Overexpression of Crry Protects Mesangial Cells from Complement-Mediated Injury

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Abstract. Crry is a membrane-associated complement regulatory protein expressed on glomerular mesangial, endothelial, and epithelial cells, which reduces C3/C5 convertase activity. This study utilized an overexpression strategy to determine the functional significance of Crry in cultured rat mesangial cells. A Crry expression vector was constructed and was tagged with a c-myc epitope that allowed transfected Crry to be distinguished from the constitutively expressed protein. In stable clones, overexpressed Crry was clearly detected immunocytochemically both by anti-c-myc and anti-Crry antibody in a membrane localization. The overexpression of Crry was also confirmed by Western blotting and immunoprecipitation. To determine if overexpression of Crry by mesangial cells confers a protective effect from complement attack, complement-mediated cell lysis assays were performed. Crry-transfected mesangial cells demonstrated complete resistance to complement-mediated cell lysis, which was reversed by neutralization of Crry with both monoclonal antibody and F(ab')2 fragments of the antibody. This study also investigated the role of Crry in protecting cells from the effects of sublytic complement attack. Overexpressed Crry suppressed antibody/complement induced production of superoxide, one of the inflammatory mediators induced by sublytic complement attack. Immunocytochemical staining confirmed a reduction in C3 and C5b-9 deposition in Crry-transfected cells. These results demonstrate directly that transfected Crry functions as a potent protector of mesangial cells against complement-mediated injury. Crry may play an important role in modulating the glomerular response to immune injury *in vivo*. (J Am Soc Nephrol 8: 223–233, 1997)

The complement system comprises a family of at least 20 plasma and membrane proteins that interact in a tightly regulated cascade system to destroy invading bacteria and prevent the deposition of immune complexes in tissues. Complement can be activated through either of two distinct enzymatic cascades, referred to as the classical and alternative pathways. The classical pathway is generally initiated by the interaction of C1q with antigen/antibody complexes, whereas the alternative pathway is initiated by deposition of C3b on a variety of substrates including bacterial lipopolysaccharide. Both the classical and alternative pathways converge at C5, which is cleaved to form products with multiple proinflammatory effects and which leads to the formation of C5b-9 membrane attack complex.

Although complement plays important roles in the maintenance of health, it, like many other effector systems, is a "double-edged sword," also contributing to tissue damage in numerous diseases, including glomerulonephritis (1).

Deposition of immunoglobulins and activation of complement on the glomerular capillary wall and in the mesangium play a very important role in glomerular injury (2–4). Complement activation mediates immune tissue injury through several mechanisms, including localization of circulating inflammatory cells through generation of chemotactic factors and immune adherence mechanisms and direct injury to resident glomerular cells through membrane insertion of the membrane attack complex, C5b-9 (4,5).

Under normal circumstances, glomerular cells are protected from complement-mediated injury by circulating and cell-bound complement regulatory proteins (6). In the human glomerulus, membrane cofactor protein (MCP, CD46) (7–10), decay accelerating factor (DAF, CD55) (11–13), and CD59 (14,15) are present. The former two function to reduce C3/C5 convertase activity (13,16), whereas CD59 inhibits the formation of membrane attack complex (17–20). MCP and DAF belong to a large family of complement regulatory proteins composed of repeating units called short consensus repeats (SCR). Each SCR is made up of 60 to 70 amino acids and includes a framework of four cysteines (21,22). In addition to MCP and DAF, this family contains complement receptor 1 (CR1), complement receptor 2 (CR2), C4b binding protein (C4BP), and factor H (23). All genes encoding these proteins are present in a cluster on human chromosome 1 (24).

The rodent counterparts of CR1, CR2, C4BP, and factor H have been identified. In contrast, the protein known as Crry serves the complement regulatory role of both DAF and MCP in rodents (25–28), although its primary structure is more closely related to CR1 (29). In rats Crry is expressed on the

Received May 23, 1996. Accepted September 16, 1996.
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1046-6673/0802-0223$03.00/0
Journal of the American Society of Nephrology

membrane of glomerular mesangial, endothelial, and epithelial cells (30–32).

In this article, we investigate the functional role of Crry in cultured glomerular mesangial cells by utilizing an overexpression strategy. Overexpressed Crry significantly protects mesangial cells from complement-mediated injury.

Materials and Methods

Construction of Expression Vectors

A full-length rat Crry cDNA (33) in pBlueScript SK(−) (pBS) (Stratagene, La Jolla, CA) and pCXN2 (a generous gift from Dr. Jun-ichi Miyazaki, University of Tokyo, Tokyo, Japan) were utilized to construct a Crry expression vector.

To detect Crry protein expressed from the transgene and distinguish it from constitutively expressed Crry, the nucleotide sequence coding for c-myc was inserted at the C-terminus of rat Crry by polymerase chain reaction (PCR). The forward primer encompassed a unique BbsI restriction site at position 1615 (AGAAGACAGCTGTGTTCAGCC; BbsI recognition site is underlined). The reverse primer was used to replace nucleotides coding for the last six amino acids (SLTQEV) with nucleotide coding for c-myc epitope (EQKLISEEDL), and included a unique BsrGI restriction site located ten residues after the stop codon (GTGTACACTATTA***CAGATCCTCTTCTGAGATGAGTTTTTGTTC***ATTCCGTGCTGGGCTACTGG; BrsGI recognition site is underlined, and the nucleic acids that code for the c-myc epitope are in bold italics). PCR was performed at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 35 cycles, and produced a fragment of 110 base pairs, as expected. This fragment was confirmed by sequence analysis and was subcloned into the BbsI/BsrGI-cleaved Crry in pBS, replacing the original sequence to give vector pCrry-myc.

