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Extracellular Matrix Is Required for MAP Kinase Activation and Proliferation of Rat Glomerular Epithelial Cells

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This study examined the role of extracellular matrix (ECM) in the regulation of glomerular epithelial cell (GEC) proliferation. Epidermal growth factor (EGF) stimulated proliferation of GEC when the cells were adherent to collagen matrices, but not plastic substratum. Significant and prolonged EGF receptor (R) tyrosine autophosphorylation (which reflects EGF-R kinase activation) was induced by EGF only in GEC adherent to collagen. In addition, EGF stimulated the activity and tyrosine phosphorylation of p42 mitogen-activated protein (MAP) kinase (ERK2) in collagen-adherent GEC, but not in cells on plastic. An inhibitor of the p42 MAP kinase pathway, PD98059, blocked EGF-induced MAP kinase activity and proliferation. Thus, adhesion to ECM enables EGF to induce proliferation of GEC, by facilitating activation of EGF-R and the p42 MAP kinase pathway. Signals from ECM to growth factor receptor tyrosine kinases may regulate cell turnover in the glomerulus under normal conditions and during immune glomerular injury. © 1997 Academic Press

Proliferative responses of cells to polypeptide growth factors and differentiation can be modulated by adhesion of cells to extracellular matrix (ECM) (1,2). We and others have studied intracellular signalling mechanisms that are activated by adhesion of cells to ECM (3-7), as well as interactions of ECM with growth factors (8). Epidermal growth factor (EGF), transforming growth factor-α, and heparin-binding EGF are structurally and functionally related polypeptide growth factors, which are mitogenic for a number of cell types, in particular, epithelial cells (9,10). These growth factors bind to a 170kDa cell surface receptor (EGF-R), which possesses intrinsic tyrosine kinase activity (10,11). It is believed that the initial events, which ultimately lead to cell proliferation, involve binding of growth factor to EGF-R, followed by receptor oligomerization (10,11). This results in transmembrane activation of the cytoplasmic tyrosine kinase, and is associated with tyrosine phosphorylation of EGF-R itself ("autophosphorylation"), and phosphorylation of other substrate proteins (4-6). The signal is then transmitted to nuclear or cytoplasmic effectors through a series of serine/threonine protein kinases, collectively known as the mitogen-activated protein (MAP) kinase pathway (12-14). Briefly, receptor tyrosine kinases, including EGF-R, usually activate Ras via Grb-2/Sos. Ras induces translocation of Raf-1 to the plasma membrane, where Raf-1 is activated by an undefined kinase. Raf-1, in turn, activates MEK [MAP or extracellular signal-regulated kinase (ERK) kinase], which then activates p42 (ERK2) and/or p44 (ERK1) MAP kinases via dual phosphorylation on threonine and tyrosine. The ERKs have multiple potential actions, including the triggering of gene expression required to induce cell proliferation.

Visceral and parietal glomerular epithelial cells (GEC) are intrinsic components of the kidney glomerulus, and both cell types are in contact with ECM. Normally, the turnover of GEC is low, but proliferation of parietal and possibly visceral GEC, and expansion of the ECM may occur in immune glomerular injury, and may lead to impaired glomerular function and/or permselectivity (15,16). For example, urines from children with Henoch-Schönlein purpura nephritis (a nephritis often associated with glomerular proliferation) contain a factor that resembles transforming growth factor-α, suggesting that the presence of this factor in the glomerulus may be stimulating epithelial proliferation (17). In previous studies, we have demonstrated that adhesion to ECM triggers signals that can regulate proliferation of cultured rat GEC in a positive and negative fashion. Turnover of inositol lipids was associated with a reduction in GEC proliferation (3,4). In contrast, ECM facilitated proliferation and enhanced EGF-de-

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Abbreviations: ECM, extracellular matrix; EGF-R, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GEC, glomerular epithelial cell; MAP kinase, mitogen-activated protein kinase; MEK, MAP or ERK kinase; PMA, phorbol myristate acetate.

