

**The role of integrin mediated adhesion and signaling
during *Xenopus* retinal development**

by

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Dedicated to my son,

Runbo

and daughter,

Lisa Hebo.

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ABSTRACT

Integrins are the major family of cell adhesion receptors for extracellular matrix (ECM) components and are involved in cell-cell and cell-ECM adhesion. Integrins also participate in cytoskeletal rearrangements, activation of signal transductions, and co-regulation of growth factor activities.

In this dissertation, I have investigated the role of β_1 integrin-mediated adhesion and signaling during early *Xenopus* retinal development. First, I characterized the expression patterns of focal adhesion associated proteins, β_1 integrin, talin, vinculin, paxillin and phosphotyrosine in retinal glial cell line XR1 and in the developing *Xenopus* retina. These proteins are colocalized at focal adhesions located at the termini of F-actin filaments in XR1 cells. These proteins display spatial and temporal expression patterns, which are related with specific morphologies during retinal development. The similar and differential expression patterns suggest that focal adhesion proteins are involved in integrin-mediated adhesion and signaling, and are likely to be essential in regulating retinal morphogenesis. Second, I tested the hypothesis that protein tyrosine kinase (PTK) activity is essential for *Xenopus* retinal development. Inhibition of tyrosine kinase activity blocked focal adhesion assembly in cultures of dissociated retinal neuroepithelial cells and XR1 glial cells. Application of PTK inhibitors to embryonic retina disrupted retinal lamination and photoreceptor morphogenesis. The expression patterns of phosphotyrosine and β_1 integrins were also disrupted. Finally, I directly blocked integrin-ECM interactions with a disintegrin echistatin and observed the effects of the blockage on the retinal development. Echistatin inhibited XR1 glial cell attachment to fibronectin substrates, and also blocked cell spreading and focal adhesion assembly in XR1 cells. In addition, echistatin reduced the tyrosine phosphorylation levels of paxillin. Furthermore, application of echistatin to embryonic retina disrupted the retinal lamination and induced rosette structures with ectopic photoreceptors in the outer retina.

Taken together, these results provide evidence that integrins are likely to play an important role in selective cell-ECM interactions, cell migration, differentiation and neurite outgrowth during retinal development. Focal adhesion associated proteins and tyrosine kinase activities are involved in the modulation of integrin-mediated adhesion and signal transduction pathways. This research contributes to our understanding of the functions and mechanisms of integrin-mediated adhesion during neural development.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Animal development begins with fertilization of an oocyte by a sperm and establishes the primary organization pattern of embryo through cleavage, gastrulation and neurulation. Subsequently, organogenesis and differentiation lead to the formation of tissues and organs. During these major steps of embryogenesis, the cell associations, attachment to basement membranes, migrations and projection of neurons all require selective adhesion of cells to one another and to the extracellular matrix (ECM). Integrins are the major family of cell adhesion receptors for ECM components and are involved in cell-cell and cell-ECM adhesion. Integrins also participate in cytoskeletal rearrangements, activation of signal transductions, and co-regulation of growth factor activities, and are key molecules to regulate early development of both invertebrates and vertebrates.

The vertebrate retina, with limited cell types and well organized laminar structure, is an excellent accessible model system to study the development of central nervous system as well as the cell-cell and cell-ECM interactions during neural development. Genetic analyses in mice have been generally unhelpful in identifying integrin functions during retinogenesis, since gene knockout results in lethality before the initiation of retinal development. Amphibian embryos are large and easy to manipulate, can be obtained in large numbers in any seasons and be maintained easily and inexpensively in the laboratory. Thus, *Xenopus laevis* has long served as a paradigm for vertebrate development and has played a major role in many significant discoveries in developmental biology. Some important studies have been carried out concerning the expression patterns of integrins and their ligands during early amphibian embryonic development. Different molecules are expressed with distinct patterns in the developing embryos coincident with the onset of morphogenesis. These patterns and the results of perturbation studies indicate the functional roles of integrins in cell adhesion, ECM assembly,

cell migration during gastrulation and formation of the nervous system. However, little has been known about the expression and function of integrins during *Xenopus* retinal development. In this dissertation, I have investigated the role of integrin-mediated adhesion and signaling during early *Xenopus* retinal development.

First, I characterized the expression patterns of focal adhesion associated proteins, β_1 integrin, talin, vinculin, paxillin and phosphotyrosine in XR1 glial cell line and in the developing *Xenopus* retina. XR1 glial cell line is derived from *Xenopus* retinal neuroepithelium and is a good cell system to investigate the mechanisms of cell-ECM interactions. These proteins are colocalized at focal adhesions located at the termini of F-actin filaments in XR1 cells. Focal adhesions are characteristic of strong adhesion, and are the sites where integrins link the ECM to the cytoskeleton and bi-directionally transmit signal transduction. These proteins display a spatial and temporal expression pattern and are related with certain morphogenesis during retinal development, providing evidence that focal adhesion proteins are involved in the retinogenesis. Second, integrin mediated adhesion can activate tyrosine kinase activity and increase tyrosine phosphorylation of focal adhesion proteins. Tyrosine phosphorylation also regulates integrin-mediated adhesion, and is a major intercellular signaling event to transduce extracellular signals into cellular responses. Thus, inhibition of tyrosine kinase activity is supposed to block integrin-mediated signaling and disrupts integrin-ECM adhesion. I tested the hypothesis that tyrosine kinase activity is essential for *Xenopus* retinal differentiation and morphogenesis. Finally, I directly blocked integrin-ECM interactions with a disintegrin (or integrin antagonist) echistatin and tested the effects of the blockage on focal adhesion formation and integrin signaling as well as the effects on the retinal development. The dissertation provides evidence that focal adhesion associated proteins and tyrosine kinase activity are involved in the modulation of integrin-mediated adhesion and signal transduction pathways, and integrin-mediated adhesion and signaling play a critical role in retinal cell adhesion, migration, differentiation and synaptogenesis during retinal development.

Dissertation Organization

This dissertation comprises a general introduction, three manuscripts, and a general conclusion. The literature review following immediately includes descriptions of adhesion molecules in the embryonic development, integrin-mediated adhesion and signaling, and integrin functions during retinal development. The regulation of integrin-mediated adhesion and the interactions of multiple signal transduction pathways are also discussed. The three manuscripts for different journals are modified to conform to the requirements of the Iowa State University thesis office. I conceived the idea of investigating the role of integrins during the retinal development and designed the experiments under the direction of Dr. Don Sakaguchi. I am principally involved in the data collection and analysis, and the writing of the papers. The first paper, "Expression patterns of focal adhesion associated proteins in the development retina", documents the distribution patterns of β_1 integrin, talin, vinculin, paxillin and phosphotyrosine and their relationships in the retinal glial cell line XR1 and in the sections of *Xenopus* retina of different stages. The results suggest that focal adhesion proteins are involved in integrin mediated adhesion and signaling, and play a role in cell adhesion, migration and synaptogenesis during the retinal development. The second manuscript, "The role of tyrosine kinase activity during early retinal development", describes the effects of tyrosine kinase inhibitors on focal adhesion assembly in cultures of retinal neuroepithelial cells and XR1 retinal glial cells as well as on the early retinal development. Tyrosine kinase inhibitors block focal adhesion formation and disrupt retinal lamination and photoreceptor morphogenesis. The results indicate that the tyrosine kinase activity modulates integrin-mediated adhesion and is necessary for normal early retinal development. The third manuscript, "Inhibition of integrin mediated adhesion with echistatin disrupts *Xenopus* retinal development", presents the results of the perturbation studies with a disintegrin echistatin, providing direct evidence that integrin mediated adhesion and signaling play a critical role in retinal morphogenesis. The last chapter includes a summary of the results observed in this research, and a recommendation for future study.

Literature Review

Adhesion molecules in development

Cell adhesion molecules. During vertebrate development individual embryonic cells become integrated to form an increasingly complex system through cell-cell and cell-extracellular matrix (ECM) interactions. The simple facts are that the associations of cells in epithelia, their attachment to basement membranes, and the migrations of cells and projections of neurons all require selective adhesion of cells to one another and to ECM. The major families of cell adhesion molecules (CAMs) include integrins, cadherins, the immunoglobulin (Ig) superfamily, selectins and syndecans (Hynes, 1999; Couchman et al., 2001). Over the last decade, it has become apparent that adhesion molecules play far more than structural role. These cellular interactions with neighboring cells and the ECM profoundly influence a variety of signaling events involved proliferation, survival and differentiation (Aplin et al., 1998; Giancotti and Ruoslahti, 1999). I briefly discuss cadherin and Ig superfamily adhesion molecules, and then focus on the major ECM receptors, integrins, and integrins' differential expression and functional roles during embryonic development.

Cadherins. Cadherins are Ca^{++} dependent homophilic (like-with-like) adhesive molecules (Angst et al., 2001). About 20 classic cadherins have been identified in vertebrates, such as N, P, R, B and E cadherins (Angst et al., 2001). Their extracellular domains contain five characteristic cadherin repeats, each composing a sandwich of β sheets. Through the most distant cadherin repeat cadherins mediate cell-cell interactions. They share homologous cytoplasmic domains that link to the actin cytoskeleton through catenins. Like other adhesion receptors, clustering of cadherins is important for their functions, and multiple dimer-dimer interactions are believed to provide sufficient local avidity to mediate cell-cell adhesion. They are of importance in cell adhesion at the blastula stage (Heasman et al., 1994). Desmosomal cadherins, related to classic cadherins in their extracellular domains, have distinct cytoplasmic

domains that link to intermediate filaments (Green and Gaudry, 2000). Protocadherins, encoded by complex genetic loci, have 6 cadherin repeats and are important in the development of the nervous system (Kohmura et al., 1998; Wu and Maniatis, 1999).

Immunoglobulin superfamily of CAMs. Ig-CAMs are characterized with the presence of varying numbers of Ig related domains, which are sandwiches of two β sheets held together by hydrophobic interactions (Walsh and Doherty, 1997). It has fibronectin type III domains, which occur in ECM components, such as fibronectin and tenascin. The Ig superfamily is diverse, with more than 100 members, such as NCAM, L1, Ng CAM, fasciclin II (Crossin and Krushel, 2000). Little is known about the interactions of Ig CAMs with cytoplasmic proteins except that ankyrin may link L1 with actin (Crossin and Krushel, 2000). Different Ig-CAM members participate in homophilic interactions, as well as heterophilic interactions with other members, ECM molecules or integrins (Tessier-Lavigne and Goodman, 2000). The Ig CAMs function in a variety of cell types and many biological processes. They play important roles in axon guidance and synaptogenesis during neural development (Johansen and Johansen, 1997; Murase and Schuman, 1999).

Selectins and syndecans. Selectins, such as L-, E- and P-selectins, bind their counter-receptors and play a role in the adhesion of leukocytes to endothelium with cooperation of integrins and Ig-CAMs (Lasky, 1995). Syndecans are a family of cell surface proteoglycans, can bind collagen and fibronectin through their heparan sulfate side chains, and are involved in cell adhesion (Echtermeyer et al., 1999; Saoncella et al., 1999).

Substrate adhesion molecules. Integrins primarily act as the receptors for ECM components and are the best known ECM receptors (Hynes, 1992). Dystroglycan is a cell surface receptor for several ECM molecules including laminin, agrin and perlecan and plays a role in the formation of basement membrane during development (Williamson et al., 1997; Henry et al., 2001). ECM molecules and their cellular receptors, integrins and proteoglycans were collectively called substrate adhesion molecules (SAMs) (Edelman, 1988). Just like

CAMs, SAMs are also morphoregulatory molecules mediating gene activities and morphogenetic events (Edelman, 1988). I discuss the ECM generally, two examples of ECM components, fibronectin and laminin before I discuss their receptors, integrins

Extracellular matrix. The extracellular matrix is the substance which underlies all the epithelia and endothelia, and surrounds all connective tissue cells providing mechanical support and physical strength to tissue, organs and the organism as a whole. Extracellular matrix is composed of proteoglycans, glycosaminoglycans and glycoproteins (Ayad et al., 1992). Proteoglycans are a diverse family of molecules characterized by a core protein to which is attached one or more glycosaminoglycan side-chains. The most abundant glycosaminoglycans include chondroitin sulphate, dermatan sulphate, heparan sulphate, keratan sulphate and hyaluronan. Many of the matrix glycoproteins contain distinct and functionally active peptide domains that prescribe interactions with cell surface receptors as well as other matrix molecules. A number of these molecules have been described as adhesive glycoproteins, including collagen, laminin, fibronectin, vitronectin, thrombospondin and von Willebrand factor. The ECM provides structural support for cells, but also acts as a physical barrier or a selective filter for soluble molecules. Furthermore, the ECM sequesters growth factors, metalloproteinases and plasma proteins like albumin. During embryogenesis, ECM molecules play vital roles in a number of morphogenetic processes including cell proliferation, differentiation, adhesion, migration, and cell death (Yamada, 1983; Kalthoff, 1996).

Fibronectin. Fibronectin is widely distributed at high concentration in most extracellular matrices, and consists of two polypeptides linked near their carboxyl termini by a pair of disulfide bonds. Each polypeptide is folded into several globular domains, which have binding sites for cells, for collagen, heparin, and other ECM components (Yamada, 1991). Different variants of fibronectin that differ in solubility originate from alternative splicing of pre-mRNA. The cell binding activity depends primarily on a tripeptide sequence, the arginine-glycine-asparagine (RGD) sequence recognized by most adherent cells via the integrin

receptors. Fibronectin plays a major role in cell adhesion, migration, and neurite outgrowth (Hall et al., 1987; Yamada et al., 1991; Gumbiner, 1996).

Laminin. Laminin is plentiful in basal laminae, and consists of three polypeptides arranged in the shape of a cross and held together by disulfide bridges (Martin and Timpl, 1987; Sanes et al., 1990). The component polypeptides, α , β , and γ , are encoded by small family of different genes. Five α , three β , and three γ chains have been identified to date and these chains form at least 15 laminin isoforms (Luckenbill-Edds, 1997; Libby et al., 2000, see Table 1). Laminin-1 (consisting of α_1 , β_1 , and γ_1); laminin-2 or merosin (α_2 , β_1 , and γ_1), laminin-3 or S-laminin (α_1 , β_2 , and γ_1) are well known laminins. Like fibronectin, laminin has several domains for other ECM components, two for type IV collagen, one for heparin, and more than two cell binding regions in the center. These multiple binding sites enable these glycoproteins to crosslink other ECM elements and cells into three-dimensional meshworks. Laminin promotes adhesions of many cell types and the extension of neurites from nerve cells.

Table 1. Known laminin isoforms

	Composition	Previous names		Composition	
Laminin-1	$\alpha_1\beta_1\gamma_1$	EHS laminin	Laminin-9	$\alpha_4\beta_2\gamma_1$	
Laminin-2	$\alpha_2\beta_1\gamma_1$	Merosin	Laminin-10	$\alpha_5\beta_1\gamma_1$	
Laminin-3	$\alpha_1\beta_2\gamma_1$	s-Laminin	Laminin-11	$\alpha_5\beta_2\gamma_1$	
Laminin-4	$\alpha_2\beta_2\gamma_1$	Merosin, s-merosin	Laminin-12	$\alpha_2\beta_1\gamma_3$	
Laminin-5	$\alpha_3\beta_3\gamma_2$	Kalinin, nicein	Laminin-13	$\alpha_3\beta_2\gamma_3$	
Laminin-6	$\alpha_3\beta_1\gamma_1$	k-Laminin	Laminin-14	$\alpha_4\beta_2\gamma_3$	
Laminin-7	$\alpha_3\beta_2\gamma_1$	Ks-Laminin	Laminin-15	$\alpha_5\beta_2\gamma_3$	
Laminin-8	$\alpha_4\beta_1\gamma_1$				

Modified from Libby et al., (2000). Laminin 8 -15 have no previous names.

Integrins. The complexity of the ECM is mirrored by a heterogeneous array of cell surface receptors. The integrin family members are the major membrane receptors for ECM components and mediate cell-matrix and cell-cell interactions. Integrins play an important role in cell adhesion, migration, proliferation, cell survival and differentiation during embryonic development (Howe et al., 1998). Integrins are transmembranal glycoproteins, consisting of one α and one β subunit that associate noncovalently to form a functional receptor. 18 α subunits, 8 β subunits and a total of 24 integrin heterodimers have been identified in vertebrates (van der Flier and Sonnenberg, 2001, see Table 2). In a recent survey of the human genome, 24 α subunits, 9 β subunits may exist, which means 6 novel α and 1 novel β subunits (Venter et al., 2001). The α/β associations determine the ligand binding specificity of integrin heterodimers for various ECM molecules (Hynes, 1992; van der Flier and Sonnenberg, 2001). $\alpha_3\beta_1$ integrin interacts only with a single ECM protein, fibronectin, in RGD dependent manner; while $\alpha_6\beta_1$ is specific for different isoforms of laminin. An individual integrin may recognize several distinct ECM components, such as $\alpha_1\beta_1$ with collagen and laminin. An individual ECM component can also bind with multiple integrin receptors. For examples, integrins with α_1 , α_2 , α_3 , α_6 , or α_7 subunits are laminin-binding receptors, while integrins with α_1 , α_2 , α_{10} , or α_{11} subunits bind collagens. Integrins containing the α_4 , α_5 , α_8 , α_{11b} , or α_v subunits bind to ECM components that contain the RGD sequence, such as fibronectin and vitronectin. Laminins and collagens also contain RGD sequences, but these are normally cryptic and inaccessible. The specific cell types and environment, such as Mg^{++} and Ca^{++} concentration also determine the binding specificity and affinity. $\alpha_2\beta_1$ integrin bind to collagen not to laminin on platelets, but binds to both on other cell types. β_1 integrins are the largest subfamily since β_1 can dimerize with 12 different α subunits (See Table 2). The complexity of the β_1 integrin family is further increased with the alternative splicing of mRNAs. In humans 4 β_1 isoforms with different cytoplasmic domains were found (van der Flier et al., 1995; Zhidkova et al., 1995; Belkin et al., 1996).

Table 2. Integrin receptors and ligands.

Integrins	Ligands	Integrins	Ligands
$\alpha 1\beta 1$	Collagen, laminin	$\alpha L\beta 2$	ICAM-1, ICAM-2, ICAM-3
$\alpha 2\beta 1$	Collagen, laminin, tenascin (Tn)	$\alpha M\beta 2$	Fibrinogen, C3b, ICAM-1
$\alpha 3\beta 1$	Laminin, collagen, fibronectin, Tsp	$\alpha X\beta 2$	Fibrinogen, C3b
$\alpha 4\beta 1$	Fibronectin (Fn), osteopontin (Op)	$\alpha D\beta 2$	ICAM-1, VCAM-1
$\alpha 5\beta 1$	Fibronectin, MAdCAM	$\alpha IIb\beta 3$	Vitronectin, Fn, vWF, Tsp
$\alpha 6\beta 1$	Laminin	$\alpha v\beta 3$	Fibronectin, Op, Tn, Fn, vWF
$\alpha 7\beta 1$	Laminin	$\alpha 6\beta 4$	Laminin
$\alpha 8\beta 1$	Fibronectin, vitronectin, Tn	$\alpha v\beta 5$	Vitronectin, fibronectin, Op
$\alpha 9\beta 1$	Collagen I, laminin, Op, Tn	$\alpha v\beta 6$	Fibronectin, tenascin
$\alpha 10\beta 1$	Collagen	$\alpha 4\beta 7$	Fibronectin, VCAM-1
$\alpha 11\beta 1$	Collagen	$\alpha IEL\beta 7$	E-cadherin
$\alpha v\beta 1$	Vitronectin, fibronectin, Op	$\alpha v\beta 8$	Fibronectin, vitronectin

C3b, complement component; ICAM, intercellular adhesion molecule; MAdCAM, mucosa addressin cell adhesion molecule; Tsp, thrombospondin; VCAM, vascular cell adhesion molecule; vWF, von Willebrand factor. Modified from Darribere et al. (2000), and van der Flier and Sonnenberg (2001).

Unlike others CAMs, integrins are heterodimers and predominantly binding ECM molecules. A few also play important roles in heterotypic cell adhesion, binding counter-receptors of the Ig-CAMs (ICAMs, VCAM-1, MAdCAM-1) or in one case, a cadherin ($\alpha E\beta 7$ -E-cadherin) (van der Flier and Sonnenberg, 2001).

The ligand-binding site of integrins is present in the globular head domain formed by the α and β subunits, while the rest of the extracellular domain forms the membrane-proximal stalk-like part of the receptor. There are divalent cation binding sites on the lower surface of the globular domain, and the metal ion dependent adhesion site motif is critical for ligand binding (Kanazashi et al., 1997; Plow et al., 2000). Except β_4 , the cytoplasmic tails are conservative and small, about 30 to 50 amino acids long without catalytic motifs. The cytoplasmic domain can be connected to microfilament bundles inside the cell, via linker proteins such as talin, α -actinin

and vinculin. Integrins transmit mechanical stress to cytoskeleton and also mediate transmembranal chemical signaling, and they may interact, coordinate and integrate between different signaling pathways in order to induce new gene expression and then regulate cellular processes (Chicurel et al., 1998).

Differential expression of Integrins in the developing embryos. Amphibian is a classic model system to study development, and is also a good model system to study cell-ECM interactions. Some important studies have been carried out concerning integrin expression patterns during early amphibian embryonic development: β_1 (Gawantka et al., 1992); β_1 , β_2 , β_3 , and β_6 (Ransom et al., 1993); α_2 and α_3 , (Meng et al., 1997); α_4 (Whittaker and Desimone, 1998); α_5 (Joos et al., 1995); α_6 (Lallier et al., 1996); α_v (Alfandari et al., 1995; Joos et al., 1998). Multiple integrins are expressed at early stages of *Xenopus* development coincident with the onset of morphogenesis. For examples, the β_1 mRNA is maternally provided, and mature β_1 integrin subunit is inserted into the newly formed plasma membranes of all cells during cleavage. The levels of β_1 integrin subunit rise gradually, and are most abundant in ectoderm and mesoderm during gastrulation (Gawantka et al., 1992; Ransom et al., 1993). Integrin α_5 mRNA and protein are also expressed in oocytes, eggs and throughout development; during neurulation, the α_5 subunit disappears from the outer layer of ectoderm, the notochord and the neural tube, but accumulates in the sensorial layer of the ectoderm, the somites and the neural crest cells (Joos et al., 1995). Furthermore, like their surface receptors, ECM proteins are also regulated differentially and related with specific morphological events during embryonic development (Krotoski and Bronner-Fraser, 1990; Yost, 1992). These results suggest the functional roles of integrins during embryonic development.

Integrin functions during development. Studies with anti-functional antibodies, peptide inhibitors of integrin-ligand interactions and genetic mutations demonstrate that integrins are key regulators of embryogenesis (Darribere et al., 2000). At gastrulation, mesoderm founder cells involute, ingress and then actively spread and migrate to the future anterior part of the

embryo. Injection of RGD containing peptides and antibodies directed against β_1 integrin subunit in the blastocoel affects gastrulation movements. Injection of antibodies against the $\alpha_5\beta_1$ integrin or against α_v subunit also disturbs gastrulation movements (Skalski et al., 1998). In addition, injection of a full-length α_3 transcript into cells that normally do not express results in embryonic defects in paraxial mesoderm, suggesting that a normal position-specific expression of integrins is important to maintain the proper organization of tissues during gastrulation (Meng et al., 1997). Furthermore, it has been shown that activin-induced changes are correlated with an up-regulation of α_3 , α_4 and α_6 transcripts in animal cap cells, suggesting that integrin binding to ECM depends on mesoderm inducing factors (Whittaker and DeSimone, 1993). With antisense integrin sequences, the two integrin α_6 and α_8 subunits have been shown to be involved in migration or lamination and neuronal survival respectively in the developing chick optic tectum (Zhang and Galileo, 1998). Taken together, the perturbation experiments with integrin antibodies, RGD containing peptides or integrin oligonucleotides demonstrate the roles of integrins during development

Genetic analysis provides direct evidence that integrins are important regulators of embryogenesis (Hynes, 1996; Darribere et al., 2000). The analysis of β_1 integrin function indicates that β_1 integrins play vital biological roles in early development, differentiation and migration, hematopoiesis, tumorigenesis, and supramolecular assembly of extracellular matrix proteins (Brakebusch et al., 1997). Knockout of α_1 only leads to subtle imperfections (Gardner et al., 1996), but knockout of α_5 causes mesodermal defects and cranial neural crest apoptosis (Goh et al., 1997). The lack of α_6 subunit results in embryonic defects and death around the time of birth due to severe blistering of the skin (Georges-Labouesse et al., 1998). α_3 , α_6 single and double knockout studies demonstrate the compensation of integrin function in apical ectodermal ridge formation and organogenesis (De Arcangelis et al., 1999). Furthermore, genetic analysis of integrin function in *C. elegans* and *Drosophila* provides evidence that

integrin are involved in neuronal migration, axon guidance, fasciculation and synaptic plasticity (Baum and Garriga, 1997; Hoang and Chiba, 1998; Rohrbough et al., 2000).