To optimize translation efficiency, the N-terminal noncoding sequence was changed to a Kozak consensus sequence (34,35). The 5′-noncoding sequence of the ATG initiation codon, GTGAGGGAAATGGAG, was changed to a Kozak consensus sequence, GTGGCCACCATGGAG, using PCR (the initiation codon is underlined). A forward primer containing a PstI restriction site, Kozak sequence, and the initiation codon (CCTGCAGCCACCATGGAGGCTTCTTCGCCTCTGG), and a reverse primer at a unique SnaBI restriction site (309) (TTACGTATACACACGTCTTG) were used for PCR and PCR was performed under the same conditions as described earlier. The PCR fragment was also confirmed by sequence analysis and was subcloned into PstI/SnaBI-digested Crry-myc in pBS, generating vector pCrry-c-myc.

Crry-c-myc was subcloned into a eukaryotic expression vector, pCXN2. Essential elements for expression in pCXN2 include the cytomegalovirus immediate early enhancer fragment upstream of the chicken β-actin promoter, accompanied by a polyadenylation signal and a 3′-flanking sequence of the rabbit β-globin gene (36). Crry-c-myc was released from pBS by restriction enzyme digestion with SmaI and HincII, and pCXN2 was blunt-ended with Klenow after digestion with XhoI. The SmaI/HincII Crry-c-myc insert was ligated with the XhoI-cleaved blunt-ended pCXN2. DH5α was transformed with the recombinant vector, and plasmids were isolated from ampicillin-resistant colonies and screened for insert orientation by restriction site mapping.

A pCXN2/rat α-smooth muscle actin (α-SMA) expression vector was constructed and used to obtain a transfected rat mesangial cell line for use as control cells. Rat α-smooth muscle actin cDNA was a generous gift from Dr. Kirk M. McHugh (Thomas Jefferson University, Philadelphia, PA) (37). A construction of pCXN2/α-SMA expression vector has been described elsewhere (38).

Mesangial Cell Culture and Transfection

Rat mesangial cells were prepared from collagenase-digested glomeruli, characterized as previously described (39) and maintained in Dulbecco modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum. Cells were transfected with pCXN2/Crry-c-myc by the standard $CaPO_4$ method as previously described (38). In brief, approximately 20%-confluent mesangial cells in 100-mm culture dishes were transfected with 20 μg of DNA in serum-free media. After 18 h incubation, the medium was removed and replaced with serum-containing media. Stably transfected clones were selected with G418 (0.8 mg/mL) 72 h after transfection. Fresh G418 medium was added every 3 days, and individual clones were isolated.

After the selections, four permanent clones of Crry-c-myc/pCXN2-transfected cells were obtained. The data described later was obtained using the clone we designated Cr1, although similar results were confirmed utilizing other clones.

Solubilization of Membrane Proteins

Membrane proteins were solubilized as previously described (31).In brief, cells were washed with phosphate-buffered saline (PBS) twice, and then solubilized for 30 min at 4°C in PBS, pH 7.2, containing 10 mM EDTA, 1% NP40, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL leupeptin, and 1 μg/mL pepstatin A. Nuclei and cytoplasmic debris were then pelleted at 14,000 rpm for 10 min at 4°C.

Antibodies

Anti-c-myc 9E10 monoclonal antibody was purchased from Sigma Chemical Co. (St. Louis, MO). The characteristics of the 5I2 monoclonal antibody to Crry have been described elsewhere (40). Anti-α smooth muscle actin monoclonal antibody was purchased from Boehringer Mannheim (Indianapolis, IN). The characteristics of the 2A1 monoclonal antibody to rat C5b-9 neoantigen have also been described previously (41). Fluorescein-conjugated goat anti-rat C3 antibody was purchased from Cappel (Durham, NC). Goat anti-thymocyte serum (ATS) immunoglobulin (Ig) G was purified utilizing a caprylic acid precipitation method (42,43).

F(ab′)2 fragments of 5I2 were prepared as reported earlier (44).

Western Blotting Analysis

To detect transfected protein, Western blotting was performed using the solubilized membrane proteins of cultured cells or immunoprecipitated samples and antibodies to Crry and c-myc. The bound antibody was detected with 1 μg/mL of alkaline phosphatase-conjugated anti mouse IgG(H+L) (Promega, Madison, WI). In the case of goat ATS IgG, biotin-rabbit anti-goat IgG(H+L) (Zymed Lab., South San Francisco, CA) and alkaline phosphatase streptavidin (Vector Lab., Burlingame, CA) were used at concentrations of 1 μg/mL and 10 μg/mL, respectively. BCIP/NBT tablets (Sigma) were used as a substrate. To ensure that equal amounts of total protein were loaded in each lane, protein concentrations of the samples were first assayed by DC protein assay (BioRad, Hercules, CA). Equal amounts of protein indicated by the assay were electrophoresed, and the gel was stained with Coomassie blue. All the lanes on the stained gel were confirmed to be equivalent by visual inspection.

