM molecules recognized by integrins, particularly laminin, are expressed in pathways followed by RGC growth cones (Cohen *et al.*, 1987; Halfter and Fua, 1987; McLoon *et al.*, 1988). Function blocking anti-β1 antibodies abolish adhesion and outgrowth of retinal neurons on purified ECM molecules and partially decrease outgrowth on astrocyte cell surfaces (Hall *et al.*, 1987; Cohen *et al.*, 1987; Neugebauer *et al.*, 1988; Cohen and Johnson, 1991). In addition, a recent study showed that expression of heterologous dominant negative β1 subunits in *Xenopus* retinal neurons reduced axon and dendrite size *in vivo* (Lilienbaum *et al.*, 1995). Results from this study suggest that integrins are particularly important for RGC outgrowth, but not neuroblast migration during the formation of retinal laminae.

In contrast, other studies point to a role for integrins in the positioning and migration of retinal cells. Microinjection of function blocking anti-β1 antibody into developing chick eyes was shown to disrupt retinal morphogenesis and reduce the size and integrity of other ocular structures (Svennevik and Linser, 1993). A similar approach in *Xenopus* resulted in abnormal lamination of the retina, with rosettes of photoreceptors in ectopic locations (Leonard and Sakaguchi, 1995).

Elsewhere in the nervous system, the role of β1 integrins in neuroblast migration is unclear. Anti-β1 integrin antibody has been shown to block neural crest cell migration (Bronner-Fraser, 1985) and β1 antisense expression inhibits migration of neuroblasts in the developing tectum (Galileo *et al.*, 1992). However, a recent study of chimeric mice lacking β1 integrin in subsets of cells found normal migration of β1 null cells from the neural crest and β1 null cells in many parts of the central nervous system (Fassler and Meyer, 1995).

In this report, we show that expression of β1 integrin is widespread in developing retinal cells, including undifferentiated neuroepithelial cells, and we compare β1 mRNA expression to that of associated α subunit mRNAs. Furthermore, function blocking anti-β1 antibody is shown to inhibit migration of newly born RGCs in explanted eye cups, suggesting a role for integrins in migration of retinal neuroblasts.

MATERIALS AND METHODS

Embryo Staging and Retinal Dissection

Fertile White Leghorn chicken eggs (Rosemary Farms, Santa Maria, CA) were maintained in a forced draft, rocking incubator (G.Q.F. Manufacturing Co., Savannah, GA) at 37.5°C. Embryos were staged using the criteria of Hamburger and Hamilton (1951), and then retinal tissue was dissected in CMF-PBS (GibcoBRL, Gaithersburg, MD) taking care to remove the retinal pigment epithelium. Retinae were dissected from E5 through hatching (E21). For RNA purification, dissected tissue was then rinsed in a second bath of CMF-PBS, flash frozen in liquid nitrogen, and stored at −80°C.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated by a modification of the method of Chirgwin *et al.* (1979), as described by Clegg *et al.* (1989). Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (Clegg *et al.*, 1989). One microgram of poly(A)$^+$ RNA was subjected to reverse transcription with 200 units of Moloney murine leukemia virus reverse transcriptase (GibcoBRL) in buffer supplied by the manufacturer, supplemented with 1 μM random hexamers (New England BioLabs, Beverly, MA), 500 μM deoxyribonucleotide triphosphates (Pharmacia, Piscataway, NJ), and 40 units RNAsin (Promega) at 42°C for 1 hr. Reactions were terminated by heating to 65°C for 5 min and cDNA was frozen at −20°C until needed. PCR was carried out using up to 50 ng of cDNA taken directly from the cDNA synthesis reaction in a 1× PCR buffer that consisted of 50 mM KCl, 10 mM Tris–HCl, pH 9.0, 1.5 mM $MgCl_2$, 0.01% gelatin, and 0.1% Triton X-100 in a final volume of 100 μl. Integrin α subunit-specific primers described by Pytela *et al.* (1990), but with an *Xho*I site included, were added to a final concentration of 1 μM each, followed by 0.05 units of *Taq* polymerase (Perkin–Elmer, Foster City, CA). Reaction mixtures were overlaid with 100 μl of light mineral oil (Sigma) and subjected to 30 cycles of amplification (1 min 94°C, 1 min 48°C, 45 sec 72°C) in a Perkin–Elmer Cetus thermocycler. To facilitate the cloning of less abundant integrin cDNAs, PCR products were cleaved with selected restriction enzymes that cut more abundant cDNAs, and the remaining cDNAs were reamplified and subcloned. Amplification was dependent on input cDNA and both primers. A second α4 clone was amplified using internal, α4-specific primers. Integrin-specific PCR products

were not observed if retinal poly(A)$^+$ RNA was amplified without reverse transcription.

cDNA Cloning and DNA Sequence Analysis

PCR-amplified cDNA was treated with T4 DNA polymerase (New England BioLabs) and then isolated by electrophoresis onto strips of NA45 paper (Schleicher & Schuell, Keene, NH) using methods described in Sambrook *et al.* (1989). Blunt-ended cDNA was inserted into the *Sma*I site of pBluescript (KS$^-$) (Stratagene, La Jolla, CA) and recombinant plasmids were identified and propagated using standard procedures described in Sambrook *et al.* (1989). DNA sequences of plasmid inserts were determined using the Sequenase kit version 2.0 (United States Biochemical Corp., Cleveland OH). FastA and PileUp analyses of DNA sequences were carried out using the software package of the Genetics Computer Group (Madison, WI).

Northern Blotting

RNA samples were electrophoresed through 2.2 *M* formaldehyde agarose gels and transferred overnight onto Zeta-Probe GT nylon membrane (Bio-Rad, Richmond, CA). Hybridizations were carried out in 50% formamide, 5× SSPE, 5× Denhardts, 7% SDS, and 250 μg/ml sonicated and boiled herring sperm DNA (Sigma, St. Louis, MO). Labeled cDNA probes were prepared using the Prime-a-Gene kit (Promega, Madison, WI) to similar specific activities. After hybridization, filters were washed two times for 10 min at room temperature in 2× SSC, 0.2% SDS followed by four washes in 0.25× SSC, 0.2% SDS at 68°C. Multiple exposures of each blot were obtained to ensure that signals detected were in the linear range of the film. Estimations of lane to lane variations in amount of poly(A)$^+$ RNA loaded were determined by probing each blot with ^{32}P-labeled oligo(dT) (11–18) (Collaborative Research, Bedford, MA), as described previously (Clegg *et al.*, 1989). Each blot was repeated at least three times with independent preparations of RNA. Representative blots are shown. To quantify results (Fig. 4), blots presented in Fig. 3 were scanned using an LKB Ultroscan XL laser densitometer, and levels of specific integrin mRNAs were normalized according to the level of poly(A)$^+$ detected in each lane.