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pendent activation of EGF-R (5). Thus, EGF stimulated EGF-R autophosphorylation and proliferation in GEC adherent to collagen matrices but not to plastic substratum. The differences in autophosphorylation between substrata could not be accounted for by differences in ligand binding, EGF-R protein content or EGF-R degradation, and appeared to be due to regulation of EGF-R kinase activity and trafficking by factors extrinsic to the receptor. Moreover, EGF-R was not intrinsically defective in plastic-adherent GEC, since the immunopurified receptor from these cells could be autophosphorylated in vitro. Most likely, adhesion of GEC to ECM enhanced EGF-R activation by reducing endocytosis and dephosphorylation of the receptor by a phosphotyrosine phosphatase (5).

The aim of the present study was to determine if the enhanced autophosphorylation of EGF-R, which occurs in GEC adherent to collagen, is actually paralleled by increased activation of pathways that may transmit the mitogenic signal to the nucleus, and stimulate proliferation. We demonstrate that EGF stimulated the activity and tyrosine phosphorylation of p42 MAP kinase (ERK2) in GEC on collagen, but not on plastic, and that activation of MAP kinase was necessary for EGF to induce proliferation.

METHODS

Materials. Tissue culture media were obtained from Gibco Laboratories, Burlington, Ontario. Pepsin-solubilized bovine dermal collagen (Vitrogen) was from Collagen Corporation, Palo Alto, California. NuSerum, and EGF were purchased from Collaborative Research, Bedford, Massachusetts. Insulin, and hormone supplements were obtained from Sigma Chemical Co., St. Louis, Missouri. Anti-phospho tyrosine monoclonal antibody, PY20, was from Transduction Laboratories, Lexington, Kentucky. Mouse anti-ERK1/2 and rabbit anti-ERK2 antibodies were from Transduction Laboratories and Santa Cruz Biotechnology, Santa Cruz, California, respectively. MAP kinase assay kit was purchased from Upstate Biotechnology, Lake Placid, New York. [γ-^{32}P]ATP (3000Ci/mmol) was from New England Nuclear, Boston, Massachusetts. Electrophoresis and immunoblotting reagents were from Biorad Laboratories, Mississauga, Ontario. Rabbit anti-EGF-R antiserum, RK-2 (5), was kindly provided by Drs. B. Margolis and J. Schlessinger, New York University, New York, New York. The MEK inhibitor, PD98059 (18), was kindly provided by Dr. A. Saltiel, Parke-Davis Pharmaceutical Research, Ann Arbor, Michigan.

Extracellular matrix and GEC culture. Type I collagen gel matrices were prepared by combining RPMI-1640 (10×) medium, 7.5% $NaHCO_3$, pepsin-solubilized bovine dermal collagen (~3mg/ml in 0.012N HCl), and 0.1N NaOH, in proportions of 10:4:80:10 at 4°C. The mixture was then poured into tissue culture dishes (~0.06ml/cm^2), and allowed to gel at 37°C, as described previously (5).

Primary cultures of rat GEC were established from explants of rat glomeruli, as described previously (3-5). Studies were done with cells between passages 25 and 70. According to established criteria, the cells demonstrated polygonal shape and cobblestone appearance at confluency, cytotoxic susceptibility to low doses of aminonucleoside of puromycin, positive immunofluorescence staining for cytokeratin, and presence of junctional complexes by electron microscopy. Presently, it is not possible to determine specifically whether GEC in culture originate from visceral or parietal epithelium. Under standard conditions, GEC were cultured on collagen matrices in K1 medium, which consisted of DMEM/Ham F10 (1:1), containing 5% Nu-Serum and hormone supplements (5). For most experiments, GEC were preincubated in serum-poor medium (DMEM/Ham F10, 1:1, with 0.5% fetal calf serum). To remove GEC from collagen substrata, collagen gels with adherent cells were scraped from culture dishes into a test tube, and were incubated with collagenase and trypsin-EDTA to produce a cell suspension (5). For passaging of cultures, GEC were replated onto collagen gels; for experiments, GEC were replated onto collagen gels or plastic.

