**Integrin $\alpha_2\beta_1$ mediates interactions between developing embryonic retinal cells and collagen**

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**SUMMARY**

In the developing nervous system, the extracellular matrix provides a source of extrinsic cues to guide determination of cell fate, neuroblast migration, axon outgrowth and synapse formation. In the neural retina, undifferentiated neuroepithelial precursor cells contact extracellular matrix that contains multiple collagen types. Collagens have been shown to support retinal cell adhesion and neurite outgrowth, but the integrin receptors mediating neuronal responses are not understood. Here we provide evidence that integrin $\alpha_2\beta_1$ acts as a collagen receptor in the developing avian retina and examine its expression pattern. Using a recently described monoclonal antibody, MEP-17, $\alpha_2$ protein was detected in the developing retina by immunofluorescence in tissue sections and dissociated cells, and by immunoprecipitation. At embryonic day 4 (E4), when the majority of retinal cells are undifferentiated neuroepithelial cells, $\alpha_2$ immunoreactivity in sections was widespread and about half of cells dissociated in culture were $\alpha_2$ positive. At E6, after the retinal ganglion cell layer had differentiated, immunoreactivity in sections decreased in the central, more developed portion of the retina and 25% of dissociated cells were $\alpha_2$ positive. E6 retinal ganglion cells, identified by neurofilament immunoreactivity, did not express detectable $\alpha_2$ immunoreactivity. Immunoprecipitation experiments using E6 extracts demonstrated that the $\alpha_2$ subunit was paired with the $\beta_1$ integrin subunit. By E12, $\alpha_2$ immunoreactivity in sections was confined to the extreme peripheral retina, although the antigen may be masked since expression levels comparable to or slightly higher than E6 could be detected in dissociated cells and extracts. By employing function blocking antibodies, it was shown that $\alpha_2\beta_1$ integrin is necessary for cell adhesion and process outgrowth by embryonic retinal cells on collagens I and IV. Although $\alpha_2$ expression continued through E12, $\alpha_2$ activity was down regulated with increasing embryonic age, since $\alpha_2$-dependent adhesion and outgrowth declined. These data suggest a role for $\alpha_2\beta_1$ in neuroepithelial cell interactions with collagen rather than for axon extension by retinal ganglion cells.

Key words: integrin $\alpha_2\beta_1$, retinal development, collagen receptor, chick eye, extracellular matrix

**INTRODUCTION**

The development of the central nervous system involves both extrinsic and intrinsic signals which influence neuronal differentiation. The retina is an example of a complex multi-laminar tissue with many different neuronal cell types and non-neuronal glial cells. Development of the retina involves the differentiation and migration of these cell types from the germinal neuroepithelial cell layer along the ventricular border of the retina to the appropriate cellular layer in the mature retina. A single neuroepithelial precursor cell can divide and give rise to almost all of the cell types present in the mature retina (Fekete et al., 1994; Turner et al., 1990; Holt et al., 1988; Wets and Fraser, 1988). These neuroepithelial precursor cells lie in the ventricular margin, but extend processes that contact extracellular matrices on both the ventricular and vitreal retinal borders (the outer and inner limiting membranes, respectively). At specific developmental time points, cells become postmitotic and move their cell bodies from the ventricular zone to a distinct layer in the retina (Prada et al., 1981; Stone, 1988; Kahn, 1974). Neuroepithelial precursors do not appear to be preprogrammed to become a particular cell type, suggesting that extrinsic cues play a role in influencing cell fate. The extracellular matrix (ECM) contacted by these cells may provide not only a structural framework, but also extrinsic cues that steer the differentiation of a cell down a particular pathway (Adams and Watt, 1993; Venstrom and Reichardt, 1993).

In the avian retina, the retinal ganglion cells (RGCs) are the first cell type to become postmitotic beginning at embryonic day 3 (E3) (Kahn, 1974). They are followed by the birth of amacrine, horizontal and receptor cells, then bipolar and Müller glial cells which continue to divide as late as E12. Committed, postmitotic cells then extend axons and dendrites and make synaptic contacts as development proceeds. These
overlapping, successive waves of differentiation occur in a central-to peripheral gradient, resulting in a mature retina at the time of hatching (21 days).

