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Complement C5b-9 Induces Receptor Tyrosine Kinase Transactivation in Glomerular Epithelial Cells

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In the passive Heymann nephritis (PHN) model of membranous nephropathy, C5b-9 induces glomerular epithelial cell (GEC) injury and proteinuria, which is partially mediated via production of eicosanoids. Using rat GEC in culture, we demonstrated that sublytic C5b-9 induced tyrosine phosphorylation of the epidermal growth factor receptor (EGF-R), Neu, fibroblast growth factor receptor-2, and hepatocyte growth factor receptor. In addition, C5b-9 stimulated increases in tyrosine^{204} phosphorylation of extracellular signal-regulated kinase-2 (ERK2), as well as free [^{3}H]arachidonic acid (AA) and prostaglandin E_2 (PGE_2). Phosphorylated EGF-R bound the adaptor protein, Grb2, and the EGF-R-selective tyrphostin, AG1478, blocked the C5b-9-induced ERK2 phosphorylation, [^{3}H]AA release, and PGE_2 production by 45 to 65%, supporting a functional role for EGF-R kinase in mediating the activation of these pathways. Glomeruli isolated from rats with PHN demonstrated increases in ERK2 tyrosine^{204} phosphorylation and PGE_2 production, as compared with glomeruli from control rats, and these increases were partially inhibited with AG1478. Thus, C5b-9 induces transactivation of receptor tyrosine kinases, in association with ERK2 activation, AA release, and PGE_2 production in cultured GEC and glomerulonephritis *in vivo*. Transactivated tyrosine kinases may serve as scaffolds for assembly and/or activation of proteins, which then lead to activation of the ERK2 cascade and AA metabolism. *(Am J Pathol 1999, 155:1701–1711)*

The complement system plays an important role in mediating inflammation, cytolysis, and phagocytosis. Activation of the complement cascade near a cell surface leads to assembly of terminal components, exposure of hydrophobic domains, and insertion of the C5b-9 membrane attack complex into the lipid bilayer of the plasma membrane.[1,2] Assembly of C5b-9 results in formation of transmembrane channels or rearrangement of membrane lipids with loss of membrane integrity. Nucleated cells require multiple C5b-9 lesions for lysis, but at lower doses, C5b-9 induces sublytic injury and various metabolic effects. These may include a rise in cytosolic Ca^{2+} concentration, alterations in membrane lipids and proteins, activation of phospholipases, protein kinases, and G-proteins, and induction of growth factors and proliferation.[1–7]

The C5b-9 membrane attack complex induces injury in diverse renal glomerular diseases. For example, in the rat passive Heymann nephritis (PHN) model of membranous nephropathy, impairment of glomerular capillary wall permselectivity (proteinuria) is mediated by antibody and C5b-9.[8,9] The primary target of C5b-9 is the visceral glomerular epithelial cell (GEC), which suffers noncytolytic injury.[8,9] C5b-9 also stimulates production of glomerular eicosanoids, including prostaglandin E_2 (PGE_2) and thromboxane A_2,[10–12] and the latter may enhance proteinuria in certain models of membranous nephropathy.[11–14] This effect of thromboxane A_2 may be through an increase in glomerular transcapillary pressure, which appears to be responsible for a portion of the enhanced urine protein excretion.[15,16]

We have used well differentiated rat GEC in culture to define biochemical pathways that are activated by sublytic C5b-9. By analogy to GEC *in vivo*, in cultured GEC, sublytic C5b-9 injures plasma membranes.[17] Sublytic C5b-9 also increases cytosolic Ca^{2+} concentration,[3,18] stimulates phospholipases C, including phospholipase C-γ1, which leads to production of inositol phosphates and 1,2-diacylglycerol,[3,19,20] stimulates activities of protein kinase C (PKC) and extracellular signal-regulated kinase-2 (ERK2),[20] activates cytosolic phospholipase A_2 ($cPLA_2$), and releases arachidonic acid (AA) and eicosanoids.[18,20–22] Our studies suggest that the functional consequences of $cPLA_2$ activation include production of eicosanoids and exacerbation of complement-induced GEC injury.[22] The functional role of ERK2 activation remains to be determined.

Although we and others have characterized multiple signaling pathways that are activated by C5b-9, less is known about plasma membrane-associated events triggered by C5b-9 assembly, or the activation of very early steps in these pathways. There is no known transmembrane receptor for C5b-9, however, in B cell lines, the

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C5b-9 complex is reported to couple to heterotrimeric G-proteins, which can activate downstream effectors.[5] The present study addresses another mechanism by which assembly of C5b-9 may lead to the activation of protein kinases and phospholipases, ie, the transactivation of receptor tyrosine kinases (RTKs). Generally, RTKs function as transmembrane receptors for peptide ligands such as growth factors and contain an intrinsic tyrosine kinase as a part of their cytoplasmic domains.[23–25] It is believed that binding of a peptide ligand to the extracellular domain of the corresponding RTK leads to an increase in tyrosine kinase activity, in association with phosphorylation of the receptor on multiple cytoplasmic tyrosine residues, and phosphorylation of substrate proteins.[23–25] The signal may then be transmitted to nuclear or cytoplasmic effectors through a series of adaptor molecules and serine/threonine protein kinases known collectively as the mitogen-activated protein kinase pathway.[24,25] Alternatively, enzymes, including phospholipase C-γ1, can associate with the phosphorylated RTK and undergo activation.[24,25] Recently, it has been reported that the epidermal growth factor receptor (EGF-R) tyrosine kinase can be activated not only by EGF, but also, in the absence of natural ligand, via transactivation by G-protein-coupled receptors.[26–28] Using rat GEC in culture, we demonstrated that early signals elicited by C5b-9 assembly in the plasma membrane include tyrosine phosphorylation of various RTKs, including EGF-R. Inhibition of the EGF-R tyrosine kinase, in part, abolishes C5b-9-induced activation of ERK2 and $cPLA_2$ and production of PGE_2. Furthermore, we extended these results in cultured GEC to C5b-9-mediated injury *in vivo* by demonstrating that ERK2 activity and PGE_2 synthesis are increased in glomeruli of rats with PHN and that these increases are partially blocked after inhibition of the EGF-R tyrosine kinase.

Materials and Methods

Materials

Tissue culture reagents were obtained from Life Technologies (Burlington, ON). C8-deficient serum and purified C8 were purchased from Quidel (San Diego, CA). Lipids, phorbol myristate acetate (PMA), digitonin, AG1478, PGE_2, and anti-PGE_2 antiserum were purchased from Sigma Chemical Co. (St. Louis, MO). EGF, basic fibroblast growth factor (bFGF), and hepatocyte growth factor were from Collaborative Research (Bedford, MA). PP1 was from Biomol Research Laboratories (Plymouth Meeting, PA), and RG50864 (AG213) was from Rhone-Poulenc Rorer (Collegeville, PA). Anti-phosphotyrosine monoclonal antibody, PY20, was from Transduction Laboratories (Lexington, KY). Rabbit antibodies to Neu, FGF-R2, Met, and ERK2 and agarose-conjugated glutathione S-transferase (GST)-Grb2-(1–217) fusion protein were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phosphoERK antibody was purchased from New England Biolabs (Mississauga, ON). Rabbit anti-EGF-R antibody, RK2, and sheep anti-rat Fx1A were described previously.[29,30] [^{3}H]AA (100 Ci/mmol), and [^{3}H]PGE_2 (200 Ci/mmol) were from New England Nuclear (Boston, MA). Electrophoresis and immunoblotting reagents were from Bio-Rad Laboratories (Mississauga, ON). Male Sprague-Dawley rats were purchased from Charles River Canada (St. Constant, PQ).