Immunoprecipitation Studies

Solubilized membrane proteins were immunoprecipitated as follows: Antigens were immunoprecipitated with 0.5 μg of anti-c-myc or irrelevant monoclonal antibody at 4°C overnight, followed by the addition of 50 μL protein A Sepharose 4 Fast Flow (Pharmacia Biotech, Washington, DC). After being thoroughly washed, immuno-

precipitated proteins were dissolved in non-reducing Laemmeli sample buffer, and electrophoresed through 10% acrylamide gel.

Immunofluorescence Microscopic Characterization of Cultured Cells

Cells cultured on slide chambers were fixed with 1:1 acetone-methanol. After being fixated and washed with PBS, the mesangial cells were incubated for 1 h with 0.3 μg/mL of anti-c-myc monoclonal antibody, 3 μg/mL of anti-Crry 5I2 monoclonal antibody, 0.1 μg/mL of anti-α smooth muscle actin monoclonal antibody, or non-specific mouse IgG at room temperature. After being washed further, the cells were incubated with 1:200 biotinylated anti-mouse IgG (H+L) immunoglobulin (Vector Lab.) for 1 h, followed by washing and incubation with 1:100 streptavidin-fluorescein isothiocyanate (FITC) (Amersham, IL). After 1 h, the cells were washed with PBS and mounted using a drop of Vectashield (Vector Lab.) to preserve the fluorescence. Nail polish was used to seal a glass coverslip onto the slide. The cells were viewed using a fluorescence photomicroscope. To perform the staining with ATS, the cells were stained with 50 μg/mL of goat ATS IgG with 1 μg/mL of biotinylated rabbit anti-goat IgG (Zymed) as the secondary antibody.

Immunocytochemical Analysis of Mesangial Cells After Complement Attack

To assess deposition of C3 and C5b-9 on mesangial cells after complement attack, cells were cultured in chamber slides until they became confluent. Cells were incubated in various concentrations of rat serum in DMEM for 30 min at 37°C after sensitization by ATS for 20 min at room temperature.

To assess staining for C3, cells were fixed with 1:1 acetone-methanol, and 30 μg/mL of fluorescein-conjugated anti-rat C3 antibody was used.

To stain the cells for C5b-9, cells were fixed in 3.7% formaldehyde and were stained with 10 μg/mL of monoclonal antibody 2A1, which was identified by a DAKO APAAP kit (DAKO Corp., Carpinteria, CA) with Vector Red (Vector Lab.) as an alkaline phosphatase substrate.

Complement-Mediated Cell Lysis Assay

To determine the functional significance of Crry overexpression *in vitro*, mesangial cells were passaged into 24-well plates (approximately $10^4/cm^2$) and grown until they were almost confluent. After being washed three times with 1 mL of Hanks balanced salt solution (HBSS), the cells were sensitized with 100 μL of ATS IgG (7.5 mg/mL) for 20 min at room temperature. IgG from goat anti-thymocyte serum was purified utilizing a caprylic acid method (43). After being washed further, the cells were incubated with various dilutions of rat serum for 1.5 h at 37°C together with DMEM.

Cell lysis was quantitated by lactate dehydrogenase (LDH) assay (Sigma Diagnostics, St. Louis, MO) as follows: Kinetic determination of LDH was based on the spectrophotometric method of Wroblewski and LaDue (45). The activity of LDH was measured by monitoring the rate at which the substrate (pyruvate) was reduced to lactate. The reduction is coupled with the oxidation of NADH, which is followed spectrophotometrically in terms of reduced absorbance at 340 nm. The background value was estimated from the value of cells incubated with serum alone without ATS sensitization. After the assay, all cells were lysed with 2% Triton X-100 (LKB Instruments, Inc., Rockville, MD), and the complement-mediated cell lysis percentage was calculated. Heat-inactivated (56°C, 30 min) normal Sprague-Dawley rat serum served as a negative control.

To clarify the role of Crry in mesangial cells, lysis studies were also done on rat mesangial cells with various concentrations of 5I2 added to the stimulating ATS IgG to neutralize Crry before exposure to complement. Irrelevant mouse IgG was used as a control at the maximal concentration.

Determination of Superoxide Production

To determine if Crry-transfected cells were resistant to sublytic complement-mediated cell activation, a known product of complement-induced cell activation, superoxide anion, was determined (39). Production of superoxide ion was measured as previously described (38).In brief, mesangial cells harvested by trypsinization were seeded at a fixed density, and allowed to adhere overnight in 24-well culture plates. After the cells were washed with several changes of HBSS, mesangial cells were incubated in 300 μL of HBSS containing 80 μM cytochrome c for the measurement of superoxide production, to determine if normal or Crry transfected mesangial cells were similar in their baseline production of O_2^-.

To determine if Crry-transfected and control cells differed in their response to a non-complement–derived stimulus, 10^{-7} M phorbol myristate acetate (PMA) was added to cytochrome c solution. To investigate the effects of sublytic C5b-9–induced cell activation, sublytic C5b-9 attack was induced by first incubating cells in 3.7 mg/mL of anti-Thy1 IgG for 20 min at room temperature and then in 5% rat serum as a complement source for 30 min at 37°C in 5% CO_2 before the incubation in cytochrome c solution. Cell lysis was assessed by measuring LDH as described above. Studies of sublytic C5b-9 attack utilized concentrations of serum that resulted in less than 5% complement-mediated lysis by LDH assay. Insertion of C5b-9 was confirmed by immunocytochemistry as described above. The mixture was incubated for up to 2 h at 37°C in 5% CO_2. Cytochrome c reduction was determined spectroscopically at 550 nm. Control samples were incubated in the presence of superoxide dismutase. Reactions were run in triplicate or quadruplicate. The production of superoxide was determined with the extinction coefficient of 21.1 mM^{-1} cm^{-1}.