Immunoprecipitation and immunoblotting. For immunoprecipitation with rabbit anti-EGF-R antiserum, GEC were scraped from culture dishes and were solubilized in immunoprecipitation buffer containing 1.0% Triton X-100, 125mM NaCl, 20mM Tris, 20μM leupeptin, 20μM pepstatin, 0.2mM PMSF, 25mM NaF, 2mM Na_3VO_4, 5mM $Na_4P_2O_7$, 1mM EDTA, 1mM EGTA, pH 7.40 (4°C). For immunoprecipitation with mouse anti-ERK1/2, GEC were scraped from culture dishes and were first boiled for 5min in buffer containing 1.0% SDS, 10mM Tris, 2mM Na_3VO_4, pH 7.40. The lysate was then diluted 10-fold in immunoprecipitation buffer. Proteins were immunoprecipitated with primary antibody or non-immune IgG in controls (2h, 4°C), followed by absorption with agarose-coupled protein A or anti-mouse IgG (2h, 4°C). The immunoprecipitates were then boiled in Laemmli buffer and were subjected to SDS-PAGE under reducing conditions prior to immunoblotting. In some experiments, membrane fractions of GEC were prepared for immunoblotting (without prior immunoprecipitation), as described previously (5).

For immunoblotting, proteins were transfered onto nitrocellulose paper, blocked with 3% BSA/2% ovalbumin, and incubated with primary antibody, followed by alkaline phosphatase-conjugated secondary antibody, as described previously (5). Alkaline phosphatase activity was detected with bromochloroindolyl phosphate-nitro blue tetrazolium. In some experiments, the amount of tyrosine phosphorylation was quantitated by densitometry (LKB Ultroscan XL Laser Densitometer). In preliminary studies, it was verified that there was a linear correlation between densitometric measurements and the amounts of phosphoprotein loaded onto gels.

Assay of ERK2 activity. GEC were scraped from culture dishes and were solubilized in buffer containing 0.5% Triton X-100, 50mM β-glycerol phosphate, 2mM $MgCl_2$, 1mM DTT, 20μM leupeptin, 20μM pepstatin, 0.2mM PMSF, 1mM Na_3VO_4, 1mM EGTA, pH 7.20 (4°C). Proteins were immunoprecipitated with rabbit anti-ERK2 antibody (1h, 4°C), followed by absorption with agarose-coupled protein A (1h, 4°C). The immunoprecipitates were then assayed for ERK2 activity using a kit, according to the manufacturer's instructions. Briefly, the assay measures phosphorylation of myelin basic protein. In addition to the immunoprecipitates, the assay mixture contained 20mM MOPS, pH 7.2, 25mM β-glycerol phosphate, 5mM EGTA, 1mM Na_3VO_4, 1mM DTT, 0.5mg/ml bovine brain myelin basic protein, 7.5mM $MgCl_2$, 50μM [γ-^{32}P]ATP (10μCi), and peptide inhibitors of cAMP-dependent protein kinase, calmodulin dependent protein kinase and protein kinase C. After a 10min incubation at 30°C, the mixture was spotted onto phosphocellulose paper. The paper was washed with 0.75% phosphoric acid and acetone, and bound radioactivity was quantitated in a β-scintillation counter.

Measurement of GEC proliferation. Cell number was determined by visual counting. Cells on collagen gels were placed into single-cell suspension with collagenase and trypsin-EDTA, as described above. Cells on plastic substratum were placed into suspension by incubation with trypsin-EDTA. Suspended cells were then counted in a hemacytometer.

