NOTE TO USERS

Extracellular Matrix Regulates Glomerular Epithelial Cell Survival and Proliferation

by

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ABSTRACT

Glomerular epithelial cells (GECs) are intrinsic components of the kidney glomerulus and are in contact with extracellular matrix (ECM). Under normal conditions, there is little turnover of GECs, but in glomerular injury, apoptosis and proliferation of GECs, and expansion of ECM may lead to sclerosis and impaired glomerular function and/or permselectivity. In order to better understand how adhesion to ECM modulates GEC survival and proliferation, we monitored the activation of the intracellular signaling pathways activated by adhesion to ECM (collagen), along with their coordinate modulation of the growth factor signaling pathways. Adhesion to collagen resulted in the activation of a focal adhesion kinase (FAK)/Src/Grb2 complex, thus activating extracellular signal-regulated kinase (ERK), possibly within focal adhesions. The ERK pathway was necessary to promote survival and growth factor-dependent proliferation. ECM-induced ERK activation was also necessary to achieve and maintain a cortical F-actin structure. There appeared to be a reciprocal relationship between collagen-induced cortical F-actin assembly and collagen-dependent survival signaling, including ERK activation. Moreover, maintenance of the cortical F-actin architecture was associated with increased turnover of inositol phospholipids. Analogous signals for GEC survival are activated in experimental focal segmental glomerulosclerosis (FSGS) in vivo. These studies will provide insights into novel therapeutic approaches to preserving intact glomerular permselectivity.

RÉSUMÉ

Les cellules épithéliales glomérulaires (CEGs) sont les composantes essentielles du glomérule renal et sont en contact avec la matrice extracellulaire (MEC). Dans les conditions normales, il n'y a pas de grand changement des CEGs, mais en cas d'insulte, l'apoptose et la prolifération glomérulaires des CEGs, et l'expansion de la MEC peuvent mener à la sclérose et l'altération du fonctionnement et/ou de la sélectivité glomérulaire. Afin de mieux comprendre comment l'adhérence à la MEC (collagène) contrôle la survie et la prolifération des CEGs, nous avons examiné l'activation des voies de signalisation intracellulaires activées par l'adhérence à la MEC et leur modulation des voies de signalisation des facteurs de croissances. L'adhérence au collagène a eu comme conséquence l'activation d'un complexe contenant FAK/Src/Grb2, ce qui résulte en l'activation de la kinase, ERK, probablement dans les adhérences focales. La signalisation à travers ERK était nécessaire pour favoriser la survie et la prolifération-dependente des facteurs de croissance des CEGs. L'activation de ERK induite par la MEC était également nécessaire pour induire et maintenir une structure de F-actine corticale. Il semblait y avoir un rapport réciproque entre l'organisation de F-actine corticale induite par le collagène et la signalisation de survie provenant du collagène, y compris l'activation de ERK. D'ailleurs, le maintien de l'architecture corticale de F-actine était associé avec une augmentation de la production et l'utilisation des phospholipides d'inositol. Des signaux semblables pour la survie des CEGs sont activés dans un modèle expérimental de glomerulosclérose focale et segmentaire in vivo. Ces études fourniront des perspectives pour de nouvelles approches thérapeutiques dans le but de préserver un selectivité glomérulaire intacte.

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I am forever grateful to my parents, Flora and Artine Bijian, for their continuous interest, assistance, and enthusiasm throughout my studies. They have always supported and encouraged me in all my endeavors.

I firmly believe that the work environment enhances the greater part of the learning experience, and for this I would like to thank my colleagues who have taught me different techniques and assisted me with their useful comments and suggestions. I truly enjoyed my interactions with them.

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Last but not least, I would like to extend my sincerest gratitude to my fiancée and life partner, Claudia D'Abramo, for believing in me. Her constant love, patience and understanding has inspired and encouraged me to take these strides and bring new meaning to my academic and professional life.

PREFACE

This thesis is assembled according to McGill University guidelines (available at http://www.mcgill.ca/gps/programs/thesis/guidelines/preparation/) and consists of:

1) An Abstract in English and in French
2) Acknowledgements
3) Rationale and Objectives
4) Literature Review
5) The Experimental Research
6) Conclusion
7) References
8) Appendix containing license for animal use in research

This thesis includes two original manuscripts, one that has been published and another that will be submitted for publication. Section 2 has been published in the American Journal of Physiology Renal Physiology, 2004, 286(2): F255-66. Section 3 will soon be submitted for publication. In addition, I am a co-author in three published papers where I contributed by developing techniques, designing experiments, analyzing results, reading the manuscript and overall discussion.

1. Cybulsky AV, Takano T, Papillon J, Khadir A, Bijian K, Le Berre L. The actin cytoskeleton facilitates complement-mediated activation of cytosolic phospholipase A2. Am J Physiol Renal Physiol. 2004, 286(3), F466-76.

2. Cybulsky AV, Takano T, Papillon J, Khadir A, Bijian K, Chien CC, Alpers CE, Rabb H. Renal Expression and Activity of the Germinal Center Kinase, SK2. Am J Physiol Renal Physiol. 2004, 286(1), F16-25.

3. Peng H, Takano T, Papillon J, Bijian K, Khadir A, Cybulsky AV. Complement Activates the c-Jun N-Terminal Kinase/Stress-Activated Protein Kinase in Glomerular Epithelial Cells. J Immunol. 2002, 169(5), 2594-601.

I have also contributed a review article (book chapter):

Bijian K and Cybulsky AV. "Stress Proteins in Glomerular Epithelial Cell Injury." Cellular Stress responses in Renal Diseases. Edited by Razzaque MS and Taguchi T, published by Karger, Contributions to Nephrology. 2005, 148, 8-20.

Dr. Andrey Cybulsky has supervised the entire work presented in this thesis and assisted in the writing of the manuscripts. Dr. Tomoko Takano has assisted with helpful discussion in Sections 2 and 3. Joan Papillon assisted me in acquiring the stable clones (R4F-MEK, CD2-FAK and L^{63}RhoA) in Sections 2 and 3. Abdelkrim Khadir performed some western blots in Section 2 (Figures 5C-D, 7A, 8A-C). Dr. Ludmilla Leberre provided me with the GFP-PH(PLCδ) and GFP-Pase expression vectors in Section 3.

This thesis contains 4 sections:

Section 1: Comprehensive literature review relevant to the work presented in this thesis.

Sections 2-3: Experimental research.

Section 4: Conclusion of the work presented in this thesis.

Sections 2 and 3 contain their own reference sections, which are found at the end of each corresponding section. The references for sections 1 and 4 are compiled at the end of the thesis.

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RATIONALE AND OBJECTIVES

This thesis is based on previous work in Dr. Cybulsky's laboratory, which has established that adhesion of cultured GEC to collagen provides important physiological signals, including inositol lipid turnover, and regulation of the epidermal growth factor (EGF)-receptor-Ras-ERK pathway and proliferation. In vivo, proliferation of highly differentiated GEC is generally not observed in the normal kidney. However, GEC injury occurs in FSGS, and may include GEC apoptosis and proliferation, which along with expansion of ECM, may lead to sclerosis and impaired glomerular function and/or permselectivity.

The overall hypothesis is that ECM regulates mechanisms that promote survival of GEC in culture and that these mechanisms are analogous to those characterized as limiting apoptosis upon injury in vivo. Following GEC injury in vivo, signals from ECM and growth factor receptors maintain cell survival and prevent apoptosis. GEC survival and/or proliferation in vivo may be a beneficial response to limit injury and promote healing.

The specific objectives of this study are to:

(i) study the intracellular signaling pathways activated by ECM and analyze the interaction of these signals with growth factor signaling.

(ii) determine the relationship between actin organization and the survival phenotype.

(iii) identify the major survival and apoptotic signaling pathways activated in GEC.

(iv) verify whether analogous signaling pathways are activated in an experimental model of focal segmental glomerulosclerosis in vivo.

SECTION 1: LITERATURE REVIEW

THE KIDNEY

The kidney is functionally indispensable to the organism since it is responsible for maintaining the proper balance of water and inorganic ions in the extracellular fluid. It is also responsible for the removal of metabolic waste products and foreign chemicals from the blood, excreting them into the urine. Moreover, the kidney functions as an endocrine gland, secreting erythropoietin, renin and 1,25-dihydroxyvitamin D_3. During prolonged fasting, the kidney could also release glucose into the blood from the breakdown of amino acids. Each kidney is composed of approximately one million nephrons (functional unit of the kidney) bound together by connective tissue, containing blood vessels, nerves and lymphatics. Each nephron is composed of the renal corpuscle, which is the filtering component of the kidney and a tubule, which is the secreting and reabsorbing element of the kidney. The renal corpuscle is composed of the filtration unit of the kidney called the glomerulus, surrounded by a hollow capsule called Bowman's capsule, creating a fluid filled space for the filtrate called Bowman's space. Briefly, the glomerulus forms a filtrate free of cells and proteins which enters the tubule, where it is concentrated and excreted as urine. The glomerular capillary wall, while allowing passage of water and solute restricts passage of nearly all plasma proteins. Any defect in its function could result in the presence of protein in the urine termed proteinuria (42).

GLOMERULOGENESIS

The nephron arises from the mesenchymal metanephric blastema by induction through the ureteric bud during the fifth week of gestation. The ureteric bud induces the cluster of mesenchymal cells which undergo many mitotic cycles and differentiation stages, eventually generating a nephron. The three major stages of nephrogenesis are illustrated in figure 1. Condensation of the renal mesenchyme results in the formation of pre-tubular aggregates and renal vesicles (Fig. 1, left panel). Polarized epithelial-like cells sitting on a basement membrane line the lumen of these vesicles. At the next stage of nephron development, a cleft appears in the growing nephron, producing a comma-shaped or S-shaped profile (Fig. 1, middle panel) allowing migratory endothelial cells to invade and help with the formation of the glomerular capillary tuft. The layer of epithelial cells lining the lower lip of the cleft eventually differentiates into podocytes. After complete formation of the S-shaped body, further growth of the other segments of the nephron gives rise to characteristic appearances of a fetal glomerulus including the mesangial cells (Fig. 1, right panel) (8; 32).

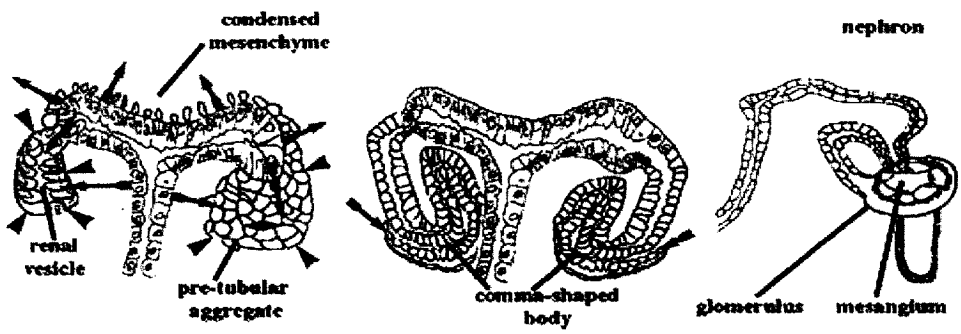
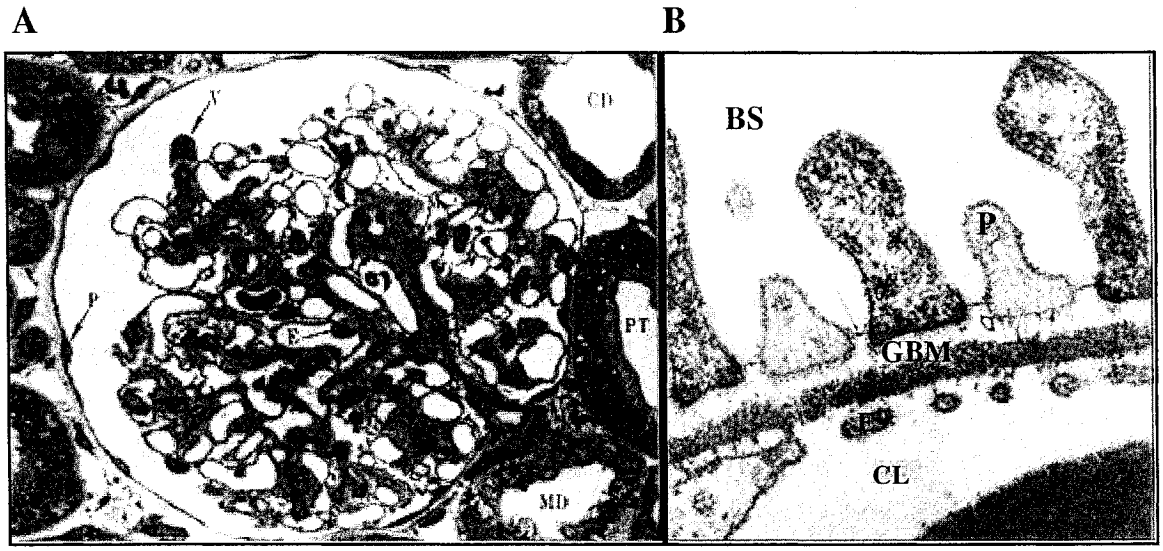


Figure 1: *Major stages of Nephrogenesis (8).*

THE GLOMERULAR CAPILLARY WALL

The glomerular capillary wall is composed of a monolayer of glomerular endothelial cells lining the capillary lumen, the glomerular basement membrane (GBM) and the visceral glomerular epithelial cells (GEC) also called podocytes, which wrap tightly around the capillary loops. The glomerular capillaries are supported by the mesangium, which is composed of mesangial cells and their surrounding matrices (Fig. 2A). The initial barrier encountered by blood is made up of the fenestrated glomerular endothelial cells. With a diameter of approximately 30nm, the fenestrations prevent the passage of blood cells into the subendothelial space. Most likely, the negative surface charge of the endothelial cells (glycocalyx) partially restricts passage of plasma proteins. After the passage of filtrate through the fenestration of the endothelial cells, the next barrier encountered by the filtrate is the GBM. With its net negative charge, the GBM is responsible for the charge and size selectivity of the glomerular filter, repelling large negative proteins. The third, and probably the most important line of defense against protein leakage is the podocytes, where foot processes extend from their large cell bodies and embed into the GBM, while interdigitating with the foot processes of adjacent podocytes forming a pore, called the slit diaphragm (Fig. 2B-C). Podocytes also exhibit a negative charge due to the negatively charged glycoproteins found on the surface of these cells (18).



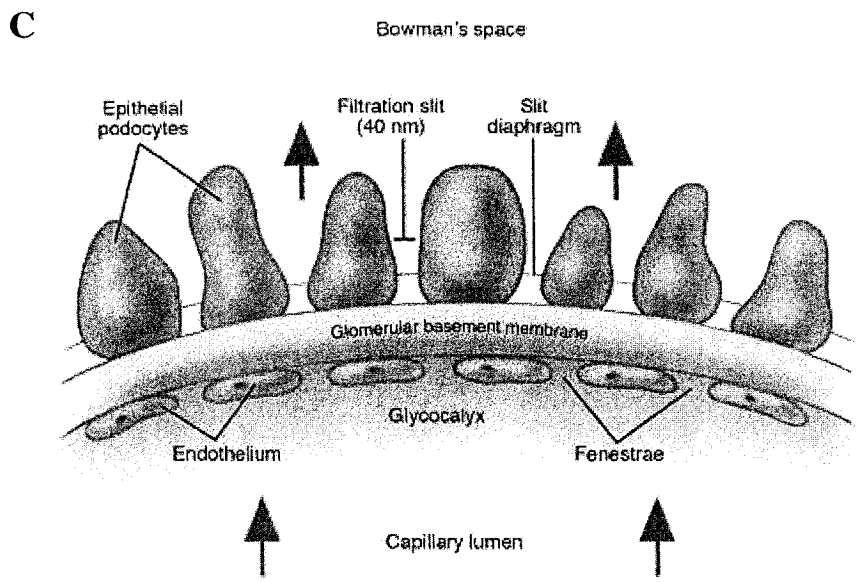


Figure 2A: *Light micrograph of a normal rat glomerulus. Major cell types found in a glomerulus are the endothelial cells (E) lining the capillary lumen, the visceral epithelial cells (V) or podocytes, the parietal epithelial cells (P) and the mesangial cells (M). Also present in this picture are other portions of the nephron: the collecting duct (CD), the proximal tubule (PT) and the macula densa (MD).*

Figure 2B: *Electron micrograph of a normal rat glomerulus displaying the structure and components of the glomerular filter from the capillary lumen (CL) to Bowman's space (BS). Notice the fenestrated endothelial cells (E), the glomerular basement membrane*

(GBM) and the podocytes (P) with its foot process (arrow head) in contact with the GBM and the formation of the slit diaphragm (arrow).

Figure 2C: *Schematic representation of the glomerular filter including the fenestrated endothelium (~30nm) with its negatively-charged cell coat (glycocalyx), the glomerular basement membrane (200nm-400nm thickness) and the glomerular epithelial cell foot processes. The foot processes form filtration slits spanned by slit diaphragms (~40nm). Arrows denote direction of filtration flow. Figure is not drawn to scale (18).*

THE GLOMERULAR BASEMENT MEMBRANE (GBM)

The GBM is indispensable for the maintenance of glomerular filtration function and structure. It is synthesized and maintained by the glomerular epithelial and endothelial cells. The GBM is composed of three layers; the central dense layer termed lamina densa and the two outer layers, lamina rara interna and lamina rara externa. The GBM is composed of a combination of basement membrane protein isoforms: laminins (laminin 11, α5, β2, γ1), collagen type IV (α3, α4, α5), entactin/nidogen, perlecan and sulphated proteoglycans (54). Perlecan is the major glycosaminoglycan present in the GBM, giving its net negative charge thus contributing to its charge selective function. Genetic alterations in the expression of some of these GBM components have been shown to disturb podocyte architecture and result in nephrotic syndrome. Mice lacking the laminin β2-chain develop podocyte foot process effacement and marked proteinuria (59). Moreover, mice deficient in the collagen IV α3-chain develop Alport syndrome, characterized by proteinuria, foot process effacement, sclerosis and renal failure (49).

THE GLOMERULAR VISCERAL EPITHELIAL CELL OR PODOCYTE

Podocytes are terminally differentiated cells, with limited ability to proliferate. They possess a characteristically complex cell architecture, which includes foot processes and a slit diaphragm. Foot processes of neighboring podocytes interdigitate (Fig. 3A-B). The slit diaphragm allows the selective paracellular flow of ultrafiltrate through the epithelium, while preventing passage of macromolecules. Maturing podocytes eventually lose their mitotic activity and begin to express podocyte specific proteins. Terminally differentiated podocytes express the slit membrane associated proteins, nephrin, podocin, CD2AP, and NEPH-1, the actin associated protein synaptopodin, cell surface protein podocalyxin, a podocyte-specific membrane protein tyrosine phosphatase, glomerular epithelial protein 1 (Glepp 1) and the intermediate filament protein vimentin. The basal cell membrane domain of the podocytes is in contact with the GBM, whereas the apical or luminal membrane domains are covered by sialoglycoproteins, which are responsible for providing the negative charge of the podocytes. At the border of the basal and luminal membranes is the slit diaphragm, which has been the focus of attention of renal researchers in recent years (Fig. 3C). The slit diaphragm bridges across the 30- to 40-nm-wide filtration slit, connecting adjacent podocytes (Fig. 2B-C). A functional slit diaphragm is necessary to maintain normal structure of foot processes. Mutational analysis of several slit diaphragm proteins has shed light on the pathogenesis of proteinuria and demonstrated the importance of the slit diaphragm in the maintenance of a functional glomerular filter. Congenital nephrotic syndrome of the Finnish type (NPHS1) is an autosomal-recessive disorder, characterized by massive proteinuria in

utero and nephrosis at birth. Mutations in the nephrin gene, NPHS1, have been linked to the impaired development and function of the kidney filtration barrier in this disease (38). CD2AP and NEPH-1 knockout mice also exhibit foot process effacement and nephrotic syndrome (21; 74). Moreover, several mutations in the gene encoding for podocin (NPHS2) were identified in patients with autosomal recessive steroid-resistant nephrotic syndrome, developing proteinuria, end-stage renal disease and focal segmental glomerulosclerosis (6). These studies along with many others demonstrate the crucial role for most of the members of the slit diaphragm proteins in the formation and maintenance of a functional glomerular filter. It is becoming increasingly clear that nephrotic syndrome is a disease of the podocyte.

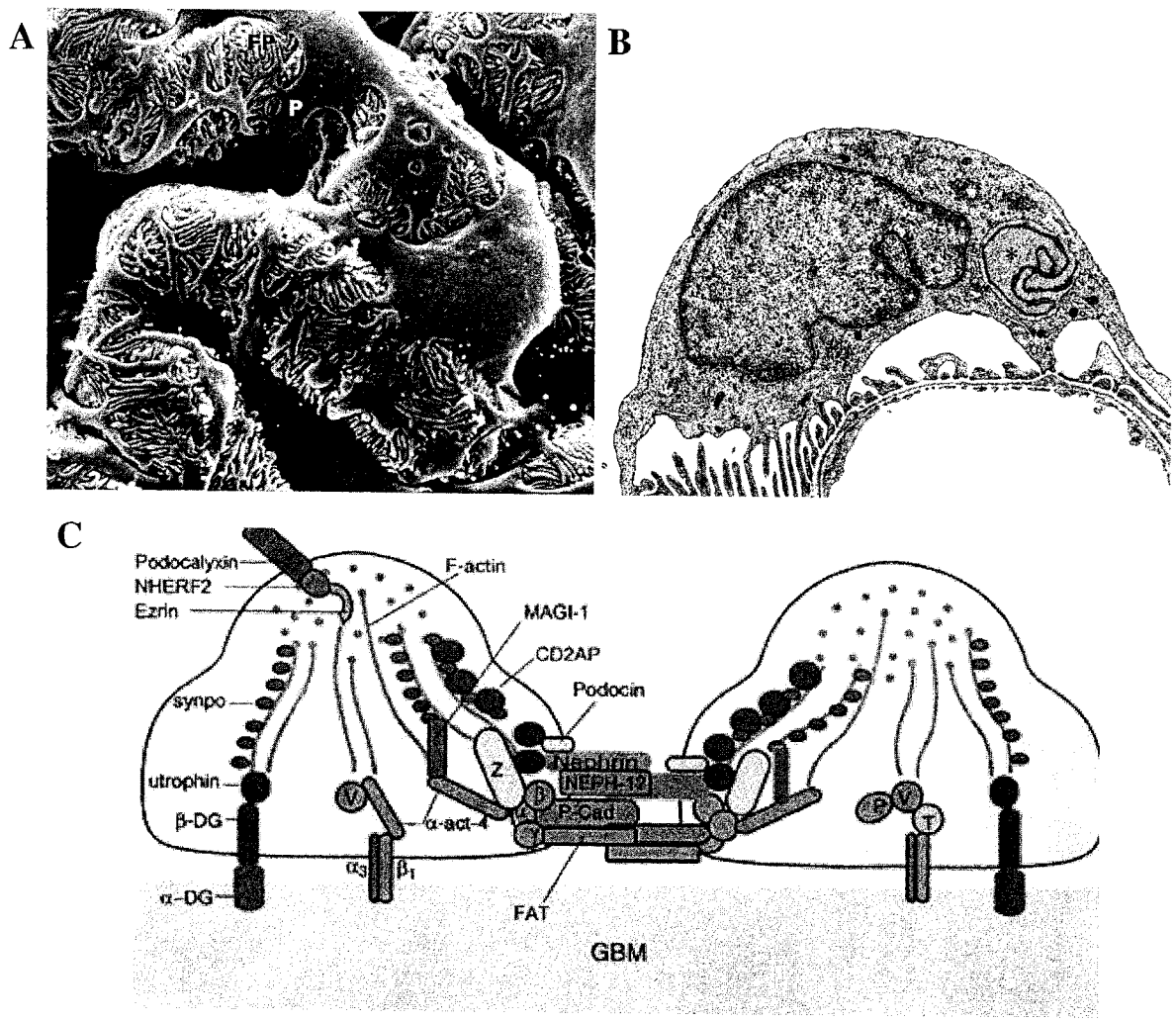


Figure 3A: *Scanning electron micrograph of a normal rat glomerulus. View from Bowman's space demonstrating the highly branched podocyte (P) and its foot processes (FP) surrounding the glomerular capillaries (61).*

Figure 3B: *Low power transmission electron micrograph of a normal rat glomerulus. The large cell body of the podocyte resting in Bowman's space connects to the capillary wall by its foot processes which interdigitate on the outer surface of the glomerular basement membrane (61).*

Figure 3C: *Molecular structure of the glomerular slit diaphragm. This schematic demonstrates the recently discovered members of the glomerular slit diaphragm; nephrin, NEPH-1, P-Cad (P-cadherin), FAT, podocin, Z (ZO-1) and CD2AP. Also*

demonstrated are the molecular components linking the apical, basal and lateral membrane domains to the actin cytoskeleton: α-act4 (α-actinin-4), α3β1 (α3β1 integrin), α-DG (α-dystroglycan), NHERF2 (Na^+/H^+ exchanger regulatory factor 2), P (paxillin), Synpo (synaptopodin), ezrin, T (talin), V (vinculin) (57).

CYTOSKELETON

The cytoskeleton is formed of three structural elements: microfilaments, intermediate filaments and microtubules. In the cell body of the podocyte, microtubules and intermediate filaments (ex: vimentin and desmin) make the bulk of the cytoskeleton, whereas cortical filamentous actin (F-actin) and microfilaments are present in the foot processes. In addition to F-actin, the foot processes contain the major protein constituent of the contractile apparatus of muscle, i.e. myosin and α-actinin, allowing the foot processes to generate contractile force, thus helping the glomerular capillaries to resist the high intraluminal hydrostatic pressure and actively modify the surface area for filtration necessary to adapt to the cyclic distension caused by each cardiac contraction (22). A functional glomerular filter is dependent upon an intact slit diaphragm and podocyte-GBM interactions. These two functionally indispensable complexes are structurally linked via the actin cytoskeleton. Genetic alterations in the expression of certain cytoskeleton-modulating proteins initiates the collapse of these structures thus leading to podocyte foot process effacement and proteinuria. A mutation in the gene encoding for α-actinin-4, which increases the affinity of α-actinin-4 for F-actin, has been associated with a familial form of autosomal dominant focal segmental

glomerulosclerosis (36; 52). Altered expression of another actin binding protein, synaptopodin, has been observed in various forms of glomerular disease (3; 37; 76). Mice deficient in Rho GDP dissociation inhibitor-α (which maintains Rho family members in their GDP bound inactive state) develop massive proteinuria and glomerulosclerosis, demonstrating the importance of the signaling pathways regulated by Rho GTPases in maintaining glomerular permselectivity (79).

EXTRACELLULLAR MATRIX RECEPTORS

Integrins are a distinct family of transmembrane heterodimeric molecules composed of an α (120-180kDa) and a β (90-110 kDa) subunit, with a long extracellular domain binding to extracellular matrix (ECM), a transmembrane domain, and a short cytoplasmic domain which associates with the actin cytoskeleton and recruits other proteins (34). There are 14 α subunits and 8 β subunits which have been cloned so far in mammals. Although there could mathematically be over 100 combinations of α and β subunit heterodimers, the actual diversity appears to be more restricted. To date 24 integrins have been identified in humans (27). These restrictions are mainly due to the fact that most α subunits could associate only with a single β subunit. Most integrins could bind to several ECM proteins, while individual matrix proteins, such as fibronectin, laminins, collagens and vitronectin could bind to several integrins. Although there appears to be some functional redundancy between integrin receptors, genetic studies have demonstrated that the loss of ~~a~~ certain integrin receptors is detrimental (35).

Integrins are capable of bi-directional signaling, i.e. integrin conformation and binding affinity is regulated from inside the cell (inside-out signaling), or upon ECM binding, integrins cluster and recruit actin filaments and signaling proteins thus transmitting the signal into the cell (outside-in signaling) (28). The association of integrins with the actin cytoskeleton is especially important when integrin ligand binding leads to the formation of specialized macromolecular signaling complexes called focal adhesions. Focal adhesions contain more than 50 structural and signaling proteins creating the primary sites of adhesion between the cell and the surrounding ECM. In addition to transmitting force or tension at adhesion sites to maintain a solid link to the underlying ECM, focal adhesions act as localized signaling centers for numerous intracellular pathways regulating cell growth, differentiation and survival (11; 67).

Given that integrins do not possess any enzymatic features, they need to recruit adapter molecules in order to transmit the extracellular signals. These adapter molecules consist of proteins that connect the integrins to the actin cytoskeleton, proteins that possess kinase activity and even transmembrane growth factor receptors. More than 20 different proteins have been shown to be recruited to focal adhesions, including the cytoskeletal molecules, paxillin, F-actin, α-actinin, vinculin and talin as well signaling molecules such as FAK, c-Src, phosphatidylinositol-3 kinase (PI3-K), Grb2, RhoA, Rac1, Ras, Raf1, and the mitogen activated kinases (MAPK)s such as extracellular-signal-regulated kinases (ERK)s (55).

THE INTEGRIN-ACTIN CONNECTION

The integrin cytoplasmic tails play a crucial role in linking the extracellular matrix proteins to the intracellular actin filaments. Upon integrin activation, the integrin cytoplasmic tails interact with actin binding proteins such as talin, filamin, α-actinin and integrin-linked kinase (ILK). Talin is recruited to focal complexes where it activates the phosphatidylinositol 4-phosphate 5-kinase type 1γ (PIPKγ), thus increasing phosphatidylinositol (4,5) bisphosphate (PIP_2) production (12; 51). Binding of PIP_2 to vinculin increases its affinity for talin; this initiates actin polymerization by recruiting ARP2/3, favoring actin nucleation (20). Talin is also involved in inside-out signaling, where binding of talin to the cytoplasmic tail of β-integrins, causes integrin conformational changes leading to the modulation of its extracellular binding affinity to ECM (78). Integrins also recruit the actin-filament-crosslinking protein filamin, which serves as a scaffold to recruit Trio, a guanine nucleotide-exchange factor (GEF) for Rho GTPases, thus controlling actin reorganization (4). α-actinin is also involved in connecting integrin cytoplasmic tails to the actin fibrils. In addition to its function of crosslinking actin filaments, it binds to many signaling proteins including vinculin, ERKs and PI3-K, thus serving as a scaffolding protein modulating integrin-cytoskeletal interactions (48; 73; 82). ILK is a serine/threonine kinase that binds to the cytoplasmic tails of β integrin subunits. ILK recruits several adaptor proteins that modulate the integrin-actin connections either directly or indirectly. ILK has been shown to bind paxillin as well as the double zinc finger domain (LIM)-only adapter proteins PINCH-1 and PINCH-2. PINCH-1 and PINCH-2 are able to recruit the adaptor protein Nck2,

which has the potential to bind through its src-homology-2 (SH2) domain to phosphorylated-tyrosine residues of different receptor tyrosine kinases, thereby crosslinking integrin signaling with growth factor signaling. Moreover, Nck2 can recruit actin regulatory proteins such as WASP (Wiskott-Aldrich Syndrome protein), DOCK180 and PAK (p21-activated kinase), thereby modulating actin organization (7).

