Expression Patterns of Focal Adhesion Associated Proteins in the Developing Retina

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ABSTRACT Adhesive interactions between integrin receptors and the extracellular matrix (ECM) are intimately involved in regulating development of a variety of tissues within the organism. In the present study, we have investigated the relationships between β1 integrin receptors and focal adhesion associated proteins during eye development. We used specific antibodies to examine the distribution of β1 integrin ECM receptors and the cytoplasmic focal adhesion associated proteins, talin, vinculin, and paxillin in the developing Xenopus retina. Immunoblot analysis confirmed antibody specificity and indicated that β1 integrins, talin, vinculin, and paxillin were expressed in developing retina and in the retinal-derived Xenopus XR1 glial cell line. Triple-labeling immunocytochemistry revealed that talin, vinculin, paxillin, and phosphotyrosine proteins colocalized with β1 integrins at focal adhesions located at the termini of F-actin filaments in XR1 cells. In the retina, these focal adhesion proteins exhibited developmentally regulated expression patterns during eye morphogenesis. In the embryonic retina, immunoreactivities for focal adhesion proteins were expressed in neuroepithelial cells, and immunoreactivity was especially strong at the interface between the optic vesicle and overlying ectoderm. At later stages, these proteins were expressed throughout all retinal layers with higher levels of expression observed in the plexiform layers, optic fiber layer, and in the region of the inner and outer limiting membrane. Strong immunoreactivities for β1 integrin, paxillin, and phosphotyrosine were expressed in the radially oriented Müller glial cells at later stages of development. These results suggest that focal adhesion-associated proteins are involved in integrin-mediated adhesion and signaling and are likely to be essential in regulating retinal morphogenesis.

Key words: focal adhesion proteins; retina; Xenopus; integrins; retinal development

INTRODUCTION

The vertebrate retina is an ideal central nervous system (CNS) structure in which to investigate cell–extracellular matrix (ECM) interactions due to its highly laminated organization and accessibility. During retinal morphogenesis, the neural tube evaginates to form a pseudostratified optic vesicle that invaginates to form the two-layered optic cup (Jacobson, 1966). The outer layer becomes the monolayered retinal pigmented epithelium (RPE), whereas the inner layer gives rise to the multilayered sensory retina (Hilfer, 1983). Retinal cell types are generated in a histologic order from the retinal neuroepithelium and migrate from the ventricular zone to their appropriate laminar position (Dowling, 1970; Turner and Cepko, 1987; Wetts and Fraser, 1988; Holt, 1989). The retina is organized into an outer nuclear layer (ONL), which contains the cell bodies of photoreceptors, an inner nuclear layer (INL), with the cell bodies of horizontal cells, bipolar cells, amacrine cells, and Müller glia, and the retinal ganglion cell layer (GCL), where the cell bodies of retinal ganglion cells reside. Between the three cellular layers are the synaptic layers: the outer plexiform and the inner plexiform layer (OPL and IPL, respectively).

During retinal development, cell–cell and cell–ECM interactions are necessary for cell adhesion, migration, proliferation, and differentiation and are likely to be critical in establishing the highly organized architecture of the retina. Integrins are the major family of cell surface receptors that mediate cell attachment to the ECM and can also mediate cell–cell interactions (Hynes, 1992, 1999). Functional integrin receptors are composed of one α and one β subunit that are associated noncovalently to form a heterodimer. At least 18 α and 8 β subunits have thus far been identified in vertebrates, giving rise to more than 24 different integrin heterodimers (van der Flier and Sonnenberg, 2001). Each subunit has a large extracellular domain, a single

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transmembrane domain, and a short conserved cytoplasmic tail. The combination of α and β subunit determines ligand specificity and intracellular signaling activity. The β1 integrins are the most prominent integrin subfamily, and the β3 subunit can interact with 12 different α subunits to form functional receptors. Alternative splicing of β1 integrin mRNA increases the diversity of the β1 integrin family (van der Flier et al., 1995).

In addition to the β2 subunit, β3 and β5 subunits have been identified in embryonic retina (Gervin et al., 1996). β3 integrins have been implicated in mediating neurite outgrowth, cell migration, and proliferation during retinal morphogenesis (Cohen et al., 1987; Cann et al., 1996; Sakaguchi and Radke, 1996; Stone and Sakaguchi, 1996). Several α subunits have been identified in the retina, including the α5, α6, αv, α4, α5, α6, α5, and α4 subunits (Cann et al., 1996; Lin and Clegg, 1998; Sherry and Proske, 2001). In addition, integrin α5/α1 subunits were found in the vitreous body in the eye (Georges-Labouesse et al., 1998). Furthermore, double knockouts of α2 and α6 subunits result in severe eye laminar defects (De Arcangelis et al., 1999).