Integrin mediated adhesions and signaling

Focal adhesions. Recent studies have provided a better understanding of the signaling pathways activated by integrins in adherent cells (Clark and Brugge, 1995; Howe et al., 1998). Integrin receptor binding with ECM molecules results in a cascade of events within the cytoplasm, including phosphorylation of proteins and the recruitment of cytoskeletal proteins that leads to formation of focal adhesions at the ventral surface of cultured cells (Craig and Johnson, 1996). Focal adhesions, characteristic of strong adhesion, are the sites where integrins link the ECM with the cytoskeleton. Focal adhesions consist of the clustered integrins and associated proteins, referred to as focal adhesion proteins (Fig. 1). These proteins include talin, vinculin, α -actinin, tensin, paxillin, focal adhesion kinase (FAK) and other phosphotyrosine proteins that mediate cell adhesion and signaling. Some of the proteins serve a structure role, and are cytoskeletal proteins that link the integrin receptors and F-actin stress fibers, while others are regulatory proteins involved in signaling (Hynes, 1992; Howe et al., 1998). Integrin-ECM binding induces phosphorylation events at focal adhesions by members of the Src protein kinase family, which leads to the recruitment and activation of downstream signaling molecules, such as Rho GTPase, extracellular regulatory kinase (ERK) and Jun N-terminal kinase (JNK) signaling cascades (Schlaepfer et al., 1998; Aplin and Juliano, 1999). Paxillin, FAK and p130^{CAS}, a CT-10-regulated kinase (Crk) associated substrate, are among the regulatory molecules with increased tyrosine phosphorylation at focal adhesions. We have characterized the expression patterns of focal adhesion proteins, β_1 integrin, talin, vinculin, paxillin and phosphotyrosine in the XR1 retinal glial cells and in developing *Xenopus* retina (Li and Sakaguchi, 2002). These proteins display co-distribution and differential expression during retinal development. I will discuss some focal adhesion associated proteins, talin, vinculin,

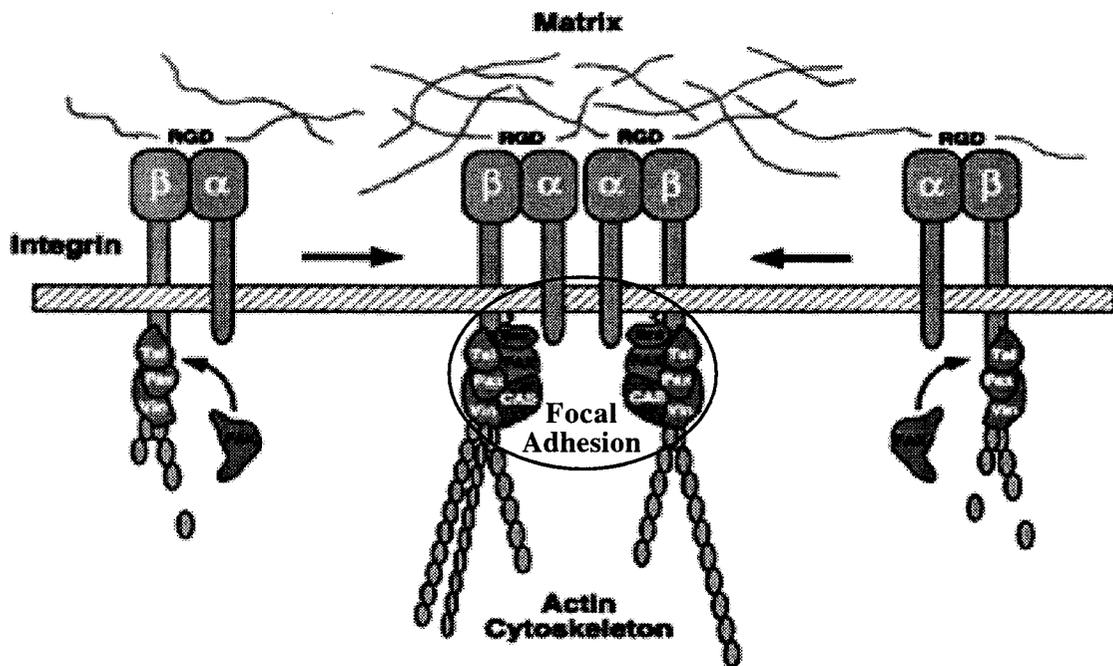


Figure 1. Ligand binding leads integrin clustering and formation of focal adhesions and actin stress fibers. RGD, Arg-Gly-Asp integrin-binding motif; Tal, talin; Vin, vinculin; Pax, paxillin; CAS, p130^{CAS}; FAK, focal adhesion kinase. Modified from Giancotti and Ruoslahti (1999).

paxillin and FAK, then discuss the integrin mediated signaling and the regulation of integrin-mediated adhesion.

Talin and vinculin. Integrin binding with the ECM regulates cytoskeletal organization and focal adhesions. Talin can bind to the cytoplasmic tail of β integrin subunits as a consequence of integrin-ligand engagement, and contributes to the formation of focal adhesions (Critchley et al., 1999). Talin has at least two actin-binding sites, and three binding sites for cytoskeletal protein, 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). The reduction in talin expression in HeLa cells using an antisense RNA strategy prevented cell spreading, and the formation of focal adhesions and stress fibers (Albiges-Rizo et al., 1995). Injection of anti-talin antibody into chicken embryonic dermal fibroblasts inhibited the spreading and migration of the fibroblasts (Nuckolls et al., 1992). Talin (-/-) mouse embryonic

stem cells were unable to assemble focal adhesions when plated on fibronectin, whereas vinculin (-/-) ES cells were able to do so (Critchley et al., 1999). In addition, in F9 embryonic carcinoma cells, vinculin deficiency might not inhibit focal adhesion formation, but reduced cell spreading (Goldmann et al., 1995; Volberg et al., 1995). Furthermore, phosphatidylinositol-4,5-bisphosphate (PIP₂) can dissociate vinculin's head-tail (N- and C-terminal domains) interaction, demasking its binding sites for talin and actin; injection of PIP₂ antibody inhibits assembly of focal adhesions and stress fibers (Gilmore and Burridge, 1996). These results indicate that talin and vinculin are important and may play distinct roles in the assembly of focal adhesions.

Paxillin. Paxillin can bind structural and signaling molecules and functions as a multi-domain adapter molecule (Turner, 2000). The Lin-11, Isl-1 and Mec-3 (LIM) 3 or LIM2 domain at the C-terminus appears involved in localizing paxillin to focal adhesions by direct association with integrin tails or through an intermediate protein (Schaller et al., 1995). LIM 3 can bind C-terminal Src kinase (CSK), an inhibitor of Src activity, and protein tyrosine phosphatase-PEST, which may dephosphorylate p130^{CAS} and then regulate focal adhesion assembly and cell migration. Paxillin leucine-aspartate repeat (LD) motifs at the N-terminal half can bind vinculin and actopaxin that can bind actin or other cytoskeletal proteins, such as α -actinin, spectrin and fimbrin (Nikolopoulos and Turner, 2000). 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. Phosphorylation of paxillin by these kinases permits its binding with downstream effector molecules such as Crk, which associates with CAS and transduces external signals into changes in cell motility, and regulates gene expression via mitogen-activated protein kinase (MAPK) cascades (Cary and Guan, 1999). Furthermore, paxillin have recently been shown to associate with integrin-linked kinase and p21-activated kinase (Hashimoto et al., 2001; Nikolopoulos and Turner, 2001). Taken together, these data indicate that paxillin play a critical role in integrin mediated signaling and the coordination of adhesion signal pathways and growth factor receptor signal pathways.

Focal adhesion kinase. Focal adhesion kinase, a cytosolic protein tyrosine kinase, was first detected at focal adhesions (Schaller et al., 1992). Focal adhesion kinase is expressed in a variety of species, including *Xenopus* (Hens and DeSimone, 1995; Zhang and Galileo, 1998), chick (Schaller et al., 1992), rodent (Hanks et al., 1992) and human (Weiner et al., 1993), indicating that it is evolutionarily conserved. Focal adhesion kinase contains a central protein tyrosine kinases (PTK) domain, which is flanked by regions showing no homology to previously identified proteins (Cary and Guan, 1999). Unlike many other cytosolic PTKs, FAK does not have a src homology (SH) 2 or SH3 domain, but it does have the SH2 and SH3 domain-interacting phosphotyrosines and proline-rich regions, respectively. The C-terminal domain is the paxillin binding sequence, which overlaps with the focal adhesion targeting sequence and talin binding sequence; The N-terminal domain binds integrin β subunits *in vitro*. Knockout mice that lack FAK or fibronectin display a very similar phenotype with defects in mesoderm formation at late gastrulation, highlighting the role of FAK in transducing signals from integrins (Furuta et al., 1995; George EL, Hynes, 1993). Evidence is accumulated and indicates that focal adhesion kinase plays an important role in early embryogenesis by mediating integrin signal pathways (Ridyard and Sanders, 1998). A novel PTK of the FAK subfamily has been identified, called proline-rich tyrosine kinase-2 (Pyk2), or related adhesion focal tyrosine kinase (RAFTK), or cell adhesion kinase β (CAK β) (Avraham et al., 1995; Lev et al., 1995; Sasaki et al., 1995).

Integrin-mediated signaling. Focal adhesion kinase is activated by most integrins. How it is activated is not well understood, but FAK can bind directly or through talin and paxillin, with cytoplasmic tail of β_1 integrin subunits (Cary and Guan, 1999). Upon activation, FAK autophosphorylates Tyr³⁹⁷, creating a binding site for the Src homology 2 domain of Src (Giancotti and Ruoslahti, 1999). The FAK/Src complex then phosphorylates a number of downstream substrates, promoting the signaling cascades. The phosphorylation of focal adhesion components, paxillin and tensin, regulates the dynamics of focal adhesions and cell

spreading. Phosphorylation of a docking protein, p130^{CAS}, which recruits Crk, activates the JNK family of MAPK. Finally, FAK also combines with, and may activate phosphoinositide 3-OH kinase (PI 3-kinase), which blocks the apoptosis through Akt. Src phosphorylates FAK Tyr⁹²⁵, creating a bind site for the complex of the growth factor receptor-bound protein-2 (Grb2) and Ras guanosine triphosphate exchange factor mSOS. The interaction activates the Ras/MAPK pathway. The Ras/MAPK pathway can also be activated by some integrin subunits such as β_1 and α_v through membrane associated protein caveolin and adapter protein, Shc, independent of FAK (Barberis et al., 2000). The Ras/MAPK pathway is also activated by growth factors (Mulder, 2000). Integrin-linked kinase, a ubiquitously expressed protein serine/threonine kinase, is located to focal adhesions and also involved in intracellular signal transduction (Li et al., 1999).

Interactions of multiple signal pathways. In addition to the MAPK members, Rho GTPases and phospholipase-C γ (PLC γ) are activated by integrin mediated-adhesion (Schlaepfer and Hunter, 1998; Aplin and Juliano, 1999). The Rho family of GTPases plays important role in cytoskeletal rearrangements (Hall, 1998). RhA promotes the formation and maintenance of stress fibers, whereas Rac and CDC 42 regulate cortical actin structures such as lamellipodia and filopodia (Nobes and Hall, 1995; Kjoller and Hall, 1999). Most significantly, integrin-cytoskeletal complexes can modulate the receptor tyrosine kinase (RTK)/Ras MAPK cascades at several levels, such as at RTKs, the linkage of Ras/Raf, and the traffic of ERK to the nucleus (Miyamoto et al., 1996; Sundberg and Rubin, 1996). Furthermore, integrin-cytoskeletal complexes also modulate signaling through G protein-coupled receptors and cytokine receptors (Rozenfurt, 1998; Slack, 1998; Frazier et al., 1999). In addition to adhesion receptors, a variety of stimuli can also induce tyrosine phosphorylation of FAK, through either specific surface receptors or intracellular molecules, such as PKC or Rho (Rodriguez-Fernandez, 1999). It was suggested that adhesion receptor clustering may be a common mechanism by which distinct factors may modulate tyrosine phosphorylation of FAK

(Rodriguez-Fernandez, 1999). Thus, integrin mediated signaling can regulate gene expression, cell cycle, and cell survival, through cooperation with other signaling pathways.

Regulation of integrin-mediated adhesion. The cytoskeleton and focal adhesions or signaling complexes are modified and strengthened by multiple interactions, which make it difficult to define the regulation of crucial primary interactions in integrin-mediated signaling (Yamada and Geiger, 1997). Tyrosine phosphorylation, PKC, Rho GTPases in the intracellular signaling pathways would modulate integrin affinity through the inside-out signaling (Hughes and Pfaff, 1998). In addition, the interactions of cytoplasmic tails with cytoskeletal and regulatory proteins as well as lateral associations with other transmembrane proteins influence ligand-binding affinity. Certain cations, such as Mn^{++} , and antibodies may activate integrins by stabilizing or inducing their active conformation (Ivins et al., 2000). Another special example is that $\alpha_5\beta_1$ integrin binds the RGD motif on fibronectin, while at the same time a heparan sulfate proteoglycan syndecan-4 binds the two heparin domains on the fibronectin. This suggests that integrins and syndecan cooperate to transduce signals for the assembly of focal adhesions and actin stress fibers in cells plated on fibronectin (Echtermeyer et al., 1999; Saoncella et al., 1999).

Tyrosine kinase activity with focal adhesions. There is no doubt that tyrosine kinase activity is important in mediating focal adhesion formation. Inhibition studies indicate that tyrosine kinase activity is required for both focal adhesion formation and cytoskeletal rearrangement in fibroblasts, endothelial cells and thyroid cells (Burrige et al., 1992; Romer et al., 1994; Yap et al., 1994). In our lab, actin polymerization, actin-myosin interactions or tyrosine kinase activity are shown to be involved in regulating focal adhesion formation in glial cells, since the inhibitors of the above activities block the assembly of focal adhesions (Folsom and Sakaguchi, 1997, 1999). Phosphorylation of FAK is correlated with focal adhesion formation and PTK inhibitors reduce the formation of focal adhesions and stress fibers, suggesting a function of FAK in the assembly of focal adhesions (Clark and Brugge, 1995; Miyamoto et al., 1995). However, cultured cells with FAK^{-/-} show little or no differences in

adhesion to fibronectin (Ilic et al., 1996). The decrease of cell motility suggests that FAK may play a role in the turnover of focal adhesions during cell migration (Ilic et al., 1995).

Other cell-ECM adhesion. Cell migration requires the precise regulation of integrin-mediated adhesion and release (Lauffenburger and Horwitz, 1996). In addition to focal adhesions that are observed in all kinds of non-neuronal cells, point adhesions or point contacts are observed in neurons, and may regulate adhesion and migration of growth cone (Arregui et al., 1994). The structure and regulation of point contacts may be different from focal adhesions. The components of point contacts are most likely different from the components in focal adhesions (Tawil et al., 1993). It is suggested that the process of cellular de-adhesion is also important for cells to participate in morphogenesis and that there most likely exists an adaptive state or intermediate cell adhesion between cell attachment and strong adhesion (Murphy-Ullrich, 2001). Surely, there exists another class of ECM proteins, such as thrombospondins, tenascins and SPARC (secreted protein, acidic and rich in cysteine), which modulate cell-matrix interactions from strong adhesion to intermediate adhesion state (Sage and Bornstein, 1991; Bornstein, 1995).

Disintegrin and metalloproteases. A novel family of extracellular matrix proteases, ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs), have been implicated in mediating cell-ECM adhesion during embryonic development, angiogenesis and wound healing (Steffensen et al., 2001; Tang, 2001). For example, ADAMTS2 is a procollagen N-proteinase; ADAMTS 4 and 5 are aggrecanases. They are different from members of ADAM family that are largely anchored on the cell surface. ADAM molecules (also referred to as MDC proteins) are related to soluble snake venom integrin ligands termed disintegrins (Weskamp and Blobel, 1994) and to snake venom metalloproteases (Bjarnason and Fox, 1995). Metalloproteases from snake venoms, called hemorrhagic toxins, can digest the components of ECM leading to local and systemic hemorrhage. The disintegrin-like domain of snake venom metalloproteases such as alternagin is responsible to inhibit collagen binding to $\alpha_2\beta_1$ integrin,

while an ADAM, jararhagin, can cleave the β_1 subunit of platelet $\alpha_2\beta_1$ integrin (Kamiguti et al., 1997; Souza et al., 2000). ADAMs have been implicated in diverse cellular processes, including fertilization and myoblast fusion, release of TNF α from the plasma membrane, and neurogenesis (Blobel, 1997). For example, four distinct ADAMs have been identified to express in a spatial and temporal pattern in early *Xenopus* development, suggesting a function utilized by different tissues or cells (Cai et al., 1998).

Disintegrins. Disintegrins are small soluble disulfide-rich, RGD containing proteins that potently inhibit platelet aggregation and cell adhesion by interacting with integrins (Gould et al., 1990; Scarborough et al., 1993). More than 30 disintegrins have been isolated and characterized from the venom of many species of snakes. They contain 49 to 84 amino acids and 8 to 14 cysteine residues linked by intramolecular disulfide bonds. The disintegrin family is grouped into two subfamilies comprising monomeric and dimeric proteins. Dimeric disintegrins include both homo- and heterodimers linked by two disulfide bonds. The activity depends on the structure of an RGD containing loop maintained in an appropriate conformation by disulfide bridges. There is evidence that amino acids adjacent to RGD and amino acids in the C-terminal region contribute to the modulation of disintegrin activity (Scarborough et al., 1993; Wright et al., 1993; Marcinkiewicz et al., 1997). Disintegrins are powerful tools to study integrin-mediated adhesion and signaling. For example, contortrostatin, a homodimeric disintegrin, induces $\alpha_v\beta_3$ -mediated tyrosine phosphorylation of CAS and FAK in tumor cells (Ritter et al., 2000). The monomeric disintegrin echistatin from the saw-scaled viper *Echis carinatus* has been shown to induce disassembly of focal adhesions and stress fibers, and to decrease phosphorylation of FAK and paxillin (Staiano et al., 1997; Della Morte et al., 2000).

The expression and function of Integrins during Retinal development

Evidence has accumulated that integrins play an essential role in neuronal cell differentiation, migration, cell positioning, neurite outgrowth and axon pathfinding (Bradshaw et

al., 1995; Baum and Garriga, 1997; Georges-Labouesse et al., 1998; Jacques et al., 1998). The vertebrate retina, with accessibility and its limited number of cell types organized in a stereotypical laminar pattern, is an excellent model system for developmental studies of the central nervous system (CNS). The retina is also a good model system to study the cell-cell and cell-ECM interactions. The retinal structure and development are discussed before discussing the important role of integrin-ECM interactions involved in all the cellular processes during retinal development. The involvement of multiple signaling pathways and tyrosine kinase activity in retinal development are also discussed.

Retina. Retina is a complex multi-laminar tissue with 5 different neuronal cell types, 2 non-neuronal glial cells and retinal pigment epithelium (RPE). Neuroepithelium in the neural tube grows out to form optic vesicles, and the distant part of the optic vesicle invaginates to form the optic cup in which the monolayered RPE contacts the neural retina. Neuroretinal cells differentiate and migrate from the germinal neuroepithelial cell layer along the ventricular border of the retina to the appropriate cellular layer (Dowling, 1970; Wetts and Fraser, 1988). The mature retina is composed of ganglion cells in the ganglion cell layer (GCL), amacrine, horizontal, bipolar cells and Müller glia in the inner nuclear layer (INL), and cone and rod photoreceptors in the outer nuclear layer (ONL). Between these three cellular layers are the synapses, inner plexiform layer (IPL) and outer plexiform layer (OPL), respectively. Figure 2 shows the retina structure of stage 42 *Xenopus* tadpole.

Retinogenesis. Histogenesis and morphogenesis of the retina is closely similar in the vertebrates except the time period. The retina is an outgrowth of the neural tube, initially follows the basic pattern of histogenesis in the vertebrate CNS, and at later stage, takes a different pattern from CNS (Angevine, 1970). The primitive optic vesicle consists of pseudostratified neuroepithelial stem cells, or ventricular cells, which are jointed together at both outer limiting membrane (OLM) and inner limiting membrane (ILM) (Sheffield and Fischman, 1970). During mitotic division, ventricular cells release their attachment to the ILM, and round

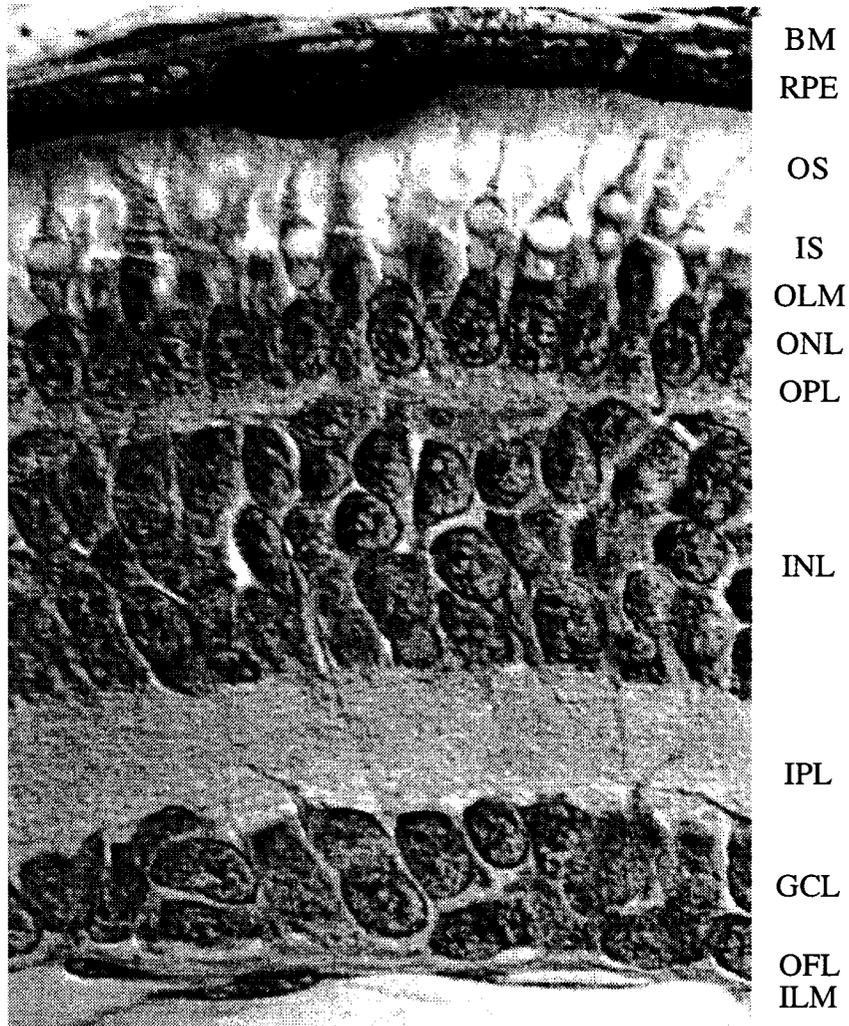


Figure 2. Retinal structure of stage 42 tadpole. BM, Bruch's membrane; RPE, retinal pigment epithelium; OS, outer segments of photoreceptors; IS, inner segments of photoreceptors; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; OFL, optic fiber layer; ILM, inner limiting membrane.

up at the OLM, the equivalent of the ventricular zone in the cerebral cortex and cerebellum.

However, the retinal ventricular cells are multipotential compared to the multiple committed lineages of ventricular cells elsewhere in the CNS (Levitt et al., 1981; Turner and Cepko, 1987; Holt et al., 1988). The retinal ganglion cells (RGCs) are first to past their final mitosis and migrate to the inner aspect of the retina. The genesis and differentiation of RGCs expands in a

central to peripheral pattern. The axons of the RGCs produce the OFL and enter into the elongating optic stalk. The dendrites of the developing RGCs start to ramify and form the presumptive IPL. At this time point, the outer retina is taken up by neuroblasts and called the neuroblast layer. The second groups of cells produced are amacrine, horizontal cells and cone photoreceptors. Rod photoreceptors and Müller glia are the last groups of cells produced. The IPL is formed before the OPL and the plexiform layers become progressively thicker and more complex. Photoreceptors possess the inner segment and then the outer segment. In addition to the center to periphery developmental gradient, retinogenesis in some species such as zebrafish take an inside to outside gradient, since the postmitotic cells first in the GCL, then in the INL and last in the ONL (Hu and Easter, 1999).

Ciliary marginal zone in the retina. In fish and amphibians, the retina grows throughout life by adding new cells of all types from the ciliary marginal zone (Wetts et al., 1989). Ciliary marginal zone (CMZ), a region at the peripheral edge of the retina, is a perpetually self-renewing proliferative neuroepithelium. In the CMZ, cells are spatially ordered with respect to cellular development, the deep stem cells being most peripheral, the proliferative retinoblasts in the middle, and the differentiating cells at the central edge. This spatial gradient in the CMZ recapitulates embryonic retinogenesis and provides a powerful system to examine the relative order of gene expression during this process (Perron et al., 1998). During metamorphosis, tadpole retina grows by proliferating asymmetrically at the CMZ, and the CMZ increases in size ventrally but not dorsally, remaining asymmetrical into adult (Beach and Jacobson, 1979). This asymmetric growth is controlled by different expression of genes in the ventral and dorsal CMZ (Beach and Jacobson, 1979).

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 tailbud 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). Retinal ganglion cell axonogenesis begins around St 28 and dendritogenesis begins around St 31, the late tail-bud stage (Holt, 1989; Sakaguchi, 1989). The RPE contacts the neural retina and pigmentation in the RPE begins around St 30. 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 morphological 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.

Cells in the developing retina must contact a variety of molecular cues in their microenvironment that are thought to guide their development (Clegg et al., 2000). Many of these cues are embedded in the surface of neighboring cells or deposited within the extracellular matrix. Adhesive molecules, including ECM molecules and CAMs may be playing a critical role in cell adhesion, polarization, migration, differentiation, survival and synaptogenesis during the retinal development. I discuss here the function of ECM components, especially laminin, during retinal development, and then the ECM receptors, integrins, which are activated by ECM molecules in the retina.

Extracellular matrix molecules in the retina. There are three basement membranes associated with the retina: the inner limiting membrane, Bruch's membrane, and the basement membrane associated with the retinal vasculature, which serve to delimit the retina from the surrounding non-neuronal tissues. Nonbasement membrane ECM components extend between the ILM and the RPE. Distinct ECMs are present within the retina and their composition changes during development (Hunter et al., 1992; Lawler and Sakaguchi, 1999). The ECM

molecules effect retinal development through direct signaling as well as indirect signaling. The direct role is that ECM molecules bind their receptor integrins, and regulate retinal development. Moreover, other extrinsic signals also control retinal development. For example, fibroblast growth factor (FGF) is involved in cell determination in the optic cup and proliferation of retinal precursors (Gensburger et al., 1987; Guillemot and Cepko, 1992; Pittack et al., 1997). Some ECM molecules can sequester soluble factors such as FGF in appropriate position and at relevant concentrations, and then facilitate the function of other extracellular molecules (McKeehan et al., 1998).

Laminin and retinal development. Different laminins are expressed in multiple locations in the retina: the ventricular surface, the vitreal border, synaptic layers, the interphotoreceptor matrix (IPM), and the ILM (Hunter et al., 1992; Lawler and Sakaguchi, 1999; Libby et al., 2000). In addition, laminin-13, 14 and 15 are only found surrounding inner segments of photoreceptors in mammals, suggesting the basic structure of the IPM surrounding the inner segments is very different from that surrounding the outer segments (Libby et al., 2000). These locations of laminin distribution associated with important developmental events suggest a diverse role for laminin in the retinal development and function. For example, laminin-3 (s-laminin) is involved in photoreceptor determination, inner and outer segment development, and photoreceptor synaptogenesis (Hunter et al., 1992; Libby et al., 2000).

As to examine the developmental and functional roles played by ECM receptors, integrins, it is reasonable to ask the following questions: 1. Which integrins are expressed in retina? Are they related with the retinal developmental events or special morphologies? 2. What functions do they play? 3. What is the mechanism or which molecules are involved in the integrin mediated signaling pathways?

Integrins' differential expression in the developing retina. Many studies have been carried out concerning integrins' expression in the developing chick retina: $\alpha_3\beta_1$ and $\alpha_6\beta_1$ (de Curtis et al., 1991); $\alpha_1\beta_1$ (Duband et al., 1992); $\alpha_2\beta_1$ (Bradshaw et al., 1995); β_1 , α_2 , α_3 , α_4 , α_6

and α_8 subunits (Cann et al., 1996); $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Gervin et al., 1996). All of these studies indicate that different integrins are expressed in a distinct differential pattern during chick retinal development, suggesting their involvement in the retinal development. For example, $\alpha_6\beta_1$ integrin, a prominent laminin receptor, is developmentally regulated in chick retina (de Curtis et al., 1991). It is at high levels of expression in embryonic chick retina at embryonic day 6 (E6), when retinal cells begin to differentiate. By E12, many retinal cells have undergone their final mitosis and the RGC axons have already reached their targets in the optic tectum; both retinal ganglion cells and other retinal neurons lose selected integrin functions, including the ability to attach and extend neurites on laminin. These developing changes correspond with a 75 -80 percent decrease of α_6 mRNA and protein in retinal ganglion cells at E12 (de Curtis et al., 1991). In addition, the α_6 subunit is partially colocalized with laminin, and its two isoforms α_{6A} and α_{6B} with distinct cytoplasmic domains have quite different distribution patterns within the E6 retina (de Curtis and Reichardt, 1993). The vitronectin integrin receptor α_v , β_3 and β_5 subunit mRNAs are also identified in undifferentiated neuroepithelial precursor cells in E6 retina and are expressed throughout the developing retina, with highest levels per retina observed around E9, a time when most differentiated neurons grow processes and initiate synapse formation (Gervin et al., 1996). Moreover, β_1 subunit is highly associated with radial cells (neuroepithelial cells and Müller glial cells) spanning the entire width of the chick retina, suggesting a role of β_1 integrins in the adhesion of these cells to the ECM of the ILM (Hering et al., 2000).

