Extracellular Matrix Modulates Epidermal Growth Factor Receptor Activation in Rat Glomerular Epithelial Cells

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Abstract

To understand how glomerular epithelial cell (GEC) proliferation may be regulated in health and disease, we studied the effects of type I collagen extracellular matrices (ECM) on EGF receptor (EGF-R) activation in cultured rat GEC. EGF stimulated proliferation of GEC adherent to ECM, but not of GEC on a plastic substratum. Significant and prolonged EGF-R tyrosine autophosphorylation (which reflects receptor kinase activation) was induced by EGF in GEC adherent to collagen, but EGF did not stimulate EGF-R autophosphorylation in GEC on plastic (at 37°C). However, EGF-R autophosphorylation increased significantly in plastic-adherent GEC that were stimulated with EGF at 4°C or in the presence of vanadate, an inhibitor of phosphotyrosine phosphatases. Furthermore, dephosphorylation of EGF-R was enhanced in GEC on plastic as compared with collagen. At 4°C, [^{125}I]EGF binding was not different between substrata, and there was negligible accumulation of intracellular [^{125}I]EGF (which reflects EGF-R internalization). At 37°C, EGF-R internalization was reduced significantly in collagen-adherent GEC as compared with GEC on plastic. Thus, contact with ECM facilitates proliferation and EGF-R activation in GEC. The enhanced activity of EGF-R tyrosine kinase may be due to ECM-induced reduction in EGF-R internalization and dephosphorylation by phosphotyrosine phosphatase(s). Signals from ECM to growth factor receptors may regulate cell turnover in the glomerulus under normal conditions and during immune glomerular injury. (*J. Clin. Invest.* 1994. 94:68–78.) Key words: collagen • phosphorylation • tyrosine kinase • phosphotyrosine phosphatase

Introduction

Adhesion to extracellular matrix (ECM)[1] may directly or indirectly modulate proliferative responses of cells to polypeptide growth factors and stimulate cell differentiation (1). Several recent studies have focused on intracellular signal transduction mechanisms that may be activated by adhesion of cells to ECM (2), as well as on interactions of ECM with growth factors (3). EGF is a 53–amino acid polypeptide which is mitogenic for a number of cell types, epithelial cells in particular (4, 5). EGF binds to a 170-kD cell-surface receptor (EGF-R), which has been shown to possess tyrosine kinase activity (4, 5). EGF-R is also a receptor for TGFα, an epithelial cell mitogen that is structurally and functionally related to EGF (4). It is believed that the initial events, which ultimately result in cell proliferation, involve binding of EGF (or TGFα) to EGF-R followed by receptor oligomerization (5, 6). This leads to transmembrane activation of the cytoplasmic tyrosine kinase, which is associated with tyrosine phosphorylation of EGF-R itself ("autophosphorylation"), and of other substrate proteins (4–6). Activated EGF-R may be inactivated through dephosphorylation by a phosphotyrosine (P-tyr) phosphatase (PTPase) (7–9). Binding of EGF to EGF-R may also be associated with internalization of ligand–receptor complexes, followed by degradation in endosomal compartments or recycling to the cell surface (5), and it has been proposed that internalization may be a mechanism for inactivating EGF-R kinase activity.

Visceral and parietal glomerular epithelial cells (GEC) are intrinsic components of the kidney glomerulus, and both cell types are in contact with ECM. Under normal conditions, the turnover of these cells is low, but proliferation of parietal and possibly visceral GEC may occur in immune glomerular injury and may lead to impaired glomerular function and permselectivity (10, 11). Urines from children with Henoch-Schönlein purpura nephritis (a nephritis often associated with glomerular proliferation) contain a factor that resembles TGFα, suggesting that the presence of this factor in the glomerulus may be stimulating epithelial proliferation (12). Our studies have focused on signal transduction pathways that are activated as a result of GEC adhesion to ECM. We have demonstrated that proliferation of cultured rat GEC is stimulated by growth factors, including EGF, when GEC are adherent to collagen matrices (13). In addition, adhesion to collagen resulted in the activation of phospholipase C, and products of phospholipase C activation downregulated the mitogenic effects of growth factors (13). Thus, adhesion of GEC to ECM may regulate mitogenic signals both in a positive and negative fashion. In this study, we investigated if ECM can directly modulate EGF-R activation. We demonstrate that EGF-dependent tyrosine autophosphorylation of EGF-R (which reflects receptor activation) is enhanced in GEC adherent to collagen as compared with plastic substrata.

Methods

Materials. Tissue culture media and FCS were obtained from Gibco Laboratories (Burlington, Ontario) or ICN Biomedical (Mississauga,

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; EGF-R, EGF receptor; GEC, glomerular epithelial cell; PTPase, phosphotyrosine phosphatase; P-tyr, phosphotyrosine.

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Ontario). Pepsin-solubilized bovine dermal collagen (Vitrogen) was from Celtrix Laboratories (Palo Alto, CA). NuSerum and EGF were purchased from Collaborative Research, Inc. (Bedford, MA). Insulin and hormone supplements were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium iodide-125 (2,500 Ci/mmol) and anti-P-tyr monoclonal antibody, PY20, were from ICN Biomedical. Electrophoresis and immunoblotting reagents were from Biorad Laboratories (Mississauga, Ontario). A431 cells (a human epidermoid carcinoma cell line) were purchased from American Type Culture Collection (Rockville, MD). WB 344 cells (a liver epithelial cell line established from a primary culture of Fisher 344 rat hepatocytes) (14) were kindly provided by Dr. J. Bergeron (McGill University, Montreal, Quebec). Rabbit anti-EGF-R antiserum, RK-2 (15), was kindly provided by Dr. B. Margolis and Dr. J. Schlessinger (New York University, New York).

Extracellular matrix. Type I collagen gel matrices were prepared by combining RPMI 1640 (×10) medium, 7.5% $NaHCO_3$, pepsin-solubilized bovine dermal collagen (~ 3 mg/ml in 0.012 N HCl), and 0.1 N NaOH in proportions of 10:4:80:10 at 4°C. Then, the mixture was poured into tissue culture dishes (~ 0.06 ml/cm^2) and allowed to gel at 37°C, as described previously (13). For measurements of [^{125}I]EGF binding (see below), culture wells were coated with collagen in 0.01 N HCl (~ 0.06 ml/cm^2) and allowed to air dry at 22°C, as described previously (13).

Cell culture. Primary cultures of rat GEC were established from explants of rat glomeruli, as described previously (13). 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 DME/Ham F10 (1:1) containing 5% NuSerum and hormone supplements (13). In some experiments, GEC were grown in serum-poor medium (DME/Ham F10, 1:1, with 0.5% FCS) containing EGF. 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 (13). For passaging of cultures, GEC were replated onto collagen gels; for experiments, GEC were replated onto collagen gels or plastic. A431 cells and WB 344 cells were cultured in DME/10% FCS on plastic. For experiments, WB 344 cells were replated onto collagen gels or plastic.

Measurement of 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.