Integrin receptor binding with ECM molecule results in a cascade of events within the cytoplasm, including phosphorylation of proteins and the recruitment of cytoskeletal proteins that lead to the formation of focal adhesions at the ventral surface of cultured cells (Craig and Johnson, 1996). Focal adhesions are the sites where integrins link the ECM with the cytoskeleton. Focal adhesions consist of clustered integrins and associated proteins, referred to as focal adhesion proteins. These proteins include talin, vinculin, tensin, paxillin, and focal adhesion kinase (FAK) that mediate cell adhesion and signaling (Clark and Brugge, 1995; Howe et al., 1998).

Talin and vinculin are cytoskeletal proteins that link the integrin receptors and F-actin cytoskeleton (Critchley et al., 1999; Critchley, 2000). Talin can bind to the cytoplasmic tail of the β integrin subunit as a consequence of integrin–ligand engagement and contributes to the formation of focal adhesions (Critchley et al., 1999). Talin possesses at least two actin-binding sites and three binding sites for vinculin (Gilmore et al., 1993). Vinculin can also bind F-actin and may cross-link talin and actin, thereby stabilizing the interaction (Calderwood et al., 1999). There is evidence that talin and vinculin are involved in regulating the formation of focal adhesions and stress fibers and cell motility, although they may have distinct roles (Nuckolls et al., 1992; Albigeis-Rizo et al., 1995; Volberg et al., 1995; Goldmann et al., 1996; Critchley, 2000). Paxillin can be tyrosine phosphorylated and is among the regulatory molecules at focal adhesions. Paxillin can bind the integrin cytoplasmic tail, vinculin, or other cytoskeletal and signaling proteins (Schaller et al., 1995). Thus, paxillin provides a platform for protein tyrosine kinases such as FAK and Src, which are activated as a result of adhesion or growth factor stimulation (Giancotti and Ruoslahti, 1999). Phosphorylation of paxillin by these kinases permits binding with downstream effector molecules such as p130 Cas and transduces external signals into cellular responses by means of MAP kinase cascades (Cary and Guan, 1999). Paxillin functions as a multidomain adapter molecule and serves as a point of convergence for signals resulting from adhesion and various growth factor receptors (Turner, 2000).

Several studies have begun to investigate the regulation of focal adhesion assembly and integrin-mediated signaling in cultured cells (Miyamoto et al., 1995; Folsom and Sakaguchi, 1997, 1999; Giancotti and Ruoslahti, 1999). We previously demonstrated a functional role for β1 integrins in regulating cell spreading and neurite outgrowth in Xenopus retina (Sakaguchi and Radke, 1996) and in regulating focal adhesion assembly in Xenopus X1 retina glial cells (Folsom and Sakaguchi, 1997, 1999). Furthermore, developmentally regulated changes of β1 integrins, talin, and vinculin have been identified in embryonic tissues (Evans et al., 1990; Gawantka et al., 1992), and tyrosine phosphorylation levels of paxillin have been shown to change during embryonic development (Turner, 1991; Sorenson and Sheibani, 1999). Taken together, these results indicate that focal adhesion proteins play a critical role during embryonic development. However, the functional relationship between integrin signaling with focal adhesion proteins during neural development remains to be clearly elucidated.

In the present study, we have identified the distribution of the focal adhesion associated proteins β, integrin, talin, vinculin, and paxillin, as well as phosphotyrosine proteins, during the development of the Xenopus retina. Immunoblot analysis indicated that these focal adhesion proteins were expressed in the developing retina and the X1 retina glial cell line. Moreover, these proteins colocalized at focal adhesions associated with the termini of F-actin stress fibers in cultured X1 cells. Their expression displayed a differentially regulated pattern in retinal tissue and was related with specific morphologic events during retinal development. These results suggest that focal adhesion proteins may be involved in integrin-mediated signaling during retinal morphogenesis.