Statistics. Data are presented as mean±SEM. One-way analysis of variance (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

Effect of ECM on EGF-R Autophosphorylation

Previously, we reported that in the presence of EGF, GEC proliferate when adherent to collagen I or IV matrices, but not to laminin or plastic substrata, even though cell adhesion and spreading is not impaired in GEC on plastic or laminin, as compared with collagen (3,5). Proliferation of GEC adherent to collagen was associated with EGF-induced tyrosine autophosphorylation of EGF-R (5). In this study, we demonstrate that in serum-deprived GEC, autophosphorylation of EGF-R was evident after 1h of stimulation with EGF, and was sustained for at least 7h (Fig. 1A). In keeping with previous results (5), EGF substantially stimulated autophosphorylation of EGF-R only in GEC adherent to collagen, but not in cells on plastic substratum (Fig. 2A). This effect could not be accounted for by differences in EGF-R protein content between substrata (Fig. 2B), nor by differences in ^{125}I-EGF binding (shown previously; ref. 5). Furthermore, we reported previously that EGF-R in GEC on plastic was not intrinsically defective, as this receptor was capable of undergoing in vitro autophosphorylation (5).

Effect of ECM on MAP Kinase

The results so far suggested that the mechanism by which ECM facilitated GEC proliferation involved enhancement of EGF-R activation. However, in order to substantiate this hypothesis, it would be necessary to show that the effect of collagen on activation of EGF-R is associated with enhanced "downstream" signalling by this receptor. Thus, we monitored the effect of ECM on EGF-dependent tyrosine phosphorylation and activation of MAP kinase. Similar to EGF-R autophosphorylation, in GEC adherent to collagen, EGF stimulated tyrosine phosphorylation of the p42 MAP kinase (ERK2), and the time course of this phosphorylation paralleled that of EGF-R (Fig. 1B). There also appeared to be some increase in tyrosine phosphorylation of p44 MAP kinase (ERK1), but the amount of ERK1 in GEC was relatively minor as compared with ERK2, and ERK1 was not detected consistently. By analogy to EGF-R autophosphorylation, the EGF-dependent increase in tyrosine phosphorylation of ERK2 was evident only in GEC adherent to collagen, but not in cells on plastic substratum (Fig. 3A). Quantitation of ERK2 phosphotyrosine content was obtained using densitometry, and is based on four experiments similar to the representative immunoblot shown in Fig. 3A. In GEC on collagen, basal phosphotyrosine content of ERK2 was 29±5 units, and EGF increased tyrosine phosphorylation of ERK2 to 64±7 units ($p<0.0002$). In GEC on plastic, basal phosphotyrosine content of ERK2 was 4±2 units, and there was no detectable change with

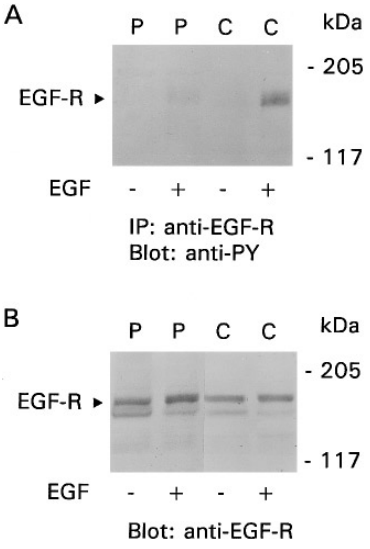

FIG. 2. Effect of ECM on EGF-dependent autophosphorylation of EGF-R. GEC adherent to plastic (P) or collagen (C) were cultured in serum-poor medium for 18h, and were then incubated with (+) or without (−) EGF (100ng/ml) for 1h at 37°C. Cell lysates were immunoprecipitated with anti-EGF-R antibody, and the immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody (panel A). Membrane fractions were immunoblotted with anti-EGF-R antibody (panel B). EGF-dependent phosphorylation of EGF-R (panel A) was substantially more pronounced in GEC on collagen. EGF-R protein content was similar in GEC on collagen and plastic (panel B).