Tyrosine phosphorylation of EGF-R. The P-tyr content of EGF-R was assessed by immunoblotting GEC membrane fractions or EGF-R immunoprecipitates with anti-P-tyr antibody. After incubation in serum-poor medium for 18 h, GEC were stimulated with EGF in serum-poor medium or were further incubated in serum-poor medium (control). Previous studies demonstrated that EGF-R autophosphorylation was evident after stimulation of GEC with EGF at 50 ng/ml; autophosphorylation was not detected consistently at lower EGF concentrations (16). Also, when whole cell lysates were subjected to SDS-PAGE, the levels of phosphorylated EGF-R were too low for detection by immunoblotting; consequently, GEC membrane fractions were prepared to enrich for EGF-R (16). After stimulation with EGF, GEC were scraped from culture dishes (two 100-mm plates or ~ 10^7 cells/sample) and were homogenized in buffer containing 50 mM Hepes, 1 mM benzamidine, 1 mM PMSF, 2 mM NaF, 2 mM Na_3VO_4, 1 mM $MgCl_2$, and 0.25 M sucrose, pH 7.40 (at 4°C). After sedimentation of unbroken cells and nuclei (3,000 *g*, 5 min), membranes were pelleted by centrifugation at 16,000 *g* for 30 min. (Although we previously pelleted membranes at 100,000 *g*, we found that recovery of [^{125}I]EGF–EGF-R complexes at 16,000 *g* as compared with 100,000 *g* was 96.1±0.3%; three experiments. Thus, the membrane pellet likely included plasma membrane and the majority of microsomal EGF-R.) Protein content was determined by the Biorad Laboratories protein assay, according to manufacturer directions. In other experiments, GEC proteins were solubilized in buffer containing 1.5% Triton X-100, 50 mM Hepes, 1 mM benzamidine, 1 mM PMSF, 40 mM NaF, 2 mM Na_3VO_4, 50 mM EDTA, and 1% BSA, pH 7.40, and were immunoprecipitated with rabbit anti-EGF-R antiserum, followed by absorption with agarose-coupled anti-rabbit IgG. In some experiments, in vitro phosphorylation was carried out in buffer containing 50 mM Hepes, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 25 μM ATP, 500 ng/ml EGF, and protease and phosphatase inhibitors described above. For immunoblotting, membrane proteins (~ 125 μg/sample) or immunoprecipitates were dissolved in 1% SDS-Tris loading buffer of Laemmli and were electrophoresed under reducing conditions in 5–15% gradient polyacrylamide slab gels. Proteins were then transferred onto nitrocellulose paper, blocked with 3% BSA/2% ovalbumin, and incubated with mouse monoclonal anti-P-tyr antibody, PY20 (100 ng/ml) (17), and goat anti-mouse IgG, alkaline phosphatase-conjugated, as described previously (16). Alkaline phosphatase activity was detected with bromochloroindolyl phosphate–nitro blue tetrazolium. The amount of EGF-R tyrosine phosphorylation was quantitated by densitometry (Ultroscan XL laser densitometer; Pharmacia LKB Biotechnology, Baie D'Urfe, Quebec); absorbance is expressed in arbitrary units, with the lowest absorbance of EGF-R in each experiment set to 1.0 (16). In preliminary studies, we observed that there was a linear correlation between densitometric measurements and the amounts of phosphoprotein loaded onto gels. Specificity of PY20 for P-tyr proteins was verified by showing that reactivity of PY20 against phosphoproteins could be abolished entirely by including 0.1 mM P-tyr in the incubation buffer. Immunoblotting with anti-EGF-R antiserum was performed in an analogous manner.

The P-tyr content of EGF-R in WB 344 cells was assessed by a protocol similar to GEC, except that whole cell lysates (~ 25–50 μg of protein/sample) instead of membrane fractions were analyzed by SDS-PAGE and immunoblotting.

EGF binding and EGF-R trafficking. Murine EGF was iodinated by the chloramine T method to a specific activity of ~ 3×10^8 cpm/μg (16). [^{125}I]EGF was separated from free iodine-125 and from high molecular mass aggregates by gel-filtration chromatography. EGF binding was measured, as described previously (16), except that in this study binding assays were carried out with cells in monolayer culture (18-mm wells). Before binding assays, monolayers of cells were placed into serum-poor medium for 18 h. Then, triplicate wells of cells were incubated for 0.5–24 h with trace [^{125}I]EGF (50,000 cpm), which was supplemented with unlabeled EGF (0.1–50 ng/ml) in some experiments. Nonspecific binding was estimated by the amount of tracer bound in the presence of excess unlabeled EGF (0.8–1.0 μg/ml). At the end of incubations, wells were washed, and bound EGF was solubilized in 0.25 N NaOH and counted in a gammacounter. In experiments that required removal of surface-bound EGF, GEC were washed rapidly three times with 10 mM sodium acetate/acetic acid buffer, pH 3.0 (4°C). Preliminary studies determined that the acid-washing protocol extracted ~ 80% of surface-bound EGF.

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 (18). Where significant differences were found, individual comparisons were made between groups by using the *t* statistic and adjusting the critical value according to the Bonferroni method. ANOVA for repeated measures was used to establish significant differences in serial measurements between groups (18).

Results

Effect of ECM on GEC proliferation. In keeping with prior results (13), a progressive increase in cell number was observed

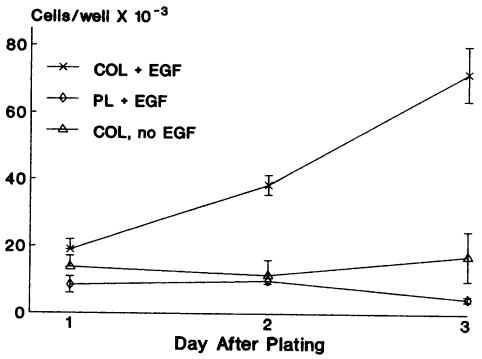

Figure 1. Effect of collagen matrices on GEC proliferation. GEC were plated into culture wells that were coated with type I collagen gels (*COL*) or were uncoated (plastic, *PL*) in medium with or without EGF (10 ng/ml). Cell number was determined 1, 2, and 3 d after plating. EGF produced significant increases in cell number in GEC adherent to collagen as compared with plastic ($P < 0.005$ COL + EGF vs PL + EGF; ANOVA). Each point represents mean±SEM of three wells. The data representing COL, no EGF are adapted from reference 13.

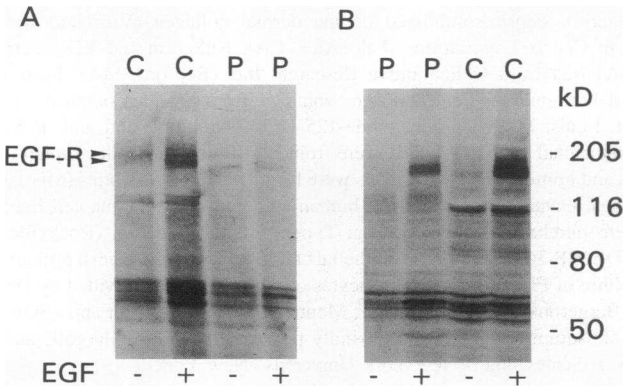

Figure 2. Immunoblots of protein tyrosine phosphorylation in collagen (*C*) and plastic (*P*)-adherent GEC. GEC, cultured in serum-poor medium for 18 h, were incubated with (+) or without (−) EGF (50 ng/ml, 30 min, 37°C) in the absence (*A*) or presence (*B*) of 1 mM Na_3VO_4. Membrane fractions of cells (see Methods) were subjected to SDS-PAGE, transferred onto nitrocellulose, and immunoblotted using anti-P-tyr antibody (also see Fig. 4).

in GEC adherent to type I collagen gels cultured in serum-poor medium (0.5% FCS) (see Methods) containing EGF (10 ng/ml). In contrast, GEC adherent to plastic substratum did not proliferate, despite the presence of EGF in the medium (Fig. 1). By light microscopy, cell adhesion or spreading was not impaired on plastic as compared with collagen. Previously, we demonstrated that EGF (1–100 ng/ml) also increases DNA synthesis ([^{3}H]thymidine incorporation) in collagen-adherent GEC (13, 16). Furthermore, besides collagen I, collagen IV (but not laminin) supported DNA synthesis in GEC, and the effect of collagen IV was comparable in magnitude with collagen I (13). However, collagen-adherent GEC did not proliferate in the absence of EGF in the medium, indicating that collagen by itself has no mitogenic activity (Fig. 1).