Cell Culture and Transfection

Rat GEC culture and characterization have been published previously.[17] GEC were cultured in K1 medium on plastic substratum. Experiments measuring free [^{3}H]AA used GEC that stably overexpress $cPLA_2$. Parental GEC or GEC that express the neomycin-resistance gene (neo) were generally used in other experiments. Production and characterization of these cell lines were described previously.[18,22]

Incubation of GEC with Complement

The standard protocol involved incubation of GEC in monolayer culture with rabbit anti-GEC antiserum (5–10% v/v) in modified Krebs-Henseleit buffer containing 145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L $MgSO_4$, 1 mmol/L Na_2HPO_4, 0.5 mmol/L $CaCl_2$, 5 mmol/L glucose, and 20 mmol/L Hepes, pH 7.4, for 40 minutes at 22°C.[3,4,19–22] GEC were then incubated at 37°C with normal human serum (2.5% v/v in Krebs-Henseleit buffer), or with heat-inactivated (decomplemented) human serum (56°C) in controls. In some experiments, antibody-sensitized GEC were incubated with 2.5% C8-deficient human serum (C8DS) supplemented with purified C8 (80 μg/ml undiluted serum), or with 2.5% C8DS alone in controls. We have generally used heterologous complement to facilitate studies with complement-deficient sera and to minimize possible signaling via complement-regulatory proteins; however, in previous studies, results of several experiments were confirmed with homologous (rat) complement.[18] Sublytic concentrations of complement (<5% normal human serum) were established previously.[22] In GEC, complement is not activated in the absence of antibody.[17]

Sublytic complement cytotoxicity was monitored by measuring release of biscarboxyethyl carboxyfluorescein (BCECF) as detailed previously.[17,22] Complement lysis was determined by measuring release of lactate dehydrogenase, similarly to the method described previously.[17]

Induction of PHN

PHN was induced by a single intravenous injection of 0.4 ml of sheep anti-rat Fx1A antiserum.[30] Urine was collected overnight at day 13–14 postinjection. Glomeruli were isolated by differential sieving on day 14[30] and were suspended in modified Krebs-Henseleit buffer for experiments.

Tyrosine Phosphorylation

After incubation with antibody and complement, ~6 × 10^6 GEC were lysed, and proteins were immunoprecipi-

tated with primary rabbit antisera directed to specific RTKs, as described previously.[25] After immunoprecipitation, immune complexes were incubated with agarose-coupled protein A. For analysis of GST-Grb2-associated proteins, ~2 × 10^7 GEC were lysed and incubated with agarose-conjugated GST-Grb2 fusion protein (4 μg) for 3 hours at 4°C. Complexes were boiled in Laemmli sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteins were then electrophoretically transferred onto nitrocellulose paper, blocked with 3% bovine serum albumin/2% ovalbumin, and incubated with antibody to phosphotyrosine. Blots were developed using alkaline phosphatase-conjugated secondary antibody and colorimetric detection.[31] To assess ERK2 activation, ~1 × 10^6 GEC were lysed and 25 μg of lysate were subjected to SDS-PAGE. Activation of ERK2 was monitored by immunoblotting with anti-phosphoERK antibody, ie, antibody that reacts with ERK phosphotyrosine[204]. RTK tyrosine phosphorylation and ERK2 tyrosine[204] phosphorylation were quantitated by densitometry, as described previously.[29] Tyrosine phosphorylation of glomerular proteins was monitored in an analogous manner. Immunoblotting for RTK or ERK2 proteins was carried out as described above, except that blots were developed with RTK- or ERK2-specific antibodies.

Measurement of Free [^{3}H]AA

GEC phospholipids were labeled to isotopic equilibrium with [^{3}H]AA for 48 to 72 hours, as detailed previously.[3,18–22] Lipids were extracted from ~1 × 10^6 cells and cell supernatants. Methods for extracting and separating radiolabeled lipids by thin layer chromatography are published.[3,18–22]

Measurement of PGE_2

PGE2 was measured by radioimmunoassay in lipid extracts of GEC plus culture supernatants, or in glomerular supernatants, using anti-PGE_2 antiserum and [^{3}H]PGE_2 tracer, as described previously,[7] and according to the manufacturer's protocol (Sigma). For GEC, [^{3}H]PGE_2 (1000 cpm) was added just before extraction to correct for extraction efficiency. Briefly, samples were incubated with [^{3}H]PGE_2 and anti-PGE_2 antibody for 1 hour at 4°C, after which unbound PGE_2 was removed by the addition of activated charcoal. The radioactivity of the supernatant was counted in a β-scintillation counter, and PGE_2 concentration was calculated from standard formulas. The range of the standard curve in the assay was 15 to 500 pg of PGE_2 per 100-μl sample.

Statistics

Data are presented as mean ± SE. The *t* statistic was used to determine significant differences between two groups. For more than two groups, 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.

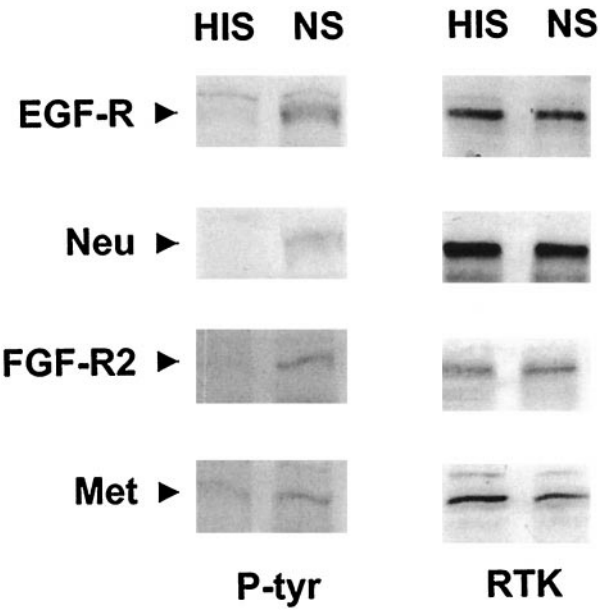

Figure 1. Complement stimulates tyrosine phosphorylation of RTKs in GEC. GEC were incubated with anti-GEC antibody and normal serum (NS; to form C5b-9) for 40 minutes at 37°C, or heat-inactivated serum (HIS) in controls. **Left panel:** Cell lysates were immunoprecipitated with antibodies to EGF-R (170 kd), Neu (185 kd), FGF-R2 (135 kd), or Met (hepatocyte growth factor receptor; 145 kd), and were subjected to SDS-PAGE and immunoblotting with antibody to phosphotyrosine (P-tyr). **Right panel:** Cell lysates were immunoblotted with antibodies to RTKs.

