In the passive Heymann nephritis (PHN) model of membranous nephropathy, complement C5b-9 induces glomerular epithelial cell (GEC) injury, proteinuria, and activation of cytosolic phospholipase A2 (cPLA2). This study addresses the role of endoplasmic reticulum (ER) stress proteins (bip, grp94) in GEC injury. GEC that overexpress cPLA2 (produced by transfection) and “neo” GEC (which expresses cPLA2 at a lower level) were incubated with complement (40 min), and leakage of constitutively expressed bip and grp94 from ER into cytosol was measured to monitor ER injury. Greater leakage of bip and grp94 occurred in complement-treated GEC that overexpress cPLA2, as compared with neo, implying that cPLA2 activation perturbed ER membrane integrity. After chronic incubation (4–24 h), C5b-9 increased bip and grp94 mRNAs and proteins, and the increases were dependent on cPLA2. Expression of bip-antisense mRNA reduced stimulated bip protein expression and enhanced complement-dependent GEC injury. Glomerular bip and grp94 proteins were up-regulated in proteinuric rats with PHN, as compared with normal control. Pretreatment of rats with tunicamycin or adriamycin, which increase ER stress protein expression, reduced proteinuria in PHN. Thus, C5b-9 injures the ER and enhances ER stress protein expression, in part, via activation of cPLA2. ER stress protein induction is a novel mechanism of protection from complement attack.

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 sublethal (sublytic) injury and various metabolic effects (1–8). An example of sublytic C5b-9-mediated cell injury in vivo is passive Heymann nephritis (PHN) in the rat, a widely accepted model of human membranous nephropathy (9). In PHN, antibody binds to visceral glomerular epithelial cell (GEC) antigens and leads to the in situ formation of subepithelial immune complexes (9, 10). C5b-9 assembles in GEC plasma membranes, “activates” GEC, and leads to proteinuria and sublytic GEC injury (9–14). Based on studies in GEC culture and in vivo, C5b-9 assembly induces transactivation of receptor tyrosine kinases (15), an increase in cytosolic free Ca2+ concentration ([Ca2+]i), and activation of protein kinase C, as well as cytosolic phospholipase A2 (16). cPLA2 is an important mediator of C5b-9-dependent GEC injury. First, arachidonic acid (AA) released by cPLA2 is metabolized in GEC via cyclooxygenases-1 and -2 to prostaglandin E2 and thromboxane A2 (20), and inhibition of prostanooid production reduces proteinuria in PHN (21–25) and in human membranous nephropathy (26). Second, cPLA2 may mediate GEC injury more directly (16).

cPLA2 (group IV PLA2) is regulated by [Ca2+]i, and phospholipase (27, 28). Stimuli that raise [Ca2+]i, in the submicromolar range may induce translocation of cPLA2 from cytosol to an intracellular membrane, where cPLA2 would bind via its N-terminal Ca2+-dependent lipid binding or C2 domain, gaining access to phospholipid substrate. We have demonstrated that cPLA2 is the major PLA2 in GEC and that complement enhances cPLA2 phosphorylation and catalytic activity (16). Moreover, C5b-9 increases free AA in GEC, and release of AA is amplified by overexpression of cPLA2 (16, 17). Recently, we demonstrated that cPLA2 localizes and hydrolyzes phospholipids at the plasma membrane in GEC, the membrane of the endoplasmic reticulum (ER), and the nuclear envelope but not at mitochondria or Golgi (29). Thus, the activation of cPLA2 and release of AA are compartmentalized to specific organelles, but it is presently unknown if hydrolysis of membrane phospholipids by cPLA2 leads to injury of these organelles in GEC.

Based on studies in cell culture, complement attack may injure cells but may also activate pathways that restrict injury or facilitate recovery. One mechanism of protection from complement attack is “ectocytosis” (shedding) of C5b-9 complexes from cell membranes (1, 2). There are undoubtedly other recovery mechanisms that require delineation. For example, ex-
posure of cells to environmental stress increases expression of stress proteins in cellular compartments such as the ER. There are several types of ER stress responses, including the “unfolded protein response” (30–32). ER stress proteins are believed to be induced by accumulation of abnormal proteins or depletion of ER Ca$^{2+}$ stores and include the glucose-related proteins (grp), grp94, bip (grp78), and erp72. Tunicamycin, a nucleoside antibiotic that blocks N-linked glycosylation, is believed to cause an accumulation of unfolded proteins in the ER, and the Ca$^{2+}$ ionophore, ionomycin, which can deplete Ca$^{2+}$ from intracellular stores, can induce ER stress proteins (30–32). Under normal conditions, ER stress proteins might serve as protein chaperones for exocytosis from ER, and they might complex with defective proteins and target them for degradation. During stress, the induction of ER stress proteins may limit accumulation of abnormal proteins in cells (31, 32). Generally, regulation of ER stress proteins appears to be controlled at the transcriptional level, and requires several hours and de novo protein synthesis. Moreover, exposure of cells to mild stress, which induces ER stress proteins, may be protective to additional insults (33, 34), although prolonged or more substantial ER stress may lead to cell death by apoptosis (35).

The aim of the present study was to determine if C5b-9-mediated activation of cPLA$_2$ and GEC injury are associated with induction of the ER unfolded protein response. We demonstrate that C5b-9 may damage the ER and enhance expression of ER stress proteins via activation of cPLA$_2$. Induction of ER stress proteins limits complement-dependent GEC injury in culture and in PHN.