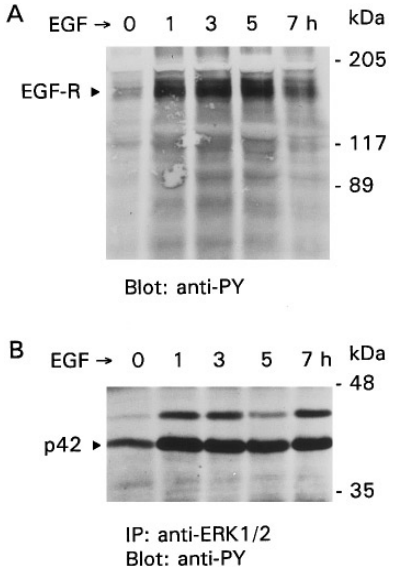

FIG. 1. EGF-dependent tyrosine phosphorylation of EGF-R (panel A) and ERK2 (panel B) in GEC adherent to collagen. GEC, cultured in serum-poor medium for 18h, were stimulated with EGF (100 ng/ml; 37°C) for 1-7h, or were unstimulated (0). Membrane fractions were immunoblotted with anti-phosphotyrosine (PY) antibody (panel A), or cell lysates were immunoprecipitated (IP) with anti-ERK1/2 antibody and the immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody (panel B). Tyrosine phosphorylation of EGF-R and ERK2 (p42) was evident at 1h, and persisted until 7h.

EGF. Although the basal levels of ERK2 tyrosine phosphorylation appeared to be greater in collagen-adherent GEC, as compared with plastic, this apparent difference was at least in part due to non-specific immunoadsorption of a 42kDa protein in collagen-adherent GEC (Fig. 3B and 3C), and was not reflected by significant changes in basal ERK2 activity (see below). Thus, adhesion of cells to ECM in the absence of EGF probably did not phosphorylate ERK2 substantially. Immuno blotting showed that there were no significant differences in the amounts of ERK2 protein between substrata (Fig. 3D).

The increased ERK2 tyrosine phosphorylation induced by EGF in GEC on collagen was paralleled by an increase in ERK2 activity. The activity was measured by immunoprecipitating cell lysates with anti-ERK2 antibody and monitoring phosphorylation of myelin basic protein by the immune precipitates, in the presence of inhibitors of other protein kinases (Methods). EGF increased ERK2 activity two-fold in GEC on collagen, but there was no detectable increase in GEC on plastic (Fig. 4A). Basal ERK2 activity tended to be greater in GEC on collagen, as compared with plastic, but the difference did not reach statistical significance. We also tested if inhibition of MEK (the kinase just "upstream" of ERK2) could block the EGF-stimulated ERK2 activity. In the presence of the MEK inhibitor, PD98059 (18), the stimulated ERK2 activity was reduced by 80±10% (Fig. 4B).

To determine if the effect of collagen on EKR2 activation was a general versus more restricted occurrence, we monitored changes in ERK2 activity in response to phorbol myristate acetate (PMA), an exogenous activator of protein kinase C. Unlike EGF, PMA (250ng/ml) effectively stimulated ERK2 activity in both GEC adherent to collagen (2.3±0.3 fold-increase above basal) and plastic (2.1±0.6 fold-increase above basal; 4 experiments). Thus, biochemical processes are preserved in GEC adherent to plastic, even though plastic does not allow proliferation.