Results

C5b-9 Induces Tyrosine Phosphorylation of RTKs in GEC

Sublytic C5b-9 activates phospholipases and protein kinases in cultured GEC.[18–22] To determine how sublytic C5b-9 induces activation of these enzymes, we examined if assembly of C5b-9 in the plasma membrane may result in the transactivation of RTKs, by monitoring tyrosine phosphorylation of RTKs. Compared with control incubations (heat-inactivated serum), activation of complement (normal serum) produced marked increases in tyrosine phosphorylation of EGF-R, Neu (erbB-2, an EGF-R family member),[32] FGF-R2,[33] and Met,[34] the receptor for hepatocyte growth factor (Figure 1). Quantitation of RTK tyrosine phosphorylation by densitometry (Table 1) confirmed the visual impression in Figure 1. Complement, however, had no effect on the expression of RTK proteins (Figure 1), indicating that RTK phosphorylation was most likely due to posttranslational modification.

To confirm that the normal serum-induced increase in EGF-R tyrosine phosphorylation, shown in Figure 1, was actually due to formation of C5b-9, GEC were incubated with anti-GEC antibody and 2.5% C8DS that had been reconstituted with purified C8 or with C8DS alone to form

Table 1. Effect of Complement on Phosphotyrosine Content of RTKs in GEC

RTK	Phosphotyrosine (% control)
EGF-R	204 ± 12*
Neu	384 ± 83†
FGF-R2	344 ± 153‡
Met	296 ± 61†

GEC were incubated with anti-GEC antibody and normal serum to form C5b-9, or with heat-inactivated serum in controls (as in Figure 1). Lysates were immunoprecipitated with antibodies to RTKs and immunoblotted with antibody to phosphotyrosine. Phosphotyrosine content was measured by densitometry.

*$P < 0.0001$, †$P < 0.01$, ‡$P = 0.05$ *versus* control; each group consists of 3 to 4 experiments.

the C5b-7 complex, which is inactive in GEC. In two experiments, EGF-R phosphotyrosine content was increased an average of 1.7-fold in C8DS+C8 incubations, as compared with C8DS alone.

Activated EGF-R can recruit several substrate proteins, eg, Grb2, that bind via SH2 domains to receptor phosphotyrosine residues. To determine whether complement-induced EGF-R tyrosine phosphorylation correlated with EGF-R activation, we examined if EGF-R interacted with a GST-Grb2 fusion protein. A phosphoprotein of ~170 kd associated with GST-Grb2 after incubation of GEC with complement (normal serum), but not in control incubations (Figure 2, middle panel). This phosphoprotein was recognized by anti-EGF-R antibody (Figure 2, left panel), and on SDS-PAGE, it co-migrated with a phosphoprotein that associated with GST-Grb2 after treatment of GEC with EGF (Figure 2, right panel). These data indicate that the 170-kd protein was most likely EGF-R. Figure 2 (middle panel) also demonstrates some fainter phosphoprotein bands in complement-treated cells. These phosphoproteins may represent other RTKs or RTK substrates; however, it was not possible to identify these proteins definitively, because Grb2 interactions with RTKs tend to be weak as compared with, for example, antigen-antibody interactions,[24] and in GEC, they were at the lower limit of detectability.

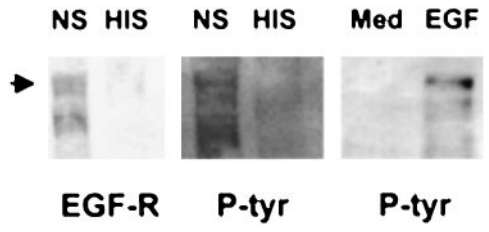

Figure 2. Complement induces Grb2 association with EGF-R. **Left** and **middle panels:** GEC were incubated with antibody and normal serum (NS), or heat-inactivated serum (HIS) in controls, as in Figure 1. **Right panel:** GEC were incubated with EGF (100 ng/ml, 60 minutes, 37°C). Cell lysates were incubated with agarose-conjugated GST-Grb2 fusion protein and subjected to SDS-PAGE and immunoblotting with antibodies to EGF-R (**left panel**) or to phosphotyrosine (P-tyr; **middle** and **right panels**). The **arrow** points to the 170-kd EGF-R. The lower band in the **left panel** probably represents an EGF-R degradation product.

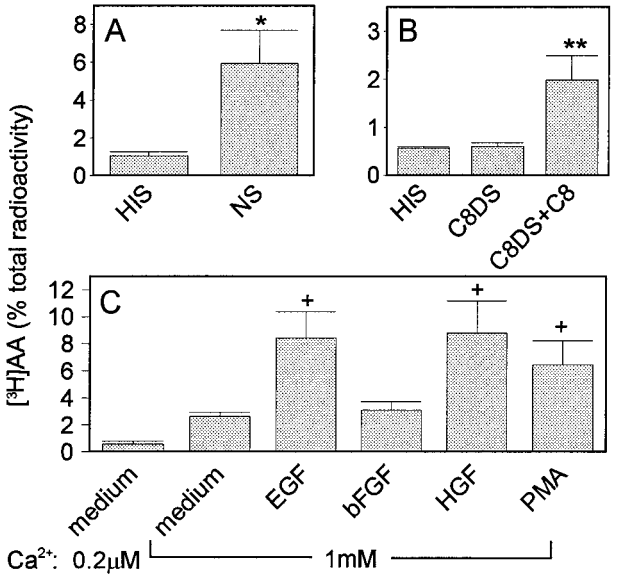

Figure 3. [^{3}H]AA release in GEC. **A:** [^{3}H]AA-labeled GEC were incubated with anti-GEC antibody and normal human serum (NS) to form C5b-9, or with heat-inactivated serum (HIS) in controls, for 40 minutes at 37°C (*$P < 0.01$ NS *versus* HIS, 5 experiments performed in duplicate). **B:** Antibody-sensitized GEC were incubated with C8DS+C8, C8DS, or heat-inactivated serum for 40 minutes at 37°C. Significant differences were present among groups (ANOVA $P < 0.03$; **$P < 0.02$ C8DS+C8 *versus* C8DS; 3 experiments performed in duplicate). **C:** GEC were incubated with medium alone, or with EGF 100 ng/ml, bFGF 50 ng/ml, or hepatocyte growth factor (HGF) 25 ng/ml for 60 minutes, or PMA 250 ng/ml for 30 minutes at 37°C. Cells were then permeabilized with medium containing digitonin and 0.2 μmol/L or 1 mmol/L free Ca^{2+} concentration. Significant differences were present among groups (ANOVA $P < 0.01$; +$P < 0.02$ EGF, HGF, or PMA *versus* medium with 1 mmol/L [Ca^{2+}]; 3 experiments performed in duplicate). It should be noted that EGF and PMA do not increase free [^{3}H]AA when the free Ca^{2+} concentration is maintained at 0.2 μmol/L.[20]