Statistical Analysis

Values were reported as mean ± SD. Data were analyzed using one-way analysis of variance–repeated measures. Values were considered significant if P was <0.05.

Results

Detection of Crry-c-myc in Transfected Mesangial Cells

Western blots. To determine if the construct expressed the Crry-c-myc fusion protein, immunoblot analyses of membrane extracts of transfected mesangial cells and control mesangial cells were performed. Electrophoresis was performed under non-reducing conditions, because 5I2 does not work under reducing conditions (40). Monoclonal antibody against c-myc detected a band in transfected cells corresponding to the molecular weight of the fusion protein (Figure 1A). No c-myc band was detected in normal mesangial cells (Figure 1A). Western blots utilizing antibody against Crry demonstrated that the total amount of Crry was also significantly increased in transfected cells, compared with that detected in control cells (Figure 1B). To further confirm specific overexpression of Crry in our Crry-c-myc–transfected cells, we also performed Western blotting of mesangial cells stably transfected with pCXN2/rat α-smooth muscle actin. The expression level of

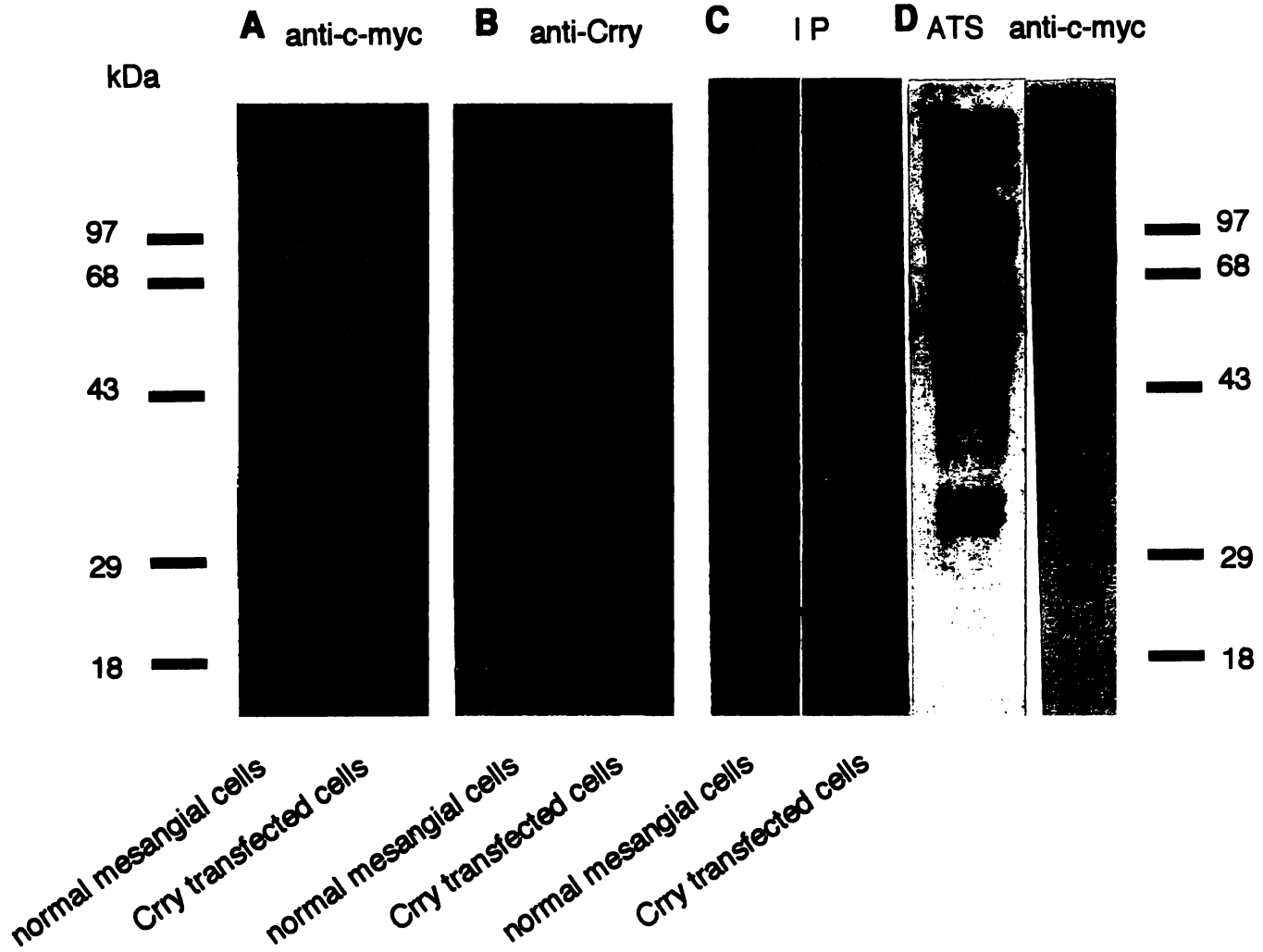

Figure 1. Western blotting comparing expression of Crry in normal and Crry-c-myc–transfected rat mesangial cells. Protein extracts or immunoprecipitated samples of cultured cells were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were electrotransferred. Markers are expressed in kilodaltons. (A) Anti-c-myc monoclonal antibody recognizes a specific band only in Crry-c-myc–transfected cells. Fifteen micrograms of solubilized membrane proteins were loaded onto each lane. (B) Immunoblotting with anti-Crry monoclonal antibody of solubilized membrane proteins shows that the total amount of Crry is markedly increased in Crry-c-myc–transfected cells compared with that in normal mesangial cells. (C) Immunoblotting with anti-Crry monoclonal antibody of immunoprecipitated samples with anti-c-myc demonstrates that Crry-c-myc fusion protein was successfully overexpressed in our transfected cells. (D) ATS antibody detected several bands that are different from those of Crry in cells overexpressing Crry-c-myc. Ten micrograms of crude extracts were used for the Western blot. Note that the c-myc band appears at a higher position compared with that in (A) because the electrophoresis was performed under reducing conditions.

Crry in stable transformants of rat α-smooth muscle actin was the same as that of normal mesangial cells (data not shown). Thus we decided to use normal mesangial cells as a control in the studies described below.

To examine if the ATS we used crossreacts with Crry, a Western blot was performed with ATS. Because the Western blot with ATS under non-reducing conditions was indistinct, we compared the blot with ATS and with anti-c-myc under reducing conditions. ATS did not react with a protein of comparable size to the Crry-c-myc protein, and therefore it is unlikely ATS contains reactivity with Crry (Figure 1D).