In Situ Hybridization

Eyes or whole heads of staged embryonic chicks were fixed in 4% paraformaldehyde for 1 hr at 0°C followed by cryoprotection in 10% sucrose at 4°C for 48 hr. Specimens were embedded in Histoprep (Fisher, Pittsburg, PA) and flash frozen in liquid nitrogen, and 20-μm cryostat sections collected on gelatin-coated slides. Sense and antisense digoxygenin (DIG)-labeled probes were transcribed from linearized plasmids in reactions composed of the full-length β1 cDNA (a kind gift of Dr. Clayton Buck) or β-tubulin cDNA (Cleveland *et al.*, 1980), T3, T7, or SP6 polymerase, and DIG-labeled UTP (Boehringer Mannheim, Indianapolis, IN). Labeled probes were hydrolyzed under alkaline conditions to obtain fragments of about 150 bases in length before use. Prior to hybridization, sections were treated with 1 mg/ml proteinase K (Sigma) at 37°C for 12–45 min followed by fixation in 4% paraformaldehyde for 5 min at room temperature. Sections were then washed sequentially in PBS, 0.1 *M* triethanolamine (Sigma), followed by a 10-min incubation in 0.1 *M* triethanolamine, 0.25% acetic anhydride. Slides were then washed in PBS, ddH_2O and then air dried. Hybridizations were carried out with probe concentrations of 0.5 μg/ml in 4× SSC, 1× Denhardt's solution, 500 μg/ml salmon sperm DNA, 500 μg/ml poly yeast tRNA, 500 μg/ml poly(A) at 50–55°C in a mineral oil bath for 8–16 hr. Nonhybridized probe was removed by washing sections in 2× SSC three times for 10 min followed by a 1-hr incubation in 60 μg/ml RNase A (Sigma), 1 U/ml RNase T1 in 10 m*M* Tris, pH 8, 1 m*M* EDTA, 150 m*M* NaCl at 37°C. Following ribonuclease treatment sections were washed in four changes of 2× SSC for 1 hr, four changes of 0.1× SSC, 50% formamide for 2 hr, and 0.1× SSC at 50°C for 30 min.

To detect hybridized probe, sections were first blocked with 2% normal goat serum diluted in 0.3% Triton X-100, 150 m*M* NaCl, 100 m*M* Tris–HCl, pH 7.5, for 3–16 hr at 4°C. Alkaline phosphatase-conjugated anti-DIG Fab fragments (Boehringer Mannheim) were diluted in 1% normal goat serum, 0.3% Triton X-100, 150 m*M* NaCl, 100 m*M* Tris–HCl, pH 7.5, and applied to sections at 25°C for 2 hr in a humidified chamber. Sections were then washed in 150 m*M* NaCl, 100 m*M* Tris–HCl, pH 7.5, followed by incubation in 100 m*M* NaCl, 100 m*M* Tris–HCl, pH 9.5, for 10 min. Bound antibody was detected using an alkaline phosphatase color reaction kit (Boehringer Mannheim) supplemented with 10% polyvinyl alcohol (Sigma) as outlined by De Block and Debrouwer (1993) for 30 min to 22 hr at 30°C. Reactions were stopped with 100 m*M* Tris, pH 7.5, 10 m*M* EDTA and then sections dehydrated in an ethanol series, followed by xylenes. One slide of each developmental time point was stained with a 1:6 dilution of 0.25% toluidine blue O, 0.25% azure II, 0.50% sodium borate and then counterstained with a 1:6 dilution of 0.05% basic fuschin to demonstrate tissue organization. Sections were viewed and photographed using Nomarski optics on an Olympus IMT-2 inverted microscope (Olympus, Tokyo, Japan).

Retinal Cell and Eye Cup Culture

To obtain retinal cells, retinae were dissected from staged chick embryos in Hanks' balanced salt solution (GibcoBRL) and triturated to a single cell suspension. Retinal cells were grown on 12 m*M* glass coverslips that had been coated with 100 μg/ml poly-D-lysine (PDL, Sigma) or PDL followed by 10 μg/ml laminin (Sigma). Retinal cells were cultured overnight on laminin-coated coverslips in F12 nutrient mixture, 1× antibiotic–antimycotic (GibcoBRL) and 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 5 ng/ml sodium selenite (Sigma) or allowed to settle for 90 min onto PDL-coated coverslips before staining.

To generate eye cup cultures, retinal pigment epithelium and connective tissue were removed from E3 eyes, and the retina was left attached to the vitreous and lens, as previously described by Halfter and Deiss (1986). E3 eye cups were cultured in 300 μl of media containing DME, 10% fetal bovine serum, 2% chick embryo extract, and 1× antibiotic–antimycotic (GibcoBRL). The blocking β1 integrin monoclonal antibody CSAT was purified from ascites or tissue culture supernatent using a protein G affinity column (Pierce, Rockford, IL) and tested for blocking activity in cell adhesion assays to laminin using cultured retinal cells (Bradshaw *et al.*, 1995). The anti-α2 monoclonal antibody MEP-17 (Bradshaw *et al.*, 1995) was purified in the same fashion. CSAT, MEP-17, and control mouse IgG (Sigma, St. Louis, MO) were added to give a final concentration of 1 mg/ml (1/10 vol of antibody in PBS added to each well), and the eye cups were cultured for 20 hr in 5% CO_2 at 37°C. In each experiment, control eye cups were taken from the same embryo as the experimental eye cups.

Immunofluorescence Microscopy

To detect of cell surface β1 integrin and Thy-1, live cells were labeled by incubating retinal cells with an undiluted tissue culture supernatant containing CSAT (the hybridoma was a kind gift of Dr. Allan Horwitz; Neff *et al.*, 1982) or a 1/1000 dilution of partially purified Thy-1 antibody (a kind gift of Dr. Peter Jeffrey; Sheppard *et al.*, 1988) for 30 min at room temperature. Bound primary antibodies were then detected with rhodamine-conjugated goat anti-mouse secondary (Cappel, Durham, NC). Cells were then fixed in 5% sucrose, 4% paraformaldehyde, mounted onto microscope slides in FITC guard (Testog Inc., Chicago IL).

Cultured eyecups were fixed in 4% paraformaldehyde in PBS for 1 hr at room temperature, followed by two rinses in PBS. The eye cups were then subjected to dehydration through a series of graded ethanol washes and xylenes and then immersed in 100% paraffin (X-tra paraplast, Fisher) for 1–1.5 hr at 65°C. Eye cups were oriented with the lens up, and 7-μm sagittal sections (parallel to the median plane) were generated on a microtome. Sections were mounted on Probe-on-treated slides (Fisher), then deparaffined in the microwave (1 min, high), and rehydrated by three washes with xylenes, followed by an ethanol gradient, and then two washes with H_2O. To improve neurofilament staining, the sections were subjected to heat-induced epitope retrieval (protocol developed by Biotek Solutions, Goleta, CA) where the sections were autoclaved in 8 m*M* citrate buffer, pH 5.5, for 7 min on the liquid cycle. The sections were allowed to come to room temperature and prepared for immunofluorescence to visualize RGCs.

Sections of cultured retinas were blocked with 3% BSA in PBS with a 1:100 dilution of normal goat serum (Sigma) for 15 min at room temperature, followed by incubation with a 1:500 dilution of polyclonal anti-neurofilament (Chemicon, Temecula, CA) antibody in blocking solution for 1 hr at room temperature. The sections were then rinsed twice in PBS and incubated with a 1:200 dilution of secondary antibody (Texas red-conjugated goat anti-rabbit; Cappel, Durham, NC) for 1 hr at room temperature. The sections were rinsed twice in PBS and once in H_2O, dried, and mounted with FITC guard (Testog, Chicago, IL). All fluorescence microscopy was carried out using an Olympus IMT-2 microscope.

