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Characterization of Glomerular Epithelial Cell Matrix Receptors

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Integrin matrix receptors on glomerular epithelial cells (GEC) may play an important role in adhesion of GEC to the glomerular basement membrane (GBM) and in the maintenance of normal glomerular permeability. Therefore, the author determined the types of matrix receptors present on cultured rat GEC and examined their interactions with several components of the extracellular matrix. Beta$_1$ integrin matrix receptors were detected on all three glomerular cell types in rat kidney in vivo ***and at areas of cell–cell contact on cultured GEC. Glomerular epithelial cell adhesion to types I and IV collagen was slightly greater than to laminin and fibronectin. Adhesion to fibronectin was significantly inhibited by a synthetic peptide containing the RGD adhesion sequence. Immunoprecipitation of lysates of surface-iodinated GEC showed the presence of $\alpha_3\beta_1$ integrin. Chromatography of lysates on immobilized collagen showed $\alpha_3\beta_1$ integrin and a 70- to 75-kd protein band as the collagen receptors on GEC. Chromatography on the 120-kd cell-binding fragment of fibronectin disclosed only $\alpha_3\beta_1$ as a specific fibronectin receptor. Antibody to the β_1 integrin chain inhibited adhesion to laminin and collagen. These studies demonstrate that*** in vitro, ***as*** in vivo, ***GEC appear to express only $\alpha_3\beta_1$ integrin. Furthermore, this matrix receptor is capable of mediating GEC adhesion to collagen, fibronectin, and laminin, components of the GBM, and presumably plays a similar role in promoting GEC adhesion to GBM*** in vivo. ***(Am J Pathol 1992, 141:571–578)***

Several different families of cell receptors for components of the extracellular matrix (ECM) that play an important role in morphogenesis and regulation of cellular functions have been described.[1–4] One such family is the integrins, noncovalently linked heterodimeric protein complexes composed of α- and β-chains, which function as matrix receptors on a variety of cell types. Several classes of integrin receptors have been identified based on the different β-chains, each of which may associate with one of several α-chains, substrate specificity generally being associated with the specific α chain.[1–4] Several β_1 integrin receptors have been detected in the adult renal glomerulus, where they presumably play a role in interactions between glomerular cells and components of the mesangial matrix and glomerular basement membrane (GBM).[5–8]

$\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins, which bind laminin (LM) and collagen (COL), are found on glomerular capillary endothelium and in the mesangium.[6,7] $\alpha_3\beta_1$ integrin, which binds fibronectin (FN), LM, and COL, has been detected on glomerular mesangial, endothelial, and visceral and parietal epithelial cells, but is localized predominantly to the epithelial cell podocytes,[5–7] where it may function in attachment of epithelial cells to the GBM. $\alpha_5\beta_1$ integrin, the FN receptor, is absent or present weakly on glomerular endothelium and mesangium.[5–7] $\alpha_6\beta_1$ integrin, an LM receptor, was similarly detected only weakly on glomerular endothelium in one report[6] and was absent in the glomerulus in another.[7] Studies using an antibody to the β_1 integrin chain and immunoelectron microscopy have demonstrated an increased density of staining for the β_1 integrin on the surfaces of glomerular endothelial and epithelial cells adjacent to the GBM, suggesting a role of these receptors in adherence to the GBM.[8]

The ligands of the integrin receptors have been determined experimentally using isolated cells or receptors.[1,2] The role of a particular receptor on a cell in mediating adhesion to components of the ECM *in vivo,* however, cannot necessarily be inferred from its presence on the cell. For example, studies of cultured human mesangial cells demonstrated that antibody to the α_5 integrin chain, but not to the α_3 chain, blocked adhesion of mesangial cells to FN even though both $\alpha_3\beta_1$ and $\alpha_5\beta_1$ are present on these cells and can bind to FN *in vitro.*[5] Adhesion of glomerular epithelial cells (GEC) to the GBM may play an important role in maintenance of glomerular

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permeability, and detachment of GEC is seen in association with proteinuria in experimental models of glomerular injury (see Discussion). Therefore, we studied integrin expression by cultured rat GEC and their role in adhesion of GEC to components of the GBM.