The ECM contacted by retinal neuroepithelial precursor cells has been shown to contain a variety of molecules that could guide development, including laminins, proteoglycans, and collagens (Halfter and Fua, 1987; Halfter and Von Boxborg, 1992; Van Der Rest and Garrone, 1991). Both fibrillar (collagen I) and sheet-forming (collagen IV) collagens have been detected in the inner limiting membrane and both have been shown to support adhesion and neurite outgrowth from embryonic retinal cells (Hall et al., 1987; Halfter and Von Boxborg, 1992; Sarthy, 1993; Carri et al., 1992). At very early times, when the optic cup is just forming (E2), collagen IV is detected in the basal lamina of the optic primordia which will become the inner limiting membrane at the vitreal border of the retina (Hilfer and Randolph, 1993). At embryonic day 7 in the avian as well as the murine retina, collagen IV has been localized to the inner limiting membrane, which is in direct contact with both the neuroepithelial endfeet and the newly born retinal ganglion cells (Halfter and Fua, 1987; Sarthy, 1993). Elsewhere in the nervous system, collagen IV is expressed along migratory pathways of neural crest cells and along early axon pathways and peripheral nerve roots in Drosophila (Perris et al., 1991; Mirre et al., 1992). Collagen IV has also been shown to support adhesion and process outgrowth by PC12 cells and embryonic rat sympathetic neurons in culture (Lein et al., 1991; Turner et al., 1987). Although collagens are likely to play a role in adhesion, migration and axon outgrowth by neurons, the receptors mediating cellular responses are not completely understood.

Retinal cells have been shown to bind and extend processes on collagens via a integrin receptors; however, the a subunits involved have not been identified (Hall et al., 1987). The integrins, which mediate cell-matrix and cell-cell interactions, are heterodimeric proteins consisting of an a and a subunit. To date, 15 a and 8 b subunits have been identified (Hynes, 1992). Embryonic retinal cells express a broad repertoire of integrin a subunits capable of forming heterodimers with b1, mRNAs encoding a2, a3, a4, a6, a9, and a11 have been detected (Cann et al., unpublished data; DeCurtis et al., 1991; Bossy et al., 1991), and a1 immunoreactivity has also been reported (Duband et al., 1992; Brem et al., 1994). To date, functions have only been assigned to a0b1, which acts as a laminin receptor, and the a4 subunit, which combines with other b subunits to form vitronectin receptors (Neugebauer et al., 1991). Based on studies of rat and human cell lines, three b1 heterodimers, a0b1, a2b1 and a3b1, are capable of acting as collagen receptors (Hynes, 1992).

Retinal expression of a2 is controversial, however, since a2 protein could not be detected in western blots of chick retina (DeCurtis et al., 1991) and a2 mRNA was not detected by in situ hybridization to mouse retina (Wu and Santoro, 1994). Using more sensitive methods, however, we were able to show an a2-related mRNA in developing chick retina (Cann et al., unpublished data). a2b1 can interact with as many as three ligands: collagens and laminins in the extracellular matrix and integrin a2b1 on cell surfaces (Elices and Hemler, 1989; Kirchofer et al., 1990; Symington et al., 1993). Here, we have utilized an anti-a2 monoclonal antibody (McNagny et al., 1992) to characterize expression of a2 protein in the developing chick retina. To investigate functions of a2, we have used the antibody to show that a2 is necessary for interactions with collagen I and IV, implicating a2b1 as a major collagen receptor on developing retinal neurons.

MATERIALS AND METHODS

Materials

The anti-a2 monoclonal antibody, MEP-17 (designated anti-a2, McNagny et al., 1992) was purified on protein G columns used according to manufacturer’s guidelines (Pharmacia, Piscataway, NJ). The anti-1 antibody CSAT, a generous gift from A. Horwitz (Neff et al., 1982) was purified from ascites fluid on protein A columns (Cappel, Durham, NC and Sigma, St. Louis, MO). The neurofilament polyclonal antibody was from Chemicon (Temecula, CA). The collagen IV polyclonal antibody was from Gibco (Grand Island, NY). Collagen I purified from calf skin, collagen IV and laminin both purified from mouse EHS sarcoma, were purchased from Sigma and Gibco. White leghorn eggs were purchased from Rosemary Farms, (Santa Maria, CA) and incubated for the appropriate length of time in a humidified incubator at 37°C. F12 media and antibiotics were purchased from Gibco. All other materials, unless otherwise specified, were from Sigma.