A few studies have been carried out concerning integrins' expression in retina of other animal systems. α_2 , α_3 , α_4 , α_5 , α_6 , α_v and β_1 , β_2 , β_3 subunits are detected in unique distribution patterns in the human retina (Brem et al., 1994). In addition, β_1 , α_1 , α_2 , α_3 , α_4 , α_5 , and α_v are highly expressed in normal human retinal cells, which adhere to and spread on laminin, fibronectin, and type IV collagen; whereas only α_4 and β_1 are highly expressed in Y79 retinoblastoma cells, which only adhere moderately to fibronectin (Skubitz et al., 1994). In

Xenopus, α_5 , α_v and β_1 integrin subunits are expressed in the developing retina and β_1 is highly associated with Müller glia and astrocytes inside the retina (Li and Sakaguchi, 1999). In the tiger salamander retina each of α integrin subunits 1-6 has been documented to have a distinct distribution, suggesting that each subunit may mediate a unique adhesive interaction and have a distinct role (Sherry and Proske, 2001).

Integrin function in the retina. The best defined role for integrins in the retina is in mediating adhesion and axon/dendrite outgrowth. β_1 integrins were shown to be required for retinal cell neurite outgrowth and adhesion on a number of ECM components (Cohen et al., 1987; Sakaguchi and Radke, 1996). Their importance for RGC neurite outgrowth was confirmed by the study, in which chimeric expression of β_1 integrins impaired process *in vivo* (Lilienbaum et al., 1995). α_v -containing integrins also mediate attachment and neurite outgrowth of retinal neurons on vitronectin (Neugebauer et al., 1991). In addition, the expressions of α_v subunit and vitronectin are colocalized in the developing chick retina; vitronectin can sustain both proliferation and differentiation of cultured neuroepithelial cells from E5 retina, and support survival and neurite outgrowth of most differentiated neurons at later stages (Martinez-Morales et al., 1995). Moreover, when explanted eye cups were incubated in the presence of function blocking anti- β_1 antibody, the retinal ganglion cell migration from the ventricular zone to the vitreal border was significantly inhibited (Cann et al., 1996). This suggests that the β_1 integrins play a role in neuroblast migration in the retina. A role for β_1 integrins in retinal migration is also suggested since injection of anti- β_1 integrin antibodies disrupted retinal morphology in developing chick and *Xenopus* embryos (Svennevik and Linser, 1993; Leonard and Sakaguchi, 1996). β_1 integrins have also been shown to be important in neural crest cell migration and CNS neuroblast migration (Galileo et al., 1992; Jacques et al., 1998). Furthermore, β_1 integrin antisense RNA containing viruses reduced the size of retinal cell clones (Skeith et al., 1999). And integrin α_6 ^{-/-} mice displayed abnormality in the laminar organization of the developing cerebral cortex and retina, and ectopic neuroblastic

outgrowths were observed in the brain and in the vitreous of these mice (Georges-Labouresse et al., 1998). These studies leave no doubt that integrins play a major role during retinal differentiation and morphogenesis.

Integrins with retinal pigment epithelium. The retinal pigment epithelium that lies at the interface between the neural retina and the choroid. Retinal pigment epithelium is a highly specialized epithelium derived from the embryonic neural tube, but retains the ability to proliferate and transdifferentiates into neural retinal cells (Okada, 1980). The RPE cells are different from other simple epithelia in that the apical surface is not free but makes direct contact with the photoreceptor outer segments and interphotoreceptor matrix. Thus, RPE performs critical transport, barrier, and phagocytic support functions, and the polarity of RPE proteins are important for the functions. Early in chick development, β_1 integrin subunit resides in the apical (facing the neural retina) and basolateral (facing the choroid) membranes of RPE, but late in development, it resides only in the basolateral membranes. This suggests that neural retina may determine the distribution of individual integrins (Rizzolo and Heiges, 1991; Rizzolo et al., 1994). However, β_1 integrin subunit is present in both apical and basal membranes of *Xenopus* RPE (Chen et al., 1997). The difference may be due to the identification of different isoforms of the β_1 subunit. Most importantly, vitronectin and its integrin receptor $\alpha_v\beta_5$ are present at the photoreceptor-RPE interface, and participate in phagocytosis of rod outer segments by mammalian RPE cells (Anderson et al., 1995; Miceli et al., 1997; Lin and Clegg, 1998). In addition, the neural cell adhesion molecule (NCAM) is also present on the apical surface of the RPE (Marmorstein et al., 1998). Furthermore, normal photoreceptor function and survival relies on the neural retina closely adherent to the RPE. Lost of retinal adhesion, known as retinal detachment, or the separation of neural retina from the RPE leads to photoreceptor degeneration (Lewis et al., 1991). The mouse with vitiligo mutation of the microphthalmia (Mtf) gene displayed decreased adhesion between the neural retina and the RPE. The mutation disrupted the outer segment/ RPE interdigitation and then led to

progressive loss of the photoreceptors (Bora et al., 1999). Taken together, integrins are involved in the function of RPE cells and the interactions between RPE and neural retina as well as these cells with ECM components in the interphotoreceptor matrix.

Cooperations of multiple adhesive molecules and other factors during retinal development. Retinal tissue is also a good model to study the cooperations and signaling pathways of different adhesive molecules and growth factors. It is obvious that integrin functions need cooperation with other adhesive molecules in the retinal development. For example, different cadherins have been documented to have unique distributions in the mouse retina (Honjo et al., 2000). Injection of antibodies against β_1 integrin receptors and N-cadherin perturbs retinotectal projection, whereas injection of one individually antibody is insufficient to cause obvious pathfinding errors (Stone and Sakaguchi, 1996). In addition, β_1 integrins cooperate with F11 of the Ig superfamily CAMs to induce neurite outgrowth in chick retina (Treubert and Brummendorf, 1998). Furthermore, disruption of cells with laminin-heparan sulfate proteoglycan interaction blocks Rana retinal regeneration (Nagy and Reh, 1994), while disruption of retinal basal lamina consisting of laminin-1, nidogen-1, collagens IV and XVII, perlecan, and agrin leads to dramatic aberrations in the retinal histogenesis (Halfter et al., 2001). 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. Moreover, cross talk between integrin and cadherin signaling is possible (Arregui et al., 2000; von Schlippe et al., 2000). Through different associated proteins, cell adhesive receptors may organize cytoskeletal structures that serve as scaffolds for signaling cascades and ultimately regulate cellular processes (Juliano, 2002).

Insulin-like growth factor-I (IGF-1) stimulated neurogenesis in cultures of neuroepithelial cells of chick E5 or E9 retina by up-regulating the α_6 mRNA expression (Frade et al., 1996). On the other hand, integrin-mediated adhesion and FAK can regulate insulin receptors substrate-1 (IRS-1) expression, through FAK mediated signaling to JNK in

fibroblasts, suggesting that the integrins could modulate insulin and IGF-1 signaling pathways by regulating IRS-1 (Lebrun et al., 2000). In addition, the activation of integrins is necessary for their function. During late embryonic development, retinal neurons lose the ability to attach and extend neurite on laminin-1, although the integrin receptors for laminin-1 are expressed. Integrin activation through extracellular factor Mn^{++} or intracellular signal R-ras can reverse the responsiveness and promote neurite outgrowth (Ivins et al., 2000).

Tyrosine Kinase activity with retinal development. It is essential to gain more complete understanding of the mechanisms regulating cell-cell and cell-ECM interactions and the signal transduction events during retinal development. A major intracellular signaling event to transduce the extracellular cues into meaningful signals that mediate cellular responses, is the phosphorylation of proteins on tyrosine residues by receptor and nonreceptor protein tyrosine kinases (Maness and Cox, 1992). In the vertebrate visual system phosphotyrosine is abundant in the process-rich layers of the embryonic retina and optic tract. In the developing *Xenopus* retina, phosphotyrosine is differentially regulated and highly expressed in the OFL, IPL and OPL as well as the Müller glial cells (Li and Sakaguchi, 2002). Furthermore, receptor and nonreceptor PTKs are also expressed in the developing visual pathway as well as the retina (Biscardi et al., 1991; Worley and Holt, 1996). For example, the neurotrophic factor receptors, TrkB (Jelsma et al., 1993) and BDNFR (Cohen and Fraser, 1994), are expressed in retina and brain. In addition, nonreceptor PTKs (also referred to as cytoplasmic PTKs) including Src, FAK and Abl subfamilies are highly expressed in the brain as well as in the retina (Sorge et al., 1984; Hoffmann, 1989; Ingraham et al., 1992; Hens and DeSimone, 1995). Furthermore, nonreceptor PTKs may transduce signals into the cells from receptors that lack intrinsic tyrosine kinase activity. Integrins, cadherins and Ig-superfamily adhesion molecules that regulate cell-cell and cell-ECM adhesions mediate signaling through nonreceptor tyrosine kinases. Due to the importance of PTK activities involved in adhesion-mediated signaling, it is necessary to further investigate the role of tyrosine kinase activity during retinal development.

Inhibitors of tyrosine kinase activity have been applied to cultured neurons or whole embryos to address the roles of tyrosine kinases in the control of neurite outgrowth and axonal pathfinding. Inhibition of PTK activity reduced neurite extension in dissociated *Xenopus* retinal cultures (Worley and Holt, 1996), while potentiated substrate-induced neurite growth in chick forebrain neurons and ciliary ganglion neurons (Bixby and Jhabvala, 1992). In addition, when applied to developing *Xenopus* embryos, PTK inhibitors reduced the rate of outgrowth of retinal axons into the optic tectum, but failed to induce pathfinding errors in the optic tract (Worley and Holt, 1996). However, PTK inhibitors applied to grasshopper embryos produced specific alterations in axon guidance (Menon and Zinn, 1998). The different responses of neurons to PTK inhibition may reflect species and/or cell type differences. It is yet to know the role of PTK activity in other cellular processes during retinal development.

Summary. Integrins, the major adhesive receptors for extracellular matrix, are important regulatory molecules of embryogenesis. They can be present at a particular location and time of development. Perturbation experiments and genetic analysis provide evidence that integrins play a critical role in selective adhesion, cell migration, and interactions with cytoskeletal and signaling molecules. Integrins are involved in cell proliferation, polarization, differentiation as well as basement membrane assembly and cell survival. Integrins also display developmentally regulated expression, and play a decisive role in retinal cell adhesion, migration, proliferation, differentiation, survival, neurite outgrowth and synaptogenesis during retinal development as well as in the maintenance of retinal function. All the cellular function may need the cooperation of integrins with other adhesive molecules and soluble factors. First, integrin-mediated adhesion is modulated through extracellular factors, clustering, associated proteins, organized cytoskeleton and intracellular signals. Second, integrin-mediated adhesion can activate multiple signaling pathways that crosstalk with the signaling pathways initiated by other adhesion receptors through cytoskeleton organization. Last and most importantly, integrins cooperate with conventional receptors at multi-levels. For examples, ECM molecules can

sequester growth factors, integrin aggregation can activate nonreceptor PTKs as well as receptor PTKs, and both integrins and growth factors can activate Ras/MAPK cascades. The retina would be a good model system to elucidate the molecular details and mechanisms in the cellular processes during neural development.

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CHAPTER 2. 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 β_1 integrin receptors and focal adhesion associated proteins during eye development. We used specific antibodies to examine the distribution of β_1 integrin ECM receptors and cytoplasmic focal adhesion associated proteins, talin, vinculin and paxillin in the developing *Xenopus* retina. Immunoblot analysis confirmed antibody specificity and indicated that β_1 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 β_1 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

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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 β_1 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), while the inner layer gives rise to the multilayered sensory retina (Hilfer, 1983). Retinal cell types are generated in a histological 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 transmembrane domain, and a short conserved cytoplasmic tail. The combination of α and β subunit determines ligand specificity and intracellular signaling activity. The β_1 integrins are the most prominent integrin subfamily and the β_1 subunit can interact with 12 different α subunits to form functional receptors. Alternative splicing of β_1 integrin mRNA increases the diversity of the β_1 integrin family (van der Flier et al., 1995).

In addition to the β_1 subunit, β_3 and β_5 subunits have been identified in embryonic retina (Gervin et al., 1996). β_1 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). A number of α subunits have been identified in the retina including the α_1 , α_2 , α_3 , α_4 , α_5 , α_6 , α_8 , and α_v subunits (Cann et al., 1996; Lin and Clegg, 1998; Sherry and Proske, 2001). In addition, integrin $\alpha_6^{-/-}$ mice displayed abnormal laminar organization in the retina, and ectopic neuroblastic outgrowths were found in the vitreous body in the eye (Georges-Labouesse et al., 1998). Furthermore, double knockouts of α_3 and α_6 subunits result in severe eye lamination 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 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; Albiges-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 via MAP kinase cascades (Cary and Guan, 1999). Paxillin functions as a multi domain adapter molecule and serves as a point of convergence for signals resulting from adhesion and various growth factor receptors (Turner, 2000).

A number of 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 β_1 integrins in regulating cell spreading and neurite outgrowth in *Xenopus* retina (Sakaguchi and Radke, 1996), and in regulating focal adhesion assembly in *Xenopus* XR1 retinal glial cells (Folsom and Sakaguchi, 1997, 1999). Furthermore, developmentally regulated changes of β_1

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_1 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 XR1 retinal glial cell line. Moreover, these proteins colocalized at focal adhesions associated with the termini of F-actin stress fibers in cultured XR1 cells. Their expression displayed a differentially regulated pattern in retinal tissue and was related with specific morphological events during retinal development. These results suggest that focal adhesion proteins may be involved in integrin-mediated signaling during retinal morphogenesis.

Materials and Methods

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 MgSO_4 , 1 mM NaHCO_3 , 0.4 mM CaCl_2 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 conducted 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 Hank's 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 tetra-acetic 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 (RBS-35; Pierce, Rockford, IL) washed 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 PMSF). XR1 cells were scraped from the flasks and placed in lysis buffer. Samples were homogenized, and protein concentration determined using a Bio-Rad assay kit. Protein samples were boiled in SDS-sample buffer (0.5 M Tris-HCl, 10% SDS, 10% glycerol, 2.5% bromophenol blue) with or without 5% β-mercaptoethanol (non-reducing conditions), and separated on 7.5% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose, blocked overnight in 1.5% 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 one hour. After washing in TBS with 0.1% tween-20, the membranes were incubated with

1:5000 goat anti-mouse IgG-HRP for 45 min. The staining was detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ).

Immunohistochemistry. *Xenopus* embryos, larvae, and froglets and cultured cells were fixed in 4% paraformaldehyde in 0.1M phosphate buffer for 24 h (animals) or 30 min. (cells). The animals were fixed in Dent's fixative (20% dimethyl sulphoxide 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 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 were diluted in blocking solution and preparations incubated overnight at 4°C. On the following day the preparations were rinsed with PBS and incubated with appropriate secondary antibodies conjugated to Alexa 488 or FITC for 90 min. at room temperature and subsequently rinsed and mounted using Vectashield mounting media (Vector Labs, Burlingame, CA). Double and triple labeling was performed in this study. For double-labeling a biotinylated goat anti-mouse IgG (1:300, Vector Laboratories Inc.) and avidin-AMCA (1:1000, Vector Laboratories Inc.) were used following the second primary antibody incubation. These preparations were subsequently triple-labeled with rhodamine-phalloidin (1:300, 30 min., Molecular Probes, Eugene, OR) to visualize the F-actin cytoskeleton. As a control, single label studies were performed using the same antibodies to rule out that similar patterns were not produced due to bleed-through and the other fluorescence channels were also examined to ensure that no bleed-through occurred. Negative controls were performed in parallel by omission of the primary or secondary antibodies. No antibody labeling was observed in the controls.

Antibodies. β_1 integrin receptors were identified 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- β_1 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- β_1 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-phosphotyrosine monoclonal antibody, 4G10 (1: 200), was purchased from Upstate Biotechnology Inc. Anti-GFAP ,G-A-5, was purchased from ICN Immunobiologicals, 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 using a Nikon Microphot-FXA photomicroscope (Nikon Inc. Garden City, NY) -equipped with epifluorescence. Images were captured with a Kodak Megaplug CCD camera connected to a Perceptics Megagrabber 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).

Results

Focal adhesion proteins are expressed in the developing retina and in the XR1 glial cell line

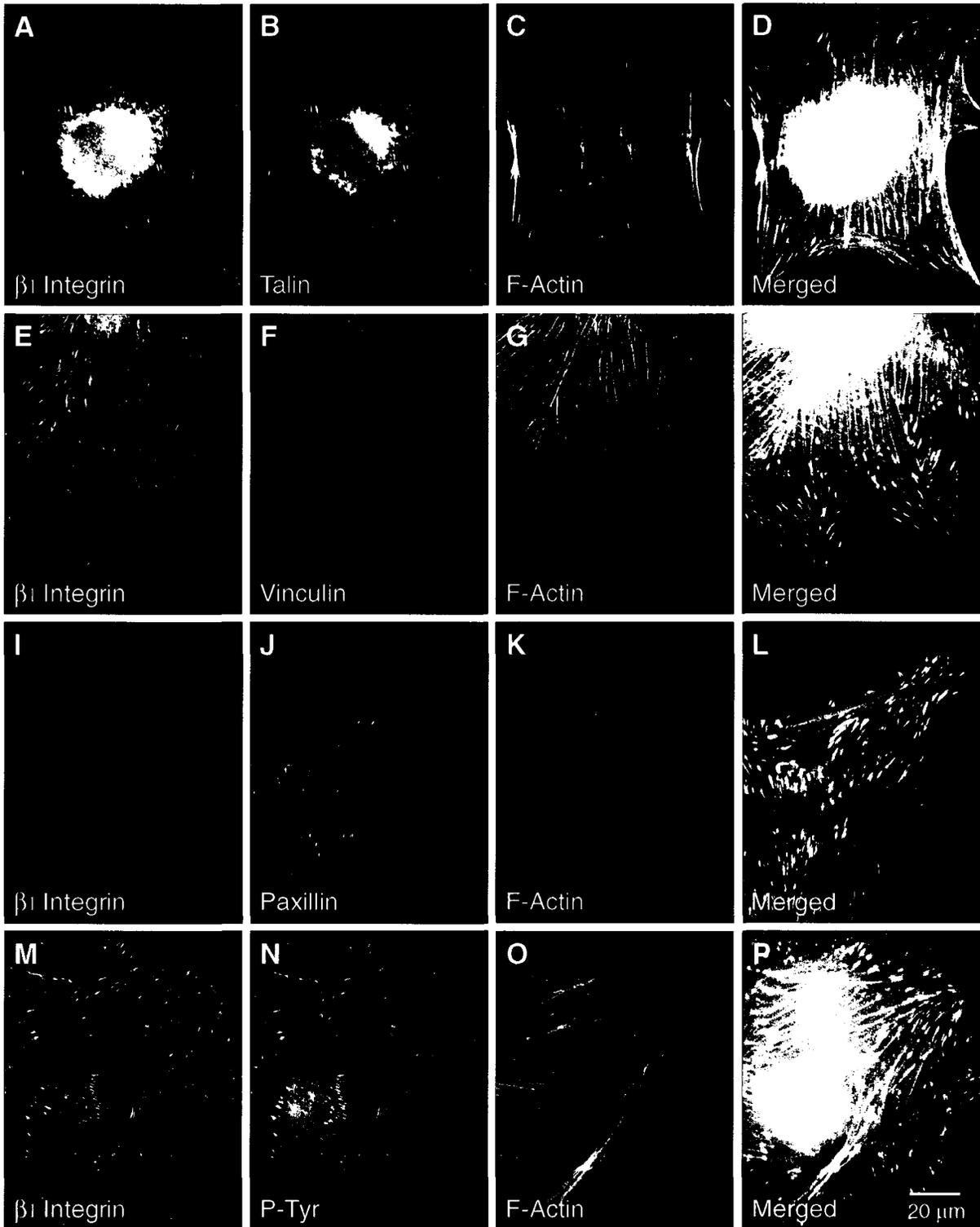
To verify the specificity of the antibodies directed against focal adhesion associated proteins used in this study we performed an immunoblot analysis. The immunoblot analysis was performed with samples from stage (St) 40 *Xenopus* eyes and from *Xenopus* retinal derived XR1 glial cells. The monoclonal anti- β_1 integrin antibody was generated against β_1 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 non-reducing 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 (Fig.1, 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 β_1 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

Figure 1. Immunoblot analysis using monoclonal antibodies against focal adhesion proteins β_1 integrin, talin, vinculin, and paxillin. Protein homogenates of St 40 *Xenopus* eye and XR1 cells were analyzed. A band of approximately 115 kDa was observed in homogenates from St 40 eyes (lane 1) and XR1 cells (lane 2) with β_1 integrin monoclonal antibody under non-reducing conditions. Bands of 235 and 225 kDa were observed with anti-talin antibody in both samples under reducing conditions (lane 3, 4). Anti-vinculin antibody identified a band of approximately 116 kDa in both samples (lane 5, 6), and anti-paxillin antibody, identified a band of approximately 68 kDa (lane 7, 8) in both samples under reducing conditions.

Figure 2. Triple-labeling fluorescence analysis revealing focal adhesion proteins in *Xenopus* XR1 retinal glial cells. Focal adhesions were characterized as discrete streak-like patterns of immunoreactivity (IR). A, E, I, M: the pattern of β_1 integrin-IR. B, F, J, N: the pattern of talin-, vinculin-, paxillin- and phosphotyrosine-IR, respectively for the same cell pictured to the left. C, G, K, O: the pattern of F-actin filaments labeled with rhodamine phalloidin in the same cell as in A, E, I, M, respectively. D, H, L, P: the merged images, indicating talin, vinculin, paxillin and phosphotyrosine are each colocalized with β_1 integrins and colocalize at the termini of F-actin filaments. Abbreviation: P-Tyr, phosphotyrosine. Scale bar = 20 μm .



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. β_1 integrin-IR (Fig. 2A, E, I, M) colocalized with talin- (Fig. 2B), vinculin- (Fig. 2F), paxillin- (Fig. 2J) or phosphotyrosine-IR (Fig. 2N), 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 co-localization 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.

A 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). Retinal ganglion cell 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 morphological 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. β_1 integrin-IR was present throughout the optic vesicle and appeared to be associated with the membranes of the neuroepithelial cells that spanned the width 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 β_1 integrin-IR in neuroepithelial cells, and strong IR at the interface between the optic vesicle and ectoderm (Fig. 4A, G).

By St 30 the eyecup was well formed and the lens placode has formed from the sensorial layer of the ectoderm. β_1 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. β_1 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. 4 B, H). At this time the presumptive

RPE was contacting the neural retina and β_1 integrin- and talin-IRs were expressed in these cells (Fig. 3B, H).

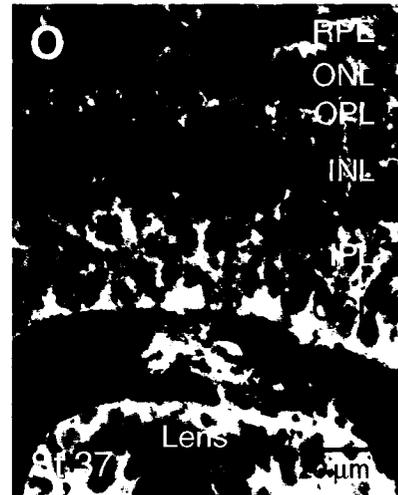
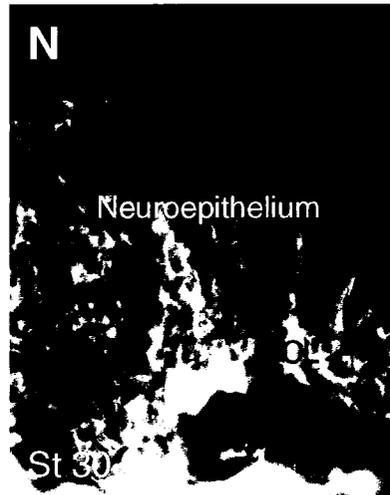
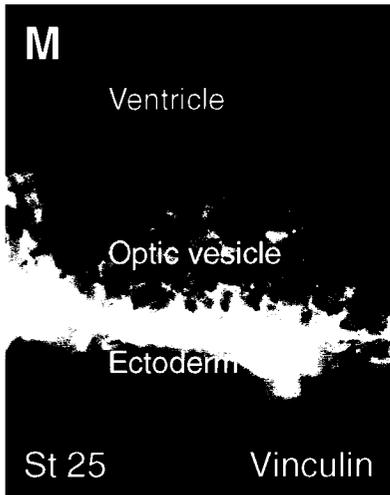
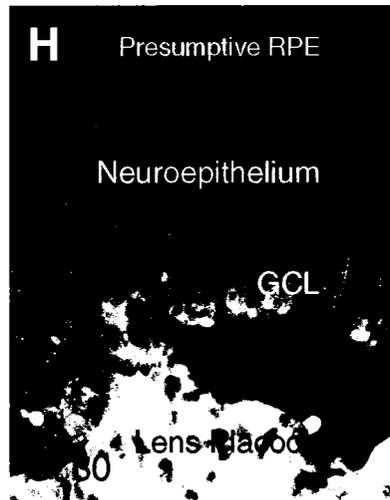
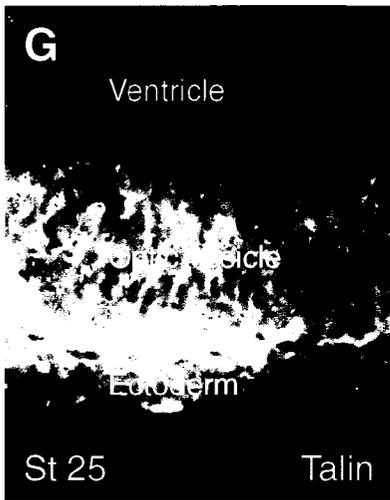
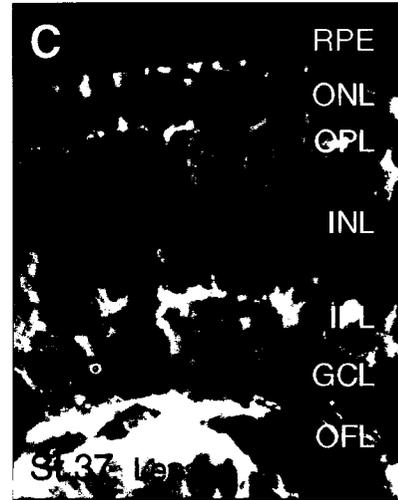
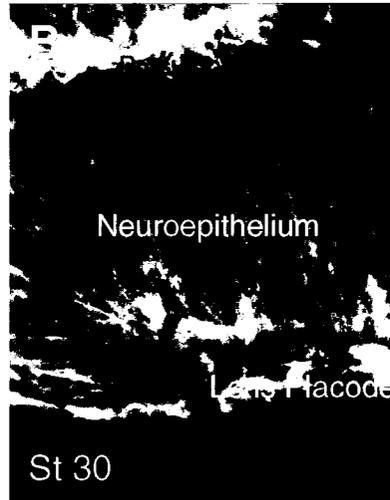
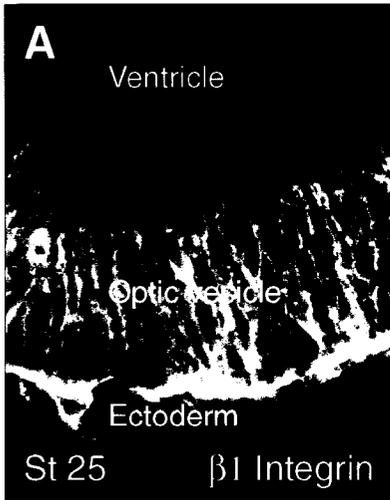
By St 37 the retina was relatively well differentiated. β_1 -IR was still widespread, and cell bodies in the ONL, INL and GCL were clearly outlined, and IR was strong in the nascent outer and inner plexiform layers (OPL and IPL), as well as optic fiber layer (OFL) (Fig. 3C). Talin- and vinculin-IRs (Fig. 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 β_1 -integrin (Fig. 3D), talin (Fig. 3J), vinculin (Fig. 3P), paxillin (Fig. 4D) and phosphotyrosine (Fig. 4J). β_1 -, paxillin- and phosphotyrosine-IRs were present in radially oriented cells with morphologies reminiscent of the Müller glial cells (Fig. 3D, Fig. 4D, J).