Effect of ECM on EGF-R tyrosine kinase in GEC. Since EGF stimulated proliferation only when GEC were adherent to collagen, we examined if this effect was associated with enhanced EGF-R activation in cells on collagen matrices. EGF-R tyrosine autophosphorylation (which reflects receptor activation; see below) was studied by subjecting GEC membrane fractions to SDS-PAGE and immunoblotting with anti-P-tyr antibody. Incubation of collagen-adherent GEC with EGF (50 ng/ml for 30 min at 37°C) induced a significant increase in the P-tyr content of a protein doublet of 160–170 kD, with the lower band containing more P-tyr than the upper (Fig. 2 *A*, first two lanes). We reported previously that in GEC EGF-R migrated as a doublet in 5–15% acrylamide gradient gels, suggesting that there are two activatable forms of EGF-R in GEC (16). To confirm that the 160–170-kD protein (Fig. 2 *A*) that was tyrosine phosphorylated in an EGF-dependent manner is EGF-R, collagen-adherent GEC and, for comparison, A431 cells (a tumor cell line that overexpresses EGF-R) were stimulated with EGF, and detergent-solubilized proteins were immunoprecipitated with anti-EGF-R antibody, RK-2. Immunoblotting of the immunoprecipitates with anti-P-tyr antibody demonstrated a single prominent band in GEC (160 kD; Fig. 3), consistent with the lower band of the protein doublet shown in Fig. 2 *A*. The upper band of the doublet was less apparent in the immunoprecipitation experiments, possibly because the immunoprecipitation was not as sensitive as direct immunoblotting of membrane proteins. The 160-kD protein in GEC also comigrated with a highly prominent band in EGF-R antibody immunoprecipitates of A431 cells (Fig. 3).

Quantitation of EGF-R P-tyr content (160–170-kD protein doublet) was obtained using densitometry and is based on several experiments similar to the representative immunoblot shown in Fig. 2 *A*. In collagen-adherent GEC, the time course of EGF-R autophosphorylation (ranging from 0 to 120 min) is presented in Table I (the 10- and 30-min time points are also presented in Fig. 4 *A*). An upward trend in EGF-R autophosphorylation was evident after 10 min of incubation with EGF. Autophosphorylation was enhanced significantly after 30 min

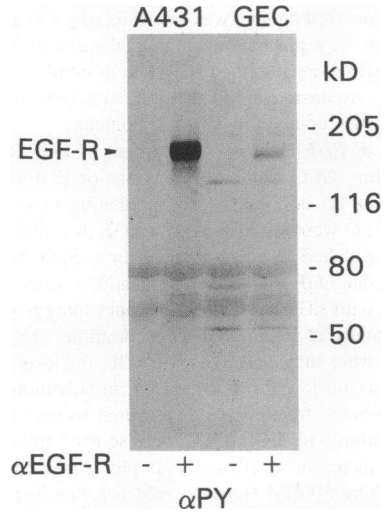

Figure 3. Identification of activated EGF-R in GEC. A431 cells (~ 2×10^6 cells; first and second lanes) and collagen-adherent GEC (~ 2.5×10^7 cells; third and fourth lanes) were cultured in serum-poor medium for 18 h and then were incubated with EGF (50 ng/ml, 30 min, 37°C). Proteins were solubilized in detergent-containing buffer and were immunoprecipitated with 2 μl of rabbit anti-EGF-R antiserum RK-2 (*αEGF-R* +) or 2 μl of nonimmune rabbit serum (−) in controls. Immune complexes were absorbed with agarose anti-rabbit IgG and were subjected to SDS-PAGE, followed by immunoblotting with anti-P-tyr antibody (*αPY*). Specific bands of 160–170 kD were demonstrated in both cell types.

Table I. Time Course of EGF-R Autophosphorylation in GEC Adherent to Collagen

Incubation time	P-tyr
0 min	1.0
10 min	1.66±0.23
30 min	3.01±0.34*
60 min	4.19±2.00*
120 min	2.80±0.74

GEC were incubated with EGF (50 ng/ml) at 37°C. The P-tyr content of EGF-R was measured by densitometry and is expressed in arbitrary units; see legend to Fig. 4. Values are mean±SEM of three to seven experiments; $P < 0.002$, ANOVA, * $P < 0.01$ vs 0 min.

of incubation and increased further at 60 min. A decline in the level of autophosphorylation was evident at 120 min.

Incubation of plastic-adherent GEC with EGF for 10 min (37°C) produced an upward trend in EGF-R autophosphorylation (188±24% of basal levels), similar to cells adherent to collagen (166±23% of basal; Fig. 4 *A*). However, when plastic-adherent GEC were incubated with EGF for 30 min (37°C), EGF-R autophosphorylation declined toward basal levels (165±33% of basal), whereas autophosphorylation had increased further in GEC on collagen (307±59% of basal; Figs. 2 *A* and 4 *A*). The absence of autophosphorylation in plastic-adherent GEC was not due to degradation of EGF-R (discussed below). Basal phosphorylation of EGF-R was not significantly different between substrata (Fig. 2 *A*). In addition to EGF-R, a small number of other proteins (115 and 50–65 kD) was tyrosine phosphorylated in an EGF-dependent manner; these proteins probably represent EGF-R substrates (Fig. 2 *A*). Similar to EGF-R, EGF-dependent tyrosine phosphorylation of the substrate proteins was enhanced in GEC on collagen as compared with plastic. (A representative immunoblot is shown in Fig. 2 *A*, and quantitation of P-tyr in the 115-kD substrate protein is presented in Fig. 4 *B*.) Thus, the pattern of EGF-R and substrate phosphorylation suggested that activation of EGF-R kinase increased progressively in collagen-adherent GEC reaching a maximum at 60 min, while in GEC on plastic, EGF-R kinase was either not activated effectively or was dephosphorylated rapidly.

Three approaches were used to determine if dephosphorylation of EGF-R (via a PTPase) may have been responsible for diminishing receptor P-tyr content in GEC adherent to plastic. EGF-R internalization (see below) and PTPase activity are known to be temperature dependent (8). Thus, we first assessed autophosphorylation of EGF-R in GEC incubated with EGF at 4°C (30 min). In contrast to the incubations at 37°C (Fig. 4 *A*), at low temperature autophosphorylation was stimulated equally in collagen (237±56% of basal levels) and plastic-adherent GEC (277±71% of basal; Fig. 4 *C*). The increase in EGF-R autophosphorylation in GEC on collagen at 4°C was ~ 25% lower than at 37°C (Fig. 4, *A* vs *C*), probably reflecting ~ 25% less binding of ligand to EGF-R at low temperature (see below and Fig. 9). Second, GEC were preincubated (at 37°C) with vanadate to inhibit PTPase activity (9, 19). Basal levels of P-tyr in EGF-R were not affected by vanadate, although the basal levels in GEC on collagen tended to be approximately twofold