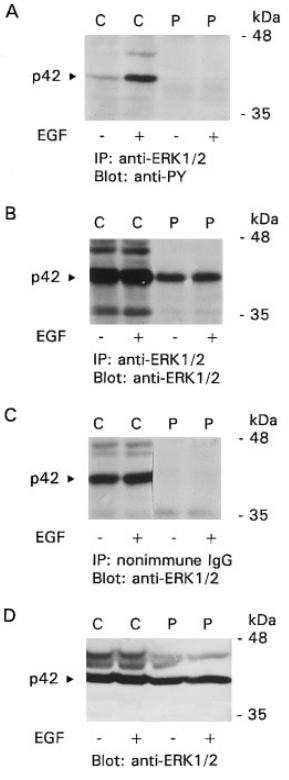

FIG. 3. Effect of ECM on EGF-dependent tyrosine phosphorylation of ERK2 (p42). GEC adherent to collagen (C) or plastic (P), were cultured in serum-poor medium for 18h, and were then incubated with (+) or without (−) EGF (100ng/ml) for 1h at 37°C. Cell lysates were immunoprecipitated with anti-ERK1/2 antibody, and the immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody (panel A) or anti-ERK1/2 antibody (panel B). In panel C, lysates were immunoprecipitated with non-immune IgG, and immunoblotted with anti-ERK1/2 antibody. Cell lysates were immunoblotted with anti-ERK1/2 antibody (panel D). EGF stimulated phosphorylation of ERK2 (panel A) in GEC on collagen but not on plastic. A portion of the 42kDa phosphoprotein in GEC on collagen was immunoprecipitated non-specifically (compare panels B with C). ERK2 protein content was similar in GEC on collagen and plastic (panel D).

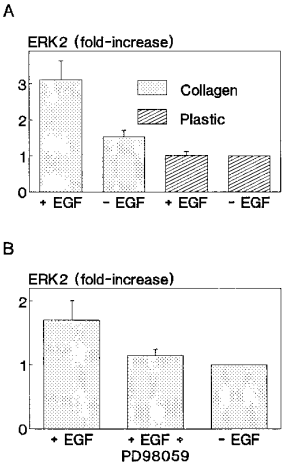

FIG. 4. Effect of ECM on EGF-stimulated ERK2 activity (panel A). GEC adherent to collagen or plastic, were cultured in serum-poor medium for 18h, and were then incubated with (+) or without (−) EGF (100ng/ml) for 1h at 37°C. Cell lysates were immunoprecipitated with anti-ERK2 antibody, and the immunoprecipitates were assayed for ERK2 activity by monitoring phosphorylation of myelin basic protein. Significant differences were present among groups ($p<0.001$, ANOVA). EGF stimulated ERK2 activity only in GEC adherent to collagen ($p<0.0025$). Values are mean±SEM of 4-5 experiments performed in duplicate. Panel B demonstrates that preincubation with the MEK inhibitor, PD98059 (50μM; 30min), significantly inhibits EGF-dependent ERK2 activity. Values are mean±SEM of 3 experiments performed in duplicate.

Effect of ECM on Proliferation

The MEK inhibitor was employed to determine if activation of the EGF-R - ERK2 pathway in collagen-

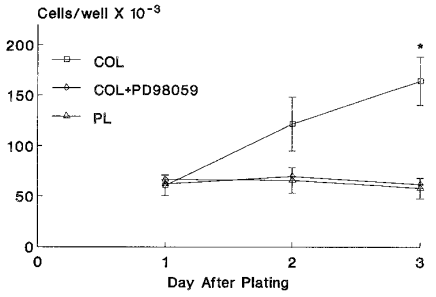

FIG. 5. Role of MAP kinase in GEC proliferation. GEC were plated into culture wells that were coated with collagen (COL), or were uncoated (plastic; PL), in the presence or absence of the MEK inhibitor, PD98059 (50μM). Medium contained EGF (100ng/ml). Cell number was determined 1-3 days after plating. In the absence of PD98059, there was a significant increase in cell number in GEC adherent to collagen, but not in cells on plastic. Proliferation on collagen was abolished with PD98059 (*$p<0.0002$ COL vs PL and COL vs COL+PD98059; $p<0.0003$ ANOVA). Each point represents mean±SEM of 3-6 wells.

adherent GEC was necessary for proliferation. Previously, we demonstrated that in the absence of EGF or other growth factors, GEC adherent to collagen do not proliferate, i.e. collagen does not independently induce proliferation (3). In keeping with prior results (3,5), EGF induced proliferation of GEC adherent to collagen, but there was no change in cell number in GEC adherent to plastic (Fig. 5). Proliferation on collagen was completely abolished with PD98059, implying that activation of the EKR2 pathway is necessary for EGF-induced GEC proliferation (Fig. 5). PD98059 had no apparent effect on adhesion, spreading or morphology of GEC on collagen, consistent with the prior demonstration that this inhibitor is not toxic to cells (18). Although PMA was able to induce ERK2 activation in GEC on collagen and plastic, PMA was, however, unable to stimulate GEC proliferation on either substratum (data not shown).