The retina was well differentiated by St 47. Immunoreactivities for β_1 -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. β_1 integrin-, paxillin- and phosphotyrosine-IRs were prominent in the radially oriented pattern observed at St 40 (Fig. 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- β_1 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- β_1 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

Figure 3. The changing patterns of expression of β_1 integrin-, talin- and vinculin-IRs during retinal development. Fluorescence images of β_1 integrin- (A-F), talin- (G-L) and vinculin-IR (M-R). Each series of images represent retinal tissue sections from *Xenopus laevis* at St 25, 30, 37, 40, 47 and 65, respectively. Abbreviations: RPE, retinal pigment epithelium; OS, outer segments of photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; OFL, optic fiber layer. Scale bar = 20 μm . (including page 69 and 70).



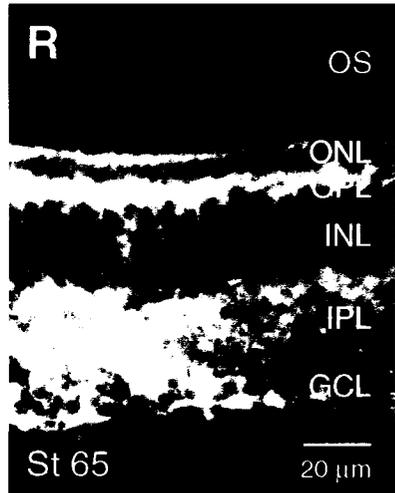
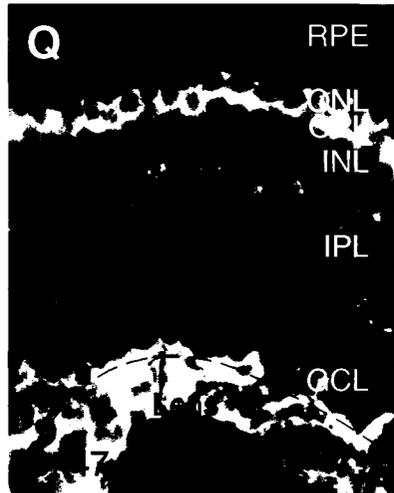
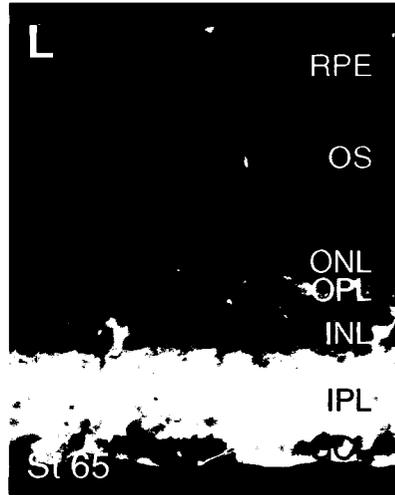
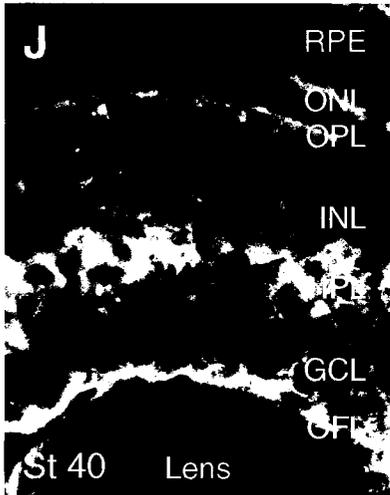
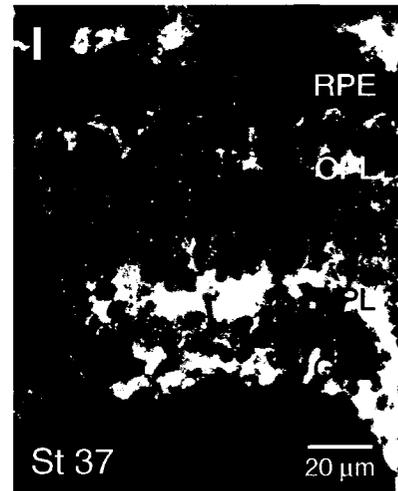
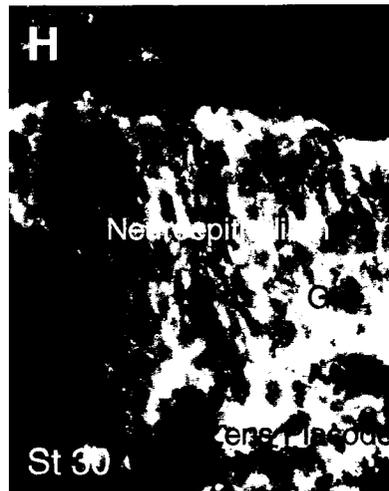
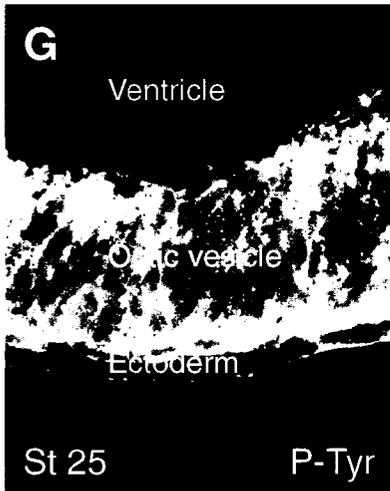
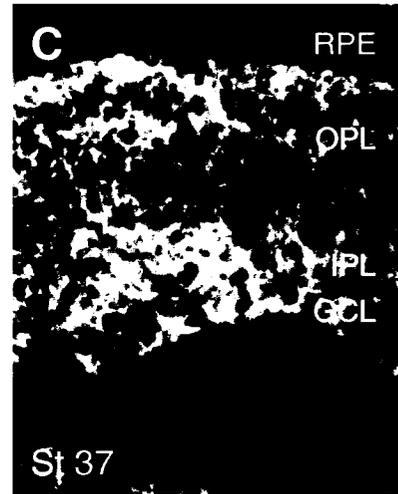
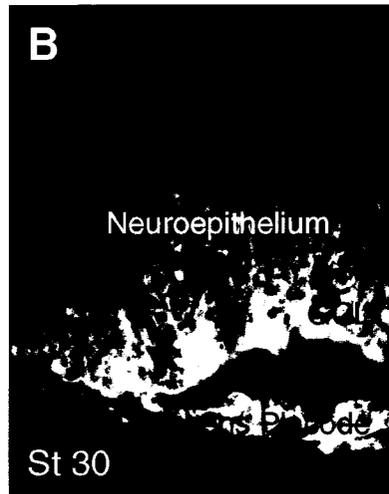
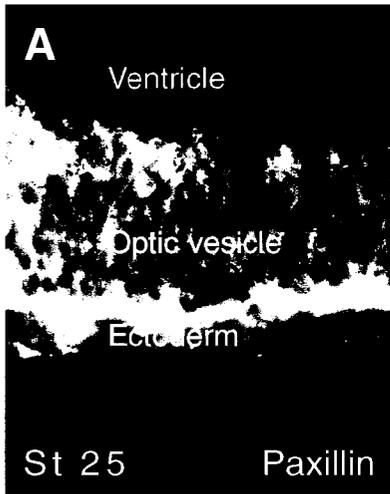


Figure 4. The changing patterns of expression of paxillin- and phosphotyrosine-IRs during retinal development. Fluorescence images of paxillin- (A-F) and phosphotyrosine-IRs (G-L). Each row of images represents retinal tissue sections from *Xenopus laevis* at St 25, 30, 37, 40, 47 and 65, respectively. Abbreviations: RPE, retinal pigment epithelium; OS, outer segments of photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; OFL, optic fiber layer. Scale bar = 20 μm . (including page 72 and 73).



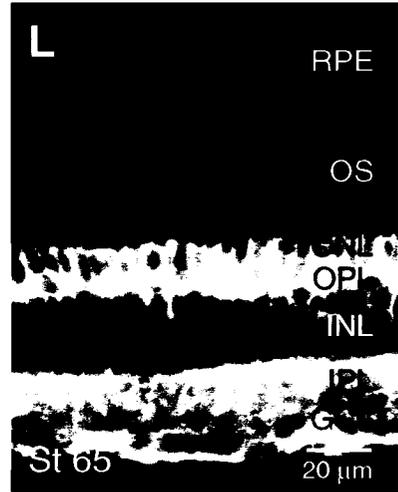
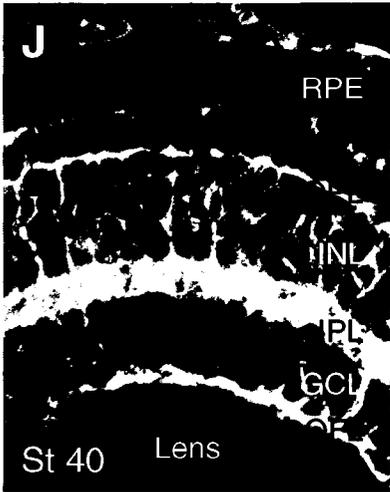
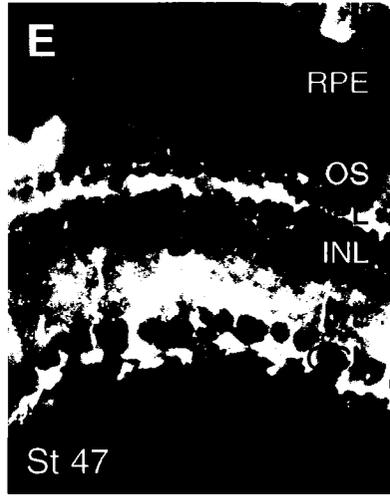
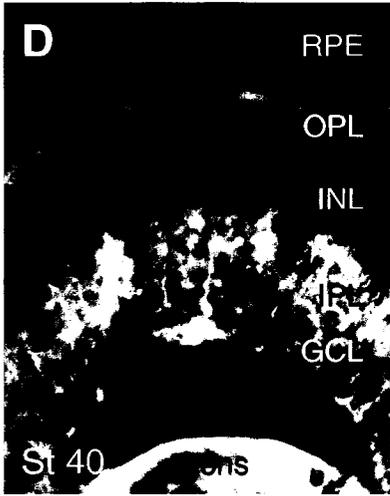
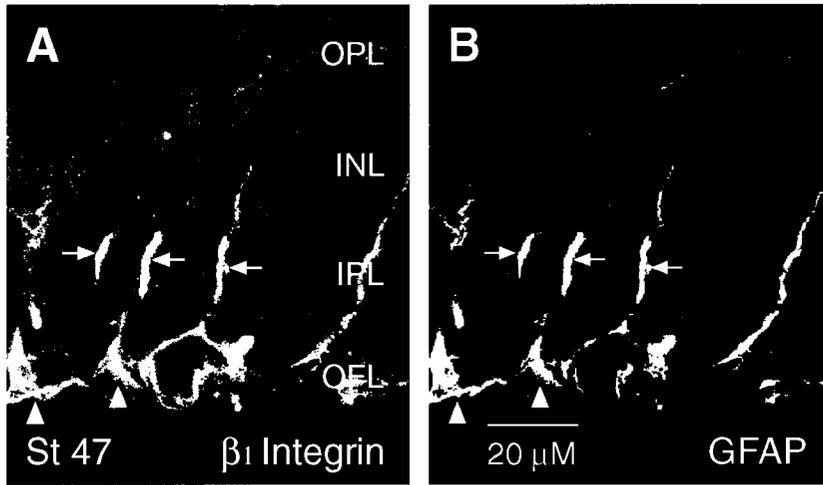


Figure 5. Confocal images illustrating colocalization of β_1 integrin- and GFAP-IRs in Müller glial cells. Cryostat sections of St 47 *Xenopus laevis* were double-labeled with anti- β_1 integrin (A) and GFAP (B) antibodies. A precise colocalization of the immunoreactivity was observed in the radially oriented Müller cells (arrows) and in the astrocytes in the OFL (arrowheads). Abbreviations: OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; OFL, optic fiber layer. Scale bar = 20 μm .



cells and astrocytes. Figure 5 shows colocalization of β_1 -IR in GFAP-IR 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 β_1 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 β_1 -, 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 β_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 β_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, β_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 β_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, β_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 β_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. β_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 β_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 β_1 integrin receptors during *Xenopus* retinal development is similar to the expression of β_1 integrins in the developing chick retina (Rizzolo and Heiges, 1991; Cann

et al., 1996; Hering et al., 2000). β_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). β_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 β_1 integrin expression in the apical membranes of St 47 RPE with the polyclonal anti- β_1 antibody (3818). This 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 β_1 integrin-IR. However, we detected β_1 integrin expression in cultured RPE cells dissociated from St 47 eye and in St 65 RPE homogenate with immunocytochemistry and westernblot analysis, respectively (data not shown).

The differential distribution of β_1 integrins suggests they have an important role in mediating cell adhesion and signaling during retinal morphogenesis. β_1 integrin-IR was present in the neuroepithelial cells, in particular at the interface between the optic vesicle and ectoderm, suggesting that β_1 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 β_1 integrin receptor ligands, such as laminin and fibronectin, have been identified in the region of the ILM (Sakaguchi, unpublished). Studies inhibiting β_1 integrin function suggest an important role for these receptor complexes during retinal development (Svennevik and Linser, 1993). Injection of β_1 integrin function blocking antibody and RGD peptides into early optic vesicle of E2 chick prevented invagination of the optic vesicle and resulted in the reduction of retinal size (Svennevik and Linser, 1993). Furthermore, infection with β_1 integrin antisense RNA virus caused the reduction of β_1 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 eyecups were cultured in the presence of function

blocking anti- β_1 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 β_1 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 β_1 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 β_1 integrin, as well as N-cadherin, perturbed the development of the *Xenopus* retinotectal projection (Stone and Sakaguchi, 1996), and expression of chimeric β_1 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 which ultimately regulate cellular processes (Juliano, 2002).

β_1 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 β_1 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 β_1 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 β_1 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 β_1 integrins to the F-actin cytoskeleton. Focal adhesions provide a platform, where integrins link ECM and cytoskeleton, and serve as bi-directional 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 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 since they are in a three-dimensional environment (3D) 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*.

Acknowledgments

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CHAPTER 3. THE ROLE OF TYROSINE KINASE ACTIVITY DURING EARLY RETINAL DEVELOPMENT

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Abstract

In the present study, we have begun to investigate the role of tyrosine kinase activity during early retinal development in *Xenopus laevis*. The protein tyrosine kinase (PTK) inhibitors lavendustin A and genistein were used to investigate the possible role of tyrosine kinase activity during retinal development *in vitro* and *in vivo*. Immunocytochemical analysis revealed the presence of focal adhesions in dissociated retinal neuroepithelial cells isolated from stage 25 embryos. Application of the PTK inhibitors blocked focal adhesion assembly in these primary cultured cells. To further investigate the regulation of focal adhesions by PTK activity we examined the effect of lavendustin A on cultured XR1 glial cells. Lavendustin A produced a dose-dependent decrease in the proportion of XR1 cells displaying focal adhesions. Application of the inhibitors to early embryonic retina disrupted the lamination of developing retina in the treated embryos. The plexiform layers were no longer apparent, and photoreceptor morphogenesis was disrupted. Taken together, these results suggest that tyrosine kinase activity may be essential for regulating neuroepithelial cell adhesion, migration and morphogenesis during retinal development. Furthermore, the disruption of retinal development may, in part, be due to the inhibition of integrin-mediated signaling.

Key words: *Protein Tyrosine Kinase, Genistein, Lavendustin, Integrins, Focal Adhesions, Retina, Xenopus, Retinal Development*

Introduction

During morphogenesis of the nervous system migrating neurons and glia contact a variety of molecular cues that are thought to guide their development. Many of these molecules are attached to the neighboring cell surfaces or are deposited within the surrounding extracellular matrix (ECM) (Howe et al., 1998; Venstrom and Reichardt, 1993).

Phosphorylation of proteins on tyrosine residues by receptor and nonreceptor protein tyrosine kinases (PTKs) appears to be a major intracellular signaling event that is required to translate these extracellular cues into meaningful signals and mediates cellular responses (Maness and Cox, 1992). In the vertebrate visual system phosphotyrosine is abundant in the process-rich layers of the embryonic retina and optic tract. Receptor and nonreceptor PTKs are also expressed in the developing visual system (Biscardi et al., 1991; Worley and Holt, 1996). For example, nonreceptor PTKs including Src, FAK and Abl subfamilies are expressed in the brain as well as in the retina (Hens and DeSimone, 1995; Hoffmann, 1989; Ingraham et al., 1992; Sorge et al., 1984). Nonreceptor PTKs may transduce signals into the cells from receptors that lack intrinsic tyrosine kinase activity. Integrin, cadherin and Ig-superfamily adhesion molecules are these cell receptors that require the nonreceptor PTKs to regulate cell-cell and cell-ECM adhesion and signaling.

Integrins are the major family of cell surface receptors for ECM components (Hynes, 1992). 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 24 different integrin heterodimers (van der Flier and Sonnenberg, 2001). β_1 integrins are the most prominent integrin subfamily since β_1 subunit can interact with 12 different α subunits to form functional receptors.

Integrin receptor binding with ECM ligands leads to the formation of focal adhesions. Focal adhesions are clustered integrins and associated proteins that link ECM with the actin cytoskeleton (Clark and Brugge, 1995; Miyamoto et al., 1995). The formation of focal

adhesions is believed to play critical roles in stabilizing cell adhesion and regulating cell morphology and motility (Burrige et al., 1988; Hynes, 1992). Furthermore, integrin binding to ECM can activate PTKs, such as FAK, Src and Ab1 family kinases, and stimulate an intracellular increase in tyrosine phosphorylation of focal adhesion proteins (Lewis et al., 1996; Wary et al., 1998). The tyrosine phosphorylation of focal adhesion proteins regulates integrin-mediated adhesion and signal transduction pathways that are required for cellular functions (Chicurel et al., 1998; Giancotti and Ruoslahti, 1999; Kumar, 1998). Inhibition of tyrosine kinase activity blocked focal adhesion formation providing further evidence for a role of tyrosine phosphorylation in the signal pathways mediated by integrin receptors (Folsom and Sakaguchi, 1997).

Integrin-mediated adhesive interactions are intimately implicated in neural cell migration, differentiation, neurite outgrowth and axon pathfinding during neural development (Baum and Garriga, 1997; Bradshaw et al., 1995; Georges-Labouesse et al., 1998; Jacques et al., 1998). Retina, with accessibility and well-organized laminar structure, is a good model system to study the cell-cell and cell-ECM interactions in the central nervous system. β_1 integrins are developmentally regulated, and highly expressed in Müller cells and astrocytes during retinal development (Hering et al., 2000). The XR1 glial cell line, derived from *Xenopus* retinal neuroepithelium, is an ideal cellular model system to investigate the mechanisms that regulate focal adhesion assembly *in vitro* (Sakaguchi et al., 1989). Evidence is accumulated indicating that tyrosine phosphorylation is one of the major factors that mediate integrin affinity and focal adhesion formation, and is also a key step to initiating the signaling cascades that regulate cell behavior (Hughes and Pfaff, 1998).

While it is clear that tyrosine kinase activity influences neurite outgrowth *in vitro* and *in vivo* (Menon and Zinn, 1998; Worley and Holt, 1996), it is not known what role tyrosine kinases play during the development of the vertebrate retina. In the present study we have tested the hypothesis that tyrosine kinase activity is required for the normal development of the

vertebrate retina and may be involved with neuroepithelial cell adhesion, neural differentiation and synaptogenesis during retinal development. Lavendustin A and genistein, two tyrosine kinase inhibitors with broad specificity, effect the activities of many receptor and nonreceptor tyrosine kinases, but with trivial effects on protein kinase A or C (Akiyama et al., 1987; Onoda et al., 1989). We have applied lavendustin A and genistein to dissociated retinal neuroepithelial cells, retinal derived XR1 glial cells and the optic vesicle of *Xenopus* embryos. When compared with controls, the lavendustin A and genistein treated cells displayed a decrease in number of cells with focal adhesions. *In vivo* analysis revealed that both lavendustin A and genistein treatment disrupted retinal lamination and photoreceptor morphogenesis. These results suggest that tyrosine kinase activity is likely to play a critical role during retinal development.

Materials and Methods

Animals. *Xenopus laevis* frogs were obtained from a colony maintained at Iowa State University. Embryos were produced from human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO; unless otherwise stated, all reagents were purchased from Sigma) 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 were staged according to the normal *Xenopus* table of Nieuwkoop and Faber (1967). All animal procedures were carried out 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). XR1 cells were grown in tissue culture flasks (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) in 60% L15 media 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. For all experiments XR1 cells were detached from subconfluent cultures by exposure to Hank's 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 tetra-acetic 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 (RBS-35; Pierce, Rockford, IL) washed 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) until prepared for analysis.

Dissociated retinal cultures. Embryos of stage 23-25 were dejellied and rinsed three times in Hanks Buffered Salt Solution (HBSS, 1.2 mM CaCl₂, 5.37 mM KCl, 0.44 mM KH₂PO₄, 0.81 mM MgSO₄·7H₂O, 0.23 mM Na₂HPO₄·7H₂O, 137 NaCl, 9.0 mM Dextrose, 0.04 mM Phenol Red) with 5.0 µg/ml fungibact and 2.5 µg/ml penicillin/streptomycin. Using sterile techniques, the overlying ectoderm was removed and the eye primordia dissected in HBSS. The dissected eye primordia were rinsed twice in HBSS and subsequently incubated with Ca/Mg free Steinberg's solution (60 mM NaCl, 0.67 mM KCl, 10 mM HEPES and 0.4 mM EDTA, pH 7.5) for 15 minutes to facilitate dissociation. The eye primordia were dissociated by gentle trituration using flame pulled glass Pasteur pipettes and plated onto ECL-coated 12 mm coverslips in the same culture medium used for XR1 cells.

Protein tyrosine kinase inhibitors. The protein tyrosine kinase inhibitors, lavendustin A and genistein, were used to investigate a possible role of tyrosine kinase activity in retinal differentiation and morphogenesis. Stock solutions of lavendustin A and genistein (Calbiochem, La Jolla, CA) were prepared in dimethyl sulfoxide (DMSO) at 50 mM and 100

mM, respectively and stored at -20°C . Lavendustin B and daidzein (Calbiochem), inactive analogs of lavendustin A and genistein, respectively were used as controls and were diluted in the same fashion as the inhibitors. Inhibitors and control drugs were diluted to their final concentrations using L15 culture media (for *in vitro* analysis) or Holtfreter's solution (for *in vivo* studies).

Protein tyrosine kinase inhibitor studies: In vitro. To determine the role of tyrosine kinase activity in regulating the assembly of focal adhesions, lavendustin A was diluted to a final concentration of 20, 50, 100 or 250 μM and applied to the XR1 cultures after the cells were plated for two hours. Lavendustin A at 100 μM was applied at 0.5, 1, 2, 6, or 24 h after plating. Control cultures received lavendustin B at the same concentrations or vehicle control media with no added drugs. The cultures were incubated for 5 h in the dark and subsequently rinsed in buffer and fixed with 4% paraformaldehyde for the analysis of focal adhesions. Dissociated retinal neuroepithelial cells were cultured on ECL substrates for 4 h and then treated with lavendustin A, B, genistein or daidzein at 100 μM for 5 h and subsequently prepared for analysis of focal adhesions. The concentration range and treatment duration for the inhibitors was chosen according to their potencies as reported in the literature (Bixby and Jhabvala, 1992; Miyamoto et al., 1995; Worley and Holt, 1996; Yap et al., 1994). All studies using inhibitors were carried out blind to eliminate any experimental bias.

Protein tyrosine kinase inhibitor studies: In vivo. Embryos between stage 23 -25 were anesthetized by immersion in 100% modified Ringer's solution (100 mM NaCl, 2 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 5 mM HEPES) containing 1:10,000 MS222 (ethyl 3-aminobenzoate, methanesulfonic acid salt, Aldrich, Milwaukee, WI). The skin overlying the optic vesicle was carefully removed, and the embryos placed in Holtfreter's solution with 2.5 $\mu\text{g/ml}$ fungibact and 2.5 $\mu\text{g/ml}$ penicillin/streptomycin in the presence of the PTK inhibitors (lavendustin A at 50

μM and genistein at 2.5 μM) or appropriate controls (inactive analogs at equal concentrations or vehicle) for 48 hours in the dark.