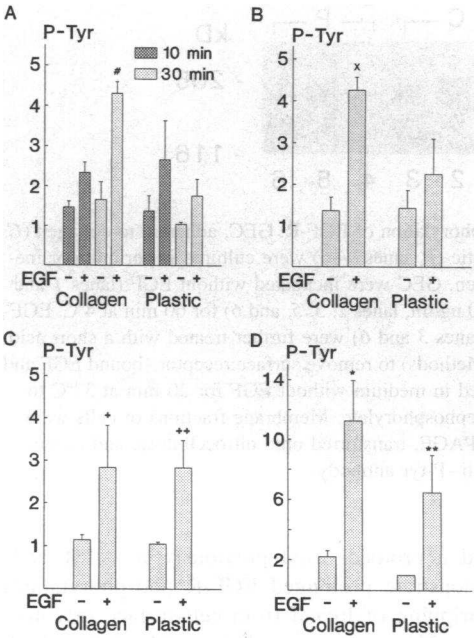

Figure 4. P-tyr content of EGF-R (*A, C,* and *D*) and of a 115-kD protein (*B*). The P-tyr content of EGF-R (160–170-kD protein doublet; see Fig. 2) or 115-kD protein was measured by densitometry and is expressed in arbitrary units (mean±SEM). GEC, on collagen or plastic, cultured in serum-poor medium for 18 h, were incubated with (+) or without (−) EGF (50 ng/ml) for 10 or 30 min at 37°C (*A*; four experiments), 30 min at 37°C (*B*; four experiments), 30 min at 4°C (*C*; three experiments), and 30 min at 37°C in the presence of 1 mM Na_3VO_4 (*D*; three experiments). Membrane fractions of cells were subjected to SDS-PAGE, transferred onto nitrocellulose, and immunoblotted using anti–P-tyr antibody. Significant differences were present among groups: *A*, $P < 0.0001$; *B*, $P < 0.025$; *C*, $P < 0.01$; *D*, $P < 0.02$ (ANOVA). EGF significantly stimulated EGF-R autophosphorylation in collagen-adherent GEC after 30 min of incubation at 37°C (*A*, $^{\#}P < 0.001$ vs unstimulated) and 4°C (*C*, $^{+}P < 0.03$ vs unstimulated), and in plastic-adherent GEC at 4°C (*C*, $^{++}P < 0.025$ vs unstimulated). At 37°C, in the presence of vanadate (*D*), EGF significantly stimulated EGF-R autophosphorylation in GEC on both collagen (*$P < 0.01$ vs unstimulated) and plastic (**$P < 0.04$ vs unstimulated). Similar to EGF-R (*A*, 30 min), EGF significantly stimulated tyrosine phosphorylation of a 115-kD protein in GEC adherent to collagen (*B*, $^{\times}P < 0.005$ vs unstimulated), but not plastic.

higher than in cells on plastic (Fig. 4 *D*). In vanadate-treated GEC on collagen, EGF-dependent receptor autophosphorylation was more pronounced (548±133% of basal; Figs. 2 *B* and 4 *D*) as compared with untreated GEC (Figs. 2 *A* and 4 *A*). Furthermore, in the presence of vanadate, EGF induced significant EGF-R autophosphorylation in GEC on plastic (647±249% of basal; Figs. 2 *B* and 4 *D*). Although the P-tyr content of EGF-R in collagen-adherent GEC showed a greater stimulated increase as compared with cells on plastic, the percent increase in receptor autophosphorylation was similar in cells on the two substrata, as basal levels of phosphorylation tended to be different (Fig. 4 *D*).

In a third series of experiments, dephosphorylation of EGF-R was assessed more directly. Phosphorylation of EGF-R in GEC is sustained for at least 2 h after addition of EGF to culture medium (Table I). Thus, to accelerate EGF-R dephosphoryla-

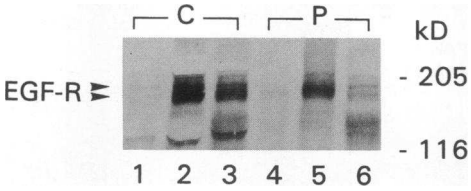

Figure 5. Dephosphorylation of EGF-R. GEC, adherent to collagen (*C*, lanes *1–3*) or plastic (*P*, lanes *4–6*) were cultured in serum-poor medium for 18 h. Then, GEC were incubated without EGF (lanes *1* and *4*) or with EGF (50 ng/ml, lanes *2, 3, 5,* and *6*) for 60 min at 4°C. EGF-stimulated GEC (lanes *3* and *6*) were further treated with a short acid wash at 4°C (see Methods) to remove surface receptor–bound EGF and then were incubated in medium without EGF for 20 min at 37°C to allow EGF-R to dephosphorylate. Membrane fractions of cells were subjected to SDS-PAGE, transferred onto nitrocellulose, and immunoblotted using anti–P-tyr antibody.

tion, we adopted a protocol used previously in A431 cells (which also demonstrate prolonged EGF-R phosphorylation) involving acid-stripping of ligand from cell-surface receptors (20). To achieve sufficient phosphorylation of EGF-R in both collagen- and plastic-adherent GEC and to prevent internalization of ligand–receptor complexes (see below), cells on collagen or plastic were incubated with EGF at 4°C (as in Fig. 4 *C*, except for 60 min). This resulted in a 583% increase in EGF-R P-tyr content in GEC on collagen and 619% on plastic (Fig. 5, lanes *2* and *5*) as compared with basal levels (Fig. 5, lanes *1* and *4*). Surface-bound EGF was removed by a short acid wash at 4°C (see Methods). Then, GEC were incubated in medium without EGF for 20 min at 37°C to allow EGF-R to dephosphorylate. (Preliminary studies demonstrated that the acid wash alone did not affect the P-tyr content of EGF-R significantly.) Substantial loss of P-tyr in EGF-R was evident in plastic-adherent GEC after 20 min (Fig. 5, lane *6*), while dephosphorylation of EGF-R in GEC on collagen during the same period was lower (Fig. 5, lane *3*). Densitometric measurements, based on four experiments similar to the one presented in Fig. 5, showed that in GEC on plastic P-tyr content of EGF-R after the 20-min incubation at 37°C declined to 37±13% of the P-tyr in the stimulated receptor (i.e., lane *6* vs *5*), while in GEC on collagen the decline represented 77±12% of the stimulated value (lane *3* vs *2*; $P = 0.035$ collagen vs plastic). In an additional set of experiments, we assessed the effect of temperature on the dephosphorylation of EGF-R in collagen-adherent GEC. As in Fig. 5, GEC were incubated with EGF at 4°C and then were acid washed to remove surface-bound EGF. Then, EGF-R was allowed to dephosphorylate at 37°C for 20 min (as in Fig. 5) or at 4°C. In these experiments, densitometry measurements showed that at 37°C P-tyr content of EGF-R declined to 54±13% of the P-tyr in the stimulated receptor, but at 4°C it declined to only 83±5% of the stimulated value ($P < 0.04$; three experiments), indicating that dephosphorylation was temperature dependent. (Although the amount of EGF-R dephosphorylation at 37°C tended to be greater in this set of experiments than in the experiments described above, i.e., 54±13 vs 77±12%, this difference was not statistically significant.) Together, these results indicate that EGF-R dephosphorylation is likely to be enzymatic (i.e., due to PTPase activity) and that in GEC on plastic there is more PTPase activity directed towards EGF-R as compared with GEC on collagen.

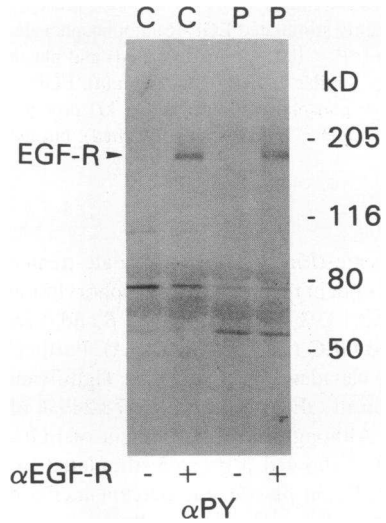

Figure 6. In vitro EGF-R autophosphorylation. Collagen (*C*) and plastic (*P*)-adherent GEC (four 100-mm culture plates of each) were cultured in serum-poor medium for 18 h. Proteins were solubilized in detergent-containing buffer, and equal amounts of soluble proteins from GEC on collagen and plastic were immunoprecipitated with 2 μl of rabbit anti–EGF-R antiserum RK-2 (*αEGF-R +*) or 2 μl of nonimmune rabbit serum (−) in controls. Immune complexes were absorbed with agarose anti–rabbit IgG and then were incubated for 20 min at 22°C in buffer containing EGF (500 ng/ml) and ATP (25 μM). Proteins were subjected to SDS-PAGE, followed by immunoblotting with anti–P-tyr antibody (*αPY*). Specific bands at ~ 170 kD of similar intensity were demonstrated in cells from both substrata, indicating that there were no substantial differences in EGF-R autophosphorylation in vitro.