DISCUSSION

This study demonstrates that adhesion of GEC to ECM facilitates the activation of EGF-R and MAP kinase by EGF, and enables proliferation. In GEC on collagen, EGF effectively stimulated EGF-R tyrosine autophosphorylation, as well as ERK2 tyrosine phosphorylation and activity. EGF also induced an increase in cell number, which was abolished by inhibition of the ERK2 pathway. In contrast, EGF was unable to induce proliferation and did not stimulate ERK2 phosphorylation and activity in GEC adherent to plastic substratum. These differences between substrata could not be accounted for by differences in EGF-R or ERK2 protein expression.

Our earlier studies demonstrated that collagen enhanced EGF-dependent receptor autophosphorylation in GEC (5). Previously, it was shown that in GEC on plastic, EGF-R autophosphorylation was poorly stimulated at physiological temperature (37°), but that autophosphorylation could be stimulated more effectively at 4°C, or in the presence of vanadate, an inhibitor of phosphotyrosine phosphatases. Furthermore, after removal of ligand, the phosphorylated EGF-R was dephosphorylated rapidly in plastic-adherent GEC, while dephosphorylation occurred slowly in GEC on collagen. Ligand-dependent EGF-R endocytosis was reduced in collagen-adherent GEC as compared with GEC on plastic. Based on these observations, we had concluded that the enhanced autophosphorylation of EGF-R may have been due to ECM-induced reduction in EGF-R endocytosis and dephosphorylation by phosphotyrosine phosphatases(s) (5). Although our earlier study addressed the effects of ECM on EGF-R activation, it did not assess whether autophosphorylation of EGF-R was associated with the activation of downstream effectors and cell proliferation. The present study, thus, represents an important extension of our earlier work, as it demonstrates that enhancement of EGF-R autophosphorylation in GEC on collagen is coupled to the activation of a downstream pathway to the nucleus, and induces proliferation. Precise characterization of the mechanism by which ECM modulates EGF-R, e.g. defining the relevant phosphatase (19), will require further study. Our findings are in keeping with another study, which also demonstrated that the activity of growth factor receptor tyrosine kinases or substrates may be regulated by ECM. However, in that study, collagen matrices inhibited platelet-derived growth factor-stimulated receptor phosphorylation and proliferation in fibroblasts (20).

In the presence of EGF, autophosphorylation of EGF-R in GEC on collagen was sustained for up to 7h. In many cells, EGF-R phosphorylation is lost rapidly, due to internalization and degradation of EGF-R, or due to dephosphorylation of the receptor at the plasma membrane or within organelles. For example, autophosphorylation disappeared almost completely within 10-30 minutes after exposure to EGF in human fibroblasts and in WB 344 cells (a rat liver epithelial cell line), and in the latter, EGF-R was internalized and degraded completely within 2h (21,22). The pattern in A431 cells (where autophosphorylation persisted for up to 6h) resembles GEC (21). However, A431 cells are a human epidermoid carcinoma cell line that overexpresses EGF-R ($\sim 2 \times 10^6$ receptors/cell), and internalizes EGF-R inefficiently (21). GEC on collagen also internalize EGF-R inefficiently, but they contain a relatively small number of receptors ($\sim 3 \times 10^4$ EGF-R/cell) (5). The time course of ERK2 activation in collagen-adherent GEC paralleled autophosphorylation of EGF-R, suggesting a close coupling of the two processes. Furthermore, the sustained phosphorylation of ERK2 suggests that there was negligible dephosphorylation of ERK2