RESULTS
Focal Adhesion Proteins Are Expressed in the Developing Retina and in the X1 Glial Cell Line

To verify the specificity of the antibodies directed against focal adhesion-associated proteins used in this study, we performed an immunoblot analysis. The immunoblot analysis was performed with samples from stage (St) 40 Xenopus eyes and from Xenopus retinal-derived X1 glial cells. The monoclonal anti-β1 integrin antibody was generated against β1 integrin-enriched proteins from Xenopus A6 and XTC cells (Gawantka et al., 1992) and labeled a single band with molecular weight of approximately 115 kDa in both
samples under nonreducing conditions (Fig. 1, lane 1, 2). The anti-talin antibody labeled two bands of 235 and 225 kDa under reducing conditions (Fig. 1, lane 3, 4). The anti-vinculin antibody identified a band of approximately 116 kDa (Fig. 1, lane 5, 6), and the anti-paxillin antibody identified a band of approximately 68 kDa (lane 7, 8) in both samples under reducing conditions. These molecular weights are consistent with previously published molecular weights for these protein from other species (Turner et al., 1990; Sydor et al., 1996). Although produced against avian talin and paxillin or human vinculin, these antibodies exhibited specific cross-reactivity with *Xenopus* tissues.

**Immunolocalization of Focal Adhesion Proteins in XR1 Retinal Glial Cells**

Focal adhesions are a discrete streak-like complex of clustered integrins and associated proteins that link the ECM with the cytoskeleton and mediate cell adhesion and signaling. To identify focal adhesions and characterize the relationship among focal adhesion proteins and the F-actin cytoskeleton, we have used the *Xenopus* retinal-derived XR1 glial cell line as a model cell system. Triple-labeling studies using the XR1 cells with β₁ integrin antisera, and either anti-talin, vinculin, paxillin, or phosphotyrosine antibodies, and rhodamine-phalloidin were carried out to examine the relationship of these proteins at focal adhesions in *Xenopus* cells. As illustrated in Figure 2, the focal adhesion protein immunoreactivities (IRs) were localized to focal adhesions at the termini of the F-actin filaments in XR1 cells. β₁ integrin-IR (Fig. 2A,E,I,M) colocalized with talin- (Fig. 2B), vinculin- (Fig. 2F), paxillin- (Fig. 2J), or phosphotyrosine- (Fig. 2N) IRs, and these focal adhesion-associated protein-IRs colocalized with rhodamine-phalloidin–labeled F-actin filaments (Fig. 2C,G,K,O) in the XR1 cells (Fig. 2D,H,L,P). The colocalization of these proteins at the termini of actin stress fibers confirms their localization to focal adhesions. Furthermore, in *Xenopus* retinal-derived cells, their localization suggests that they are involved in focal adhesion formation and cytoskeletal organization, as well as signal transduction at focal adhesions.

**Distribution of Focal Adhesion Proteins in the Developing Xenopus Retina**

The functional relationships of focal adhesion-associated proteins have been extensively characterized in cultured cells (Miyamoto et al., 1995; Folsom and Sakaguchi, 1997, 1999). However, little is known about their relationships in vivo. To characterize the expression pattern of focal adhesion associated proteins and their relationships during retinal development in vivo,
tissue sections from *Xenopus* embryos, larvae, and froglets were stained with antibodies directed against focal adhesion associated proteins. Immunoreactivities for these focal adhesion proteins were present in all retinal cells throughout development. Although the patterns changed during the course of development, similarities in immunoreactivities were clearly apparent between the different focal adhesion associated proteins.

**Summary of Xenopus Eye Development:** Neurulation of the *Xenopus* embryo ends at St 20. The primary optic vesicle is produced from the diencephalic neuroepithelium by St 23, the early tail bud stage. The eye cup starts to form at the anterodorsal margin at St 26. At St 26, the first retinal ganglion cells (RGCs) are produced in the central retina, and at St 27, the lens placode begins to form from the sensorial layer of the ectoderm (Hausen and Riebesell, 1991). RGC axonogenesis begins around St 28, and dendritogenesis begins around St 31, the late tail bud stage (Holt, 1989; Sakaguchi, 1989). The tail bud embryo begins to hatch into a freely swimming larva at St 32, when the first optic axons have reached the chiasm. Hatching finishes at St 35/36, when the early RGC axons have arrived at the mid-optic tract region. The first optic axons reach the tectum around St 37/38 (Sakaguchi and Murphey, 1985). The retina is highly laminated, and the RGC axons have begun elaborating terminal arborization in the tectum by St 40, when the first visual responses are detected on the tectum (Holt and Harris, 1983). At St 47, morphologic classes of RGCs can be identified (Sakaguchi et al., 1984), and metamorphosis is nearly complete by St 65, and the retina is mature and similar in overall structure to the adult retina.