INTEGRIN MITOGENIC SIGNAL TRANSDUCTION

Besides being a very important modulator of the structural and architectural component of a cell, integrins are also involved in the signal transduction of multiple biological functions including cell growth, differentiation and survival. Integrin receptors have the potential to signal alone, i.e. direct signals are transmitted as a result of integrin ligation, or indirectly with the collaboration of other membrane receptors such as growth factor receptors. Normal cell growth requires the concomitant activation of signals emanating from growth-factors and from adhesion to ECM. Intracellular signals modulating cell growth and differentiation usually involve a family of closely related proline-rich serine-threonine protein kinases, known as the mitogen-activated protein kinases (MAPKs). MAPKs could be divided into three families: extracellular signal-regulated kinases (ERKs), c-Jun NH2-terminal kinases (JNKs) and p38 protein kinases (9). It is recognized that the ERK pathway is typically involved in growth-related signals, whereas the JNK and p38 MAP kinase cascades are activated by various stress stimuli. The ERK pathway (Ras/Raf/MEK/ERK) is a vital pathway conserved

throughout evolution that is activated in mammalian cells via stimulation of receptor tyrosine kinases, G-protein coupled receptors and integrins. These cell surface signals induce the conversion of Ras to its GTP-bound form, which then recruits the serine/threonine protein kinase Raf. Localization of Raf to the plasma membrane leads to its activation via mechanisms that are incompletely understood. Raf is then able to activate mitogen-activated protein kinase kinase (MEK) by phosphorylating it on two serine residues. Active MEK then activates ERK by phosphorylating it on a tyrosine and a threonine residue. Activated ERK phosphorylates numerous intracellular substrates that participate in cell cycle regulation, including the transcription factor Elk-1 and $p90^{RSK}$ (63). Understanding how integrin activation influences the ERK pathway is of utmost importance and will be discussed in the following text.

Upon integrin engagement it was shown that multiple intracellular proteins underwent tyrosine phosphorylation (44). A predominant protein which was shown to undergo rapid tyrosine phosphorylation upon integrin activation was a 125kDa non-receptor protein tyrosine kinase called focal adhesion kinase (FAK) (68). FAK co-localizes with integrin receptors at sites of ECM attachment. FAK knockout in mice is embryonic lethal, and these mice demonstrate a similar phenotype to fibronectin deficient mice, reinforcing the theory that FAK lies within the integrin signaling pathway (24; 26). Upon activation, FAK autophosphorylates at tyrosine 397 creating a docking site for multiple SH2 domain-containing proteins including the Src family of tyrosine kinases, PI3-K, phospholipase C-δ (PLCδ) and Grb7 (29; 64; 69; 86). Src family kinases can further activate FAK by phosphorylating other FAK tyrosine residues. Src

phosphorylates two tyrosine residues (Y576 andY577) in the activation loop of the catalytic domain which enhance the catalytic activity of FAK. Src also phosphorylates FAK tyrosine 925, creating a binding site for Grb2, thus recruiting Grb2/SOS which may be one of the mechanisms that FAK may employ to activate Ras and the ERK pathway (71). The FAK C-terminal domain contains several proline-rich sequences which serve as binding sites for src-homology-3 (SH3) domain containing proteins including the Crk-associated protein ($p130^{cas}$) and the GTPase regulator associated with FAK (GRAF) (31; 62). Tyrosine phosphorylation of $p130^{cas}$ by FAK or indirectly by Src results in the recruitment of the Crk and Nck adaptor proteins, which associate with SOS, and thus provides another mechanism for the stimulation of the Ras/ERK pathway (70). FAK has also been shown to stimulate the JNK pathway through the recruitment of $p130^{cas}$/Crk (60). FAK activation could also promote the tyrosine phosphorylation of Shc, which also recruits Grb2/SOS linking once again to the ERK pathway (72). The ERK pathway could be stimulated by FAK through the recruitment of PI3-K which activates protein kinase C (PKC) and consequently Raf-1 (41). Alternatively, integrins are able to stimulate the ERK pathway independently of FAK. One such mechanism includes the ability of certain α integrin subunits to bind to a membrane protein called caveolin, leading to the recruitment of Src family kinases and Shc to this complex. Shc then recruits Grb2/Sos and is able to signal to the ERK pathway (84). Moreover, an indirect interaction between growth factor receptors and integrins via FAK/paxillin/talin has also been demonstrated to activate ERK (65).

SURVIVAL SIGNALING AND THE MAP KINASE PATHWAY

The physiological significance of integrin-mediated ERK activation is not clear, since the stimulation of ERK1 and ERK2 by integrins alone is not sufficient to promote cell cycle progression. It has been demonstrated in many different cell types that adhesion signals require additional signals from growth-factors and their receptors to promote cell proliferation and survival. Moreover, the sole presence of growth factors is also not sufficient to promote cell proliferation and requires additional signaling from integrin mediated cell adhesion. It is becoming increasingly clear that integrin and growth factor signaling pathways are interconnected and it is almost impossible to consider their effects on cell proliferation independently. The crosstalk between these two signaling pathways has been the subject of extensive research and will be discussed briefly in the following text.

Many studies have established that adding soluble mitogens to cells in suspension triggers a weak and transient activation of ERK, whereas a strong and sustained activation is observed in adherent cells. From a biological standpoint, optimal activation of the ERK pathway is required to promote cell proliferation and survival, whereas non-optimal activation may have no affect and may eventually lead to cell death. Collaboration between growth factor receptors and integrin receptors has been demonstrated at the level of the receptors themselves, as well as at multiple sites within the Ras/Raf/MEK/ERK signaling pathways downstream of the receptors. Cooperation at the level of the receptors was demonstrated in a study by Miyamoto et al., where

epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) receptor aggregation and tyrosine phosphorylation required both aggregation and occupancy of integrin receptors (56). In contrast, Lin and colleagues determined that although EGF and PDGF–induced activation of the ERK pathway is impaired in NIH 3T3 cells in suspension, as compared to cells adherent to fibronectin, growth factor receptor phosphorylation and Ras activation are similar in adherent and suspended cells, suggesting the presence of an anchorage-dependent step between Ras and Raf (50). On the other hand, Renshaw et al. examined the growth factor induced activation of Ras and Raf in NIH 3T3 cells adherent to fibronectin vs suspended cells. Their results showed that Ras and Raf activity were similar in adherent and suspended cells in response to growth factors, whereas MEK activity was inhibited in suspended cells. Moreover, NIH 3T3 cells transiently transfected with constitutively active mutants of Ras and Raf were still adhesion-dependent, whereas cells expressing a constitutively active mutant of MEK were able to stimulate cell growth in suspension. These results suggest the presence of an adhesion dependent step between Raf and MEK (66). Moreover, in NIH 3T3 fibroblasts, cell adhesion to fibronectin diminished the association of the GTPase-activating protein of Ras (RasGAP) with the PDGF-receptor, favoring the accumulation of GTP bound Ras (active), as compared to cells adherent to polylysine. This was due to the increased association of the receptor with SHP-2, which dephosphorylates the RasGAP tyrosine binding site on the receptor. These results demonstrate the possible adhesion dependent step to be upstream of Ras (19). Another study on NIH 3T3 fibroblasts analyzed the translocation of activated ERK from the cytoplasm to the nucleus, where it is responsible for the phosphorylation of its nuclear

substrate Elk-1. This study demonstrated that the growth factor induced phosphorylation of Elk-1 was dependent upon adhesion to fibronectin. Moreover, ERK translocation to the nucleus and consequent phosphorylation of Elk-1 was dependent on an intact actin cytoskeleton, as fibronectin adherent fibroblasts failed to display Elk-1 phosphorylation after cytochalasin D treatment. This study suggests that adhesion to ECM leads to a certain organization of the actin cytoskeleton which modulates the localization of active ERK in these cells (2).

One can appreciate the diversity of the signaling properties observed in the above studies, which reinforce the importance of determining the limiting adhesion dependent step or steps unique to each cell type and experimental condition.

PROGRAMMED CELL DEATH OR APOPTOSIS

Apoptosis is an essential physiological process required for normal development and maintenance of tissue homeostasis. However, dysregulation of apoptosis has been associated with a variety of diseases. Excess apoptosis is observed in diseases such as in T cell depletion in HIV infection, neurodegenerative diseases (Alzheimer's) and hepatocellular degeneration (Wilson's). In contrast, impaired apoptosis is usually observed in most cancers (16). Apoptosis may be induced or is preprogrammed into cells leading to the death of an individual cell, in contrast to necrotic cell death where groups of cells within a tissue are affected. Apoptosis may be triggered in cells by insufficient exposure to growth factors, disruption of the cytoskeleton, inadequate ECM-cell contacts

(anoikis) and other factors (23). There are currently three main pathways leading to apoptotic cell death in mammalian cells (Fig. 4), the mitochondrial apoptotic pathway, the death domain receptor pathway and the endoplasmic reticulum (ER) apoptotic pathway, which are linked to the activation of inducer caspases-9, -8 and -12, respectively. These inducer caspases amplify the apoptosis signal by activating effector caspases such as caspase-3. Caspase-3 is able to degrade regulatory proteins and activate endonucleases and other proteases required for apoptosis.

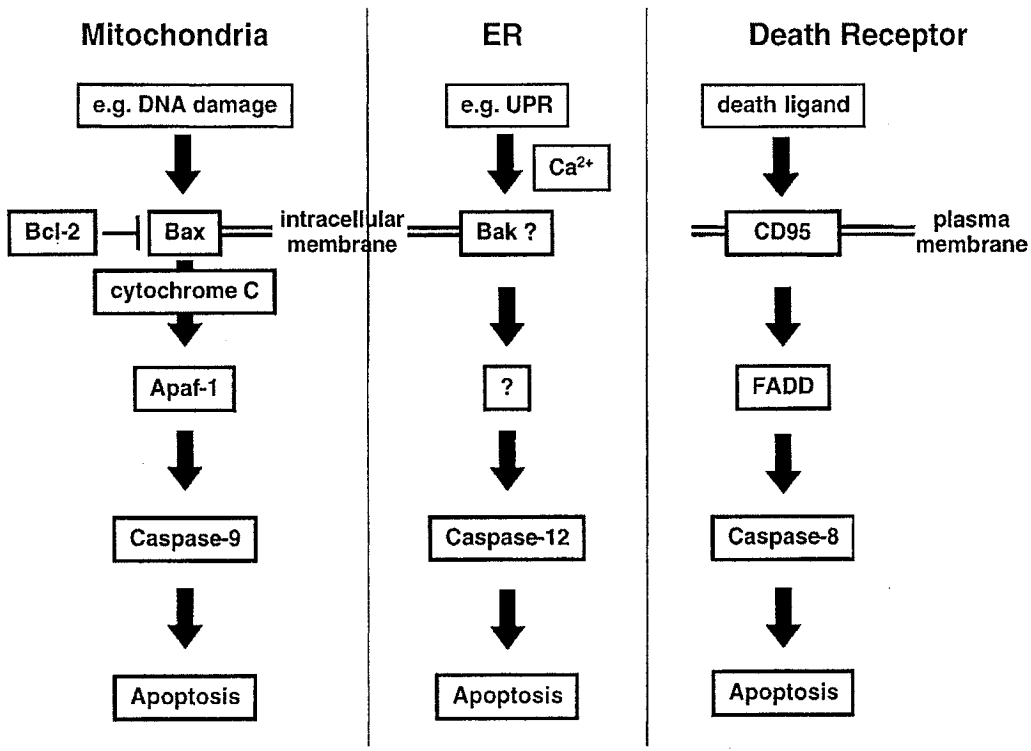


Figure 4: *Apoptosis signaling pathways in mammalian cells (16).*

The mitochondrial apoptotic pathway is modulated by the expression of Bcl-2 family members which exert their effects on the mitochondrial membrane. Anti-apoptotic Bcl-2 family members (ex: Bcl-2, Bcl-X_L) reside on the mitochondrial outer

membrane preventing the release of apoptogenic factors, such as cytochrome c, from the intermembrane space into the cytoplasm. In contrast, DNA damage could activate pro-apoptotic Bcl-2 family members (e.g. Bax, Bak), which reside in the cytoplasm, and following an apoptotic signal, translocate to the mitochondrial membrane causing the release of cytochrome c and thus instigating the activation of caspase-9. Alternatively, ligation of death receptors such as CD95 (Fas) upon binding to their ligand (Fas ligand), results in the formation of death-inducing signaling complexes by recruiting death domain contain proteins (Fas-associated death domain;FADD). FADD recruits multiple procaspase-8 molecules leading to their cleavage (active form) and subsequent activation of the apoptotic caspase cascade (16; 87). The ER apoptotic pathway is activated by conditions leading to the perturbation of ER calcium homeostasis or accumulation of unfolded proteins in the ER. This may lead to ER stress and the activation of the unfolded protein response (UPR), resulting in the activation of caspase-12 and apoptosis.

While the activation of the three apoptosis signaling pathways leads to a similar outcome (cell death by apoptosis), they have been demonstrated to be non-redundant in the apoptosis program of mammalian cells. For example, embryonic thymocytes derived from caspase-9 deficient mice proved to be resistant to mitochondrial disturbing stimuli requiring the activation of the mitochondrial apoptotic pathway (47). In embryonic fibroblasts derived from caspase-8 deficient mice, death ligands were unable to induce apoptosis through the death domain receptor apoptosis pathway, while apoptosis was successfully induced by other intracellular stresses (81). Moreover, embryonic fibroblasts derived from caspase-12 deficient mice were resistant to ER stress-induced

apoptosis, such as brefeldin A, tunicamycin and thapsigargin treatment, whereas they showed similar sensitivity to non-ER stress-induced apoptotic signals as their wild type counterpart (58).

Although these signaling pathways are independent and non-redundant as mentioned above, caspases are able to activate each other. For example depletion of caspase-9 from Jurkat cell extracts inhibited the cytochrome c-induced activation of caspases-2, -3, -6, -7, -8 and -10, suggesting that these caspase are all active as a result of caspase-9 activation, which is the initial caspase activated (75).

PODOCYTE SPECIFIC SIGNALING

There are two major adhesion complexes involved in the interactions between the podocyte and the GBM, $\alpha3\beta1$ integrin and the α, β dystroglycan. $\alpha3\beta1$-integrin, found at the sole of the podocytes foot processes along the GBM, is the major integrin expressed by podocytes (43). It has been demonstrated that $\alpha3\beta1$-integrins bind to laminin, collagen type IV ($\alpha3$, $\alpha4$, $\alpha5$ isoforms), and entactin/nidogen, which are components of the GBM, as well as fibronectin (17). The importance of the $\alpha3\beta1$ integrin in podocytes is demonstrated in $\alpha3$ knockout mice that are unable to assemble podocyte foot processes and die soon after birth due to glomerular dysfunction. Moreover, lack of the $\alpha3\beta1$ integrin caused damage to the GBM, suggesting a role for the $\alpha3\beta1$ integrin in maintaining the structural organization of the GBM (46).

In order to understand GEC growth and survival in greater depth, our lab has been studying the effect of ECM on the signaling events responsible for proliferation of GEC. As illustrated in figure 5, epidermal growth factor (EGF) stimulated the EGF receptor (EGF-R) and the Ras/ERK pathway, and proliferation in GEC, when cells were adherent to collagen matrices, but not plastic substratum. In GEC on collagen, EGF induced sustained activation of EGF-R and ERK, in association with reduced EGF-R dephosphorylation by phosphatases. On plastic, EGF-R activation was weak or transient, as dephosphorylation appeared to be rapid (13). This study was among the first to demonstrate that the activation of a growth factor receptor was regulated by ECM. Similarly, Cybulsky et al. further demonstrated that bFGF, hepatocyte growth factor (HGF) and thrombin stimulated GEC proliferation only when adherent to collagen matrices, but not plastic substratum. HGF and thrombin activated ERK in collagen adherent cells, while ERK activation was weak or transient in plastic adherent cells (15).

Stable transfection of GEC with a constitutively active mutant of Ras supplanted the requirement of ECM, thus supporting EGF-induced proliferation and ERK activation in GEC adherent to plastic substratum (15). These results support the hypothesis that the adhesion dependent signal(s) in the growth factor pathway leading to proliferation in GEC are mediated via Ras.

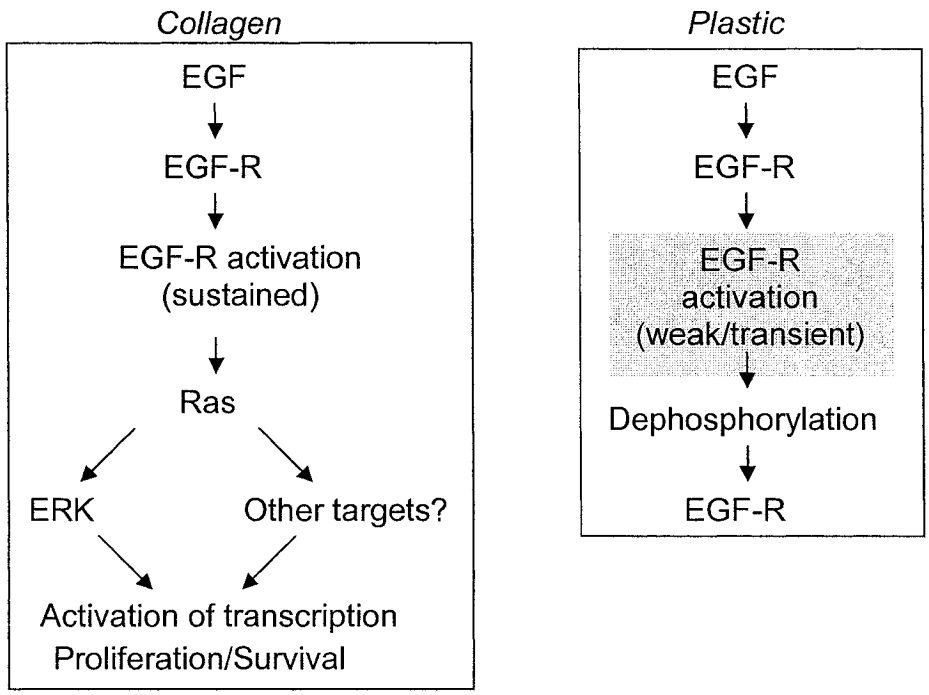


Figure 5: *Model for EGFR regulation by ECM.*

Adhesion of GEC to collagen was also shown to increase the production of PIP_2, as well as its breakdown products 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG), independently of growth factors. The increase in PIP_2 production was due to increased activity of phosphatidylinositol-4-phosphate 5-kinase, whereas the increased production of IP_3 and DAG was due to the increased presence of PLC in the membrane fractions of GEC adherent to collagen matrices, as compared to GEC on plastic (14).

Taken together, in GEC, there is substantial support for the involvement of the ECM signaling pathway in modulating intracellular protein kinases responsible for cell

growth and survival. Understanding the precise interplay among ECM receptors and growth factor receptors will help us identify specific intracellular targets that may eventually serve as a target for therapy.

ABSTRACT

Glomerular epithelial cell (GEC) injury and apoptosis may contribute to sclerosis in glomerulonephritis. The present study addresses signals that regulate survival of GEC in culture, and in the acute puromycin aminonucleoside nephrosis (PAN) model of GEC injury in vivo. Compared with GEC on plastic substratum, adhesion to collagen increased activation of focal adhesion kinase (FAK), c-Src and extracellular signal-regulated kinase (ERK), and facilitated survival (prevented apoptosis). GEC on plastic exhibited increased caspase 8 and 9 activities, increased expression of the pro-apoptotic protein, Bax, and decreased anti-apoptotic protein, Bcl-X_L, as compared with collagen. Stable expression of constitutively-active mutants of FAK (CD2-FAK) or mitogen-activated protein kinase kinase (R4F-MEK) activated the ERK pathway, and supplanted the requirement of collagen for survival. In contrast, expression of a Ras mutant that activates phosphatidylinositol 3-kinase, but blocks ERK activation, or pharmacological inhibition of the ERK pathway decreased survival on collagen. Glomeruli isolated from rats with PAN revealed increased β1 integrin expression, along with increased activation of FAK, c-Src and ERK, as compared with controls. EGF receptor activation was undetectable in PAN. Therefore, adhesion to collagen, resulting in activation of FAK and the Ras/ERK pathway, supports GEC survival. Analogous signals for GEC survival are activated in PAN.

Key words: apoptosis, glomerulonephritis, protein kinases, signal transduction

INTRODUCTION

Cell homeostasis reflects a balance of proliferation, apoptosis and differentiation (20,21,26). Apoptosis may be triggered by insufficient exposure to growth factors, disruption of the cytoskeleton, inadequate extracellular matrix (ECM)[1]-cell contact (also known as "anoikis"), and other factors (20). Mechanisms reported to regulate cell survival (prevention of apoptosis/anoikis) may involve adhesion receptors, such as integrins, and protein or lipid kinases, including focal adhesion kinase (FAK), extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)-Akt, or integrin-linked kinase pathways, as well as multiple downstream effectors, including Bcl-2 family proteins, caspases, nuclear factor-κB, and others (6,20,21,26,38,41). Signals from ECM may directly affect cellular functions, or may interact with signals from growth factors (41). FAK appears to play a major role in mediating signals emanating from ECM via β1 integrins. In cultured cells, FAK may be recruited to focal contacts directly or through cytoskeletal proteins, talin and paxillin, where FAK interacts with the cytoplasmic tail of the β1 integrin subunit (6). Activation of FAK leads to autophosphorylation at tyrosine^{397}, creating a binding site for c-Src. Upon binding, c-Src phosphorylates tyrosine^{925} of FAK, allowing the adaptor protein, Grb2, to bind to the motif surrounding tyrosine^{925}. Grb2, which exists as a complex with Sos, may link integrin signals to the Ras-ERK pathway (6,24,39,40). Phosphorylation/activation of FAK may affect various cellular responses (6). For example, activation of FAK was able to prevent apoptosis, although the mechanism for this effect remains unclear. One possibility involves the binding of the Src homology 2 (SH2) domain of PI3K to tyrosine^{397} of FAK, thus promoting survival through the PI3K-Akt pathway, or by the

inhibition of pro-apoptotic proteins, such as p53. The role of ERK in mediating downstream signals from FAK has not been well-defined.

Glomerular visceral epithelial cells (GEC) or podocytes are intrinsic components of the kidney glomerulus and play a key role in the maintenance of glomerular permselectivity (19,27,35). Under normal conditions, where GEC are in contact with ECM and are exposed to trivial concentrations of growth factors, there appears to be little turnover of GEC. Certain forms of glomerulonephritis feature GEC injury, which may lead to apoptosis or proliferation, and in association with ECM expansion to glomerulosclerosis, and/or impaired glomerular function or permselectivity. Crosstalk between ECM receptors and growth factor receptors may modulate GEC proliferation and/or survival. We have previously determined that epidermal growth factor (EGF), basic fibroblast growth factor, and hepatocyte growth factor stimulate proliferation of cultured GEC that are adherent to collagen, but not plastic substratum (12,14). Moreover, in GEC on collagen, EGF was able to induce sustained activation of the EGF receptor (EGF-R) and ERK, in association with reduced EGF-R dephosphorylation by phosphatases. On plastic, EGF-R activation was weak or transient, as dephosphorylation appeared to be rapid (12,14).

In vivo, GEC injury occurs in human focal segmental glomerulosclerosis (FSGS), in the context of ECM expansion (4,5,36,46). Familial forms of FSGS, associated with mutations in GEC structural proteins, further support the view that FSGS is a disease of the GEC (19,27,35). FSGS may feature apoptosis, as well as proliferation; the latter is observed in the collapsing variant of FSGS, where the proliferating cells appear to be visceral GEC with dysregulated phenotype and loss of the p27 cyclin-dependent kinase

inhibitor. Experimental rat models, including acute and chronic puromycin aminonucleoside nephrosis (PAN) have provided a better understanding of the pathophysiology of GEC injury in FSGS (22). These models generally feature GEC injury with heavy proteinuria, sclerosis and some GEC detachment, expansion of ECM components, as well as collapse of glomerular capillaries in chronic PAN (18,32,42). DNA synthesis in GEC occurred early and late in the course of PAN, with increasing GEC apoptosis, and loss of GEC as the lesion became more chronic (42). Actually, it has been proposed that GEC apoptosis may lead to "podocytopenia" and consequently sclerosis in FSGS (19,27,28,35). Alterations in expression of GEC structural proteins and filtration slit-diaphragm components have also been reported (19,27,32). Together, these changes suggest that in PAN, there may be altered signaling from the ECM to GEC, but precisely how such signals may regulate GEC apoptosis or proliferation requires further study.

The aim of the present study was to address the signals that regulate survival/proliferation of GEC in culture, and to determine if analogous signals are activated in the PAN model of GEC injury in vivo. We demonstrate that adhesion of GEC to collagen enhances activation of FAK, c-Src and ERK, and prevents apoptosis via the ERK pathway. Stable expression of constitutively-active mutants of FAK, or mitogen-activated protein kinase kinase-1 (MEK) supplanted the requirement of ECM for promoting survival, and in the presence of growth factors, proliferation. Analogous signals for GEC survival are activated in glomeruli of rats with PAN.

MATERIALS AND METHODS

Materials

Tissue culture reagents, Transfinity $CaPO_4$ transfection system, G418 (geneticin), RNAse A and proteinase K were obtained from Invitrogen (Burlington, Ontario). Pepsin-solubilized bovine dermal collagen I (Vitrogen) was from Cohesion (Palo Alto, California). EGF, thrombin, puromycin aminonucleoside, Engelbreth-Holm-Swarm sarcoma laminin, LY294002, and rabbit anti-actin antibody were obtained from Sigma Chemical Co. (St. Louis, Missouri). Rabbit anti-phospho-ERK1/2 (threonine^{202}/tyrosine^{204}) and rabbit anti-ERK1/2 antibodies were purchased from New England Biolabs (Mississauga, Ontario). NuSerum, collagen IV, and mouse monoclonal antibodies to phosphotyrosine (PY20), Ras, Bax (6A7), and Bcl-X_L were obtained from BD Biosciences (Mississauga, Ontario). GST-Grb2 (1-217) fusion protein, rabbit anti-FAK, and rabbit anti-Grb2 polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, California). Rabbit anti-MEK1 was purchased from Stressgen Biotechnologies Co. (Victoria, British Columbia). Protein A-agarose and mouse anti-Src monoclonal antibody were obtained from Upstate Biotechnology (Lake Placid, New York). Rabbit anti-phospho-Src (tyrosine^{418}) was purchased from Biosource International (Camarillo, California). Bisbenzimide H33342 fluorochrome, propidium iodide, caspase 8 substrate (granzyme B substrate I), caspase 9 substrate II, LEHD-CHO, PP2, and PD98059 were obtained from Calbiochem (La Jolla, California). Rabbit anti-EGF-R antibody, RK2, was described previously (14). Anti-β1 integrin rabbit antiserum was kindly provided by Dr. Michael DiPersio (Albany Medical College, Albany, New York) (34). Electrophoresis and immunoblotting reagents were from Bio-Rad Laboratories

(Mississauga, Ontario). Male Sprague-Dawley rats (150 g) were purchased from Charles River Canada (St. Constant, Quebec). An expression plasmid containing the G12V/Q43E/D54N/E63K Ras mutant (J25 Ras) cDNA was kindly provided by Dr. Anne Vojtek (University of Michigan, Ann Arbor Michigan) (48). Plasmid CDM8 containing constitutively-active FAK (CD2-FAK) cDNA was kindly provided by Dr. Alejandro Aruffo (Bristol Myers Squibb, Princeton, New Jersey) (7). R4F-MEK cDNA was provided by Dr. Natalie Ahn (University of Colorado, Boulder, Colorado) (33).

Extracellular matrix and GEC culture

Type I collagen gel matrices were prepared as described previously (9). Collagen IV solution was applied to culture wells at 0.02mg/cm^2 and was allowed to air-dry at 22°C. Collagen I was used in all experiments, except where indicated. Primary cultures of rat GEC were established from explants of rat glomeruli, and have been characterized previously (8,9,12,14). Unlike some other GEC culture lines, the cells used in the present study do not contain the SV40 large T antigen, and thus, in our view are better suited for studies of cell survival. Experiments were done with cells between passages 25 and 70. Under standard conditions, GEC were cultured on collagen I matrices in K1 medium, which consisted of DMEM/Ham F10 (1:1), containing 5% NuSerum and hormone supplements (9,12). GEC were maintained in culture by passaging onto collagen gels; for experiments, GEC were replated onto collagen, laminin or plastic substrata in serum-poor (DMEM-0.5% fetal calf serum) or K1 media.

GEC transfection

GEC on collagen were co-transfected with the expression vector of interest (2 μg of DNA per 100 mm plate), and a neomycin resistance gene pRc/RSV (molar ratio 12.5:1), using the $CaPO_4$ technique, as described previously (13). GEC were then cultured on collagen in K1 medium containing 0.5 mg/ml G418. GEC clones resistant to G418 were isolated and replated onto plastic substratum. Clones that proliferated on plastic in K1 medium were selected, passaged, and assessed for the expression of the specific protein by immunoblotting. A soft agar assay was used to test for anchorage-independent proliferation (13).

Measurement of GEC proliferation

Cell number was determined by visual counting. Cells adherent to collagen gels (35 mm plates) were placed into single-cell suspension with collagenase and trypsin-EDTA. Cells on plastic substratum were placed into suspension by incubation with trypsin-EDTA. Suspended cells were then counted in a hemacytometer (13,14).

Induction of PAN in rats

PAN was induced in Male Sprague-Dawley rats (150 g) by a single intravenous injection of 80 mg/kg of puromycin aminonucleoside (22). Urine was collected on day 14, and rats were then sacrificed, and glomeruli were isolated by differential sieving (15).

Immunoprecipitation and immunoblotting

Preparation of GEC and glomerular lysates was described previously (15).

Briefly, after treatment, ~6 × 10^6 GEC were lysed in immunoprecipitation buffer, containing 1.0% Triton X-100, 125 mM NaCl, 20 mM Tris, 20 μM leupeptin, 20 μM pepstatin, 0.2 mM PMSF, 25 mM NaF, 2 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 1 mM EDTA, pH 7.4 (4°C). The mixture was centrifuged at 14,000 x g for 10 minutes, and the supernatant was then used for immunoprecipitation or immunoblotting. Glomeruli were centrifuged, and resuspended in immunoprecipitation buffer, as above. After adjusting protein concentrations, proteins were immunoprecipitated with primary antiserum under quantitative conditions, as described previously (15). Immune complexes were incubated with agarose-coupled protein A. Complexes were boiled in Laemmli sample buffer, and subjected to SDS-PAGE under reducing conditions. Proteins were then electrophoretically transferred onto nitrocellulose paper, blocked with 3% BSA/2% ovalbumin, and incubated with primary antibody, and then with horseradish peroxidase-conjugated secondary antibody. The blots were developed using the enhanced chemiluminescence technique (ECL, Amersham Pharmacia Biotech AB). Protein content was quantified by scanning densitometry, using NIH Image software (15). Preliminary studies demonstrated that there was a linear relationship between densitometric measurements and the amounts of protein loaded onto gels.

DNA fragmentation

Adherent cells were collected in PBS and centrifuged 5 minutes at 500 x g at 4°C. Cell pellets were resuspended in buffer, containing 10 mM Tris-HCl, 1 mM EDTA, 0.2% Triton X-100, pH 7.4 and incubated for 30 minutes (4°C). The mixture was centrifuged at 14,000 x g for 10 minutes. The supernatant was collected and incubated with 100 μg/ml

of RNase A and 100 μg/ml of proteinase K for 1 hour (37°C). Ice cold 5 M NaCl and isopropanol were added to the mixture in order to precipitate the DNA overnight (-20°C). The pellet was then washed in ice cold ethanol and dissolved in Tris-EDTA buffer. After adjusting DNA concentrations, 3 μg of DNA was separated on a 1.2% agarose gel prestained with ethidium bromide.