Role of RTKs in C5b-9-Stimulated [^{3}H]AA and PGE_2 Release in GEC

In previous studies, we demonstrated that C5b-9 increases free [^{3}H]AA via activation of $cPLA_2$. Using GEC that stably overexpress $cPLA_2$, we showed that $cPLA_2$ activation is dependent on a rise in cytosolic Ca^{2+} concentration and on the activation of PKC, but is independent of the Ras-ERK pathway.[18,20,22] In keeping with previous results, we demonstrate that incubation of antibody-sensitized GEC with normal serum stimulated a marked increase in free [^{3}H]AA as compared with heat-inactivated serum (Figure 3A). By analogy, incubation with C8DS (C5b-7) had no significant effect on basal levels of [^{3}H]AA, but when C8DS was reconstituted with purified C8, free [^{3}H]AA increased markedly (Figure 3B). Complement-induced release of [^{3}H]AA release occurred within 30 minutes, and elevated levels of free [^{3}H]AA persisted for at least 3 hours (Figure 4). In these experiments (and in studies of ERK2 activation, described below), C8DS+C8 and normal serum were used at the same final concentrations (2.5% v/v); however, the effects of C8DS+C8 on [^{3}H]AA release were generally less potent than those of normal serum. Studies carried out to assess sublytic GEC injury, ie, BCECF release,[17,22] demonstrated that specific BCECF release was lower with C8DS+C8, as compared with normal serum (Table

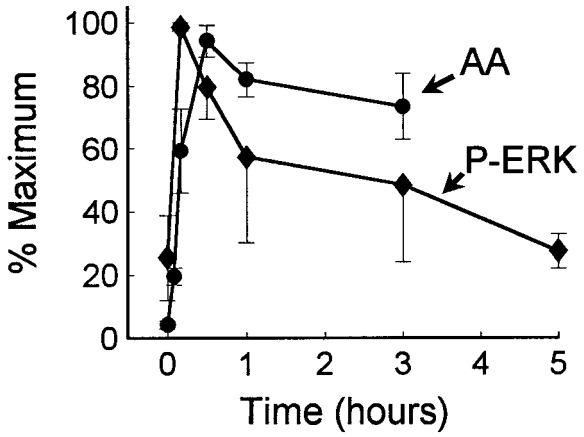

Figure 4. Kinetics of [^{3}H]AA release and ERK2 tyrosine^{204} phosphorylation (P-ERK). GEC were incubated with antibody and complement for up to 5 hours. ERK2 phosphorylation was monitored by immunoblotting with antibody to phosphoERK (phosphotyrosine^{204}), and was quantitated by densitometry (see Figure 6).

2A), indicating that the C8-reconstituted C8DS had less complement activity than normal serum. Thus, the more potent [^{3}H]AA release by normal serum could be accounted for by greater complement activity.

To determine whether C5b-9-induced activation of $cPLA_2$ occurred secondary to RTK transactivation, we studied [^{3}H]AA release in the presence of the tyrphostin, AG1478, a compound that is a highly selective inhibitor of the EGF-R tyrosine kinase.[26–28,35] C5b-9-induced [^{3}H]AA release was inhibited significantly with AG1478, 300 nmol/L (Table 3), supporting the involvement of the EGF-R kinase in this pathway. To further evaluate the role of protein kinases in $cPLA_2$ activation, we previously developed a system to study release of [^{3}H]AA in GEC

Table 2. GEC Cytotoxicity

A	BCECF-specific release (%)		
	5% vol/vol	2.5% vol/vol	1.25% vol/vol
Normal serum	77 ± 4*	52 ± 4*	20 ± 3*
C8DS+C8	55 ± 7	17 ± 7	9 ± 5

B	LDH-specific release (%)		
	10% vol/vol	5% vol/vol	2.5% vol/vol
Normal serum	61 ± 9	17 ± 8	0 ± 0
Normal serum + AG1478	67 ± 11	19 ± 7	0 ± 0

A: GEC were incubated with BCECF-acetoxymethyl ester and then incubated with either antibody and complement (normal serum or C8DS+C8) or heat-inactivated serum in controls. Supernatants were collected and cells were permeabilized with digitonin to release remaining BCECF. BCECF content was measured by spectrofluorometry. Specific release is calculated as $(E - C)/(100 - C)$, where E is the percent released in complement-treated cells and C is the percent released in control incubations (heat-inactivated serum). Significantly greater BCECF release was present in normal serum groups, as compared with C8DS+C8 groups (*$P < 0.0001$ ANOVA; 3 experiments).

B: GEC were incubated with or without AG1478, 300 nmol/L and with antibody and normal serum, or with heat-inactivated serum in controls. Supernatants were collected and cells were permeabilized with digitonin to release remaining lactate dehydrogenase (LDH). LDH activity was assayed in supernatants and digitonin-treated cells, and specific release was calculated as above. There are no significant differences between AG1478-treated and untreated groups (4 experiments).

Table 3. Effect of Tyrosine Kinase Inhibitors or EGF-R Down-Regulation on [^{3}H]AA Release in GEC

Stimulus	Inhibitor	Inhibition (%)	*N*
Complement	AG1478, 300 nmol/L	45 ± 10*	5
	AG1478, 30 nmol/L	22 ± 18	3
	RG50864, 5 μmol/L	−19 ± 5#	3
	PP1, 10 μmol/L	−21 ± 9	3
	EGF-R down-regulation	60 ± 10*	5
EGF	AG1478, 300 nmol/L	100 ± 0+	3
	AG1478, 30 nmol/L	71 ± 14++	3
	AG1478, 3 nmol/L	13 ± 17	3
	EGF-R down-regulation	68 ± 13*	4
HGF	AG1478, 300 nmol/L	−8 ± 36	6
PMA	AG1478, 300 nmol/L	0 ± 3%	3
	EGF-R down-regulation	26 ± 10**	4

[^{3}H]AA-labeled GEC were either untreated or preincubated with AG1478, RG50864, or PP1 for 15 minutes at 37°C. To down-regulate EGF-R, GEC were preincubated with EGF (100 ng/ml) for 24 hours. GEC were then incubated with complement, EGF, hepatocyte growth factor (HGF), or PMA. The cells incubated with EGF, HGF, or PMA were permeabilized with buffer containing digitonin and 1 mmol/L free Ca^{2+} (as in Figure 3). Inhibition of [^{3}H]AA release is determined by comparing stimulated increases in [^{3}H]AA with and without tyrosine kinase inhibitor or EGF-R down-regulation.

*$P < 0.015$, +$P < 0.0001$, ++$P < 0.02$, **$P < 0.05$ *versus* untreated.

#negative values indicate a trend toward stimulation in the presence of inhibitor.