Immunohistochemistry

Embryonic chicks were staged according to Hamburger and Hamilton (1951) and their eyes dissected. The eyes were then fixed at 0°C for 1 hour in 4% paraformaldehyde in PBS. The tissue was then transferred to 10% sucrose in PBS for 16 hours at 4°C followed by 25% sucrose in PBS for 24 hours at 4°C. 20 mm cryostat sections were obtained after the tissue was embedded with Histosep (Fisher, Pittsburgh, PA) and flash frozen in LN2. The sections were cut mounted onto gelatin-coated slides for immunohistochemistry. For collagen IV staining the sections were treated with pronase reagent (Biomedica, Foster City, CA) for 15 minutes at room temperature prior to antibody incubation. The sections were first incubated with a 3% BSA in PBS blocking solution for 15 minutes at room temperature followed by a 1 hour incubation with a 1:500 dilution of purified anti-a2 antibody in blocking solution. Control parallel sections were incubated with a 1:500 dilution of purified nonspecific mouse IgG (Sigma) also in blocking solution. After washing, the sections were incubated with a rhodamine-conjugated secondary antibody (Cappel) at a dilution of 1:200 in blocking solution. The sections were then cover slipped and viewed with an Olympus IMT-2 inverted microscope equipped for epifluorescence.

Embryonic retinal cells were dissected free of retinal pigmented epithelia, mechanically dissociated and plated onto collagen-coated coverslips (10 mg/ml Collagen I or IV). Cells were grown overnight at 37°C in 5% CO2 in F12 media containing 5 µg/ml bovine insulin, 5 µg/ml human transferrin and 5 ng/ml sodium selenite (ITSE, Sigma) and incubated alive with undiluted anti-a2 tissue culture supernatant after blocking with PBS containing 3% BSA and a 1:100 dilution of normal goat serum (Sigma). A 1:200 dilution of appropriate second antibody conjugated to Texas Red (Molecular Probes, Eugene, OR) was used for a2 detection. For double labeling experiments, a 1:200 dilution of appropriate second antibody conjugated to fluorescein (Cappel) was used for neurofilament detection. The coverslips were then mounted in FITC guard (Testog Inc, Chicago, IL) and viewed with an Olympus IMT-2 or a Zeiss Axiosvert 10.

Immunoprecipitations

Immunoprecipitation experiments were carried out as described (Choi et al., 1994) with the following modifications: cells were
metabolically labeled by culturing dissected whole retina in 3 ml of methionine-free DME supplemented with ITSE containing 100 µCi/ml [35S]methionine (NEN Dupont, Boston, MA) for 16 hours. Cells were harvested and lysed in 10 mM Tris acetate (pH 8.0), 150 mM NaCl, 0.5 mM MgCl2, 0.5 mM CaCl2, 0.5% NP-40 (IP buffer) with protease inhibitors. Cell extracts were incubated with the appropriate antibodies (30 µg of purified IgG) and subjected to the outlined protocol (Choi et al., 1994). For immunodepletion experiments, the supernatant from the first immunoprecipitation was incubated with 30 µg purified anti-α2 antibody for 2 hours at 4°C, followed by a 1 hour incubation with 75 µl goat anti-mouse sepharose beads. Immunoprecipitates were eluted with non-reducing SDS sample buffer at 95°C for 5 minutes and then separated on a 3-10% gradient polyacrylamide SDS gel (Laemmli, 1970). The gel was treated for fluorography and exposed to NEF-146 film (NEN Dupont) at −70°C.