**EXPERIMENTAL PROCEDURES**

**Materials**

Tissue culture media, TRIzol reagent, and a Random Primer DNA Labeling System were obtained from Invitrogen (Burlington, Canada). Complement-deficient sera and purified C8 were purchased from Quidel (San Diego, CA). [3H]AA (100 Ci/mmol) and [32P]dCTP (3000 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). ADI, porcymycin, aminonucleoside, tunicamycin, ionomycin were from Sigma Chemical Co. (St. Louis, MO). Methyl arachidonyl fluorophosphonate (MAFP) was from BIOMOL (Plymouth Meeting, PA). Antibodies to bip, grp94, and erp72 were purchased from Stressgen (Victoria, BC). Fluorescein-conjugated antibodies were from Cedarlane Laboratoires Ltd. (Hornby, Ontario). Electrophoresis and immunoblotting reagents were from Bio-Rad Laboratories (Mississauga, Ontario). Scintillation cocktails were purchased from Amersham (Oakville, Ontario). Immunofluorescence microscopy reagents were purchased from Microscience Canada Ltd. (Mississauga, Ontario). Antibodies to serum were purchased from Santa Cruz Biotechnology. Phosphoric acid (85%) was from Fisher Scientific (Nepean, ON). Reverse transcriptase (M-MLV) was purchased from Promega (Madison, WI). Ficoll-Paque was purchased from Amersham (Buckinghamshire, UK). Radioactive materials were purchased from Amersham (Buckinghamshire, UK). DNA topoisomerase I was purchased from New England Biolabs (Beverly, MA). Subcellular organelle and cytoskeleton markers were purchased from Invitrogen (Burlington, Canada). Electrodes used during experiments were purchased from Warner Instruments (Hamden, CT). Glass capillaries were purchased from Kimble (Vineland, ON). Anti-GEC antiserum (5% v/v) in modified Krebs-Henseleit buffer containing 145 mM NaCl, 5 mM KCl, 0.5 mM MgSO$_4$, 1 mM Na$_2$HPO$_4$, 0.5 mM CaCl$_2$, 5 mM glucose, and 20 mM Heps, pH 7.4, for 40 min at 22°C (16, 17). GEC were then incubated with normal human serum (diluted in Krebs-Henseleit buffer in acute incubations), or K1 medium in chronic incubations) or with heat-inactivated (decomplemented) human serum (56°C) in controls, at 37°C. In some experiments, antibody-sensitized GEC were incubated with C8-deficient human serum or C8-deficient serum supplemented with purified C8 (80 µg/ml undiluted serum). We have generally used heterologous complement to facilitate studies with complement-deficient sera, and to minimize possible signaling via complement-regulatory proteins, however, in earlier studies results of several experiments were confirmed with homologous (rat) complement. There was some variability in sublytic and lytic concentrations of complement among batches of sera. In GEC, complement is not activated in the absence of antibody, and antibody does not independently affect free [3H]AA release (18–19).

**Induction and Characterization of PHN—Sheep anti-rat Fx1A was provided as described previously (37). Male Sprague-Dawley rats (150 g, Charles River, St. Constant, Quebec) were injected with 350 µl of sheep anti-Fx1A antisera. This batch of antisera caused little proteinuria in the heterologous phase (days 5 or earlier) but induced significant proteinuria in the autologous phase (days 7–14). Rats were sacrificed at various intervals, and glomeruli were isolated by differential sieving (37).

**Immunofluorescence microscopy for sheep IgG, rat IgG, and rat C3** was performed as described previously (11). Briefly, 4-µm cryostat kidney sections were stained with fluorescein-conjugated IgG fractions of monospecific antisera. The immunofluorescence signals in whole glomeruli were evaluated using a Leitz immunofluorescence microscope with visual output connected to a Nikon UFX-II photomultiplier and camera, similar to a method described earlier (38). Densitometry readings were done under immersion oil, and the biopsy material was magnified 400 times, such that the glomerular cross-section filled the majority of the densitometry field. The time required to collect an image from a glomerulus is inversely proportional to immunofluorescence intensity. Times required to collect images from three representative glomeruli in each tissue section were recorded and averaged. Serum creatinine measurements were performed in the clinical biochemistry laboratory of the Royal Victoria Hospital (Montreal, Quebec).

**Isolation of Free [3H]AA—GEC phospholipids were isolated and labeled to isotopic equilibrium with [3H]AA for 48–72 h, as described previously (16–20). Lipids were extracted from ~1 × 10$^6$ cells and cell supernatants. Methods for extraction and separation of radiolabeled lipids by thin layer chromatography were published previously (16–20).**

**Northern Hybridization and Immunoblotting—Northern hybridization was performed as described previously (20). Briefly, total RNA was extracted from GEC using the TRIzol reagent. RNA was separated by gel electrophoresis (1% agarose containing 1.9% formaldehyde) and was transferred to a nylon membrane. Coding regions of rat bip, grp94, and erp72 cDNAs were radiolabeled with [32P]dCTP using the Random Primer DNA Labeling System. Membranes were hybridized in buffer containing 5% bovine serum albumin, 7% SDS, 0.5 mM sodium phosphate, pH 6.8, 1 mM EDTA, and 1–5 × 10$^4$ cpm/ml of radiolabeled probe for 16 h at 42°C. Membranes were washed in buffer containing 0.5% bovine serum albumin, 5% SDS, 40 mM sodium phosphate, pH 6.8, 1 mM EDTA twice for 20 min at 65°C, and then buffer containing 1% SDS, 40 mM sodium phosphate, pH 6.8, 1 mM EDTA four times for 20 min at 65°C. Membranes were exposed to x-ray film with an intensifying screen at ~70°C for 48–72 h.

For immunoblotting, GEC or glomerular lysates were mixed with Laemmli sample buffer and were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose paper, blocked with 5% fat-free dry milk in 20 mM Tris, 50 mM NaCl, pH 7.5, with 0.05% Tween 20 (15–17). After blocking, primary antibodies were added to the nitrocellulose paper and blotted for 1 h at room temperature. Tris-buffered saline/Tween 20 solution, blots were incubated with secondary antibody and developed using the enhanced chemiluminescence technique.

**Quantification of Northern blots and immunoblots** was performed by densitometry. Blots were scanned, specific bands of interest were selected, and the density of the bands was measured using National Institutes of Health Image software. Results are expressed in arbitrary units. Preliminary studies demonstrated that there was a linear relationship between densitometric measurements and the amounts of protein loaded onto gels.