N, number of experiments.

permeabilized with digitonin at clamped $[Ca^{2+}]$.[20] At 0.2 μmol/L free $[Ca^{2+}]$, levels of free [^{3}H]AA were low in GEC (Figure 3C). In contrast, when free $[Ca^{2+}]$ was clamped at 1 mmol/L, [^{3}H]AA release was stimulated (Figure 3C). This stimulation was enhanced greatly by incubation of GEC with EGF, hepatocyte growth factor, or PMA, but not bFGF (Figure 3C). As expected, AG1478 inhibited EGF-dependent [^{3}H]AA release (Table 3). AG1478 was more effective in inhibiting EGF-dependent [^{3}H]AA release, as compared to the activation by complement; however, it should be recognized that complement-dependent [^{3}H]AA release may be mediated via EGF-R and also through other RTKs that would not be susceptible to AG1478 inhibition, eg, Met and possibly others (Figure 1). So far, there are no known specific inhibitors of Met. AG1478 had no effect on hepatocyte growth factor-induced or PMA-induced [^{3}H]AA release, which occur independently of EGF-R (Table 3), suggesting that the inhibitory effect of AG1478 in these short-term experiments was not simply cytotoxic or nonspecific. Furthermore, it was demonstrated that AG1478 had no significant effect on complement-mediated lysis of GEC (lactate dehydrogenase release; Table 2B). This result confirms that AG1478 was not toxic; in addition, it is unlikely that the inhibitory effect of AG1478 on complement-mediated [^{3}H]AA release could have been due to impairment of C5b-9 assembly.

Another series of experiments was carried out to provide additional support for the role of EGF-R in mediating C5b-9-induced [^{3}H]AA release. First, to further verify the specificity of AG1478 for EGF-R, we tested the effects of two other tyrosine kinase inhibitors on complement-induced [^{3}H]AA release. The tyrphostin, RG50864, is related to AG1478, but is ~300-fold less potent in inhibiting

EGF-R, as compared with AG1478.[35] At a concentration of 5 μmol/L (17-fold greater than AG1478), RG50864 had no significant effect on complement-induced [^{3}H]AA release (Table 3), unlike AG1478. PP1 is an inhibitor of cytoplasmic tyrosine kinases of the Src family, and is reported to inhibit Src at a concentration of 10 μmol/L.[27] At this concentration, PP1 had no significant effect on complement-induced [^{3}H]AA release (Table 3). Based on these results, it is unlikely that the inhibitory effect of AG1478 was due to cross-reactivity with Src family kinases. Second, another way to abrogate effects mediated by EGF-R is to down-regulate the receptor before stimulation of cells. Prolonged preincubation of GEC with EGF down-regulates EGF-R expression.[36] Consequently, after down-regulation of EGF-R in GEC, the acute release of [^{3}H]AA by EGF was inhibited significantly (Table 3). EGF-R down-regulation also produced a large decrease in the complement-induced release of [^{3}H]AA (Table 3), in keeping with the effect of AG1478. The stimulating effect of PMA on free [^{3}H]AA was not expected to decrease after EGF-R down-regulation, but we observed that EGF-R down-regulation actually led to a modest reduction in the PMA-induced release of [^{3}H]AA (Table 3). Possibly, the down-regulation of EGF-R by prolonged incubation with EGF also led to partial down-regulation of EGF-R downstream effectors, including PKC,[20] the target of PMA. Partial down-regulation of EGF-R effectors may also explain the greater potency of EGF-R down-regulation in inhibiting complement-mediated [^{3}H]AA release, as compared with AG1478, which would not be expected to affect EGF-R effectors (Table 3).

Incubation of GEC for 18 hours with PMA (2 μg/ml) leads to complete depletion (down-regulation) of PKC.[20] In keeping with previous results,[20] C5b-9-mediated release of [^{3}H]AA was inhibited by 67 ± 5% in GEC depleted of PKC ($P < 0.005$, 3 experiments). Treatment of PKC-depleted GEC with AG1478 (300 nmol/L) had no additional inhibitory effect on complement-mediated release of [^{3}H]AA (75 ± 4% inhibition; 3 experiments, *P* not significant). Together, the results suggest that complement-induced transactivation of EGF-R and activation of PKC occur within the same pathway, but because PKC-induced release of [^{3}H]AA was insensitive to AG1478, PKC activation occurs downstream of EGF-R.

Release of AA due to C5b-9-mediated activation of $cPLA_2$ is coupled to production of PGE_2.[22] These results were confirmed in the present study (Figure 5). In addition, we demonstrate that AG1478 blocked the complement-mediated increase in PGE_2, in keeping with its effect on AA release (Figure 5).

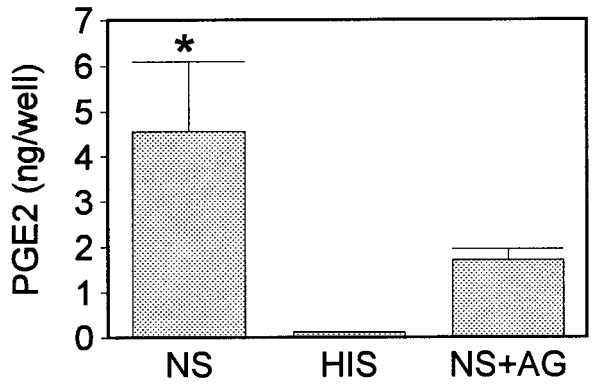

Figure 5. PGE_2 production in GEC. GEC were preincubated with or without AG1478 (AG), 300 nmol/L, for 15 minutes at 37°C, and then incubated with antibody and normal serum (NS) to assemble C5b-9 or with heat-inactivated serum (HIS) in controls (40 minutes at 37°C). PGE_2 was measured in cell extracts plus supernatants. Significant differences were present among groups (ANOVA $P = 0.01$; $^*P < 0.003$ NS *versus* HIS, $P = 0.04$ NS *versus* NS+AG1478; 6 experiments performed in duplicate).

Role of RTKs in C5b-9-Mediated ERK2 Phosphorylation in GEC

In a previous study, we used an immune complex kinase assay to demonstrate that sublytic C5b-9 stimulates ERK2 activity, as well as ERK2 tyrosine phosphorylation.[20] In keeping with these results, the present study shows that C5b-9 induced ERK2 tyrosine[204] phosphorylation, which correlates with ERK2 activation (Figure 6, Table 4). Incubation of GEC with antibody and C8DS reconstituted with purified C8 induced ERK2 tyrosine[204] phosphorylation, whereas in incubations with C8DS alone (C5b-7), ERK2 phosphorylation was weak, comparable to basal levels observed in unstimulated GEC (Figure 6A). The effect of the exogenous PKC activator, PMA, on ERK2 phosphorylation was greater than the effect of C8DS+C8 (Figure 6A). By analogy, incubation of antibody-sensitized GEC with normal serum markedly increased ERK2 phosphorylation, as compared with heat-inactivated serum (Figure 6B and Table 4). Complement-induced ERK2 tyrosine[204] phosphorylation occurred within 30 minutes, and elevated levels of free