Hoechst H33342 staining

To quantitate apoptosis, adherent cells were stained with Hoechst H33342 dye (1 μg/ml) for 10 minutes at 37°C without fixation (31). After washing with PBS, cells were stained with propidium iodide (5 μg/ml) to visualize necrotic or late-apoptotic cells. Nuclei of apoptotic cells were condensed and stained brightly with H33342 dye while excluding propidium iodide. Cells were photographed using a Nikon Diaphot immunofluorescence microscope and Nikon Coolpix 995 digital camera, and the number of H33342-positive/propidium iodide-negative cells were quantified by visual counting.

Caspase activity assay

Intracellular caspase 8 and 9 activities were measured using a colorimetric assay with p-nitroaniline (pNA)-labeled substrates, according to the manufacturer's instructions. Adherent cells were lysed in buffer containing 50 mM Hepes, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 100 μM EDTA, pH 7.4 (4°C). The mixture was centrifuged at 14,000 x g for 10 minutes. Supernatants were collected and adjusted for protein concentration. The reaction mixture contained 50 mM Hepes, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 100 μM EDTA, 10% glycerol , pH 7.4 (37°C) with 2 mM

substrate, i.e. Ac-Leu-Glu-His-Asp-pNA (caspase 9), or Ac-Ile-Glu-Thr-Asp-pNA (caspase 8). The absorbance of the reaction was read at 405 nm every 5 minutes for 2 hours. After subtraction of background activity, the slope of absorbance versus time is proportional to caspase activity.

Immunofluorescence microscopy

Cryostat kidney sections (4 μm) were fixed with ether-ethanol (1:1, 10 minutes), followed by ethanol (20 minutes) at 4°C. Sections were incubated with rabbit anti-phospho-ERK IgG (2.5 μg/ml) overnight at 4°C. Non-immune rabbit IgG was used in control incubations. After washing, sections were incubated with fluorescein-conjugated goat anti-rabbit IgG for 1 hour at 22°C. The immunofluorescence signals were evaluated using a Nikon Diaphot immunofluorescence microscope with visual output connected to a Nikon Coolpix 995 digital camera.

Statistics

Data are presented as mean±SEM. The t statistic was used to determine significant differences between two groups. One-way ANOVA was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the t statistic, and adjusting the critical value according to the Bonferroni method.

RESULTS

Collagen facilitates GEC survival

In keeping with previous results (12,14), addition of growth factors (K1 medium) or EGF to cultured GEC adherent to collagen resulted in proliferation (as reflected by a progressive increase in cell number) (Fig. 1). Cell number remained constant in GEC on collagen in serum-poor medium. However, following adhesion, cell number decreased steadily on plastic in the presence of serum-poor medium, and the decrease was slightly attenuated by addition of growth factor-replete medium or EGF (Fig. 1). By light microscopy, there were no significant differences in cell spreading between collagen and plastic (not shown).

To determine if the decline in GEC number in the absence of collagen was due to apoptosis, we analyzed DNA isolated from parental GEC plated onto collagen or plastic in the presence or absence of growth factors. DNA fragmentation ("laddering"), a feature consistent with apoptosis, was evident in parental GEC adherent to plastic after 72 hours of plating, but not in GEC plated onto collagen (Fig. 2A). DNA laddering occurred whether or not growth factors were present in the medium. In addition, we quantified the amount of apoptosis by staining GEC with Hoechst 33342 dye (H33342) and propidium iodide. In this assay, nuclei of apoptotic cells (which show chromatin condensation and fragmentation) stain brightly with H33342, but these cells generally do not stain with propidium iodide, since apoptotic cells usually possess intact plasma membranes. However, in cell culture, propidium iodide-positive cells are generally "late-apoptotic", as apoptotic cells are not phagocytosed and may proceed to necrosis. H33342-positive GEC were evident almost exclusively in monolayers of cells adherent to plastic (Fig. 2B

and 2C), consistent with the DNA laddering assay. The presence of growth factors did not affect the amount of apoptosis in GEC on plastic (Fig. 2C), even though growth factors slightly attenuated the decline in cell number (Fig. 1). Possibly the H33342 assay was not sufficiently sensitive to detect minor differences. Substitution of collagen with laminin was unable to promote GEC survival, shown by increased numbers of H33342-positive cells in GEC plated onto laminin, as compared with GEC on collagen (Fig. 2D). These results highlight a key role for collagen in promoting survival.

Effect of collagen on caspase activity and expression of Bax and Bcl-X_L

The morphologic changes of an apoptotic cell are induced by the activation of caspases (43), and may be modulated by pro- or anti-apoptotic actions of Bcl-2 family proteins. Caspase-9 activity (linked to release of caspase-9-activating proteins from mitochondria into cytosol) was increased in GEC adherent to plastic, as compared with collagen (Fig. 3A). Interestingly, caspase-8 activity (typically linked to "death domain" receptors) was also increased in GEC adherent to plastic (Fig. 3A). Pretreatment of GEC with the caspase 9 inhibitor, LEHD-CHO (30), blocked both plastic-stimulated caspase 9 and caspase 8 activities (Caspase 9: Collagen 0.11±0.02, Plastic 0.76±0.14, Plastic + inhibitor 0.33±0.05; Caspase 8: Collagen 0.11±0.03, Plastic 0.77±0.15, Plastic + inhibitor 0.34±0.02; N=4, $P<0.0025$ Plastic + inhibitor vs Plastic for both caspases; activity is presented in arbitrary units). Finally, analysis of the expression of two proteins of the Bcl-2 family revealed a significant increase in the pro-apoptotic protein, Bax, and a significant decrease in the anti-apoptotic protein, Bcl-X_L, in GEC plated onto plastic substratum, as compared with GEC on collagen (Fig. 3B).

Adhesion to collagen induces activation of FAK and ERK

The next series of studies addressed collagen-induced signals that may mediate survival. First, we examined activation of FAK. FAK protein expression and tyrosine phosphorylation (which reflects FAK activation) were increased on collagen, as compared with plastic substratum (Fig. 4A). The change in FAK phosphorylation exceeded the increase in expression (Fig. 4A, right panel). FAK activation was due to a direct effect of collagen, as EGF or K1 medium were unable to activate FAK (data not shown).

The nonreceptor protein tyrosine kinase, c-Src, is reported to regulate FAK activity (6). In parallel with FAK, c-Src tyrosine^{418} phosphorylation (reflecting activation of Src) was increased significantly in GEC adherent to collagen, as compared with plastic substratum (Fig. 4B). Treatment of parental GEC on collagen with PP2, a selective inhibitor of the Src family of protein tyrosine kinases (25), significantly inhibited FAK phosphorylation, as compared with untreated GEC (Fig. 4C).

In addition to FAK, adhesion of GEC to collagen stimulated a ~2-fold increase in ERK1 and ERK2 threonine^{202}/tyrosine^{204} phosphorylation (Fig. 4D), which reflects ERK activation (13,14). Collagen did not alter ERK protein expression significantly (Fig. 4D). The phosphorylation of ERK was a direct effect of collagen, and was not dependent on the presence of growth factors in the culture medium. The reduction in FAK phosphorylation by the PP2 Src inhibitor (Fig. 4C) was paralleled by a reduction in ERK phosphorylation (Fig. 4E; 40% inhibition by densitometry). These results suggest that FAK is linked to and may signal via the ERK pathway.

Expression of constitutively active FAK and MEK

To determine the downstream targets of FAK, and if FAK activation was functionally important, GEC on collagen were stably co-transfected with a neomycin-resistance gene plus a constitutively-active FAK cDNA (CD2-FAK). Of 68 G418-resistant clones that proliferated on collagen in K1 medium, 34 were also able to proliferate on plastic, and all of these clones expressed CD2-FAK at various levels (representative clones are shown in Fig. 5A). Thus, expression of CD2-FAK supplanted the requirement for collagen shown in Fig. 1. In the next series of experiments, we tested if ERK is a potential target of endogenous FAK and CD2-FAK, i.e. if activated FAK or CD2-FAK could bind Grb2, an adapter protein that would link FAK to the Ras-ERK pathway. FAK was immunoprecipitated from parental GEC on collagen or plastic, or from CD2-FAK-transfected GEC, and the immunoprecipitates were blotted with either anti-phosphotyrosine antibody or GST-Grb2, followed by anti-Grb2 antibody. The results demonstrate that CD2-FAK, as well as FAK in GEC on collagen, were intensely tyrosine phosphorylated (activated), and that only the intensely phosphorylated CD2-FAK and FAK bound Grb2 (Fig. 5B).

To examine the functional role of the Ras-ERK pathway, GEC on collagen were stably co-transfected with a neomycin-resistance gene plus constitutively-active MEK (R4F-MEK; N3/S218E/S222D). Among multiple G418-resistant clones that proliferated on collagen in K1 medium, 17 clones were also able to proliferate on plastic substratum in a growth factor-dependent manner, and all of these clones expressed R4F-MEK. Three representative clones are shown in Fig. 5C (it should be noted that endogenous MEK was not readily detectable by immunoblotting). In the absence of exogenous EGF (or other

mitogens), ERK phosphorylation in the three R4F-MEK-expressing GEC clones was at least 5-fold greater, as compared with parental GEC on collagen (4 experiments) (Fig. 5D). By analogy to the stimulatory effect of collagen in parental GEC, as well as the effect of R4F-MEK expression, ERK phosphorylation was readily detectable in the CD2-FAK-expressing cells in the absence of exogenous EGF (Fig. 5E, four left lanes).

FAK and the ERK pathway mediate GEC survival

Adhesion to collagen induced ERK activation, whereas expression of constitutively-active FAK and MEK supplanted the collagen effect. In parallel, R4F-MEK- or CD2-FAK-expressing GEC plated onto plastic showed levels of apoptosis (determined by H33342 staining) similar to parental GEC on collagen, i.e. levels of apoptosis in these clones were significantly lower, as compared with parental GEC on plastic (Fig. 6, Untreated). In addition, DNA fragmentation was not observed in the GEC that express CD2-FAK (Fig. 2A). To verify that the pro-survival signaling of collagen was actually mediated through the ERK pathway, GEC clones were treated with the MEK inhibitor, PD98059 (17), and apoptosis was quantified after 24 hours (Fig. 6). For comparison, GEC were also treated with the PI3K inhibitor, LY294002 (47). Inhibition of the ERK pathway significantly increased the amount of apoptosis in parental GEC, as well as in R4F-MEK- and CD2-FAK-expressing clones, as compared with untreated cells (Fig. 6, PD98059). It should be noted that treatment of GEC plated on plastic substratum with PD98059 did not further enhance apoptosis, thus excluding a potential nonspecific or cytotoxic effect of this inhibitor (data not shown). PI3K inhibition significantly increased the level of apoptosis only in the CD2-FAK clones (Fig. 6, LY294002). There

were no significant increases in apoptosis in LY294002-treated R4F-MEK GEC, nor in parental GEC on collagen, although an upward trend occurred in the latter.

Growth factor signals are regulated by collagen

The experiments described above focus on signals induced by collagen, which regulate GEC survival. Previously, we demonstrated that in the presence of growth factors, including EGF, GEC proliferate when adherent to collagen I or IV matrices, but not plastic substratum, and that stable transfection of constitutively-active Ras (V^{12}Ras) supplanted the requirement for collagen, but not for growth factors (12-14). Similar to previous results (12), EGF further stimulated ERK activation in GEC on collagen I, but was not able to activate ERK significantly on plastic (Fig. 7A and 7B). EGF also stimulated ERK phosphorylation on collagen IV (a more physiological substratum for GEC) (Fig. 7A). Moreover, in the CD2-FAK-expressing GEC and in the R4F-MEK-expressing clones, ERK phosphorylation was further stimulated by EGF (Fig. 5E and 7B), similar to ERK activation in collagen-adherent parental GEC, but unlike parental GEC on plastic (Fig. 7B). These results are similar to those observed earlier in GEC that were stably transfected with V^{12}Ras, a constitutively-active Ras mutant (13). Thus, constitutively active Ras, FAK or MEK provided sustained ERK activation, and enabled EGF to further stimulate ERK activity in the absence of collagen.

Proliferation of R4F-MEK- and CD2-FAK-expressing clones occurred independently of collagen, but was nonetheless dependent on adhesion to plastic (i.e. anchorage-dependent), and required addition of exogenous growth factors (Fig. 7C and 7D). Thus, R4F-MEK and CD2-FAK were able to supplant the requirement for collagen,

but not growth factors, and the ability of EGF to induce proliferation correlates with its ability to induce ERK activation (Fig. 7B). R4F-MEK- and CD2-FAK-expressing GEC clones did not proliferate in soft agar (data not shown), indicating that these cells were not transformed.

ERK is the principal Ras effector that mediates GEC survival

In addition to the ERK pathway, collagen or V^{12}Ras (which supplants collagen) (13) may potentially activate other downstream effectors. To determine if the PI3K pathway may be activated by collagen, or alternatively, if constitutive PI3K activation would supplant the signal from collagen, GEC on collagen were stably transfected with J25 Ras (Q43E/D54N/E63K Ras), a Ras mutant that constitutively activates PI3K, but is unable to activate the ERK pathway (Fig. 8A). In the presence of growth factors, J25 Ras-expressing GEC were unable to proliferate or survive on plastic, suggesting that activation of non-ERK Ras effectors (e.g. PI3K) is insufficient to supplant collagen. J25 Ras expression significantly reduced EGF-dependent ERK activation (on collagen), which proceeds via EGF-R-Grb2-Ras (Fig. 8B), but not activation by thrombin, which stimulates ERK via a G-protein receptor, independently of Ras (Fig. 8C and 8D). In the absence of EGF or thrombin, ERK phosphorylation was lower in J25 Ras-expressing GEC on collagen, as compared with parental cells (Fig. 8B and 8C). These results imply that J25 Ras functioned as a dominant inhibitor of the Ras-ERK pathway, blocking both EGF- and collagen-stimulated ERK phosphorylation.

Culture of J25 Ras-expressing GEC in growth factor-poor medium (on collagen) resulted in a marked decrease in cell number, whereas cell number remained constant in

parental GEC under similar conditions (Fig. 8E). Together with the observed reduction in ERK phosphorylation in J25 Ras-expressing GEC on collagen (Fig. 8B, no EGF), these results indicate that J25 Ras most likely blocked a signal for GEC survival from the collagen matrix, involving the Ras-ERK pathway. This inhibitory action of J25 Ras was unrelated to growth factor-induced Ras-ERK activation, since growth factors were absent in these experiments (Fig. 8E). The above results demonstrate the importance of the ERK pathway in mediating the survival signals emanating from collagen. Possibly, PI3K may also contribute to GEC survival (Fig. 6, LY294002), but this pathway is unable to mediate survival independently of the ERK pathway.

In the presence of growth factors (K1 medium), GEC that express J25 Ras proliferated on collagen, but at a reduced rate, as compared with parental cells. Parental GEC passaged at a ratio of 1:10 reached 50% confluency at 4.3±0.1 days (N=11), while J25 Ras-expressing GEC passaged at a ratio of 1:3 reached 50% confluency only at 7.7±1.2 days (N=6, $P<0.001$). The result confirms that J25 Ras most likely functioned as a dominant inhibitor of endogenous Ras, and blocked EGF-induced activation of the ERK pathway.

Signals for GEC survival are activated in PAN

The above studies characterized signaling by ECM and growth factors in cultured GEC, but it is important to demonstrate that analogous signaling pathways may be activated in GEC in vivo. To address this question, we employed the acute PAN model of GEC injury (22). First, we demonstrated that puromycin aminonucleoside markedly enhanced the apoptosis of cultured GEC on plastic (Fig. 9). On collagen, there was an

upward trend in apoptosis in the presence of puromycin aminonucleoside, but adhesion to collagen largely protected GEC from puromycin aminonucleoside-induced apoptosis (Fig. 9). These results suggest that the collagen-induced survival signals characterized above (Fig. 4-6) can limit GEC injury. Treatment of GEC on collagen with puromycin aminonucleoside did not enhance the level of collagen-induced ERK phosphorylation significantly (puromycin aminonucleoside: 92% of untreated at 30 min, 115% at 2 hours, and 99% at 24 hours; N=4). In vivo, two weeks after administration of puromycin aminonucleoside, rats demonstrated marked proteinuria, a hallmark of GEC injury (Control 8±3, PAN 246±29 mg protein/24 hours). Expression of β1 integrin was increased almost 2-fold in glomeruli isolated from rats with PAN, as compared with controls (Fig. 10A), suggesting that in PAN, there may be amplification of those signals from ECM, which are mediated via β1 integrins. Protein expression and phosphorylation of FAK (Fig. 10B), c-Src (Fig. 10C) and ERK (Fig. 10D) were detectable in normal (control) glomeruli, and significant increases in activation and expression of these proteins were evident in glomeruli isolated from rats with PAN, as compared with controls. In contrast, EGF-R protein expression was not significantly different between control and PAN, and EGF-R tyrosine phosphorylation was not detected consistently in either group (Fig. 10E). Thus, the protein kinases that are activated by collagen and support survival in cultured GEC are also activated in vivo.

By immunofluorescence microscopy, glomeruli of rats with PAN showed specific, although faint staining for phospho-ERK, with focal and patchy accentuation (Fig. 11A). Staining of glomerular capillary loops was evident, particularly at the periphery of glomeruli, in keeping with ERK activation in GEC (Fig. 11B). Phospho-

ERK staining was also detectable in normal rat glomeruli, but was weaker than in PAN (not shown).

DISCUSSION

This study demonstrates that adhesion of GEC to collagen facilitates survival, principally via activation of FAK and a Ras-ERK pathway. After plating of GEC onto collagen in the absence of growth factors, cell number remained constant, whereas cell number declined on plastic substratum, and GEC demonstrated features of apoptosis (Fig. 1 and 2). It should be noted that the pro-apoptotic effect of plastic was not due to the inability of GEC to attach and spread on plastic. Surprisingly, adhesion of GEC to laminin did not facilitate survival (Fig. 2D) even though GEC express laminin-binding cell surface receptors (1,10,11). The major cell surface receptors for ECM proteins are integrins, and GEC in culture and in vivo express the $\alpha 3\beta 1$ integrin (1,10,11). Cultured GEC adhere to collagen I, collagen IV, laminin and fibronectin, and show greater adhesion to the collagens, as compared with the other substrata (1,11). Anti-$\beta 1$ integrin antibody significantly inhibited adhesion to ECM proteins (1,11). Thus, the anti-apoptotic effect of collagen may be related to the greater efficiency of GEC adhesion to this ECM protein.

FAK, a key intermediate protein kinase that transmits some of the signals from ECM that are relayed by integrins, was activated in parental GEC on collagen (Fig. 4A). FAK protein expression was also enhanced on collagen, suggesting that increased synthesis or decreased degradation of FAK protein may contribute to the regulation of activity. Stable expression of a constitutively-active FAK (CD2-FAK) supplanted the effect of collagen in facilitating GEC survival (Fig. 2A and 6), implying that FAK

activation is anti-apoptotic. As reported in other cells (40), collagen-induced FAK activation in GEC was, at least in part, dependent on the activation of c-Src (Fig. 4B and 4C). Activated endogenous FAK in parental GEC on collagen and CD2-FAK were able to bind Grb2, but nonphosphorylated FAK in parental GEC on plastic did not (Fig. 5B). In addition, adhesion of GEC to collagen or expression of CD2-FAK increased ERK phosphorylation (Fig. 4D and 5E), while inhibition of Src reduced FAK and ERK phosphorylation in parallel (Fig. 4C and 4E). Together, these results imply that transmission of signals from collagen may occur via FAK and the ERK pathway.

Evidence for a functional role of the Ras-ERK pathway in blocking apoptosis was provided by stable transfection of constitutively-active MEK (R4F-MEK). R4F-MEK increased ERK phosphorylation (Fig. 5E), and facilitated survival (Fig. 6). Conversely, inhibition of the ERK pathway with PD98059 increased apoptosis in parental GEC on collagen, as well as in R4F-MEK- and CD2-FAK-expressing GEC clones (Fig. 6). Moreover, expression of the J25 Ras mutant, which blocked the ERK pathway by dominant inhibition of Ras (Fig. 8B), blocked GEC survival (Fig. 8E). Together these results confirm the importance of the ERK pathway in mediating the survival signals emanating from collagen. By analogy to GEC, fibronectin rescued a carcinoma cell line from serum depletion-induced apoptosis through the ERK pathway, and cell survival on fibronectin was significantly reduced by treatment with PD98059, or by expression of a dominant-negative mutant of MEK1 (23). In other cells, survival appears to be dependent on the activation of the PI3K-Akt pathway (20), or a combination of PI3K and ERK (16). In GEC, inhibition of the PI3K pathway with LY294002 significantly increased the level of apoptosis only in the CD2-FAK-expressing clones, but not in parental cells on

collagen nor in GEC that express R4F-MEK (Fig. 6). Furthermore, the J25 Ras mutant, which constitutively activates the PI3K pathway, did not facilitate survival, suggesting that activation of PI3K downstream of Ras is insufficient to supplant signals from collagen. Thus, the ERK cascade appears to be the major pathway that mediates the survival signal emanating from collagen.

There are two major apoptotic pathways in mammalian cells, the death domain receptor pathway, which leads to activation of caspase 8, and the mitochondrial pathway, linked to caspase 9 (38). Both caspase 8 and 9 activities were increased in GEC adherent to plastic, as compared with collagen (Fig. 3A), but inhibition of caspase 9 inhibited caspase 8 in parallel. This result suggests that activation of caspase 9 may have been responsible for secondary activation of caspase 8 (43). Alternatively, a possible role for death domains in triggering anoikis, perhaps by self-association of death domain receptors or interaction with endogenous death ligands, has been proposed (20). Another explanation may be that caspase 8 was activated as a result of non-ligated integrins, as recently shown in T24E carcinoma cells (45). Involvement of the mitochondrial pathway in GEC apoptosis was further supported by changes in the expression of Bax and Bcl-X_L, which exert some of their functional effects on the mitochondria (38). There was a significant increase in the pro-apoptotic protein Bax and a significant decrease in the anti-apoptotic protein Bcl-X_L in GEC plated onto plastic substratum (Fig. 3B). Together, these results substantiate an anti-apoptotic or survival-promoting effect of collagen. The precise interplay among ECM, Bcl-2 family members and caspases will require further study.

In earlier studies, we demonstrated that EGF stimulated ERK activation and

proliferation of cultured GEC only when the cells were adherent to collagen matrices, and that proliferation was abolished by PD98059 (12,14). GEC adherent to laminin were unable to proliferate (9). In addition, stable transfection of a constitutively-active mutant of Ras (V^{12}Ras) supplanted the requirement for collagen, but not for growth factors (13). These results imply that EGF most likely activated the ERK pathway through endogenous Ras, whereas V^{12}Ras had activated a separate ERK pathway. In the present study, expression of constitutively-active mutants of MEK or FAK did not induce GEC proliferation independently of growth factors (Fig. 7C and 7D). However, by analogy to V^{12}Ras, R4F-MEK or CD2-FAK enabled exogenously-added growth factors to further stimulate ERK phosphorylation above resting levels (Fig. 7B), and to induce proliferation of GEC in the absence of collagen (Fig. 7C and 7D). These experiments further support the view that constitutively-active FAK and MEK mutants supplant ERK activation by collagen, while growth factor-induced ERK activation occurs via a distinct Ras-ERK pathway. Morever, a survival signal from collagen, or from constitutively-active protein kinases that supplant this collagen effect, is a prerequisite for EGF-dependent activation of EGF-R and the ERK pathway, as well as proliferation. In fibroblasts, EGF-dependent ERK activation was dependent on adhesion to ECM and an intact cytoskeleton (2). ECM enabled EGF to induce ERK translocation to the nucleus and phosphorylation of transcription factors, whereas EGF was ineffective in suspended cells. Further studies will be required to characterize the ERK pathways in GEC. For example, activation of ERK by collagen might occur at focal adhesions and the target of ERK may be cytosolic or nuclear, while growth factor induced ERK activation may occur near the receptor and the target may be in the nucleus.

To verify that signals in cultured GEC are relevant to GEC in vivo, we studied PAN, an experimental model of GEC injury that has been employed to obtain a better understanding of FSGS pathophysiology (4,5,36,46). In PAN, GEC foot processes are effaced, such that a greater area of the cell surface is in contact with the glomerular basement membrane. Furthermore, expression of α3 and β1 integrin subunits, as well as paxillin (a FAK-interacting protein), was upregulated in early PAN (29,32,44), and increases in laminin and types I and IV collagen were evident as the lesion progressed (18). Integrin-mediated signals may be amplified by increased integrin expression or engagement by ECM (e.g. due to increased amounts of ECM proteins or adhesivity) (3). Consequently, it is reasonable to propose that in PAN, there may be enhanced signaling from the ECM to GEC (via β1 integrins). In keeping with the earlier report (32), we demonstrate that glomerular β1 integrin expression was increased in rats with PAN, as compared with controls (Fig. 10A). FAK, c-Src and ERK protein expression and phosphorylation were detected at relatively low levels in normal glomeruli, and expression and activation were increased in PAN (Fig. 10B-10D, Fig. 11). These results are in keeping with the view that integrin signaling was amplified in PAN. Puromycin aminonucleoside did not enhance ERK phosphorylation in cultured GEC, and it is unlikely that changes in ERK phosphorylation in vivo were due to a direct effect of this compound. These results contrast with findings in mesangial cells adherent to collagen gels, where puromycin aminonucleoside enhanced tyrosine phosphorylation, including phosphorylation of FAK (49). Based on our observation that adhesion to collagen limits puromycin aminonucleoside-induced GEC injury in culture (Fig. 9), the role of enhanced ECM signaling in PAN (including activation of FAK and ERK) may be to limit apoptosis

of GEC, and development of sclerosis. Under resting conditions, a weaker signal from ECM/integrins may be sufficient to block apoptosis. We cannot exclude activation of FAK by growth factors (e.g. EGF) in PAN, but we did not observe EGF-R activation in acute PAN (Fig. 10E), and growth factors could not activate FAK in cultured GEC. EGF-R activation might occur during the chronic phase of PAN, when there is increased expression of heparin binding-EGF mRNA (putative ligand for EGF-R in vivo) and evidence for cell proliferation (37). Our in vivo results support the pathophysiological relevance of the signaling pathways characterized in cultured GEC. However, further studies, including development of animal models where glomerular signaling pathways are disrupted, will be required to address the functional role of these signals in the pathophysiology of GEC injury in vivo.

FOOTNOTES

1. Abbreviations: ECM, extracellular matrix; EGF-R, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FSGS, focal segmental glomerulosclerosis; GEC, glomerular visceral epithelial cells; MEK, mitogen-activated protein kinase kinase-1; PAN, puromycin aminonucleoside nephrosis; PI3K, phosphatidylinositol 3-kinase; SH2, Src homology 2.

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REFERENCES

1. Adler S. Integrin receptors in the glomerulus: potential role in glomerular injury. *Am J Physiol* 262: F697-F704, 1992.

2. Aplin AE, Stewart SA, Assoian RK, and Juliano RL. Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. *J Cell Biol* 153: 273-281, 2001.

3. Asthagiri AR, Nelson CM, Horwitz AF, and Lauffenburger DA. Quantitative relationship among integrin-ligand binding, adhesion, and signaling via focal adhesion kinase and extracellular signal-regulated kinase 2. *J Biol Chem* 274: 27119-27127, 1999.

4. Barisoni L, Kriz W, Mundel P, and D'Agati V. The dysregulated podocyte phenotype: a novel concept in the pathogenesis of collapsing idiopathic focal segmental glomerulosclerosis and HIV-associated nephropathy. *J Am Soc Nephrol* 10: 51-61, 1999.

5. Barisoni L, Mokrzycki M, Sablay L, Nagata M, Yamase H, and Mundel P. Podocyte cell cycle regulation and proliferation in collapsing glomerulopathies. *Kidney Int* 58: 137-143, 2000.

6. Cary LA, and Guan JL. Focal adhesion kinase in integrin-mediated signaling. *Front Biosci* 4: D102-113, 1999.

7. Chan PY, Kanner SB, Whitney G, and Aruffo A. A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to pp125FAK. *J Biol Chem* 269: 20567-20574, 1994.

8. Coers W, Reivinen J, Miettinen A, Huitema S, Vos JT, Salant DJ, and Weening JJ. Characterization of a rat glomerular visceral epithelial cell line. *Exp Nephrol* 4:184-192, 1996.

9. Cybulsky AV, Bonventre JV, Quigg RJ, Wolfe LS, and Salant DJ. Extracellular matrix regulates proliferation and phospholipid turnover in glomerular epithelial cells. *Am J Physiol* 259: F326-F337, 1990.

10. Cybulsky AV, Carbonetto S, Cyr MD, McTavish AJ, and Huang Q. Extracellular matrix-stimulated phospholipase activation is mediated by β_1 integrin. *Am J Physiol* 264: C323-C332, 1993.

11. Cybulsky AV, Carbonetto S, Huang Q, McTavish AJ, and Cyr MD. Adhesion of glomerular epithelial cells to extracellular matrices: role of β1 integrins. *Kidney Int* 42: 1099-1106, 1992.

12. Cybulsky AV, McTavish AJ, and Cyr MD. Extracellular matrix modulates epidermal growth factor receptor activation in rat glomerular epithelial cells. *J Clin Invest* 94: 68-78, 1994.

13. Cybulsky AV, McTavish AJ, Papillon J, and Takano T. Role of extracellular matrix and Ras in regulation of glomerular epithelial cell proliferation. *Am J Pathology* 154: 899-908, 1999.

14. Cybulsky AV, and McTavish AJ. Extracellular matrix is required for MAP kinase activation and proliferation of rat glomerular epithelial cells. *Biochem Biophys Res Comm* 231: 160-166, 1997.

15. Cybulsky AV, Takano T, Papillon J, and McTavish AJ. Complement C5b-9 induces receptor tyrosine kinase transactivation in glomerular epithelial cells. *Am J Pathol* 155: 1701-1711, 1999.

16. Danilkovitch A, Donley S, Skeel A, and Leonard EJ. Two independent signaling pathways mediate the antiapoptotic action of macrophage-stimulating protein on epithelial cells. *Mol Cell Biol* 20: 2218-2227, 2000.

17. Dudley DT, Pang L, Decker SJ, Bridges AJ, and Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci USA* 92: 7686-7689, 1995.

18. Ebihara I, Suzuki S, Nakamura T, Fukui M, Yaguchi Y, Tomino Y, and Koide H. Extracellular matrix component mRNA expression in glomeruli in experimental focal glomerulosclerosis. *J Am Soc Nephrol* 3: 1387-1397, 1993.

19. Endlich K, Kriz W, and Witzgall R. Update in podocyte biology. *Curr Opin Nephrol Hypert* 10: 331-340, 2001.

20. Frisch SM, and Screaton RA. Anoikis mechanisms. *Curr Opin Cell Biol* 13: 555-562, 2001.

21. Giancotti FG, and Ruoslahti E. Integrin signaling. *Science* 285: 1028-1033, 1999.

22. Grond JG, Weening JJ, and Elema JD. Glomerular sclerosis in nephrotic rats. Comparison of the long-term effects of adriamycin and aminonucleoside. *Lab Invest* 51: 277-285, 1984.

23. Gu J, Fujibayashi A, Yamada KM, and Sekiguchi K. Laminin-10/11 and fibronectin differentially prevent apoptosis induced by serum removal via phosphatidylinositol 3-kinase/Akt- and MEK1/ERK-dependent pathways. *J Biol Chem* 277: 19922-19928, 2002.

24. Guan JL. Role of focal adhesion kinase in integrin signaling. *Int J Biochem Cell Biol* 29: 1085-1096, 1997.

25. Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, and Connelly PA. Discovery of a novel, potent, and src family-selective tyrosine kinase inhibitor. Study of lck- and fyn-dependent T cell activation. *J Biol Chem* 271: 695-701, 1996.