At St 25, the primary eye vesicle was fully developed and consisted primarily of retinal neuroepithelial cells. β₁ integrin-IR was present throughout the optic vesicle and appeared to be associated with the membranes of the prospective sensory retina. However, the strongest IR was detected at the interface between the vesicle and overlying ectoderm, the future location of the inner limiting membrane (ILM) and the lens placode (Fig. 3A). Talin- and vinculin-IR were also strong at the interface between the optic vesicle and ectoderm (Fig. 3G,M). The immunoreactivity for paxillin and phosphotyrosine displayed similar patterns with β₁ integrin-IR.
in neuroepithelial cells and strong IR at the interface between the optic vesicle and ectoderm (Fig. 4A,G).

By St 30, the eye cup was well formed and the lens placode has formed from the sensorial layer of the ectoderm. H9252 integrin-IR was present outlining retinal cells, including the undifferentiated neuroblasts and the first generated ganglion cells along the inner retina adjacent to the lens placode. H9252 integrin-IR was highly expressed on newly generated ganglion cells as well as in the lens placode (Fig. 3B). Talin- and vinculin-IR appeared to be expressed in all retinal cells and was stronger on the nascent ganglion cells in the inner retina (Fig. 3H,N). Strong paxillin- and phosphotyrosine-IRs were also expressed within the inner retina in the newly generated ganglion cells and the lens placode (Fig. 4B,H). At this time, the presumptive RPE was contacting the neural retina, and H9252 integrin- and talin-IRs were expressed in these cells (Fig. 3B,H).

By St 37, the retina was relatively well differentiated. H9252 integrin-IR was still widespread, and cell bodies in the ONL, INL, and GCL were clearly outlined, and IR was strong in the nascent outer and inner plexiform layers (OPL and IPL), as well as optic fiber layer (OFL; Fig. 3C). Talin- and vinculin-IRs (Fig. 3I,O) and paxillin- and phosphotyrosine-IRs (Fig. 4C,I) clearly outlined cell bodies and were stronger in the plexiform layers.

Retinal lamination was clearly present by larval St 40. The cell bodies in the ONL, INL, and GCL were clearly outlined by immunoreactivities, and the OFL as well as the IPL and OPL displayed more intense levels of immunoreactivity by H9252 integrin (Fig. 3D), talin (Fig. 3J), vinculin (Fig. 3P), paxillin (Fig. 4D), and phosphotyrosine (Fig. 4J). H9252 integrin-, paxillin- and phosphotyrosine-IRs were present in radially oriented cells with morphologies reminiscent of the Müller glial cells (Figs. 3D, 4D,J).

The retina was well differentiated by St 47. Immunoreactivities for H9252 integrin (Fig. 3E), talin (Fig. 3K), vinculin (Fig. 3Q), paxillin (Fig. 4E), and phosphotyrosine (Fig. 4K) were expressed in the OFL as well as the IPL and OPL. H9252 integrin-, paxillin-, and phosphotyrosine-IRs were prominent in the radially oriented pattern observed at St 40 (Figs. 3E, 4E,K). Formation of the plexiform layers begins centrally and spreads peripherally to the mitotically active ciliary marginal zone at the rim of the eye (Perron et al., 1998). At later stages, the expression patterns for these focal adhesion proteins at the ciliary marginal zone were similar to the patterns at early stages (data not shown).

The radial pattern of immunoreactivity observed with the anti-H9252 integrin, -paxillin, and -phosphotyrosine antibodies was similar to the pattern of retinal Müller glial cells. To investigate this possibility, we double-labeled retinal sections from late stage Xenopus with polyclonal anti-H9252 integrin antibody and an antibody directed against glial fibrillary acidic protein (GFAP) as illustrated in Figure 5. In Xenopus, the anti-GFAP antibody labels Müller cells and astrocytes. Figure 5 shows colocalization of H9252-IR in GFAP-immunoreactive Müller cells and astrocytes along the ILM. These results provide strong evidence that Müller cells express relatively high levels of focal adhesion associated proteins.

The retina was relatively mature by St 65. Immunoreactivities for H9252 integrin (Fig. 3F), talin (Fig. 3L), vinculin (Fig. 3R), paxillin (Fig. 4F), and phosphotyrosine (Fig. 4L) were present in the OLM, OPL, IPL,
and OFL. The radial pattern of $\beta_1$ integrin-, paxillin-, or phosphotyrosine-IR in Müller glial cells was still present. Immunoreactivities for these focal adhesion associated proteins were low at the outer segments of the photoreceptors and at the apical membranes of RPE, the same as the IRs at St 47. At St 40, 47, and 65, vinculin- and talin-IRs were rarely observed in radially oriented processes. The expression patterns of these focal adhesion proteins at St 65 were similar to their patterns in adult retina (data not shown).