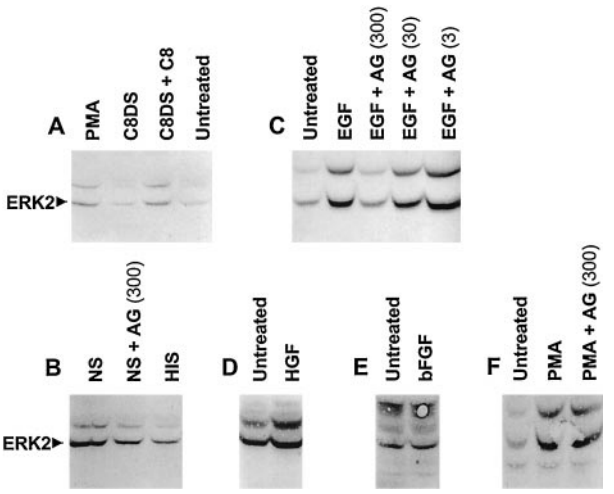

Figure 6. Effect of C5b-9 on ERK2 activation in GEC. **A:** GEC were incubated anti-GEC antibody and C8DS+C8 (to form C5b-9) or C8DS (C5b-7) for 40 minutes at 37°C. GEC that were untreated or that were incubated with PMA (250 ng/ml, 40 minutes, 37°C) are shown for comparison. **B:** GEC were incubated with anti-GEC antibody and normal serum (NS) or heat-inactivated serum (HIS) for 40 minutes at 37°C. In one group, GEC were pretreated with the EGF-R-specific tyrphostin, AG1478 (AG), 300 nmol/L, for 15 minutes at 37°C. **C-F:** For comparison, GEC were incubated with EGF (100 ng/ml, 60 minutes, 37°C), hepatocyte growth factor (HGF; 50 ng/ml, 60 minutes, 37°C), bFGF (25 ng/ml, 60 minutes, 37°C), or PMA (250 ng/ml, 30 minutes, 37°C). In some incubations, GEC were also pretreated with 3, 30, or 300 nmol/L of AG1478 (AG). ERK2 activation was monitored by immunoblotting with antibody to phosphoERK (phosphotyrosine[204]).

Table 4. ERK2 Tyrosine^{204} Phosphorylation in GEC

Stimulus	Phosphotyrosine (% control)
Normal serum	1252 ± 469*
C8DS+C8	198 ± 36*
EGF	227 ± 46†
PMA	560 ± 183‡

GEC were incubated with normal serum (heat-inactivated serum in controls), C8DS+C8 (C8DS in controls), EGF, or PMA, as in Figure 6. Cell lysates were immunoblotted with antibody to phosphoERK, and phosphotyrosine^{204} content was measured by densitometry. Each group consists of 4 experiments (*$P < 0.025$, †$P < 0.04$, ‡$P < 0.05$ *versus* control).

[^{3}H]AA persisted for ~3 hours, thereafter declining to baseline (Figure 4).

To determine whether C5b-9-induced activation of ERK2 was associated with RTK transactivation, we studied ERK2 tyrosine^{204} phosphorylation in the presence of AG1478. The C5b-9-induced activation of ERK2 was inhibited significantly with AG1478 (Figure 6B and Table 5), suggesting that transactivation of EGF-R kinase was involved in the activation of ERK2. As anticipated, activation of EGF-R by its natural ligand, EGF, stimulated ERK2 tyrosine^{204} phosphorylation (Figure 6C and Table 4). Hepatocyte growth factor also stimulated ERK2 tyrosine^{204} phosphorylation (Figure 6D), although bFGF did not activate ERK2 significantly (Figure 6E). As expected, AG1478 inhibited EGF-dependent activation of ERK2 (Figure 6C and Table 5). By analogy to [^{3}H]AA release, AG1478 was more effective in inhibiting EGF-dependent ERK2 activation, as compared to activation by complement. However, C5b-9-dependent activation of ERK2 may be mediated via EGF-R plus other RTKs not inhibited with AG1478. AG1478 had no effect on PMA-induced ERK2 tyrosine^{204} phosphorylation, which occurs independently of EGF-R (Table 5).

Production of PGE_2 in PHN

The above studies used GEC in culture to characterize biochemical pathways activated by C5b-9. However, it is also important to demonstrate that analogous pathways are activated *in vivo*, specifically, in the PHN model of membranous nephropathy, where C5b-9 assembles in GEC plasma membranes and induces injury.[8,9] Studies were carried out in rats with autologous phase PHN (day 14), which is known to be complement-mediated.[37] At the 14-day time point, rats with PHN excreted 434 ± 23 mg of urinary protein per 24 hours ($n = 12$), as compared with 12 ± 2 mg per 24 hours in normal control rats ($n = 8$, $P <$ 0.0001 PHN *versus* control).

Table 5. Effect of AG1478 on ERK2 Tyrosine^{204} Phosphorylation in GEC

Stimulus	AG1478 (nmol/L)	Inhibition (%)	*N*
Complement	300	62 ± 7*	4
EGF	300	100 ± 1*	4
	30	52 ± 24†	4
	3	0	2
PMA	300	−15 ± 10‡	4

GEC were untreated or were preincubated with AG1478 for 15 minutes at 37°C, then incubated with complement, EGF, or PMA (as in Figure 6). ERK2 tyrosine^{204} phosphorylation was monitored by immunoblotting and quantitated by densitometry. Inhibition was determined by comparing stimulated increases in ERK2 tyrosine^{204} phosphorylation with and without AG1478. *$P < 0.0001$, †$P = 0.06$ *versus* untreated.

‡Negative value indicates a trend toward stimulation in the presence of AG1478.

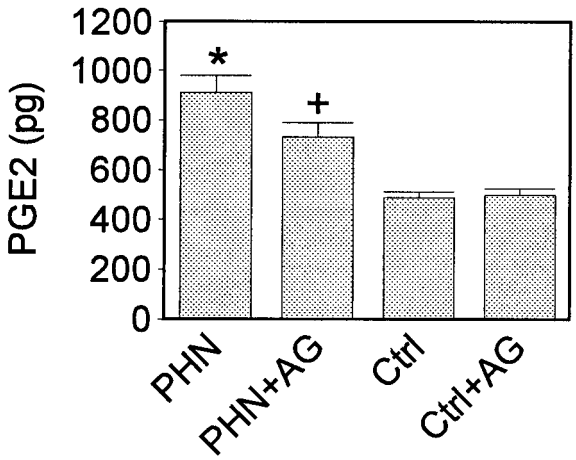

Figure 7. Glomerular PGE_2 production. Glomeruli were isolated from seven rats with PHN on day 14 and from seven control rats (Ctrl). Glomeruli from each rat were divided into two aliquots and preincubated with or without AG1478 (AG), 300 nmol/L, for 15 minutes at 37°C. The incubation buffer was changed, and incubations were continued with or without AG1478 for 30 minutes at 37°C. PGE_2 was then measured in glomerular supernatants by radioimmunoassay. Significant differences were present among groups ($P <$ 0.0001 ANOVA). *$P < 0.025$ PHN *versus* PHN+AG1478 and $P < 0.0001$ PHN *versus* control: +$P < 0.0025$ PHN+AG1478 *versus* control.

In short-term incubations, glomeruli isolated from rats with PHN synthesized eicosanoids at a rate greater than isolated normal glomeruli, indicating that the C5b-9 assembled *in vivo* remained active after glomerular isolation.[10–12] Thus, we carried out experiments to determine whether the mechanism for the C5b-9-stimulated PGE_2 production in PHN may involve the EGF-R tyrosine kinase. Glomeruli were isolated from rats with PHN on day 14 and from control rats, and were incubated briefly with or without AG1478. PGE_2 production was then measured in glomerular supernatants. PGE_2 production was greater in glomeruli from rats with PHN, as compared with controls, and was reduced significantly when glomeruli from PHN rats were incubated in the presence of AG1478 (Figure 7). AG1478 did not, however, affect PGE_2 production significantly in control glomeruli (Figure 7).