26. Ingber DE, and Folkman J. How does extracellular matrix control capillary morphogenesis. *Cell* 58: 803-805, 1989.

27. Kerjaschki D. Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis. *J Clin Invest* 108: 1583-1587, 2001.

28. Kim YH, Goyal M, Kurnit D, Wharram B, Wiggins J, Holzman L, Kershaw D, and Wiggins R. Podocyte depletion and glomerulosclerosis have a direct relationship in the PAN-treated rat. *Kidney Int* 60: 957-968, 2001.

29. Koukouritaki SB, Tamizuddin A, and Lianos EA. Enhanced expression of the cytoskeletal-associated protein, paxillin, in experimental nephrotic syndrome. *J Invest Med* 46: 284-289, 1998.

30. Kurita-Ochiai T, Ochiai K, and Fukushima K. Butyric acid-induced apoptosis is mediated by caspase-8 and -9 activation in a Fas-independent manner. *Clin Diagn Lab Immunol* 8: 325-332, 2001.

31. Lieberthal W, Menza SA, and Levine JS. Graded ATP depletion can cause necrosis or apoptosis of cultured mouse proximal tubular cells. *Am J Physiol* 274: F315-F327, 1998.

32. Luimula P, Sandstrom N, Novikov D, and Holthofer H. Podocyte-associated molecules in puromycin aminonucleoside nephrosis of the rat. *Lab Invest* 82: 713-718, 2002.

33. Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande Woude GF, and Ahn NG. Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* 265: 966-970, 1994.

34. Marcantonio EE, and Hynes RO. Antibodies to the conserved cytoplasmic domain of the integrin β1 subunit react with proteins in vertebrates, invertebrates and fungi. *J Cell Biol* 106: 1765-1772, 1988.

35. Mundel P, and Shankland SJ. Podocyte biology and response to injury. *J Am Soc Nephrol* 13: 3005-3015, 2002.

36. Nagata M, Hattori M, Hamano Y, Ito K, Saitoh K, and Watanabe T. Origin

and phenotypic features of hyperplastic epithelial cells in collapsing glomerulopathy. *Am J Kidney Dis* 32: 962-969, 1998.

37. Paizis K, Kirkland G, Khong T, Katerelos M, Fraser S, Kanellis J, and Power DA. Heparin-binding epidermal growth factor-like growth factor is expressed in the adhesive lesions of experimental focal glomerular sclerosis. *Kidney Int* 55: 2310-2321, 1999.

38. Reed JC. Mechanisms of apoptosis. *Am J Pathol* 157: 1415-1430, 2000.

39. Schlaepfer DD, Hanks SK, Hunter T, and Van der Geer P. Integrin-mediated signal transduction linked to Ras pathway by Grb2 binding to focal adhesion kinase. *Nature* 372: 786-791, 1994.

40. Schlaepfer DD, Jones KC, and Hunter T. Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: Summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. *Mol Cell Biol* 18: 2571-2585, 1998.

41. Schwartz MA, and Baron V. Interactions between mitogenic stimuli, or a thousand and one connections. *Curr Opin Cell Biol* 11: 197-202, 1999.

42. Shiiki H, Sasaki Y, Nishino T, Kimura T, Kurioka H, Fujimoto S, and Dohi K. Cell proliferation and apoptosis of the glomerular epithelial cells in rats with puromycin aminonucleoside nephrosis. *Pathobiol* 66: 221-229, 1998.

43. Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Green DR, and Martin SJ. Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol* 144: 281-292, 1999.

44. Smoyer WE, Mundel P, Gupta A, and Welsh MJ. Podocyte α-actinin induction precedes foot process effacement in experimental nephrotic syndrome. *Am J Physiol* 273: F150-F157, 1997.

45. Stupack DG, Puente XS, Boutsaboualoy S, Storgard CM, and Cheresh DA. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *J Cell Biol* 155: 459-470, 2001.

46. Valeri A, Barisoni L, Appel GB, Seigle R, and D'Agati V. Idiopathic collapsing focal glomerulosclerosis: a clinicopathologic study. *Kidney Int* 50: 1734-1746, 1996.

47. Vlahos CJ, Matter WF, Hui KY, and Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-

4-one (LY294002). *J Biol Chem* 269: 5241-5248, 1994.

48. Winkler DG, Johnson JC, Cooper JA, and Vojtek AB. Identification and characterization of mutations in Ha-Ras that selectively decrease binding to cRaf-1. *J Biol Chem* 272: 24402-24409, 1997.

49. Zent R, Ailenberg M, Downey GP, and Silverman M. ROS stimulate reorganization of mesangial cell-collagen gels by tyrosine kinase signaling. *Am J Physiol* 276: F278-287, 1999.

FIGURE 1

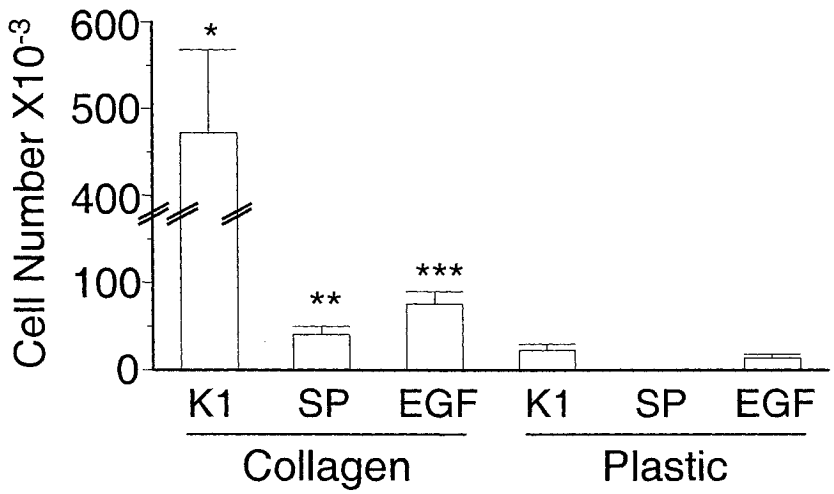

Figure 1. GEC number is sustained on collagen. Parental GEC (50,000 cells) were plated onto collagen or plastic, and were cultured in growth factor-replete (K1) medium, serum-poor (SP) medium + EGF (100 ng/ml), or serum-poor medium alone for five days. Cell number increased on collagen in the presence K1 medium or EGF. In the absence of growth factors, cell number was sustained on collagen. Cell number decreased on plastic, independently of the culture media. *P<0.001 collagen K1 vs plastic K1, **P<0.001 collagen SP vs plastic SP, ***P<0.001 collagen EGF vs plastic EGF (8 experiments).

FIGURE 2

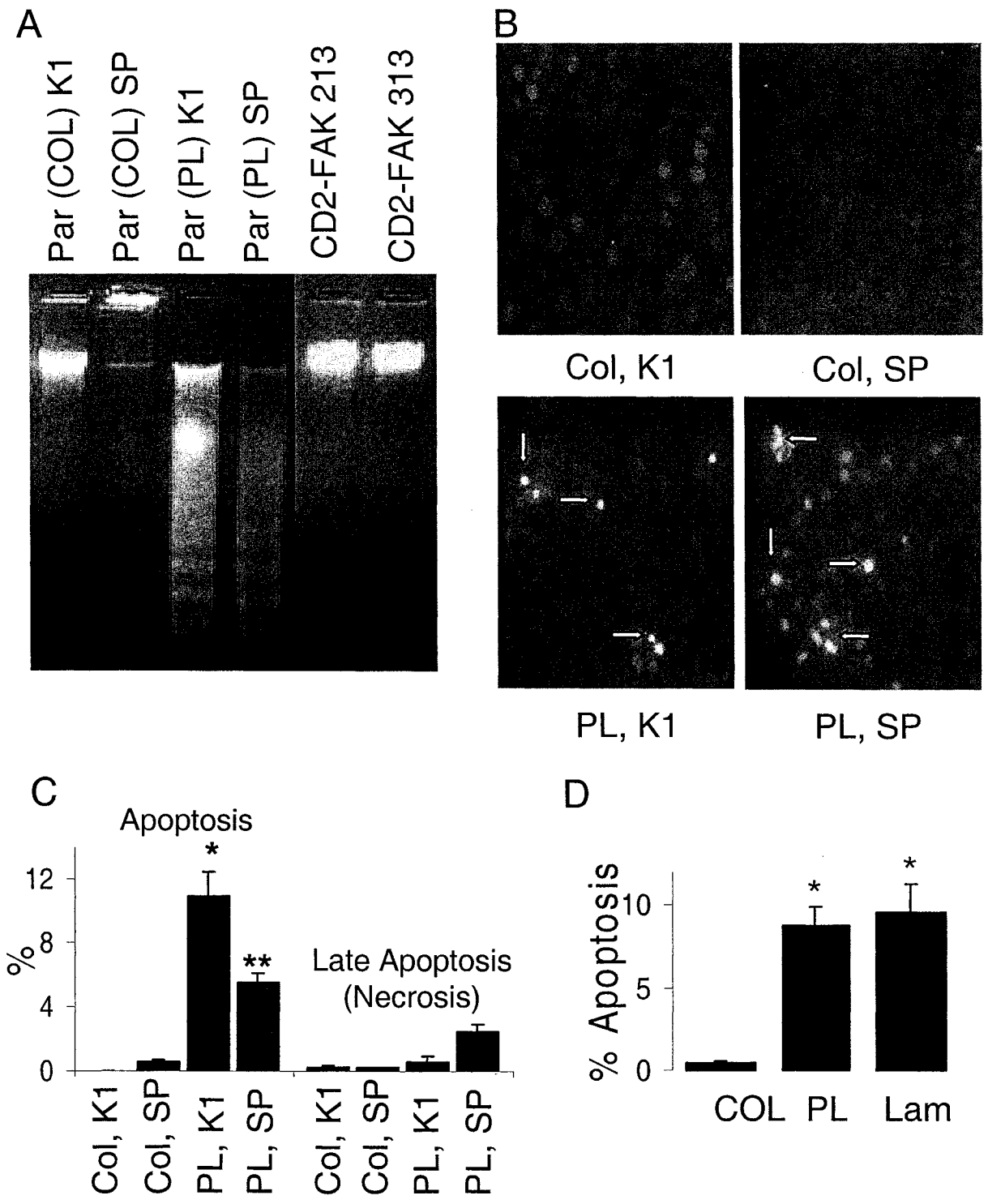
A
Par (COL) K1
Par (COL) SP
Par (PL) K1
Par (PL) SP
CD2-FAK 213
CD2-FAK 313
B
Col, K1
Col, SP
PL, K1
PL, SP
C
Apoptosis
Late Apoptosis (Necrosis)
%
12
8
4
0
*
**
Col, K1
Col, SP
PL, K1
PL, SP
Col, K1
Col, SP
PL, K1
PL, SP
D
% Apoptosis
10
5
0
*
*
COL
PL
Lam

Figure 2. Collagen facilitates GEC survival (decreases apoptosis). A) Total DNA was isolated from parental (Par) GEC plated on collagen (COL) or plastic (PL) in K1 or serum-poor (SP) media for 72 hours, and from CD2-FAK-expressing GEC. DNA laddering is evident in parental GEC adherent to plastic in K1 or serum-poor media. B) Parental GEC plated on collagen or plastic in K1 or serum-poor media for 48 hours were stained with Hoechst H33342 and propidium iodide. The lower panels show photomicrographs of propidium iodide-negative cells with H33342-positive nuclei (arrows). C) Quantification of H33342-positive nuclei (apoptotic cells), as well as propidium iodide-positive cells with H33342-positive nuclei (late apoptotic/necrotic cells). *P<0.0001 collagen K1 vs plastic K1, **P<0.0001 collagen SP vs plastic SP (3-9 experiments). D) Parental GEC were plated on collagen, plastic or laminin (Lam) in serum-poor medium for 48 hours. Apoptosis was identified as H33342-positive nuclei, propidium iodide-negative cells. *P<0.0001 vs collagen (5-6 experiments performed in duplicate).

FIGURE 3

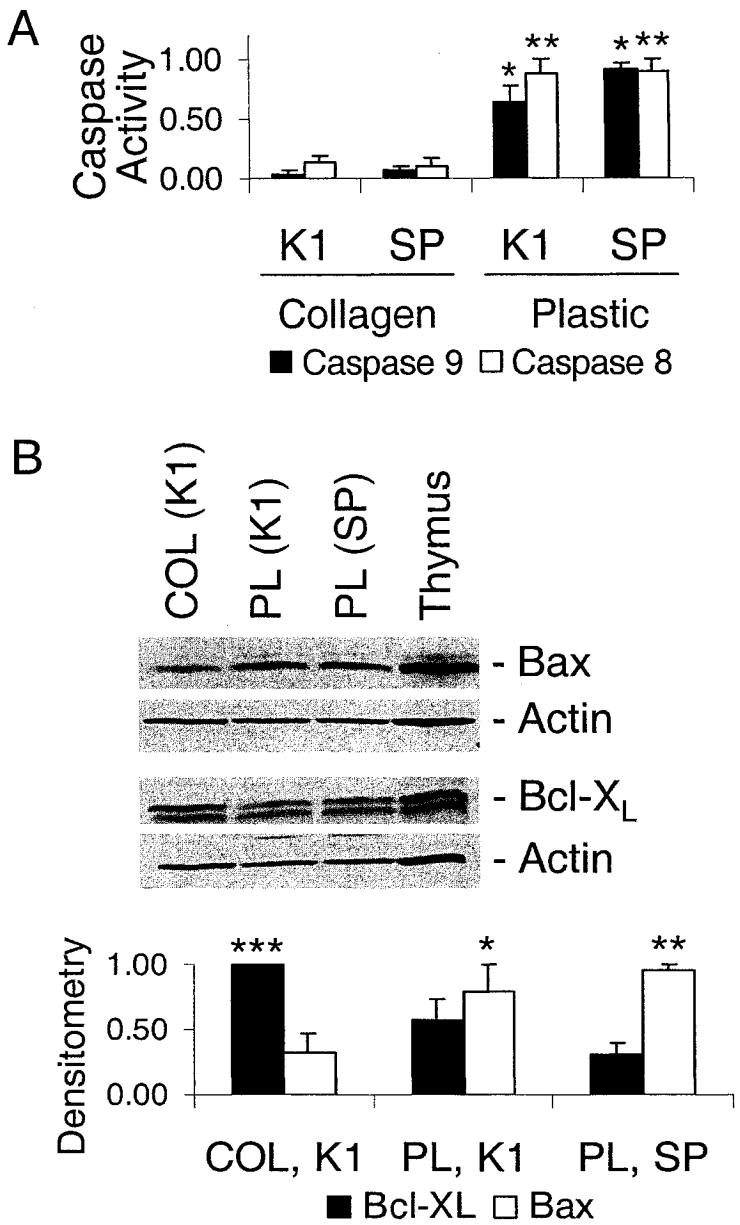
A
Caspase Activity
1.00
0.50
0.00
*
**
***
K1
SP
K1
SP
Collagen
Plastic
Caspase 9
Caspase 8
B
COL (K1)
PL (K1)
PL (SP)
Thymus
- Bax
- Actin
- Bcl-X$_L$
- Actin
Densitometry
1.00
0.50
0.00
***
*
**
COL, K1
PL, K1
PL, SP
Bcl-XL
Bax

Figure 3. A) Caspase 8 and 9 activities are increased in GEC on plastic. Parental GEC were cultured in K1 or serum-poor (SP) media for 24 hours. *P<0.0001 collagen K1 vs plastic K1 (caspase 9), *P<0.0001 collagen SP vs plastic SP (caspase 9), **P<0.0001 collagen K1 vs plastic K1 (caspase 8), **P<0.0001 collagen SP vs plastic SP (caspase 8) (5-6 experiments). B) Collagen enhances expression of Bcl-X_L, and reduces expression of pro-apoptotic Bax. Lysates of parental GEC that had been plated onto collagen or plastic in K1 or serum-poor media (24 hours) were immunoblotted with antibodies to Bax and Bcl-X_L. Rat thymus was used as positive control. The lysates were also immunoblotted for actin to demonstrate equal loading. *P<0.03 collagen K1 vs plastic K1 (Bax), **P<0.01 collagen K1 vs plastic SP (Bax), ***P<0.007 collagen K1 vs plastic K1 (Bcl-X_L), P<0.001 collagen K1 vs plastic SP (Bcl-X_L) (4 experiments).

FIGURE 4

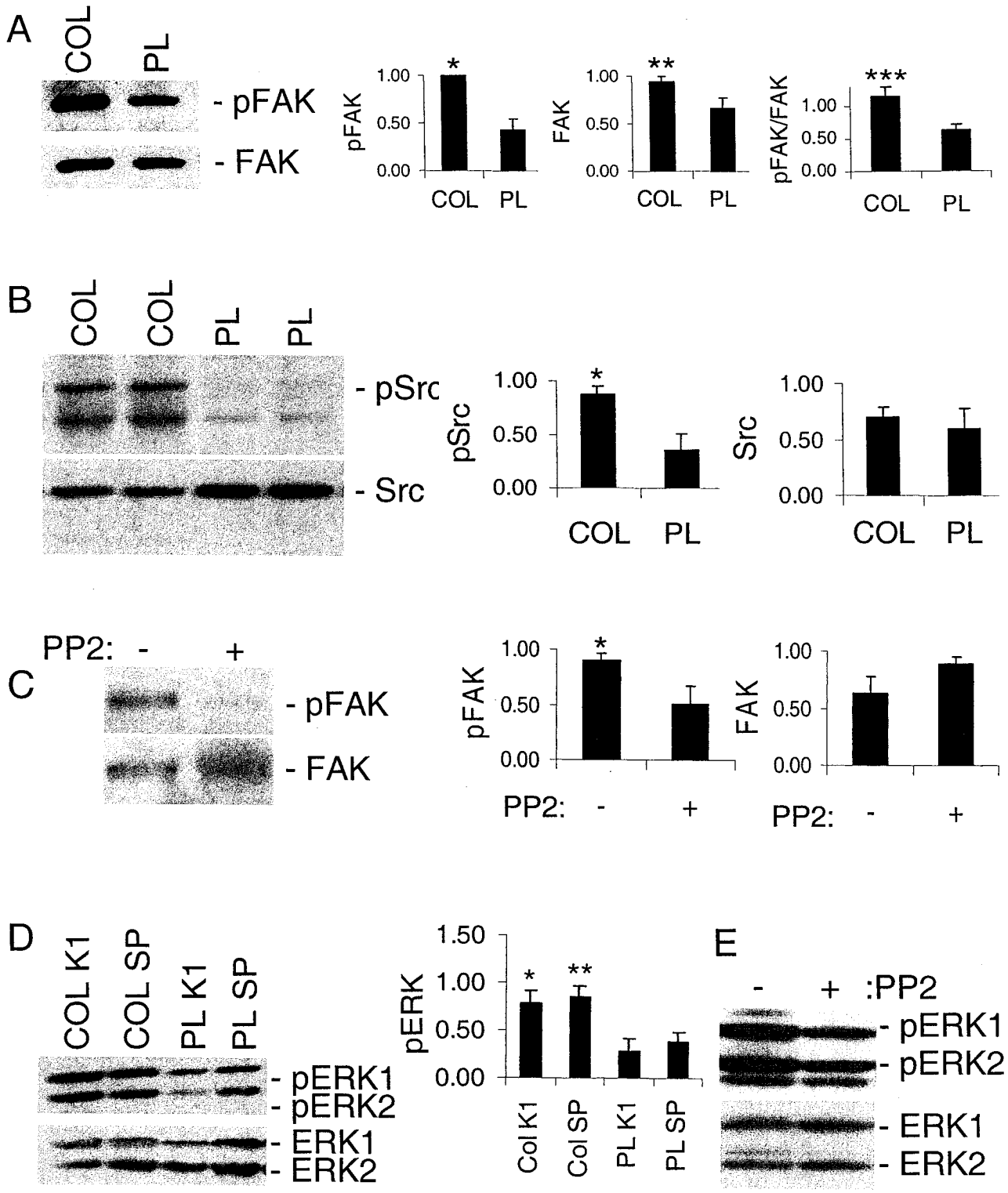
A
COL
PL
- pFAK
- FAK
pFAK
FAK
pFAK/FAK
1.00
0.50
0.00
*
**
***
COL
PL
B
COL
COL
PL
PL
- pSrc
- Src
pSrc
Src
C
PP2: - +
- pFAK
- FAK
D
COL K1
COL SP
PL K1
PL SP
- pERK1
- pERK2
- ERK1
- ERK2
pERK
1.50
Col K1
Col SP
PL K1
PL SP
E
- + :PP2
- pERK1
- pERK2
- ERK1
- ERK2

Figure 4. Activation of FAK and ERK in GEC (representative immunoblots and densitometric quantification). A) Lysates of parental GEC on collagen (COL) or plastic (PL) (24 hours after plating) were immunoprecipitated with antibody to FAK, and were immunoblotted with antibodies to phosphotyrosine (upper panel; pFAK) or FAK (lower panel). *P<0.001, **P<0.03, ***P<0.015 COL vs PL (5-7 experiments). B) Lysates of parental GEC were immunoblotted with antibodies to Src phosphotyrosine^{418} (pSrc; upper panel) or Src (lower panel). *P<0.02 COL vs PL. There were no significant differences in Src protein expression (6 experiments). C) Lysates of parental GEC, treated with or without PP2 (10 μM) for 24 hours, were immunoprecipitated with antibody to FAK, and were immunoblotted with antibodies to phosphotyrosine (pFAK; upper panel) or FAK (lower panel). *P<0.04 PP2 vs untreated. There were no significant differences in FAK protein expression (4 experiments). D) Lysates of parental GEC that had been plated onto collagen or plastic in K1 or serum-poor (SP) media for 24 hours were immunoblotted with antibodies to ERK1/2 phospho-threonine^{202}/tyrosine^{204} (pERK; upper panel) or to ERK1/2 (lower panel). *P<0.01 COL vs PL (K1), **P<0.02 COL vs PL (SP; 4-6 experiments). E) Lysates of parental GEC, treated with or without PP2 (10 μM) for 24 hours, were immunoblotted with antibodies to phosphoERK (upper panel) or ERK (lower panel). PP2 decreased ERK phosphorylation, but not protein expression.

FIGURE 5

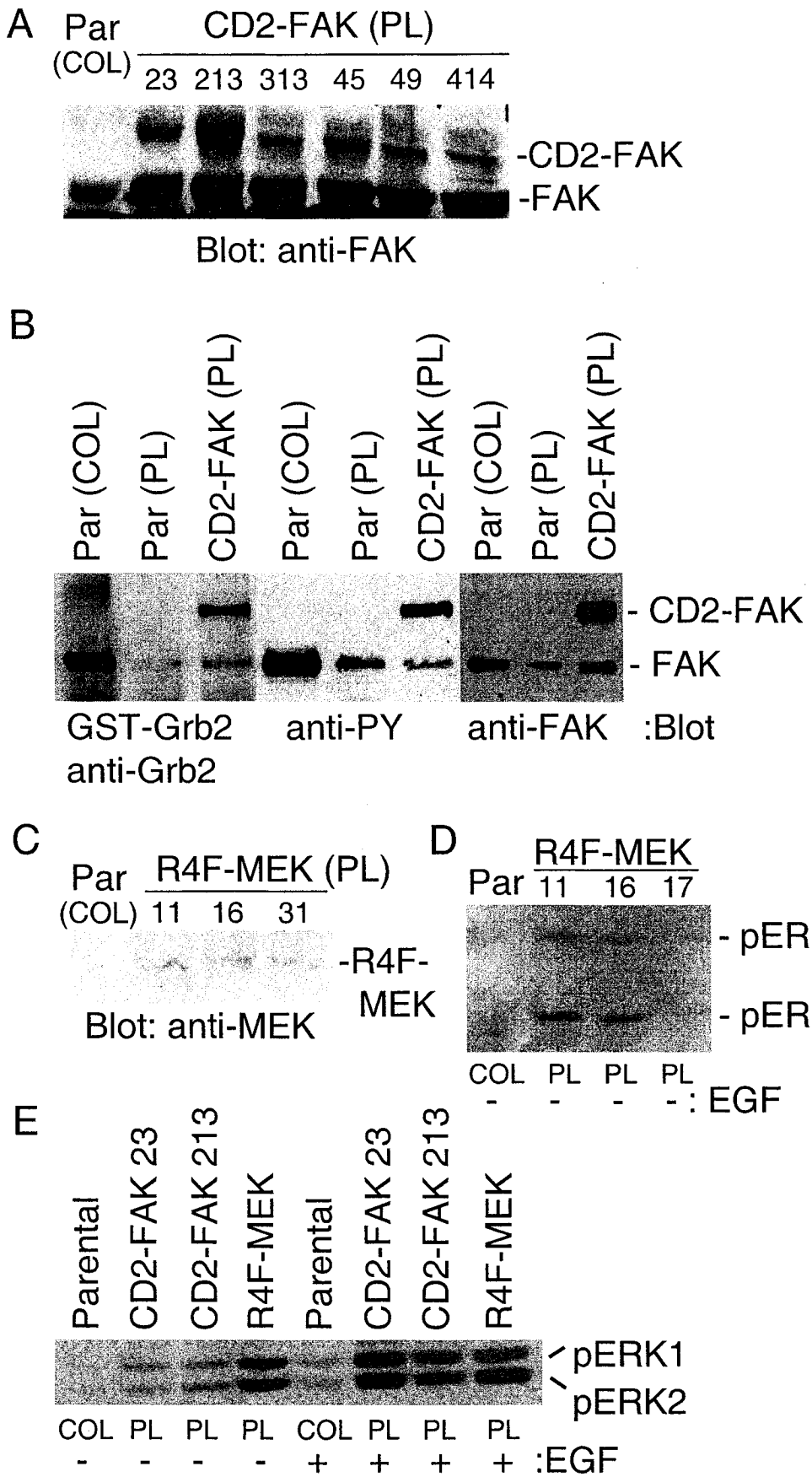
A
Par (COL)
CD2-FAK (PL)
23 213 313 45 49 414
-CD2-FAK
-FAK
Blot: anti-FAK
B
Par (COL)
Par (PL)
CD2-FAK (PL)
Par (COL)
Par (PL)
CD2-FAK (PL)
Par (COL)
Par (PL)
CD2-FAK (PL)
- CD2-FAK
- FAK
GST-Grb2
anti-Grb2
anti-PY
anti-FAK
:Blot
C
Par (COL)
R4F-MEK (PL)
11 16 31
-R4F-MEK
Blot: anti-MEK
D
Par
R4F-MEK
11 16 17
- pERK1
- pERK2
COL PL PL PL
- - - - : EGF
E
Parental
CD2-FAK 23
CD2-FAK 213
R4F-MEK
Parental
CD2-FAK 23
CD2-FAK 213
R4F-MEK
pERK1
pERK2
COL PL PL PL COL PL PL PL
- - - - + + + + :EGF

Figure 5. Expression of constitutively active FAK and MEK. Parental (Par) GEC on collagen (COL) were stably co-transfected with a neomycin-resistance gene and constitutively-active FAK (CD2-FAK, 160 kDa) (A), or constitutively-active MEK (R4F-MEK, 43 kDa) (C). Panel A shows several representative GEC clones that stably express CD2-FAK. Equal amounts of total protein were loaded into each lane (PL, plastic). B) Lysates of parental GEC on collagen or plastic (24 hours after plating), or of GEC stably transfected with CD2-FAK, clone 213 (on plastic) were immunoprecipitated with antibody to FAK, and immunoblotted with GST-Grb2 and anti-Grb2 antibody (left panel), or antibodies to phosphotyrosine (PY; middle panel) or FAK (right panel). CD2-FAK is tyrosine phosphorylated. GST-Grb2 binds to tyrosine phosphorylated FAK and CD2-FAK. Panel C shows representative GEC clones that stably express R4F-MEK. D) ERK phosphorylation in parental GEC on collagen, and three clones of GEC that stably express R4F-MEK on plastic in the absence of EGF (immunoblot with antibody to phosphoERK). E) Parental GEC were plated onto collagen; R4F-MEK-expressing GEC (clone 16) and CD2-FAK-expressing GEC (clones 23 and 213) were plated onto plastic for 24 hours in serum-poor medium. Then, GEC were treated with (+) or without (-) EGF (100 ng/ml) for 30 minutes. Equal amounts of total protein were loaded into each lane prior to immunoblotting with anti-phosphoERK antibody. Both R4F-MEK- and CD2-FAK-expressing GEC showed higher levels of ERK phosphorylation, as compared with GEC on collagen (in the absence of EGF). EGF further stimulated ERK phosphorylation in all cell lines.

FIGURE 6

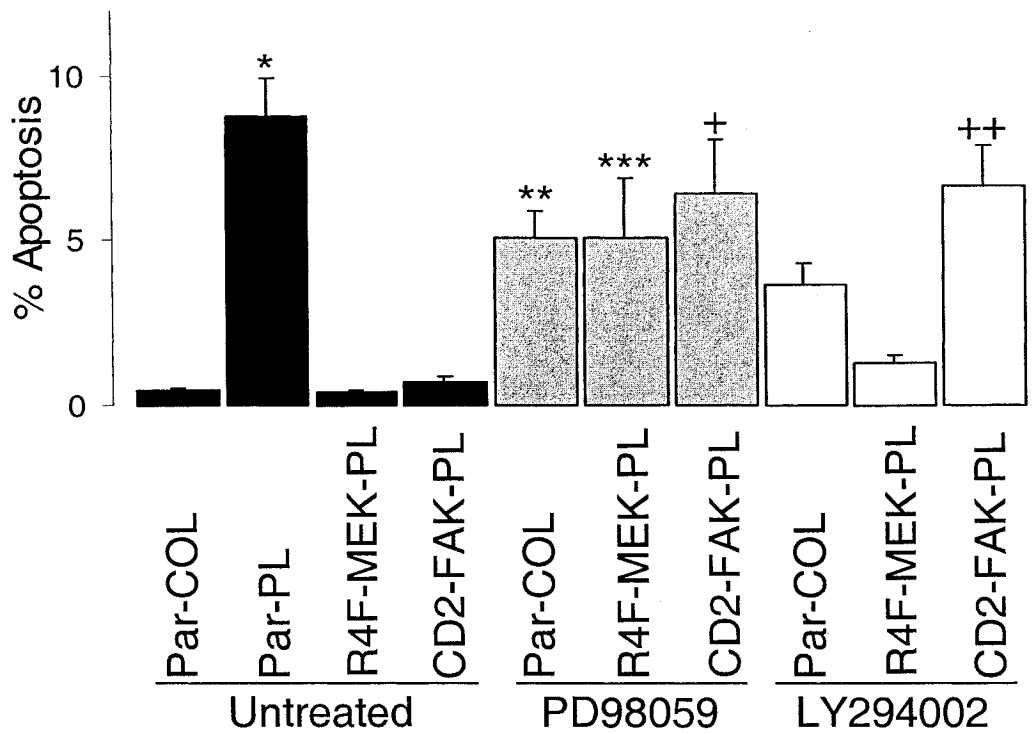

Figure 6. Inhibition of the ERK pathway increases apoptosis. Parental (Par) GEC on collagen or plastic, GEC that express R4F-MEK (clone 16), and GEC that express CD2-FAK (clone 213) were plated in serum-poor medium, with or without the MEK inhibitor, PD98059 (75 μM), or the PI3K inhibitor, LY294002 (10 μM), for 24 hours. Apoptosis was monitored as H33342-positive nuclei. *P<0.001 Parental plastic vs Parental collagen, R4F-MEK and CD2-FAK. **P<0.04 Parental (collagen) PD98059 vs untreated, ***P<0.03 R4F-MEK PD98059 vs untreated, +P<0.02 CD2-FAK PD98059 vs untreated. ++P<0.001 CD2-FAK LY294002 vs untreated (5-6 experiments performed in duplicate).