RGC Migration Assay

To quantify RGC migration from the ventricular to vitreal retina, sections spanning the entire eyecup were analyzed. Progressive sagittal sections were inspected using phase-contrast microscopy, and all sections that contained the lens were further analyzed to determine the location of neurofilament-positive RGCs. The lens was used as a marker of position so that the same region in control and experimental eye cups was analyzed. Fluorescence photomicrographs of the peripheral regions of the section were taken at 100×. Cells were classified as ventricular if the cell body was in contact with the border of the retina adjacent to the RPE or vitreal if the cell body was in contact with the vitreal perimeter. Cells were classified as migrating if the cell body itself was not in contact with a border regardless of cellular processes. Processes present in sections without an associated cell body were not counted. The total number of neurofilament-positive cells per frame was quantified and percentages of ventricular, vitreal, and migrating cells were calculated. For each experimental determination, approximately 100–500 cells were counted per retina (10–50 microscopic fields).

RESULTS

Widespread β1 Integrin Expression in the Developing Retina

To examine spatial patterns of β1 integrin mRNA expression in the developing chick retina, *in situ* hybridizations were performed using digoxigenin-labeled antisense RNA transcribed from a full-length chicken β1 cDNA. Sections were hybridized to a β-tubulin probe as a positive control and a β1 integrin sense probe as a negative control. At E4, the chicken retina is composed of undifferentiated neuroepithelial cells of homogeneous morphology, except for differentiating RGCs at the vitreal border, which are slightly larger. (Fig. 1A). β1 Expression was detected throughout the undifferentiated neuroepithelial cells, with slightly higher levels of expression in the RGC layer (Fig. 1C). Tubulin expression was especially robust in the RGC layer (Fig. 1B). Little hybridization was detected using a β1 sense probe (Fig. 1D).

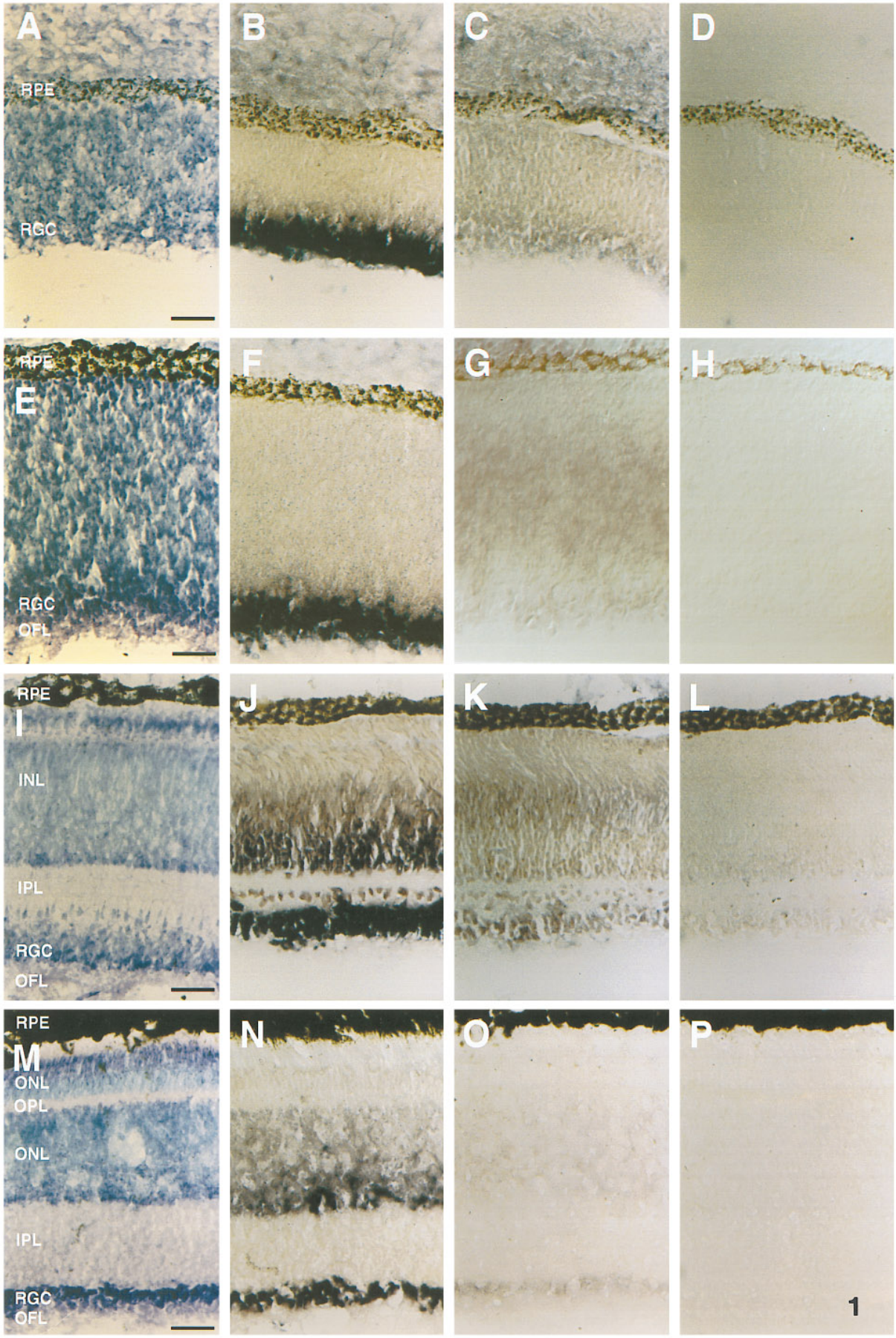
By E6, toluidine blue staining (Fig. 1E) revealed a distinct band of RGCs and a thickening optic fiber layer (OFL) along the vitreal border of the retina. β1 mRNA was again detected in both neuroepithelial cells and RGCs (Fig. 1G). However, lower signal was detected along the ventricular surface of the retina when compared with β1 expression at E4 retina (compare Figs. 1C and 1G).

At E12, all the cellular and plexiform layers that are present in the adult were observed (Fig. 1I). β1 Message was detected throughout most of the retina at E12, with highest levels observed in RGCs (Fig. 1K). β1 Expression was also observed in cells embedded in the inner plexiform layer, which are probably displaced amacrine cells migrating to the ganglion cell layer (Spira *et al.*, 1987). In the inner nuclear layer (INL), a central band of strong β1 expression was observed. These may be differentiating Muller glia or bipolar cells. In contrast, tubulin expression in the INL was highest in cells along the vitreal edge (Fig. 1J).

Expression of β1 at P1 was reduced, although still detectable in RGCs and in cells populating the INL (Fig. 1O). A similar but more robust pattern of expression was observed for tubulin mRNA (Fig. 1N).