The distribution of $\beta_1$ integrin and focal adhesion associated proteins displayed similar spatial and temporal patterns of expression during retinal development. Immunoreactivities for these focal adhesion associated proteins were present in neuroepithelial cells and were especially strong in the outer and inner limiting membranes, plexiform layers, and optic fiber layer in the retinal tissue. Immunoreactivities for $\beta_1$ integrin, paxillin, and phosphotyrosine were intensively displayed in the radially oriented Müller glial cells at later stages. These results suggest that focal adhesion proteins may be involved in regulating integrin-mediated adhesion and signaling during retinal development.

**DISCUSSION**

This study is the first systematic analysis of the distribution of the focal adhesion-associated proteins, $\beta_1$ integrin, talin, vinculin, paxillin, and phosphotyrosine during the development of the retina. Characterizing their patterns of expression during retinal development is essential to gain a better understanding of their roles during eye morphogenesis. These focal adhesion-associated proteins displayed similar and differentially regulated expression patterns. Immunoreactivities for these proteins were localized at the interface between the optic vesicle and ectoderm and the plexiform layers as well as the outer limiting membrane and optic fiber layer. Immunoreactivities for $\beta_1$ integrin, paxillin, and phosphotyrosine were highly expressed in the radially oriented Müller glial cells. These results suggest that these focal adhesion associated proteins may play a vital role in cell adhesion, migration, differentiation, and neurite outgrowth during retinal development in *Xenopus*.

Immunoblot analysis confirmed the specificity of the antibodies and showed that the focal adhesion associated proteins, $\beta_1$ integrins, talin, vinculin, and paxillin, were expressed in developing retina and XR1 retinal glial cells. Immunocytochemical analysis revealed that talin, vinculin, paxillin, and phosphotyrosine proteins colocalized with $\beta_1$ integrins at focal adhesions located at the termini of F-actin filaments in XR1 cells. Frozen sections from *Xenopus* larvae at stages 25, 30, 37, 40, 47, and 65 were immunostained with antibodies against these focal adhesion proteins. $\beta_1$ integrin-, talin-, vinculin-, paxillin-, and phosphotyrosine-IRs were present in the retina at all stages analyzed. At early stages, the immunoreactivities were localized to the radial neuroepithelial cells that spanned the width of the prospective sensory retina. Immunoreactivities appeared strongest at the interface between the optic vesicle and ectoderm, the region of the future ILM. The immunoreactivities were strong in the newly generated ganglion cells and in the newly formed plexiform layers. Strong immunoreactivities were maintained in the plexiform layers as well as the ILM and OLM at later stages (St 47 and 65). During the late stages, the immunoreactivities for $\beta_1$ integrin, paxillin, and phosphotyrosine were highly expressed in the radially oriented Müller glial cells, spanning the width of the neural retina. The similarities and the changing patterns of distribution for these focal adhesion associated proteins during development suggest that the regulation of focal adhesions may be essential during retinal morphogenesis.

The distribution of $\beta_1$ integrin receptors during *Xenopus* retinal development is similar to the expression of $\beta_1$ integrins in the developing chick retina (Rizzolo and Heiges, 1991; Cann et al., 1996; Hering et al., 2000). $\beta_1$ integrins were expressed in the undifferentiated neuroepithelial cells and persisted in most retinal cells during retinogenesis and synaptogenesis and were highly displayed in the Müller glial cells (Cann et al., 1996; Hering et al., 2000). $\beta_1$ integrins were also expressed in the RPE progenitor cells and resided in the basal membranes at later stages in *Xenopus*, as well as in chick retina (Rizzolo and Heiges, 1991). We identified $\beta_1$ integrin expression in the apical membranes of St 47 RPE with the polyclonal anti-$\beta_1$ antibody (3818). This finding is consistent with another study on *Xenopus* RPE (Chen et al., 1997). The differences from different antibody labeling may be due to the antibody specificity or the expression of different isoforms. Furthermore, the pigmentation in RPE cells...
may mask some fluorescence of β1 integrin-IR. However, we detected β1 integrin expression in cultured RPE cells dissociated from St 47 eye and in St 65 RPE homogenate with immunocytochemistry and Western blot analysis, respectively (data not shown).