by a MAP kinase phosphatase (23), and the prolonged phosphorylation of EGF-R implies that ERK2 activation probably did not lead to the induction of an EGF-R-directed phosphatase (24). The magnitude of the EGF-induced increase in ERK2 activity was, however, relatively small as compared with other cell types, although similar to another renal epithelial cell line (25). Sustained activation of EKR2 in GEC may be required for EGF to induce a proliferative response (26). By analogy, it has been shown that fibroblast growth factor-induced DNA synthesis in BALB/c 3T3 cells is associated with sustained tyrosine phosphorylation of various substrate proteins (27). It should also be noted that in the absence of EGF, adhesion to ECM did not increase ERK2 activity significantly, although we have shown that ECM independently activates inositol lipid breakdown in GEC, via β_1-integrins (3,4). This finding is distinct from results in various fibroblast lines, where adhesion to fibronectin induced activation of ERKs (7). However, it is possible that ECM activated ERK2 transiently, or at early time points after plating of cells.

Adhesion to collagen was essential for the activation of ERK2 via EGF-R, but PMA was able to bypass the requirement for ECM, as PMA activated ERK2 in GEC on collagen as well as plastic (presumably via protein kinase C). This result implies that activation of the ERK2 pathway by ECM is mainly "upstream" of Raf or Ras (the most likely targets of protein kinase C) (14), and probably occurs solely at the level of EGF-R. The failure of PMA to induce proliferation suggests that although ERK2 is necessary for proliferation, there may also be a requirement for the activation of other pathways by EGF-R. For example, we attempted to investigate whether ECM might modulate EGF-dependent activation of DNA-binding proteins in GEC (28,29), but we were unable to detect activation of latent transcription factors (Stat1) by use of gel-mobility shift assays or by monitoring tyrosine phosphorylation. However, similar to earlier reports (29), we were able to detect such activity in EGF-stimulated A431 cells (unpublished observations). Another potential pathway by which ECM and EGF-R may regulate proliferation is the activation of cyclin D1 or cyclin E-associated kinase (30). Further studies will also be required to determine which other tyrosine kinase or non-tyrosine kinase receptors are expressed in GEC, and whether their activity and mitogenic signalling are modulated by ECM.

In the present study, we used collagen I as the ECM for GEC. Contact of GEC with collagen I in vivo is generally limited to pathological conditions (e.g. glomerular inflammation), while in the normal glomerulus, GEC are adherent to collagen IV. In cultured GEC, collagens I and IV exert similar effects on DNA synthesis (3). However, it was not possible to directly assess the effect of collagen IV on EGF-R or MAP kinase activation, because it is not feasible to produce collagen IV in amounts sufficient for these experiments. One can speculate on how the effects of ECM on EGF-R signalling might regulate GEC proliferation in vivo. There appears to be minimal turnover of GEC, and there is a low concentration of epithelial growth factors in normal glomeruli. Proliferation of GEC may occur in pathological states, including experimental membranous nephropathy in rats (Heymann nephritis) (31). Following initial sublethal injury of GEC by the complement membrane attack complex, GEC express proliferating cell nuclear antigen, in the absence of inflammatory cell infiltrate (31). At present, the endogenous growth factor(s) responsible for this proliferative response have not been defined. Nevertheless, exogenously-administered basic fibroblast growth factor can increase the number of mitoses in the injured GEC in experimental membranous nephropathy (32). In other types of glomerulonephritis, glomeruli may become infiltrated with inflammatory cells, e.g. macrophages, or platelets, which are sources of epithelial growth factors, including transforming growth factor-α and EGF (33). Accumulation of basement membrane and interstitial collagens is often evident in glomerulopathies. As a result, the concentration of factors that can potentially modulate EGF-R activation may increase, and may lead to enhanced GEC proliferation. The present study may provide further insights into the mechanisms of GEC proliferation in glomerular injury.

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