The β1 *in situ* hybridization patterns indicated that β1 subunit mRNA expression was more widespread than β1

FIG. 1. *In situ* hybridization of integrin β1 mRNA in developing chick retina. E4, E6, E12, and P1 chick retinal sections were stained with toluidine blue (A, E, I, M), hybridized to a β-tubulin probe (B, F, J, N), hybridized to a chicken β1-antisense probe (C, G, K, O), or hybridized to a chicken β1 sense probe (D, H, L, P). RPE, retinal pigmented epithelium; RGC, retinal ganglion cells; OFL, optic fiber layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Bars, 50 μm.


A
RPE
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B
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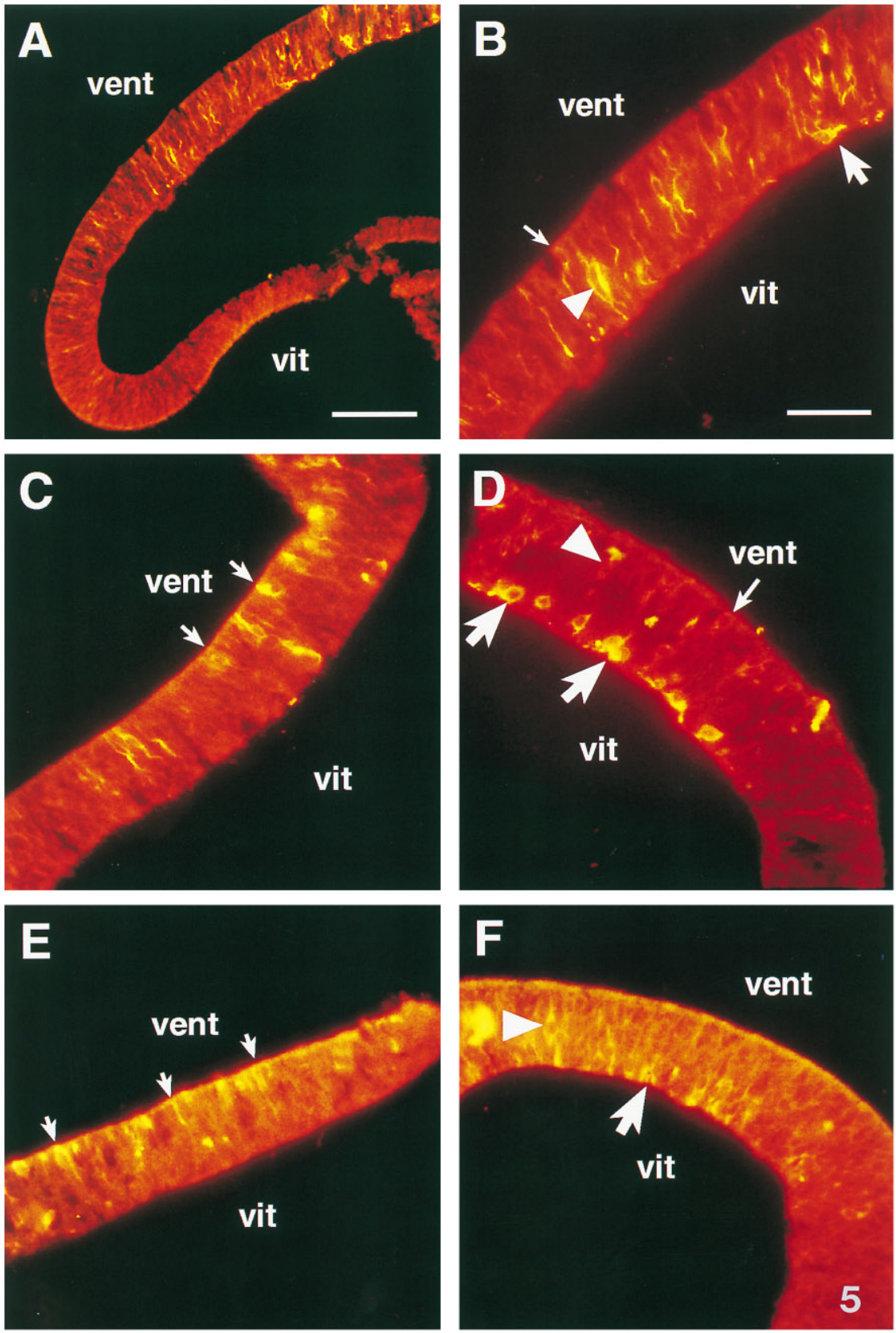

protein immunoreactivity previously detected in sections (Cohen *et al.,* 1987). Because function blocking antibodies were used in the previous study, some β1 epitopes may have been masked if occupied by ligand. To avoid this problem, retinal cells were dissociated from E6 retina, cultured overnight in serum-free media, and then stained with the the anti-β1 monoclonal antibody CSAT (Neff *et al.,* 1982) or the RGC marker, Thy-1 (Sheppard *et al.,* 1988). Sixty-four percent of retinal cells were found to be β1 positive, while 22% of retinal cells were Thy-1 positive (n = 350). To control for the possibility that the culture conditions used might be inducing β1 expression, cells were dissociated from an E6 retina and plated on PDL-coated coverslips and immediately stained for β1 or Thy-1. Over 50% of retinal cells freshly dissociated from an E6 retina were β1 positive, while 11% were Thy-1 positive (n = 600). These results suggest that β1 protein is expressed on the surfaces of at least 50% of E6 chick retinal cells, consistant with the widespread distribution of β1 mRNA.

Integrin α Subunit mRNAs Expressed in E6 Retina

To examine the repertoire of integrin α subunit mRNAs expressed in the E6 chick retina, E6 RNA was subjected to RT–PCR. E6 RNA was chosen since β1 expression is widespread in the retina at this time (Fig. 1G), and numerous developmental events which may require integrin function are also occurring. Using E6 retinal cDNA and degenerate, inosine containing primers—designed to amplify conserved sequences present in the metal binding regions of multiple α subunits (Pytela *et al.,* 1990)—chick integrin encoding PCR products were amplified, subcloned, and sequenced.

Sequence analysis revealed the isolation of partial cDNA clones encoding chick integrins α6, α8, and αv (data not shown) and chick homologues of integrins α2 and α4 (Fig. 2). The clones for α6, α8, and αv were 100% identical to previously published sequences (de Curtis *et al.,* 1991; Bossy *et al.,* 1991, 1990). The predicted chick α2 amino acid sequence (Fig. 2A) was 70% identical to mouse and human α2, and 73% identical to bovine α2. The predicted sequence for chick α4 was 81% identical to mouse and human α4 and 77% identical to the *Xenopus* α4 homologue. In both cases, the next nearest sequences were other integrin α subunits, which showed about 50% identity.

Developmental Regulation of Integrin mRNA Expression

Temporal patterns of α subunit expression were analyzed by Northern blotting. Figure 3 shows a series of blots prepared from panels of poly(A)$^+$ RNA isolated from chick retinae ranging in age from E6 to P1 (newly hatched). To correct for variations in loading and purity of poly(A)$^+$ RNA preparations, each blot was stripped and rehybridized to ^{32}P-labeled oligo(dT) to measure actual hybridizable poly(A)$^+$ RNA in each lane. (Figs. 3B, 3E, and 3H). For β1 mRNA, a 4.2-kb transcript was detected (Fig. 3A), comparable in size with that previously reported for chicken β1 in other tissues (Tamkun *et al.,* 1986). α2, α4, α6, and α8 transcript bands were measured at 7.0, 6.0, 5.6, and 6.3 kb, respectively. The sizes of these transcripts are in accord with sizes determined previously for these mRNAs in human and chicken (Takada and Hemler, 1989; Takada *et al.,* 1989; de Curtis *et al.,* 1991; Bossy *et al.,* 1991; Bossy and Reichardt, 1990). Additional hybridizing bands were detected in blots of β1 and α8. These may represent alternative splice products rather than other integrin subunits, since wash stringencies were predicted to allow less than 5% mismatch (see Methods and Materials).