For some experiments, retinal membrane proteins were labeled with 0.3mg/ml NHS-LC biotin (Pierce, Rockford, IL) according to manufacturer’s instruction. Cells were subjected to 3 rounds of freeze-thaw in IP buffer and the membranes pelleted by centrifugation at 10,000 g for 10 minutes. The pellet was resuspended in IP buffer with 0.5% NP-40 and treated as described. Quantification of the membrane proteins was performed by amido schwarz assay (Schaffner and Weissman, 1973). Equal amounts of membrane protein were then subjected to immunoprecipitation (above). Immunoprecipitates were separated on non-reducing SDS gels and transferred to nitrocellulose (S&S, Keene, New Hampshire). Immunoprecipitated proteins were detected by a 1:500-1:1000 dilution of strepavidin/horse radish peroxidase conjugate (Amersham, Buckinghamshire, England) using enhanced chemiluminescence (Amersham).

Cell adhesion assays
Retinae were labeled with 10-20 µCi/ml Trans-label (a mixture of [35S]methionine and [35S]cysteine; ICN) in F12, ITSE for 4-6 hours at 37°C. Care was taken to remove all remnants of the retinal pigment epithelium since these cells are known to express α2β1 (Hunt et al., 1994). Retinae were washed three times with CMF-PBS and triturated to a single cell suspension. 105 cells in 100 µl of F12-ITSE were added to polystyrene wells coated with substrate (100 µl of 10 µg/ml of purified laminin, collagen I, or collagen IV in PBS) overnight and then blocked with BSA (Turner et al., 1987). Cells were allowed to adhere 90 minutes, then washed with media, then quantified by scintillation counting (Choi et al., 1991). Where indicated, background adhesion to BSA coated wells was subtracted.

Measurement of process outgrowth
Staged embryonic retinae were dissected, triturated to a single cell suspension and resuspended at 7×104 cells/ml in serum-free F12 media containing ITSE (see above). A volume of 100 µl was added to each coated well and plates were incubated in 5% CO2 at 37°C overnight. Process outgrowth was recorded by photography and quantified using a computerized bitpad

and the program Sigma scan (Jandel Scientific, Corte Madera, CA) as described in Choi et al. (1994).

RESULTS

Expression of α2 integrin in developing retina
The MEP-17 monoclonal antibody (designated anti-α2) was generated by immunizing balb/c mice with a pool of embryonic day-2 chicken blastodermis transfected with the acute avian leukemia virus E26 (McNagny et al., 1992). This antibody recognized an integrin-like heterodimer expressed at high levels by E26-transformed progenitor cells. Although originally thought to recognize integrin α4 subunit, MEP-17 was subsequently shown to recognize the chicken homologue of integrin α2 by peptide sequence analysis of the antigen (McNagny et al., unpublished data).

To determine if integrin α2 protein was expressed in the developing retina, an immunoprecipitation was performed using biotinylated E6 retinal cells. As shown in Fig. 1A, the anti-α2 antibody specifically precipitated two major bands at M1 140 and 120 x 103 from E6 retinal extracts, corresponding to α2 and β1 integrin subunits, respectively (McNagny et al., 1992). The identity of the β1 subunit was determined by using an anti-β1 monoclonal antibody (CSAT; Neff et al., 1982) to immunoprecipitate from metabolically labeled E6 retinal

![Image](Image 1)