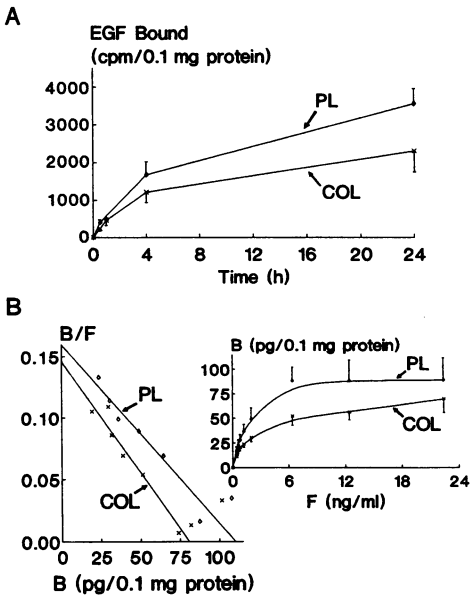

Figure 7. (*A*) [^{125}I]EGF binding in GEC adherent to collagen (*COL*) and plastic (*PL*). GEC, cultured in serum-poor medium for 18 h, were incubated with trace amounts of [^{125}I]EGF (50,000 cpm) at 4°C for 0.5–24 h. There were no significant differences in [^{125}I]EGF binding between groups (ANOVA). Values are mean±SEM of four experiments performed in triplicate. (*B*) Scatchard analysis of [^{125}I]EGF binding in GEC adherent to collagen and plastic (representative experiment). The high affinity component of EGF binding is indicated. Inset depicts amount of ligand bound (*B*) versus the concentration of free (*F*) ligand (mean±SEM of three experiments performed in triplicate). Collagen- and plastic-adherent GEC were incubated with [^{125}I]EGF (50,000 cpm) and various amounts of unlabeled EGF at 4°C for 24 h.

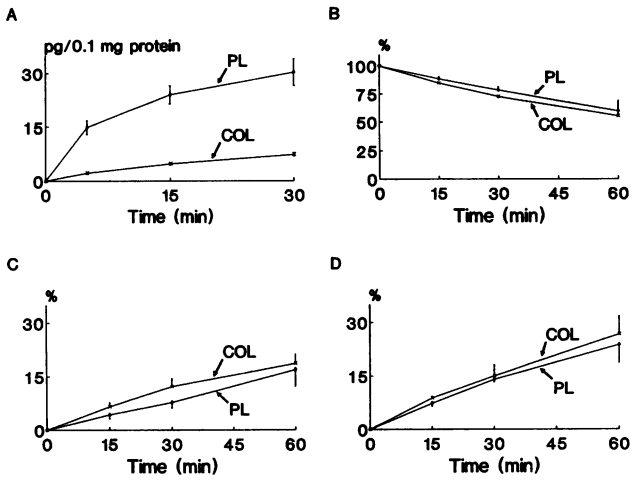

Figure 8. EGF-R trafficking. (*A*) Internalization of EGF-R. GEC, adherent to collagen (*COL*) or plastic (*PL*), were incubated with [^{125}I]EGF (2 ng/ml, 37°C) for serial time intervals. Intracellular radioactivity was determined by acid washing cells to remove surface-bound EGF and then solubilizing cells with NaOH. Values represent intracellular EGF and are mean±SEM of three to six experiments performed in triplicate. Significantly more intracellular EGF was found in GEC adherent to plastic as compared with collagen ($P < 0.001$ collagen vs plastic, ANOVA). (*B–D*) Degradation and recycling of EGF-R. GEC, adherent to collagen or plastic, were loaded with [^{125}I]EGF (2 ng/ml, 37°C, for 30 min). Then, GEC were acid washed and chased with unlabeled EGF (100 ng/ml) for 60 min at 37°C. At serial time intervals, medium was collected, and EGF in the medium was precipitated with TCA (final concentration of 10% vol/vol). Intracellular EGF was determined by solubilizing cells with NaOH (*B*). In the medium, TCA-precipitable radioactivity (*C*) represents intact EGF released from cells, which reflects EGF-R recycling, and TCA-soluble radioactivity (*D*) represents degraded EGF released from cells, which reflects EGF-R degradation. Values represent the percentage of total radioactivity (mean±SEM of four experiments performed in triplicate). There were no significant differences in these measurements between GEC adherent to collagen and plastic.

Table II. EGF-R Content in GEC

	EGF-R
Unstimulated	
Collagen	1.07±0.07
Plastic	1.51±0.25
EGF-stimulated	
Collagen	1.09±0.08
Plastic	2.05±0.13

GEC, cultured in serum-poor medium for 18 h, were incubated in serum-poor medium (30 min, 37°C; *Unstimulated*) or with EGF (50 ng/ml, 30 min, 37°C) as in Fig. 2 *A*. Membrane fractions of cells were subjected to SDS-PAGE, transferred onto nitrocellulose, and immunoblotted using anti-EGF-R antibody RK-2 (not shown). The content of EGF-R protein (160–170-kD bands) was quantitated by densitometry. Results are expressed in arbitrary units. Values are mean±SEM of three experiments. There are no significant differences in EGF-R content between unstimulated GEC adherent to collagen and plastic and between stimulated and unstimulated GEC adherent to collagen or plastic.

An additional study was carried out to confirm that the low level of EGF-R autophosphorylation in plastic-adherent GEC (at 30 min, 37°C) was not because of an intrinsic defect in EGF-R. GEC that had been plated onto collagen or plastic were solubilized in detergent-containing buffer, and equal amounts of cell proteins were immunoprecipitated with anti-EGF-R antibody. Then, EGF-R in the immunoprecipitates was autophosphorylated in vitro in the presence of excess EGF and ATP. P-tyr antibody immunoblotting demonstrated that there were no significant differences in autophosphorylation between EGF-R immunoprecipitated from GEC that had been adherent to collagen and plastic (Fig. 6), suggesting that the receptor from GEC on plastic was not intrinsically defective.

Effect of ECM on EGF binding and EGF-R trafficking in GEC. [^{125}I]EGF-binding studies were carried out (at 4°C) to determine if differences in EGF binding could account for differences in EGF-R autophosphorylation between substrata. Using trace concentrations of [^{125}I]EGF, we found that EGF binding tended to be greater in GEC adherent to plastic as compared with collagen (1.1–1.7-fold at time points ranging from 30 min to 24 h), but these differences were not statistically significant (Fig. 7 *A*). Scatchard analysis (Fig. 7 *B*) demonstrated a high affinity binding site with an apparent K_d of 247±82 pM and 9,444±2,028 binding sites/cell in GEC on collagen, and a K_d of 317±49 pM and 15,777±2,555 binding sites/cell in GEC on plastic (differences are not statistically significant; three experiments performed in triplicate). There also appeared to be a low affinity binding site in GEC on both substrata (collagen, K_d of 1,186±522 pM and 17,055±3,156 binding sites/cell; plastic, K_d of 1,111±747 pM and 24,889±4,168 binding sites/cell). The results of the [^{125}I]EGF-binding studies were confirmed in experiments that estimated EGF-R content by immunoblotting with anti-EGF-R antibody. These experiments demonstrated that there were no significant differences in the total amount of EGF-R protein between membranes of collagen- and plastic-adherent GEC (Table II). Thus, differences in EGF binding were unlikely to account for differences in EGF-R autophosphorylation between substrata (Fig. 4). Incubation with vanadate did not significantly alter [^{125}I]EGF binding in GEC on collagen (95±8% of untreated) or plastic (85±4% of untreated; three experiments performed in triplicate). Also, direct binding of [^{125}I]EGF to the collagen matrices or to the plastic substratum (in the absence of cells) was not detectable (data not shown).