**Measurement of Complement-dependent Cytotoxicity**—Complement-mediated cell lysis was determined by measuring release of lactate dehydrogenase (LDH), as described previously (15, 36). Specific release of LDH was calculated as [NS – HIS]/100 – HIS, where NS represents the percentage of total LDH released into cell supernatants in incubations with normal serum, and HIS is the percentage of total LDH
Representative immunoblot is shown in Fig. 1A overexpress cPLA2, there were no significant increases in bip and grp94 in the cytosol of GEC that did not induce significant increases in total ER stress protein (Table II, neo GEC expressed in the cytosol represented only a minor proportion of the total (2%)). Resting GEC express the ER membrane phospholipids at the membrane of the ER (29). We assessed whether hydrolysis of ER membrane phospholipids may induce injury to the ER compartment. Resting GEC express the ER stress proteins, bip and grp94, and these proteins are localized in the ER compartment. Brief incubation of GEC with complement (2.5% normal serum, 40 min) induced significant increases in the amounts of bip or grp94 in the cytosol of the GEC that overexpress cPLA2 (a representative immunoblot is shown in Fig. 1A, and densitometric quantification is given in Table IIA). The amount of bip or grp94 in the cytosol represented only a minor proportion of the total (<15%), and brief incubation with complement did not induce significant increases in total ER stress protein expression (Fig. 1). At the concentration of complement that induced increases in bip and grp94 in the cytosol of GEC that overexpress cPLA2, there were no significant increases in bip or grp94 in the cytosol of neo GEC (Table IIA). However, increases in bip and grp94 could be detected in the cytosol of neo GEC when the concentration of complement was increased (4.0% normal serum, Fig. 1A and Table IIB). These results suggest that activation of cPLA2 by complement perturbed the ER membrane sufficiently to allow a small portion of bip and grp94 to leak into the cytosol. Sublytic complement did not cause bip to leak out of cells, i.e. through the plasma membrane (not shown).

Overexpression of cPLA2 in GEC increased complement-mediated cytotoxicity (16). To assess the role of endogenous cPLA2 in complement-mediated injury, antibody-sensitized neo GEC were incubated with serially increasing doses of complement in controls (a representative experiment is shown in Fig. 1A). An increase in bip and grp94 in the digitonin fraction of complement-treated GEC that overexpress cPLA2 (seventh and eighth lanes from the left) B. antibody-sensitized neo GEC were incubated with 4.0% normal serum or heat-inactivated serum in controls, for 40 min. After collection of supernatants, GEC plasma membranes were briefly permeabilized with digitonin (37 µg/ml) to recover the cytosol. Then, membranes and contents of the ER were solubilized by the addition of 1% Triton X-100. Densitometry of bip and grp94 were immunoblotted with antibodies to bip or grp94 (in duplicate). An increase in bip and grp94 is present in the digitonin fraction of complement-treated GEC that overexpress cPLA2 (seventh and eight lanes from the left).

Table I

| C5b-9-induced release of [3H]AA | Neo 2.5 | Neo 4.0 | Neo 6.7 |
|--------------------------------|---------|---------|---------|
| Free [3H]AA                   | 2.96 ± 0.66 | 4.57 ± 0.47 | 5.47 ± 0.47 |
| C8DS + CS                     | 1.57 ± 0.42 | 1.25 ± 0.20 | 1.25 ± 0.20 |
| HIS                           | 0.86 ± 0.16 | 0.47 ± 0.07 | 0.20 ± 0.04 |

*p < 0.01 versus HIS.

*p < 0.005 versus C8DS and HIS; three experiments performed in duplicate.

Table II

| GEC | NS | Densitometry of cytosolic fractions |
|-----|----|-----------------------------------|
|     | %  | fold                             |
| bip | grp94 |
| A)  | neo | 0.94 ± 0.18 | 1.10 ± 0.14 |
| cPLA2 | 2.5 | 1.33 ± 0.20 | 1.52 ± 0.23 |
| B)  | neo | 1.36 ± 0.28 | 1.56 ± 0.43 |

*p < 0.04.

*p < 0.0025, NS versus HIS (A and B: five experiments performed in duplicate).
Complement Enhances Expression of ER Stress Proteins in GEC—Assembly of C5b-9 may result in cell injury and may in parallel activate mechanisms that restrict or limit the amount of injury. Because C5b-9-induced activation of cPLA2 appeared to disrupt the integrity of the ER membrane, as a consequence, the function of the ER may have been altered. Thus, we proceeded to assess whether exposure of GEC to complement attack would lead to increased expression of ER stress proteins. Incubation of antibody-sensitized GEC that overexpress cPLA2 with sublytic complement for 4 h resulted in increased mRNA levels of bip, grp94, and erp72 (Fig. 2A). The signals on Northern blots were quantified by densitometric analysis. Bip, grp94, and erp72 mRNAs increased 1.7 ± 0.5-fold (n = 4, p < 0.02), 1.4 ± 0.2-fold (n = 6, p = 0.01), and 1.3 ± 0.2-fold (n = 4, p < 0.04), respectively, as compared with control. Complement had no effect on the mRNA level of the cytosolic stress protein, Hsp70 (not shown). Incubation of GEC with C5b-9 induces an increase in [Ca2+]i, and activates protein kinases (17, 18). To determine the effect of increased [Ca2+]i, on bip, grp94, and erp72 mRNAs, GEC were incubated with the Ca2+ ionophore, ionomycin, for 24 h. Ionomycin increased bip, grp94, and erp72 mRNAs (Fig. 2B), 1.7-, 2.3-, and 1.4-fold, respectively, as compared with control (two experiments). The effect of tunicamycin, one of the most potent inducers of ER stress proteins, is shown for comparison (Fig. 2B). Tunicamycin increased bip, grp94, and erp72 mRNAs 5.5-, 5.4-, and 3.1-fold, respectively, as compared with control (two experiments). The next series of experiments addressed changes in protein levels of bip, grp94, and erp72 in GEC, and the role of cPLA2. Incubation with ionomycin for 24 h increased ER stress protein expression (Fig. 3). The effect of ionomycin was more prominent in the GEC that overexpress cPLA2 (1.6- to 2.7-fold increases), as compared with neo (1.3- to 1.7-fold increases). Tunicamycin (shown for comparison as a positive control) was generally a more potent inducer of ER stress proteins, and its effect was similar in neo GEC and GEC that overexpress cPLA2 (1.7- to 2.9-fold increases). These results indicate that the effect of ionomycin is, at least in part, mediated via activation of cPLA2, whereas changes secondary to tunicamycin are cPLA2-independent. Chronic incubation of antibody-sensitized GEC with complement (4–24 h) induced increases in bip and grp94 protein expression (Fig. 4, A–C). In these experiments, the increases in protein levels (30–50% above control at 24 h) were evident mainly in the GEC that overexpress cPLA2. An upward trend was present in neo GEC, but the change did not reach statistical significance. It should be noted that complement-induced increases in [Ca2+]i are similar in magnitude in neo GEC and in GEC that overexpress cPLA2 (17). Thus, the effect of complement on bip and grp94 expression is, at least in part, mediated via activation of cPLA2. To address the involvement of endogenous cPLA2, antibody-sensitized neo GEC were incubated with a higher concentration of complement, with or without the cPLA2 inhibitor, MAFP. At the higher dose, significant complement-induced increases in bip and grp94 protein levels were evident in neo GEC, and the increases were inhibited by MAFP (Fig. 4D). The complement-induced increases in bip and grp94 were functionally important, as demonstrated in the studies of complement-induced injury (discussed below). We did not study the effect of complement on erp72, because preliminary studies demonstrated that complement-induced changes in erp72 protein levels were not readily detectable. Increases in the expression of ER stress proteins could be due to cPLA2-mediated phospholipid hydrolysis and membrane injury or, secondarily, to signals triggered by cPLA2-generated lipid products or their metabolites (prostanoids). GEC that overexpress cPLA2 were incubated with ionomycin or ionomycin plus the cyclooxygenase inhibitor, indomethacin (10 µM), at a concentration known to inhibit cyclooxygenase activity (20). Ionomycin increased bip and grp94 expression (as in Fig. 3), and this effect was not inhibited by indomethacin, suggesting that prostanoid production was not involved in ER stress protein up-regulation (Fig. 5). Products of PLA2-induced phospholipid hydrolysis include AA and lysophospholipid. Addition of AA (10 µM) did not enhance bip or grp94 expression (Fig. 5), although a similar concentration of AA can induce prostanoid generation in GEC (20). Lysophosphatidylcholine was also ineffective in enhancing ER stress protein expression (Fig. 5). Thus, increases in bip and grp94 are most likely triggered by cPLA2-mediated phospholipid hydrolysis and membrane perturbation.