Immunohistochemistry. *Xenopus* embryos, larvae, and cultured cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h (animals) or 30 min. (cultured cells). The animals were rinsed with buffer and cryoprotected in 30% sucrose in 0.1 M PO_4 buffer overnight, and then frozen in OCT medium (Tissue-Tek, Sakura Finetek U.S.A., Inc. Torrance, CA). The frozen specimens were sectioned at 16 μm using a cryostat (Reichert HistoSTAT) and sections were mounted on Superfrost microscope slides (Lewis et al.). Tissue sections and cultures were rinsed in phosphate buffer saline (PBS, 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 , 1.47 mM KH_2PO_4) and blocked in 5% goat serum supplemented with 0.2% BSA, 0.1% Triton X-100 in PBS. Primary antibodies were diluted in blocking solution and preparations incubated overnight at 4°C. On the following day the preparations were rinsed with PBS and incubated with appropriate secondary antibodies conjugated to Alexa 488 or RITC for 90 min. at room temperature and subsequently rinsed and mounted using Vectashield mounting media (Vector Labs, Burlingame, CA). The actin filaments in XR1 cells were visualized with rhodamine-phalloidin (1:300, 30 min., Molecular Probes, Eugene, Oregon) following the antibody labeling procedures. For double labeling, single labeling was performed to rule out that similar patterns were not produced due to bleed-through and the other fluorescence channels were also examined to ensure that no bleed-through occurred. Negative controls were performed in parallel by omission of the primary or secondary antibodies. No immunoreactivity was observed in the controls. Tissue sections or cultured cells were examined using a Nikon Microphot-FXA photomicroscope (Nikon Inc. Garden City, NY) -equipped with epifluorescence. Images were captured with a Kodak Megaplug CCD camera connected to a Perceptics Megagrabber 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). Figures were prepared with Adobe Photoshop version 4.0 and macromedia Freehand Version 9 for Macintosh. Final output was produced using a Tektronix Phaser continuous tone color printer (Tektronix, Beverton, OR).

Antibodies. β_1 integrin receptors were identified using monoclonal antibody 8C8 purchased from Developmental Studies Hybridoma Bank (diluted 1:10, DSHB, University of Iowa, Iowa City, IA) or rabbit anti- β_1 integrin antibody 3818 (obtained from K. Yamada, National Cancer Institute). Photoreceptors were identified using the anti-*Xenopus* photoreceptor antibody, XAP-1 (diluted 1:20, Sakaguchi et al., 1997). Anti-synaptic vesicle protein, SV2 antibody was purchased from DSHB (diluted 1:20), anti-phosphotyrosine monoclonal antibody, 4G10, was purchased from Upstate Biotechnology Inc (diluted 1:100). Goat anti-mouse IgM or IgG secondary antibodies conjugated with RITC were purchased from Southern Biotechnology (Birmingham, AL) and Alexa 488 from Molecular Probes. The secondary antibodies were diluted to a final concentration of 1:200 with blocking solution.

Focal adhesion assay and cell area measurements. XR1 glial cells were allowed to adhere to ECL-coated coverslips, exposed to inhibitors or control solutions, and fixed and processed for immunocytochemistry after defined time periods. Seventy-two microscope fields of 180 μm x 140 μm were examined for identification of focal adhesions in each condition from cells prepared from three separate culturing sessions. In previous studies we have identified focal adhesions on XR1 cells as streak-like patterns of immunoreactivity (IR) where β_1 integrins were colocalized with vinculin or phosphotyrosine-IR at the termini of F-actin filaments (Folsom and Sakaguchi, 1997). As such, in this communication we have defined focal adhesions as discrete streaks of β_1 integrin-IR. In each field, cells were scored as positive if focal adhesions were present and negative if focal adhesions were absent. The proportion of cells displaying focal adhesions was calculated for each group. Cell area measurements were

obtained from captured images by using NIH Image 1.58 VDM software. A known distance 100 μm was measured for calibration, and outlining the cell perimeter produced a calculation of cell area. Forty-four cells from twenty-four fields of 360 x 280 μm were examined for each condition. Data were represented as mean \pm SEM and were analyzed using the Student's t-test.

Results

Regulation of focal adhesion assembly by tyrosine kinase activity in cultures of retinal neuroepithelial cells and XR1 retinal glial cells

It is likely that the interaction of integrin receptors with the ECM activates tyrosine kinases and tyrosine phosphorylation, and that the tyrosine phosphorylation may be essential to focal adhesion assembly and integrin-mediated signal transduction (Romer et al., 1994). In the present analysis, focal adhesions on cultured cells were identified based on the discrete, streak-like patterns of β_1 integrin-immunoreactivity (IR) and phosphotyrosine-IR (Folsom and Sakaguchi, 1997). To determine if retinal neuroepithelial cells were capable of forming focal adhesions in culture, optic vesicles were dissected, dissociated, cultured for 9 h and subsequently stained with anti- β_1 integrin and phosphotyrosine antibodies. At the time of culturing the St 23-25 eyebud is an optic vesicle consisting of undifferentiated neuroepithelial cells. Cells dissociated from the optic vesicle often had elongated and flattened morphologies. As illustrated in Figure 1, focal adhesions were observed in the cultured retinal neuroepithelial cells as streaks of β_1 integrin- and phosphotyrosine-IR (Fig. 1A-D). The protein tyrosine kinase inhibitors lavendustin A or genistein were used to determine if the focal adhesions observed on the primary retinal neuroepithelial cultures were susceptible to tyrosine kinase inhibition. Compared with controls, we found that inhibitor-treated neuroepithelial cells displayed fewer focal adhesions (Fig. 1E-H).

To more carefully investigate the regulation of focal adhesions by tyrosine kinase activity we used the retinal derived XR1 glial cell line as an *in vitro* model system (Folsom and

Sakaguchi, 1997; Henderson and Sakaguchi, 1993; Sakaguchi et al., 1989). Lavendustin A was applied to XR1 cells and control cultures received lavendustin B, an inactive analog, or vehicle solution (DMSO) in culture media. Cultures that received lavendustin B resembled vehicle control cultures, with β_1 integrin-IR and phosphotyrosine-IR in their characteristic discrete streak-like focal adhesion patterns (Fig. 2A-D). In contrast, XR1 cells treated with lavendustin A, in general, failed to form focal adhesions (Fig. 2E-F). No change in the proportion of cells displaying focal adhesions was observed in control cultures, whereas cultures treated with lavendustin A displayed a dose-dependent reduction in the proportion of cells with focal adhesions (Fig. 3A). The reduction was significant when the XR1 cells were incubated with 100 and 250 μ M lavendustin A (Fig. 3A, $P < 0.05$). To observe the cytoskeletal organization in the XR1 cells, the F-actin filaments were visualized with rhodamine-phalloidin. In the control cultures, the phalloidin staining revealed well organized actin cytoskeleton in the XR1 cells (Fig. 2B). However, in the lavendustin A-treated cells that failed to exhibit focal adhesions, the F-actin cytoskeleton was severely disorganized (Fig. 2E). These results suggest that the inhibition of tyrosine phosphorylation in XR1 cells by the tyrosine kinase inhibitor, lavendustin A, was effective in disrupting focal adhesion formation mediated by β_1 integrins.

To investigate how tyrosine activity regulates the assembly of focal adhesions in XR1 glial cells, the cells were subjected to lavendustin A or B at varying time points (0.5, 1, 2 or 6 h) after plating. In the control cultures the proportion of cells displaying focal adhesions increased with each successive time point. Similar to controls, the lavendustin A treated cultures displayed an increasing proportion of cells with focal adhesions over time, but there was a significantly lower proportion of cells displaying focal adhesions at 0.5, 1 and 2 h time points when compared with the controls (Fig. 3B, $p < 0.05$). Treatment with lavendustin A after 6 h in culture resulted in no significant difference with the controls (Fig. 3B). Furthermore, no significant difference was obtained when the drugs were applied after the cells were plated for 24 h (data not shown). XR1 focal adhesions appear to be well formed and stable after 6 h, and

Figure 1. Focal adhesions are present on retinal neuroepithelial cells in vitro.

Fluorescence photomicrographs illustrating examples of focal adhesions in neuroepithelial cells and the disruption of focal adhesions following treatment with lavendustin A and genistein. Primary cultures of retinal neuroepithelial cells were plated for 9 hours or 4 hours plus 5 hours with inhibitors. Focal adhesions were identified with β_1 integrin antibody (A, C) and phosphotyrosine antibody (B, D) in the elongated cells. Focal adhesions were absent in lavendustin A and genistein-treated cells (E, F, G, H). Inset images are a higher magnification of the boxed region. Abbreviation: P-Tyr, phosphotyrosine; LA, lavendustin A; GNS, genistein. Scale bar = 20 μm , bar in insets = 10 μm .

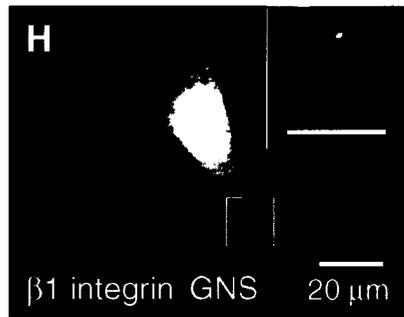
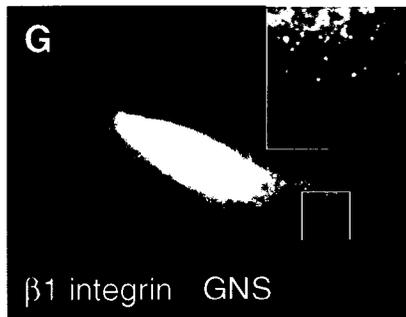
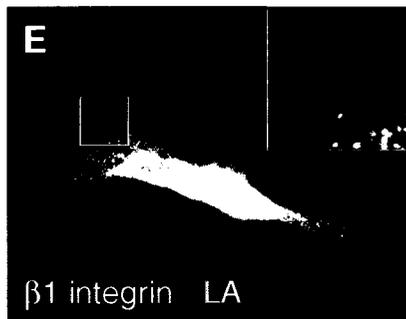
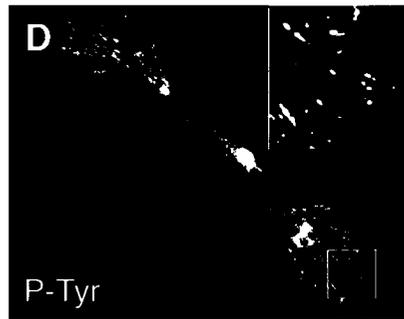
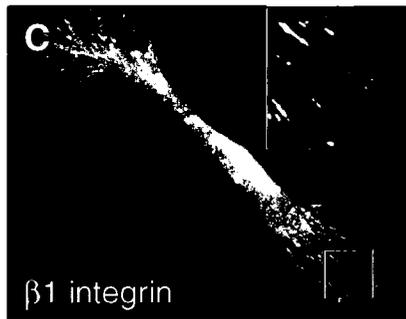
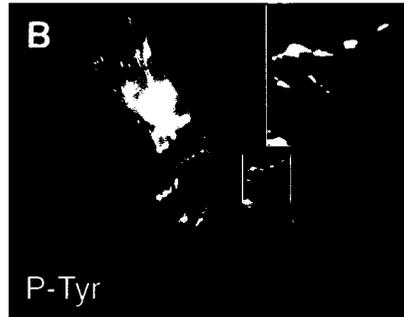
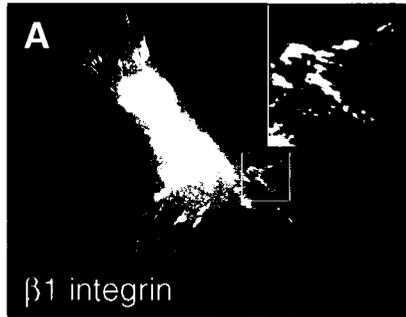


Figure 2. Fluorescence photomicrographs revealing examples of the disruption of focal adhesions and the actin cytoskeleton following treatment of XR1 glial cells with lavendustin A (LA). XR1 cells were plated for 2 hours and then incubated with the inhibitors for 5 hours. Control cells were treated 100 μ M lavendustin B (LB) (A, B, C) and the inhibitor treated cell used with 100 μ M LA (D, E, F). Focal adhesions were identified with β_1 integrin antibody (A, D) and phosphotyrosine antibody (C, F). The F-actin cytoskeleton was labeled with rhodamine phalloidin (B, E). Images in (A and B) and D and E) are of the same cell. Note β_1 integrin and phosphotyrosine-IR were absent from focal adhesions and the actin cytoskeleton was disrupted in LA treated cells. Scale bar = 20 μ m.

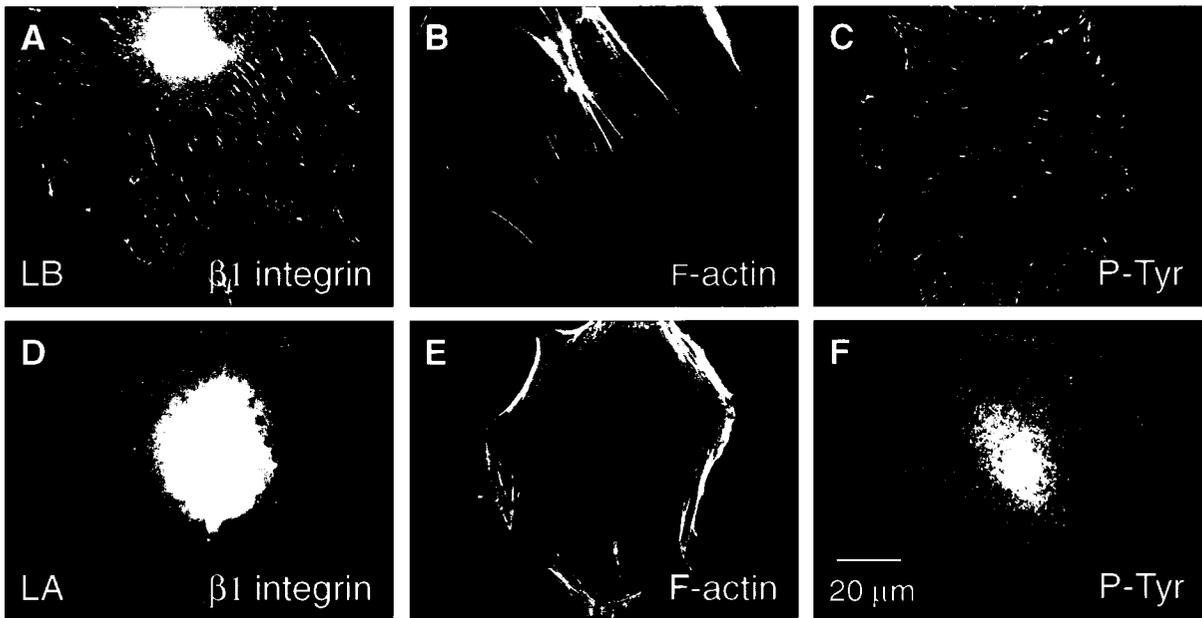
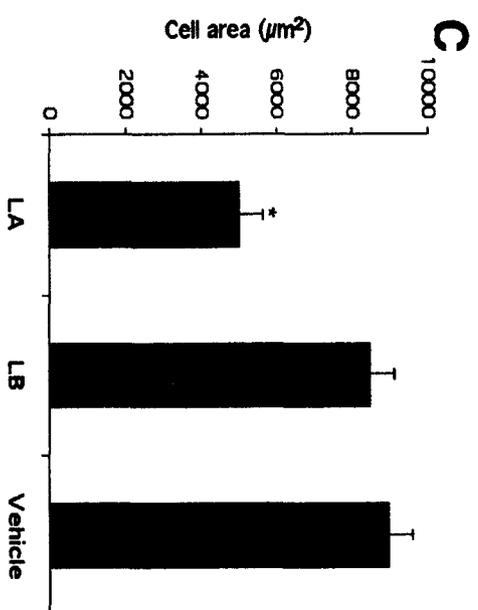
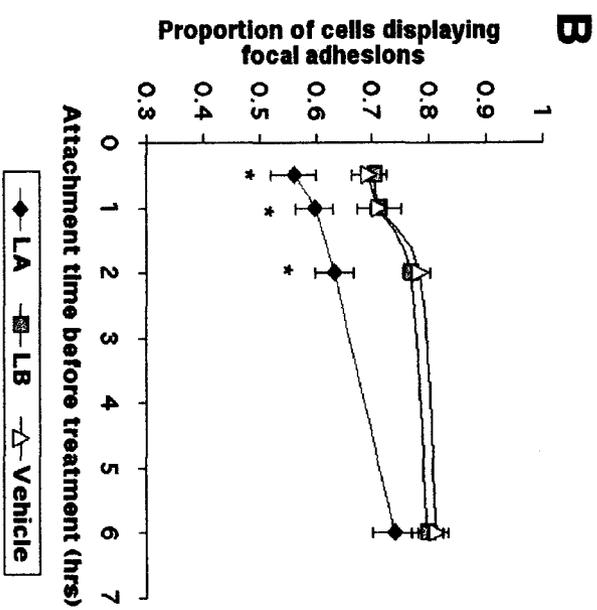
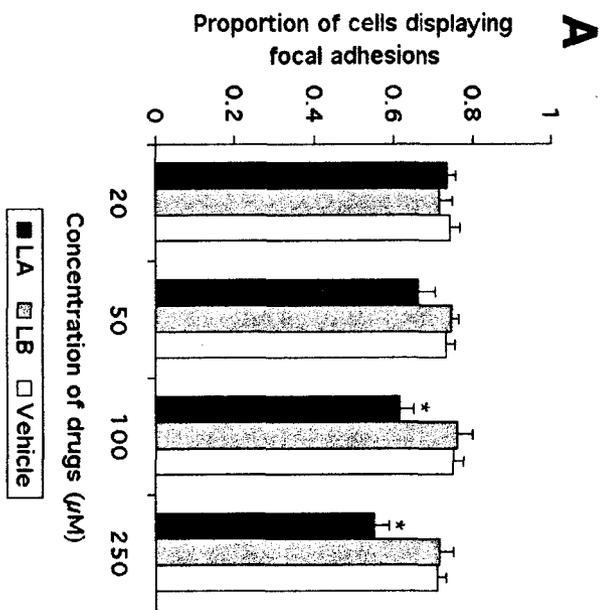


Figure 3. Lavendustin A inhibits focal adhesion assembly and produced a decrease in cell area in XR1 glial cells. A: XR1 cells were allowed to attach and spread for 2 h and then incubated with lavendustin A (20, 50, 100 and 250 μM), an equal concentration of lavendustin B, or culture media with DMSO for 5 h. The proportion of cells displaying focal adhesions was then determined using anti- β_1 antibodies. B: XR1 cells were allowed to attach for 0.5, 1, 2 or 6 h and subsequently incubated with 100 μM LA, LB or vehicle media for 5 h. Focal adhesions were identified with β_1 integrin-IR. The values are expressed as the percentage of cells displaying focal adhesions from three experiments. At least 150 cells were examined for each treatment. C. Cell area measurements were obtained from captured images ($n = 44$) with NIH Image 1.58 VDM software at conditions in which cells were plated for 2 h and incubated with 100 μM drugs for 5 h. Error bars represent means \pm S.E.M.; *, statistically significant at $p < 0.05$.



thus resistant to inhibitor treatment. Tyrosine kinase activity appears to be necessary for the initial formation of focal adhesions, but seems to be less crucial to the maintenance of focal adhesions.

In addition to the decreased proportion of cells displaying focal adhesions, many XR1 cells that were subjected to the lavendustin A treatment exhibited a round or spindle shaped morphology, rather than their usual flattened morphology as in the control cultures. Cell area measurements revealed a significant decrease in the average XR1 cell area following treatment with lavendustin A at 100 μM (Fig. 3C, $p < 0.05$). These results indicate that tyrosine kinase activity appears to be involved in regulating cell spreading on ECM substrates. These results using lavendustin A are consistent with our previous studies using genistein, another tyrosine kinase inhibitor (Folsom and Sakaguchi, 1997). Thus, the inhibition of focal adhesion assembly and cell spreading by lavendustin A and genistein indicates that tyrosine kinase activity is intimately involved in integrin-mediated cell-ECM interactions.

Inhibition of tyrosine kinase activity disrupts retinal development

Nonreceptor tyrosine kinases have been identified to be developmentally regulated in the chick neural retina (Ingraham et al., 1992; Sorge et al., 1984). The distribution of phosphotyrosine proteins was mapped with an anti-phosphotyrosine antibody during *Xenopus* retinal development. Phosphotyrosine-IR was detected throughout the retinal neuroepithelium and overlying ectoderm at stage 25, and was especially intense at the interface between the optic vesicle and ectoderm (Fig. 4A). As the retina continues to develop, phosphotyrosine-IR was highly expressed in the optic fiber layer (OFL) and the nascent inner plexiform layer (IPL) at stage 37 (Fig. 4B). In addition to the IPL and OFL, phosphotyrosine-IR was highly present in the outer plexiform layer (OPL) at stage 40 and 47 (Fig. 4C, 4D). In addition, the somata comprising the nuclear layers were also outlined with phosphotyrosine-IR (Fig. 4C, D). These

results suggest the differential regulation of phosphorylation of tyrosine proteins during *Xenopus* retinal development.

To investigate the possibility that protein tyrosine kinases (PTKs) are important in regulating embryonic retinal development *in vivo*, we bath-applied two inhibitors of PTK activity, lavendustin A and genistein, directly to the optic vesicle beginning at St 23 to St 25. Genistein and lavendustin A are competitive inhibitors of adenosine triphosphate in the kinase reaction, with specific effects on PTK, but not on PKA or PKC. The exposed eye preparation, similar to the exposed brain preparation (Chien et al., 1993; McFarlane et al., 1995; Worley and Holt, 1996) permits direct access of reagents to the optic vesicle during eye morphogenesis. At this stage of development the eye is a relatively undifferentiated neuroepithelium. Embryos were incubated in the presence of the inhibitors, or control drugs, until stage 40 at which time the retina is normally well differentiated, exhibiting its distinct laminar organization (Fig. 4).

Embryos incubated with the inhibitors appeared healthy and developed at a normal rate when compared to control embryos. However, those eyes exposed to the PTK inhibitors displayed severe defects in the pattern of retinal lamination. The histogenesis was analyzed with anti-synaptic vesicle protein SV2 and anti-photoreceptor protein antibodies, SV2 and XAP-1, respectively. Synaptic vesicle protein SV2 is a transmembrane transporter in the vesicle and dominantly associated at the nerve terminal (Feany et al., 1992); and XAP-1 protein correlates with outer segment assembly of photoreceptors (Wohabrebbi et al., 2002). In control-treated retinas (vehicles and lavendustin B) SV2-IR clearly demarcated the OPL and IPL (Fig. 5A, B), and the discrete band of photoreceptor outer segments was labeled with the XAP-1 antibody (Fig. 5D, E). In striking contrast, in lavendustin A-treated eyes, SV2-IR was observed scattered throughout the retina rather than localized to discrete layers (Fig. 5C). In addition, the pattern of photoreceptors was disrupted in the lavendustin A-treated when compared with control retinas. Although XAP-1-IR was localized to the outer retina, the labeling was discontinuous and patchy (Fig. 5F).

Figure 4. The expression pattern of phosphotyrosine proteins during retinal development. A-D is tissue section of St 25, 37, 40, and 47, respectively, labeled with phosphotyrosine antibody. Phosphotyrosine-IR was detected in neuroepithelial cells, with strong immunoreactivity at the interface between the neuroepithelium and ectoderm (arrows, A). As retinal development continues, phosphotyrosine-IR was highly expressed in the IPL, OPL and OFL (C, D). Lower levels of expression were observed in the outer segment of the photoreceptors (C). Abbreviation: RPE, retina pigment epithelium; OS, outer segment of photoreceptors; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; RGC, retinal ganglion cells; OFL, optic fiber layer. Scale bar = 20 μ m

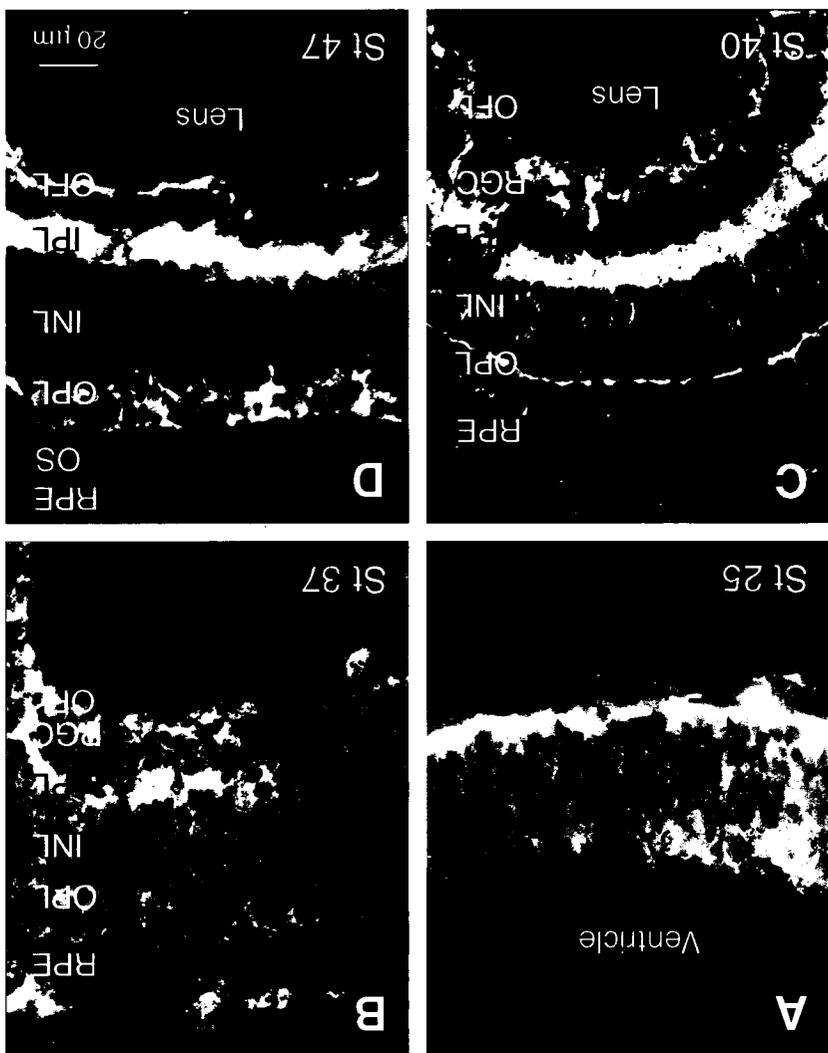


Figure 5. Fluorescence photomicrographs revealing the disruption of retinal lamination following treatment of *Xenopus* embryos with 50 μ M lavendustin A (LA). The inhibitors were applied to St 25 embryos and they were allowed to survive for 48 hours to stage 40. Immunohistochemistry was performed with SV2 antibody (A, B, C) and photoreceptor antibody, XAP-1 (D-F). The control, lavendustin B (LB)-treated retina displayed the same pattern (B, E) as the vehicle-treated retinas (A, D). In LA treated retinas the plexiform layers were no longer organized in a distinct IPL and OPL (C), and photoreceptors were disrupted (F). Abbreviation: RPE, retinal pigment epithelium; OS, outer segment of photoreceptors; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 20 μ m.