Quantification of Northern blots is presented in Fig. 4. Levels of β1, α2 and α6 declined significantly from E6 - P1. In contrast, changes in the levels of α4 and α8 during development showed more complex patterns. In the case of α8, mRNA levels displayed a biphasic pattern during development, with levels decreasing from E6 to E9 by about 80% and then increasing to a level 45% higher than its E6 value at hatching. α4 mRNA decreased slightly from E10-12, then increased through hatching.

The decrease in levels of integrin mRNAs between E6 and E9 (Fig. 4A) could represent a coordinate downregulation in retinal cells expressing them, or it could reflect a dilution of these mRNAs by increased expression of other messages. Cell number in the retina increases 10-fold between E6 and E10 (Dütting *et al.,* 1983; Morris and Cowan, 1984), so new cells not expressing integrins could give rise to an apparent decrease in integrin mRNA levels on a Northern blot. In order to address this question, we examined how levels of individual α and β subunits change on a per retina basis. The total amount of poly(A)$^+$ RNA per retina was determined at specific time points during development and these values were then used to convert integrin mRNA per microgram of poly(A)$^+$ RNA to integrin mRNA per retina. Between E6 and E10, the amount of poly(A)$^+$ RNA per retina increased

FIG. 5. Effects of blocking anti-β1 antibody on RGC migration. Migrating RGCs were visualized with anti-neurofilament antibody. (A) Low-magnification view of RGCs in various states of migration from E3 eyecups cultured 20 hr in media alone. Bar, 84 μm. (B) Higher-magnification view of another E3 retina cultured under the same conditions. Note the cellular processes which span the retina during the migration of RGCs. Small arrows indicate cell bodies along the ventricular border, arrowheads indicate migrating cell bodies, and large arrows point out cell bodies along the vitreal border. Bar, 50 μm. E3 retinae were incubated in the presence of anti-β_1 antibody (C and E) or control, nonspecific mouse IgG (D and F). Small arrows point to cell bodies present along the ventricular border, arrowheads indicate migrating cells, and large arrows highlight cells along the vitreal border; vent; ventricular; vit, vitreal. Panels C–F are The same magnification as B.

A

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                1                                                    50
chick   α2   SVVCSVDVNK DSVTDVLLVG APMFMNDMKK EEGRVYMFSI TKGILDQQEL
human   α2   SVLCSVDVDK DTITDVLLVG APMYMSDLKK EEGRVYLFTI KKGILGQHQF
murine  α2   SVLCSVDVDK DTITDVLLVG APTYMNDLKK EEGKVYLFTI TKGILNQHQF
bovine  α2   SVLCAVDVNK DTITDVLLVG APMYMNDLKK EEGRVYLFTI TKGILNWHQF

                51                          74        Identity
chick   α2   LQGPQGSENA RFGSAI•ALT DIDL                   -
human   α2   LEGPEGIENT RFGSAIAALS DINM                  70%
murine  α2   LEGPEGTGNA RFGSAIAALS DINM                  70%
bovine  α2   LEGPNGLENA RFGSAIAALS DINM                  73%
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B

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                1                                                    50
chick    α4   ASVCAVDLNS DGLSDLLVGA PMESTIREEG RVYVYINSGS KAEMVELDIE
human    α4   ASVCAVDLNA DGFSDLLVGA PMQSTIREEG RVFVYINSGS GAVMNAMETN
murine   α4   ASVCAVDLNA DGFSDLLVGA PMQSTIREEG RVFVYINSGM GAVMVEMERV
xenopus  α4   •AVCAADLNC DGLSDLLVGA PIQSTIREEG RVFVYMNTGS GA•MEELKFE

                51                           73        Identity
chick    α4   LSGSDSYAAR FGESITNLGD IDN                   -
human    α4   LVGSDKYAAR FGESIVNLGD IDN                  81%
murine   α4   LVGSDKYAAR FGESIANLGD IDN                  81%
xenopus  α4   LSGSDLYAAR FGETIANLGD IDN                  77%
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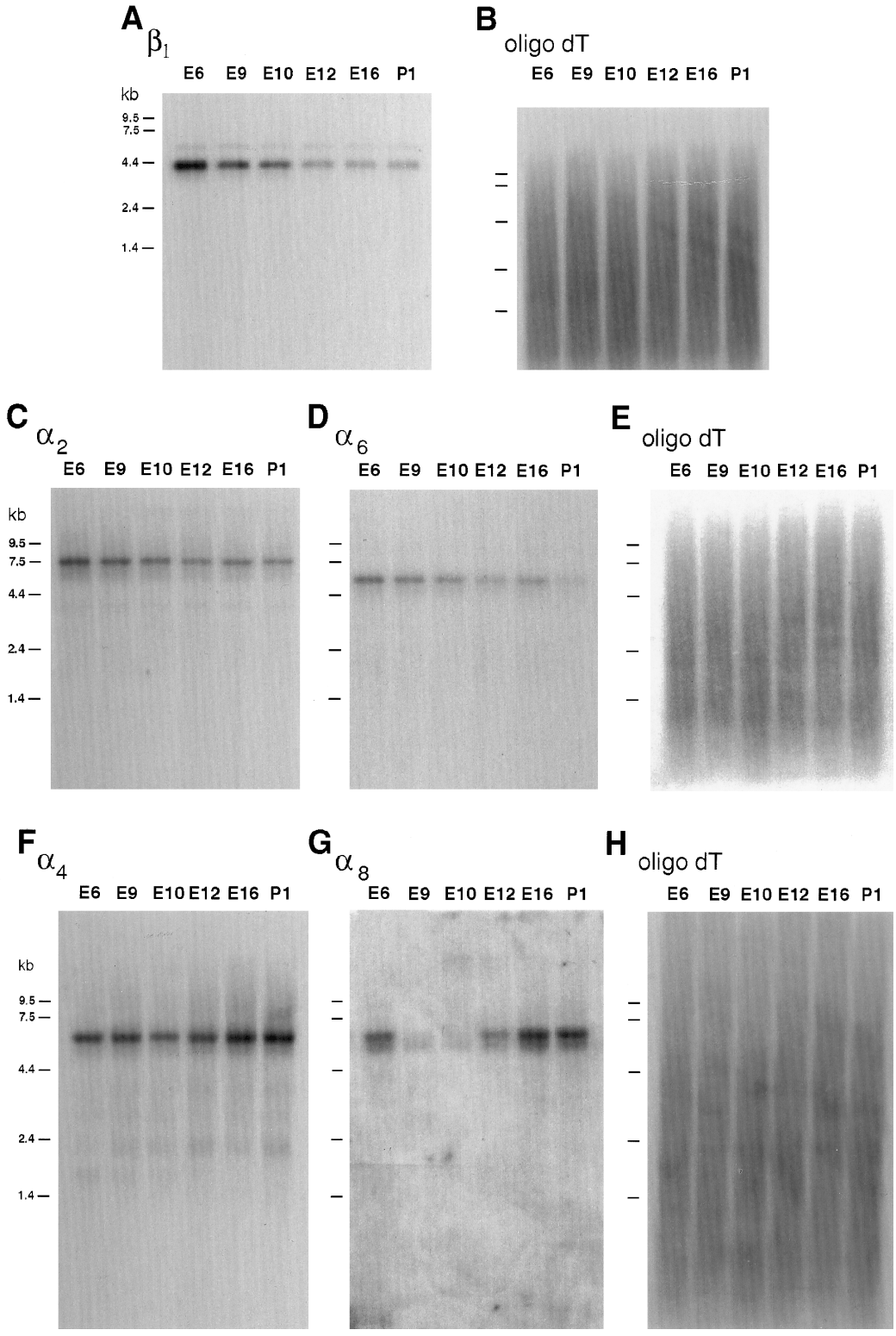
FIG. 2. Predicted amino acid sequences of chick α2 and α4 aligned with homologues from other species. (A) Alignment of the predicted chick α2 amino acid sequence with sequences from human, mouse, and cow α2 (Takada and Hemler, 1989; Edelman *et al.*, 1994; Kamata *et al.*, 1994). (B) Alignment of the predicted chick α4 amino acid sequence with sequences from human, mouse, and *Xenopus* α4 (Takada *et al.*, 1989; Neuhaus *et al.*, 1991; Whittaker and DeSimone, 1993). Alignments were created with the GCG programs PileUp and FastA, and residues in bold letters show sequence positions conserved in at least three species. The percentage identity with the chick sequence is listed. In all cases the sequences shown represented the highest scoring alignments found in database searches. Numbers refer to the predicted chick amino acid sequence.