The differential distribution of β1 integrins suggests they have an important role in mediating cell adhesion and signaling during retinal morphogenesis. β1 integrin-IR was present in the neuroepithelial cells, in particular at the interface between the optic vesicle and ectoderm, suggesting that β1 integrins may be involved in the adhesion of the neuroepithelial stem cells to the basal lamina of the ILM. Furthermore, relatively high concentrations of potential β1 integrin receptor ligands, such as laminin and fibronectin, have been identified in the region of the ILM (Sakaguchi, unpublished observations). Studies inhibiting β1 integrin function suggest an important role for these receptor complexes during retinal development (Svennevik and Linser, 1993). Injection of β1 integrin function blocking antibody and RGD peptides into early optic vesicle of embryonic day 2 chick prevented invagination of the optic vesicle and resulted in the reduction of retinal size (Svennevik and Linser, 1993). Furthermore, injection with β1 integrin antisense RNA virus caused the reduction of β1 integrin expression and also produced retina of small size (Skeith et al., 1999). In the chick retina, ganglion cell migration from the ventricular zone was significantly inhibited when explanted eye cups were cultured in the presence of function blocking anti-β1 integrin antibody (Cann et al., 1996). Together, these results suggest that integrin-mediated adhesion is critical for proliferation, differentiation, and migration of the retinal neuroblasts.

Numerous studies provide evidence that integrins are involved in the regulation of neurite outgrowth and synaptic morphology (Condic and Letourneau, 1997; Ivins et al., 2000; Rohrbough et al., 2000; Condic, 2001). The coincident presence of β1 integrins and other focal adhesion-associated proteins within the plexiform layers and OFL suggests that focal adhesions may be important during neurite outgrowth and synaptogenesis in the vertebrate retina. In previous studies, β1 integrins have been implicated in mediating retinal neurite outgrowth during development and regeneration on ECM substrates (Sakaguchi and Radke, 1996). Furthermore, injection of function blocking antibodies against β1 integrin, as well as N-cadherin, perturbed the development of the Xenopus retinotectal projection (Stone and Sakaguchi, 1996), and expression of chimeric β1 integrins in Xenopus embryos impaired the outgrowth of axons and dendrites from RGCs in the retina (Lilienbaum et al., 1995). In addition, different α integrin subunits have been identified to have different distributions in the tiger salamander retina (Sherry and Proske, 2001). Moreover, different cadherins have been identified to have unique distributions in the mouse retina (Honjo et al., 2000). Taken together, these results indicate that adhesion receptors are likely to play a role in selective cell–ECM and cell–cell interactions within the heterogeneous cell pool of the developing retina. Furthermore, cross-talk between integrins and cadherins is possible (Arregui et al., 2000; von Schlippe et al., 2000). Through different associated proteins, integrins and cadherins are involved in organizing cytoskeletal structures that serve as scaffolds for signaling cascades that ultimately regulate cellular processes (Juliano, 2002).

β1 integrin-, paxillin-, and phosphotyrosine-IRs were highly expressed by Müller cells, displaying a radial pattern through the retina with intense IR at the endfeet in the ILM and the OLM. This pattern suggests that regulation of β1 integrin-mediated focal adhesions may play an important role in maintaining the structural arrangement of the Müller glial cells. We have not observed strong expression of talin- and vinculin-IRs in the Müller glial cells, even though strong immunoreactivity was observed in the ILM and OLM. The retinal tissue sections cut at an oblique angle may produce a loss of the radial appearance of labeling. However, we did not observe the radial labeling patterns for talin and vinculin, even when using the same sets of tissue as for paxillin and phosphotyrosine, which displayed the radial patterns of immunoreactivity. The differences in the patterns of expressions between β1 integrin, paxillin, and phosphotyrosine with talin and vinculin may indicate that these proteins are separately regulated and each has its distinct role during retinal development, in addition to their coordinating function in integrin-mediated adhesion.