**Bip Antisense mRNA Expression and Effects on GEC Injury**—To determine if complement-mediated induction of ER stress proteins is functionally important, GEC were stably transfected with bip antisense cDNA. Three clones that demonstrated a reduction in tunicamycin-stimulated bip protein expression, as compared with neo GEC were selected for further studies (Fig. 6A). In these bip antisense clones, tunicamycin generally stimulated increases in grp94 and erp72 protein expression similar to neo GEC. The increases tended to be blunted in one of the clones (Fig. 6A); however, previous studies have reported that bip antisense mRNA expression may also decrease induction of other ER stress proteins (33, 40). When the GEC clones that express bip antisense mRNA were incubated with 1 µM ionomycin for 24 h, there was greater cytolyis...
Fig. 3. Effect of ionomycin on expression of ER stress proteins in GEC. GEC that overexpress cPLA₂ or neo GEC were incubated with ionomycin (Iono, 1 μM) or buffer alone (control; Ctrl) for 24 h. Tunicamycin (Tunic, 10 μg/ml) was employed as a positive control. Cell lysates were immunoblotted with antibodies to bip, grp94, or erp72. A, representative immunoblot. B, densitometric quantification of immunoblots. The control incubations demonstrate that there is some basal expression of bip, grp94, and erp72 proteins in GEC. Ionomycin stimulated increases in grp94, bip, and erp72, and the effect of ionomycin was more prominent in the GEC that overexpress cPLA₂, whereas upward trends in bip and grp94 expression occurred in neo GEC (six experiments). Thus, inhibition of the ionomycin-induced increase in bip expression by bip antisense mRNA results in enhanced cytolysis, indicating that induction of bip plays a functionally important role in limiting the amount of complement-dependent injury. In other experiments, neo GEC and the GEC that express bip antisense mRNA were incubated with antibody and serially increasing doses of complement that induced minimal to moderate cell lysis at 18 h. This protocol allows for C5b-9 to increase ER stress protein expression (Fig. 4), but with increasing incubation time and complement dose, a portion of the cells will undergo lyse. After 18 h of incubation, lysis (LDH release) was consistently greater in the GEC clones that express bip antisense mRNA (Fig. 6B). Thus, attenuation of the C5b-9-induced increase in bip expression by bip antisense mRNA results in enhanced cytolysis.
stimuli that induce ER stress protein expression would affect complement-mediated GEC injury. We observed that some stimuli provided protective effects, whereas others enhanced complement lysis. GEC in culture are particularly sensitive to the cytotoxic effect of puromycin aminonucleoside (36), and injection of this compound into rats may induce GEC injury (41). Incubation of cultured GEC with puromycin aminonucleoside increased expression of bip at 2 and 4 h, however, expression returned to basal levels at 24 h (Fig. 7A). In GEC that were preincubated with puromycin aminonucleoside for 3 h, complement-dependent cytolyis was reduced significantly (Fig. 7B). We then examined the effects of pretreatment with tunicamycin and ionomycin (Fig. 3). Unlike puromycin aminonucleoside, 24-h preincubation with tunicamycin (a potent inducer of ER stress proteins) enhanced complement-mediated lysis (Table VI). Preincubation with tunicamycin for only 6 h did not affect complement-mediated GEC injury significantly, but this shorter preincubation time did not increase expression of ER stress proteins consistently (data not shown). Ionomycin pretreatment tended to enhance complement-mediated injury, although the change was not statistically significant (Table VI). We also evaluated if exposure of GEC to an in vitro model of ischemia-reperfusion injury (chemical anoxia) would increase expression of ER stress proteins and protect from subsequent complement attack. Incubation of GEC with 2-deoxyglucose + antimycin A for 90 min (to inhibit glycolytic and/or oxidative metabolism), followed by re-exposure to glucose-replete culture medium for 24 h resulted in increased expression of bip and grp94 (Fig. 8). This protocol did not protect, but rather enhanced, complement-mediated injury significantly (Table VI).

Expression of ER Stress Proteins Is Enhanced in GEC Injury in Vivo—The above experiments demonstrated that C5b-9 increases bip and grp94 protein expression in cultured GEC, but it is important to determine if analogous changes occur in C5b-9-mediated GEC injury in vivo. To address this question, we assessed levels of bip and grp94 in PHN, where GEC injury is due to C5b-9 assembly, and is associated with cPLA2 activation and production of prostanoids. In our model of PHN, proteinuria begins to appear at approximately day 7 and is well-established at days 13 and 14 (see below). On day 14, expression of glomerular bip and grp94 was increased in rats with PHN, as compared with control (Fig. 9, A and B). Increases in levels of bip and grp94 proteins were not detected consistently on days 3 and 7 (data not shown). By analogy to puromycin aminonucleoside, GEC in vivo are sensitive to the cytotoxic effects of adriamycin, and injection of rats with adriamycin may lead to GEC injury, in association with proteinuria.