ERK2 Tyrosine^{204} Phosphorylation in PHN

Glomeruli were isolated from normal rats and from rats with PHN (day 14), and glomerular proteins were subjected to immunoblotting with anti-phosphotyrosine antibody. Multiple phosphoproteins were present in both PHN and control glomeruli. However, a band at ~42 kd was significantly more prominent in PHN glomeruli as compared with control (Figure 8A). The molecular mass of this protein suggested that it was ERK2. Glomerular proteins were then immunoblotted with anti-phosphoERK

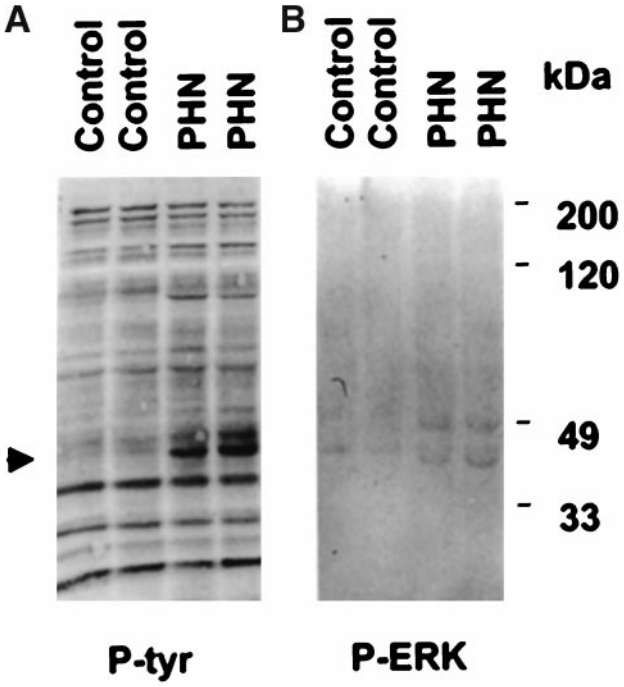

Figure 8. Glomerular protein tyrosine phosphorylation and ERK2 tyrosine^{204} phosphorylation. Glomeruli were isolated from normal (control) rats and from rats with PHN on day 14. Representative immunoblots showing two animals per group are presented. Glomerular proteins were subjected to SDS-PAGE and immunoblotting with anti-phosphotyrosine (P-tyr, **A**), or anti-phosphoERK antibodies (P-ERK, **B**). A band at 42 kd is significantly more prominent in PHN glomeruli, as compared with control (**A, arrow**). ERK2 tyrosine^{204} phosphorylation is enhanced in PHN glomeruli, as compared with control (**B**).

antibody. There was weak basal ERK2 tyrosine^{204} phosphorylation in control glomeruli, but phosphorylation was enhanced in PHN glomeruli (Figure 8B). There were, however, no differences in the expression of ERK2 protein between PHN and control glomeruli (results not shown). To confirm the visual impression in Figure 8B, glomerular ERK2 tyrosine^{204} phosphorylation and protein content, were quantitated by densitometry. The phosphoERK-to-ERK ratio was 0.23 ± 0.06 units in control glomeruli ($n = 5$) and was increased to 0.36 ± 0.04 units in PHN glomeruli ($n = 6$, $P < 0.04$ *versus* control). It should be noted that the 42-kd band identified by anti-phosphotyrosine antibody in PHN glomeruli is markedly more intense than in control glomeruli (Figure 8A), whereas the difference in the relative intensities of the bands recognized by anti-phosphoERK antibody was not as dramatic (Figure 8B). This result suggests that the 42-kd phosphoprotein may not be exclusively ERK2.

A second set of experiments was carried out to determine whether C5b-9-dependent ERK2 phosphorylation in PHN glomeruli was mediated via EGF-R. By analogy to the PGE_2 measurements (above), glomeruli were isolated from rats with PHN and from control rats, and were incubated with or without the EGF-R-specific tyrphostin, AG1478. Immunoblotting with anti-phosphoERK antibody and analysis by densitometry demonstrated that in glomeruli from PHN rats, C5b-9, assembled *in vivo*, maintained an increase in ERK2 tyrosine^{204} phosphorylation as compared with control glomeruli, but this increase was reduced significantly in the presence of AG1478 (Table 6). AG1478 did not, however, affect ERK2 tyrosine^{204} phosphorylation significantly in control glomeruli.

Table 6. Effect of AG1478 on ERK2 Tyrosine^{204} Phosphorylation in Glomeruli

Incubation	Phosphotyrosine (arbitrary units)
PHN	0.57 ± 0.02*
PHN + AG1478	0.44 ± 0.03
Control	0.43 ± 0.03
Control + AG1478	0.37 ± 0.06

Glomeruli were isolated from four rats with PHN on day 14 and from four control rats. Glomeruli from each rat were divided into two aliquots and incubated with or without AG1478, 300 nmol/L, for 45 minutes at 37°C. Lysates were subjected to SDS-PAGE and immunoblotting with anti-phosphoERK antibody as in Figure 8B. ERK2 tyrosine^{204} phosphorylation was quantitated by densitometry and is expressed in arbitrary units. Significant differences were present among groups ($P < 0.03$ ANOVA).

*$P < 0.04$ PHN *versus* PHN + AG1478; $P = 0.03$ PHN *versus* control.

Discussion

Sublytic amounts of the C5b-9 membrane attack complex may induce cell injury and various biochemical or metabolic effects.[1–7] Assembly of C5b-9 in plasma membranes of cells results in formation of transmembrane channels or rearrangement of membrane lipids; however, the mechanism by which the assembled C5b-9 complex induces activation of downstream effectors, such as protein kinases, is poorly understood. An earlier study demonstrated that C5b-9 can interact with and activate G-proteins,[5] which can then potentially couple to downstream effectors. The present study proposes a novel mechanism of C5b-9 signaling, the transactivation of RTKs. Assembly of sublytic C5b-9 in GEC resulted in tyrosine phosphorylation of EGF-R, Neu, FGF-R2 and Met, without altering RTK protein expression (Figure 1 and Table 1). These four proteins are members of three distinct RTK families.[38] In keeping with earlier results, we also demonstrated that C5b-9 induced activation of ERK2 and $cPLA_2$, the latter leading to release of AA and PGE_2. Ligand-induced tyrosine phosphorylation of RTKs typically correlates with activation,[24] and thus, it was possible the C5b-9-dependent activation of ERK2 and $cPLA_2$ may have been mediated via RTKs. Support for this conclusion was provided by experiments showing that GST-Grb2 fusion protein bound the complement-transactivated EGF-R (Figure 2), and that complement-dependent ERK2 and $cPLA_2$ activation were blocked by AG1478, a selective inhibitor of EGF-R tyrosine kinase (Figure 6, Tables 3 and 5). The possibility that AG1478 may have inhibited enzymes other than EGF-R, or acted nonspecifically cannot be excluded entirely, but is unlikely. For example, the effect of AG1478 on AA release was reproduced by down-regulating EGF-R, whereas another tyrphostin or a Src family kinase inhibitor could not reproduce this effect (Table 3). Furthermore, C5b-9 does not appear to activate Src in GEC (unpublished observations). In addition, AG1478 did not affect activation of

$cPLA_2$ by hepatocyte growth factor, nor activation of ERK2 or $cPLA_2$ by PMA, which stimulates PKC independently of RTKs (Figure 6, Table 3), and AG1478 did not impair complement activation (Table 2). Although we also considered using dominant-inhibitory mutants of EGF-R, at this stage such an approach was not practical. Because the mechanism of RTK transactivation by C5b-9 has not been established precisely (see below), it may be necessary to test various mutants targeting distinct regions of EGF-R. In addition, transfection efficiency of GEC is very low, and thus it would be necessary to develop methods that would allow stable or inducible expression of dominant-negative EGF-R at a high level without altering cell phenotype or ability to proliferate.