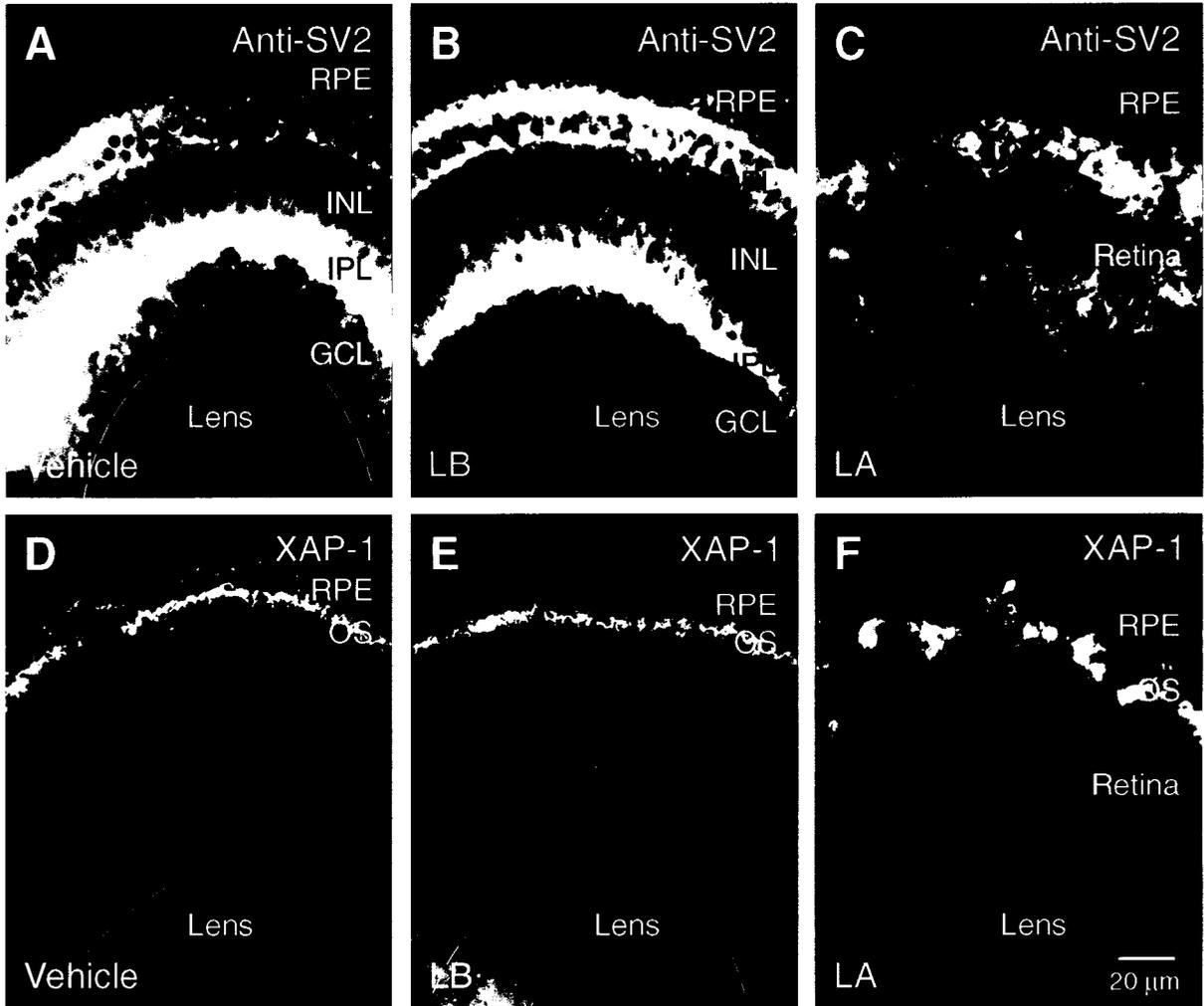
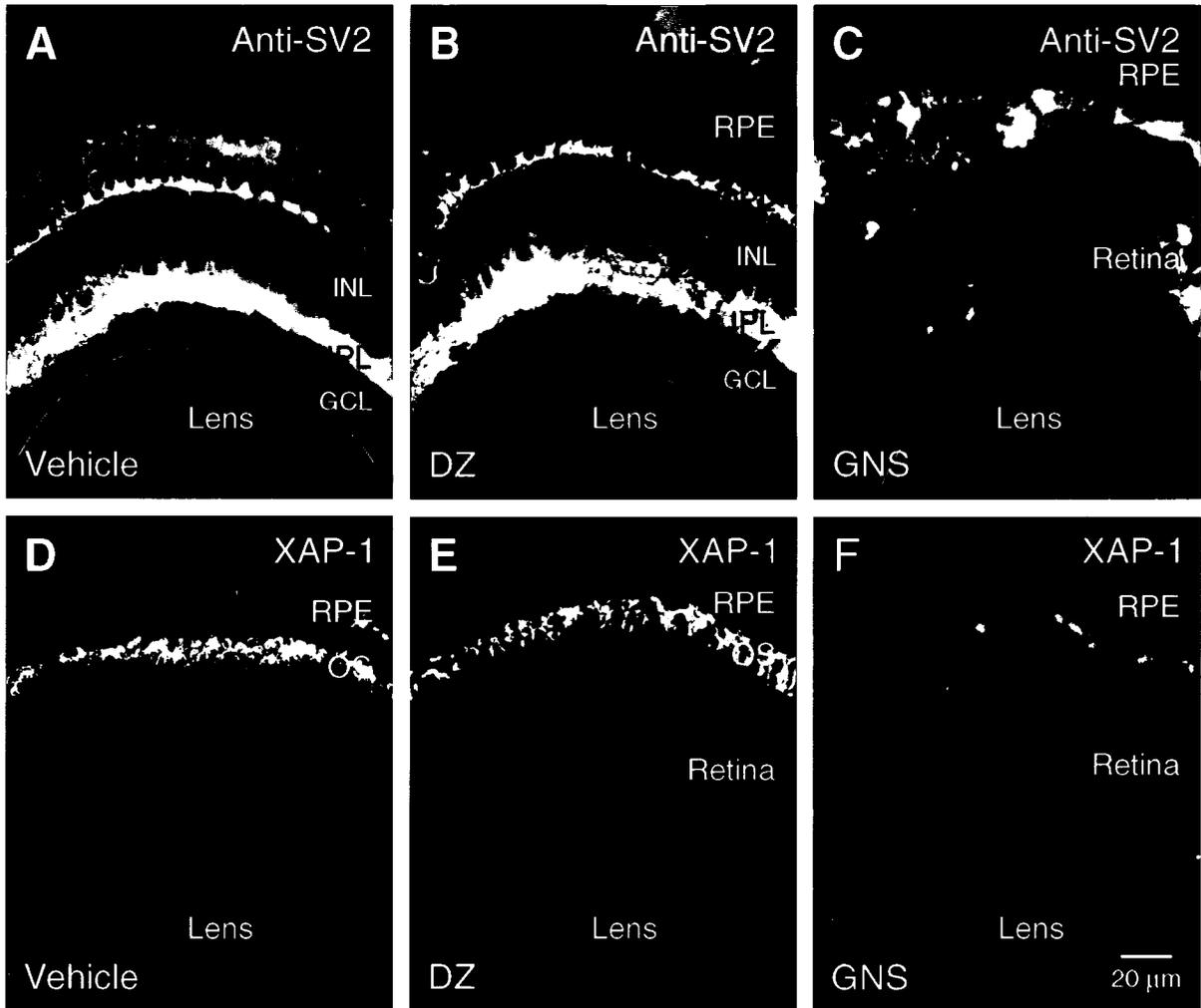


Figure 6. Fluorescence photomicrographs revealing the disruption of retinal lamination following treatment of *Xenopus* embryos with 2.5 μ M genistein (GNS). The inhibitors were applied to St 25 embryos and they were allowed to survive for 48 hours to stage 40. Immunohistochemistry was performed with SV2 antibody (A-C) and photoreceptor antibody, XAP-1 (D-F). The control, daidzein (DZ)-treated retinas displayed the same pattern (B, E) as the vehicle-treated retinas (A, D). In genistein (GNS)-treated retina the plexiform layers were no longer organized to a distinct IPL and OPL (C), and photoreceptors were disrupted (F). RPE, retinal pigment epithelium; OS, outer segment of photoreceptors; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar = 20 μ m.



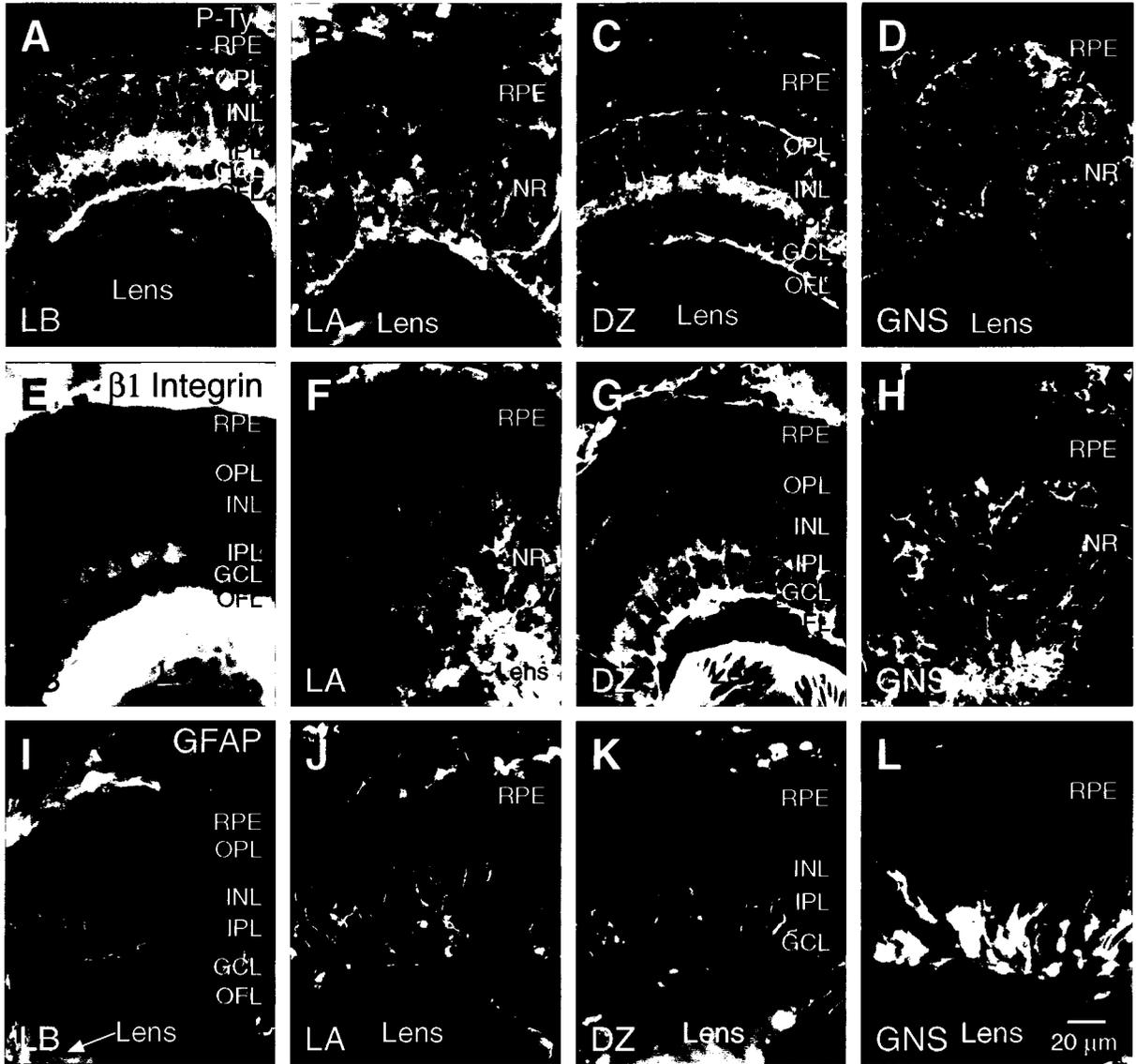
Since genistein also blocked focal adhesion formation in XRI glial cells (Folsom and Sakaguchi, 1997), we therefore applied genistein to embryonic retinas to confirm that the disruption of retinal development was due to the inhibition of tyrosine kinase activity. Treatment of the optic vesicles with genistein also produced severe disruption of retinal lamination and photoreceptor differentiation (Fig. 6). In contrast to the controls, in the genistein-treated retinas SV2-IR was located predominantly in the outer half of the retina but failed to localize to a discrete OPL or IPL (Fig. 6C). Furthermore, analysis of photoreceptors showed that the normal pattern of XAP-1-IR was severely disrupted, with sparse IR in the outer retina in the genistein-treated embryos (Fig. 6D).

Although severe defects in retinal lamination were observed in the PTK inhibitor treated animals, there were no obvious deleterious effects on general development, since the embryos themselves appeared to develop normally. In addition, when the inhibitors lavendustin A and genistein were applied to older embryos at St 33/34 for 24 hours (to stage 40), the lamination of the retinas were not effected, and the retinas appeared indistinguishable from controls (data not shown). This result is consistent with the study by Worley and Holt in which St 32 *Xenopus* embryos were incubated with PTK inhibitors and retinal lamination was not disrupted (Worley and Holt, 1996). Taken together, our results indicate that tyrosine kinase activity was essential for neuroepithelial cells in synaptogenesis and photoreceptor morphogenesis during early retinal development and the disruption in the patterning of the developing retina was not due to nonspecific drug cytotoxicity.

Inhibition of PTK activity alters the expression pattern of phosphotyrosine and β_1 integrins

Our results demonstrate an important role for tyrosine kinase activity during early retinal development. To investigate possible changes in the pattern of phosphotyrosine protein expression in the inhibitor-treated retinas, we labeled retinas with anti-phosphotyrosine antibody. In the control, lavendustin B- and daidzein-treated retinas, phosphotyrosine-IR was

Figure 7. Fluorescence photomicrographs revealing the disruption of the pattern of phosphotyrosine-, β_1 integrin- and GFAP-IRs in the retina following treatment of *Xenopus* embryos with 50 μ M lavendustin A and 2.5 μ M genistein. The inhibitors were applied to St 23 to 25 embryos and they were allowed to survive for 48 hours to stage 40. Immunohistochemistry was performed with phosphotyrosine (A-D), GFAP (E-H) and β_1 integrin (H-K) antibodies. Abbreviation: LA, lavendustin A; LB, lavendustin B; GNS, genistein; DZ, daidzein; RPE, retina pigment epithelium; NR, neural retina; OS, outer segment of photoreceptors; OPL, outer plexiform layer; IPL, inner plexiform layer; OFL, optic fiber layer. Scale bar = 20 μ m.



expressed in all retinal cells, and clearly outlined the somata in the nuclear layers, and was intense in the OPL, IPL and OFL (Fig. 7A, C). In contrast, the pattern of phosphotyrosine-IR in the inhibitor-treated retinas was disrupted and the IR was no longer prevalent in distinct inner and outer plexiform layers (Fig. 7B, D).

β_1 integrins have been shown to be vital for glial cell attachment and spreading on ECM substrates (Sakaguchi and Radke, 1996). Our *in vitro* results indicate that PTK inhibitors disrupted focal adhesion formation mediated by β_1 integrins in XR1 glial cells. Moreover, β_1 integrins are highly expressed in the Müller glial cells in the developing *Xenopus* retina (Li and Sakaguchi, 2002). We examined the expression pattern of β_1 integrins to determine if the pattern was altered in the PTK inhibitor-treated retinas. In the control retinas, β_1 integrin-IR was strongly expressed in Müller cells and astrocytes in the retina, displaying a radially oriented pattern of IR (Fig. 7E, G). In contrast, although β_1 integrin-IR was highly expressed in the inhibitor-treated retinas, no radial pattern of organization was observed and β_1 integrin-IR was in disorder (Fig. 7F, H).

The radially oriented Müller glial cells are likely to be involved in establishing and maintaining the columnar organization of the retina (Willbold and Layer, 1998). To investigate if Müller cell differentiation may be effected by inhibition of tyrosine kinase activity we used anti-GFAP antibodies to label *Xenopus* retina Müller glial cells, as well as the astrocytes in the inner limiting membrane (ILM). In normal, as well as in control-treated retinas, GFAP-IR was expressed in a regular pattern in the radially oriented Müller cells (Fig. 7I, K). In contrast, GFAP-IR was highly disordered in the PTK inhibitor-treated retinas (Fig. 7J, L) and it appeared that in some cases, the expression of GFAP in Müller cells was more intense within the PTK inhibitor-treated eyes (Fig. 7L). This implies that the cell-cell and cell-ECM interactions were disrupted.

Neural retinal detachment from the RPE was frequently observed in the PTK inhibitor-treated eyes. Retinal detachment was observed in 7 of 9 lavendustin A-treated embryos, and in

11 of 12 genistein-treated embryos, while detachment was seldom observed in the untreated or control-treated eyes (chi-squared analysis, $P < 0.001$). The retinal detachment was highly correlated with the disruption of expression pattern of phosphotyrosine-, β_1 integrin- and GFAP-IRs in the inhibitor-treated retina. It is possible that inhibition of tyrosine kinase activity may produce a decrease in adhesion of the neural retina to the retinal pigment epithelium, or of these cells to the extracellular matrix.

Discussion

Two inhibitors of PTKs, lavendustin A and genistein, were used to investigate the possible role of tyrosine kinase activity during retinal development *in vitro* and *in vivo*. Focal adhesions were identified in primary cultures of retinal neuroepithelial cells and application of the PTK inhibitors to these cultures blocked focal adhesion assembly. To more carefully examine the roles of tyrosine kinase activity in regulating focal adhesion assembly we used *Xenopus* XR1 glial cells as a model cellular system. Lavendustin A produced a dose-dependent decrease in the proportion of XR1 cells displaying focal adhesions. The role of tyrosine kinase activity was also investigated during early retinal development in *Xenopus* embryos. We found that inhibition of tyrosine kinase activity with lavendustin A or genistein disrupted the establishment of retinal lamination and also effected photoreceptor differentiation during early retinal development. These results suggest that tyrosine phosphorylation is necessary to regulate neuroepithelial cell migration, cell differentiation and neurite outgrowth during retinal development.

Lavendustin A and genistein are considered to be broad-spectrum protein tyrosine kinase inhibitors, effecting the activities of many receptor and nonreceptor tyrosine kinases *in vitro*, including Src, Yes, the PDGFR and EGFR, but with trivial effects on protein kinase A or C (Akiyama et al., 1987; Onoda et al., 1989). Both are competitive inhibitors of ATP in the reaction, but noncompetitive with the protein substrates. The inactive analogs of lavendustin A

and genistein, lavendustin B and daidzein, respectively, were used as controls. The drugs were dissolved in DMSO, and DMSO has been found to stimulate tyrosine phosphorylation (Earp et al., 1983). Thus, control cultures received either lavendustin B, daidzein, or vehicle DMSO, at the same concentration as the drugs. The controls displayed no obvious effects, while both lavendustin A and genistein produced similar defects in our *in vitro* and *in vivo* studies, suggesting that the blocking of focal adhesion formation and the disruption in retinal development was due to the inhibition of tyrosine kinase activities.

Our *in vitro* studies show that focal adhesions are formed by cultured retinal neuroepithelial cells and lavendustin A and genistein were effective inhibitors of focal adhesion assembly. The treatment of XR1 retinal glial cells with these inhibitors produced a dose-dependent decrease in the proportion of cells displaying focal adhesions. The treatment also disrupted the actin cytoskeleton and blocked cell spreading. This indicates that tyrosine kinase activity was required for both focal adhesion assembly and stress fiber formation in XR1 glial cells. However, these inhibitors of tyrosine kinase activity do not completely disrupt focal adhesion formation in XR1 glial cells thus providing evidence that tyrosine kinases are not the only factors regulating focal adhesion assembly and integrity. Our studies are consistent with others suggesting that multiple factors such as protein kinase C and Rho are involved in regulating focal adhesion assembly (Crowley and Horwitz, 1995; Defilippi et al., 1995; Yap et al., 1994).

Recent studies provide a better understanding of the signaling pathways activated by integrin adhesion receptors. Integrin receptor binding to ECM and clustering leads to the formation of focal adhesions and the activation of multiple signaling pathways (Critchley, 2000; Giancotti and Ruoslahti, 1999). Integrins activate protein tyrosine kinases, including FAK, Src family kinase, and Abl and increase tyrosine phosphorylation of focal adhesion associated proteins. Thus, integrin-mediated signaling cooperates and integrates with other signaling

pathways to regulate cellular processes including gene expression, cell cycle control and cell survival (Giancotti and Ruoslahti, 1999).

Inhibition of tyrosine kinase activity blocked integrin-based focal adhesion formation and cell spreading in *Xenopus* retinal neuroepithelial cells and XR1 glial cells. Thus, tyrosine kinase activity is involved in the integrin-mediated adhesion and signaling. At least eight integrin subunits have been identified to be highly expressed in the developing retina and they may play vital roles in regulating cell migration, determination of cell fate, neurite outgrowth and synaptogenesis (Clegg, 2000). When the tyrosine kinase inhibitors were applied to *Xenopus* retinas, it is likely that integrin-mediated protein tyrosine kinase activity was inhibited. The plexiform layers failed to form in the inhibitor-treated retinas and therefore it is likely that neurite outgrowth was inhibited. This is consistent with the results of Worley and Holt (Worley and Holt, 1996) in which lavendustin A inhibited axon extension through the embryonic optic tract and reduced neurite outgrowth in *Xenopus* retinal cultures. There are similarities in the phenotypes produced by the inhibition of tyrosine kinase activity and by blocking integrin function in the retina. Expression of chimeric β_1 integrin in *Xenopus* embryos impaired the outgrowth of axons and dendrites from retinal ganglion cells (Lilienbaum et al., 1995). Functionally, β_1 integrins have been implicated in mediating neurite outgrowth during retinal development and regeneration (Sakaguchi and Radke, 1996). Another possible effect is the inhibition of retinal cell migration from the ventricular border to the vitreal border by PTK inhibitors. This effect may be similar to that produced by inhibition of integrin function with blocking antibodies (Cann et al., 1996).

The expression pattern of phosphotyrosine proteins and β_1 integrins was severely altered, and the detachment of neural retina from the RPE frequently occurred in the inhibitor-treated retinas. The retinal detachment caused by lavendustin A and genistein treatment is likely due to the decrease of phosphorylation of intracellular proteins that results in the decrease of adhesion, including integrin binding to their appropriate substrates. Normal photoreceptor

development and function relies on the neural retina closely adherent to the RPE or interphotoreceptor matrix (Libby et al., 2000). The retinal detachment is one of the important causes leading to photoreceptor degeneration (Guerin et al., 1993). The blockage of photoreceptor inner and outer segment assembly is likely due to the retinal detachment or directly due to the inhibition of PTK activity that can be initiated by extracellular signals including integrin-mediated adhesion. In addition, GFAP expression appeared to be up regulated in Müller glial cells in some cases. The increase of GFAP expression is a sign of reactive glia that protect the retina from disruption of cell-cell and cell-ECM interactions. The radially oriented Müller glial cells have been hypothesized to be involved in organizing and maintaining the columnar organization of the retina (Willbold and Layer, 1998). Much evidence indicates that retinal Müller cells could protect photoreceptors and ganglion cells from stress stimuli, which induced dramatic upregulation of GFAP in retinal Müller cells (Chu et al., 1998; de Raad et al., 1996; Lewis et al., 1994; Peterson et al., 2000). The defects observed in treated retinas indicate that the PTK inhibitors disrupted cell-cell and cell-ECM interactions.

It is likely that integrin-mediated adhesion activates tyrosine kinase activity *in vivo*, as *in vitro*, and regulates a variety of cellular processes. Many extracellular and intracellular signals contribute to retinal development (Clegg et al., 2000). Our studies indicate that inhibition of tyrosine kinase activity blocked focal adhesion formation in retinal cells *in vitro* and disrupted normal retinal development *in vivo*. These results suggest that tyrosine kinase activity plays an essential role for neuroepithelial cell adhesion, differentiation and neurite outgrowth during retinal development, and the integrin-mediated adhesion and signaling may be involved in all these cellular processes.

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CHAPTER 4. INHIBITION OF INTEGRIN MEDIATED ADHESION WITH ECHISTATIN DISRUPTS *XENOPUS* RETINAL DEVELOPMENT

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Abstract

Integrins are the major family of cell adhesion receptors that mediate cell adhesion to extracellular matrix (ECM). Integrin-mediated adhesion and signaling play important roles in neural development. In this study we have used echistatin, a RGD containing short monomeric disintegrin, to investigate the role of integrin-mediated adhesion and signaling during retinal development in *Xenopus*. Application of echistatin to *Xenopus* retinal derived XR1 glial cells inhibited the three stages of integrin-mediated adhesion: cell attachment, cell spreading, and formation of focal adhesions and stress fibers. XR1 Cell attachment and spreading increased tyrosine phosphorylation of paxillin, a focal adhesion associated protein, while echistatin significantly decreased phosphorylation levels of paxillin. Application of echistatin to early *Xenopus* retina disrupted retinal lamination and produced rosette structures with ectopic photoreceptors in the outer retina. These results indicate that integrin mediated cell-ECM interactions play a critical role in cell adhesion, migration and morphogenesis during retinal development.

Key words: *Disintegrin, Echistatin, Integrin, Paxillin, Focal Adhesion, Retinal Development*

Introduction

Integrins are the most prominent family of cell adhesive receptors for extracellular matrix (ECM) molecules. Each integrin forms a heterodimer that contains an α and a β subunit

(Hynes, 1992). In vertebrates 18 α and 8 β subunits have thus far been identified which can form 24 functional integrin receptors (van der Flier and Sonnenberg, 2001). The combination of the α and β subunits determines the ligand binding specificity, affinity, and intracellular signaling activity of the integrin receptors (Hynes, 1992). β_1 is a prominent subunit, which can associate with 12 α subunits to form heterodimers (van der Flier and Sonnenberg, 2001). β_1 subunit can combine with α_4 , α_5 , α_8 , or α_v subunits to form heterodimer receptors, which bind to RGD (Arg-Gly-Asp) containing ECM components, such as fibronectin, vitronectin.

Integrin ligand binding leads to the formation of focal adhesions where integrins link the ECM to intracellular cytoskeletal complexes and bundles of actin filaments (Critchley, 2000). These protein assemblies play important roles in stabilizing cell adhesion and regulating cell shape and motility. Integrins also mediate transmembrane signal transduction via signaling molecules recruited to focal adhesions (Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999). Protein tyrosine phosphorylation is one of the intracellular events that transmit extracellular cues into cellular responses (Maness and Cox, 1992; Juliano and Haskill, 1993). For example, tyrosine phosphorylation of focal adhesion kinase and paxillin has been observed in many cell types in response to cell attachment to fibronectin or other ECM proteins (Burrige et al., 1992). Focal adhesion kinase (FAK) is a 125 kD nonreceptor tyrosine kinase, which is recruited to focal adhesions and plays an important role in integrin signaling (Cary and Guan, 1999). Paxillin is a 68 kD focal adhesion associated adapter protein implicated in the regulation of integrin signaling and organization of the actin cytoskeleton (Cary and Guan, 1999; Turner, 2000).