Materials and Methods

Reagents

Media and additives for culture of rat glomerular epithelial cells were obtained from Gibco Laboratories (Grand Island, NY), and Sigma Chemical Co. (St. Louis, MO). Tissue culture plates and dishes were obtained from Becton Dickinson and Co. (Oxnard, CA). Types I (rat-tail) and IV (EHS tumor) collagen, laminin (EHS tumor), and fibronectin (human) were obtained from Collaborative Research Inc. (Bedford, MA). Glucose oxidase, lactoperoxidase, and protein-A Sepharose were obtained from Sigma. Phenylmethylsulfonyl fluoride (PMSF) and leupeptin were obtained from Calbiochem Corp. (La Jolla, CA). Triton X-100 was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). The adhesion peptide GRGDSP (gly-arg-gly-asp-ser-pro), the control GRGESP (gly-arg-gly-glu-ser-pro) and the 120-kd cell-binding chymotryptic fragment of fibronectin were obtained from Telios Pharmaceuticals (San Diego, CA). ^{125}-Iodine (carrier free) and thymidine, [methyl-^{3}H] were obtained from New England Nuclear (Boston, MA). Rabbit antisera reactive with the cytoplasmic domains of α_2, α_3, α_5, and β_1 integrin chains, which recognize integrins from several species[9,10] and monoclonal antibodies reactive with rat α_1 and α_4 chain were the gifts of Drs. E. Marcantonio, R. Hynes, T. Issekutz, D. Turner, and J. Edelman. Rabbit antiserum to the rat β_1 chain[11] was the gift of Dr. T. Borg.

Epithelial Cell Culture

Rat glomerular epithelial cell (GEC) cultures were established from the kidneys of male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) as previously described.[12] Cells were grown and passaged on type I collagen in a 50:50 mixture of K1 medium (a defined epithelial cell growth mixture) and conditioned medium from Swiss mouse 3T3 fibroblasts (Dulbecco's minimum essential medium [DMEM] with 10% heat-inactivated fetal calf serum).[13] Cultures exhibited the typical polygonal morphology described by others as well as sensitivity to puromycin aminonucleoside.[13] Immunofluorescent staining was negative with antibodies to factor VIII (Atlantic Antibodies, Scarborough, ME), rat common leukocyte Ag (Cooper Biomedical, Malvern, PA), and rat Thy-1 (a marker of mesangial cells). Staining was positive with anti-Fx1A antibody and with anti-keratin (ICN Immunobiologicals, Lisle, IL). Electron microscopy showed the presence of cilia and junctional complexes. Experiments were performed on cells in their 40th through 60th passages.

Studies of GEC Adhesion

Glomerular epithelial cells were labeled with ^{3}H-thymidine (0.5 μCi/ml) for 48 hours during log phase growth followed by a 24-hour washout period in K1-3T3 media. Cell suspensions were prepared by incubating cells in 0.5 mmol/l (millimolar) ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) at 37°C, washing in PBS, and resuspending in binding medium at 200,000 cells/ml. Multi-well tissue culture plates (96 well) were coated with varying amounts of fibronectin, laminin, and types I or IV collagen dissolved in DMEM (0.1 to 50 μg/ml) for at least 1 hour at 37°C as described previously[14] and then blocked with DMEM with 0.25% bovine serum albumin for 1 to 2 hours. The wells were then washed with PBS containing 1 mmol/l $MgCl_2$ and 1 mmol/l $CaCl_2$ followed by the addition of labelled cells in 100 μl of DMEM with 0.25% bovine serum albumin. Cells were allowed to adhere to the various substrates for 1 hour at 37°C followed by washing, addition of trypsin, and harvesting onto glass fiber filters using a cell harvester (Cambridge Technology, Inc., Cambridge, MA). For competition studies, wells were coated with substrate at 20 μg/ml and competing peptides (GRGDSP or GRGESP), antibody, or normal IgG were added to quadruplicate wells at the indicated concentrations simultaneously with the cells. Filters were counted in Optifluor in a liquid scintillation counter (Packard Instruments, Downers Grove, IL). Aliquots of known numbers of cells were also harvested from plates in an identical manner and demonstrated a linear relationship between cell number and cpm of ^{3}H.

Immunoprecipitation of GEC Integrins

Confluent cultures of GEC were surface iodinated with carrier-free ^{125}-I sodium iodide (60 μCi/ml) in PBS containing 20 mmol/l glucose, 20 μg/ml lactoperoxidase, and 200 mU/ml glucose oxidase for 30 minutes at room temperature. Cells then were lysed in 20 mmol/l TRIS-Cl, pH 7.4, with 1% Triton X-100, 10 μg/ml leupeptin, and 2 mmol/l PMSF (lysis buffer) with 110 mmol/l NaCl, 1 mmol/l $CaCl_2$, and 1 mmol/l $MgCl_2$, for 1 hour at 0 to 4°C. The lysate was precleared by incubation with protein A-Sepharose beads, followed by incubation with rabbit antise-

rum to α_3, α_5, and β_1 integrin chains (9–11) or normal rabbit serum. Immune complexes were harvested by incubation with protein A-Sepharose, washed with lysis buffer plus 0.5 mol/l NaCl, lysis buffer plus 0.1% sodium dodecyl sulfate (SDS), and PBS followed by boiling in SDS-PAGE (polyacrylamide gel electrophoresis) sample buffer with or without β-mercapto-ethanol as indicated. Samples were run on 7.5% SDS-PAGE gels followed by auto-radiography on Kodak XAR-5 film.