FIGURE 7

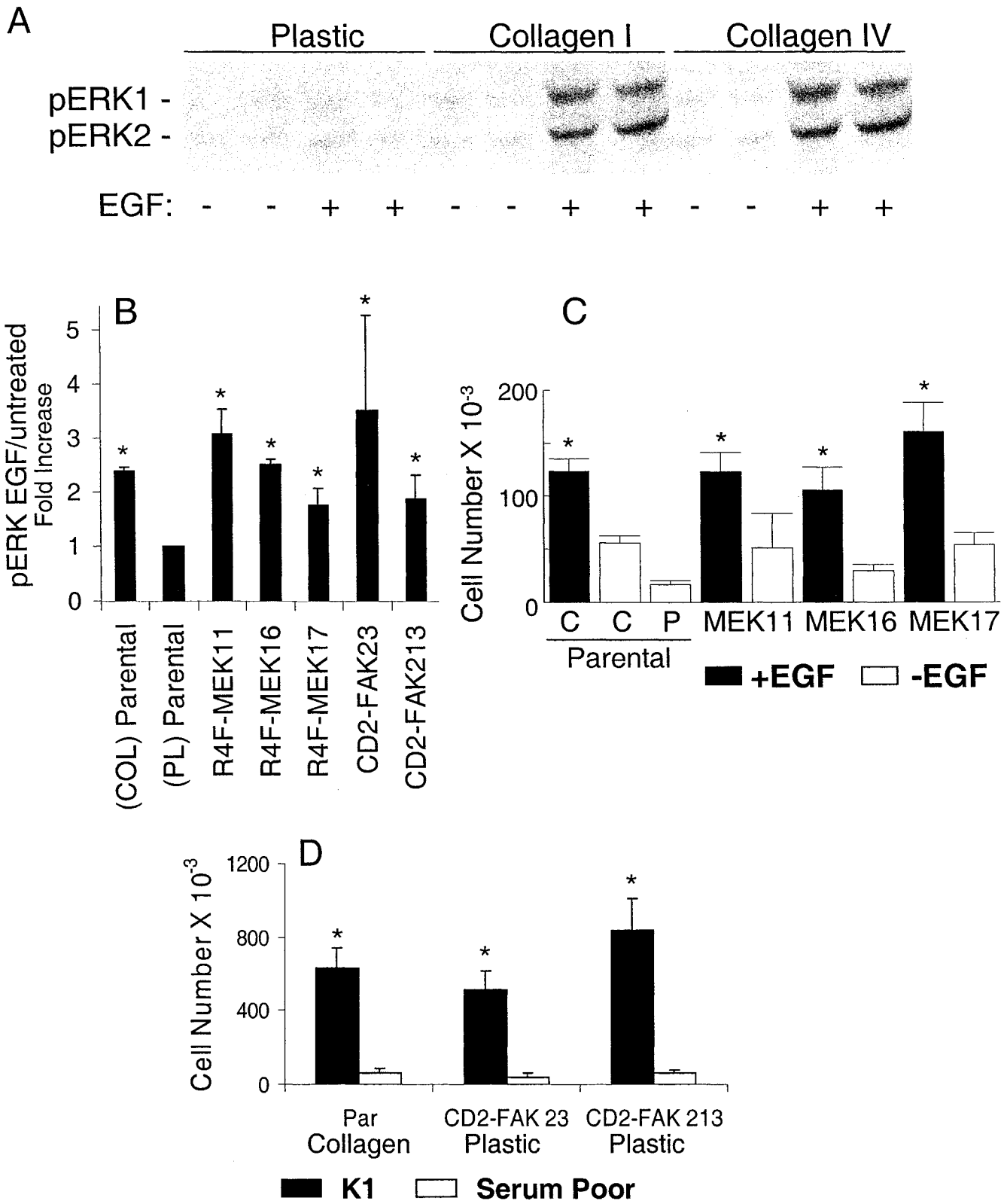
A
Plastic
Collagen I
Collagen IV
pERK1 -
pERK2 -
EGF: - - + + - - + + - - + +
B
pERK EGF/untreated
Fold Increase
0
1
2
3
4
5
(COL) Parental
(PL) Parental
R4F-MEK11
R4F-MEK16
R4F-MEK17
CD2-FAK23
CD2-FAK213
C
Cell Number X 10-3
0
100
200
C C P
Parental
MEK11 MEK16 MEK17
+EGF
-EGF
D
Cell Number X 10-3
0
400
800
1200
Par Collagen
CD2-FAK 23 Plastic
CD2-FAK 213 Plastic
K1
Serum Poor

Figure 7. Effect of collagen on growth factor-induced ERK activation and proliferation. A) Parental GEC were plated onto collagen I, collagen IV, or plastic for 24 hours, in serum-poor medium. Then, GEC were treated with (+) or without (-) EGF (100 ng/ml) for 30 minutes. Equal amounts of total protein were loaded into each lane prior to immunoblotting with anti-phosphoERK antibody. Adhesion to collagen induced small increases in ERK phosphorylation, while further increases occurred with EGF. B) Densitometric quantification of EGF-induced ERK phosphorylation in parental GEC, as well as R4F-MEK and CD2-FAK-expressing GEC (also see representative immunoblot in Fig. 5E). *P<0.001 EGF vs no EGF (4-8 experiments). C) Parental GEC were plated onto collagen (C) or plastic (P) and three clones of R4F-MEK-transfected GEC (MEK) were plated onto plastic (~50,000 cells/well). GEC were cultured with or without EGF, 100 ng/ml, in serum-poor medium. Cell number was determined at 72 hours by visual counting. *P<0.002 EGF vs no EGF. There were no significant differences among the groups of cells cultured without EGF (7 experiments). D) Parental GEC on collagen and two clones of CD2-FAK-transfected GEC on plastic were plated at ~50,000 cells/well and were cultured in growth factor-replete K1 medium or serum-poor medium. Cell number was determined at 72 hours by visual counting. *P<0.02 K1 vs Serum Poor (3-5 experiments).

FIGURE 8

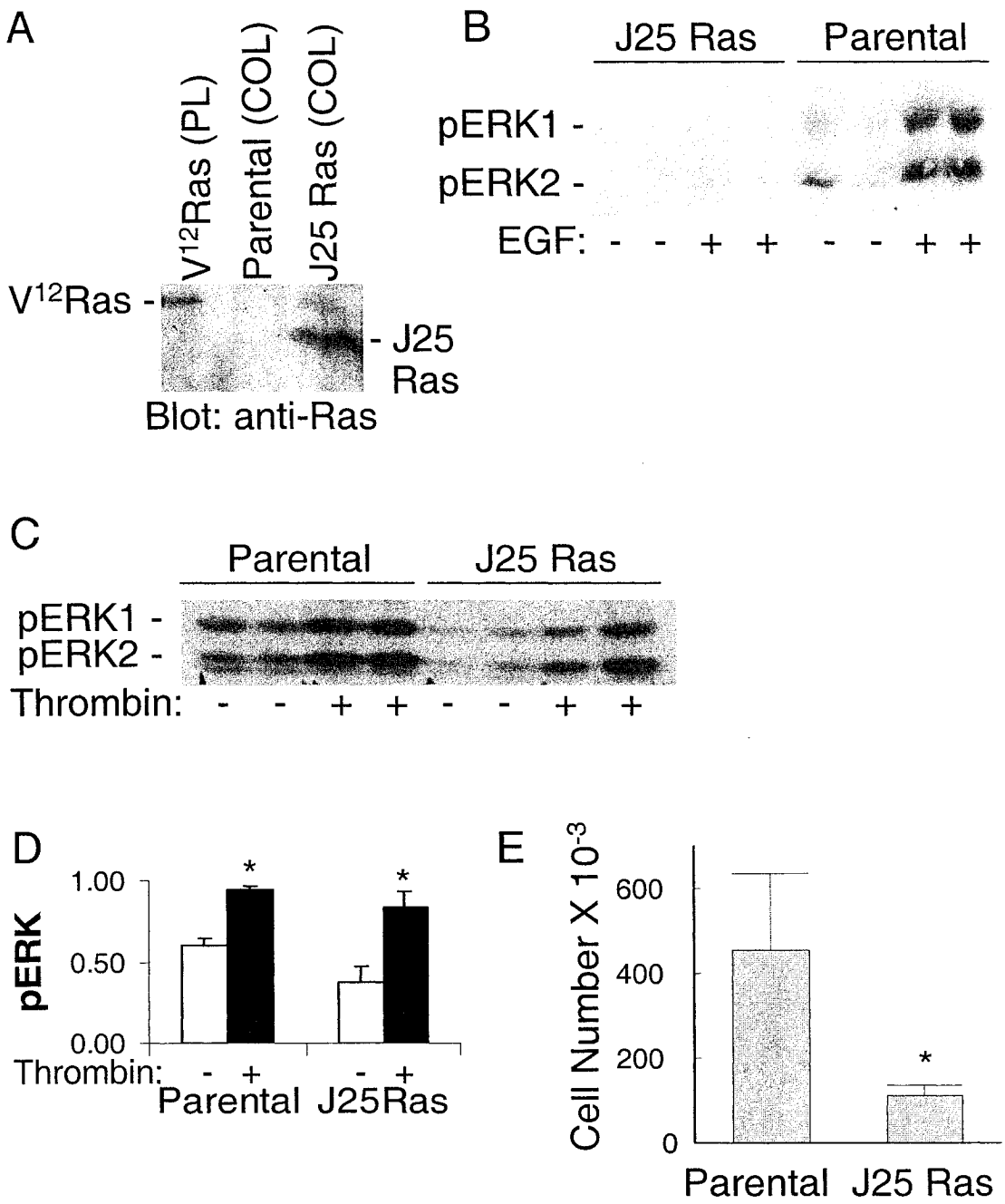
A
V12Ras (PL)
Parental (COL)
J25 Ras (COL)
V12Ras -
- J25 Ras
Blot: anti-Ras
B
J25 Ras
Parental
pERK1 -
pERK2 -
EGF: - - + + - - + +
C
Parental
J25 Ras
pERK1 -
pERK2 -
Thrombin: - - + + - - + +
D
pERK
1.00
0.50
0.00
Thrombin: - + - +
Parental J25Ras
E
Cell Number X 10-3
600
400
200
0
Parental J25 Ras

Figure 8. Stable transfection of GEC with J25 Ras. A) Parental (Par) GEC on collagen (COL) were co-transfected with a neomycin-resistance gene and J25 Ras (Q43E/D54N/E63K Ras, ~21 kDa). Transfection of constitutively-active Ras (V^{12}Ras) (13) is shown for comparison (PL, plastic). B-D) Clones of parental GEC and GEC that express J25 Ras were plated onto collagen in serum-poor medium. Then, GEC were treated with (+) or without (-) EGF (100 ng/ml; B) or thrombin (5 units/ml; C and D) for 30 minutes. ERK phosphorylation (without EGF or thrombin) was greater in parental GEC, as compared with J25 Ras-expressing cells. EGF was unable to stimulate ERK phosphorylation in GEC that express J25 Ras. Thrombin activated ERK equally in J25 Ras-expressing and parental GEC. *$P<0.002$ thrombin vs unstimulated (8 experiments). E) Parental or J25 Ras-transfected GEC were plated onto collagen at ~5 x 10^5 cells/well, and were cultured in serum-poor medium (that contained thrombin, 5 units/ml) for 72 hours. *$P=0.05$ parental vs J25 Ras (4 experiments).

FIGURE 9

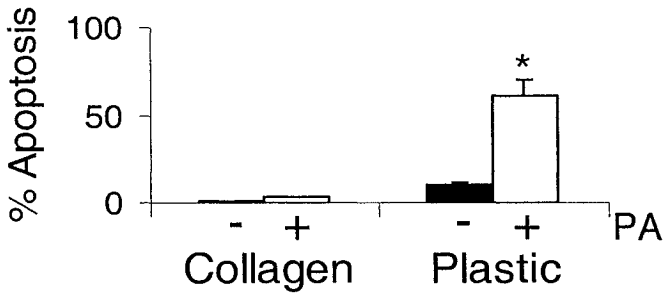

Figure 9. Collagen attenuates puromycin aminonucleoside (PA)-induced apoptosis. Parental GEC were plated onto collagen or plastic and were cultured in serum-poor medium with or without puromycin aminonucleoside (50 μg/ml) for 24 hours. Apoptosis was measured as H33342-positive nuclei (as in Fig. 2B and 2C). $P<0.001$ *PA plastic vs collagen (3 experiments performed in duplicate).

FIGURE 10

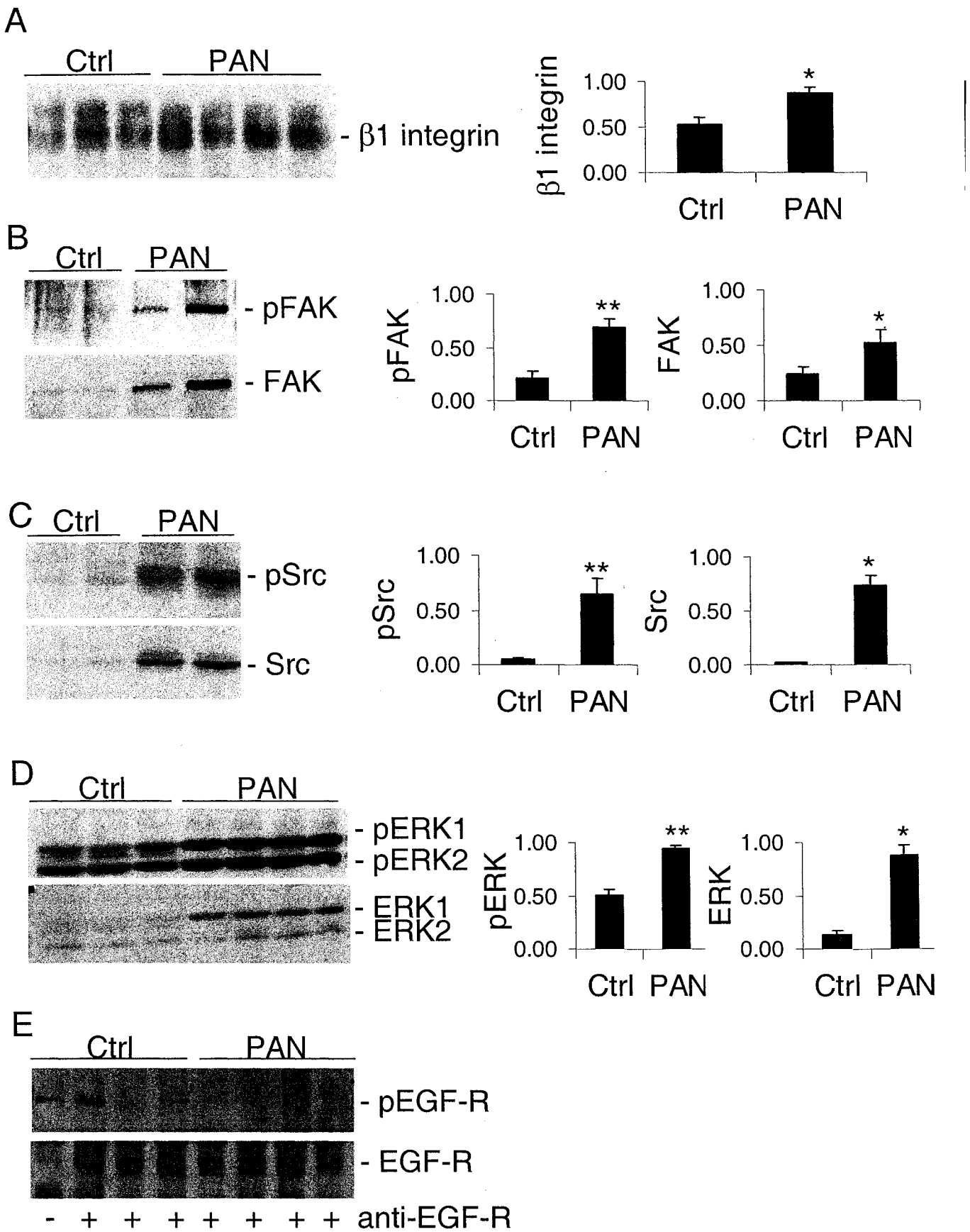
A
Ctrl
PAN
- β1 integrin
β1 integrin
1.00
0.50
0.00
*
Ctrl
PAN
B
Ctrl
PAN
- pFAK
- FAK
pFAK
1.00
0.50
0.00
**
Ctrl
PAN
FAK
1.00
0.50
0.00
*
Ctrl
PAN
C
Ctrl
PAN
- pSrc
- Src
pSrc
1.00
0.50
0.00
**
Ctrl
PAN
Src
1.00
0.50
0.00
*
Ctrl
PAN
D
Ctrl
PAN
- pERK1
- pERK2
- ERK1
- ERK2
pERK
1.00
0.50
0.00
**
Ctrl
PAN
ERK
1.00
0.50
0.00
*
Ctrl
PAN
E
Ctrl
PAN
- pEGF-R
- EGF-R
- + + + + + + + anti-EGF-R

Figure 10. Activation of anti-apoptotic pathways in GEC in vivo. Glomeruli were isolated from normal control (Ctrl) rats, or rats injected with puromycin aminonucleoside (80 mg/kg; PAN) on day 14. A) Glomerular lysates were immunoblotted with anti-β1 integrin antibody. B) Glomerular lysates were immunoprecipitated with antibody to FAK, and were immunoblotted with antibodies to phosphotyrosine (pFAK) or FAK. C) Glomerular lysates were immunoblotted with anti-phospho-Src^{418} (pSrc) or anti-Src antibodies. D) Glomerular lysates were immunoblotted with antibodies to ERK1/2 phospho-threonine^{202}/tyrosine^{204} (pERK) or ERK1/2. E) Glomerular lysates were immunoprecipitated with antibody to EGF-R, and were immunoblotted with antibodies to phosphotyrosine (pEGF-R) or EGF-R. There were significant increases in the expression of β1 integrin (A, *P<0.04), as well as in the expression and phosphorylation of FAK (B, *P<0.03, **P<0.0001), Src (C, *P<0.001, **P<0.01) and ERK (D, *P<0.001, **P<0.001) in PAN, as compared with control (7-14 experiments). There were no differences in EGF-R protein expression between groups, and EGF-R tyrosine phosphorylation was not detected consistently.

FIGURE 11

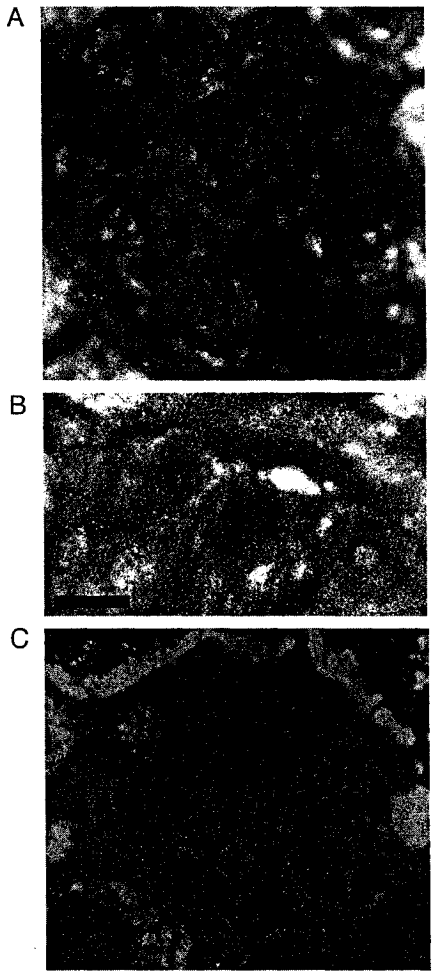

Figure 11. Phospho-ERK in glomeruli of rats with PAN. A) Glomerulus shows specific staining for phospho-ERK with focal and patchy accentuation. B) Higher power view of a portion of a glomerulus showing staining of glomerular capillary loops at the periphery of a glomerulus (arrowheads), adjacent to Bowman's space ("B"). The asterisks denote capillary lumena. C) Negative control (i.e. incubation with non-immune rabbit IgG). Tubular staining adjacent to the glomeruli (seen in all panels) is nonspecific. The bars denote 10 μm.

Analysis of the intracellular signaling pathways activated by collagen in GEC revealed that collagen facilitated survival, via activation of FAK and a Ras-ERK pathway. Constitutively active protein kinases supplanted the ERK activation by collagen, but failed to support EGF-induced ERK activity. These results indicate that there are two separate ERK pathways activated in GEC: i) activation of ERK by collagen, perhaps occurring within focal adhesions (necessary for GEC survival), and ii) EGF-induced ERK activation (necessary for GEC proliferation). A survival signal from collagen is a prerequisite for EGF-dependent activation of the EGF-R and the ERK pathway, as well as proliferation. The next study addresses the relationship between the survival phenotype and actin organization.

ABSTRACT

Adhesion of rat glomerular epithelial cells (GEC) to collagen results in activation of focal adhesion kinase (FAK) and the Ras-extracellular signal-regulated kinase (ERK) pathway, and supports GEC survival (prevents apoptosis). The present study addresses the relationship between actin organization and the survival phenotype. Parental GEC (adherent to collagen), and GEC stably transfected with constitutively active mutants of mitogen-activated protein kinase kinase (R4F-MEK) or FAK (CD2-FAK) (on plastic) showed ERK activation, low levels of apoptosis, and a cortical distribution of F-actin. Parental GEC adherent to plastic substratum showed increased apoptosis, disorganization of cortical F-actin, and formation of prominent stress fibers. Assembly of cortical F-actin was, at least in part, mediated via the ERK pathway. However, disruption of the actin cytoskeleton with cytochalasin D or latrunculin B in parental GEC adherent to collagen, as well as in GEC that express R4F-MEK and CD2-FAK on plastic led to decreased activation of ERK and increased apoptosis. Stable expression of a constitutively active RhoA (L^{63}RhoA) induced assembly of cortical F-actin, promoted ERK activation, and supplanted the requirement of collagen for survival. Adhesion of GEC to collagen increased phosphatidylinositol-4,5-bisphosphate (PIP_2). Downregulation or sequestration of PIP_2 by stable transfection with an inositol 5'-phosphatase or the plextrin-homology domain of phospholipase C-δ reduced F-actin content and decreased GEC survival. These results demonstrate a reciprocal relationship between collagen-induced cortical F-actin assembly and collagen-dependent survival signaling, including ERK activation.

INTRODUCTION

Adhesion of cells to extracellular matrix (ECM) via integrins modulates cell growth, differentiation, survival, and morphology (19; 20). Integrins are heterodimeric transmembrane molecules consisting of α and β subunits. In addition to providing a structural link between ECM proteins and the actin cytoskeleton, integrins are often involved in signal transduction pathways in cooperation with growth factor receptors. Among the many intracellular signaling pathways that may be activated independently or synergistically by integrins and growth factor receptors, the Ras-extracellular-signal-regulated kinase (ERK) pathway is one of the central pathways regulating cell proliferation and survival. The level(s) at which crosstalk occurs may be dependent on the cell type, i.e. crosstalk has been demonstrated at the level of growth factor receptors, upstream of Ras, from Ras to Raf-1, from Raf-1 to mitogen-activated protein kinase kinase (MEK), and at the nuclear translocation of activated ERK (27).

Glomerular epithelial cells (GEC), or podocytes are highly differentiated cells that play a key role in maintaining glomerular permselectivity (18; 25; 29; 32). The major integrin expressed in GEC is $\alpha 3\beta 1$, which preferentially binds to laminin, as well as collagen, fibronectin, and entactin/nidogen (1; 2; 16; 17; 22; 26). GEC are anchorage-dependent cells, as they require attachment to ECM via integrins for survival, and soluble growth factors for proliferation. Under normal conditions, where GEC are in contact with ECM, there is little GEC turnover. Podocyte injury occurs in many forms of human and experimental glomerular diseases (4; 5; 30; 38). Injury, leading to foot process

effacement and proteinuria, may be caused, at least in part, by disruption of the actin cytoskeleton. For example, mutations in a gene encoding for α-actinin-4, which increase the affinity of α-actinin-4 for filamentous (F)-actin, has been associated with a familial form of autosomal dominant focal segmental glomerulosclerosis (23; 28). Altered expression of another actin binding protein, synaptopodin, has been observed in various forms of glomerular disease (4; 24; 34). Other factors that have been shown to affect the organization of the actin cytoskeleton include Rho GTPases (7) and phosphatidylinositol-4,5-bisphosphate (PIP_2) (39). Mice deficient in Rho GDP dissociation inhibitor-α (which maintains Rho family members in their GDP bound inactive state) develop massive proteinuria and glomerulosclerosis, demonstrating the importance of signaling pathways regulated by Rho GTPases in maintaining glomerular permselectivity (36). PIP_2 has been shown to modulate the activity and targeting of actin regulatory proteins, promoting actin polymerization (39). In addition to proteinuria, podocyte injury may also result in apoptosis or proliferation, and in glomerulosclerosis (18; 25; 29; 32).

We have previously demonstrated that adhesion of GEC to collagen results in the activation of focal adhesion kinase (FAK), a key mediator of integrin signaling. In GEC, FAK activates the ERK pathway, which supports survival (prevents apoptosis) (6). In contrast, apoptosis is markedly enhanced in GEC adherent to plastic substratum. Stable expression of constitutively active mutants of FAK (CD2-FAK) or MEK (R4F-MEK) in GEC activated the ERK pathway, and supplanted the requirement of collagen for survival, whereas inhibition of the ERK pathway decreased survival of GEC on collagen. Analogous signals for GEC survival were activated in experimental focal segmental

glomerulosclerosis, a model of GEC injury in vivo (6). Earlier, we showed that adhesion to ECM increased PIP_2 levels in GEC, possibly through the activation of phosphatidylinositol 4-phosphate 5-kinase, although the involvement of PIP_2 in the regulation of GEC actin cytoskeleton has yet to be demonstrated (13).

The aim of the present study was to address the relationship between GEC actin organization and the survival phenotype. We demonstrate that in GEC adherent to plastic (i.e. the apoptosis phenotype), F-actin is organized predominantly into stress fibers. On collagen, and in GEC that express CD2-FAK, R4F-MEK, or a constitutively active mutant of RhoA (i.e. the survival phenotype), there is a switch to cortical actin organization. Disruption of the actin cytoskeleton with actin depolymerizing drugs decreased ERK activation, and promoted GEC death. Moreover, F-actin content and survival were decreased by downregulating/sequestering PIP_2 levels through the expression of an inositol 5'-phosphatase (Pase) or the plextrin homology domain of phospholipase C-δ [PH(PLCδ)].

MATERIALS AND METHODS

Materials

Tissue culture reagents, Transfinity $CaPO_4$ transfection system and G418 (geneticin) were obtained from Invitrogen (Burlington, Ontario). NuSerum was obtained from BD Biosciences (Mississauga, Ontario). Fugene 6 transfection reagent was obtained from Roche Molecular Biochemicals (Indianapolis, Indiana). Pepsin-solubilized bovine dermal collagen I (Vitrogen) was from Cohesion (Palo Alto, California). Epidermal growth

factor (EGF), LY294002, cytochalasin D, latrunculin B and caspase-3 substrate were obtained from Sigma Chemical Co. (St. Louis, Missouri). Bisbenzimide H33342 fluorochrome, propidium iodide, caspase-8 substrate (granzyme B substrate I), caspase-9 substrate II, PD98059, and Y27632 were obtained from Calbiochem (La Jolla, California). Electrophoresis and immunoblotting reagents were from Bio-Rad Laboratories (Mississauga, Ontario). Rhodamine conjugated-phalloidin and Alexa-Fluor 488 conjugated-deoxyribonuclease I (DNase I) were obtained from Molecular Probes (Eugene, Oregon). Rabbit anti-phospho-ERK1/2 (threonine^{202}/tyrosine^{204}) and rabbit anti-ERK1/2 antibodies were purchased from New England Biolabs (Mississauga, Ontario). Mouse anti-RhoA and mouse anti-green fluorescent protein (GFP) antibodies were from Santa Cruz Biotechnology (Santa Cruz, California). R4F-MEK, CD2-FAK and L^{63}RhoA cDNAs have been described previously (6; 15). pcDNA3-GFP-Pase and pEGFP-PH(PLCδ) cDNAs were kindly provided by Dr. Tobias Meyer (Stanford University, Palo Alto, California) (33; 35).

Extracellular matrix and GEC culture

Type I collagen gel matrices were prepared as described previously (10). For practical reasons, collagen I was used in all experiments in this study, but in previous studies, some key observations were confirmed using collagen IV substrata (6; 15) Primary cultures of rat GEC were established from explants of rat glomeruli, and have been characterized previously (9-12). Unlike some other GEC culture lines, the cells used in the present study do not contain the SV40 large T antigen, and thus, in our view are better suited for studies of cell survival. Experiments were done with cells between

passages 25 and 70. Under standard conditions, GEC were cultured on collagen I matrices in K1 medium, which consisted of DMEM/F12 (1:1), containing 5% NuSerum and hormone supplements (10; 11). GEC were maintained in culture by passaging onto collagen gels; for experiments, GEC were replated onto collagen or plastic substrata in serum-poor (DMEM-0.5% fetal calf serum) or K1 media.

GEC transfection

Production of GEC that stably express CD2-FAK, R4F-MEK and L^{63}RhoA was described previously (6; 15). Briefly, GEC on collagen were co-transfected with the expression vector of interest (2 μg of DNA per 100 mm plate), and a neomycin resistance gene pRc/RSV (molar ratio 12.5:1), using the $CaPO_4$ technique, as described previously (14). GEC were then cultured on collagen in K1 medium containing 0.5 mg/ml G418. GEC clones resistant to G418 were isolated and replated onto plastic substratum. Clones that proliferated on plastic in K1 medium were selected, passaged, and assessed for the expression of the specific protein by immunoblotting. A soft agar assay was used to test for anchorage-independent proliferation (14). GEC on collagen were also co-transfected with GFP-PH(PLCδ) or GFP-Pase (10 μg of plasmid DNA per 100 mm plate) and a neomycin resistance gene pRc/RSV (2.6 μg) using Fugene 6 transfection reagent, according to the manufacturer's instructions. GEC were then cultured on collagen in K1 medium containing 0.5 mg/ml G418. GEC clones resistant to G418 were isolated, sorted by flow cytometry, and GFP positive GEC were expanded on collagen.

Measurement of GEC proliferation

Cell number was determined by visual counting. Cells adherent to collagen gels (35 mm plates) were placed into single-cell suspension by incubation with collagenase and trypsin-EDTA. Cells on plastic substratum were placed into suspension by incubation with trypsin-EDTA. Suspended cells were then counted in a hemacytometer (11; 14).

Immunoblotting

GEC lysates were subjected to SDS-PAGE under reducing conditions, as detailed previously (6). Proteins were then electrophoretically transferred onto nitrocellulose paper, blocked with 3% BSA/2% ovalbumin, and incubated with primary antibody, and then with horseradish peroxidase-conjugated secondary antibody. The blots were developed using the enhanced chemiluminescence technique (ECL, Amersham Pharmacia Biotech AB). Protein content was quantified by scanning densitometry, using NIH Image software (6). Preliminary studies demonstrated that there was a linear relationship between densitometric measurements and the amounts of protein loaded onto gels.

Hoechst H33342 staining

Staining with Hoechst H33342 dye was used to quantify apoptosis, as described previously (6). Briefly, adherent cells were stained with H33342 (1 μg/ml) for 10 min at 37°C without fixation. After washing with PBS, cells were stained with propidium iodide (5 μg/ml) to identify necrotic or late-apoptotic cells. Cells were examined using a Nikon Diaphot fluorescence microscope, and the number of H33342-positive/propidium iodide-negative cells was quantified by visual counting. Previously, we demonstrated that

apoptosis, as assessed by H33342 staining, correlated with the DNA laddering assay (6).