In recent years disintegrins have become powerful tools to investigate the functional roles of integrin mediated adhesion and signaling (Staiano et al., 1997; Della Morte et al., 2000; Chavis and Westbrook, 2001). Disintegrin is a family of low molecular weight, disulfide-rich, RGD containing proteins derived from the venom of various snakes (Gould et al., 1990). They can bind to integrin receptors on the cell membrane and are potent inhibitors of platelet

aggregation and integrin mediated cell adhesion (Dennis et al., 1990). Echistatin is a 5400 D monomeric disintegrin derived from the venom of the saw-scaled viper, *Echis carinatus* (Gan et al., 1988). Echistatin expresses an RGD sequence at the apex of the integrin binding loop with four disulfide bounds and is an inhibitor of RGD dependent integrins, including $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ (Marcinkiewicz et al., 1996; Thibault, 2000; Smith et al., 2002)

We previously demonstrated a functional role for β_1 integrins in regulating cell spreading, neurite outgrowth and axon pathfinding in *Xenopus* retina (Sakaguchi and Radke, 1996; Stone and Sakaguchi, 1996). In this study we have used echistatin to investigate the functional role of integrins during retinal morphogenesis. We have observed the effects of echistatin on *Xenopus* retinal derived XR1 glial cell attachment, focal adhesion formation and integrin mediated signaling on fibronectin substrates and also analyzed the effect of echistatin on early retinal development *in vivo*. These results indicate that integrin mediated adhesion and signaling are essential for retinal development.

Materials and Methods

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). All animal procedures were carried out 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). 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% embryonic 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 Hank's 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 tetra-acetic 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 (RBS-35; Pierce, Rockford, IL) washed glass coverslips (Fisher Scientific Co., Pittsburgh, PA) coated with 10 µg/ml fibronectin substrate (Upstate Biotechnology). Cultures were grown at room temperature (~ 24 °C).

Cell adhesion assay. Resuspended XR1 cells were diluted to 1.0×10^5 cells/ml after counting and cell viability evaluation with trypan blue exclusion. Cell suspension was added into 35 mm plastic dishes containing four 10 µg/ml fibronectin-coated coverslips. Echistatin (Sigma), GRGDSP or GRGESP peptides (Life Technologies-Gibco BRL, Grand Island, NY) were added into the dishes to final concentrations of 2.5, 5, and 10 µg/ml, 50, 100, and 200 µg/ml, respectively, immediately after the cells were plated. The plated cells received nothing as a control. The cells were allowed to attach for 30 min. and subsequently the cultures were fixed and stained with rhodamine-phalloidin. On each of the 4 coverslips images from 16 fields using a 20x objective were captured and the number of adherent cells was counted. The data were normalized as the percentage of attached cells in treated groups versus the attached cells in the control group.

Focal adhesion assay. XR1 glial cells were allowed to adhere to fibronectin-coated coverslips for one hour, and exposed to 2.5 $\mu\text{g/ml}$ echistatin for 2 hours, and fixed and processed for immunocytochemistry with anti- β_1 integrin antibody. Cultures were examined using a 40 X oil immersion objective. In previous studies we identified focal adhesions on XR1 cells as discrete streak-like patterns of immunoreactivity where β_1 integrins were colocalized with vinculin or phosphotyrosine immunoreactivity at the termini of F-actin filaments (Folsom and Sakaguchi, 1997). As such, in this communication we have defined focal adhesions as discrete streaks of β_1 integrin-IR. Seventy-two microscope fields of 180 μm x 140 μm were examined for each condition from cells prepared from three separate culturing sessions. In each field, cells were scored as positive if focal adhesions were present and negative if absent. The proportion of cells displaying focal adhesions was calculated for each group. Data were represented as means \pm SEM and analyzed using the Student's t-test.

Cell area measurements. XR1 cultures were examined using a 20 X objective and images were captured as described above. Cell area measurements were obtained from captured images using NIH Image 1.58 VDM software (Wayne Rasband, National Institutes of Health, Bethesda, MD). A known distance 100 μm was measured for calibration, and outlining the cell perimeter produced a calculation of cell area. Twenty-four fields of 360 x 280 μm with more than 50 cells were examined for each condition. Data were represented as means \pm SEM and were analyzed using the Student's t-test.

Immunoprecipitation and western blot analysis. XR1 glial cells were plated onto fibronectin-coated dishes for one hour, and exposed to 2.5 $\mu\text{g/ml}$ echistatin for 2 hours. Cells were scraped from the bottom of the dishes and placed in lysis buffer (0.1 M NaCl, 10 mM Tris, pH 7.6, 1 mM EDTA, 0.2% NP-40, 1 $\mu\text{g/ml}$ aprotinin, 2 mM Na_3VO_4 and 1 mM PMSF). Samples were

homogenized, and protein concentration determined using a Bio-Rad protein assay kit. Protein samples were also obtained from cells in suspension, cells attached for one and three hours on fibronectin-coated dishes. Anti-paxillin antibody was added to the cell lysate, and the preparation was gently rocked at 4°C overnight. A Protein G agarose bead slurry was added and incubated at 4°C for 2 hours. Beads were collected by pulsing 5 seconds in microcentrifuge at 14,000 rpm, and rinsed three times with ice-cold cell lysis buffer. The agarose beads were resuspended in SDS-sample buffer (0.5 M Tris-HCl, 10% SDS, 10% glycerol, 2.5% bromophenol blue, 5% β -mercaptoethanol). Protein samples were boiled and separated on 7.5% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose, blocked overnight with 1.5% BSA in Tris-buffered saline (TBS, 10 mM Tris-HCl, 150 mM NaCl, pH 8.0), and incubated with antibodies directed against phosphotyrosine for one hour. Control blots using anti-paxillin antibody were run to confirm equal loading of paxillin in the precipitates. After washing in TBS with 0.1% tween-20, the membranes were incubated with 1:5000 goat anti-mouse IgG-alkaline phosphatase for 45 min. The blots were visualized with NBT/BCIP (Promega, Madison, WI). Densitometric analysis was performed with NIH Image 1.58 VDM software.

In vivo treatment with echistatin. Embryos between stages 23 -25 were anesthetized by immersion in 100% modified Ringer's solution (100 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES) containing 1:10,000 MS222 (ethyl 3-aminobenzoate, methanesulfonic acid salt, Aldrich, Milwaukee, WI). The skin overlying the left optic vesicle was carefully removed, and the embryos placed in Holtfreter's solution with 2.5 μ g/ml fungibact and 2.5 μ g/ml penicillin-streptomycin in the presence of 10 μ g/ml echistatin or nothing for 48 hours up to St 40. 5 animals of St 40 from each treated and control group were transferred into Holtfreter's solution and allowed to develop up to St 47. The tadpoles were processed for immunohistochemical analysis.

Immunohistochemistry. *Xenopus* larvae and cultured cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h (animals) or 30 min. (cells). The specimens were rinsed with buffer and cryoprotected in 30% sucrose in 0.1 M PO_4 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 using a cryostat (Reichert HistoSTAT) and sections were thaw mounted on Superfrost microscope slides (Fisher). For antibody labeling procedures the tissue sections and cultures were rinsed in phosphate buffered saline (PBS, 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 , 1.47 mM KH_2PO_4) and blocked in 5% goat serum, containing 0.4% BSA and 0.2% Triton X-100 in PBS. Primary antibodies were diluted in blocking solution and preparations incubated overnight at 4°C. On the following day the preparations were rinsed with PBS and incubated with appropriate secondary antibodies conjugated to Alexa 488 or RITC (diluted 1:200 in blocking solution) for 90 min. at room temperature and subsequently rinsed and mounted under glass coverslips. For double-labeling immunocytochemistry, a biotinylated goat anti-mouse IgG (1:300, Vector Laboratories Inc.) and avidin-AMCA (1:1000, Vector laboratories Inc.) were used following the second primary antibody incubation. These preparations were subsequently triple-labeled with rhodamine-phalloidin (1:300, 30 min. from Molecular Probes, Eugene, Oregon) to visualize the F-actin cytoskeleton. As a control, single label studies were performed parallel to the multi-labeling to rule out that similar patterns were due to bleed-through and the other fluorescence channels were also examined to ensure that no bleed-through occurred. Negative controls were performed in parallel by omission of the primary or secondary antibodies. No antibody labeling was observed in the control.

Antibodies. β_1 integrin receptors were identified using monoclonal antibody 8C8, purchased from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) and diluted 1:10 with blocking solution and polyclonal anti- β_1 integrin (3818, a gift from Dr. K. Yamada,

Lab of Molecular Biology, NCI, Bethesda, MD, diluted to 20 µg/ml). Anti-paxillin, clone 439 (Transduction Laboratory) was diluted at 1:100, anti-phosphotyrosine monoclonal antibody, 4G10 (Upstate Biotechnology Inc.) was diluted at 1:200. Photoreceptors were identified using anti-*Xenopus* photoreceptor antibody. XAP-1 diluted 1:20 (Sakaguchi et al., 1997). Anti-synaptic vesicle protein, SV2 antibody (Developmental Studies Hybridoma Bank (university of Iowa, Iowa City, IA) was at diluted 1:20. Goat anti-mouse IgG, IgM or goat anti-rabbit IgG secondary antibodies conjugated with Alexa 488 or RITC were purchased from Southern Biotechnology (Birmingham, AL), or Molecular Probes.

Analysis of fluorescence images. Tissue sections or cultured cells were examined using a Nikon Microphot-FXA photomicroscope (Nikon Inc. Garden City, NY) -equipped with epifluorescence. Images were captured with a Kodak Megaplug 1.4 CCD camera connected to a Perceptics Megagrabber framegrabber using NIH Image 1.58 VDM software in a Macintosh computer (Apple Computer, Cupertino, CA). Analysis of multi-labeled tissues was performed on a Leica TCS-NT confocal scanning laser microscopy (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).

Results

Echistatin inhibits XR1 retinal glial cell attachment to fibronectin

Echistatin, a disintegrin, is a potent inhibitor of integrin mediated cell adhesion and has been shown to inhibit rabbit retinal pigment epithelial cell attachment and mouse melanoma cell attachment to fibronectin and vitronectin (Staiano et al., 1995; Yang et al., 1996). α_v , α_5 and β_1 integrin subunits have been identified to be expressed on XR1 retinal glial cells (Folsom and Sakaguchi, 1997; Sakaguchi, unpublished observation). Integrin $\alpha_v\beta_1$ is a receptor for

fibronectin and vitronectin, while $\alpha_5\beta_1$ is a receptor for fibronectin. Both fibronectin and vitronectin contain the classic integrin binding motif, the RGD sequences. To identify whether echistatin and RGD containing peptides could disrupt integrin-fibronectin interaction in XR1 cells, a cell adhesion assay was performed. XR1 cells were plated to fibronectin coated coverslips in the presence of different concentrations of echistatin or GRGDSP peptides. GRGESP peptide-treated or untreated cells served as control. Figure 1 shows the inhibitory effect of echistatin and GRGDSP peptides on XR1 cell attachment to fibronectin substrates. GRGDSP and echistatin inhibited XR1 cell attachment to fibronectin in a dose-dependent manner while control GRGESP peptides did not inhibit XR1 cell attachment (Fig. 1). At 5 or 10 $\mu\text{g/ml}$, echistatin inhibited cell binding to fibronectin by more than 98%. At 2.5 $\mu\text{g/ml}$ echistatin blocked XR1 cell adhesion by approximately 90%, which was more effective than GRGDSP peptides at a concentration of 200 $\mu\text{g/ml}$ (86%). On a molar basis echistatin was approximately 700 times more potent than GRGDSP peptide at inhibiting XR1 cell attachment to fibronectin.

This echistatin induced inhibition of cell attachment was not due to cytotoxicity. When the non-attached cells were collected, rinsed and replated, almost all the cells attached and spread onto fibronectin coated coverslips within 30 minutes. In addition, trypan blue exclusion analysis indicated that there were no differences about cell viability between the XR1 cells treated with 10 $\mu\text{g/ml}$ echistatin for 2 hours and the untreated cells.

Echistatin blocks the formation of focal adhesion and stress fibers in XR1 glial cells

Focal adhesions are discrete streak-like complexes, composed of clustered integrins and associated structural and signaling proteins that link the ECM to the cytoskeleton and mediate cell adhesion and signaling. To investigate the possibility that echistatin may disrupt focal adhesion formation, we cultured XR1 cells for 1 h on fibronectin substrate and then applied echistatin, or GRGDSP peptide to the cultures for 2 h. Control cultures received GRGESP

peptide or nothing in culture media. Focal adhesions were identified with antibodies directed against β_1 integrin, paxillin and phosphotyrosine, and the F-actin filaments were visualized with rhodamine-phalloidin. Figure 2 showed the images in which the formation of focal adhesions and stress fibers was disrupted by echistatin. In the control cultures, immunoreactivities (IRs) for β_1 integrin, paxillin and phosphotyrosine were colocalized to focal adhesions located at the termini of well formed F-actin stress fibers (Fig. 2A-H). In contrast, in the echistatin treated XR1 cells, focal adhesions rarely formed and β_1 integrin-, paxillin- and phosphotyrosine-IRs did not localize to discrete streaks (Fig. 2I, J, M, N). Moreover, F-actin cytoskeleton was severely disorganized (Fig. 2K, O). Focal adhesions and the actin cytoskeleton appeared normal in the GRGDSP peptide treated XR1 cells and no differences were observed between the peptide-treated and -untreated cells (data not shown). Quantitative analysis of focal adhesions in XR1 cells showed that no significant difference in the proportion of cells displaying focal adhesions was observed between GRGDSP peptides treated cultures and the controls (Fig. 3A). However, cultures treated with echistatin displayed a significant reduction in the proportion of cells displaying focal adhesions (Fig. 3A, $p < 0.01$). These results suggest that echistatin effectively blocked β_1 integrin, paxillin or other focal adhesion associated phosphotyrosine proteins from localizing to focal adhesions.

In addition to the decreased proportion of cells displaying focal adhesions, many of the echistatin treated XR1 cells exhibited a round or spindle shaped morphology, rather than their usual flattened morphology (Fig. 2). Cell area measurements revealed a significant decrease in the average XR1 cell area following treatment with echistatin at 2.5 $\mu\text{g/ml}$ (Fig. 3B, $p < 0.01$). At higher concentration of echistatin cell retracting and detachment was frequently observed. These results indicate that echistatin disrupted integrin-mediated XR1 cell-ECM interactions, which blocked cell spreading, and assembly of focal adhesions and actin stress fibers.

Figure 1. Echistatin inhibits XR1 cell attachment to fibronectin substrate in a dose-dependent fashion. Freshly suspended XR1 cells were plated onto fibronectin coated coverslips in the presence of echistatin, GRGDSP or GRGESP peptides in the culture media at concentration of 2.5, 5, and 10, 50, 100, and 200, respectively. After 30 minutes of exposure, attached cells were fixed and stained with rhodamine-phalloidin, and cells were counted. The number of cells was normalized as a percentage of adhered cells in the treated groups compared with the adhered cells in the control group. Data reported as the mean \pm SEM from three experiments from separate culturing sessions. *, statistically significant from the control group at $p < 0.01$.

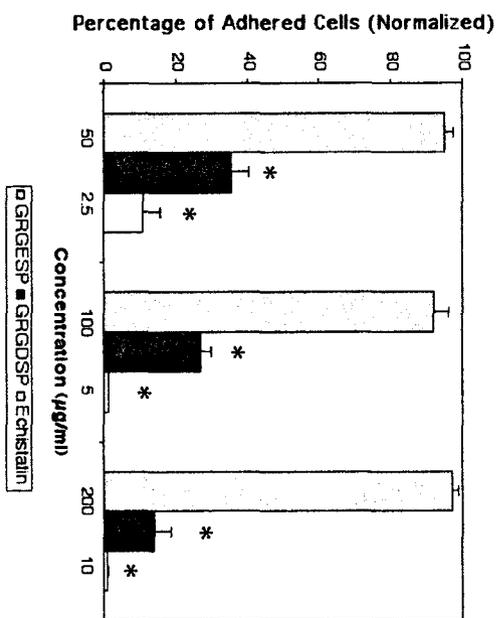


Figure 2. Echistatin disrupts focal adhesion assembly in XR1 cells.

Fluorescence photomicrographs reveal the disruption of focal adhesions and the F-actin stress fibers following echistatin treatment of XR1 glial cells. XR1 cells were plated for 1 h and then incubated with 2.5 $\mu\text{g/ml}$ echistatin for 2 h. Focal adhesions were identified with β_1 integrin antibody (A, E, I, M), paxillin antibody (B, J) and phosphotyrosine antibody (F, N). F-actin filament was labeled with rhodamine phalloidin (C, G, K, O). D, H, L, P were the merged images of the left triple labeling. Note that β_1 integrin-, paxillin- and phosphotyrosine-IRs were absent from focal adhesions and actin stress fibers were not formed in echistatin treated cells (I-P) compared with the control (A-H). Abbreviations: P-Tyr, phosphotyrosine; Ech, echistatin. Scale bar = 20 μM .

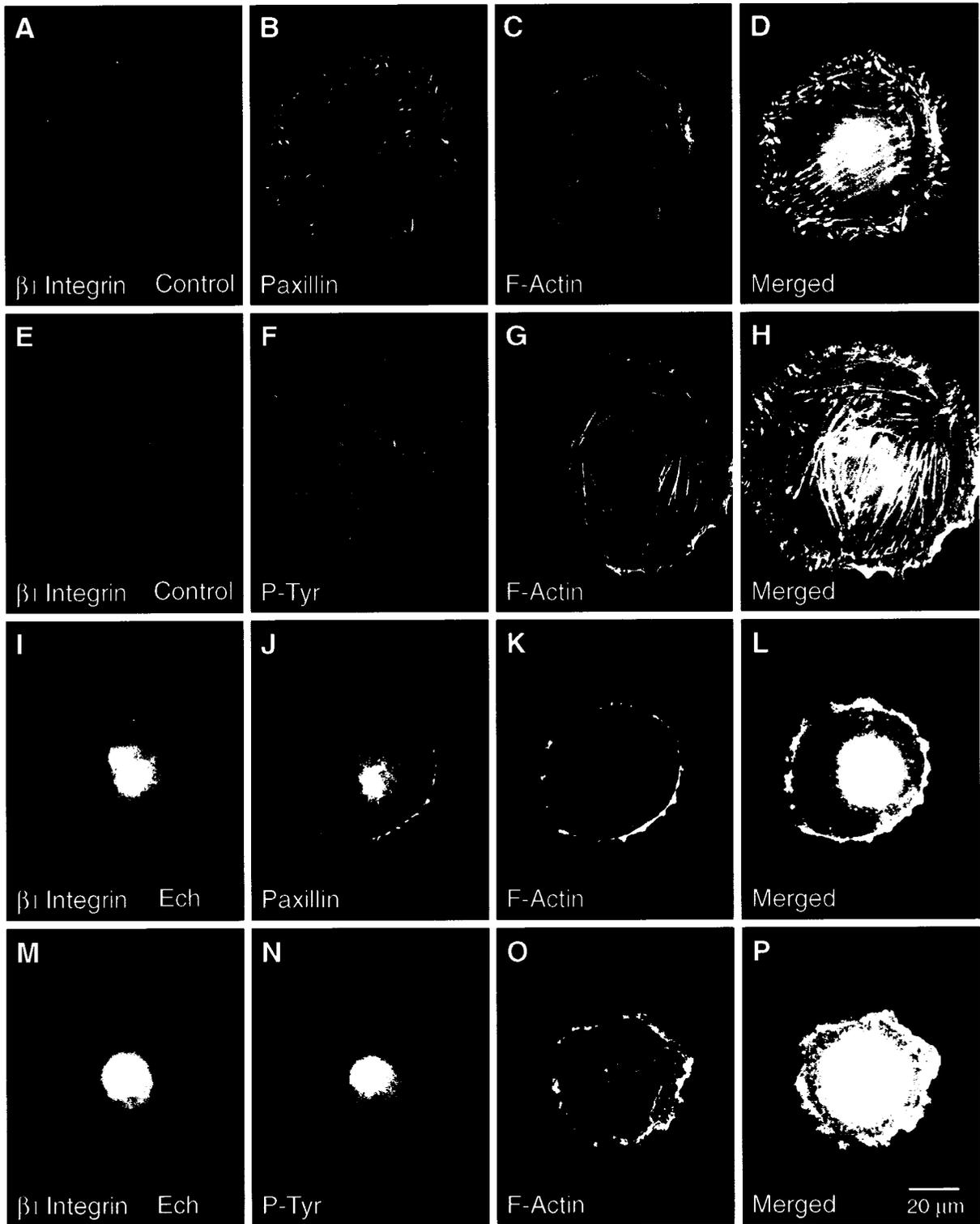
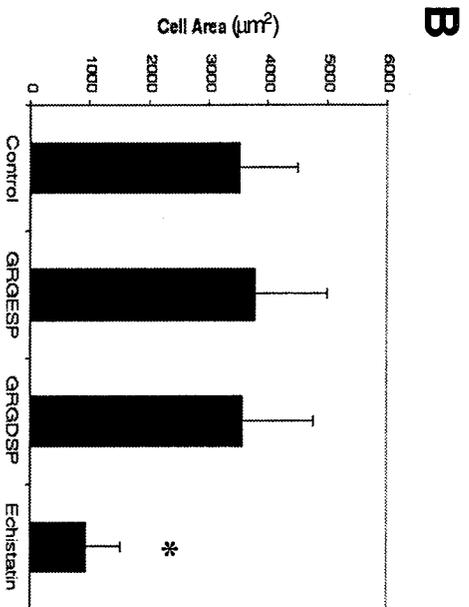
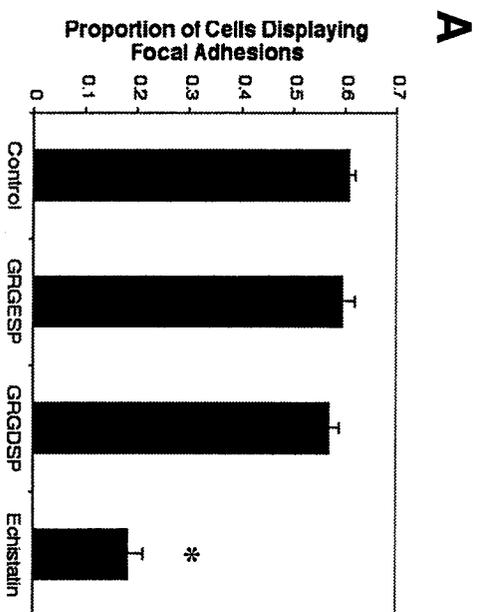


Figure 3. Echistatin blocks focal adhesion assembly and XR1 cell spreading.

XR1 cells were allowed to attach and spread for 1 h and incubated with echistatin at 2.5 $\mu\text{g/ml}$ in the culture media for 2 h. A. Focal adhesions were identified with β_1 integrin-IR. The values were expressed as the percentage of cells displaying focal adhesions from three experiments. At least 150 cells were counted for each treatment. B. Cell area measurements were obtained from captured images ($n = 50$) with NIH image 1.58 VDM software. Error bars represent \pm SEM; *, statistically significant at $p < 0.01$.



Echistatin reduces tyrosine phosphorylation levels of paxillin

Cell adhesion to substrates activates protein tyrosine kinases and leads to an increase of tyrosine phosphorylation of several focal adhesion associated proteins (Burrige et al., 1992; de Curtis and Malanchini, 1997). As we showed above, echistatin inhibited XR1 cell adhesion of all the stages: attachment, cell spreading and formation of focal adhesions and actin stress fibers. Paxillin and other phosphotyrosine proteins did not localize to focal adhesions in the echistatin treated XR1 cells (Fig. 2). To investigate if echistatin disrupted integrin signaling in the XR1 cells, we assessed the tyrosine phosphorylation levels of paxillin (Fig. 4). XR1 cells were allowed to adhere to fibronectin substrates for 1 h and then exposed to echistatin for 2 h. Phospho-paxillin was just detectable in suspended cells, while the phosphorylation levels of paxillin had significantly increased after the cells were attached for 1 h. The levels had no more increase after XR1 cells were attached for 3 h, however, the phosphorylation levels of paxillin were significantly decreased when the XR1 cells were exposed to echistatin (Fig. 4A, C). This result indicates that echistatin blocked integrin mediated signaling.

Echistatin disrupts retinal lamination

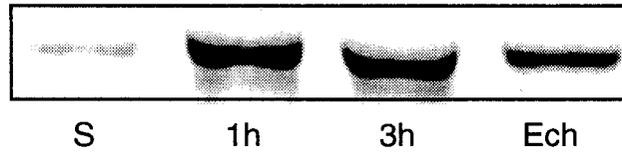
β_1 integrins and focal adhesion associated proteins have been identified to be differentially regulated during *Xenopus* retinal development (Li and Sakaguchi, 2002). To investigate the roles of β_1 integrin mediated adhesion and signaling during early retinal development *in vivo*, we bath-applied echistatin to the optic vesicle. The exposed eye preparation, similar to the exposed brain preparation (Chien et al., 1993; McFarlane et al., 1995; Worley and Holt, 1996) permitted the direct access of the echistatin to the optic vesicle. At the optic vesicle stage the retina is a relatively undifferentiated neuroepithelium. These embryos were incubated in the presence of echistatin until stage 40, when the retina is normally well differentiated, exhibiting its distinct laminar organization.

Figure 4. Echistatin reduces paxillin phosphorylation in fibronectin adherent XR1 glial cells. XR1 cells were in suspension for 1h or allowed to adhere to fibronectin coated dishes for 1 h and then exposed to 2.5 $\mu\text{g/ml}$ echistatin for 2 h. Cell lysates were immunoprecipitated with anti-paxillin antibody and subsequently separated by electrophoresis. After blotting, tyrosine phosphorylated proteins were probed with anti-phosphotyrosine antibody (A), and paxillin was probed with anti-paxillin (B). The absorbance of bands corresponding to phosphorylated paxillin in A was determined by densitometric analysis and the values on y axis represent the means \pm SEM in arbitrary units from three separate experiments of identical design (C). The absorbance values of paxillin bands in B were with less than 7% difference.