Fig. 1. Immunoprecipitation of E6 retinal extracts with anti-α2. Immunoprecipitates were separated on a 3-10% gradient gel under non-reducing conditions and the gel processed for fluorography. (A) Lane 1, immunoprecipitation from surface-biotinylated extract with purified anti-α2. Lane 2, control with no primary antibody added. Arrowheads indicate major bands corresponding to α2 and β1 polypeptides. (B) Immunodepletion of α2 antigen by β1 antibody. A metabolically labeled extract was divided into 3 equal parts and subjected to one or two sequential immunoprecipitations (IP1, IP2). Lane 1, immunoprecipitation with anti-β1. Lane 2, sequential immunoprecipitation with anti-β1 followed by anti-α2. Lane 3, immunoprecipitation with anti-α2 antibody. Lane 4, control immunoprecipitation without primary antibody. Arrowheads indicate integrin polypeptides. Positions of molecular weight markers (x103) are indicated on the right of both panels.
extracts (Fig. 1B). CSAT immunoprecipitated the 120×10^3 M_r β_1 band (as well as a lower molecular weight band likely to be pre-β_1 (Choi et al., 1994)) along with associated α subunit bands, including the 140×10^3 M_r α_2 band. To confirm that α_2 was forming heterodimers with β_1 (and not other β subunits) in E6 retinal cells, an immunodepletion experiment was performed using the anti-β_1 antibody (Fig. 1B). When E6 retinal extracts from metabolically labeled cells were pre-cleared with the anti-β_1 antibody, no detectable α_2 subunit remained in the supernatant, indicating that the α_2β_1 heterodimer is the only detectable α_2 integrin in the developing retina. The two labeling methods employed resulted in different relative intensities of the α_2 and β_1 bands. The surface biotinylation appeared to preferentially label the α_2 band, whereas the metabolic labeling with [35S]methionine appeared to preferentially label the β_1 band. Integrin α subunits are known to turn over more rapidly than the β subunits (see discussion in Choi et al., 1994).

To determine when and where α_2 is expressed, cryostat sections from different ages were stained with purified anti-α_2 antibody. At embryonic day 4 (Hamburger and Hamilton stage 24) strong immunoreactivity was observed throughout the retina (Fig. 2A) with a slightly higher level of expression at the ventricular border as compared to the vitreal margin. Parallel control sections stained with purified mouse IgG showed no significant immunoreactivity. The widespread expression pattern was also observed in dissociated cells, where approximately 50% of E4 were α_2 positive (Fig. 3). α_2 immunoreactivity in E6 retina was restricted to the peripheral retina; the central more developed portion of the retina showed significantly less α_2 immunoreactivity (Fig. 2B). A diffuse, punctate staining pattern was observed, with a stronger signal associated with cells along the ventricular border. Little or no immunoreactivity was detected on the large cell bodies at or near the vitreal border where postmitotic RGCs reside (McLoon and Barnes, 1989; Watanabe et al., 1991). By E12, staining was only detectable in the outermost tip of the retina (Fig. 2B). At P1 (1 day after hatching), α_2 immunoreactivity could not be detected in the retinal sections (data not shown).

Although α_2 immunoreactivity appeared to decline from E6-E12 cryostat sections, expression levels were comparable when other methods were employed. If retinas were dissociated to single cells and live staining was carried out, equal percentages of E6 and E12 cells were immunoreactive for α_2 (~25%; Fig. 3). When E6 and E12 retinas were surface biotinylated and equal amounts of membrane protein were subjected to immunoprecipitation with anti-α_2, E12 extracts contained slightly higher levels of α_2 (Fig. 4). However, quantification by densitometry of 4 separate experiments, after subtracting background, showed no significant difference between the two ages (data not shown). In these experiments, variable amounts of the β_1 subunit were coprecipitated, which may reflect dissociation of the heterodimer during the experiment. This was also observed by McNagny et al. (1992). The epitope may be differentially sensitive to fixation, since greater E12 immunoreactivity could be detected in unfixed retina (data not shown). Alternatively, the epitope maybe masked at E12 due to occupation of the receptor. The MEP-17 anti-α_2 antibody blocks function, so it may not bind to the receptor when the ligand is bound (see below). Attempts to stain sections with a polyclonal antibody to a cytoplasmic domain peptide from human α_2 were not successful (see also DeCurtis et al., 1991).