Caspase activity assay

Intracellular caspase-3, -8 and -9 activities were measured using a colorimetric assay with p-nitroaniline (pNA)-labeled substrates, as described previously (6). Adherent cells were lysed in buffer containing 50 mM Hepes, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 100 μM EDTA, pH 7.4 (4°C). The mixture was centrifuged at 14,000 x g for 10 min. Supernatants were collected and adjusted for protein concentration. The reaction mixture contained 50 mM Hepes, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 100 μM EDTA, 10% glycerol , pH 7.4 (37°C) with 2 mM substrate, i.e. Ac-Asp-Glu-Val-Asp-pNA (caspase-3), Ac-Leu-Glu-His-Asp-pNA (caspase-9), or Ac-Ile-Glu-Thr-Asp-pNA (caspase-8). The absorbance of the reaction was read at 405 nm every 5 min for 2 h. After subtraction of background activity, the slope of absorbance versus time is proportional to caspase activity.

Fluorescence microscopy

Cells adherent to uncoated or collagen-coated glass coverslips were fixed with 3% paraformaldehyde in PBS and were permeabilized with 0.5% Triton X-100. After washing, cells were incubated with rhodamine-phalloidin (0.043 μg/ml) to visualize F-actin and Alexa-Fluor 488 conjugated-DNase I (9 μg/ml, diluted in 3% BSA in PBS) to visualize G-actin for 20 min. Coverslips were mounted onto glass slides and were examined using both rhodamine and fluorescein filters in a Nikon Diaphot fluorescence microscope. Slides were photographed using a Nikon Coolpix 995 digital camera locked

at a constant exposure. Fluorescence intensity was then quantified using Adobe Photoshop software.

Statistics

Data are presented as mean±SEM. The t statistic was used to determine significant differences between two groups. One-way ANOVA was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the t statistic, and adjusting the critical value according to the Bonferroni method.

RESULTS

Role of F-actin in GEC survival

Previously, we showed that adhesion of GEC to collagen induced ERK activation, which was essential for GEC survival, while GEC adherent to plastic showed limited ERK phosphorylation and underwent apoptosis. Second, stable expression of constitutively active mutants of FAK (CD2-FAK) or MEK (R4F-MEK) both activated the ERK pathway, and supplanted the requirement of collagen for survival (6). To study the relationship between actin organization and the survival phenotype, we analyzed the actin cytoskeleton in various GEC lines. Parental GEC adherent to plastic had significantly greater levels of F-actin, as compared with parental GEC on collagen (Fig. 1A). In parental GEC on collagen, F-actin was distributed in a cortical pattern (Fig. 1B), whereas parental GEC adherent to plastic demonstrated a complete loss of the cortical F-

actin structure and formation of extensive stress fibers (Fig. 1B). GEC expressing CD2-FAK or R4F-MEK on plastic had levels of F-actin comparable with parental GEC on collagen (Fig. 1A). In addition, the GEC expressing CD2-FAK or R4F-MEK on plastic showed a clear cortical actin distribution, comparable to parental GEC on collagen, and in keeping with their survival phenotype (Fig. 1B). These results demonstrate the association of a cortical actin cytoskeletal structure with GEC survival.

In a previous study, we demonstrated that the MEK inhibitor, PD98059, induced apoptosis in parental GEC adherent to collagen, and in CD2-FAK- and R4F-MEK-expressing GEC (on plastic) (6). Thus, survival of GEC was dependent on the activation of ERK. To determine if ERK pathway activation was associated with reorganization of the cytoskeleton, we examined F-actin staining in collagen-adherent GEC that were treated with PD98059. Inhibition of the ERK pathway in parental GEC on collagen resulted in a partial loss of the cortical F-actin staining pattern, and formation of stress fibers (Fig. 1B), resembling F-actin staining in parental GEC adherent to plastic (Fig. 1B). This result suggests that the ERK pathway contributes toward cortical F-actin organization, which is associated with the survival phenotype.

In the next series of experiments we addressed the importance of the cytoskeleton in facilitating ECM-induced survival signaling. GEC were treated with two distinct inhibitors of actin polymerization, cytochalasin D or latrunculin B. Cytochalasin D binds to the growing end of actin filaments preventing stress fiber formation, whereas latrunculin B sequesters G-actin monomers disrupting both actin stress fibers as well as

cortical actin filaments. In GEC, both compounds were shown to disassemble F-actin extensively (15). Incubation of GEC with cytochalasin D (20 μM) or latrunculin B (1 μM) for 24 h, significantly reduced ERK activation in parental GEC, as well as in R4F-MEK- and CD2-FAK-expressing clones, as compared with untreated cells (Fig. 2, A-C). Thus, there appears to be a reciprocal relationship between ERK activation and cortical F-actin assembly, i.e. ERK activation is associated with cortical F-actin organization (Fig. 1B), while disassembly of F-actin impairs ERK activation.

F-actin disruption also led to increased levels of apoptosis in parental GEC, as well as in R4F-MEK- and CD2-FAK-expressing clones (Fig. 2D). Moreover, analysis of intracellular caspase activities revealed increased caspase-8 and -9 activities in R4F-MEK- and CD2-FAK-expressing GEC treated with cytochalasin D or latrunculin B, as compared with untreated cells (Fig. 2E). Parental GEC showed upward trends in caspase-8 and -9 activities, which did not reach statistical significance, possibly due to lower apoptosis rates and/or sensitivity limitations of these assays.

Expression of constitutively active RhoA (L^{63}RhoA)

Rho GTPases are known to regulate the structure of the actin cytoskeleton (7). To address the effects of RhoA on GEC survival and cytoskeletal structure, GEC were stably transfected with constitutively active RhoA (L^{63}RhoA). Two representative clones are demonstrated in Fig. 3A. In keeping with previous results, parental GEC proliferated only on collagen, but not plastic substratum (Fig. 3B). In contrast, the two L^{63}RhoA-expressing clones (as well as several other L^{63}RhoA clones that are not shown) were also

able to proliferate on plastic substratum (Fig. 3B). Proliferation of L^{63}RhoA-expressing GEC on plastic was dependent upon the presence of growth factors in the medium (Fig. 3C, two left bars) and on the activation of the ERK pathway (Fig. 3C, PD98059). Moreover, proliferation of L^{63}RhoA-expressing GEC on plastic was adhesion-dependent, as demonstrated by inability to form colonies in soft agar (not shown). Thus, expression of L^{63}RhoA supplanted the requirement of GEC for collagen in supporting survival and proliferation.

GEC expressing L^{63}RhoA (on plastic) showed levels of F-actin comparable to parental GEC on collagen (Fig. 1A). Moreover, the GEC expressing L^{63}RhoA showed a cortical actin distribution, comparable to parental GEC on collagen (Fig. 1B), and in keeping with the survival phenotype. In addition, the L^{63}RhoA clones had some stress fiber formation, although these stress fibers were substantially smaller than those observed in parental GEC adherent to plastic.

In the absence of mitogens (serum-poor medium), basal ERK phosphorylation in the L^{63}RhoA-expressing clones on plastic was approximately 4-fold greater as compared with parental GEC on plastic, reaching ~50% of the level seen in parental cells on collagen (Fig. 3D). Moreover, basal ERK phosphorylation in L^{63}RhoA GEC on plastic could be further stimulated by EGF (Fig. 3E), similar to parental GEC adherent to collagen, but unlike parental GEC on plastic, where lack of signals emanating from the ECM prohibited efficient activation of the EGF receptor and ERK pathway by EGF (6; 11). Survival of L^{63}RhoA GEC was dependent on the ERK pathway, as inhibition of

MEK with PD98059 increased apoptosis and caspase-3 activation (Fig. 3F and G). L^{63}RhoA-expressing GEC on plastic were relatively resistant to cytochalasin D- or latrunculin B-induced apoptosis, demonstrated by reduced levels of apoptosis at similar drug concentrations, when compared with parental GEC on collagen (Fig. 3H). Nevertheless, cytochalasin D and latrunculin B treatments led to increased caspase-3 activity in L^{63}RhoA-expressing GEC (Fig. 3G). For comparison, L^{63}RhoA-expressing GEC were also treated with the Rho kinase (ROCK) inhibitor, Y27632, or with the phosphatidylinositol 3-kinase inhibitor, LY294002. Y27632 and LY294002 had no significant effects on apoptosis or caspase-3 activation (Fig. 3F and G), and did not affect ERK phosphorylation (not shown). Thus, stable expression of L^{63}RhoA in GEC induced an actin organization that permitted efficient activation of ERK in the absence of ECM, and thus supplanted the requirement of collagen for survival.

Downregulation of PIP_2 in GEC

Adhesion of GEC to collagen was shown to increase the mass of PIP_2 (13). To address the role of PIP_2 in regulating cell survival, PIP_2 was downregulated or sequestered by stably transfecting GEC on collagen with an inositol 5'-specific phosphatase (GFP-Pase), or the plextrin-homology domain of phospholipase C-δ [GFP-PH(PLCδ)], which binds to PIP_2 with high affinity and specificity (33; 35). The expression of GFP-PH(PLCδ) and GFP-Pase are demonstrated in Fig. 4A. We observed a significant increase in apoptosis in GFP-PH(PLCδ) and GFP-Pase GEC, as compared with parental cells, although the level of apoptosis observed in GFP-PH(PLCδ) or GFP-

Pase GEC was less than in parental GEC adherent to plastic substratum (Fig. 4B). There were no significant differences observed among the proliferation rates of parental, GFP-PH(PLCδ) and GFP-Pase-expressing GEC (Fig. 4C), however, this assay may not have been sufficiently sensitive to detect small differences. Intracellular caspase-3 activity was significantly increased in GFP-PH(PLCδ) and GFP-Pase-expressing GEC, as compared with parental GEC (Fig. 4D), in keeping with apoptotic cell counts (Fig. 4B). In addition, basal ERK activity was not significantly different among the three cell lines (not shown). The importance of the ERK pathway in promoting cell survival was further demonstrated by treating GEC with the MEK inhibitor, PD98059, and quantifying caspase-3 activity. In addition to the increased levels of caspase-3 observed in GFP-PH(PLCδ) and GFP-Pase-expressing GEC, there was a further increase in apoptosis in the PD98059-treated GEC, as compared with untreated (Fig. 4E). This result suggests that sequestration of PIP_2 and inhibition of the ERK cascade may lead to apoptosis via separate pathways, since the inhibitory effects on the two pathways were additive.

GEC expressing GFP-PH(PLCδ) adherent to collagen had reduced levels of F-actin, as compared with parental GEC on collagen (Fig. 1A). A trend toward lower F-actin content was observed in GEC expressing GFP-Pase, although the result was not statistically significant (Fig. 1A). GEC expressing GFP-PH(PLCδ) or GFP-Pase demonstrated cortical actin staining, however, at reduced intensity, as compared with parental GEC on collagen (Fig. 1B).

DISCUSSION

This study demonstrates the importance of an organized cortical actin cytoskeleton in transmitting survival signals emanating from ECM. Adhesion of GEC to collagen induced FAK and ERK activation, as well as survival (6), in association with the assembly of a cortical actin cytoskeleton (Fig. 1 and 5). In contrast, adhesion to plastic, which enhances apoptosis (Fig. 4B) (6), was associated with disorganization of cortical actin, and formation of prominent stress fibers (Fig. 1). Assembly of cortical F-actin was, at least in part, mediated via the ERK pathway (Fig. 5), as treatment of collagen-adherent GEC with PD98059 resulted in loss of cortical actin and appearance of stress fibers, resembling the distribution of F-actin in GEC adherent to plastic (Fig. 1B). However, there also appeared to be a reciprocal relationship between ERK activation and cortical F-actin assembly (Fig. 5). Thus, disruption of the actin cytoskeleton by cytochalasin D or latrunculin B in parental GEC adherent to collagen, as well as in GEC that express R4F-MEK and CD2-FAK led to decreased activation of ERK and reduced cell survival (increased apoptosis) (Fig. 2). Cytochalasin D and latrunculin B had no effect on FAK activation in parental GEC (data not shown), suggesting functional integrin-FAK signaling despite cytoskeletal disruption. In GEC, collagen-induced FAK activation was dependent on Src, and resulted in binding of Grb2, and activation of the Ras-ERK cascade (Fig. 5) (6). Thus, the cytoskeleton-depolymerizing drugs most likely acted downstream of FAK. Together, the results demonstrate the dependence of efficient Ras-ERK pathway activation and transmission of survival signals from collagen on the proper organization of the actin cytoskeleton.

The role of the cytoskeleton in ERK activation has also been addressed in other cells. In fibroblasts, EGF-dependent ERK activation was dependent on adhesion to ECM and an intact cytoskeleton (3). In cells in suspension or in cells treated with cytochalasin D, the nuclear translocation of activated ERK was absent because of the disruption of the actin cytoskeleton (3). Insulin-induced Ras and ERK activation were inhibited by cytochalasin D in L6 myotubes (37). Moreover, in human intestinal epithelial cells (HT-29 or Caco-2), disruption of the actin cytoskeleton with cytochalasin D induced the activation of NF-κB and p38 kinase, demonstrating the concomitant activation of certain pathways resulting from actin disassembly (31).

GEC signaling, survival and organization of the actin cytoskeleton were modulated by stable expression of L^{63}RhoA. Survival of L^{63}RhoA-expressing cells adherent to plastic was comparable with that of parental GEC on collagen, and in the presence of growth factors, the L^{63}RhoA-expressing cells were able to proliferate on plastic substratum, thus supplanting the requirement for ECM (Fig. 3). Basal ERK phosphorylation was increased in L^{63}RhoA-GEC, as compared with parental GEC on plastic (Fig. 3), indicating that L^{63}RhoA stimulated a pathway that resulted in ERK activation (Fig. 5). Also, stable expression of L^{63}RhoA in GEC induced cortical distribution of F-actin and some stress fiber formation (Fig. 1 and 5), in keeping with the results of a recent study (15). L^{63}RhoA could potentially activate various effectors, which include Rho kinase/ROK/ROCK, the myosin-binding subunit of myosin phosphatase, protein kinase N/PRK1, rhophilin, rhotekin, citron, p140 mDia, or phosphatidylinositol 4-phosphate 5'-kinase. Some or all of these effectors could influence actin polymerization

(Fig. 5), stress fibers formation, focal adhesion formation, cytokinesis, smooth muscle contraction and cell motility (21). Disassembly of the actin cytoskeleton with cytochalasin D or latrunculin B led to increased apoptosis in parental GEC on collagen (Fig. 3), while L^{63}RhoA GEC proved to be more resistant to these drugs, displaying relatively lower levels of apoptosis at every concentration of cytochalasin D or latrunculin B (Fig. 3). In our earlier study, treatment of L^{63}RhoA GEC with latrunculin B resulted in relatively less disassembly of the cortical actin structure, although the stress fibers disappeared (15). This result reinforces the importance of the cortical actin structure in maintaining cell survival. In L^{63}RhoA GEC, the dependence of survival on the ERK, phosphatidylinositol 3-kinase-Akt and Rho-kinase pathways was analyzed using specific inhibitors for these cascades, PD98059, LY294002 and Y27632 respectively. GEC apoptosis and caspase-3 activity (an instigator of apoptosis) was increased only after PD98059 treatment (Fig. 3), in keeping with previous results, demonstrating that the ERK cascade is the major pathway that mediates the survival signaling emanating from collagen (6). The absence of increased caspase-3 activity in L^{63}RhoA–GEC treated with Y27632 suggests that the survival signals emanating from L^{63}RhoA are not exclusively mediated through Rho kinase, implying the involvement of one or more of the other aforementioned Rho effectors. It should be noted that we were unable to detect activation of endogenous RhoA in parental GEC on collagen or plastic (results not shown). Lack of detection may be related to a low expression level of endogenous RhoA (Fig. 3), or other technical reasons, but it is also possible that assembly of cortical F-actin and survival signaling in parental GEC may not involve RhoA. Thus, the expression and function of L^{63}RhoA may be unlike those of CD2-FAK,

and R4F-MEK, both of which appear to be supplanting the endogenous GEC kinases activated by ECM.

It is becoming increasingly apparent that phosphoinositides, particularly PIP_2, may regulate the actin cytoskeleton by modulating the activity and targeting of actin regulatory proteins. PIP_2 promotes actin polymerization by activating proteins, including WASP, gelsolin, cofilin, which favor the formation of positive ends on actin nuclei that are necessary for the addition of actin monomers. Moreover, PIP_2 activates many actin cross-linking proteins (α-actinin, filamin, ezrin/radixin/moesin, talin, vinculin), further facilitating actin polymerization (39). We have previously determined that adhesion of GEC to collagen increases PIP_2 levels, possibly through the activation of phosphatidylinositol 4-phosphate 5-kinase (Fig. 5) (13). In the present study, we reduced/sequestered PIP_2 by stable transfection of GFP-Pase or GFP-PH(PLCδ). GEC expressing either of these two proteins demonstrated increased levels of apoptosis (Fig. 4), although the levels of apoptosis were much lower, as compared with parental GEC on plastic. In addition, GFP-PH(PLCδ) and GFP-Pase cell lines had reduced levels of F-actin (Fig. 1), as compared with parental GEC. However, the cortical F-actin pattern, although reduced, was maintained in the GFP-PH(PLCδ) or GFP-Pase-expressing cell lines (Fig. 1). These results indicate that PIP_2 facilitated survival, and was involved in F-actin organization (Fig. 5). The results are in agreement with a recent study in 3T3-L1 adipocytes, where the induction of insulin resistance in these cells was paralleled by a marked loss of cortical F-actin and a decrease in PIP_2 levels. Interestingly, cortical F-actin and insulin responsiveness was restored after replenishing the plasma membrane

with PIP_2 (8).

GEC injury or genetic alterations in the expression of certain cytoskeleton-modulating proteins are observed in different forms of focal segmental glomerulosclerosis, in association with proteinuria and foot process effacement. Expanding our knowledge about these factors that control GEC survival will help us understand the pathophysiology of GEC injury in vivo. Eventually, these insights will provide novel therapeutic approaches to preserving a functional glomerular filter with intact permselectivity.

FOOTNOTES

1. Abbreviations: ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; GEC, glomerular epithelial cell; GFP, green fluorescent protein; MEK, mitogen-activated protein kinase kinase; Pase, inositol 5'-phosphatase; PH(PLCδ), plextrin-homology domain of phospholipase C-δ; PIP_2, phosphatidylinositol-4,5-bisphosphate.

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REFERENCES

1. Adler S. Characterization of glomerular epithelial cell matrix receptors. *Am J Pathol* 141: 571-578, 1992.

2. Adler S. Integrin receptors in the glomerulus: potential role in glomerular injury. *Am J Physiol* 262: F697-F704, 1992.

3. Aplin AE, Stewart SA, Assoian RK and Juliano RL. Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. *J Cell Biol* 153: 273-282, 2001.

4. Barisoni L, Kriz W, Mundel P and D'Agati V. The dysregulated podocyte phenotype: a novel concept in the pathogenesis of collapsing idiopathic focal segmental glomerulosclerosis and HIV-associated nephropathy. *J Am Soc Nephrol* 10: 51-61, 1999.

5. Barisoni L, Mokrzycki M, Sablay L, Nagata M, Yamase H and Mundel P. Podocyte cell cycle regulation and proliferation in collapsing glomerulopathies. *Kidney Int* 58: 137-143, 2000.

6. Bijian K, Takano T, Papillon J, Khadir A and Cybulsky AV. Extracellular matrix regulates glomerular epithelial cell survival and proliferation. *Am J Physiol Renal Physiol* 286: F255-F266, 2004.

7. Bishop AL and Hall A. Rho GTPases and their effector proteins. *Biochem J* 348 Pt 2: 241-255, 2000.

8. Chen G, Raman P, Bhonagiri P, Strawbridge AB, Pattar GR and Elmendorf JS. Protective effect of phosphatidylinositol 4,5-bisphosphate against cortical filamentous actin loss and insulin resistance induced by sustained exposure of 3T3-L1 adipocytes to insulin. *J Biol Chem* 279: 39705-39709, 2004.

9. Coers W, Reivinen J, Miettinen A, Huitema S, Vos JT, Salant DJ and Weening JJ. Characterization of a rat glomerular visceral epithelial cell line. *Exp Nephrol* 4: 184-192, 1996.

10. Cybulsky AV, Bonventre JV, Quigg RJ, Wolfe LS and Salant DJ. Extracellular matrix regulates proliferation and phospholipid turnover in glomerular epithelial cells. *Am J Physiol* 259: F326-F337, 1990.

11. Cybulsky AV and McTavish AJ. Extracellular matrix is required for MAP kinase activation and proliferation of rat glomerular epithelial cells. *Biochem Biophys Res Commun* 231: 160-166, 1997.

12. Cybulsky AV, McTavish AJ and Cyr MD. Extracellular matrix modulates epidermal growth factor receptor activation in rat glomerular epithelial cells. *J Clin Invest* 94: 68-78, 1994.

13. Cybulsky AV, McTavish AJ and Papillon J. Extracellular matrix stimulates production and breakdown of inositol phospholipids. *Am J Physiol* 271: F579-F587, 1996.

14. Cybulsky AV, McTavish AJ, Papillon J and Takano T. Role of extracellular matrix and Ras in regulation of glomerular epithelial cell proliferation. *Am J Pathol* 154: 899-908, 1999.

15. Cybulsky AV, Takano T, Papillon J, Khadir A, Bijian K and Le Berre L. The actin cytoskeleton facilitates complement-mediated activation of cytosolic phospholipase A2. *Am J Physiol Renal Physiol* 286: F466-F476, 2004.

16. Dedhar S, Jewell K, Rojiani M and Gray V. The receptor for the basement membrane glycoprotein entactin is the integrin alpha 3/beta 1. *J Biol Chem* 267: 18908-18914, 1992.

17. Elices MJ, Urry LA and Hemler ME. Receptor functions for the integrin VLA-3: fibronectin, collagen, and laminin binding are differentially influenced by Arg-Gly-Asp peptide and by divalent cations. *J Cell Biol* 112: 169-181, 1991.

18. Endlich K, Kriz W and Witzgall R. Update in podocyte biology. *Curr Opin Nephrol Hypertens* 10: 331-340, 2001.

19. Frisch SM and Screaton RA. Anoikis mechanisms. *Curr Opin Cell Biol* 13: 555-562, 2001.

20. Giancotti FG and Ruoslahti E. Integrin signaling. *Science* 285: 1028-1032, 1999.

21. Kaibuchi K, Kuroda S and Amano M. Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. *Annu Rev Biochem* 68: 459-486, 1999.

22. Kanwar YS, Wada J, Lin S, Danesh FR, Chugh SS, Yang Q, Banerjee T and Lomasney JW. Update of extracellular matrix, its receptors, and cell adhesion molecules in mammalian nephrogenesis. *Am J Physiol Renal Physiol* 286: F202-F215, 2004.

23. Kaplan JM, Kim SH, North KN, Rennke H, Correia LA, Tong HQ, Mathis BJ, Rodriguez-Perez JC, Allen PG, Beggs AH and Pollak MR. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet* 24: 251-256, 2000.

24. Kemeny E, Durmuller U, Nickeleit V, Gudat F and Mihatsch MJ. Distribution of podocyte protein (44 KD) in different types of glomerular diseases. *Virchows Arch* 431: 425-430, 1997.

25. Kerjaschki D. Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis. *J Clin Invest* 108: 1583-1587, 2001.

26. Kreidberg JA and Symons JM. Integrins in kidney development, function, and disease. *Am J Physiol Renal Physiol* 279: F233-F242, 2000.

27. Lee JW and Juliano R. Mitogenic signal transduction by integrin- and growth factor receptor-mediated pathways. *Mol Cells* 17: 188-202, 2004.

28. Mathis BJ, Kim SH, Calabrese K, Haas M, Seidman JG, Seidman CE and Pollak MR. A locus for inherited focal segmental glomerulosclerosis maps to chromosome 19q13. *Kidney Int* 53: 282-286, 1998.

29. Mundel P and Shankland SJ. Podocyte biology and response to injury. *J Am Soc Nephrol* 13: 3005-3015, 2002.

30. Nagata M, Hattori M, Hamano Y, Ito K, Saitoh K and Watanabe T. Origin and phenotypic features of hyperplastic epithelial cells in collapsing glomerulopathy. *Am J Kidney Dis* 32: 962-969, 1998.

31. Nemeth ZH, Deitch EA, Davidson MT, Szabo C, Vizi ES and Hasko G. Disruption of the actin cytoskeleton results in nuclear factor-kappaB activation and inflammatory mediator production in cultured human intestinal epithelial cells. *J Cell Physiol* 200: 71-81, 2004.

32. Pavenstadt H, Kriz W and Kretzler M. Cell biology of the glomerular podocyte. *Physiol Rev* 83: 253-307, 2003.

33. Raucher D, Stauffer T, Chen W, Shen K, Guo S, York JD, Sheetz MP and Meyer T. Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion. *Cell* 100: 221-228, 2000.

34. Srivastava T, Garola RE, Whiting JM and Alon US. Synaptopodin expression in idiopathic nephrotic syndrome of childhood. *Kidney Int* 59: 118-125, 2001.

35. Stauffer TP, Ahn S and Meyer T. Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. *Curr Biol* 8: 343-346, 1998.

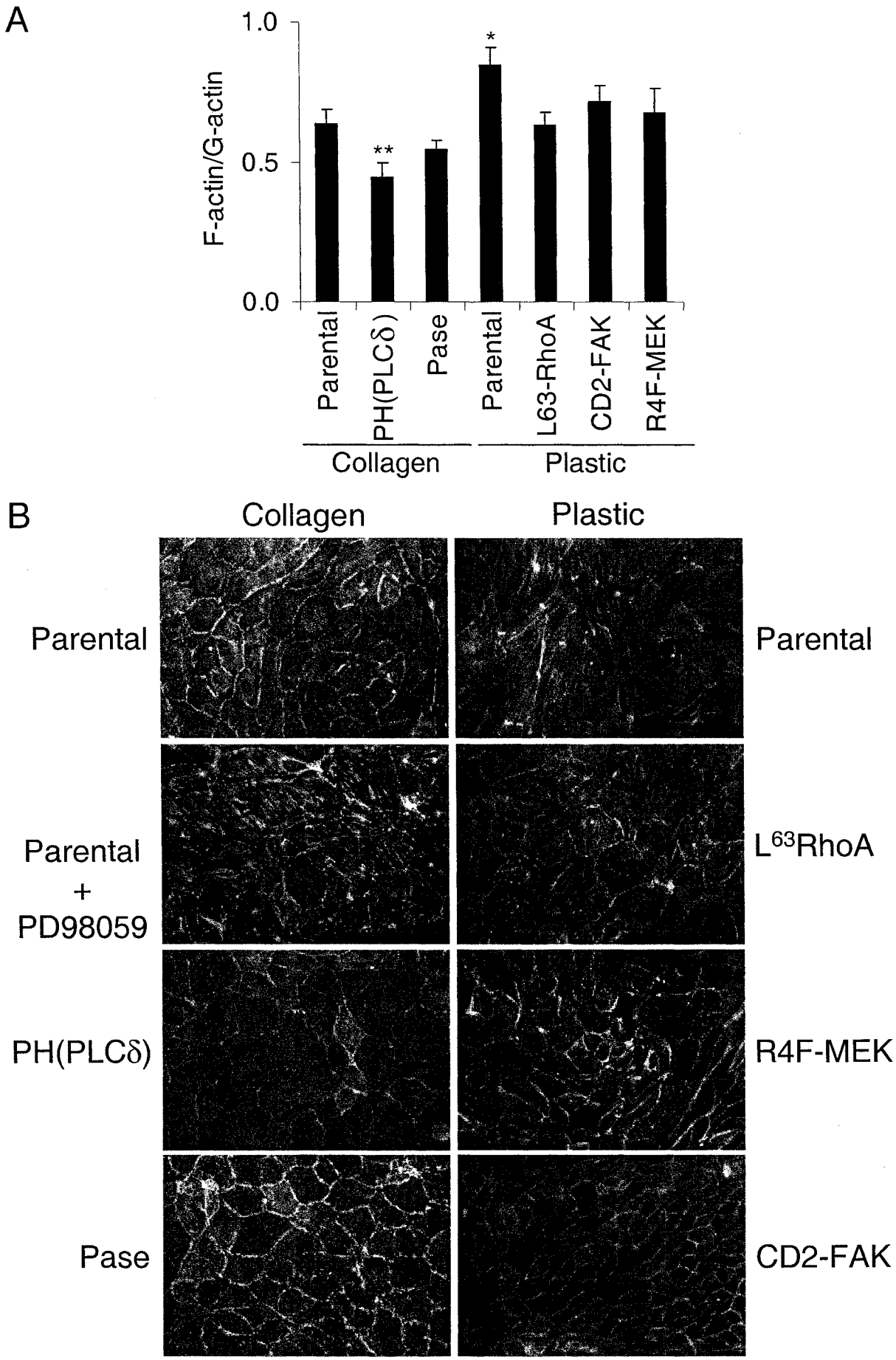
36. Togawa A, Miyoshi J, Ishizaki H, Tanaka M, Takakura A, Nishioka H, Yoshida H, Doi T, Mizoguchi A, Matsuura N, Niho Y, Nishimune Y, Nishikawa S and Takai Y. Progressive impairment of kidneys and reproductive organs in mice lacking Rho GDIalpha. *Oncogene* 18: 5373-5380, 1999.

37. Tsakiridis T, Tsiani E, Lekas P, Bergman A, Cherepanov V, Whiteside C and Downey GP. Insulin, insulin-like growth factor-I, and platelet-derived growth factor activate extracellular signal-regulated kinase by distinct pathways in muscle cells. *Biochem Biophys Res Commun* 288: 205-211, 2001.

38. Valeri A, Barisoni L, Appel GB, Seigle R and D'Agati V. Idiopathic collapsing focal segmental glomerulosclerosis: a clinicopathologic study. *Kidney Int* 50: 1734-1746, 1996.

39. Yin HL and Janmey PA. Phosphoinositide regulation of the actin cytoskeleton. *Annu Rev Physiol* 65: 761-789, 2003.

FIGURE 1


A
1.0
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F-actin/G-actin
**
*
Parental
PH(PLCδ)
Pase
Parental
L63-RhoA
CD2-FAK
R4F-MEK
Collagen
Plastic
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PD98059
L63RhoA
PH(PLCδ)
R4F-MEK
Pase
CD2-FAK

Figure 1. Actin cytoskeleton in GEC. The actin cytoskeleton was analyzed in parental GEC on collagen or plastic, in GEC that express GFP-PH(PLCδ) or GFP-Pase on collagen, and in GEC that express constitutively active RhoA (L^{63}RhoA), FAK (CD2-FAK) or MEK (R4F-MEK) on plastic. A: F-actin content was quantified in these GEC lines by rhodamine-phalloidin labeling of F-actin, normalized for Alexa-Fluro-488-conjugated DNase1-labeled G-actin. Parental GEC on plastic had significantly greater levels of F-actin, as compared with parental GEC on collagen (*P<0.007 parental plastic vs. parental collagen, 8 experiments). GEC expressing GFP-PH(PLCδ) or GFP-Pase had reduced levels of F-actin, as compared with parental GEC on collagen, but the decrease reached statistical significance only in GEC expressing GFP-PH(PLCδ) (**P<0.02 GFP-PH(PLCδ) collagen vs. parental collagen, 8 experiments). GEC expressing L^{63}RhoA, CD2-FAK or R4F-MEK on plastic had similar levels of F-actin as parental GEC on collagen. B: Fluorescence micrographs of rhodamine-phalloidin labeled GEC. Rhodamine-phalloiding staining of parental GEC on collagen (row 1, column 1) demonstrates cortical F-actin distribution with faint cytosolic staining. Parental GEC plated on plastic substratum (row 1, column 2) demonstrate extensive stress fibers with loss of organized cortical staining. Parental GEC on collagen treated with the MEK inhibitor (PD98059, 75 μM) for 24 h (row 2, column 1) had a similar F-actin staining pattern as parental GEC on plastic with loss of the cortical F-actin, and formation of stress fibers. GEC expressing GFP-PH(PLCδ) (row 3, column 1) or GFP-Pase (row 4, column 1) on collagen demonstrate reduced levels of cortical staining, as compared with parental GEC on collagen. GEC expressing L^{63}RhoA (row 2, column 2) or R4F-MEK (row 3, column 2) or CD2-FAK (row 4, column 2) on plastic demonstrate obvious cortical staining, comparable to parental GEC on collagen.