It has been proposed that EGF-R internalization may lead to inactivation of EGF-R and that internalization may be controlled by kinase activity. To assess whether effects of ECM on EGF-R autophosphorylation were associated with internalization of EGF-R, we monitored changes in cell surface-bound (acid-extractable) and intracellular [^{125}I]EGF at 37°C. (Morphological and biochemical studies have shown that the majority of EGF–EGF-R complexes do not dissociate intracellularly, and endocytic and recycling pathways are common for ligand and receptor [21–23].) In the presence of EGF (2 ng/ml), there was a time-dependent increase in intracellular EGF, and significantly more EGF was found intracellularly in GEC adherent to plastic as compared with collagen (4.0–6.8-fold at time points ranging from 5 to 30 min; Fig. 8 *A*). As indicated above, EGF-binding studies (4°C, 24 h) showed that there were no significant differences in the number of EGF-binding sites between collagen and plastic substrata, although an upward trend in EGF binding was observed in plastic-adherent GEC (a differ-

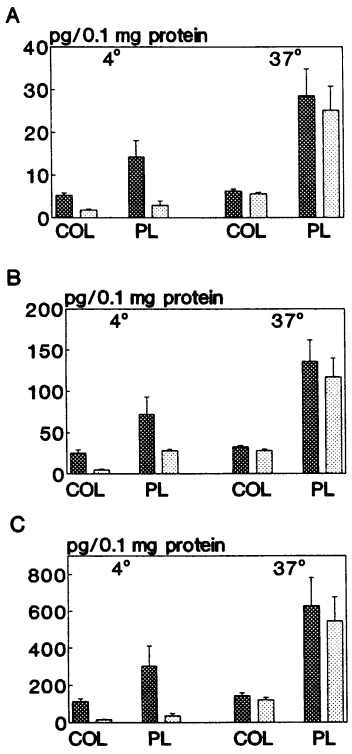

Figure 9. Surface binding and internalization of [^{125}I]-EGF. GEC, adherent to collagen (*COL*) or plastic (*PL*), were incubated with [^{125}I]-EGF at concentrations of 2 ng/ml (*A*), 10 ng/ml (*B*), or 50 ng/ml (*C*) for 30 min at 4 or 37°C. Total (*hatched bars*) cell-bound [^{125}I]EGF represents surface-bound (acid-extractable) [^{125}I]EGF + internalized (*dotted bars*) [^{125}I]EGF. Values are mean±SEM of four experiments performed in triplicate. Significant differences in total EGF binding and internalized EGF between collagen and plastic were present in the incubations at 37°C at the three concentrations of EGF ($P < 0.001$, ANOVA; $P < 0.015$ collagen vs plastic). Differences at 4°C were not significant.

ence of 1.7-fold; Fig. 7 *A*). To exclude that this upward trend in EGF binding accounted for the greater amount of internalized EGF in cells on plastic, we reanalyzed the differences in intracellular EGF between the two substrata, after reducing the values of plastic-adherent GEC (shown in Fig. 8 *A*) by a factor of 1.7, and determined that the differences remained statistically significant ($P < 0.001$). We also evaluated the amounts of surface-bound and intracellular [^{125}I]EGF at 30 min, using higher concentrations of EGF in the incubation medium (10 and 50 ng/ml). By analogy to the results in Fig. 7 *A*, at 4°C total cell-associated EGF tended to be higher in GEC adherent to plastic, as compared with collagen, at all concentrations of EGF, but the differences were not statistically significant (Fig. 9, *A–C*). In all groups of cells, most of the EGF appeared to be on the cell surface, and only a small portion of the EGF was intracellular, indicating that there was minimal internalization of EGF-R (Fig. 9). When cells were incubated at 37°C, total cell-associated EGF increased slightly in collagen-adherent GEC (as compared with 4°C), while a marked rise was noted in GEC on plastic. Furthermore, by analogy to Fig. 8 *A*, ≥ 85% of total EGF was intracellular (Fig. 9, *A–C*). The approximately four-fold differences in intracellular EGF between GEC on collagen and plastic suggest that internalization of ligand–receptor complexes was greater in GEC on plastic. Also, it should be noted that accumulation of intracellular EGF was not affected by vanadate (data not shown).

Although the results in Figs. 8 *A* and 9 suggest that adhesion of GEC to ECM most likely reduced internalization of EGF–EGF-R complexes, it is possible, however, that the lower amount of intracellular EGF in collagen-adherent GEC was due to increased receptor degradation or enhanced recycling to the cell surface rather than reduced internalization. To test for this possibility, GEC were preloaded with [^{125}I]EGF, and the fate of intracellular EGF was then monitored at serial time intervals. When expressed as a proportion of initial intracellular EGF, ~ 75% of this EGF was present after 30 min, and there were no significant differences in the rate of disappearance of intracellular EGF between collagen- and plastic-adherent GEC (Fig. 8 *B*). Furthermore, at 30 min, appearance of extracellular intact or degraded EGF was similar between substrata and was relatively small (Fig. 8, *C* and *D*). Consequently, differences in EGF-R recycling or degradation did not account for the large differences in accumulation of intracellular EGF-R (Figs. 8 *A* and 9). This profile of EGF-R degradation was confirmed by analyzing EGF-R content. In these experiments, GEC on collagen or plastic were incubated in the presence of cycloheximide to prevent new receptor synthesis, with or without EGF (24). The amount of EGF-R present in GEC membranes was assessed by immunoblotting with anti–EGF-R antibody (Table III). In GEC on both collagen and plastic, there was no significant decrease in EGF-R content (i.e., no significant degradation) after 0.5, 1, and 2 h of incubation with EGF. EGF-R content declined by ~ 60% at 4 h in both groups of cells. In the absence of EGF, there was no significant EGF-R degradation at 2 h (Table III). These experiments also indicate that the failure to observe EGF-R phosphorylation in GEC on plastic (30-min incubation with EGF at 37°C; Figs. 2 and 4 *A*) was not due to degradation of EGF-R in these cells.

Effect of ECM on proliferation and EGF-R tyrosine kinase in WB 344 cells. To substantiate the conclusion that the effect of ECM on EGF-R activation relates to its effect on proliferation, we also studied EGF-R autophosphorylation and proliferation in the rat hepatocyte-derived cell line WB 344. These cells contain 200,000–300,000 EGF-R per cell of single high affinity (14). Activation of EGF-R tyrosine kinase has been well characterized in WB 344 cells, which grow on plastic substrata under

Table III. Degradation of EGF-R in GEC

		EGF-R	
EGF	Time	Collagen	Plastic
	h	%	%
	0	100	100
+	0.5	91±5	100±0
+	1.0	88±10	86±13
+	2.0	76±18	71±19
+	4.0	40±10*	37*
—	2.0	87±16	150±51

GEC, cultured in serum-poor medium for 18 h, were incubated in serum-poor medium in the presence of cycloheximide (20 μg/ml) with (+) or without (—) EGF (50 ng/ml). Membrane fractions of cells were subjected to SDS-PAGE, transferred onto nitrocellulose, and immunoblotted using anti–EGF-R antibody RK-2 (not shown). The content of EGF-R protein (160–170-kD bands) was quantitated by densitometry. Values are mean±SEM of three to four experiments, except plastic at 4 h (+ EGF), which represents the average of two experiments. In the presence of EGF, significant differences in EGF-R content were present within each group ($P < 0.001$, ANOVA), but not between the two groups (i.e., collagen and plastic). In each group, EGF-R content was decreased significantly at 4 h (* $P < 0.01$ vs time 0 h). There were no significant differences in EGF-R content in the absence of EGF.