Affinity Chromatography of GEC on Immobilized Matrix

Type I collagen and the 120-kd chymotryptic cell adhesion fragment of fibronectin (Telios Pharmaceuticals) were coupled to CNBr-activated Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) using the manufacturer's protocol. Glomerular epithelial cells were surface iodinated as described above and lysed in lysis buffer containing 1 mmol/l $MnCl_2$. Lysates were applied to affinity columns in the same buffer and the flow-through collected. Collagen-Sepharose was washed with lysis buffer containing 1 mmol/l $MnCl_2$ and 0.1% Triton X-100 until counts in the eluate were back to baseline. Bound material was then eluted in lysis buffer with 10 mmol/l EDTA. Equal counts of eluted material, cell lysate, and the original column flow-through were pre-cleared, immunoprecipitated, and analyzed by SDS-PAGE and autoradiography as described above.

For chromatography on fibronectin-Sepharose, lysates of surface iodinated cells were prepared, applied to the column and washed as described above. The column was eluted with GRGESP (1 mg/ml in lysis buffer), washed with lysis buffer, and finally eluted with GRGDSP (1 mg/ml in lysis buffer).

Immunofluorescence Studies

Frozen sections of normal rat kidney were fixed in ether-alcohol and stained as previously described.[15] Glomerular epithelial cells were passaged onto type IV collagen (Collaborative Research) coated multi-well glass slides (Carlson Scientific, Inc., Peotone, IL) and grown for 24 to 48 hours before staining. Slides were fixed in fresh 4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were incubated for 45 minutes with rabbit antibody to the whole integrin β_1 chain or appropriate controls, washed, incubated with fluoresceinated goat anti-rabbit IgG antibody (Cappel, Malvern, PA) for 30 minutes, washed, mounted, and examined on a Nikon microscope equipped for epifluorescence.

Statistical Analysis

All results are expressed as mean ± standard error of the mean. Comparisons of the means of groups were made using Student's *t*-test or one-way analysis of variance with post-hoc testing using the Tukey-Kramer test as appropriate, employing Systat statistical software (Systat Inc., Evanston, IL). P values < 0.05 were regarded as significant.

Results

Immunofluorescence Studies

Staining of normal rat kidney with antibody to the intact rat β_1 integrin chain[11] showed a diffuse distribution of integrin receptors in the kidney (Figure 1A), similar to the pattern seen in normal human kidney. In the glomerulus, staining was most prominent in the mesangium, but definite staining was also present along the capillary wall and a double line of staining along the GBM was evident in some areas, indicating the presence of receptors on both glomerular endothelial and epithelial cells. Staining for integrins was also present along tubular basement membranes and vessel walls.

Cultured GEC stained with the same antibody displayed predominantly membrane staining with accentuation along areas of cell–cell contact (Figure 1B). Permeabilization of cells with 0.1% Triton X-100 before staining did not alter this pattern of reactivity (not shown).

GEC Adhesion to Substrates

When tissue culture dishes were coated with a variety of ECM materials, there was a dose-dependent increase in the adhesion of labeled GEC to the various substrates used in comparison to adhesion to untreated wells (Figure 2). Types I and IV collagen resulted in slightly greater adhesion than laminin and fibronectin. The potential importance of binding of cells to RGD sites in the substrate was evaluated by performing adhesion studies in the presence of the synthetic adhesion peptide GRGDSP, which can competitively inhibit binding to the RGD sequence, or the control peptide GRGESP. Adhesion to fibronectin, which contains an RGD sequence, was significantly inhibited by GRGDSP but not the control peptide (Figure 3). Adhesion to the other substrates studied was unaffected by either peptide.