Table IV

| GEC       | Specific LDH release in U/10^6 GEC |
|-----------|-----------------------------------|
|           | Ionomycin | Tunicamycin |
| bipAS-1   | 6.1 ± 2.0 | 4.6 ± 2.9  |
| bipAS-2   | 8.0 ± 1.4 | 3.1 ± 1.6  |
| bipAS-3   | 3.4 ± 2.0 | 4.5 ± 1.5  |
| neo       | 0.1 ± 0.08| 0           |

*p < 0.025.

*p < 0.005 bipAS versus neo.
Complement-mediated cytolysis (acute incubation) in GECs that express bip antisense mRNA

 neo GEC and three clones of GECs that express bip antisense mRNA were incubated with anti-GEC antibody (40 min) and normal serum (or heat-inactivated serum in controls) for 40 min. Complement-mediated cytolysis was determined by measuring release of LDH into cell supernatants. There were no significant increases in cytolysis in the bip antisense clones, as compared with neo. Values represent 6–10 experiments.

| Normal serum | 15% Specific LDH release | 10% Specific LDH release | 5% (v/v) Specific LDH release |
|--------------|--------------------------|--------------------------|-----------------------------|
| neo          | 70 ± 4 54 ± 8 23 ± 6      |                          |                             |
| bipAS-1      | 40 ± 7 44 ± 8 34 ± 8      |                          |                             |
| bipAS-2      | 40 ± 5 44 ± 5 31 ± 7      |                          |                             |
| bipAS-3      | 40 ± 6 31 ± 7 21 ± 5      |                          |                             |

A second group of rats was injected with a subnephrotoxic dose of adriamycin, i.e. a dose that did not induce proteinuria for up to 14 days. Glomeruli of these rats showed a significant increase in grp94 expression, whereas bip expression showed an upward trend (Fig. 5B). Thus, levels of ER stress proteins also increased when GEC were injured in vivo by a toxin-mediated mechanism. Moreover, the results with adriamycin suggest that development of proteinuria is not essential for enhanced ER stress protein expression in GEC. Finally, injection of rats with tunicamycin enhanced glomerular expression of bip and grp94 (Fig. 9C), without inducing proteinuria.

The experiments presented in Figs. 6 and 7 show that ER stress proteins restrict complement-mediated cytolysis in cultured GEC. In the next series of experiments, we assessed whether stimuli shown to induce ER stress protein expression would limit C5b-9-mediated GEC injury (i.e. proteinuria) in vivo. Rats were treated with a subnephrotoxic dose of adriamycin, or tunicamycin, prior to the induction of PHN. Proteinuria developed in untreated rats with PHN (on days 7, 9, and 13), whereas the amount of proteinuria was significantly lower in the rats with PHN that had been pretreated with adriamycin or tunicamycin (Fig. 10). Thus, increased ER stress protein expression can reduce C5b-9-mediated GEC injury in vivo. By immunofluorescence microscopy and/or immunoblotting, there were no apparent differences in the amounts of glomerular sheep antibody IgG, rat IgG, and rat C3 among the three groups of rats (Fig. 11 and Table VII), indicating that adriamycin and tunicamycin did not decrease proteinuria by reducing the amount of glomerular antibody deposition. Serum creatinine was not significantly different among the three groups of rats, indicating that most likely there were no significant differences in renal function (Table VII). Urine volumes ranged from 5.6 to 22.5 ml/24 h, and there was no consistent pattern among groups (data not shown). In the PHN rats pretreated with adriamycin or tunicamycin, we did not detect further increases in glomerular levels of bip and grp94 (day 14), as compared with the PHN-untreated group (data not shown).

**DISCUSSION**

In this study, we show that cPLA₂ activation in GEC was associated with leakage of luminal ER stress proteins (bip and grp94) into the cytosol (Fig. 1), suggesting that cPLA₂-induced phospholipid hydrolysis resulted in impairment of ER membrane integrity. These results are in keeping with our previous studies, which demonstrated that overexpression of cPLA₂ in GEC exacerbates complement-mediated cytotoxicity (16) and that complement-induced activation of cPLA₂ leads to phospho-

**TABLE V**

| Complement-mediated cytolysis (acute incubation) in GECs that express bip antisense mRNA |
|------------------------------------------------------------------------------------------------|
| neo GEC and three clones of GECs that express bip antisense mRNA were incubated with anti-GEC antibody (40 min) and normal serum (or heat-inactivated serum in controls) for 40 min. Complement-mediated cytolysis was determined by measuring release of LDH into cell supernatants. There were no significant increases in cytolysis in the bip antisense clones, as compared with neo. Values represent 6–10 experiments. |

**TABLE VI**

| Effects of tunicamycin, ionomycin, and chemical anoxia on complement-mediated GEC injury |
|------------------------------------------------------------------------------------------------|
| GECs that overexpress cPLA₂ were preincubated with tunicamycin (10 μg/ml, 24 h), ionomycin (1 μM, 24 h), or deoxyglucose (DG) + antimycin A (as in Fig. 8) to induce expression of ER stress proteins. Then, GECs were incubated with antibody, followed by 5, 10, or 15% normal serum (or heat-inactivated serum in controls). LDH release (reflecting cell lysis) was determined after 40 min. Pretreatment with tunicamycin or prior exposure to chemical anoxia enhanced complement-dependent lysis significantly (p < 0.01 tunicamycin versus untreated, four experiments; p < 0.04 DG + antimycin A versus untreated, three experiments). A small upward trend in complement-dependent lysis was evident with ionomycin pretreatment (five experiments). |

**TABLE VII**

| Effects of tunicamycin, ionomycin, and chemical anoxia on complement-mediated GEC injury |
|------------------------------------------------------------------------------------------------|
| GECs that overexpress cPLA₂ were preincubated with tunicamycin (10 μg/ml, 24 h), ionomycin (1 μM, 24 h), or deoxyglucose (DG) + antimycin A (as in Fig. 8) to induce expression of ER stress proteins. Then, GECs were incubated with antibody, followed by 5, 10, or 15% normal serum (or heat-inactivated serum in controls). LDH release (reflecting cell lysis) was determined after 40 min. Pretreatment with tunicamycin or prior exposure to chemical anoxia enhanced complement-dependent lysis significantly (p < 0.01 tunicamycin versus untreated, four experiments; p < 0.04 DG + antimycin A versus untreated, three experiments). A small upward trend in complement-dependent lysis was evident with ionomycin pretreatment (five experiments). |