Table IV. Effect of ECM on Proliferation of WB 344 Cells

	Cell number × 10^{-3}	
	Collagen	Plastic
Day 1	18.3±4.5	25.1±6.3
Day 2	9.2±2.0	61.7±2.3
Day 3	5.2±0.7	180.3±16.0

WB 344 cells were plated into culture wells that were coated with type I collagen gels or were uncoated (*Plastic*) in serum-poor medium containing EGF (50 ng/ml). Cell number was determined 1, 2, and 3 d after plating. EGF produced significant increases in cell number in WB 344 cells adherent to plastic as compared with collagen ($P < 0.0001$ collagen vs plastic, ANOVA). Each point represents mean±SEM of three wells.

Table V. Effect of ECM on EGF-R P-tyr Content in WB 344 Cells

	EGF-R P-tyr	
	Collagen	Plastic
Unstimulated	1.1±0.1	4.2±1.6
EGF, 1 min	1.1±0.3	21.8±1.2*
EGF, 5 min	2.2±1.6	15.7±4.0‡

The P-tyr content of EGF-R (170-kD protein; see reference 14) was measured by densitometry. Results are expressed in arbitrary units. WB 344 cells, on collagen or plastic, cultured in serum-poor medium for 18 h, were incubated without EGF (*Unstimulated*) or with EGF (50 ng/ml) for 1 or 5 min at 37°C. Cell lysates were subjected to SDS-PAGE, transferred onto nitrocellulose, and immunoblotted using anti-P-tyr antibody (not shown). Values are mean±SEM of three experiments. Significant differences were present between substrata ($P < 0.0001$, ANOVA). EGF significantly stimulated EGF-R autophosphorylation in plastic-adherent WB 344 cells after 1 and 5 min of incubation (‡ $P = 0.05$, * $P < 0.001$ EGF vs unstimulated).

standard culture conditions (14). In contrast to GEC, a progressive increase in the number of WB 344 cells was measured when the cells were adherent to plastic and cultured in serum-poor medium containing EGF (Table IV). However, when WB 344 cells were plated onto collagen gels, the cells adhered but did not proliferate (Table IV). At 24 h after plating (day 1), there were no differences in viability (trypan blue exclusion) between WB cells adherent to collagen and plastic, and the collagen-adherent cells were able to proliferate when they were placed into suspension and replated onto plastic (data not shown). A decline in the number of WB 344 cells on collagen after 24 h suggests eventual detachment (Table IV).

EGF-R tyrosine autophosphorylation was studied by subjecting WB 344 cell lysates to SDS-PAGE and immunoblotting with anti-P-tyr antibody, as described previously (14). In preliminary studies using plastic-adherent cells that had been cultured in serum-poor medium for 18 h, we observed that EGF (50 ng/ml, 37°C) stimulated tyrosine phosphorylation in a 170-kD protein, consistent with EGF-R. Phosphorylation was maximal at 1 min, declined at 5 min, and returned to basal levels at 15 min (data not shown). This profile is identical to that reported previously in this cell type (14) and is clearly distinct from GEC (Table I). We then assessed the effect of ECM on EGF-R autophosphorylation. WB 344 cells were plated onto plastic or collagen gels and were cultured in serum-poor medium for 18 h. Subsequent incubation of plastic-adherent WB 344 cells with EGF induced a fivefold increase in the P-tyr content of EGF-R at 1 min and a fourfold increase at 5 min (Table V). In contrast, EGF did not stimulate EGF-R autophosphorylation significantly in WB 344 cells adherent to collagen (Table V). Thus, as in GEC, the effect of ECM on EGF-R activation in WB 344 cells was related to the effect on proliferation, but unlike GEC the effect of ECM was inhibitory in WB 344 cells. The differences in the effects of ECM on EGF-R autophosphorylation could not be accounted for by differences in ligand binding. After 2 h of incubation at 4°C (as in reference 14), bound [^{125}I]EGF constituted 2,737±610 cpm/0.1 mg protein in plastic-adherent WB 344 cells and 3,173±415 cpm/0.1 mg protein in cells on collagen (P = not significant, three experiments). It should also be noted that immunoblotting of WB 344 cell lysates with anti-EGF-R antibody demonstrated that there were no significant differences in EGF-R protein content between collagen- and plastic-adherent cells (not shown).

Discussion

We have demonstrated that ECM modulates EGF-dependent proliferation, receptor autophosphorylation, and receptor trafficking. EGF stimulated proliferation of GEC adherent to type I collagen matrices but not to plastic substratum (Fig. 1). At physiological temperature (37°C), tyrosine autophosphorylation of EGF-R was stimulated by EGF in GEC on collagen but not plastic (Figs. 2 and 4). However, in plastic-adherent GEC, EGF-R autophosphorylation was demonstrable at 4°C and at 37°C in the presence of vanadate, an inhibitor of PTPases (9, 19). The differences in EGF-R autophosphorylation between substrata could not be accounted for by differences in ligand binding, EGF-R content, or receptor degradation (Fig. 7 *A*, Tables II and III) and appeared to be due to regulation of EGF-R tyrosine kinase activity 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 (Fig. 6). In contrast to GEC, we have demonstrated that in WB 344 cells adhesion to collagen inhibited both proliferation and EGF-R activation (Tables IV and V). Therefore, the effect of ECM on EGF-R activation is associated with the effect of ECM on cell proliferation. To our knowledge, this is the first report demonstrating that adhesion of cells to ECM can modulate EGF-R autophosphorylation. Furthermore, our findings are in keeping with a recent study, which demonstrated that adhesion of human foreskin fibroblasts to collagen matrices inhibited platelet-derived growth factor–stimulated receptor phosphorylation and proliferation as compared with fibroblasts on plastic substrata (24). Thus, it appears that the activity of other growth factor receptor tyrosine kinases may also be regulated by ECM.

EGF-R autophosphorylation sites are located in the carboxyl terminus (25). Studies that used EGF-R carboxyl-terminal deletion mutants or amino acid substitutions of tyrosines at autophosphorylation sites suggest that EGF-R kinase activity decreases with increased amounts of receptor truncation or increasing number of tyrosine substitutions (23, 26, 27). Thus, with one possible exception (28), autophosphorylation is be-

lieved to be indicative of EGF-R activation and leads to increased tyrosine kinase activity, perhaps by release of competitive inhibition of substrate binding to EGF-R. Based on the changes in EGF-R autophosphorylation, it is reasonable, therefore, to conclude that in GEC adhesion to ECM increased EGF-R tyrosine kinase activity. Further support for this conclusion is provided by the observation that EGF-dependent tyrosine phosphorylation of other cellular proteins (which are presumably EGF-R substrates) was also enhanced in GEC adherent to collagen (Figs. 2 and 4).