Immunoprecipitation. As shown in Figures 1A and 1B, anti-c-myc antibody seemed to recognize one band with the lowest mobility in electrophoresis, whereas anti-Crry antibody recognized several bands with different mobilities, all of which were overexpressed compared with control cells. As the electrophoresis was performed under non-reducing conditions, proteins of different higher conformations run to different positions. We speculate that the fusion protein has several different conformations, as does endogenous Crry (shown in the Western blot of normal mesangial cells), and that the antigenic c-myc epitope is revealed primarily, or only, on the fusion protein, which migrates at the position where the anti-c-myc detects it while anti-Crry detects several other conformations.

To confirm this speculation, we performed immunoprecipitation studies of normal mesangial cells and Crry-c-myc–transfected cells using anti-c-myc monoclonal antibody. Immunoprecipitated samples were electrophoresed, and Western blots

of the samples with anti-Crry were performed. Anti-Crry detected the bands corresponding to the fusion protein in immunoprecipitated samples of Crry-c-myc–transfected cells, whereas it detected no corresponding bands in normal mesangial cells (Figure 1C). As another control, we also performed immunoprecipitation of Crry-c-myc–transfected cells with irrelevant monoclonal antibody, which did not react with anti-Crry on Western blot (data not shown). These results support our speculation described above.

Immunostaining of transfected mesangial cells. Transfected mesangial cells stained strongly with both anti-Crry antibody and anti-c-myc antibody, displaying a bright homogenous membrane staining pattern distributed over the entire cell surface (Figure 2, A and C). Normal mesangial cells did not show positive stainings for Crry or c-myc (Figure 2, B and D). Normal mouse IgG did not stain either normal mesangial cells or Crry-transfected cells (data not shown). Crry-transfected cells retained normal morphologic characteristics in cell culture and displayed standard mesangial phenotypic markers such as α-smooth muscle actin (Figure 3A). Normal and transfected mesangial cells stained similarly with ATS IgG (Figure 3 B, C).

Complement-Mediated Cell Lysis is Inhibited in Crry-Transfected Cells

To examine the functional consequences of overexpressed Crry, we performed complement-mediated cell lysis assays. Mesangial cells sensitized with ATS antibody demonstrated cell lysis that was dependent on the concentration of rat serum used as a complement source. The sensitizing ATS antibody did not exhibit reactivity with Crry by Western blotting (Figure 1C). Less than 5% complement-mediated cell lysis was observed when either sensitized normal mesangial cells or Crry-transfected cells were incubated with 5% normal rat serum (3.0 ± 2.0% and 1.0 ± 1.0%, respectively; $P > 0.01$). Sensitized normal mesangial cells showed 15.3 ± 2.5 and 25.3 ± 8.5% lysis with 10 and 20% rat serum, respectively. In contrast, sensitized Crry-transfected mesangial cells showed remarkable resistance to complement attack with 0.7 ± 0.6 and 2.0 ± 2.0% lysis at 10 and 20% rat serum, respectively (mean ± SD; $N = 3$, $P < 0.001$) (Figure 4). Both normal and Crry-transfected mesangial cells showed no complement-mediated cell lysis when incubated with 20% heat-inactivated serum (0.7 ± 1.2% and 2.3 ± 4.0%, respectively; $N = 3$, $P > 0.01$).