FIGURE 2

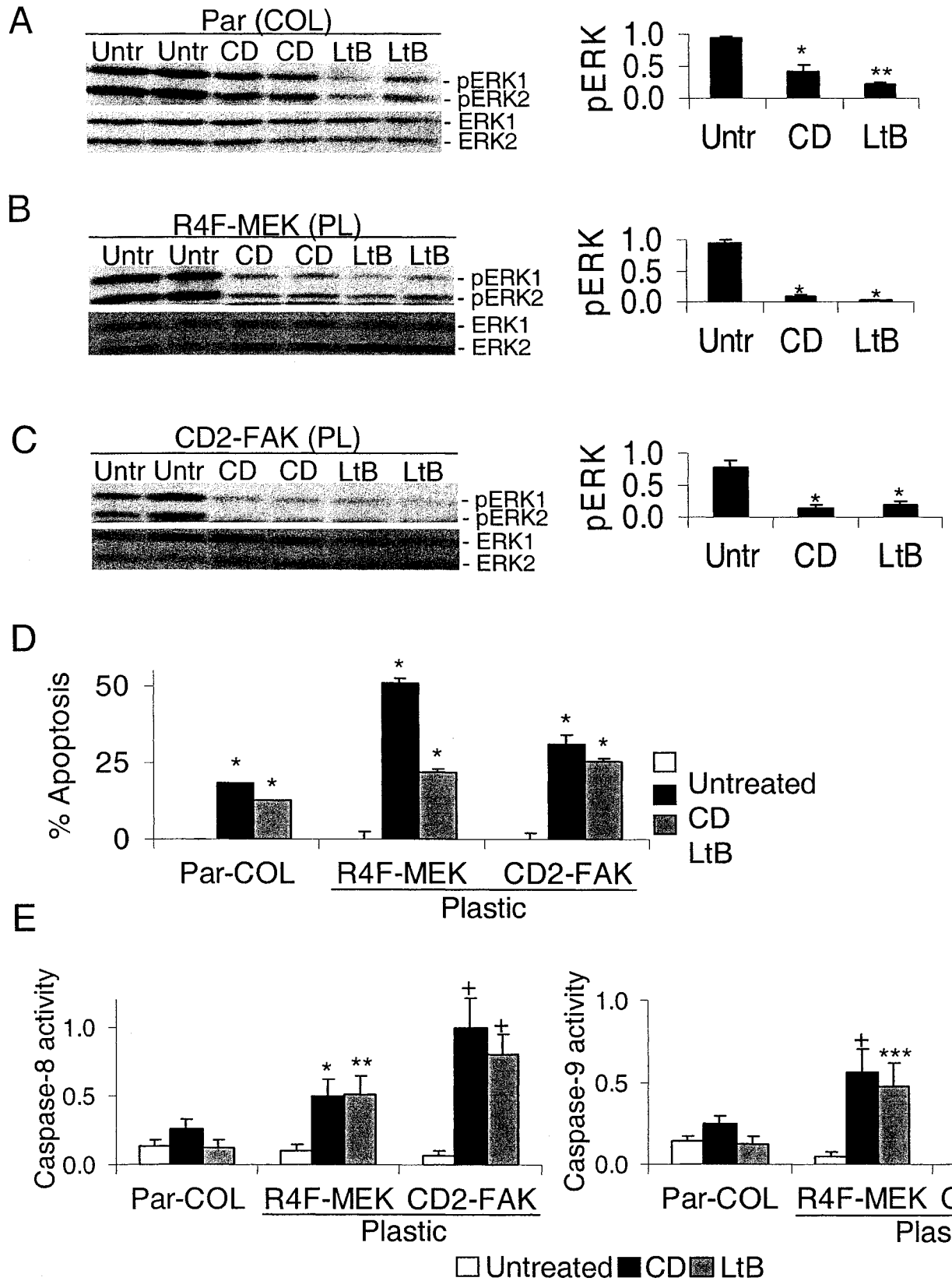
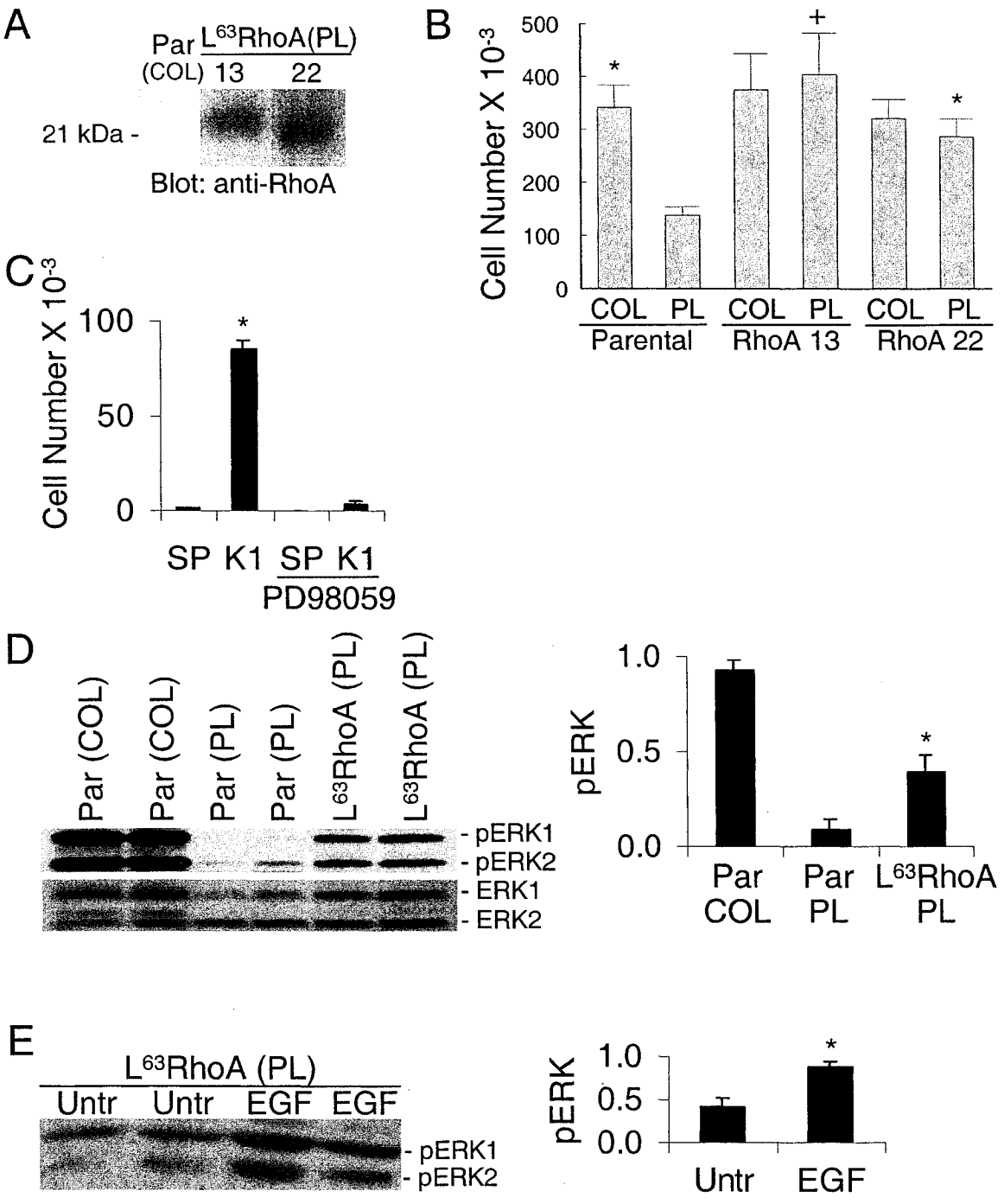

A
Par (COL)
Untr Untr CD CD LtB LtB
- pERK1
- pERK2
- ERK1
- ERK2
pERK
1.0
0.5
0.0
Untr CD LtB
B
R4F-MEK (PL)
Untr Untr CD CD LtB LtB
- pERK1
- pERK2
- ERK1
- ERK2
pERK
1.0
0.5
0.0
Untr CD LtB
C
CD2-FAK (PL)
Untr Untr CD CD LtB LtB
- pERK1
- pERK2
- ERK1
- ERK2
pERK
1.0
0.5
0.0
Untr CD LtB
D
% Apoptosis
50
25
0
Par-COL
R4F-MEK
CD2-FAK
Plastic
Untreated
CD
LtB
E
Caspase-8 activity
1.0
0.5
0.0
Par-COL
R4F-MEK
CD2-FAK
Plastic
Caspase-9 activity
1.0
0.5
0.0
Par-COL
R4F-MEK
CD2-FAK
Plastic
Untreated CD LtB

Figure 2. Survival signals emanating from collagen are dependent upon an intact actin cytoskeleton. Parental (Par) GEC on collagen (COL) (A), R4F-MEK GEC on plastic (PL) (B) or CD2-FAK GEC on plastic (C) were untreated (Untr), or were treated with cytochalasin D (CD, 20 μM) or latrunculin B (LtB, 1 μM) for 24 h in serum-poor medium. Cell lysates were immunoblotted with antibodies to ERK1/2 phospho-threonine^{202}/tyrosine^{204} (pERK; upper panel) or to ERK1/2 (lower panel). Representative immunoblots are shown on the left, and densitometric quantification on the right. ERK activation was decreased significantly in CD- or LtB-treated GEC, as compared with untreated. A) *P<0.002 CD vs. untreated, **P<0.001 LtB vs. untreated (3-5 experiments); B) *P<0.001 CD or LtB vs. untreated (6 experiments); C) *P<0.001 CD or LtB vs. untreated (6 experiments). There were no significant differences in ERK protein expression. D: Apoptosis was monitored as H33342-positively stained nuclei in the three GEC lines treated with or without CD or LtB. There were significant increases in apoptosis in CD or LtB-treated GEC, as compared with untreated. *P<0.001 CD or LtB vs. untreated (4 experiments). E: Caspase-8 and -9 activities were monitored in the three GEC lines treated with or without CD or LtB. There were significant increases in caspase-8 and -9 activities in GEC expressing R4F-MEK or CD2-FAK, treated with CD or LtB, as compared with untreated. *P<0.02 CD vs. untreated (R4F-MEK; caspase-8), **P<0.03 LtB vs. untreated (R4F-MEK; caspase-8), +P<0.001 CD or LtB vs. untreated (CD2-FAK; caspase-8 and caspase-9), CD vs. untreated (R4F-MEK; caspase-9), ***P<0.004 LtB vs. untreated (R4F-MEK; caspase-9) (4-6 experiments). There were no significant increases in caspase-8 or -9 activities in parental GEC treated with CD or LtB.

FIGURE 3


A
Par
(COL)
L63RhoA(PL)
13
22
21 kDa -
Blot: anti-RhoA
B
Cell Number X 10-3
500
400
300
200
100
0
COL PL COL PL COL PL
Parental
RhoA 13
RhoA 22
C
Cell Number X 10-3
100
50
0
SP K1 SP K1
PD98059
D
Par (COL)
Par (COL)
Par (PL)
Par (PL)
L63RhoA (PL)
L63RhoA (PL)
- pERK1
- pERK2
- ERK1
- ERK2
pERK
1.0
0.5
0.0
Par COL
Par PL
L63RhoA PL
E
L63RhoA (PL)
Untr
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EGF
EGF
- pERK1
- pERK2
pERK
1.0
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Untr
EGF

FIGURE 3, Continued

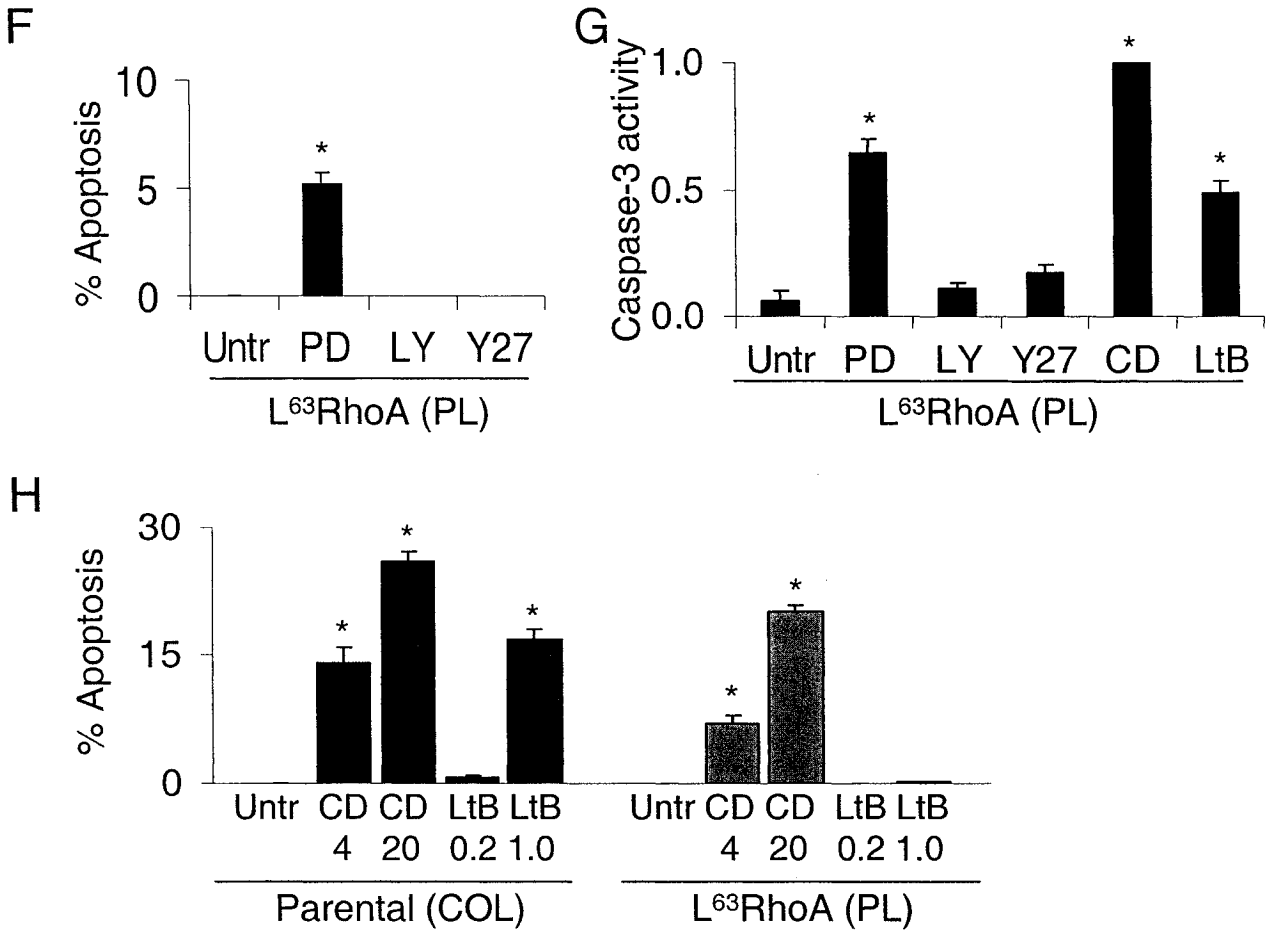
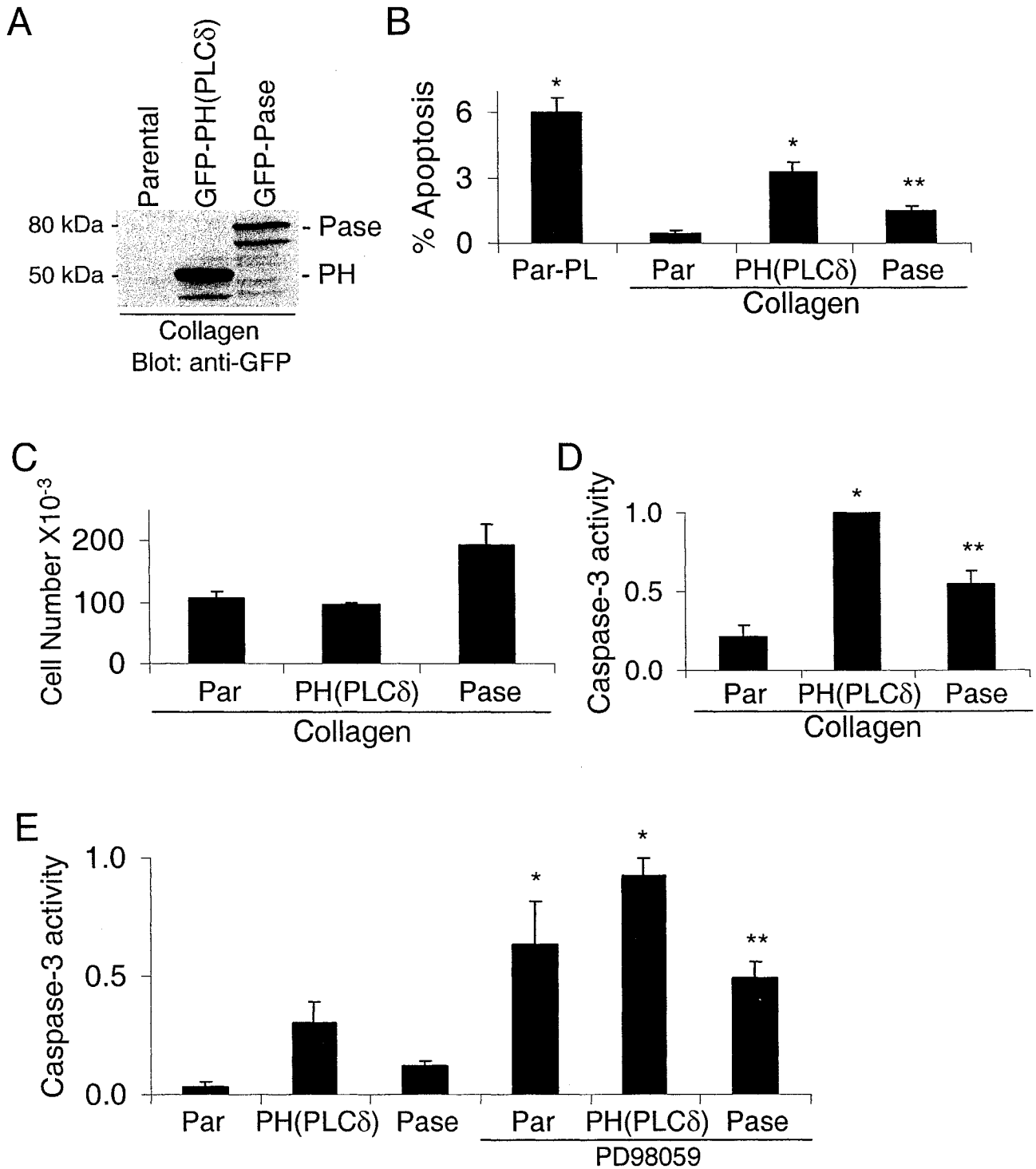


Figure 3. Stable transfection of GEC with constitutively active RhoA (L^{63}RhoA). A: Two representative GEC clones (clones 13 and 22) that stably express L^{63}RhoA, adherent to plastic (PL), and parental GEC on collagen are presented. Equal amounts of total protein were loaded into each lane. Endogenous RhoA was not detectable by immunoblotting at this exposure, but was detected in other experiments (not shown). B: Parental or L^{63}RhoA-transfected GEC (clones 13 and 22) were plated at 2 x 10^5 cells/well and cultured on collagen (COL) or plastic (PL) in K1 medium for 72 h. Parental GEC proliferated only on collagen (*$p<0.005$ parental COL vs. parental PL, 11 experiments). L^{63}RhoA clones proliferated on collagen and plastic (*$p<0.005$, +$p<0.04$ L^{63}RhoA PL vs. parental PL, 11 experiments). *Continued on following page*

C: L^{63}RhoA transfected GEC (50,000 cells) on plastic were cultured in serum-poor (SP) medium, or growth factor-replete (K1) medium for 5 days. Cell number increased in the presence of K1 (*p<0.001 K1 vs. SP, 8 experiments). Addition of PD98059 (75 μM) abolished proliferation. D: Parental (Par) GEC on collagen (COL) or plastic (PL) and L^{63}RhoA-transfected GEC on plastic (clone 13) were cultured in serum-poor medium for 24 h. Lysates were immunoblotted with antibodies to ERK1/2 phospho-threonine^{202}/tyrosine^{204} (pERK; upper panel) or to ERK1/2 (lower panel) (representative immunoblots and densitometric quantification). ERK activation was low in parental GEC on plastic, while ERK was activated in GEC on collagen. ERK phosphorylation was increased in L^{63}RhoA GEC, as compared with parental GEC on plastic (*P<0.004 L^{63}RhoA PL vs. Parental PL, 4 experiments). E: L^{63}RhoA-transfected GEC on plastic were cultured in serum-poor medium for 24 h. Cells were then treated with or without EGF (100 ng/ml) for 30 min. Equal amounts of total protein were loaded into each lane prior to immunoblotting with anti-phosphoERK antibody. Basal L^{63}RhoA ERK phosphorylation was further stimulated by EGF (*P<0.002 EGF vs. untreated, 6 experiments). F: Apoptosis (H33342-positively stained nuclei) in L^{63}RhoA GEC on plastic (PL) was monitored after treatment with or without PD98059 (PD; 75 μM), the phosphatidylinositol 3-kinase inhibitor LY294002 (LY; 10 μM) or the Rho kinase (ROCK) inhibitor Y27632 (Y27; 10 μM) in serum-poor medium for 24 h. There was a significant increase in apoptosis only in PD98059-treated cells (*P<0.001 vs. untreated, 8 experiments). G: Caspase-3 activity was monitored in L^{63}RhoA transfected GEC on plastic (PL) cultured in serum-poor medium with or without PD98059 (PD, 75 μM), LY294002 (LY, 10μM), Y27632 (Y27, 10 μM), cytochalasin D (CD, 20 μM), or latrunculin B (LtB, 1 μM) for 24 h. Caspase-3 activity was increased in the L^{63}RhoA GEC treated with PD98059, cytochalasin D and latrunculin B, as compared with untreated (*P<0.001 vs. untreated, 4 experiments). H: Apoptosis of parental GEC on collagen (COL) and L^{63}RhoA GEC on plastic (PL) was monitored after treatment with or without cytochalasin D (CD; 4 or 20 μM) or latrunculin B (LtB; 0.2 or 1.0 μM) in serum-poor medium for 24 h. Treatment with CD (4 or 20 μM) significantly increased apoptosis in both cell lines, whereas treatment with 1.0 μM LtB significantly increased apoptosis only in parental GEC (*P<0.001 vs. untreated Parental or untreated L^{63}RhoA; 8 experiments).

FIGURE 4


A
Parental
GFP-PH(PLCδ)
GFP-Pase
80 kDa -
50 kDa -
- Pase
- PH
Collagen
Blot: anti-GFP
B
% Apoptosis
0
3
6
*
*
**
Par-PL
Par
PH(PLCδ)
Pase
Collagen
C
Cell Number X10-3
0
100
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Par
PH(PLCδ)
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Collagen
D
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0.0
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Par
PH(PLCδ)
Pase
PD98059

Figure 4. Stable transfection of GEC with GFP-PH(PLCδ) or GFP-Pase. A: Lysates of parental GEC and of GEC clones that stably express GFP-PH(PLCδ) or GFP-Pase were immunoblotted with anti-GFP antibody. Equal amounts of total protein were loaded into each lane. B: Apoptosis of parental (Par) GEC on plastic (PL) or collagen and of GEC expressing GFP-PH(PLCδ) or GFP-Pase cultured in serum-poor medium for 24 h was quantified by monitoring H33342 staining of nuclei. Parental GEC on plastic substratum had increased levels of apoptosis, as compared with parental GEC on collagen. GEC expressing GFP-PH(PLCδ) or GFP-Pase had significantly greater levels of apoptosis as compared with parental GEC on collagen (*P<0.001 PH(PLCδ) COL vs. parental COL, parental COL vs. parental PL, **P<0.03 Pase COL vs parental COL, 6 experiments). C: Parental (Par) GEC, GEC expressing GFP-PH(PLCδ) or GFP-Pase (50,000 cells) were cultured in K1 medium. Cell number was quantified at 72 h. There were no significant differences among groups. D: Caspase-3 activity was significantly increased in GEC expressing GFP-PH(PLCδ) or GFP-Pase, as compared to parental (Par) GEC (*P<0.001 PH(PLCδ) vs. parental, **P<0.003 Pase vs. parental, 5 experiments). E: Caspase-3 activity of parental (Par) GEC on collagen and of GEC expressing GFP-PH(PLCδ) or GFP-Pase on collagen cultured in K1 medium was monitored after treatment with PD98059 (75 μM) for 24 h. Addition of PD98059 resulted in further increases in caspase-3 activity in all cell lines, as compared with untreated (*P<0.001 parental PD98059 vs. parental untreated, PH(PLCδ) PD98059 vs. PH(PLCδ) untreated,**P<0.03 GFP-Pase PD98059 vs. GFP-Pase untreated, 3 experiments).

FIGURE 5

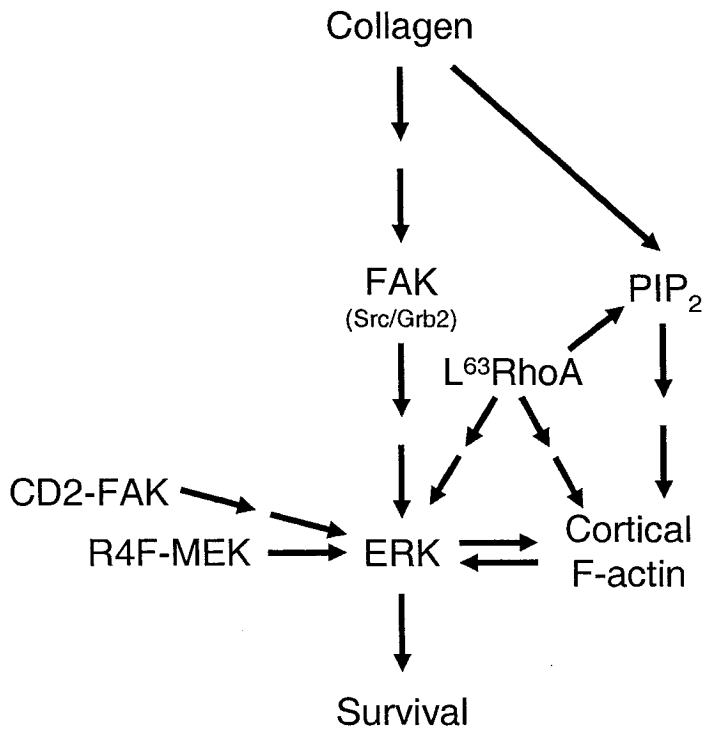

Figure 5. Schematic representation of the reciprocal relationship between ERK activation and the cortical F-actin structure involved in the intracellular survival signals emanating from the ECM. See text for details.

SECTION 4: CONCLUSION

The discovery that mutations in podocyte specific proteins cause hereditary kidney diseases, has reinforced the current understanding that among the cells present in the glomerulus, the podocyte is the major cell type responsible for the progression of many glomerular diseases. Certain forms of glomerulonephritis feature GEC injury, which may lead to apoptosis or proliferation, and, in association with ECM expansion, to glomerulosclerosis and/or impaired glomerular function or permselectivity. Understanding the factors that modulate GEC proliferation and/or survival, and the mechanisms utilized will help to identify future therapeutic targets toward improving the renal survival of patients with glomerular disease. The objective of this study was to identify and characterize the major intracellular signaling pathways that modulate GEC proliferation and/or survival, and are activated by adhesion to ECM and involved in the crosstalk between ECM receptors and growth factor receptors.

Of primary importance is the work, which revealed that adhesion of GEC to collagen increased the activation of FAK, c-Src, and ERK, and facilitated survival (prevented apoptosis). In contrast, GEC adherent to plastic underwent apoptosis, characterized by increased oligonucleosomal DNA fragmentation, increased caspase-8 and -9 activities, chromatin condensation, increased expression of the active proapoptotic protein, Bax, and decreased expression of the antiapoptotic protein, Bcl-X_L. The result also suggests the involvement of the mitochondrial apoptotic pathway. Moreover, the survival signals emanating from collagen were supplanted by the stable expression of

constitutively active mutants of FAK or MEK, which successfully activated ERK in GEC clones on plastic, without replacing the requirement of growth factors for cell survival and proliferation. This study supports the view that collagen and constitutively active FAK and MEK mutants activated an ECM-specific ERK pathway (perhaps within focal adhesions), whereas growth factor-induced ERK activation occurred via a distinct Ras-ERK pathway, as illustrated in Fig. 1. In a previous study, stable transfection of a constitutively active mutant of Ras (V^{12}Ras) supplanted the requirement for collagen, but not for growth factors (15), further supporting this view.

The importance of the ERK pathway in supporting cell survival was further demonstrated by the expression of a Ras mutant that constitutively activates PI-3K but blocks ERK activation or by the use of a pharmacological inhibitor of the ERK pathway (PD98059). In spite of the presence of ECM, inhibition of the ERK pathway led to increased GEC apoptosis. The inability of the PI3-K pathway to promote GEC survival is of a special interest since the PI3-K pathway has been viewed as an important survival pathway in different cell types (10). Our studies revealed that the ERK pathway is necessary to promote ECM-induced GEC survival, whereas the PI3-K pathway is neither sufficient nor necessary for GEC survival.