**FIG. 7.** Effects of puromycin aminonucleoside on bip expression (A) and complement-mediated GEC injury (B). A, GEC that overexpress cPLA₂ were incubated with puromycin aminonucleoside (PA; 50 μg/ml) for 2, 4, or 24 h. An increase in bip expression is evident at 2 and 4 h (representative immunoblot). The effect of tunicamycin is shown for comparison. B, GEC were preincubated without (Ctrl) and with puromycin aminonucleoside for 3 h (to induce bip expression) and were then incubated with antibody and normal serum (heat-inactivated serum in controls). LDH release (reflecting cell lysis) was determined after 40 min. Pretreatment with puromycin aminonucleoside reduced complement-dependent lysis significantly (p < 0.002 PA versus Ctrl, five experiments).

**FIG. 8.** Effect of chemical anoxia on ER stress protein expression (representative immunoblot). GEC were incubated with glucose-free measurement buffer for 40 min at 37 °C. Then, GEC were incubated with 10 μM 2-deoxyglucose (DG), 10 μM 2-deoxyglucose + 10 μM antimycin A, or buffer alone (Ctrl). Supernatants were removed after 90 min at 37 °C, and GEC were placed into culture medium for 24 h. Lysates were immunoblotted with antibodies to bip or grp94. Deoxyglucose with or without antimycin A induced an increase in bip. There was a smaller increase in grp94 with deoxyglucose + antimycin A. The effect of tunicamycin is shown for comparison.
lipid hydrolysis at the membrane of the ER (29). Furthermore, we show that inhibition of endogenous cPLA2 can attenuate complement-mediated cytotoxicity in GEC (Table III). The mechanism of bip and grp94 leakage from the ER may have contributed to the increased proteinuria in PHN-untreated groups (42), but it will require further delineation. Another consideration is that complement-induced cPLA2 activation is associated with increased retrograde translocation of abnormal proteins from the ER (the abnormal proteins being coupled with bip or grp94) (42), although it is less likely that retrograde translocation could have occurred in an acute time frame. Impairment of ER membrane integrity could potentially be injurious by causing leakage of Ca2+ and other ER luminal components as well as impairment in ER Ca2+ uptake. Although association of cPLA2 with membranes of cell organelles has been established in other cell types (27), there is little information on potential damage to organelle membranes due to phospholipid hydrolysis. In addition to the present study, overexpression of cPLA2 in LLC-PK1 kidney epithelial cells was associated with disruption of the Golgi (43).

C5b-9 Increases ER Stress Proteins

Fig. 9. Expression of ER stress proteins in vivo. Glomeruli were isolated from normal rats (control) and from rats with PHN on day 14. Glomerular lysates were immunoblotted with antibodies to bip or grp94 (A, representative immunoblots; B, densitometric quantification). Bip and grp94 expression was increased in glomeruli isolated from rats with PHN, as compared with control (*, p < 0.002; **, p < 0.005 PHN versus control; 7–8 rats per group). B also shows the densitometric quantification of grp94 and bip expression in rats injected with subnephrotogenic adriamycin, 6 mg/kg intravenously (ADR, day 14, **, p < 0.001 adriamycin versus control; 9–12 rats per group), as well as in rats injected with tunicamycin, 1 mg/kg intraperitoneally (Tun, 24 h), the dots represent the values of two individual rats in each group.

Fig. 10. Pretreatment of rats with a subnephrotogenic dose of adriamycin or tunicamycin reduces proteinuria in PHN. Rats were untreated or were injected with adriamycin, 6 mg/kg intravenously (ADR), or tunicamycin, 1 mg/kg intraperitoneally (Tun) to up-regulate ER stress proteins (Fig. 9). Four days later, rats were injected with anti-Fx1A to induce PHN (day 0). Urine protein was measured on days 0, 7, 9, and 13. Compared with the PHN-untreated group (4 rats), rats with PHN show increased glomerular activity of cPLA2 (Fig. 9-induced GEC injury, it was demonstrated that glomerular bip and grp94 proteins were up-regulated in proteinuric rats with PHN, as compared with control (7–8 rats per group). Therefore, cPLA2-null mice could not be used to address the functional role of cPLA2.

Chronic incubation of GEC with complement (4–24 h) enhanced expression of bip and grp94 mRNAs and proteins (Figs. 2 and 4), although there was no detectable increase in the cytosolic stress protein, Hsp70. The increases in ER stress proteins were dependent on C5b-9 assembly (Fig. 4C). At lower doses, complement-induced increases in bip and grp94 expression were seen mainly in the GEC that overexpress cPLA2, but at a higher dose, expression was also induced in neo GEC, and the increases were blocked with MAFP (Fig. 4). Thus, increases in bip and grp94 were, at least in part, dependent on the activation of cPLA2. Further support for the role of cPLA2 in ER stress protein induction was provided by experiments in which GECs were incubated chronically with the Ca2+ ionophore, ionomycin. Bip and grp94 protein expression was enhanced by ionomycin, and the changes were significantly greater in the GEC that overexpress cPLA2 (Fig. 3). Increases in ER stress proteins were not limited to cultured GEC but also occurred in vivo. Using the PHN model of C5b-9-induced GEC injury, it was demonstrated that glomerular bip and grp94 proteins were up-regulated in proteinuric rats with PHN (day 14), as compared with normal controls (Fig. 9). Rats with PHN show increased glomerular activity of cPLA2, and AA metabolites (19, 20). At present, there are no specific inhibitors of cPLA2 that can be used in experimental animals (44). Thus, although bip and grp94 up-regulation in PHN is associated with the activation of cPLA2, proof for the functional importance of cPLA2 in vivo will require development of suitable cPLA2 inhibitors. Moreover, we have not been successful in establishing a proteinuric model of PHN in mice, and, consequently, cPLA2-null mice (45) could not be used to address the functional role of cPLA2.