by 300% and then plateaued. On a per retina basis, integrin mRNAs increased between E6 and E9, with the exception of α8 (Fig. 4B). From E9 to hatching, levels of α2, α6, and β1 mRNA decreased. In contrast, levels of α4 and α8 mRNA increased during this period. These data show that individual integrin subunit mRNA levels fluctuate in a complex manner and that the levels of β1 and its associated α subunits are not always coordinately regulated. Furthermore, the data indicate that all integrin mRNAs examined are expressed throughout development.

β1 Integrin Role in RGC Migration

The expression of β1 integrins in undifferentiated neuroepithelial cells suggested that they might be involved in functions other than axon and dendrite extension. To assess the role of β1 integrins in retinal neuroblast migration, explanted eye cups were incubated in the presence of blocking β1 antibody, and migration of newly born RGCs was assayed. We used the organ culture system documented by Halfter and Deiss (1986), in which eye cups consisting of retina, lens, and vitreous body are cultured overnight, and retinal layers develop in a normal pattern (see also Pollerberg and Beck-Sickenger, 1993).

Newborn RGCs were visualized by staining for neurofilament, an RGC-specific marker at early ages (Bennet and Dilullo, 1985). RGC markers begin to be expressed soon after the cessation of mitosis, while cells are still in the ventricular zone (Waid and McLoon, 1995). We focused on the distribution of migrating RGCs in the periphery of organ-cultured E3-4 eye cups. In control eye cups, RGCs were visible in various stages of migration (Fig. 5). Neurofilament-positive cell bodies were observed in contact with the ventricular border (ventricular), in the process of migration (migrating) and along the vitreal border in contact with the ILM (vitreal). When eye cups were incubated in the presence of function blocking anti-β1 antibody, a significant decrease was observed in the number of neurofilament-positive cells associated with the vitreal border. Results from two separate experiments are shown (Fig. 5). In eye cups treated with anti-β1, the majority of neurofilament-positive cell bodies are associated with the ventricular border of the retina (Figs. 5C and 5E). In control eye cups taken from the same embryo and treated with purified mouse IgG, the majority are associated with the vitreal border (Figs. 5D and 5F).



In six of nine retinas, blocking integrin β1 function resulted in increased ventricular RGCs. In one of three retinas without increased ventricular RGCs, significantly more RGCs were associated with the migratory phase in the anti-β1 retina than in the control (Table 1). After all nine experiments were averaged, the mean number of neurofilament-positive cells at the ventricular border for eye cups cultured with anti-β1 was significantly greater than controls (43.1% vs 22.1%). Fewer neurofilament-positive cells were also noted at the vitreal border, although the differences were not as great. A purified anti-integrin α2 monoclonal antibody (MEP-17; Bradshaw *et al.*, 1995), purified in the same manner as CSAT, did not affect RGC position. The percentages of ventricular, migrating, and vitreal cells in MEP-17-treated eye cups (13.7 ± 3.4; 68.0 ± 7.8; 18.4 ± 8.2) were not significantly different from control mouse IgG-treated eye cups (20.5 ± 4.5; 62 ± 8.2; 17.5 ± 4.6). This argues against a trace contaminant from the CSAT ascites being the cause of the inhibition.

DISCUSSION

Integrin β1 Expression in Retinal Neuroepithelial Cells

To investigate the roles of β1 integrins in the developing retina, we first examined the expression patterns of the β1 subunit. Earlier studies had shown expression of integrin β1 by RGCs in the developing retina (Cohen *et al.*, 1987). The results presented here support and extend that observation by describing a more widespread pattern of β1 mRNA and protein expression in developing retinal neuroepithelial cells. The expression patterns observed are consistent with the striking effects of anti-β1 antibodies on attachment and process outgrowth by early retinal cells previously observed (e.g., Hall *et al.*, 1987) and with reports of α subunit expression. Subunit α8 is expressed on the axons of RGCs and by cells of the INL (Bossy *et al.*, 1991), and α6 immunoreactivity is found throughout the retina at E6 (de Curtis and Reichardt, 1993). The expression patterns suggest that β1 integrins may play important roles beyond axon extension by RGCs, especially in early events involving undifferentiated neuroblasts.

Repertoire of α Subunits Expressed

How many α subunits pair with β1 to carry out its functions in developing retina? Previous studies have detected α1, α3, α6, and α8 associated with β1 in chick retina (de Curtis *et al.*, 1991; Duband *et al.*, 1992; Bossy *et al.*, 1991). We identified five α subunit mRNAs expressed by RT–PCR, and four of the five (α2, α4, α6, and α8) are likely to pair with the β1 subunit, since αvβ1 was not detected in the developing chick retina (Neugebauer *et al.*, 1991). α1 and α3 mRNAs were not detected in this study, perhaps due poor hybridization of primers or low abundance of message.