There are several possible mechanisms by which ECM may have modulated activation of EGF-R. Most likely, adhesion of GEC to ECM resulted in enhanced EGF-R activation by preventing dephosphorylation of the receptor by PTPase(s) (7, 8). These enzymes have been implicated in maintaining P-tyr content of intracellular proteins at low levels, and inhibition of PTPases would be expected to enhance indirectly the activity of tyrosine kinases that are stimulated through autophosphorylation. In GEC adherent to plastic, an upward trend in EGF-R autophosphorylation occurred after 10 min of stimulation with EGF, but unlike cells on collagen this was followed by loss of autophosphorylation at 30 min (Fig. 4 *A*). Loss of autophosphorylation was not evident in the presence of the PTPase inhibitor vanadate (Fig. 4 *D*). Moreover, in GEC on collagen, autophosphorylation at 30 min was approximately twofold higher in vanadate-treated cells, suggesting that maximum autophosphorylation had not been obtained in untreated cells. Although vanadate may have effects on biochemical processes other than PTPase inhibition (19), these other reported effects are unlikely to affect the EGF-R. EGF-R autophosphorylation in GEC on plastic was also enhanced when cells were incubated with EGF at 4°C (Fig. 4 *C*). Thus, low temperature may have directly inhibited PTPase activity or may have blocked internalization of EGF-R into endosomes, preventing accessibility of the receptor to PTPase(s) (discussed below). Furthermore, when GEC containing tyrosine-phosphorylated EGF-R were incubated at 37°C, EGF-R was dephosphorylated more rapidly in plastic-adherent GEC than in GEC on collagen (Fig. 5). There appear to be two types of PTPases, transmembrane receptor–like proteins and cytoplasmic enzymes with domains that may direct subcellular distribution and attachment to membrane lipids or receptors (7, 8). The extracellular domains of some transmembrane PTPases contain regions of similarity with fibronectin (7, 8), suggesting that the activity of these PTPases might be regulated by cell–ECM or cell–cell interactions. Thus, in GEC, collagen might interact directly with the extracellular domain of a PTPase and might diminish PTPase activity of the cytoplasmic domain of the molecule. Alternatively, PTPase activity or PTPase accessibility to EGF-R might be regulated further "downstream." For example, adhesion of cells to ECM may induce changes in cell shape, cytoskeletal structure, or distribution of intracellular enzymes, which may lead to alterations in physiological processes (2) such as receptor internalization into endosomes (see below). It should be noted that an endosomal PTPase appears to be responsible for dephosphorylating EGF-R in rat liver (9); consequently, internalization of EGF-R into endosomes may render the receptor accessible to dephosphorylation and inactivation. The EGF-R–directed PTPase in liver endosomes appears to be anchored firmly to endosomal membranes and it is not recognized by antisera to PTPases that have been identified so far (9). Further studies will be required to characterize PTPase activities and their location in GEC, as well as cell-surface adhesion molecules that may potentially regulate the relevant PTPases.

The proposed mechanism for EGF-R dephosphorylation in liver endosomes (9) may be applicable to the regulation of EGF-R kinase activity in GEC. Our results demonstrate that EGF-R internalization was reduced in GEC adherent to collagen as compared with plastic (Figs. 8 and 9). In the absence of EGF, it is believed that unoccupied EGF-R are distributed diffusely on the cell surface. After binding EGF, EGF-R oligomerize, become activated, and then accumulate in coated pits, followed by internalization within endosomes (5). Internalization of ligand–receptor complexes may lead to inactivation of EGF-R kinase activity; ligand and receptor may undergo degradation in lysosomal compartments, or, alternatively, inactive EGF-R may recycle back to the plasma membrane (21, 23, 29). Intrinsic properties of EGF-R, including the presence of a cytoplasmic internalization domain (30), are at least in part responsible for directing EGF-R trafficking. Also, in several cell lines, it appears that the rate of EGF-R internalization correlates with the amount of receptor kinase activity, although there has been debate about whether EGF-R kinase activity and autophosphorylation determine the rate of ligand-induced internalization versus the rate of EGF-R recycling (21, 23, 27, 30). In our study, the amount of EGF-R internalization was related inversely to autophosphorylation, suggesting that factors other than EGF-R kinase activity influenced EGF-R internalization in GEC. Thus, the amount of EGF-R internalization appeared to be determined primarily by signals from ECM, perhaps via the cytoskeleton. This view is supported by the recent demonstration that EGF-R is an actin-binding protein (31) and that EGF-R trafficking may be associated with receptor–cytoskeleton interactions (32, 33). By analogy to EGF-R metabolism in the liver (9), the increased rate of EGF-R internalization in GEC on plastic may have rendered the receptor more accessible to an EGF-R–directed endosomal PTPase, with consequent dephosphorylation. Of interest, a recent study (34) showed that EGF-dependent proliferation and metabolism of EGF were altered in heparin-treated GEC and suggested that the heparan sulfate component of ECM may accelerate EGF-R internalization and degradation.

In addition to PTPases, other possible mechanisms by which ECM may have modulated EGF-R activation should also be considered. EGF-R kinase activity is known to be regulated by the composition of plasma membrane lipids, e.g., glycosphingolipids and lysophospholipids (35). We have shown previously that adhesion of GEC to ECM stimulates phospholipases and phospholipid turnover (13). Thus, changes in phospholipase activity might modify the membrane lipid microenvironment of EGF-R and enhance its activation (35). Phosphorylation of EGF-R via protein kinase C (on threonine-654) blocks EGF-R tyrosine kinase activity (4) and is unlikely, therefore, to explain the effects of ECM on EGF-R. Finally, some growth factors are known to bind to ECM components (3). This allows ECM to concentrate these growth factors at their site of action, or, alternatively, ECM may prevent proteolytic degradation of growth factors, thereby leading to a higher growth factor concentration. However, this mechanism did not account for the enhanced effect of EGF in GEC adherent to ECM since we could not demonstrate binding of EGF to collagen.

While we have demonstrated that collagen I enhances EGF-R activation, it should be noted that contact of GEC with colla-

gen I in vivo is limited generally to pathological conditions (e.g., glomerular inflammation); under normal circumstances, GEC are adherent to collagen IV. In cultured GEC, collagens I and IV exert similar effects on DNA synthesis (13). Unfortunately, it was not possible to assess the effect of collagen IV on EGF-R activation because it is not feasible to produce collagen IV in amounts sufficient for such experiments. Second, while both EGF-dependent proliferation of GEC and EGF-R activation were facilitated by adhesion to ECM, the enhancement of EGF-R activation may not be the sole mechanism by which ECM promotes GEC proliferation. Although not as potent as EGF, FCS (which does not contain EGF) can induce proliferation of GEC (13, 16), and the mitogenic effect of FCS is also facilitated by contact of GEC with ECM (13). Thus, other signals from ECM may also be involved in facilitating proliferation. For example, it has been reported that adhesion of cells to substrata can directly induce expression of early growth-response genes (36). It should also be noted that, unlike EGF-R activation, other pathways in GEC are not affected by contact with ECM. For example, endothelin-1 synthesis does not differ between GEC adherent to collagen and plastic (37).

One can speculate on how the effects of ECM on EGF-R might regulate GEC proliferation in vivo. Under normal conditions, there appears to be little turnover of GEC, and there is a low concentration of epithelial growth factors in glomeruli. However, proliferation of GEC may occur in pathological states, such as experimental membranous nephropathy in rats (Heymann nephritis) (38). After initial injury of GEC by the complement membrane attack complex, positive immunostaining for proliferating cell nuclear antigen was demonstrated in GEC, in the absence of inflammatory cell infiltrate (38). At present, the growth factors responsible for this proliferative response have not been defined. In other forms of glomerulonephritis, glomeruli may become infiltrated with inflammatory cells, e.g., macrophages, or platelets, which are sources of epithelial growth factors, including TGFα and EGF (39). In addition, 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 facilitate enhanced GEC proliferation. Thus, our observations may provide further insight into the mechanisms of cell proliferation after glomerular injury.

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