Further support for the role of RTKs in mediating C5b-9-dependent ERK2 activation and release of AA was provided by experiments demonstrating that the natural ligands of EGF-R and Met (ie, EGF or hepatocyte growth factor, respectively) could also activate these two pathways in GEC (Figures 3 and 6). Thus, EGF-R and Met actually couple with ERK2 and $cPLA_2$ pathways in GEC. It should also be noted that in GEC, EGF induces tyrosine phosphorylation of both EGF-R and Neu, a RTK that is related to EGF-R, but does not bind EGF directly.[32] This result (unpublished observation) indicates that Neu phosphorylation may occur secondarily to that of EGF-R. bFGF did not appear to stimulate ERK2 activation or AA release. Possibly, FGF-R2 does not couple to these effectors in GEC. So far, we have not examined complement transactivation of any other RTKs in GEC.

Transactivation of EGF-R by G-protein-coupled receptors has been reported recently.[26–28] In these studies, agonists that bind to receptors coupled with G-proteins, including endothelin-1, lysophosphatidic acid, or thrombin, activated ERK and induced mitogenesis via phosphorylation of EGF-R and Neu tyrosine kinases. These effects appeared to be specific to EGF-R (or Neu), and did not, for example, involve the platelet-derived growth factor receptor. In another study, ultraviolet light or osmotic stress were reported to induce activation of the c-Jun amino terminal kinase, in part, via EGF-R tyrosine phosphorylation.[39] Results of the G-protein-coupled receptor studies differ somewhat from ours in that RTK transactivation via C5b-9 was not restricted to a single RTK or one RTK family. It has been proposed that transactivation of RTKs enables the RTK to serve as a scaffold and permit docking of molecules that lead to activation of effector pathways.[26–28] Our earlier studies have demonstrated that C5b-9-induced activation of $cPLA_2$ is dependent on the activation of PKC, but is independent of Ras-ERK2 and is associated with tyrosine phosphorylation of phospholipase C-γ1, and 1,2-diacylglycerol production.[20] C5b-9-induced activation of ERK2 is mediated via both the PKC pathway, as well as independently of PKC, probably via Ras.[20] Therefore, transactivation of EGF-R by C5b-9 likely results in binding of Grb2-Sos by the phosphotyrosine residues of the cytoplasmic domain (Figure 2), leading to activation of Ras, Raf, and the ERK2 pathway, and it could also result in binding/activation of phospholipase C-γ1, followed by diacylglycerol production and stimulation of PKC.[24,25]

Presently, it is unknown whether RTK transactivation is due to a direct molecular interaction between proteins within the C5b-9 complex and RTKs, whether C5b-9 alters the composition of the plasma membrane such that RTK enzymatic activity increases, or if there may be activation of an intermediary tyrosine kinase by C5b-9, which then secondarily phosphorylates RTKs. Similarly, the mechanism by which G-protein-coupled receptors transactivate EGF-R[26–28] has not been established. Stimulation of cells with ultraviolet light or osmotic stress appeared to induce clustering of multiple receptors, including EGF-R, in the plasma membrane, and it was proposed that receptor clustering was required for activation of c-Jun amino terminal kinase.[39] We attempted to localize EGF-Rs in GEC before and after C5b-9 stimulation, using immunofluorescence microscopy, however, we were not able to detect EGF-R consistently (unpublished observations), probably because GEC express EGF-R at relatively low levels.[36] We also considered that C5b-9 may have induced production of reactive oxygen species,[40] which led to inhibition of phosphotyrosine phosphatases, with a consequent increase in RTK activity.[41] However, inclusion of reactive oxygen species scavengers in incubations did not affect C5b-9-dependent release of [^{3}H]AA (unpublished observations), suggesting that phosphatase inhibition was not involved. The precise mechanism for induction of RTK phosphorylation by C5b-9 will require further study.

The results that demonstrated RTK transactivation by C5b-9 in cultured GEC were extended to the PHN model of membranous nephropathy, an *in vivo* model of GEC injury.[8,9] In PHN, C5b-9 assembles in GEC plasma membranes and induces injury and proteinuria.[8,9] We and others have shown that in brief incubations, glomeruli isolated from rats with PHN synthesize eicosanoids at a rate greater than in isolated normal glomeruli, indicating that the effect of C5b-9 assembled *in vivo* persists after glomerular isolation. Moreover, treatment of rats with PHN or PHN kidneys perfused *ex vivo* with inhibitors of cyclooxygenase or thromboxane synthase can substantially reduce urinary protein excretion.[10–14] In this study, it was not practical to undertake chronic blockade of RTKs in rats with PHN; however, we demonstrated that in brief incubations, the complement-mediated increase in PGE_2 production in glomeruli isolated from rats with PHN was attenuated by AG1478 (Figure 7). Basal PGE_2 production, ie, in control glomeruli, was not affected by AG1478 (Figure 7). Thus, C5b-9-induced PGE_2 production in glomeruli *in vivo* may be, at least in part, mediated via EGF-R (and EGF-R activation persists after glomerular isolation). However, basal glomerular PGE_2 production is EGF-R-independent.

By analogy to GEC in culture, ERK2 tyrosine[204] phosphorylation was enhanced in PHN glomeruli *in vivo* (Figure 8). Using an approach analogous to the PGE_2 studies, examination of complement-mediated ERK2 tyrosine[204] phosphorylation after incubation of glomeruli with AG1478 demonstrated a significant attenuation of phosphorylation in glomeruli from rats with PHN, but no significant change in glomeruli from control rats (Table 6). To our knowledge, this is the first report of ERK2 activa-

tion in PHN, although ERK2 activation was previously observed in glomeruli from rats with proliferating glomerular injury (anti-glomerular basement membrane nephritis).[42] At present, the role of C5b-9-induced ERK2 activation in GEC is not known. GEC proliferation is not a prominent feature of PHN, although in PHN there is expression of proliferation-associated genes.[43] Studies have demonstrated the induction of various other genes and/or proteins during the course of PHN, including platelet-derived growth factor B-chain,[42] cytochrome b_{558},[44] matrix metalloproteinase-9,[45] and cyclooxygenase-2.[46] Thus, C5b-9-induced activation of pathways that potentially mediate transcription, such as the ERK2 cascade, may be necessary for the induction of these genes. Additional studies will be required to further define the role of ERK2 in C5b-9-mediated glomerular injury.

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