Characterization of GEC Matrix Receptors

Immunoprecipitation of lysates of surface-iodinated GEC with anti-β_1 antibody demonstrated the presence of two

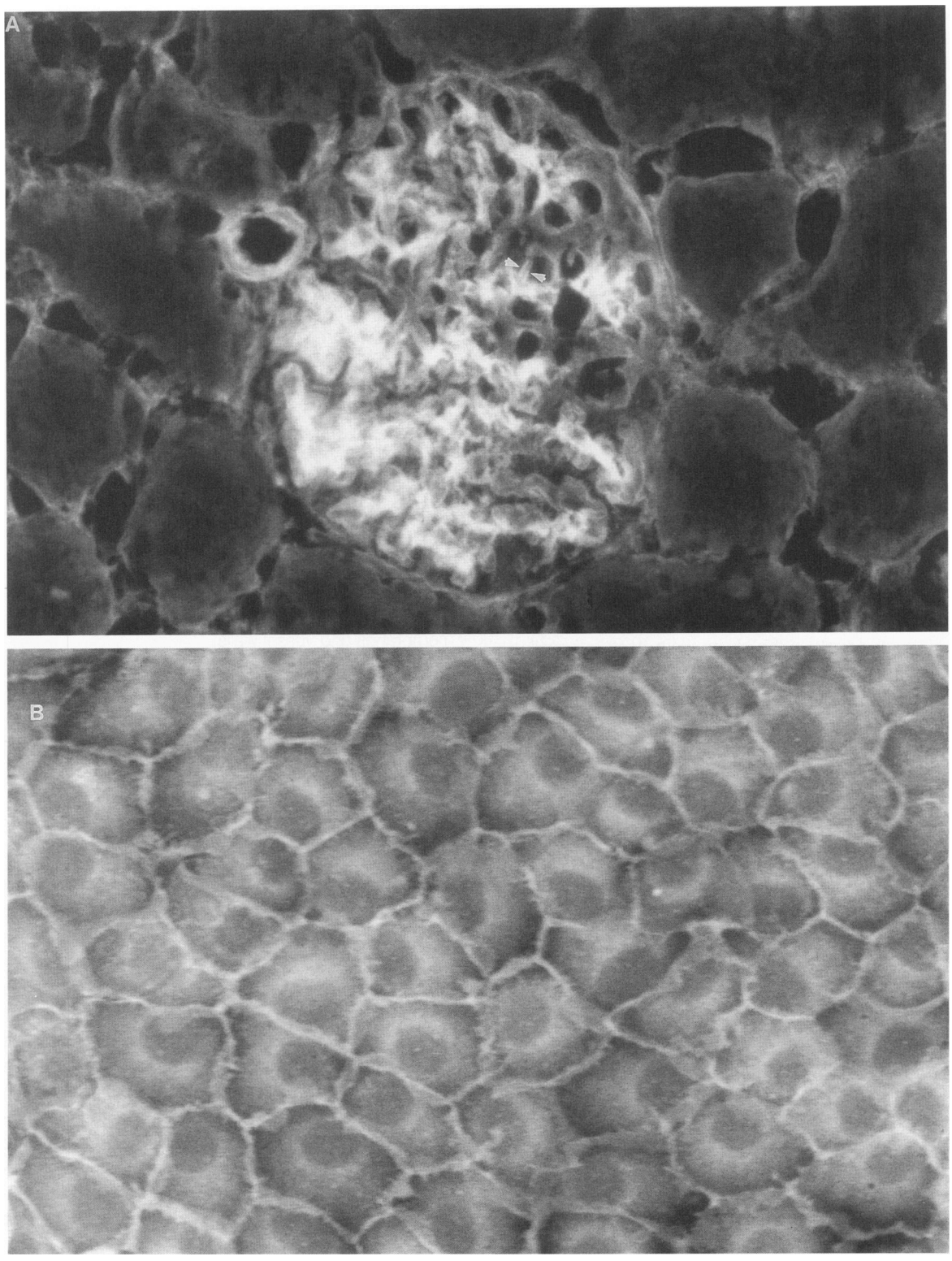

Figure 1. *Normal rat kidney* **(A)** *and cultured GEC* **(B)** *stained with anti-*β_1 *integrin antibody. In the glomerulus, staining is prominent in the mesangium but is also evident on the glomerular capillary wall. Arrowheads indicate an area of staining of the inner and outer aspects of the GBM. In culture, staining is most prominent along the cell membrane at points of cell-cell contact (Original magnification, ×400).*

bands of radioactivity after analysis by SDS-PAGE under nonreducing conditions and autoradiography (Figure 4, lane 4) with approximate mobilities of 135 and 115 kd representing the β_1 integrin chain (115 kd) and an associated α chain. If lysates were exposed to dissociating conditions (0.1% SDS, no Ca^{++} or Mg^{++}), only the β_1