Detection of α2 and α4 subunit mRNAs is of interest since previous studies have suggested that these subunits are not expressed in the developing vertebrate retina. Previous studies did not detect α2 protein in E6 or E12 chick retinal tissue by Western blotting (de Curtis *et al.*, 1991), and α2 mRNA was not detected in embryonic mouse retina by *in situ* hybridization (Wu and Santoro, 1994). However, we have shown that α2 cDNAs can be amplified from retinal RNA and α2 transcripts can be detected by Northern blotting. In addition, we have recently characterized the expression and function of the chick α2 protein in the retina (Bradshaw *et al.* (1995), using a blocking monoclonal antibody to show that α2β1 mediates interactions between developing retinal cells and collagens I and IV. Other investigators have noted α2 immunoreactivity in the adult human retina (Duguid *et al.*, 1992; Brem *et al.*, 1994).

Immunohistochemical studies of E2.5 chick retina failed to detect expression of α4 (Stepp *et al.*, 1994). However, α4 immunoreactivity has been detected in the retinae of early mouse embryos (Sheppard *et al.*, 1994). An obvious difference between our study and that of Stepp *et al.* (1994) is that we examined much older retinal tissue (E6 as opposed to E2.5) and this may account for the difference between our two studies especially since our data shows that levels of α4 mRNA increase during development.

The function of the α4 subunit in developing retina is not yet clear. A large body of evidence exists describing the expression and function of α4 containing heterodimers on the surfaces of cells of hematopoietic origin (Hemler *et al.*, 1990) and recent studies suggest that α4 and its ligands are expressed in the developing mouse and chick embryo (Rosen *et al.*, 1992; Sheppard *et al.*, 1994; Stepp *et al.*, 1994). Genetic studies demonstrate that the presence of α4 is essential for developmental events such as allantois–chorion fusion during placental formation and epicardium formation in mouse embryos (Yang *et al.*, 1995). Subunit α4 has been shown to associate with two different β subunits, β1 and β7 (Ruegg *et al.*, 1992) and to mediate both cell–cell and cell–ECM interactions. RT–PCR experiments designed to examine integrin β subunit expression in E6 retina have

FIG. 3. Temporal expression patterns of β1, α2, α6, α4, and α8 subunit mRNAs in the developing chick retina. Northern blots are shown, with the probe indicated above each blot. (A) Subunit β1, detected after a 20-hr exposure using 1 μg of poly(A)$^+$ RNA; (B) blot used to detect β1 hybridized with labeled oligo(dT); (C) α2 detected after a 4.5-day exposure using 5 μg of poly(A)$^+$ RNA; (D) α6 detected after a 2-day exposure using the same blot after removal of the α2 probe; (E) blot used to detect α2 and α6 hybridized with labeled oligo(dT); (F) α4 detected after a 60-hr exposure using 12 μg of poly(A)$^+$ RNA; (G) α8 detected after a 9-day exposure using the same blot after removal of the α4 probe; (H) blot used to detect α4 and α8 hybridized with labeled oligo(dT).

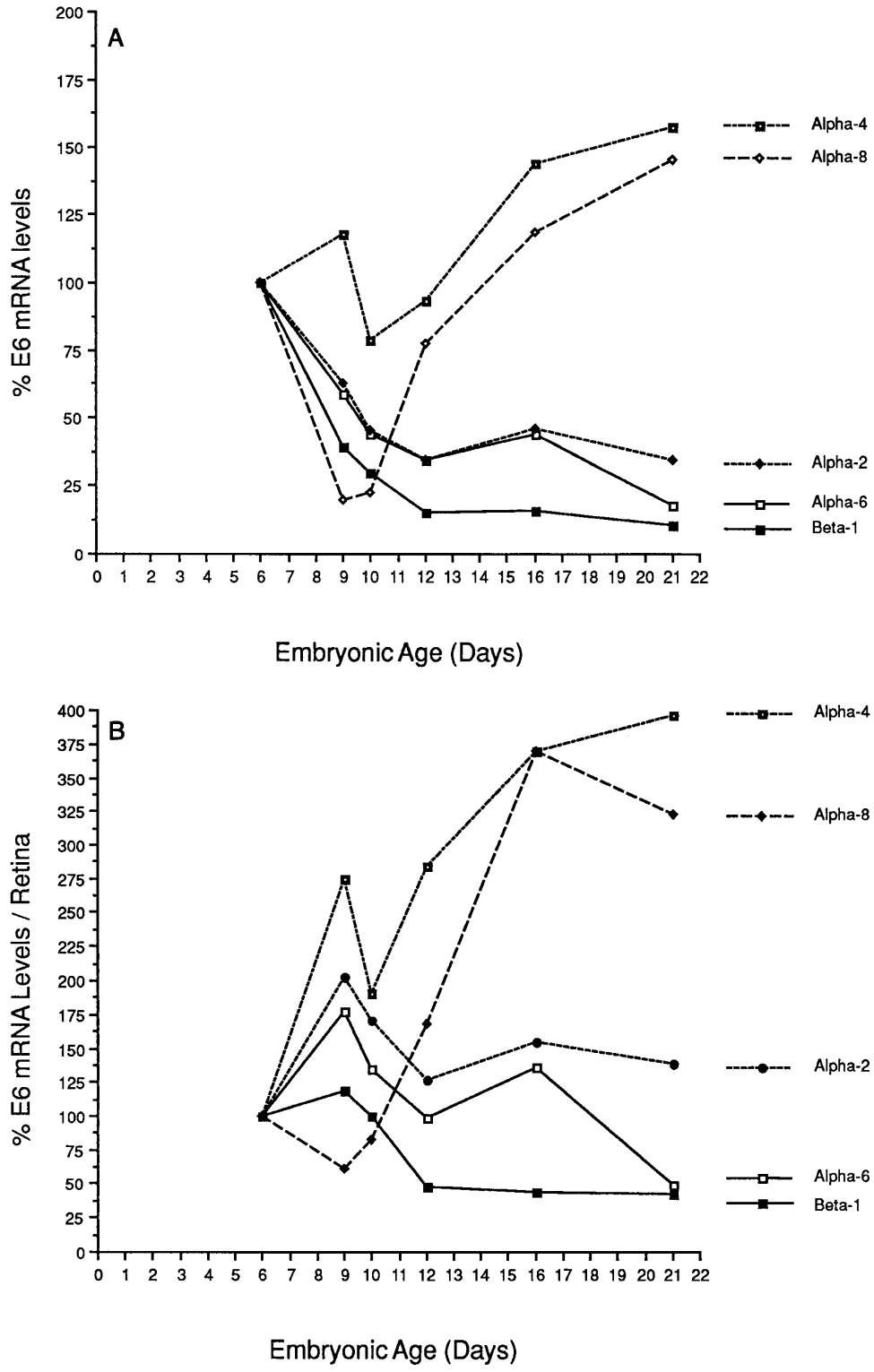


FIG. 4. Developmental time course of integrin mRNA levels in chick retina. Autoradiographs of each blot in Fig. 3 were scanned with a laser scanning densitometer, and levels of each subunit mRNA are normalized to the level of poly(A)$^+$ RNA detected in each lane. (A) mRNA levels are expressed as a percentage of their E6 values. (B) Levels of integrin mRNA per retina are plotted versus age.