Our in vitro studies in XR1 retinal glial cells revealed that β1 integrins colocalized with talin, vinculin, paxillin, and phosphotyrosine at focal adhesions located at the termini of actin stress fibers. Talin and vinculin serve as structural molecules that link β1 integrins to the F-actin cytoskeleton. Focal adhesions provide a platform, where integrins link ECM and cytoskeleton, and serve as bidirectional signal transduction receptors (Clark and Brugge, 1995; Miyamoto et al., 1995). In the retina, these focal adhesion proteins showed a general diffuse distribution and did not reveal obvious streak-like patterns of focal adhesion. This finding is consistent with other studies on integrin subunits (Hering et al., 2000; Sherry and Proske, 2001). In vivo cells may be less likely to form focal adhesions, because they are in a three-dimensional (3D) environment, unlike the cultured cells that are constrained on two dimensional substrates. Furthermore, the resolution limitation for imaging may be a barrier to observe focal adhesions in vivo. An in vitro 3D matrix system that may be more biologically related to living organism is needed to study cell–ECM interactions (Cukierman et al., 2001). Moreover, the developing cells are most likely in an adaptive state of intermediate cell adhesion and are less likely to form strong adhesions during morphogenesis as the cells in culture (Murphy-Ullrich, 2001).
In vitro studies examining the formation of focal adhesions in cultured retinal glia demonstrate an important role for tyrosine kinase activity in regulating focal adhesions and in maintaining cell shape (Folsom and Sakaguchi, 1997; Li and Sakaguchi, unpublished data). Inhibitors of tyrosine kinases block recruitment of a large set of signaling molecules to focal adhesion complexes in cultured fibroblasts (Miyamoto et al., 1995). Tyrosine kinase inhibitors can also block axonal extension from retinal ganglion cells in vitro and in vivo (Worley and Holt, 1996). The application of tyrosine kinase inhibitors to the developing embryonic retina disrupted the formation of the lamination of the retina. The plexiform layers did not appear in the tyrosine kinase inhibitor-treated retina (Li and Sakaguchi, manuscript in preparation). Taken together, these results indicate that tyrosine phosphorylation, initiated by integrin receptors, is a major factor that mediates integrin affinity and focal adhesion formation and can then transduce extracellular cues into meaningful signals that mediate cellular behavior (Maness and Cox, 1992).

This study provides important new information and contributes to our understanding of the relationships between these focal adhesion-associated proteins during neural development. These focal adhesion proteins analyzed here displayed similar and developmentally regulated expression patterns during *Xenopus* retinal development. These results suggest that these focal adhesion proteins are involved in regulating integrin-mediated adhesion and signaling and play a critical role in regulating retinal cell adhesion, migration, proliferation, and neurite outgrowth during retinogenesis. Information about subcellular localization and the relationships between focal adhesion proteins in the developing retina will help elucidate the mechanisms of integrin-mediated adhesion and signaling in vivo.

**EXPERIMENTAL PROCEDURES**

**Animals**

*Xenopus laevis* frogs were obtained from a colony maintained at Iowa State University. Embryos were produced from human chorionic gonadotropin (Sigma-Aldrich, St. Louis, MO)-induced matings and were maintained in 10% Holtfreter's solution (37 mM NaCl, 0.5 mM MgSO4, 1 mM NaHCO3, 0.4 mM CaCl2, and 0.4 mM KCl) at room temperature. Embryos and larvae were staged according to the normal *Xenopus* table of Nieuwkoop and Faber (1967). Laboratory procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and had the approval of the Iowa State University Committee on Animal Care.

**XR1 Cell Cultures**

The XR1 cell line is an immortal glial cell line derived from *Xenopus* retinal neuroepithelium (Sakaguchi et al., 1989; Sakaguchi and Henderson, 1993). XR1 cells were grown in tissue culture flasks (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) in 60% L15 media (Sigma) containing 10% fetal bovine serum (Upstate Biotechnology, Inc, Lake Placid, NY, 1% embryo extract (Sakaguchi et al., 1989), 2.5 μg/ml fungibact and 2.5 μg/ml penicillin/streptomycin (Sigma). XR1 cells were detached from subconfluent cultures by exposure to Hanks' dissociation solution (5.37 mM KCl, 0.44 mM KH2PO4, 10.4 mM Na2HPO4, 137.9 mM NaCl, 9.0 mM D-glucose, 0.04 mM Phenol Red) supplemented with 2.5 μg/ml fungibact, 2.5 μg/ml penicillin/streptomycin, 0.2 mg/ml ethylenediamine tetraacetic acid (EDTA), and 0.5 μg/ml trypsin. Detached cells were collected, pelleted by centrifugation, resuspended in culture media, and seeded onto 12-mm detergent-washed (RBS-35; Pierce, Rockford, IL) glass coverslips (Fisher Scientific Co., Pittsburgh, PA) coated with 10 μg/ml Entactin-Collagen IV-Laminin (ECL) substrate (Upstate Biotechnology). Cultures were grown at room temperature (~24°C).