**Identification of cell types expressing α_2**

Before E6, the avian retina consists mostly of undifferentiated neuroepithelial precursor cells and a layer of RGCs, the first neuronal cell type to become postmitotic (Kahn, 1974). To determine which of these cell types expressed α_2, E5 retinal
sections were stained with either anti-α2 antibody or anti-neurofilament antibody. Neurofilament is expressed by RGCs, but not by neuroepithelial precursor cells or by any other differentiated neural cell in the retina (Bennet and DiLullo, 1985). The anti-neurofilament antibody reacted with RGC axon structures present along the vitreal border of the retina (Fig. 5B). Although the anti-α2 antibody also showed immunoreactivity along this retinal border, the α2-positive structures did not have the same morphology as the neurofilament-positive bundles (Fig. 5A) and were probably neuroepithelial endfeet which are known to contact the inner limiting membrane ECM at the vitreal border. This ECM was strongly immunoreactive for collagen IV at E4 (Fig. 5C). To further investigate whether the RGCs express α2, double labeling experiments with dissociated cells were performed. To ensure that the antigens were not being induced upon prolonged incubation in culture, E6 cells were dissected and processed for immunofluorescence directly using two specific antibodies: the monoclonal anti-α2 antibody and a polyclonal antibody directed against neurofilament. Neurofilament is expressed by RGCs, but not by neuroepithelial precursor cells or by any other differentiated neural cell in the retina (Bennet and DiLullo, 1985). The variation in the collagen IV experiments may be due to variable amounts of denatured collagen IV which supports cell adhesion 50-70%. In both cases maximal inhibition was observed with 10 μg/ml anti-α2.

The α2β1-mediated adhesion to both collagen I and collagen IV was developmentally down regulated, with adhesion being consistently higher at earlier ages (E4-E6) than later ages (E9-E12; Fig. 7). Adhesion of retinal cells to collagen I was completely blocked by anti-α2 and by anti-β1 antibodies indicating that the α2β1 integrin mediated adhesion at all developmental times tested. Developmental regulation of cell adhesion to collagen IV also appeared to be down regulated; however, this result was less consistent. In 1/5 experiments, there was no significant difference in terms of adhesion between cells dissected from E6 and cells from E12. In the remaining experiments, a significant decrease was observed between younger and older cells in varying degrees. A representative experiment is shown in Fig. 7, where both anti-α2 and anti-β1 antibodies inhibit adhesion 50-70%.

The variation in the collagen IV experiments may be due to variable amounts of denatured collagen IV which supports cell adhesion not mediated by α2β1. We found that denatured collagen IV will support adhesion of retinal cells as noted previously for PC12 cells (Turner et al., 1987). Anti-α2 (and anti-β1) had no effect on retinal cell adhesion to denatured collagen IV (Fig. 8). It is known that the interaction of human α2β1 with collagen is dependent upon the native conformation of the

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**Fig. 3.** Quantification of α2-positive cells in dissociated culture. Embryonic retinal cells were plated on collagen I-coated coverslips, stained with anti-α2 antibody and the percent of immunoreactive cells were determined. Error bars represent the standard error of the mean.

**Fig. 4.** Immunoprecipitation of α2 from surface-labeled E6 and E12 retinal extracts. Equal amounts of surface-biotinylated membrane protein extracted from E6 and E12 retina were subjected to immunoprecipitation with anti-α2 antibody and detected by autoradiography using enhanced chemiluminescence. Lane 1: E6, anti-α2; lane 2: E6 control, no primary antibody; lane 3: E12, anti-α2; lane 4: E12 control, no primary antibody. Arrows indicate α2 and β1 polypeptides, and positions of molecular weight markers are indicated on the left. The bands that appear between α2 and β1 were not reproducible from experiment to experiment and were also present in control immunoprecipitations.
collagen proteins as well (Kuhn and Eble, 1994). Thus, the α2β1-independent adhesion to collagen IV may reflect the extent of denatured collagen IV on the substratum.

**Process outgrowth**

Because the neuroepithelial cells expressing α2 extend processes that contact the collagen containing matrix in vivo, the role of α2 integrins in process outgrowth on collagen was also investigated. Approximately 30% of E6 retinal cells extended processes on both collagen I and collagen IV (Fig. 9). Both substrata supported an average process length of ~30-40 μm. The vast majority of cells that extended processes on collagen were α2 positive and neurofilament negative, which suggested that almost all of the outgrowth observed was by neuroepithelial precursors rather than RGCs. On rare occasions, neurofilament-positive cells were observed that extended neurites on collagen. Process outgrowth on both collagens was down regulated in agreement with previous experiments carried out by Hall et al. (1987) on collagen IV. By embryonic day 12, no neuronal cells were able to extend processes on the collagen substratum. However, a small number of non-neuronal flat cells were able to extend short filopodial projections.