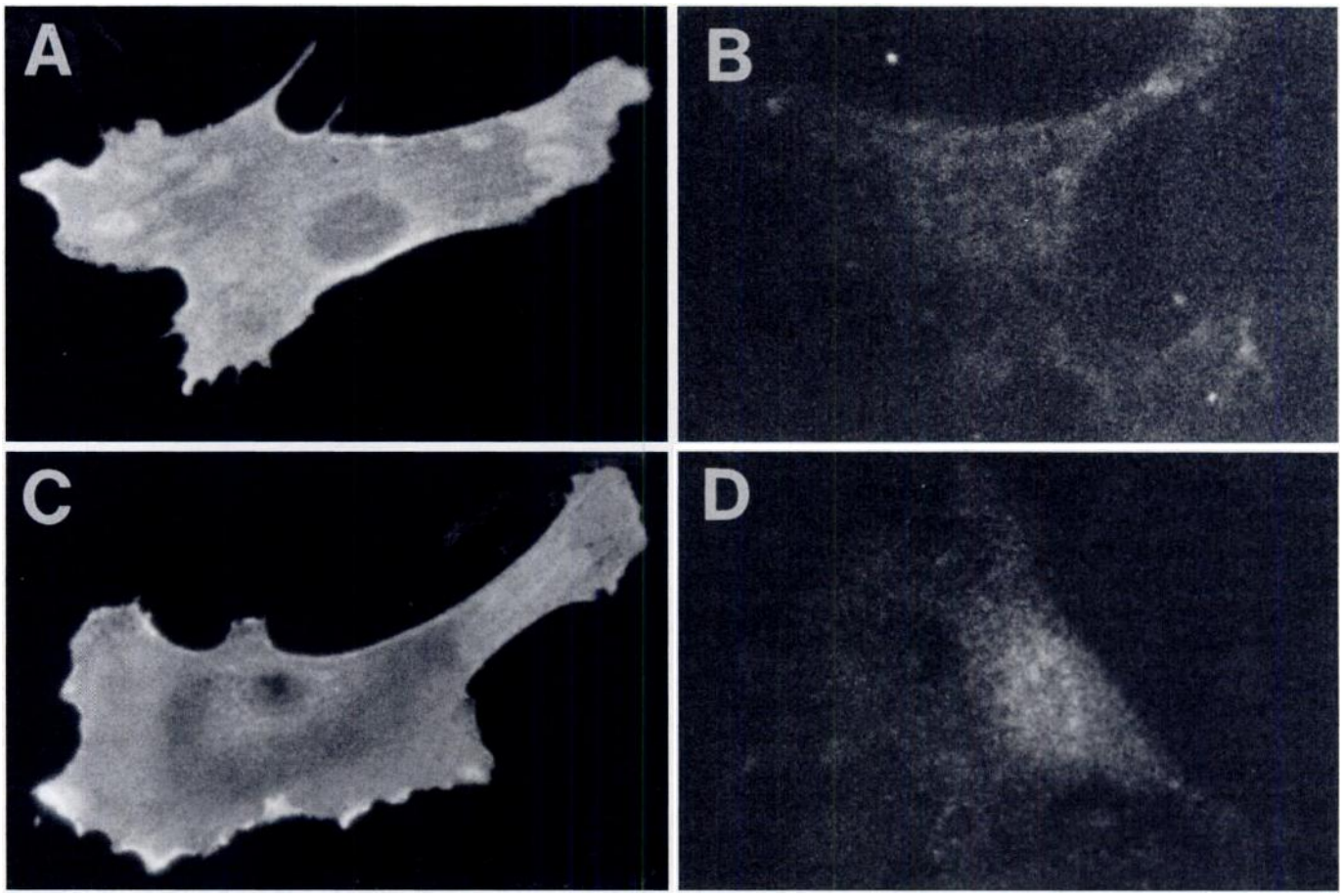

Figure 2. Immunofluorescent staining for Crry and c-myc in normal and Crry-c-myc–transfected mesangial cells. (A) Crry-transfected cells stained with anti-c-myc. A homogenous membranous staining pattern is observed over the entire cell. (Original magnification, ×400.) (B) Normal mesangial cells showed no staining with anti-c-myc antibody. The photograph is overexposed to show the background staining of the cell. (Original magnification, ×400.) (C) Crry-transfected cells stained with anti-Crry antibody. The same homogenous staining pattern as in (A) is demonstrated. (Original magnification, ×400.) (D) Normal mesangial cells stained with anti-Crry. The staining was very faint, and the photograph is overexposed to show the background staining of the cell. (Original magnification, ×400.)