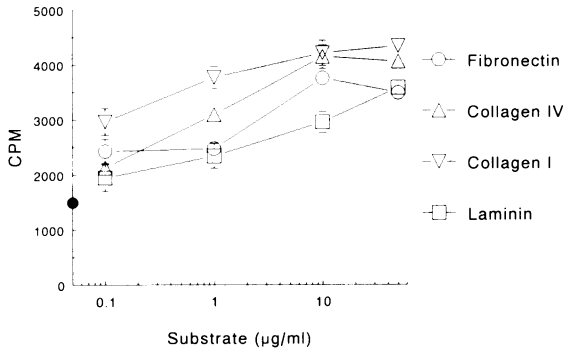

Figure 2. *Adhesion of GEC to substrates. Cells labeled with ^{3}H-thymidine were allowed to adhere to albumin-coated tissue culture plates or to wells coated with the indicated concentrations of substrate. The symbol on the ordinate represents binding to albumin treated wells. Each bar represents the mean ± SE of substrates evaluated in triplicate in two separate experiments.*

chain was immunoprecipitated (not shown), confirming the noncovalent linking of the α and β_1 chains. The identity of the associated α chain was confirmed as α_3 by immunoprecipitation with anti-α_3 antibody (Figure 4, lane 2). Immunoprecipitation with anti-α_5 antibody demonstrated the absence of α_5 chains (Figure 4, lane 3). Immunoprecipitation with anti-α_1, -α_2 and -α_4 was also negative (not shown). When anti-β_1 immunoprecipitates of GEC were analyzed under reducing conditions (Figure 5, right lane), the characteristic shift of mobility of the α_3 and β_1 chains was demonstrated, further confirming the identity of the GEC integrin receptor as $\alpha_3\beta_1$.

Analysis of the matrix-binding surface proteins of GEC was next performed by passage of lysates of surface-iodinated cells over columns of Collagen I or the 120-kd chymotryptic fragment of fibronectin immobilized on Sepharose. When surface-iodinated GEC proteins that had bound to collagen-Sepharose were eluted with buffer containing 10 mmol/l EDTA, proteins with mobilities similar to α_3 and β_1 integrins were detected as well as a

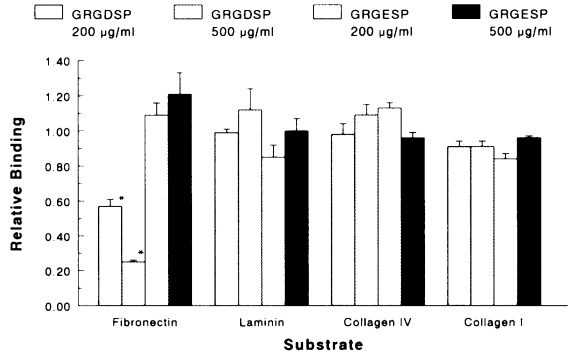

Figure 3. *Effect of adhesion peptides on GEC adhesion to substrates. GEC labeled as in Figure 2 were allowed to adhere to substrates (20 μg/ml) for 1 hour in the presence of the synthetic adhesion peptide GRGDSP or the control peptide GRGESP. Relative binding of cells to the indicated substrates was compared in the presence or absence of peptides. Each bar represents the mean ± SE of quadruplicate determinations in two experiments (*P < 0.05 vs. control).*