A

IP: Paxillin

Probe: P-tyr

**B**

Probe: Paxillin

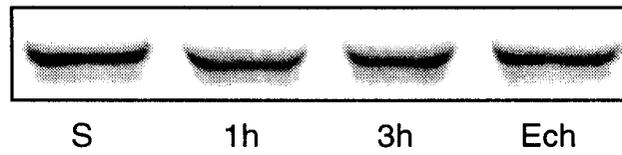
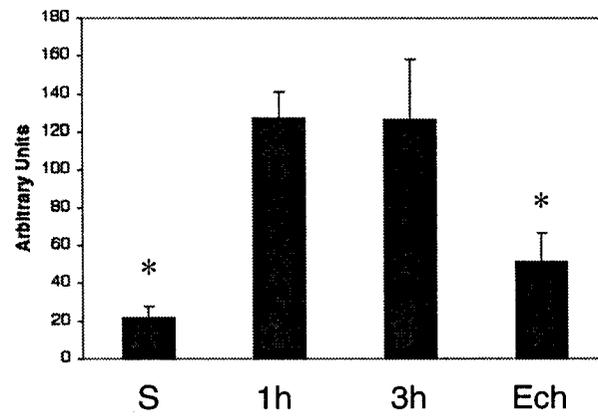
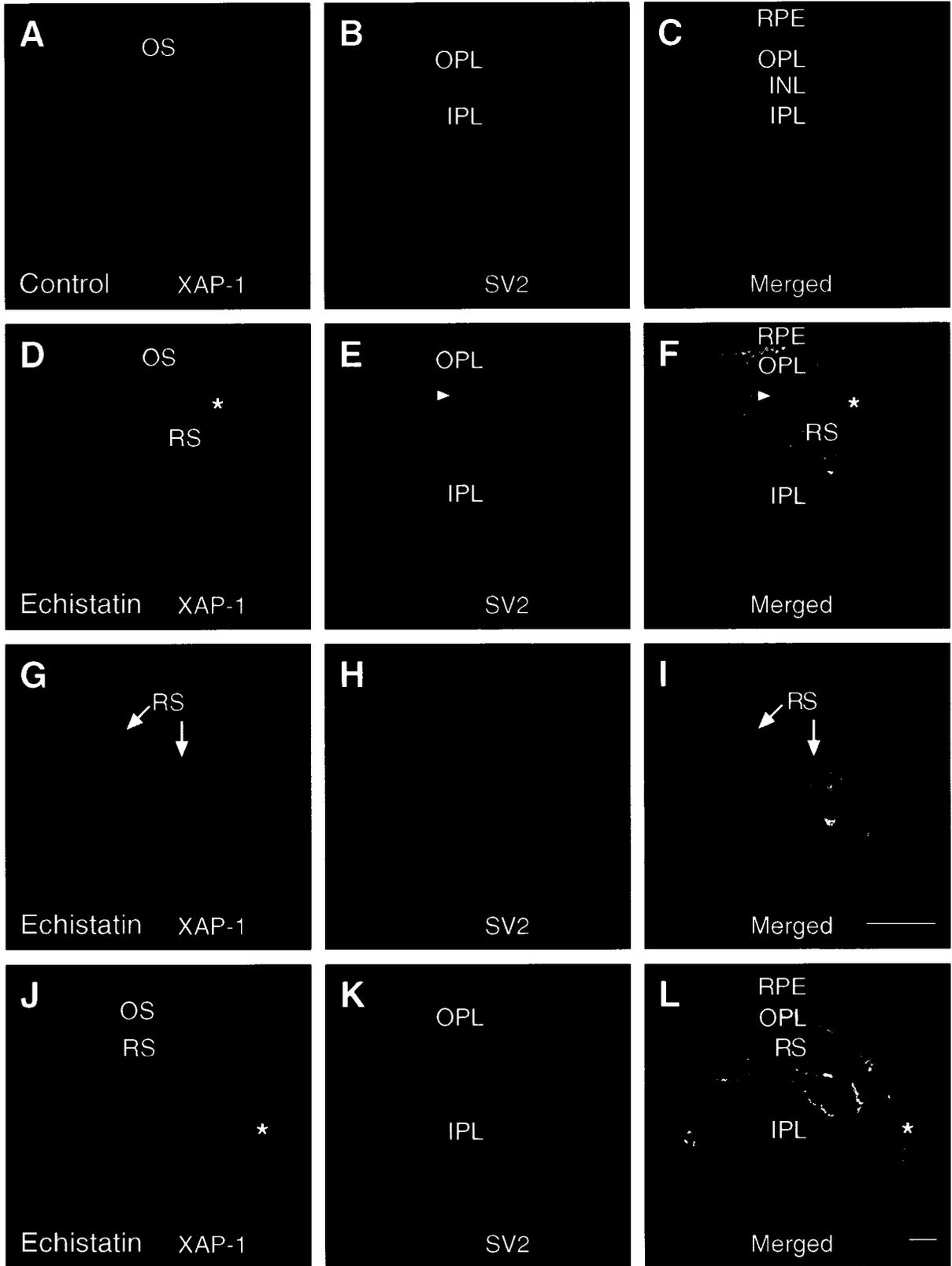
**C**

Figure 5. Echistatin disrupts retinal development. Confocal images reveal the disruption of retinal lamination following echistatin treatment of *Xenopus* embryos. Embryonic retinas at stage 25 were treated with 10 $\mu\text{g/ml}$ echistatin and survived for 48 hours to stage 40. Immunohistochemical analysis was processed with anti-synaptic protein antibody, SV2 (A, D, G) and anti-photoreceptor antibody, XAP-1 (B, E, H). A-C, the untreated contralateral retina; D-I, echistatin treated retina. G, H, I are higher magnification images corresponding to D, E, F. Note that rosette structures (RS, arrows in G-I) were produced, and extra plexiform layer (arrowhead) and a break of XAP-1-IR (asterisk) in the outer segment of photoreceptors was produced in echistatin treated retina. Abbreviations: OPL, outer plexiform layer; IPL, inner plexiform layer; RS, rosette structures. Calibration scale bar = 20 μM (in L applied to A-F and J-L; in I applied to G-I).



Embryos incubated with echistatin appeared healthy and developed at a normal rate when compared to control embryos. However, those eyes exposed to echistatin displayed severe defects in the pattern of retinal lamination. The histogenesis of retina was analyzed with anti-synaptic protein SV2 and anti-photoreceptor protein antibodies, SV2 and XAP-1, respectively. Synaptic vesicle protein SV2 is a transmembrane transporter in vesicles that are located predominantly to the nerve terminal (Feany et al., 1992); while XAP-1 protein correlates with inner and outer segment assembly of photoreceptors (Wohabrebbi et al., 2002). In control retinas the labeling pattern for SV2 antibodies clearly demarcated the OPL and IPL (Fig. 5A, C), while the XAP-1 antibody labeled the discrete band of photoreceptor outer segments (Fig. 5B). In 20 out of 23 echistatin-treated retinas, retinal lamination was strikingly disrupted, particularly in the outer retina (Fig. 5D-L). In all the defective retinas ectopic photoreceptors were observed usually forming circular clusters of cells or rosette structures (Fig. 5D, G, J), and XAP-1-IR was not continuous in the outer segments of photoreceptors (Fig. 5D, J). Infoldings of the photoreceptor layer were often observed (data not shown). Displaced plexiform layer was formed between the normal OPL and rosette structures (Fig. 5E, F). In 4 out of the 5 treated animals that were allowed to develop to St 47 in normal solution, rosette structures with extended outer segments of photoreceptors were observed, but the defects in the retinas seem not as severe as the defects in the treated retinas at St 40.

Discussion

Cellular interactions with the ECM and neighboring cells profoundly influence a variety of signaling events including those involved in adhesion, migration, proliferation, survival and differentiation (Giancotti and Ruoslahti, 1999; Hynes, 1999). In this paper we have investigated the functional role of ECM major receptor integrins during early retinal development. Echistatin, a RDG containing disintegrin, is a useful molecule in the *in vitro* and *in vitro*

perturbation studies. We have shown that echistatin disrupted the interactions between integrin receptors and ECM substrates. Echistatin inhibited retinal XR1 glial cells attachment to fibronectin, and also blocked cell spreading and focal adhesion assembly in XR1 cells. The inhibitory activity reduced paxillin tyrosine phosphorylation, an important event of integrin mediated signaling. Moreover, application of echistatin to embryonic retina disrupted retinal lamination and induced the formation of ectopic photoreceptors in the outer retina. These results indicate that integrin mediated adhesion and signaling is essential for retinal morphogenesis.

Cell adhesion occurs in three stages: attachment, spreading, and formation of focal adhesions and actin stress fibers (Burrige et al., 1988). Focal adhesions, characteristic of strong cell adhesion, consist of clustered integrins and associated structural and signaling molecules that link the ECM and actin cytoskeleton (Jockusch et al., 1995). At the initial stage, cell attachment involves the interactions between integrins and ECM substrates, and the integrin activation that induces integrin clustering and increases integrin affinity. At the intermediate stage, cells increase their surface contact area on the ECM substrates through cell spreading. All these lead the formation of focal adhesions and stress fibers, which needs appropriate extrinsic and internal signals (Humphries, 1996; Hughes and Pfaff, 1998; Schoenwaelder and Burrige, 1999). Through focal adhesions and stress fibers integrins bi-directionally transmit mechanical and biochemical signals that are extracellular and intracellular in origin (Howe et al., 1998; Giancotti and Ruoslahti, 1999).

Perturbation studies have shown that integrin-mediated selective adhesion plays a critical role in regulating cellular processes during early development (Darribere et al., 2000). Recently, echistatin, GRGDSP and integrin functional blocking antibody were reported to block synaptic maturation at a hippocampal synapse *in vitro* (Chavis and Westbrook, 2001). In our study, although both echistatin and GRGDSP peptides inhibited XR1 cell attachment to fibronectin, echistatin is about 700 fold more effective than RGD containing peptides at

inhibiting cell attachment. This is consistent with other studies, in which echistatin were about 200 times potent than RGD containing peptides in inhibiting RPE cell attachment to fibronectin substrates, and about 1000 times at inhibiting platelet aggregation (Gould et al., 1990; Yang et al., 1996). Furthermore, echistatin effectively blocked cell spreading and focal adhesion formation in XR1 cells, but GRGDSP peptides were ineffective. This difference of inhibitory effect is most likely due to the different configuration of the molecules. The optimal conformation of the RGD loop as well as to the amino acid sequences flanking the RGD locus in echistatin determine the specificity and affinity against the RGD dependent integrins (McLane et al., 1996; Marcinkiewicz et al., 1997; Wierzbicka-Patynowski et al., 1999; Smith et al., 2002).

Echistatin has been shown to bind with high affinity to $\alpha_v\beta_3$ or $\alpha_5\beta_1$ integrin receptor (Kumar et al., 1997; Wierzbicka-Patynowski et al., 1999) as well as $\alpha_3\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$ (Thibault, 2000). In addition to β_1 subunit, α_v and α_5 subunits are expressed in XR1 glial cells (Sakaguchi, unpublished observation). In the presence of 2.5 $\mu\text{g/ml}$ echistatin, XR1 cell adhesion was reduced and most of the cells began rounding up or retracting, and cell detachment was occasionally observed, while at higher concentration of 10 $\mu\text{g/ml}$ echistatin a large part of the cells detached from fibronectin coated coverslips. It is most likely that echistatin competes for integrin receptors at the cell surface, and focal adhesions organized by β_1 containing integrins may represent the privileged site of its action. Staiano and colleagues (1997) has reported that echistatin caused disassembly of focal adhesions and detachment of well attached melanoma cells under serum-free medium. Under serum-free medium, GRGDSP peptides could also disrupt focal adhesion formation in XR1 cells (data not shown). It is likely that RGD containing peptides at moderate concentration can inhibit the initial stage of adhesion or weak adhesion without facilitation of other attenuating signals.

In addition to the inhibition of focal adhesion formation, echistatin reduced the tyrosine phosphorylation levels of paxillin. Paxillin, a focal adhesion associated adapter protein, is

implicated in the regulation of integrin signaling (Turner, 2000). The decrease of paxillin phosphorylation indicates that echistatin inhibited integrin signaling in XR1 cells. Ligand binding promotes the conformational change that allows intracellular interactions of integrin tails with cytoskeletal molecules and induces the formation of focal adhesions and current signaling (Clark and Brugge, 1995; Cary and Guan, 1999). For example, in many types of cells, attachment to ECM substrates causes an increase of phosphorylation of focal adhesion kinase pp125^{FAK} and paxillin (Burrige et al., 1992; de Curtis and Malanchini, 1997). Adhesion of XR1 cells to fibronectin substrates induces a rapid increase of tyrosine phosphorylation of paxillin. Echistatin interaction with engaged integrins on cell surface could cause a conformational change that inactivates integrin molecules or reverses the adhesion process. This disruption of integrin mediated adhesion may result in a subsequent blocking of signaling cascade, including tyrosine kinase inactivation, and disassembly of focal adhesions and actin stress fibers. The Staiano group has reported that exposure of melanoma cells to echistatin inhibits paxillin and FAK phosphorylation and causes a dramatic disassembly of focal adhesions with disappearance of both FAK and paxillin (Staiano et al., 1997; Della Morte et al., 2000).

The FAK appears to play a central role in regulating focal adhesion formation and integrin signaling (Richardson and Parsons, 1995; Cary and Guan, 1999). Developmental expression of FAK has been characterized in *Xenopus* (Hens and DeSimone, 1995). In addition, we have detected FAK in different stages of the developing retina with immunocytochemistry and immunoblot (data not shown). Focal adhesion kinase was also expressed in retinal dissociated cultures, and localized to focal adhesions in some flattened glial-like cells. As a control, FAK was detected in focal adhesions in mouse primary astrocyte cultures, however, it is undetectable in XR1 glial cells and no FAK was localized to focal adhesions in XR1 cells. XR1 glial cells are stationary and FAK was likely down regulated. This agrees with other studies, in which FAK deficiency decreased cell motility, suggesting that

FAK may play a role in the turnover of focal adhesions during cell migration (Ilic et al., 1995). It is likely that some homologues or isoforms of FAK may function in the place of the FAK in the integrin signaling. Otherwise, integrin signaling in XR1 cells is independent of FAK.

Application of echistatin to early embryonic retina disrupted retinal lamination and induced rosette structures with ectopic photoreceptors in outer retina. Displaced plexiform layers between the original outer plexiform layer and the rosette structures were also observed. How the rosettes were induced is not clear. Echistatin is membrane impermeable and the removed skin covering optic vesicle grows back in 2 to 4 hours. Only during this period the retinal cells be exposed to the echistatin. In tissue sections of most treated retinas, XAP-1 immunoreactivity did not localize to some parts of the outer segments of photoreceptors, which formed a break. In addition, infoldings of XAP-1-IR were often observed in the treated retinas. It is most likely that the disruption of the interactions between integrins and the ECM caused an invagination of photoreceptor progenitors. All the retinal layers around the rosettes seem in reversed orientation. Rosettes are characteristic structures that are of great concern in developmental biology and medicine, because they have also been observed in retinoblastomas, naturally occurring malformations or in grafts of transplanted embryonic retinas (Liu et al., 1983; Bogenmann, 1986; Ohira et al., 1994; Seiler et al., 1995). In the dissociated cultures of chick retina, rosette structures were formed, however, Müller glial cells or Müller cell-derived factors as well as RPE cells could reorganize dissociated cells into corrected laminated retinal-like structure (Rothermel et al., 1997; Willbold et al., 2000). This indicates that the cell-cell and cell-ECM interactions may have an important role in organizing and maintaining the columnar organization of the retina. These rosettes caused by echistatin may result from the disruption of cellular interactions between neuroepithelial cells with RPE and Müller glial cells or between all the cells with the surrounding ECM. Furthermore, β_1 integrin antibodies and RDG peptides have been shown to disrupt eye morphogenesis after being microinjected into preoptic regions of chick embryos (Svennevik and Linser, 1993). Injection of β_1 integrin function blocking

antibodies into *Xenopus* optic vesicles also caused similar rosette structures in the retinas (Sakaguchi, unpublished data). Taken together, these results suggest that integrin-mediated adhesion and signaling may play a decisive role in determining the position and polarity of retinal cells as well as retinal morphogenesis during development.

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CHAPTER 5. GENERAL CONCLUSIONS

Summary

The long goal of the ongoing research in the Sakaguchi laboratory is to better understand the development and plasticity of the vertebrate nervous system. The retina, with limited cell types and highly organized laminar structure, is easily accessible for experimental manipulation. Thus, retina is an ideal model system for neural developmental studies. Integrins are the major family of cell adhesion receptors for ECM components and are involved in cell-cell and cell-ECM interactions. Integrins also participate in cytoskeletal rearrangements, activation of signal transductions, and co-regulation of growth factor activities. It is clear that integrins are important regulators in cell adhesion, migration, proliferation, differentiation, survival and neurite outgrowth during embryonic development. However, their functions in the developing retina are poorly understood.

In this dissertation, I have investigated the function and mechanism of cell-extracellular matrix interactions during early *Xenopus* retinal development through in vitro and in vivo perturbation experiments. I documented the expression patterns of focal adhesion proteins in the developing retina and the retinal derived cell line, and then carried out a series of perturbation experiments. The results in this dissertation provide evidence that integrin mediated cell-ECM interactions play a critical role in a variety of cellular processes during retinal development. These studies help us get insight into the functions and mechanisms of integrin-mediated adhesion during neural development.

We first characterized the expression patterns of focal adhesion associated proteins, β_1 integrin, talin, vinculin, paxillin and phosphotyrosine in XR1 glial cell line and in the developing *Xenopus* retina (Li and Sakaguchi, 2002). XR1 glial cell line is derived from *Xenopus* retinal neuroepithelium and is a good cell system to investigate the mechanisms of cell-ECM interactions. These proteins are colocalized at focal adhesions located at the termini of F-actin

filaments in XR1 cells. Focal adhesions are characteristic of strong adhesion, and are the sites where integrins link the ECM to the cytoskeleton and bi-directionally transmit signals (Yamada, 1997; Critchley, 2000). These proteins display a spatial and temporal expression pattern and are related with certain morphogenetic events during retinal development. Immunoreactivities for focal adhesion proteins were expressed in neuroepithelial cells, and were especially strong at the interface between the optic vesicle and overlying ectoderm, where the induction of lens occurs, and the future inner limiting membrane forms. These proteins were expressed throughout all retinal layers with higher levels of expression in the plexiform layers, optic fiber layer and in the region of inner and outer limiting membranes at later stages. This suggests that integrin mediated adhesion may be involved in neurite outgrowth and synaptogenesis as well as retinal cell attachment to the limiting membranes. At the region of outer limiting membrane, adherens junctions are formed between Müller glial cells and inner segments of photoreceptors as well as between the cells and ECM components (Jablonski and Ervin, 2000; Libby et al., 2000). These adherens junctions are important to the development and maintenance of photoreceptor outer segment morphologies. In addition, strong immunoreactivities for β_1 integrin, paxillin and phosphotyrosine were expressed in the radially oriented Müller glial cells. The expression pattern of β_1 integrins in *Xenopus* retina is consistent with the pattern identified in chick retina (Hering et al., 2000). This pattern suggests that the integrins are involved in the glial-neuronal interactions, which may be essential for the maintenance of the columnar structure of the retina (Willbold et al., 2000). Some integrin ligands, such as laminin-1 and laminin-3 are also expressed in these areas, and colocalized with β_1 integrins (Lawler and Sakaguchi, 1999). The developmentally regulated co-distribution patterns suggest that focal adhesion proteins are involved in integrin-mediated adhesion and signaling, and are likely to be necessary in regulating retinal morphogenesis.

Integrin mediated adhesion can activate protein tyrosine kinase activity and increase tyrosine phosphorylation of focal adhesion proteins. Tyrosine phosphorylation also regulates

integrin-mediated adhesion and is a major intercellular signaling event to transduce extracellular signals into cellular responses (Romer et al., 1994). Thus, inhibition of tyrosine kinase activity is supposed to block integrin-mediated signaling and disrupts integrin-ECM adhesion. Application of PTK inhibitors blocked focal adhesion assembly in the cultures of dissociated neuroepithelial cells and XR1 glial cells. Moreover, application of the PTK inhibitors to optic vesicles disrupted the lamination of developing retina. The inner and outer plexiform layers were no longer apparent, suggesting that tyrosine kinase activity is essential for neurite outgrowth and synaptogenesis. This is consistent with other studies in which tyrosine kinase activity promoted neurite outgrowth in retinal neurons in vitro and in vivo (Worley and Holt, 1996). Furthermore, the assembly of inner and outer segments of photoreceptors was blocked in the inhibitor treated retina. This disruption was correlated with the alteration of the expression patterns of phosphotyrosine and β_1 integrins as well as the retinal detachment from the RPE caused by the inhibitors. Retinal adhesion or the ECM components such as laminin in the interphotoreceptors as well as signaling initiated by extracellular molecules may contribute to the photoreceptor development and survival (Libby et al., 2000). The disruption of photoreceptor morphologies may be due to the decrease of adhesion caused by PTK inhibitors or the inhibition of tyrosine kinase activity that can be activated by integrin-mediated adhesion. There also is a correlation between these phenotypes caused by PTK inhibitors and the phenotypes caused by disruption of integrin-ECM interactions with integrin functional blocking antibodies, or RGD containing peptides (Svennevik and Linser, 1993; Cann et al., 1996). These results suggest that tyrosine kinase activity is essential for neuroepithelial cell adhesion, migration, neurite outgrowth and photoreceptor development. These results also suggest that the disruption of retinal development may be partially due to the inhibition of integrin-mediated adhesion and signaling.

Finally, we directly blocked integrin-ECM interactions with a disintegrin (or antagonist) echistatin in the in vitro and in vivo perturbation experiments. Echistatin not only inhibited XR1

glial cell attachment to substrates, but also blocked cell spreading and focal adhesion assembly in XR1 cells. In addition, echistatin decreased the tyrosine phosphorylation levels of paxillin, indicating that integrin signaling was inhibited by echistatin. Application of echistatin to embryonic retina disrupted the retinal lamination and induced rosette structures with ectopic photoreceptors in the outer retina. These perturbation results with echistatin are similar with the results caused by injection of β_1 integrin functional blocking antibodies (Leonard and Sakaguchi, 1996). Müller cells and RPE cells as well as their secreted molecules could reorganize dissociated chicken retinal cells into correctly laminated retina in vitro (Rothermel et al., 1997; Willbold et al., 2000). The rosette formation is likely due to the disruption of cell-cell and cell-ECM interactions in the developing retina.

Taken together, these results provide evidence that integrins are likely to play an important role in selective cell-ECM interactions, cell migration, differentiation and neurite outgrowth during retinal development. Focal adhesion associated proteins and tyrosine phosphorylation are involved in the modulation of integrin-mediated adhesion and signal transduction pathways. This research contributes to our understanding of the cellular and molecular basis of neural development.

Future Research

The retina is an idea model system for investigating the cellular and molecular mechanisms for cell-cell and cell-ECM interactions. The detail profiles of what integrins, what ECM molecules are expressed in the retina, and where and when they are expressed, will provide basis for understanding the developmental and functional roles of integrins. In addition to integrin β_1 subunit, the expression patterns of integrin α_5 and α_v subunits as well as laminin-1, laminin-3 and fibronectin have been characterized in the developing *Xenopus* retina (Lawler and Sakaguchi, 1999; Li and Sakaguchi, 1999). In chick retina, seven α and three β subunits ($\alpha_1, \alpha_2, \alpha_3, \alpha_4, \alpha_6, \alpha_8, \alpha_v$ and $\beta_1, \beta_3, \beta_5$) have been documented (Clegg et al., 2000), and in tiger

salamander retina six α subunits (1 to 6) have been identified (Sherry and Proske, 2001). Each of all the molecules has a distinct distribution in the retina. These indicate that a selective adhesion is important for the retinal development and function. It is likely that different integrin receptors would have different distribution and function, and the integrins would have a co-distribution with responding ECM molecules in the developing *Xenopus* retina.

It is also important to characterize the molecules that are involved in the regulation of integrin-mediated adhesion and signaling in the developing retina. We already well know that focal adhesion proteins and cytoskeleton modulate integrin-mediated adhesion and signaling. Integrins link the ECM with cytoskeleton through focal adhesions, where integrins transmit signals bi-directionally. Talin, vinculin and α -actinin are cytoskeletal-associated proteins that link integrin with the F-actin cytoskeleton. These protein-integrin bindings may alter the conformation of integrins and then increase the integrin affinity to their ligands. The cytoskeleton can facilitate the clustering of integrins. Cell adhesion to the substrates initiates signal transduction through activating protein tyrosine kinases and increasing phosphorylation of paxillin, FAK and P130^{CAS} (Cary and Guan, 1999). These proteins are also recruited to focal adhesions and act as regulatory molecules in the integrin signaling. These focal adhesion proteins colocalize at focal adhesions that are located at the termini of actin filaments and are characteristic of strong adhesion in the cultured cells (Li and Sakaguchi, 2002).

In vivo cells in deformable three-dimensional substrata may not form strong adhesion like in cultures. This raises the question of whether events that happen at focal adhesions in vitro also occur in vivo. Do the cytoskeletal proteins regulate integrin-mediated adhesion and signaling proteins mediate integrin signaling in vivo through the same mechanism that occur at focal adhesions? The identification of the distribution patterns of focal adhesion proteins in vivo is a beginning to address such questions. Identification of the association of these proteins in tissue homogenates by co-immunoprecipitation did not produce convincing results. It is likely that the in vitro conditions are not like the microenvironment in the cells. The proper

microenvironment may be necessary for these proteins to associate and function in vivo. Multiple labeling and ultrastructural studies may provide evidence about their associated relationships in subcellular location in vivo.

Tyrosine Phosphorylation and paxillin are involved in integrin mediated adhesion and signaling in our system, which are consistent with other studies (Richardson et al., 1997; Della Morte et al., 2000). A variety of studies indicate focal adhesion kinase (FAK) mediates integrin signaling (Cary and Guan, 1999). Developmental expression of FAK has been identified in *Xenopus* (Hens and DeSimone, 1995). FAK was detected in different stages of the developing retina with immunocytochemistry and immunoblot (data not shown). The FAK was expressed in retinal dissociated cultures, and localized to focal adhesions in some flattened glial-like cells. As a control, FAK was detected in focal adhesions in mouse primary astrocyte cultures, however, it is undetectable in XR1 glial cells and no FAK was localized to focal adhesions in XR1 cells. There is evidence that FAK plays a role in the modulation of cell migration, and focal adhesion kinase deficiency decreases cell motility (Ilic et al., 1995; Ilic et al., 1996). XR1 cells are stationary and FAK is likely down regulated. It is needed to identify if other members of the FAK family or isoforms of FAK are expressed in the XR1 cells, and transgenic expression of FAK into XR1 cells may increase cell motility. XR1 cells may be a good system to study FAK gene expression, and integrin signaling to MAPK in XR1 may be independent of FAK activity.

Some molecules antagonize the pro-adhesion activities of other matrix proteins and regulate cell-ECM interactions. Tenascin-C, TSP1 and SPARC have de-adhesive effects on focal adhesions and can stimulate reorganization of actin stress fibers and disassembly of focal adhesion complexes (Murphy-Ullrich, 2001).

Recently, it has become apparent that metalloproteases modulate the extracellular microenvironment (Chang and Werb, 2001). Over 20 matrix metalloproteases (MMPs) have been identified to degrade the ECM dependent on Zn^{++} binding for proteolytic activity. Another

metalloprotease family is the metalloproteases-disintegrins (ADAMs) that are transmembrane proteins and contain disintegrin and metalloprotease domains, indicative of cell adhesion and protease activities. It is worth to mention that tissue inhibitors of metalloproteases, secreted proteins, exist to selectively antagonize metalloproteases (Chang and Werb, 2001). In addition, ADAMs may be specific to integrin receptors and ECM components (Kamiguti et al., 1997; Souza et al., 2000), and may be useful tools to study the function of certain integrins. Some ADAMs have been identified to express in *Xenopus* embryos (Cai et al., 1998). It is likely that ADAMs are expressed in the retina. The information about which metalloproteases, when and where are expressed, would shed light on the regulation of the cell adhesion, migration and axon outgrowth during retinal development.

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