TABLE 1
Inhibition of RGC Migration by Anti-β1 Antibody

Experiment No.	Ventricular	Migrating	Vitreal
	Anti-β1		
1.	46.4 ± 10	40.3 ± 7.2	13.3 ± 7.2
2.	60.2 ± 4.8	29.9 ± 4.2	9.8 ± 3.4
3.	77.0 ± 15	10.0 ± 10	13.0 ± 8.3
4.	45.8 ± 5.9	30.4 ± 5.5	23.9 ± 4.4
5.	14.6 ± 3.6	58.7 ± 3.4	26.5 ± 2.5
6.	48.7 ± 5.2	35.0 ± 6.2	16.2 ± 2.6
7.	30.4 ± 6.6	65.1 ± 7.1	4.5 ± 1.9
8.	48.4 ± 5.7	39.0 ± 4.6	12.2 ± 2.8
9.	16.8 ± 3.3	46.7 ± 4.4	36.5 ± 3.4
Average	43.1 ± 6.7**	39.5 ± 5.7	17.3 ± 4.0*
	IgG Control		
1.	20.5 ± 4.5	62.0 ± 8.2	17.5 ± 4.6
2.	15.2 ± 3.8	44.0 ± 6.0	43.1 ± 7.3
3.	25.8 ± 7.3	51.8 ± 8.3	22.3 ± 6.1
4.	16.4 ± 4.3	40.8 ± 4.0	42.8 ± 5.2
5.	21.8 ± 4.9	57.2 ± 8.2	21.8 ± 3.0
6.	39.9 ± 7.9	35.9 ± 7.2	24.0 ± 7.8
7.	8.9 ± 1.8	58.8 ± 3.5	31.9 ± 3.4
8.	21.6 ± 4.6	55.9 ± 4.3	22.5 ± 3.5
9.	29.0 ± 7.7	31.2 ± 6.7	39.7 ± 5.8
Average:	22.1 ± 5.2	48.4 ± 6.3	29.5 ± 5.2

Note. Results from nine eye cup culture experiments are presented. Retinae incubated in the presence of anti-β_1 antibodies are shown in the top panel with the corresponding control eyes incubated in the presence of nonspecific mouse IgG in the bottom panel. Each value is presented plus and minus the standard error of the mean $^{**}P < .05\%$; $^{*}P < .10\%$ (Student's two-tailed t test).

detected β1 expression but not β7, suggesting that $\alpha4\beta1$ is the relevant heterodimer in the retina (Gervin *et al.*, 1996). The $\alpha4\beta1$ heterodimer is known to bind the vascular cell adhesion molecule-1 (VCAM-1) (Elices *et al.*, 1990), the CS-1 region of plasma fibronectin (Wayner *et al.*, 1989; Guan and Hynes, 1990; Mould *et al.*, 1990), thrombospondin (Yabkowitz *et al.*, 1993), and a bacterial protein called invasion (Isberg *et al.*, 1990). $\alpha4\beta1$ may also engage in homophilic interactions (Quian *et al.*, 1994). The CS-1 peptide of Fn has been shown to support attachment but not neurite outgrowth of chick retinal cells, indirectly implying the presence of α4 protein (Neugebauer, 1991). In the chick and mouse retina however, Fn has not been detected (Halfter *et al.*, 1988; Sheppard *et al.*, 1994), suggesting that $\alpha4\beta1$ may interact with ligands other than Fn in the retina.

Levels of Integrin Subunit mRNAs during Development

Examination of the expression patterns of β1 integrin subunits in the developing retina showed that α subunit mRNAs are not all coordinately regulated and that expression persists throughout development. As a percent of total RNA, α2, α6, and β1 message levels decline during development, while levels of α4 and α8 increase after an initial dip. However, on a per retina basis, levels of all subunit mRNAs, except α8, increase between E6 and E9. This peak in integrin mRNA expression may be related physiologically to the 10-fold increase in retinal cell number between E6 and E9 (Dütting *et al.*, 1983; Morris and Cowan, 1984) and the finding that maximal numbers of RGC axons populate the optic nerve at E9 (Rager, 1980). The rise in α6 expression between E6 and E9 is probably due to increasing numbers of non-RGCs expressing α6, since RGC expression of α6 decreases at this (de Curtis *et al.*, 1991). The decline in levels of α8 mRNA between E6 and E9 may be due to downregulation of α8 mRNA synthesis by RGCs, since they appear to be the only cell type expressing α8 mRNA until E12 (Bossy *et al.*, 1991). Increases in α4 and α8 at later times may be due to new cell populations that express these integrins. The fact that mRNA expression of multiple subunits persists throughout development suggests that integrins may have functions in later events such as synapse formation by RGCs or interactions between Muller glia and matrix.

Role of β1 Integrins in Neuroblast Migration

The early expression of β1 integrins in undifferentiated retinal cells may reflect roles in the proper movement and positioning of cells to form the eventual layered retinal morphology. We found that the function blocking CSAT antibody delayed the migration of RGCs from the ventricular border of the retina. This same antibody has been shown by others to block migration of neural crest cells and myoblasts (Bronner-Fraser, 1985, 1994; Jaffredo *et al.*, 1988). These cells migrate long distances through tissues rich in extracellular matrix. In the developing tectum of the chick brain, β1 antisense expression was shown to inhibit migration of neuroblasts along radial glia (Galileo *et al.*, 1992).

Retinal neuroblasts are not thought to migrate along radial glia, and the migration event covers a much shorter distance than that traversed by neural crest or myoblast cells. Migration may be mediated by the extension of a process that contacts the vitreal border, followed by a somal translocation to eventually form the RGC layer (Prada *et al.*, 1981). However, similar mechanisms may be involved in many types of migration, namely, cell contact with extracellular matrix-mediated by β1 integrins. Retinal neuroblasts may contact matrix as they extend processes through the retina, or they may use matrix contacts as a means to anchor endfeet on the inner limiting membrane. A role in retinal cell positioning and migration would explain the dramatic effects of anti-β1 integrin antibodies on developing chick and *Xenopus* retinae (Svennevik and Linser, 1993; Leonard and Sakaguchi, 1995).

The simplest interpretation of the mechanism of antibody inhibition of migration in the retina is that a direct inhibition of cell movement occurs due to loss of adhesive contacts. This interpretation is consistent with many *in vitro* studies that have shown integrin requirements for ad-

hesion and movement of cells and growth cones over matrix and cell surfaces. Alternatively, integrins might be required for adhesion to the vitreal border matrix before migration can be initiated. Blockade of integrins in some studies has been shown to induce apoptosis (Brooks *et al.*, 1994), which could potentially explain inhibition of migration observed in this and other studies. However, we did not observe pycnotic nuclei or any visible decreases in cell number or retinal thickness, and the cells express comparable levels of neurofiliament, a marker of RGC differentiation.

Effects of blocking antibodies and antisense must be interpreted with caution, however, since perturbation of β1 integrins by other means has not interfered with migration by neural cells (Lilenbaum *et al.*, 1995; Fassler and Meyer, 1995). One possible explanation for differing results is that compensatory or ''back up'' systems are activated *in vivo* upon disruption of the primary mechanism used for migration (Shastry, 1994). Alternatively, blockade of one integrin family by an antibody may decrease expression or stability of another integrin or cell adhesion molecule (Dalton *et al.*, 1995). Further investigations of specific integrin heterodimers, using multiple approaches, will be necessary to fully understand the role these receptors play in the multiple phases of neuronal life.

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