The ER serves as a site for folding, assembly, and degradation of proteins (30–32). Membrane and secreted proteins are translocated into the lumen of the ER shortly after initiation of synthesis, and resident ER luminal proteins, including bip and grp94, mediate protein folding. Moreover, bip and grp94 are believed to bind to misfolded or abnormal proteins and prevent their aggregation, either by rescuing such proteins from irreversible damage, or by increasing their susceptibility to proteolytic attack. Other ER proteins, including erp72, may participate in disulfide Isomerization. Perturbation of the ER by stimuli, including accumulation of mutant proteins or Ca2+ depletion, may increase expression of ER stress proteins. In yeast, misfolded proteins in the ER lead to activation of Ire1p endonuclease (31, 32). Ire1p splices HAC1 (homologous to activating transcription factor (ATF) and CREB) mRNA in the...
nucleus, which is then relagitated, exits the nucleus, and is translated into Hac1p transcription factor. Hac1p translocates to the nucleus, binds to the unfolded response element, and induces expression of ER stress proteins. The mammalian unfolded protein response, although analogous to yeast, is more complex and appears to involve additional pathways and effectors (46).

C5b-9-induced sublethal cell injury may lead to a decline in cellular ATP, mitochondrial lipid perturbation, or loss of mitochondrial membrane potential (47–49), whereas at high doses, C5b-9 can induce mitochondrial damage and cell necrosis (50). Based on biochemical and morphologic observations (36, 49), it is likely that, during complement-dependent GEC injury, integral membrane and secretory proteins are altered. Such proteins may include integrins, transporters, and/or cell junctional proteins, and these alterations may contribute to the permeability defect of the glomerular capillary wall in PHN. Besides inducing cell injury, C5b-9 assembly leads to activation of mechanisms that restrict injury or facilitate recovery of cells from complement attack. One such mechanism of protection is "ectocytosis" (shedding) of C5b-9 complexes from cell membranes (1, 2). The present study identifies another mechanism that limits complement attack (Figs. 6, 8, and 10). Expression of bip antisense mRNA in GEC reduced stimulated bip protein expression, and when these GEC were incubated with complement attack. The capability of the GEC to recover or limit the severity of complement attack may depend on its capacity to re-synthesize or reassemble integral membrane proteins, which may require the presence of bip or grp94. Induction of these ER stress proteins during complement attack may also limit accumulation of abnormal proteins and help sustain physiological functions and viability (31, 32).

Exposure of cells to mild stress, sufficient to induce upregulation of ER stress proteins, may be protective to additional insults (33, 34), although progression to cell death may occur if the stress is more intense or prolonged (35). The present study demonstrates that preincubation of cultured GEC with a sublethal dose of puromycin aminonucleoside increased bip expression and protected GEC from complement attack (Fig. 7). On the other hand, prior exposure of cultured GEC to ischemia-reperfusion injury (chemical anoxia), despite increasing ER stress proteins (Fig. 8), exacerbated complement-mediated cytotoxicity (Table VI). However, cellular effects of ischemia and reperfusion are complex and are manifested by a variety of changes that include a decline in cellular ATP levels, alterations of cellular redox state and perturbations in intracellular Ca2+ homeostasis (34). In earlier studies, pretreatment with tunicamycin protected renal tubular epithelial cells from chemical anoxia or toxin-induced injury (33, 34). In contrast, ionomycin and tunicamycin, despite increasing ER stress proteins, either had no effect or exacerbated complement-mediated cytotoxicity in cultured GEC (Table VI). These compounds may have induced multiple alterations in GEC leading to substantial injury, and with subsequent exposure to complement, the cells could not withstand the additional stress. However, it is not clear why the effect of tunicamycin was cytotoxic in culture, but protective in PHN. This question will require further study.

The functional role of ER stress proteins in cultured GEC was applicable to GEC injury in vivo. Induction of ER stress proteins by pretreatment with a subnephrotoxic dose of adriamycin or tunicamycin significantly reduced proteinuria in PHN (Fig. 10), independently of changes in glomerular immunoglobulin or complement deposition (Fig. 11 and Table VII). It should be noted that, although tunicamycin was reported to induce a renal lesion resembling acute tubular necrosis in mice (51), the PHN rats that had been pretreated with tunicamycin had no significant alterations in renal function (Table VII). Our observations provide a rationale for developing non-toxic methods to induce expression of ER stress protein in vitro, which may eventually have applications for therapy of glomerular disease. The importance of these results extends beyond complement-induced GEC injury. For example, preinduction of ER stress proteins prior to xenotransplantation may potentially be a means of reducing hyperacute xenograft rejection, which is complement-dependent (52).

**REFERENCES**

1. Morgan, B. P. (1992) *Curr. Topics Microbiol. Immunol.* 176, 115–140
2. Nicholson-Weller, A., and Halperin, J. A. (1993) *Immunol. Res.* 12, 244–257
3. Niculescu, F., Rus, H., and Shim, M. L. (1994) *J. Biol. Chem.* 269, 4417–4423
4. Niculescu, F., Rus, H., van Biesen, T., and Shim, M. L. (1997) *J. Immunol.* 158, 4405–4412
5. Rus, H. G., Niculescu, F., and Shim, M. L. (1996) *J. Immunol.* 156, 4892–4900
6. Halperin, J. A., Taratukina, A., and Nicholson-Weller, A. (1995) *J. Clin. Invest.* 91, 1974–1978
7. Shankland, S. J., Pippin, J. W., and Couser, W. G. (1999) *Kidney Int.* 56, 538–548
8. Kilgore, K. S., Schmid, E., Shanley, T. P., Florý, C. M., Maheswari, V., Tramontini, N. L., Cohen, H., Ward, P. A., Friedli, H., and Warren, J. S. (1997) *Am. J. Pathol.* 150, 2019–2031
9. Tischer, C. G., and Couser, W. G. (2000) *J. Am. Soc. Nephrol.* 11, 183–188
10. Cybulsky, A. V., Foster, M. H., Quiigg, R. J., and Salant, D. J. (2000) in *The Kidney: Physiology and Pathophysiology*, 3rd Ed. (Seldin, D. W., and Giebisch, G., eds) pp. 2645–2697, Lippincott-Raven Publishers, Philadelphia
11. Cybulsky, A. V., Renneke, H. G., Feintzeig, I. D., and Salant, D. J. (1986) *J. Clin. Invest.* 77, 1096–1107
12. Cybulsky, A. V., Quiigg, R. J., and Salant, D. J. (1986) *J. Immunol.* 137, 1511–1516
13. Kerjaschki, D., Schulze, M., Binder, S., Kain, R., Ohja, P. P., Susani, M., Harvat, R., Baker, P. J., and Couser, W. G. (1989) *J. Immunol.* 143, 546–552
14. Baker, P. J., Ochi, R. F., Schulze, M., Johnson, R. J., Campbell, C., and Couser, W. G. (1989) *Am. J. Pathol.* 135, 185–194
15. Cybulsky, A. V., Takano, T., Papillon, J., and McTavish, A. J. (1999) *Am. J. Pathol.* 155, 1701–1711
16. Cybulsky, A. V., Monge, J. C., Papillon, J., and McTavish, A. J. (1995) *Am. J. Physiol.* 269, F739–F749
17. Panesar, M., Papillon, J., McTavish, A. J., and Cybulsky, A. V. (1997) *J. Im-