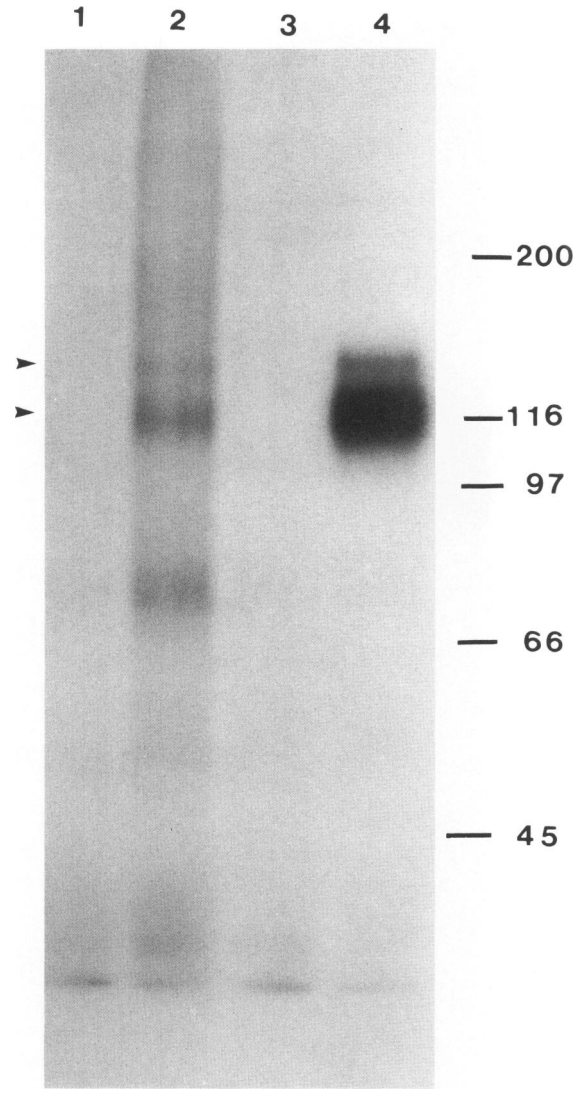

Figure 4. *Analysis of GEC integrins. Lysates of surface iodinated cells were immunoprecipitated with control antibody (lane 1), or antibodies to integrin α_3 chain (lane 2), integrin α_5 chain (lane 3) and integrin β_1 chain (lane 4). Lanes 2 and 4 demonstrate the presence of $\alpha_3\beta_1$ integrin. No α_5 chains could be demonstrated. Arrowheads indicate the positions of the α_3 (upper) and the β_1 (lower) integrin chains (7.5% SDS-PAGE; unreduced).*

broad band at approximately 70 to 75 kd, the identity of which is not clear (Figure 6, lane 1). Immunoprecipitation of equal counts of whole cell lysate, column flow-through (data not shown) and eluted proteins (Figure 6, lane 2) with anti-β_1 demonstrated significant enrichment of $\alpha_3\beta_1$ integrins in the material that bound to the column. The identity of the coprecipitating α chain as α_3 was confirmed by immunoprecipitation with anti-α_3 antibody (data not shown). This confirms the importance of the $\alpha_3\beta_1$ integrin on GEC in cell binding to collagen.

Chromatography of GEC lysates on the cell-binding fragment of fibronectin showed no material eluting non-specifically with the GRGESP control peptide (Figure 7, lane 1). The GRGDSP peptide, however, which com-

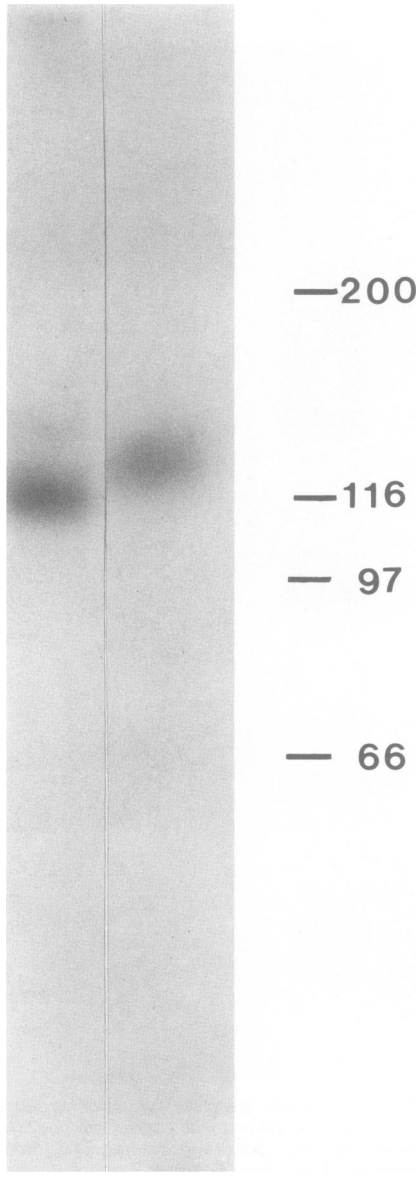

Figure 5. *Effect of reduction on GEC integrin. Lysates of surface iodinated cells immunoprecipitated with anti-β_1 antibody were run unreduced (left lane) or reduced (right lane). Shifts in mobility typical of the α_3 and β_1 chains are demonstrated.*

petes with the RGD site on the fibronectin molecule, resulted in elution of the $\alpha_3\beta_1$ chains, demonstrating that this integrin on the GEC surface can also mediate binding to fibronectin.