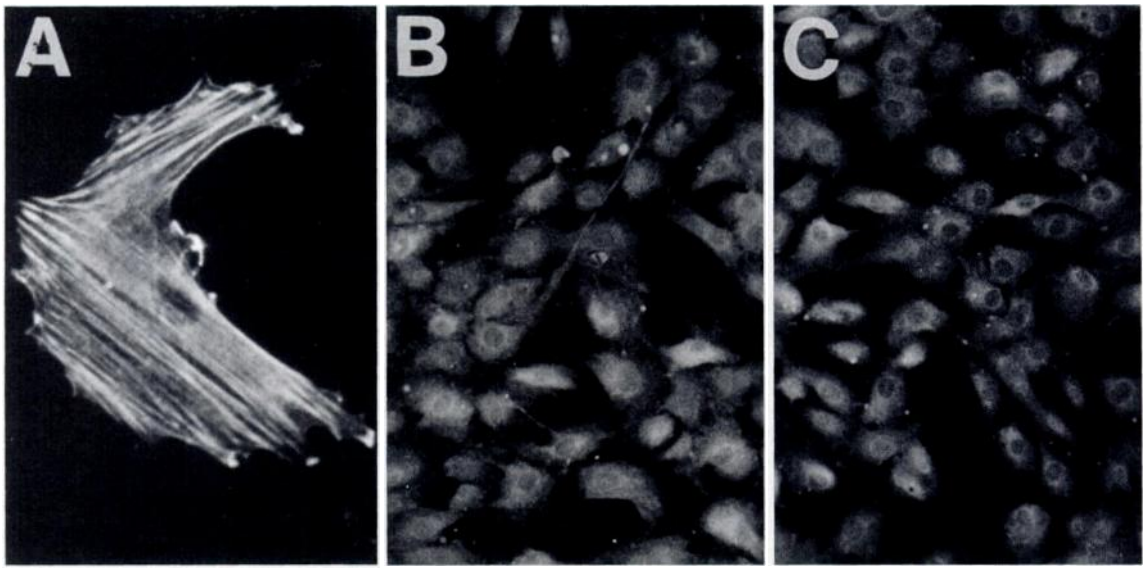

Figure 3. Immunocytochemical characterization of Crry-transfected mesangial cells. (A) Filamentous staining for α-smooth muscle actin was observed along the stress fibers in Crry-transfected cells. (Original magnification, ×400.) (B) ATS showed a fine granular staining in Crry-transfected cells. (Original magnification, ×200.) (C) The same pattern of ATS staining was observed in normal mesangial cells with the same intensity. (Original magnification, ×200.)

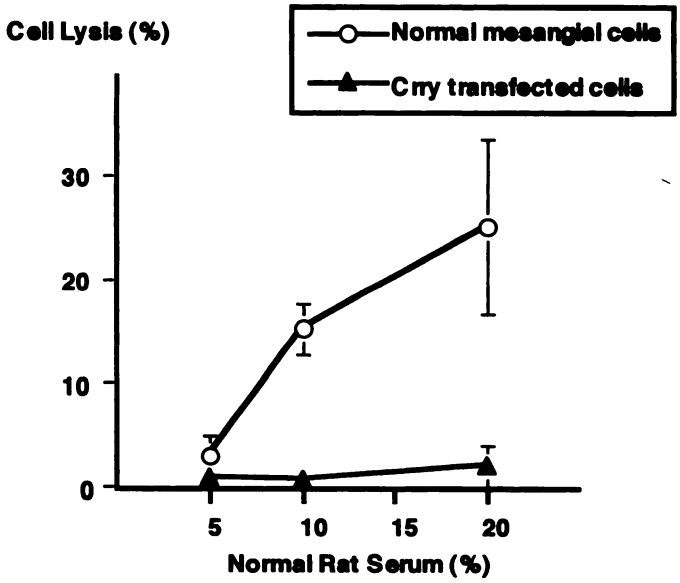

Figure 4. Crry overexpression protects mesangial cells from complement-mediated cell lysis. Normal and Crry-transfected mesangial cells were subjected to complement-mediated lysis by treatment of cells with varying dilutions of rat serum as a complement source after prior sensitization by ATS. Cell lysis was determined as LDH release after 1.5 h incubation at 37°C. Note that Crry-transfected cells showed complete resistance to complement attack at all serum concentrations.

To demonstrate whether the overexpression of Crry was responsible for the protection of the transfected mesangial cells from complement-mediated lysis, the transfected cells were treated with anti-Crry antibody before exposure to complement. As a source of complement, 20% normal rat serum was used.

Neutralization of Crry on transfected cells using a monoclonal antibody caused an increase in susceptibility to complement-mediated cell lysis. Although transfected cells demonstrated 2.0 ± 1.8% and 1.5 ± 2.4% of complement-mediated cell lysis in the absence and in the presence of 0.025 mg/mL of anti-Crry antibody, respectively, these cells showed 15.3 ± 6.1, and 21.5 ± 6.6% of complement-mediated cell lysis in the presence of 0.1 and 0.4 mg/mL of anti-Crry antibody, respectively ($N = 4$; $P < 0.01$ and $P < 0.001$, respectively) (Figure 5). Control IgG showed no effects on Crry-transfected cells at the concentration of 0.4 mg/mL (1.33 ± 1.15%; $N = 3$). To exclude the possibility that the neutralizing antibody itself activated complement and resulted in cell lysis, we also performed the neutralization study with F(ab')2 fragments of the anti-Crry monoclonal antibody. Incubation of mesangial cells with 0.2 mg/mL of F(ab')2 fragments of the antibody, followed

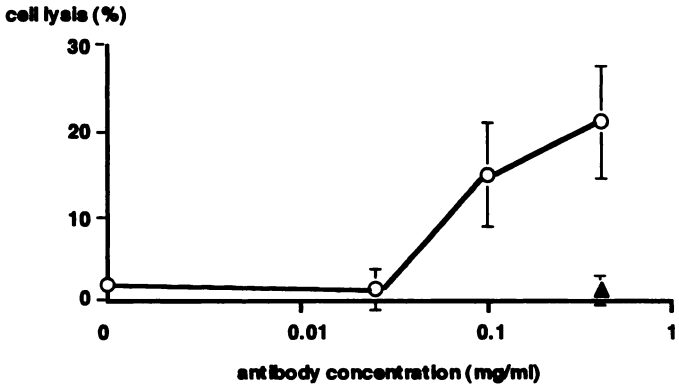

Figure 5. The protective effect of Crry is neutralized by anti-Crry antibody in Crry-transfected cells. Crry-transfected mesangial cells were incubated with various concentrations of anti-Crry monoclonal antibody together with 7.5 mg/mL ATS IgG before exposure to 20% normal rat serum. Anti-Crry enhanced complement-mediated cell lysis of transfected mesangial cells (white circles). Control IgG has no effect (black triangle).

by complement attack, also demonstrated a significant increase of mesangial cell lysis comparable with that achieved with equimolar quantities of IgG (20.5 ± 12.1%; $N = 4$, $P < 0.01$). This confirms a specific protective role for overexpressed Crry in our study.