**Western Blot Analysis**

Eyes were dissected from stage 40 larva and placed in lysis buffer (0.1 M NaCl, 10 mM Tris, pH 7.6, 1 mM EDTA, 0.2% NP-40, 1 μg/ml aprotinin, and 1 mM phenylmethyl sulfonl fluoride). XR1 cells were scraped from the flasks and placed in lysis buffer. Samples were homogenized, and protein concentration was determined by using a Bio-Rad assay kit. Protein samples were boiled in sodium dodecyl sulfate (SDS) sample buffer (0.5 M Tris-HCl, 10% SDS, 10% glycerol, 2.5% bromophenol blue) with or without 5% β-mercaptoethanol (nonreducing conditions) and separated on 7.5% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose, blocked overnight in 1.5% bovine serum albumin (BSA) in Tris-buffered saline (TBS, 10 mM Tris-HCl, 150 mM NaCl, pH 8.0), and incubated with antibodies directed against β1 integrin, talin, vinculin, or paxillin for 1 hr. After washing in TBS with 0.1% tween-20, the membranes were incubated with 1:5,000 goat anti-mouse immunoglobulin (Ig) G–horseradish peroxidase for 45 min. The staining was detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

**Immunohistochemistry**

*Xenopus* embryos, larvae, froglets, and cultured cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 hr (animals) or 30 min (cells). The animals were fixed in Dent's fixative (20% dimethyl sulfoxide and 80% methanol) for anti-GFAP staining. The specimens were rinsed with buffer and cryoprotected in 30% sucrose in 0.1 M PO4 buffer overnight and then frozen in OCT medium (Tissue-Tek, Sakura Finetek U.S.A., Inc., Torrance, CA). The frozen tissues were sectioned at 16 μm by using a cryostat (Reichert Histostat), and sections were mounted on Superfrost microscope slides (Fisher). Tissue sections and cultures were rinsed in phosphate buffered saline (PBS, 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na2HPO4, 1.47 mM KH2PO4) and blocked in 5% goat serum, containing 0.2% BSA and 0.1% Triton X-100 in PBS. Primary
Antibodies

β1 integrin receptors were identified by using monoclonal antibody 8C8, purchased from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) and diluted 1:10 in blocking solution, and polyclonal anti-β1 integrin antibody (3818, a gift from Dr. K. Yamada, Lab of Molecular Biology, NCI, Bethesda, MD). The polyclonal antibody was in limited supply and produced a higher background during immunohistochemical procedures than the monoclonal anti-β1 antibody and, therefore, was rarely used on tissue sections. Anti-talin, 8d4 (1:50), and anti-vinculin, hVIN-1 (1:100) were purchased from Sigma; anti-paxillin, clone 439 (1:100), was purchased from Transduction Laboratory; anti-phosphoryrosine monoclonal antibody, 4G10 (1:200), was purchased from Upstate Biotechnology, Inc. Anti-GFAP, G-A-5, was obtained from Upstate Biotechnology, Inc. Anti-paxillin, clone 439 (1:100), was purchased from Sigma; anti-paxillin, clone 439 (1:100), was purchased from Transduction Laboratory; anti-phosphoryrosine monoclonal antibody, 4G10 (1:200), was purchased from Upstate Biotechnology, Inc. Anti-GFAP, G-A-5, was purchased from ICN Immunobiologics (Costa Mesa, CA). Goat anti-mouse IgG secondary antibodies conjugated with FITC or Alexa 488 (diluted 1:200 in blocking solution) were purchased from Southern Biotechnology (Birmingham, AL) or Molecular Probes.

Analysis of Fluorescence Images

Tissue sections or cultured cells were examined by using a Nikon Microphot-FXA photomicroscope (Nikon, Inc. Garden City, NY) -equipped with epifluorescence. Images were captured with a Kodak Megaplus CCD camera connected to a Perceptrics Megagrabbler framegrabber in a Macintosh 8100/80 AV computer (Apple Computer, Cupertino, CA) using NIH Image 1.58 VDM software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Analysis of double-labeled sections was performed on a Leica TCS-NT confocal scanning laser microscope (Leica Microsystems, Inc., Exton, PA). Figures were prepared on an iMac (Apple, power PC G3) using Adobe Photoshop version 4.0 and Macromedia Freehand Version 9 for Macintosh. Outputs were generated on a Tectronix Phaser continuous tone, color printer (Tectronix, Beaverton, OR).

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REFERENCES


Focal Adhesion Proteins in Developing Retina

553