Effect of Anti-β_1 Antibody on GEC Binding to Substrate

Because $\alpha_3\beta_1$ integrin appears to be the only integrin expressed by GEC, the effect of anti-β_1 antibody on binding to substrates should reflect the participation of this integrin in GEC adhesion. When GEC plus anti-β_1 or normal IgG were co-incubated, adhesion of GEC to laminin and types I and IV collagen were significantly inhibited (Figure 8), demonstrating that the $\alpha_3\beta_1$ integrin of GEC plays a role in adhesion of GEC to these substrates. No effect on GEC adhesion to fibronectin was evident (see Discussion). Because the anti-α_3 antibody reacts with the cytoplasmic portion of the α_3 chain, we could not use it to specifically demonstrate the role of the α_3 chain in cell adhesion.

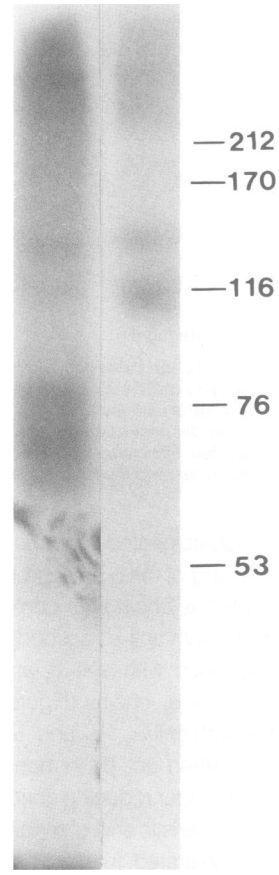

Figure 6. *Collagen-Sepharose chromatography of GEC lysates. Surface iodinated cell lysates were applied to a column of Collagen I-Sepharose and eluted with EDTA. Eluted proteins (lane 1) demonstrated several bands capable of binding to collagen. Immunoprecipitation of eluted proteins with anti-β_1 (lane 2) confirmed the enrichment of $\alpha_3\beta_1$ integrins in the collagen binding material.*

Discussion

The results presented here demonstrate that cultured GEC adhere to several components of the GBM, including types I and IV COL, LM, and FN, and express an $\alpha_3\beta_1$ integrin receptor on their surface that has been shown *in vitro* to be capable of binding to these substrates. The involvement of the GEC $\alpha_3\beta_1$ receptor in actually mediating adhesion is further supported by the ability of an RGD containing peptide and anti-β_1 antibody to inhibit adhesion to several substrates and by the isolation of

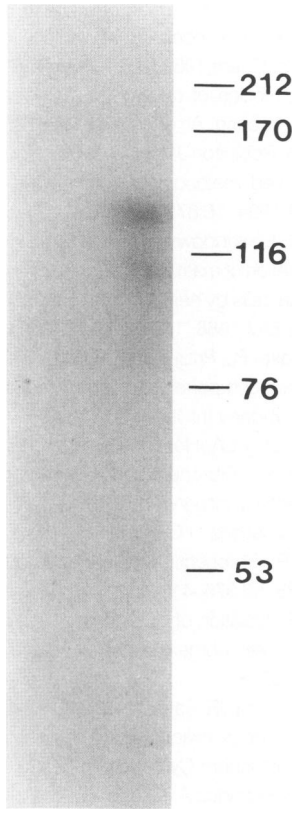

Figure 7. *Fibronectin–Sepharose chromatography of GEC lysates. Lysates were prepared as described earlier, applied to the column and bound material eluted with the control GRGESP peptide (lane 1) or with GRGDSP (lane 2).*

GEC $\alpha_3\beta_1$ receptors on columns of immobilized FN and COL.

Many proteins present in the ECM contain the tripeptide sequence RGD as their cell recognition site, including FN, vitronectin, osteopontin, collagens, and thrombospondin.[3] Until recently it was thought that of the β_1 integrins only $\alpha_5\beta_1$ and $\alpha_v\beta_1$ adhered to FN via this sequence.[1] Many of these studies, however, involved cells containing both $\alpha_5\beta_1$ and $\alpha_3\beta_1$ integrins. By removing the $\alpha_5\beta_1$ integrin or using another cell line that expresses mainly $\alpha_3\beta_1$, Elices et al[16] were able to demonstrate that $\alpha_3\beta_1$ can also bind to the RGD site on FN as well as being able to bind to non-RGD sites on LM and COL. Our data support these findings in that GEC do not express $\alpha_5\beta_1$ and their adhesion to FN but not COL or LM could be inhibited by RGD peptides. The high degree of inhibition of binding to FN (~80%) with 500 μg/ml GRGDSP and the presence of only $\alpha_3\beta_1$ in the eluate of the FN column further suggests that the $\alpha_3\beta_1$ receptor is the sole or most important GEC receptor for FN. Although anti-β_1 antibody did not interfere with GEC binding to FN, it is possible that this antibody preparation does not recognize the site that binds to the RGD sequence in FN. This binding site seems to be different from the site that mediates binding to other substrates.[16] We cannot rule out the presence of some $\alpha_v\beta_1$ integrins on GEC because of a lack of a specific antibody that recognizes rat α_v and the fact that α_3 and α_v chains show similar mobility in SDS-PAGE gels and changes in mobility on reduction.[4] However, antibodies to the α_3 or β_1 chains produce a similar ratio of α_3 to β_1 bands on immunoprecipitation (data not shown) suggesting that another α chain is not present. Another potential FN receptor on GEC is heparan sulfate proteoglycan, although we did not detect it in the FN column eluate.[17]