Anti-α2 and anti-β1 antibodies blocked process outgrowth on collagen I and IV at all ages tested. The inhibition was not just a result of blocking cell access to the substratum, since addition of antibodies after the cells attached inhibited process outgrowth but did not dislodge cells (data not shown). If antibodies were added after processes were formed, process retraction occurred on both collagen I and collagen IV. No significant process outgrowth occurred on heat-denatured collagen IV, indicating that outgrowth requires recognition of native collagens by α2β1 integrin.

**DISCUSSION**

We have presented evidence that integrin α2β1 is expressed on undifferentiated neuroepithelial precursor cells of the embryonic chick retina, where it acts as a collagen receptor,
mediating adhesion and process outgrowth on collagens I and IV. This analysis was made possible by the isolation of an anti-α2 monoclonal antibody, MEP-17 (McNagny et al., 1992), which we have used to track expression and perturb α2 function. The anti-α2 antibody inhibits cell adhesion and process outgrowth on collagens I and IV, indicating that α2 is necessary for these interactions. To our knowledge, this is the first demonstration of process outgrowth mediated by α2β1.

The expression of α2 in retina is surprising in light of previous reports that failed to detect α2 expression. DeCurtis et al. (1991) found that an anti-human-α2-C-terminal peptide antibody did not detect α2 in western blots of chick retina, but could detect immunoreactive bands in chick heart. Likewise, α2 mRNA and protein expression could not be detected in murine retina by in situ hybridization and immunohistochemistry using a peptide antibody (Wu and Santoro, 1994). In contrast, in the developing chick retina, we have detected α2 mRNA by RT-PCR and northern blotting (Cann et al., unpublished data). Cell types expressing α2 protein by immunohistochemistry and immunoprecipitation (this report) and in extracts, at similar levels. The epitope in older sections may be masked or more sensitive to fixation. Continued expression is also indicated by analysis of α2 mRNA levels, which can be detected throughout development (Cann et al., unpublished data). Cell types expressing α2 at later ages were not investigated thoroughly but, in E12 cultures, immunoreactive flat cells resembling Müller glial cells were observed. Müller cells continue to divide throughout development (Turner et al., 1990), so the observation that mitotically active cells express α2 may also apply to the retina.

Despite continued expression, integrin α2β1 heterodimers appear to be down regulated functionally, since older retinal cells can no longer interact with collagen I (Figs 7, 9) and IV (Figs 7, 9; Hall et al., 1987). In contrast to Hall et al. (1987), we find that adhesion to collagen IV is also down regulated, although results were somewhat variable, possibly due to an integrin-independent adhesion to denatured collagen IV (Fig. 8). Our results are consistent with Neugebauer and Reichardt (1991) who showed that older neurons will respond to collagen

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**Fig. 6.** Inhibition of collagen adhesion by anti-α2. A concentration of 10 μg/ml completely blocks adhesion to collagen I and maximally inhibits adhesion to collagen IV, but had no effect on adhesion to laminin. Each point represents the average of at least three determinations. Error bars are the standard error of the mean.

**Fig. 7.** Developmental regulation of cell adhesion to collagen I and collagen IV. Retinal cells from progressively older embryos showed a decreased ability to bind to collagen I and collagen IV-coated wells (no Ab; closed circles). Purified anti-α2 antibody (10 μg/ml; open diamonds) and purified anti-β1 antibody (10 μg/ml; closed triangles) showed the same level of inhibition of adhesion at all ages tested. Control wells incubated with purified mouse IgG (10 μg/ml) showed no inhibition of adhesion (not shown). Background adhesion to wells coated with BSA was subtracted from the experimental values. Each point represents the average of at least three determinations. Error bars indicate the standard error of the mean.
in the presence of the anti-\(\beta_1\) monoclonal antibody (TASC).

The mechanism of down regulation is unknown, but it appears to be post-translational and it applies to all \(\beta_1\) integrins investigated thus far (Cohen et al., 1986, 1989; Hall et al., 1987; de Curtis et al., 1991; de Curtis and Reichardt, 1993; Neugebauer and Reichardt, 1991). Post-translational down regulation of \(\alpha_2\beta_1\) has also been observed in avian B cells and in other cell types (McNagny et al., unpublished data; Hynes, 1992).