**TABLE VII**

|                  | Sheep IgG | Rat IgG | Sheep IgG | Rat IgG | Rat C3 | Creatinine |
|------------------|-----------|---------|-----------|---------|--------|------------|
| **PHN-ADR**      | 0.58 ± 0.08 | 0.83 ± 0.13 | 1.14 ± 0.08 | 1.91 ± 0.13 | 1.95 ± 0.09 | 28 ± 3      |
| **PHN-Tun**      | 0.81 ± 0.03 | 0.76 ± 0.05 | 1.37 ± 0.18 | 1.87 ± 0.12 | 1.76 ± 0.11 | 25 ± 2      |
| **PHN**          | 0.82 ± 0.07 | 0.84 ± 0.06 | 1.15 ± 0.11 | 1.78 ± 0.12 | 2.07 ± 0.29 | 25 ± 2      |
18. Cybulsky, A. V., Papillon, J., and McTavish, A. J. (1998) *Kidney Int.* **54**, 360–372
19. Cybulsky, A. V., Takano, T., Papillon J., and McTavish, A. J. (2000) *Kidney Int.* **57**, 1052–1062
20. Takano, T., and Cybulsky, A. V. (2000) *Am. J. Pathol.* **156**, 2091–2101
21. Zoja, C., Benigni A., Verroust, P., Ronco, P., Bertani, T., and Remuzzi, G. (1987) *Kidney Int.* **31**, 1335–1343
22. Kirschenbaum, M. A., Liebross, B. A., and Serros, E. R. (1985) *Prostaglandins* **30**, 295–303
23. Cybulsky, A. V., Lieberthal, W., Quigg, R. J., Renzke, H. G., and Salant, D. J. (1987) *Am. J. Pathol.* **128**, 45–51
24. Nagao, T., Nagamatsu, T., and Suzuki, Y. (1996) *Eur. J. Pharmacol.* **316**, 73–80
25. Weise, W. J., Natori, Y., Levine, J. S., O’Meara, Y. M., Minto A. W., Manning, E. C, Goldstein, D. J., Abrahamson, D. R., and Salant, D. J. (1993) *Kidney Int.* **43**, 359–368
26. Golseth, H., Black, V., Shemesh, O., and Myers, B. D. (1989) *Am. J. Physiol.* **256**, F44–F51
27. Hirabayashi, T., and Shimizu, T. (2000) *Biochim. Biophys. Acta* **1488**, 124–138
28. Dessen, A. (2000) *Biochim. Biophys. Acta* **1488**, 40–47
29. Liu, J., Takano, T., Papillon, J, Khadir, A., and Cybulsky, A. V. (2001) *Biochem. J.* **353**, 79–90
30. Lee, A. (1999) *Curr. Opin. Cell Biol.* **4**, 267–273
31. Pahl, H. L. (1999) *Physiol. Rev.* **79**, 683–701
32. Kaufman, R. J. (1999) *Genes Dev.* **13**, 1211–1233
33. Liu, H., Bowes, R. C., van de Water, B., Sillence, C., Nogelkerke, J. F., and Stevens, J. L. (1997) *J. Biol. Chem.* **272**, 21751–21759
34. Bush, K. T., George, S. K., Zhang, P. L., and Nigam, S. K. (1999) *Am. J. Physiol.* **277**, F211–F218
35. Rao, R. V., Hermel, E, Castro-Obregon, S., del Rio, G., Ellerby, L. M., Ellerby, H. M., and Brederesen, D. E. (2001) *J. Biol. Chem.* **276**, 33869–33874
36. Quigg, R. J., Cybulsky, A. V., Jacobs, J. B., and Salant, D. J. (1998) *Kidney Int.* **53**, 43–52
37. Salant, D. J., and Cybulsky, A. V. (1988) *Methods Enzymol.* **162**, 421–461
38. Pruchno, C. J., Burns, M. M., Schultz, M., Johnson, R. J., Baker, P. J., Alpers, C. E., and Couser, W. G. (1991) *Am. J. Pathol.* **138**, 203–211
39. Balsinde, J., and Dennis, E. A. (1996) *J. Biol. Chem.* **271**, 6758–6765
40. Li, L. J., Li, X., Ferrario, A., Rucker, N., Liu, E. S., Wang, S., Gomez, C. J., and Lee, A. S. (1992) *J. Cell. Physiol.* **153**, 575–582
41. Grond, J. G., Weening, J. J., and Elena, J. D. (1984) *Lab. Invest.* **51**, 277–285
42. Kopito, R. R. (1994) *Annu. Rev. Biochem.* **63**, 883–914
43. Choukroun, G. J., Marshansky V., Gustafson, C. E., McKee, M., Hajjar, R. J., Rosenzweig, A., Brown, D., and Bonventre, J. V. (2000) *J. Clin. Invest.* **106**, 983–993
44. Yedgar, S., Lichtenberg, D., and Schnitzer, E. (2000) *Biochim. Biophys. Acta* **1488**, 182–187
45. Bonventre, J. V., Huang, Z., Taheri, M. R., O’Leary, E., Li, E., Moskowitz, M. A., and Sapirstein, A. (1997) *Nature* **390**, 622–625
46. Ma, Y., and Hendershot, L. M. (2001) *Cell* **107**, 827–830
47. Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L., and Ron, D. (1998) *Genes Dev.* **12**, 982–995
48. Samstein, B., and Platt, J. L. (2001) *J. Am. Soc. Nephrol.* **12**, 182–193