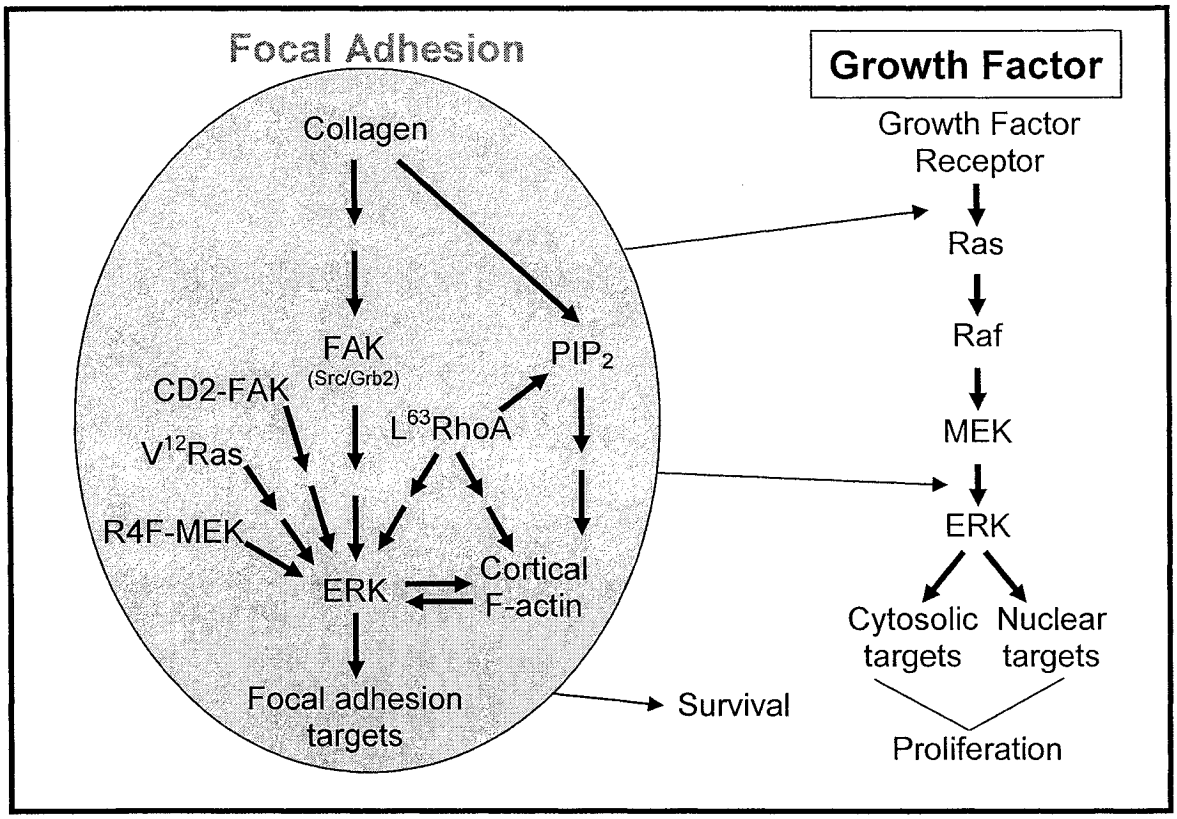

Figure 1: *Crosstalk between ECM receptors and growth factor receptors modulating GEC proliferation and/or survival in GEC. Adhesion to ECM (collagen) resulted in the activation of FAK, which led to the phosphorylation of the Grb2 binding site of FAK in a Src dependent manner. The FAK/Src/Grb2 complex was able to activate ERK within focal adhesions, which was necessary to promote cell adhesion and growth factor dependent GEC survival and proliferation. ECM-induced ERK activation was also necessary to achieve and maintain a cortical actin structure required for growth-factor induced ERK activation and adequate GEC proliferation and survival. Adhesion of GEC to ECM may also favor the maintenance of the cortical F-actin architecture necessary for GEC survival through the increased turnover of inositol phospholipids (i.e. PIP_2 production). Disruption or inhibition of any of the aforementioned signaling components leads to GEC apoptosis possibly through the mitochondrial apoptotic pathway. In contrast, constitutive activation of certain signaling components is able to supplant the requirement for ECM and thus promote survival and growth factor induced proliferation.*

Podocyte loss occurs in humans with type I diabetes, although podocyte cell density is maintained. This suggests that the glomerular filter including the podocyte is able to actively regulate its architecture to maintain the same number of cells per volume, however this compensatory mechanism is limited and eventually podocyte loss leads to the progression of the disease and renal insufficiency (77). Moreover, in experimental focal segmental glomerulosclerosis (FSGS), i.e. the puromycin aminonucleoside nephrosis (PAN) model of GEC injury in vivo, 15, 31 and 53% of podocyte depletion was observed after the administration of one, two or three injections of puromycin aminonucleoside at 30 day intervals respectively. Podocyte loss was confirmed by the identification of detached podocytes in Bowman's space, the presence of the podocyte specific proteins nephrin and GLEPP1 mRNA in urine and the presence of TUNEL positive (i.e. apoptotic) podocytes in glomeruli. Furthermore, glomerulosclerosis was observed in the area of the glomerulus that did not contain podocytes (40). In our study, glomeruli isolated from rats with PAN revealed increased β1-integrin expression, along with increased activation of FAK, c-Src, and ERK, compared with controls, indicating the presence of analogous GEC survival signals in vivo. In our case increased β1 integrin signaling through FAK/Src/ERK may be an initial protective mechanism of GEC in order to limit injury, which eventually may not be sufficient to maintain a stable GEC-GBM interaction, thus leading to GEC detachment and apoptosis (anoikis). Hence, factors that facilitate detachment of GEC from the GBM are key instigators of GEC apoptosis, leading to glomerulosclerosis and should be analyzed in greater detail.

Factors that may promote GEC detachment and apoptosis include alterations in the integrin-binding affinity, intraglomerular hypertension, immune complexes, and C5-9 mediated injury to podocytes. Moreover, factors that affect the GBM integrity such as diabetes, amyloidosis and immune complexes may also influence the GEC-GBM contact (40). Integrin binding affinity can be regulated via inside-out signaling through the cytoplasmic domain of integrin receptors. FAK and ERK signaling could modulate integrin binding affinity for different extracellular matrices. For example, in differentiated prostatic epithelial cells, FAK was shown to be present at lower levels, as compared to cells with a proliferative phenotype, suggesting that FAK may reduce integrin binding to ECM, which is required for cell proliferation (80). Activation of ERK has also been shown to reduce high affinity binding of β1 integrin to ECM. This may serve as a negative feedback loop suppressing the growth promoting signals emanating from ligated integrins (33). Moreover, integrin binding affinity could be regulated by cytoskeletal components. In our study, disruption of the actin cytoskeleton with cytochalasin D or latrunculin B in parental GEC adherent to collagen, as well as in GEC that express R4F-MEK and CD2-FAK on plastic led to decreased activation of ERK and increased apoptosis. ERK activation by collagen was necessary to maintain a cortical actin architecture, which was abolished in GEC plated on plastic substratum. This intact cortical architecture is required for efficient activation of the growth factor mediated ERK activation leading to GEC survival and proliferation. Moreover, ERK activation was also dependent upon an intact actin cytoskeleton, suggesting a reciprocal relationship between collagen-induced cortical F-actin assembly and collagen-dependent survival

signaling, including ERK activation (Fig. 1). This study demonstrated the importance of an organized cortical actin cytoskeleton in transmitting survival signals emanating from ECM. The cortical cytoarchitecture may regulate integrin adhesivity and thus modulate cell survival. However, disruption of the cortical actin may also influence growth factor receptor translocation to focal adhesions upon integrin ligation. In human umbilical vein endothelial cells (HUVEC), vascular endothelial growth factor (VEGF) is able to stimulate the tyrosine phosphorylation of FAK, however upon treatment with cytochalasin D, tyrosine phosphorylation of FAK by VEGF was completely inhibited (1).

Our studies demonstrated the ability of membrane phosphoinositides to regulate GEC architecture, and therefore GEC survival. Downregulation or sequestration of PIP_2 by stable transfection with an inositol 5'-phosphatase or the plextrin-homology domain of phospholipase C-δ reduced cortical F-actin content and decreased GEC survival. PIP_2 is able to promote F-actin assembly through many of its effectors (85). Understanding the process of ECM-induced inositol lipid production and breakdown in GEC may provide insights into the regulation of GEC survival in normal conditions and during glomerular injury.

OTHER RESULTS AND FUTURE IMPLICATIONS

It is becoming increasingly apparent that there is no single protein or mechanism responsible for the progression of FSGS, since multiple defects in podocytes result in the same clinical (proteinuria) and histopathological (foot process effacement and sclerosis) condition. Therefore, analyzing the molecular targets or pathways involved in the pathogenesis of the disease is of greatest importance for eventually attaining a therapeutic solution.

Additional studies need to address the upstream regulators of GEC survival. For example, it would be important to determine if collaboration occurs between ECM survival signaling and growth factor mediated activation of the ERK pathway at levels other that those illustrated in Fig. 1. Moreover, it would be important to identify common downstream targets of both of these signaling pathways, including cytosolic, nuclear or focal adhesions proteins. Monitoring the translocation of certain proteins may also be informative. Key protein kinases could eventually become targets for design of therapies.

In addition to the activation of caspases-3, -8 and -9 in GEC plated on plastic, preliminary results also demonstrate slight increases in the endoplasmic reticulum (ER) chaperone proteins Bip and Grp94, along with increased caspase-12 activity (unpublished observations), suggesting activation of the unfolded protein response (ER stress). Additional studies are required to address expression of GADD153 (CHOP; a gene upregulated in ER stress), and the potential roles of caspase-12 and GADD153 in

apoptosis. Monitoring cytochrome c release from mitochondria, as well as Bax translocation to mitochondrial or ER membranes will help us dissect the major apoptotic pathways activated in GEC. We have determined that caspases-3 and -9, and Bax are activated in glomeruli isolated from rats with PAN i.e. in GEC injury in vivo (unpublished observations). Increasing our knowledge of these apoptotic pathways in vivo, may eventually lead to development of specific caspase inhibitors that can be employed to maintain glomerular function or permselectivity in humans.

Another area of potential investigation may involve functional studies using GEC stably transfected with wild type (WT) and K256E mutant-α-actinin-4. This mutation increases the affinity of α-actinin-4 for filamentous (F)-actin and has recently been associated with a familial form of autosomal dominant FSGS. Mice with podocyte specific expression of the mutated α-actinin-4 develop FSGS-like features similar to humans with FSGS caused by α-actinin-4 mutations (53). Potential alterations inflicted to the actin cytoskeleton by the expression of this mutant may render GEC more susceptible to injury and consequently apoptosis through one of the above mentioned pathways.

The availability of knockout gene technology allows more precise delineation of the roles of specific mediators in podocyte injury. We carried out preliminary studies using a mouse model of experimental FSGS that features GEC injury and proliferation. This model consists of injecting mice with a sheep anti-rabbit glomerular antibody, which results in DNA synthesis and proliferation of GEC (39). Our initial results demonstrate

that mice injected with this antibody developed significant albuminuria, along with increased ERK and FAK activation (unpublished observations). To address the functional importance of these mediators, additional experiments may be performed with mice genetically deficient in Src family kinases (which can potentially amplify the activation of FAK), or podocyte specific proteins (e.g. CD2AP, α-actinin-4).

The present study has enhanced our knowledge about the factors that control GEC survival and proliferation, which will help us understand the pathophysiology of GEC injury in vivo. These findings may provide novel therapeutic approaches to preserve a functional glomerular filter and prevent renal failure. However, the impact of this study extends beyond GEC biology, since ECM-dependent regulation of growth factor signaling, protein kinases, apoptosis and proliferation is likely to be applicable to other cell types and diseases. Defective cell survival (both increased or decreased) is observed in a wide range of pathological conditions, including neurodegenerative, cardiovascular and immunological diseases, as well as in AIDS and cancer.

There is currently extensive documentation indicating irregular FAK expression in human cancers. Although FAK is not regarded as an oncogene, FAK overexpression has been reported in epithelial tumors of the breast, colon, thyroid, prostate, oral cavity, liver, stomach, and ovary as well as in tissues of mesenchymal origin such as muscle and glial cells. Under normal conditions, FAK regulates cell growth, survival and motility, all of which are dysregulated in tumors. Moreover, in certain tumors, FAK overexpression provides them the ability to undergo adhesion independent growth,

preventing anoikis and thus supporting tumor malignancy. FAK also appears to play a major role in cancer metastasis. FAK could regulate the secretion of matrix metalloproteinases (MMPs), which are able to degrade basement membrane and thus support metastasis (25). Recent studies also suggest the presence of atypical crosstalk between the ECM and growth factor signaling pathways in tumors. It has been suggested that integrin signaling through FAK could lead to the transactivation of growth-factor receptors leading to harmful consequences. A study by Wang et al. demonstrated that Met (the hepatocyte growth-factor (HGF) receptor) could be activated in hepatocytes by cell adhesion in the absence of HGF. This may explain why transgenic mice overexpressing Met in hepatocytes develop hepatocellular carcinoma, one of the human tumors in which Met has been implicated, even though HGF is not present in normal liver or in hepatocellular carcinoma tissues. The activation of the growth factor receptor by cell adhesion is misinterpreted by the cell as a growth stimulatory signal and thus leads to unwanted cellular growth, suggesting that cell adhesion can be an alternative mechanism for growth factor receptor activation in human cancers (83). However, additional studies analyzing the crosstalk between these two signaling pathways are required to eventually discover therapeutic targets for these diseases.

These findings support the view that FAK may be an important therapeutic target in human cancers. Inhibition of FAK impairs tumor growth and metastasis and induces apoptosis in certain types of cancers. Hauch et al. analyzed the function of FAK in supporting human A549 adenocarcinoma cell migration/metastasis by reducing FAK expression by use of antisense oligonucleotides or by promoting FAK dephosphorylation

through the expression of FAK-related non-kinase (FRNK). FAK antisense treatment or FRNK expression significantly reduced EGF-stimulated A549 cell motility. Moreover, the inhibition of FAK affected the extent and duration of the EGF-stimulated ERK and JNK activation (30). This is in agreement with our findings in GEC, where in the absence of ECM, and thus absence of FAK activation, EGF-stimulated EGFR and ERK activation was weak/transient (Fig. 5; section 1).

ECM modulation of growth factor-induced cell proliferation is also observed in occlusive lesions of atherosclerosis that result in myocardial infarction, stroke and peripheral vascular disease. Theses conditions result from the accumulation of monocytes and T-lymphocytes from the circulation and from migration and proliferation of smooth muscle cells (SMC) from the underlying layer of the artery. Studies have determined that the ECM surrounding SMC determines whether SMC remain quiescent or whether they migrate and proliferate in response to growth factors released from inflammatory cells. The ECM composition surrounding SMC in normal arteries is mainly fibrillar type I collagen, which has been shown to maintain SMC in their differentiated and non-proliferative state. In contrast, ECM composition in areas of developing lesions of atherosclerosis contains degraded or monomeric collagen, which may be required for SMC migration and proliferation. In a study by Koyama et al., fibrillar collagen inhibited cyclin E-associated kinase activity and cyclin-dependent kinase 2 (cdk2) phosphorylation and increased the expression of the cdk2 inhibitors $p27^{Kip1}$ and $p21^{Cip1/Waf1}$, as compared with SMC on monomeric collagen, thus inhibiting the G1/S transition in SMC (45). A recent study by Bond et al., addressed the molecular

mechanism underlying the regulation of $p27^{Kip1}$ expression in SMC. The authors were able to demonstrate that the downregulation of $p27^{Kip1}$ in SMC in the absence of appropriate ECM (i.e isolated SMC), was mediated via the S-phase-associated kinase protein-2 (Skp-2), a ubiquitin-protein ligase involved in proteasome mediated degradation. Serum stimulation of isolated SMC significantly increased Skp-2 protein expression and FAK phosphorylation at tyrosine 397, as compared to intact SMC. Overexpression of mutated FAK at tyrosine 397 or the FAK related non-kinase (FRNK) in SMC, significantly decreased Skp-2 expression, demonstrating the importance of FAK activity in regulating SMC proliferation (5). Although proteosome inhibitors have proven to be useful in preventing SMC proliferation, they are associated with some harmful side effects. Perhaps therapeutic strategies targeting FAK or any of the SH2-domain-containg kinases binding to tyrosine 397, may prove to be valuable in preventing the progression of disease.

References

1. Abedi H and Zachary I. Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J Biol Chem* 272: 15442-15451, 1997.

2. Aplin AE, Stewart SA, Assoian RK and Juliano RL. Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. *J Cell Biol* 153: 273-282, 2001.

3. Barisoni L, Kriz W, Mundel P and D'Agati V. The dysregulated podocyte phenotype: a novel concept in the pathogenesis of collapsing idiopathic focal segmental glomerulosclerosis and HIV-associated nephropathy. *J Am Soc Nephrol* 10: 51-61, 1999.

4. Bellanger JM, Astier C, Sardet C, Ohta Y, Stossel TP and Debant A. The Rac1- and RhoG-specific GEF domain of Trio targets filamin to remodel cytoskeletal actin. *Nat Cell Biol* 2: 888-892, 2000.

5. Bond M, Sala-Newby GB and Newby AC. Focal adhesion kinase (FAK)-dependent regulation of S-phase kinase-associated protein-2 (Skp-2) stability. A novel mechanism regulating smooth muscle cell proliferation. *J Biol Chem* 279: 37304-37310, 2004.

6. Boute N, Gribouval O, Roselli S, Benessy F, Lee H, Fuchshuber A, Dahan K, Gubler MC, Niaudet P and Antignac C. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* 24: 349-354, 2000.

7. **Brakebusch C and Fassler R. The integrin-actin connection, an eternal love affair.** ***EMBO J*** **22: 2324-2333, 2003.**

8. **Burrow CR. Regulatory molecules in kidney development.** ***Pediatr Nephrol*** **14: 240-253, 2000.**

9. **Cano E and Mahadevan LC. Parallel signal processing among mammalian MAPKs.** ***Trends Biochem Sci*** **20: 117-122, 1995.**

10. **Cantley LC. The phosphoinositide 3-kinase pathway.** ***Science*** **296: 1655-1657, 2002.**

11. **Carragher NO and Frame MC. Focal adhesion and actin dynamics: a place where kinases and proteases meet to promote invasion.** ***Trends Cell Biol*** **14: 241-249, 2004.**

12. **Chen HC, Appeddu PA, Parsons JT, Hildebrand JD, Schaller MD and Guan JL. Interaction of focal adhesion kinase with cytoskeletal protein talin.** ***J Biol Chem*** **270: 16995-16999, 1995.**

13. **Cybulsky AV, McTavish AJ and Cyr MD. Extracellular matrix modulates epidermal growth factor receptor activation in rat glomerular epithelial cells.** ***J Clin Invest*** **94: 68-78, 1994.**

14. **Cybulsky AV, McTavish AJ and Papillon J. Extracellular matrix stimulates production and breakdown of inositol phospholipids.** ***Am J Physiol*** **271: F579-F587, 1996.**

15. Cybulsky AV, McTavish AJ, Papillon J and Takano T. Role of extracellular matrix and Ras in regulation of glomerular epithelial cell proliferation. *Am J Pathol* 154: 899-908, 1999.

16. Daniel PT. Dissecting the pathways to death. *Leukemia* 14: 2035-2044, 2000.

17. Dedhar S, Jewell K, Rojiani M and Gray V. The receptor for the basement membrane glycoprotein entactin is the integrin alpha 3/beta 1. *J Biol Chem* 267: 18908-18914, 1992.

18. Deen WM. What determines glomerular capillary permeability? *J Clin Invest* 114: 1412-1414, 2004.

19. DeMali KA, Balciunaite E and Kazlauskas A. Integrins enhance platelet-derived growth factor (PDGF)-dependent responses by altering the signal relay enzymes that are recruited to the PDGF beta receptor. *J Biol Chem* 274: 19551-19558, 1999.

20. DeMali KA, Barlow CA and Burridge K. Recruitment of the Arp2/3 complex to vinculin: coupling membrane protrusion to matrix adhesion. *J Cell Biol* 159: 881-891, 2002.

21. Donoviel DB, Freed DD, Vogel H, Potter DG, Hawkins E, Barrish JP, Mathur BN, Turner CA, Geske R, Montgomery CA, Starbuck M, Brandt M, Gupta A, Ramirez-Solis R, Zambrowicz BP and Powell DR. Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. *Mol Cell Biol* 21: 4829-4836, 2001.

22. **Drenckhahn D and Franke RP. Ultrastructural organization of contractile and cytoskeletal proteins in glomerular podocytes of chicken, rat, and man. *Lab Invest* 59: 673-682, 1988.**

23. **Frisch SM and Screaton RA. Anoikis mechanisms. *Curr Opin Cell Biol* 13: 555-562, 2001.**

24. **Furuta Y, Ilic D, Kanazawa S, Takeda N, Yamamoto T and Aizawa S. Mesodermal defect in late phase of gastrulation by a targeted mutation of focal adhesion kinase, FAK. *Oncogene* 11: 1989-1995, 1995.**

25. **Gabarra-Niecko V, Schaller MD and Dunty JM. FAK regulates biological processes important for the pathogenesis of cancer. *Cancer Metastasis Rev* 22: 359-374, 2003.**

26. **George EL, Georges-Labouesse EN, Patel-King RS, Rayburn H and Hynes RO. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* 119: 1079-1091, 1993.**

27. **Giancotti FG. Complexity and specificity of integrin signalling. *Nat Cell Biol* 2: E13-E14, 2000.**

28. **Giancotti FG and Ruoslahti E. Integrin signaling. *Science* 285: 1028-1032, 1999.**

29. **Han DC and Guan JL. Association of focal adhesion kinase with Grb7 and its role in cell migration. *J Biol Chem* 274: 24425-24430, 1999.**

30. **Hauck CR, Sieg DJ, Hsia DA, Loftus JC, Gaarde WA, Monia BP and Schlaepfer DD. Inhibition of focal adhesion kinase expression or activity**

disrupts epidermal growth factor-stimulated signaling promoting the migration of invasive human carcinoma cells. *Cancer Res* 61: 7079-7090, 2001.

31. Hildebrand JD, Taylor JM and Parsons JT. An SH3 domain-containing GTPase-activating protein for Rho and Cdc42 associates with focal adhesion kinase. *Mol Cell Biol* 16: 3169-3178, 1996.

32. Horster MF, Braun GS and Huber SM. Embryonic renal epithelia: induction, nephrogenesis, and cell differentiation. *Physiol Rev* 79: 1157-1191, 1999.

33. Hughes PE, Renshaw MW, Pfaff M, Forsyth J, Keivens VM, Schwartz MA and Ginsberg MH. Suppression of integrin activation: a novel function of a Ras/Raf-initiated MAP kinase pathway. *Cell* 88: 521-530, 1997.

34. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69: 11-25, 1992.

35. Hynes RO. Targeted mutations in cell adhesion genes: what have we learned from them? *Dev Biol* 180: 402-412, 1996.

36. Kaplan JM, Kim SH, North KN, Rennke H, Correia LA, Tong HQ, Mathis BJ, Rodriguez-Perez JC, Allen PG, Beggs AH and Pollak MR. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet* 24: 251-256, 2000.

37. Kemeny E, Durmuller U, Nickeleit V, Gudat F and Mihatsch MJ. Distribution of podocyte protein (44 KD) in different types of glomerular diseases. *Virchows Arch* 431: 425-430, 1997.

38. Kestila M, Lenkkeri U, Mannikko M, Lamerdin J, McCready P, Putaala H, Ruotsalainen V, Morita T, Nissinen M, Herva R, Kashtan CE, Peltonen L, Holmberg C, Olsen A and Tryggvason K. Positionally cloned gene for a novel glomerular protein--nephrin--is mutated in congenital nephrotic syndrome. *Mol Cell* 1: 575-582, 1998.

39. Kim YG, Alpers CE, Brugarolas J, Johnson RJ, Couser WG and Shankland SJ. The cyclin kinase inhibitor p21CIP1/WAF1 limits glomerular epithelial cell proliferation in experimental glomerulonephritis. *Kidney Int* 55: 2349-2361, 1999.

40. Kim YH, Goyal M, Kurnit D, Wharram B, Wiggins J, Holzman L, Kershaw D and Wiggins R. Podocyte depletion and glomerulosclerosis have a direct relationship in the PAN-treated rat. *Kidney Int* 60: 957-968, 2001.

41. King WG, Mattaliano MD, Chan TO, Tsichlis PN and Brugge JS. Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. *Mol Cell Biol* 17: 4406-4418, 1997.

42. Koeppen BM and Stanton BA. *Renal physiology*. St. Louis: Mosby, 2001.

43. Korhonen M, Ylanne J, Laitinen L and Virtanen I. The alpha 1-alpha 6 subunits of integrins are characteristically expressed in distinct segments of developing and adult human nephron. *J Cell Biol* 111: 1245-1254, 1990.

44. Kornberg LJ, Earp HS, Turner CE, Prockop C and Juliano RL. Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of beta 1 integrins. *Proc Natl Acad Sci U S A* 88: 8392-8396, 1991.

45. Koyama H, Raines EW, Bornfeldt KE, Roberts JM and Ross R. Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors. *Cell* 87: 1069-1078, 1996.

46. Kreidberg JA, Donovan MJ, Goldstein SL, Rennke H, Shepherd K, Jones RC and Jaenisch R. Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis. *Development* 122: 3537-3547, 1996.

47. Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MS, Rakic P and Flavell RA. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* 94: 325-337, 1998.

48. Leinweber BD, Leavis PC, Grabarek Z, Wang CL and Morgan KG. Extracellular regulated kinase (ERK) interaction with actin and the calponin homology (CH) domain of actin-binding proteins. *Biochem J* 344 Pt 1: 117-123, 1999.

49. Lemmink HH, Mochizuki T, van den Heuvel LP, Schroder CH, Barrientos A, Monnens LA, van Oost BA, Brunner HG, Reeders ST and Smeets HJ. Mutations in the type IV collagen alpha 3 (COL4A3) gene in autosomal recessive Alport syndrome. *Hum Mol Genet* 3: 1269-1273, 1994.

50. Lin TH, Chen Q, Howe A and Juliano RL. Cell anchorage permits efficient signal transduction between ras and its downstream kinases. *J Biol Chem* 272: 8849-8852, 1997.

51. Ling K, Doughman RL, Firestone AJ, Bunce MW and Anderson RA. Type I gamma phosphatidylinositol phosphate kinase targets and regulates focal adhesions. *Nature* 420: 89-93, 2002.

52. **Mathis BJ, Kim SH, Calabrese K, Haas M, Seidman JG, Seidman CE and Pollak MR. A locus for inherited focal segmental glomerulosclerosis maps to chromosome 19q13. *Kidney Int* 53: 282-286, 1998.**

53. **Michaud JL, Lemieux LI, Dube M, Vanderhyden BC, Robertson SJ and Kennedy CR. Focal and segmental glomerulosclerosis in mice with podocyte-specific expression of mutant alpha-actinin-4. *J Am Soc Nephrol* 14: 1200-1211, 2003.**

54. **Miner JH. Renal basement membrane components. *Kidney Int* 56: 2016-2024, 1999.**

55. **Miyamoto S, Teramoto H, Coso OA, Gutkind JS, Burbelo PD, Akiyama SK and Yamada KM. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J Cell Biol* 131: 791-805, 1995.**

56. **Miyamoto S, Teramoto H, Gutkind JS and Yamada KM. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J Cell Biol* 135: 1633-1642, 1996.**

57. **Mundel P and Shankland SJ. Podocyte biology and response to injury. *J Am Soc Nephrol* 13: 3005-3015, 2002.**

58. **Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA and Yuan J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 403: 98-103, 2000.**

59. **Noakes PG, Miner JH, Gautam M, Cunningham JM, Sanes JR and Merlie JP. The renal glomerulus of mice lacking s-laminin/laminin beta 2: nephrosis**

despite molecular compensation by laminin beta 1. *Nat Genet* 10: 400-406, 1995.

60. Oktay M, Wary KK, Dans M, Birge RB and Giancotti FG. Integrin-mediated activation of focal adhesion kinase is required for signaling to Jun NH2-terminal kinase and progression through the G1 phase of the cell cycle. *J Cell Biol* 145: 1461-1469, 1999.

61. Pavenstadt H, Kriz W and Kretzler M. Cell biology of the glomerular podocyte. *Physiol Rev* 83: 253-307, 2003.

62. Polte TR and Hanks SK. Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. *Proc Natl Acad Sci U S A* 92: 10678-10682, 1995.

63. Pouyssegur J, Volmat V and Lenormand P. Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. *Biochem Pharmacol* 64: 755-763, 2002.

64. Reiske HR, Kao SC, Cary LA, Guan JL, Lai JF and Chen HC. Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase-promoted cell migration. *J Biol Chem* 274: 12361-12366, 1999.

65. Renshaw MW, Price LS and Schwartz MA. Focal adhesion kinase mediates the integrin signaling requirement for growth factor activation of MAP kinase. *J Cell Biol* 147: 611-618, 1999.

66. Renshaw MW, Ren XD and Schwartz MA. Growth factor activation of MAP kinase requires cell adhesion. *EMBO J* 16: 5592-5599, 1997.

67. Sastry SK and Burridge K. Focal adhesions: a nexus for intracellular signaling and cytoskeletal dynamics. *Exp Cell Res* 261: 25-36, 2000.

68. Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB and Parsons JT. pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc Natl Acad Sci U S A* 89: 5192-5196, 1992.

69. Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR and Parsons JT. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol* 14: 1680-1688, 1994.

70. Schlaepfer DD, Broome MA and Hunter T. Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. *Mol Cell Biol* 17: 1702-1713, 1997.

71. Schlaepfer DD, Hanks SK, Hunter T and van der GP. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* 372: 786-791, 1994.

72. Schlaepfer DD and Hunter T. Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. *J Biol Chem* 272: 13189-13195, 1997.

73. Shibasaki F, Fukami K, Fukui Y and Takenawa T. Phosphatidylinositol 3-kinase binds to alpha-actinin through the p85 subunit. *Biochem J* 302 (Pt 2): 551-557, 1994.

74. Shih NY, Li J, Karpitskii V, Nguyen A, Dustin ML, Kanagawa O, Miner JH and Shaw AS. Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* 286: 312-315, 1999.

75. Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Green DR and Martin SJ. Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol* 144: 281-292, 1999.

76. Srivastava T, Garola RE, Whiting JM and Alon US. Synaptopodin expression in idiopathic nephrotic syndrome of childhood. *Kidney Int* 59: 118-125, 2001.

77. Steffes MW, Schmidt D, McCrery R and Basgen JM. Glomerular cell number in normal subjects and in type 1 diabetic patients. *Kidney Int* 59: 2104-2113, 2001.

78. Tadokoro S, Shattil SJ, Eto K, Tai V, Liddington RC, de Pereda JM, Ginsberg MH and Calderwood DA. Talin binding to integrin beta tails: a final common step in integrin activation. *Science* 302: 103-106, 2003.

79. Togawa A, Miyoshi J, Ishizaki H, Tanaka M, Takakura A, Nishioka H, Yoshida H, Doi T, Mizoguchi A, Matsuura N, Niho Y, Nishimune Y, Nishikawa S and Takai Y. Progressive impairment of kidneys and reproductive organs in mice lacking Rho GDIalpha. *Oncogene* 18: 5373-5380, 1999.

80. Tremblay L, Hauck W, Nguyen LT, Allard P, Landry F, Chapdelaine A and Chevalier S. Regulation and activation of focal adhesion kinase and paxillin during the adhesion, proliferation, and differentiation of prostatic epithelial cells in vitro and in vivo. *Mol Endocrinol* 10: 1010-1020, 1996.

81. Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N, Beckmann JS, Mett IL, Rebrikov D, Brodianski VM, Kemper OC, Kollet O, Lapidot T, Soffer D, Sobe T, Avraham KB, Goncharov T, Holtmann H, Lonai P and Wallach D. Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity* 9: 267-276, 1998.

82. Wachsstock DH, Wilkins JA and Lin S. Specific interaction of vinculin with alpha-actinin. *Biochem Biophys Res Commun* 146: 554-560, 1987.

83. Wang R, Ferrell LD, Faouzi S, Maher JJ and Bishop JM. Activation of the Met receptor by cell attachment induces and sustains hepatocellular carcinomas in transgenic mice. *J Cell Biol* 153: 1023-1034, 2001.

84. Wary KK, Mariotti A, Zurzolo C and Giancotti FG. A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. *Cell* 94: 625-634, 1998.

85. Yin HL and Janmey PA. Phosphoinositide regulation of the actin cytoskeleton. *Annu Rev Physiol* 65: 761-789, 2003.

86. Zhang X, Chattopadhyay A, Ji QS, Owen JD, Ruest PJ, Carpenter G and Hanks SK. Focal adhesion kinase promotes phospholipase C-gamma1 activity. *Proc Natl Acad Sci U S A* 96: 9021-9026, 1999.

87. Zimmermann KC, Bonzon C and Green DR. The machinery of programmed cell death. *Pharmacol Ther* 92: 57-70, 2001.