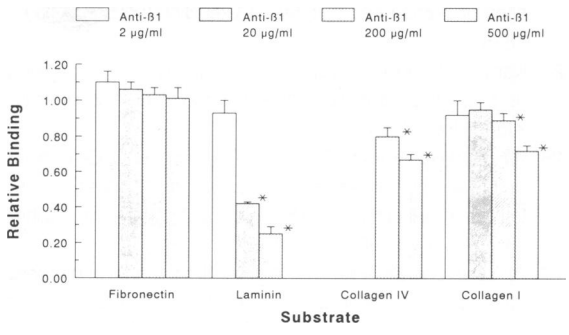

Figure 8. *Effect of anti-β_1 antibody on GEC adhesion. GEC prepared as in Figure 2 were allowed to adhere to substrates (20 μg/ml) for 1 hour in the presence of the indicated concentrations of anti-β_1 or normal IgG. Relative binding of cells was compared in the presence of antibody or normal IgG. Each bar represents the mean ± SE of four to eight measurements (*P < 0.05 vs. control).*

Chromatography of surface-iodinated GEC lysates on COL-Sepharose also showed binding of $\alpha_3\beta_1$ integrin to COL, demonstrating that the $\alpha_3\beta_1$ receptor on GEC is capable of mediating cell adhesion to COL. Anti-β_1 antibody also inhibited adhesion of GEC to types I and IV COL, albeit only up to about 30%, suggesting the presence of other proteins that might act as COL receptors, such as the COL-binding proteins with molecular weights of ~70 to 75 kd, which we detected. COL-binding proteins of similar molecular weight have been detected by others on rat heart fibroblasts and chick cornea,[11,18] but have not been further characterized.

The evidence presented here, along with previous studies, strongly suggests that $\alpha_3\beta_1$ integrin plays a major role in GEC adhesion to the GBM. *In vivo*, $\alpha_3\beta_1$ has been the only integrin identified on GEC podocytes, and it is concentrated on the portion of the podocyte adjacent to the GBM.[5–7] In culture, it is also the only β_1 integrin expressed on GEC, and it binds to FN and COL in a manner dependent on the RGD sequence of FN and on the presence of a divalent cation (Mn^{++}) for COL binding. *In vivo*, the GBM contains COL concentrated in the lamina densa and FN and LM concentrated in the laminae rarae,[19,20] although some studies have questioned the presence of FN in the GBM (reviewed in reference 21). $\alpha_3\beta_1$ on GEC also appears to play an important role in binding to LM as evidenced by approximately 80% inhibition of GEC binding to LM by anti-β_1 antibody and the absence of other LM receptors such as $\alpha_6\beta_1$ and the

non-integrin 67-kd LM receptor in the normal glomerulus.[7] A role for $\alpha_3\beta_1$ in cell–cell adhesion, particularly in epidermal keratinocytes, has been proposed[16,22] and $\alpha_3\beta_1$ is localized primarily to areas of cell–cell contact of keratinocytes *in vivo* and *in vitro*.[22–24] Similarly, immunofluorescence staining of cultured GEC with anti-β_1 antibody demonstrated predominant localization at areas of cell–cell contact, raising the possibility that $\alpha_3\beta_1$ might function similarly in the glomerulus.

Detachment of GEC from the GBM has been demonstrated as a pathologic correlate of the development of proteinuria in several models of experimental glomerular injury, including adriamycin and puromycin nephrosis, protein overload proteinuria, and infusion of neuraminidase.[25–28] Possible alterations of GEC integrins in such lesions have not been explored. The role of GEC matrix receptors in maintaining normal glomerular capillary wall permeability and their involvement in proteinuric lesions remains to be defined.

Acknowledgments

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