At all ages tested, retinal cell adhesion and process outgrowth on collagen I and, to a lesser extent, on collagen IV, was blocked by the anti-\(\alpha_2\) antibody. The antibody, MEP-17, also blocks collagen-dependent adhesion of avian thrombocytes (McNagny et al., unpublished data). The cell adhesion perturbed in vitro may reflect a role for \(\alpha_2\beta_1\) in mediating adhesion of neuroepithelial cells to collagens present at the vitreal border of the retina. Since neuroepithelial cells at E4-E6 are not active in extending axons in vivo, the process outgrowth observed in vitro may reflect the neuroepithelial processes that extend through the width of the retina to make contact with the inner limiting membrane ECM (Prada et al., 1981). \(\alpha_2\beta_1\) integrins may also mediate the very first initial axon outgrowth from newborn RGCs as they differentiate from neuroepithelial cells and growth cones move across the inner limiting membrane. Although \(\alpha_2\) immunoreactivity could not be detected on neurofilament-positive RGCs, an occasional neurofilament-positive cell could be observed extending neurites on collagen and this outgrowth was blocked by anti-\(\alpha_2\) (data not shown). Thus, it is possible that \(\alpha_2\) integrin may be expressed below the level of detection on some newborn RGCs, but is still functional.

In contrast to interactions with collagen, retinal cell interactions with laminin were not perturbed, indicating that \(\alpha_2\) function is necessary for collagen interactions but not laminin interactions. We cannot rule out, however, that \(\alpha_2\beta_1\) acts as a laminin receptor and, when blocked by antibody, other integrins fulfill this role. In addition, the anti-\(\alpha_2\) antibody may not be capable of blocking an \(\alpha_2\beta_1\) interaction with laminin. The entire cohort of laminin receptors on retinal cells has yet to be identified (de Curtis et al., 1993). Alternatively, \(\alpha_2\beta_1\) could bind \(\alpha_3\beta_1\) on other cells and mediate cell-cell interactions as shown for human T-cells (Symington et al., 1993). However, we could not detect any disruption of retinal cell association in cultures incubated with anti-\(\alpha_2\) antibody.

In rat sympathetic neurons, process outgrowth on collagen IV is mediated by \(\alpha_1\beta_1\) recognition of the NCI domain (Lein et al., 1991), so it appears that different neurons use different integrin collagen receptors. Although \(\alpha_1\) may be expressed in retina (Duband et al., 1992), blocking \(\alpha_1\) antibody is not available for chicken cells, so we could not determine the importance of \(\alpha_1\) for collagen interactions. However, because the anti-\(\alpha_2\) antibody blocks to the same degree as anti-\(\beta_1\), we conclude that \(\alpha_1\beta_1\) integrin cannot be sufficient for interactions with collagen.

What function might \(\alpha_2\beta_1\) play in vivo? Collagens I and IV are found in the inner limiting membrane on the vitreal border of the developing retina (Fig. 5C and Halfter and Fua 1987; Halfter and Boxberg 1992; Sarthy, 1993) and mRNAs have been detected in the fetal human optic nerve head (Hernandez et al., 1991). The inner limiting membrane ECM is contacted by both RGCs and neuroepithelial precursor cells. It seems
unlikely that this integrin is involved in long-term axonal extension by RGCs along the inner limiting membrane because neurofilament-positive cells in our cultures were not positive for α2. In contrast, neuroepithelial precursor cells expressing α2β1 extend processes that connect them to the vitreal border of the developing retina. Collagen may primarily provide an anchor for neuroepithelial cell endfeet, with attachment and process outgrowth mediated by α2β1. The down regulation function may accompany detachment from these borders and movement to internal retinal layers. In the epidermis, the onset of differentiation of basal cells coincides with a down regulation of integrin function (Watt et al., 1993). Further experiments, which perturb α2 function in vivo utilizing MEP-17, are currently underway to fully address the role of this integrin in retinal development.

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REFERENCES


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