Response of Crry-Transfected Mesangial Cells to Non-Complement Stimuli and Sublytic Complement Attack

To determine if overexpression of Crry would make mesangial cells resistant to sublytic Crry-mediated activation, superoxide production was selected as a mediator produced in response to complement attack that is known to be important in glomerular disease (39). As shown in Table 1, there was no significant difference between normal and Crry-transfected mesangial cells in baseline production of O_2^-. Moreover, both cell types demonstrated a similar increase in production of O_2^- in response to PMA (Table 1). However, when cells were exposed to sublytic attack by complement, sensitized Crry-transfected cells produced less than one third the O_2^- produced by sensitized normal cells (Table 1). Heat-inactivated serum also did not induce superoxide production (Table 1).

Staining of C3 and C5b-9 Is Decreased in Crry-Transfected Mesangial Cells

To confirm the inhibition of complement attack in Crry-transfected cells, we performed immunocytochemical studies utilizing anti-C3 and anti-C5b-9 antibodies. Sensitized normal mesangial cells exposed to lytic concentrations of complement demonstrated positive staining for C3 and C5b-9 (Figure 6, A and C). Sensitized cells exposed to sublytic concentrations of complement also showed positive staining for C5b-9, although the number of positive cells and the intensity of the staining was less (Figure 6D). In contrast, Crry-transfected cells did not show staining for C3 or C5b-9 under either condition (Figure 6, B and E). Normal mesangial cells without sensitization showed no staining (data not shown).

Discussion

The role of complement in mediating glomerular injury has been well established in numerous studies in which antibody-induced diseases have been substantially modified or prevented by maneuvers that block or inhibit complement activation (2–4). The primary nephritogenic effects of complement activation are attributed to release of chemotactic factors such as C5a, leading to recruitment of inflammatory cells and insertion of C5b-9 into glomerular cell membranes, which probably results in cell activation leading to local release of a variety of inflammatory mediators, including oxidants and proteases (2,4,46).

Subsequent to establishing the central role of the complement system in mediating glomerular injury, it has been recognized that complement activation *in vivo* is carefully regulated by a host of circulating and cell-bound complement regulatory proteins, which act to prevent complement-mediated tissue injury (6, 47). Among several complement regulatory proteins, the role of Crry has been emphasized recently.

Crry was originally identified because of its ability to cross-react with a polyclonal rabbit anti-human C3b receptor (CR1) antibody (48). The Crry gene was cloned in mice utilizing low-stringency hybridization with a cDNA probe specific for human CR1 gene against mouse genomic and cDNA libraries (29,49,50). Recently, rat Crry cDNA was also cloned (33,51). Rat Crry protein was shown to consist of a signal peptide, six or seven SCR, a transmembrane, and an intracytoplasmic domain.

Functionally, Crry was demonstrated to block complement activation by both the classical (26) and alternative pathways (27). The mechanism of complement regulation by Crry is reduction of C3/C5 convertase activity, like DAF and MCP. DAF acts by blocking convertase assembly and accelerating the spontaneous decay of C2a and Bb from C4b and C3b, respectively. MCP, conversely, has no decay-accelerating activity, but rather acts as a required cofactor for factor-I mediated cleavage of C3b and C4b. Recently, it was shown that Crry has the functions of both DAF and MCP (28).

Systemic administration of anti-Crry monoclonal antibody induced deposition of C3 in the capillary endothelial cells, resulting in symptoms resembling endotoxin shock in rats (decreased blood pressure, increased vascular permeability, and leukocyte infiltration in organs) (44). Crry has been demonstrated immunohistochemically in glomerular capillary walls and mesangial cells in normal rats (32) and mice (25). Quigg *et al.* utilized antibody to Crry and demonstrated that Crry exists in all three types of cultured glomerular cells and plays a complement regulatory role in these cells (30,31,52). Renal artery perfusion of F(ab')2 fragments of anti-Crry in normal rats resulted in marked renal tubular damage and mononuclear cell infiltration (53). Suppression of glomerular Crry by antibody worsened the anti-Thy1 glomerulonephritis model of rats (54). Although these functional studies emphasized the importance of Crry, all of them were based only on antibody neutralization. Overexpression of Crry has been induced in only one cell type, K562, which is a human erythroleukemia cell line (26–28).

Our studies confirm and extend prior observations by utilizing an overexpression strategy to increase Crry expression in rat mesangial cells and determine the functional significance of this process.

Normal rat mesangial cells transfected with rat Crry containing the nucleotide sequence for c-myc overexpressed Crry-

Table 1. Superoxide (O_2^-) production by normal and Crry-transfected mesangial cells in response to a pharmacological stimulus and sublytic complement attack[a]

Stimulus (*N*)	Normal	Crry-Transfected	*P* Value
Baseline (6)	0.66 ± 0.39	0.86 ± 0.49	>0.05
PMA 10^{-7} M (6)	3.07 ± 2.62	2.82 ± 2.80	>0.05
Sublytic C5b-9 (10)	2.38 ± 0.56	0.55 ± 0.54	<0.01
Heat-inactivated serum (3)	0.67 ± 0.69	0.63 ± 0.61	>0.05

[a] Values are represented as nmole $O_2^-/10^6$ cells · hour.

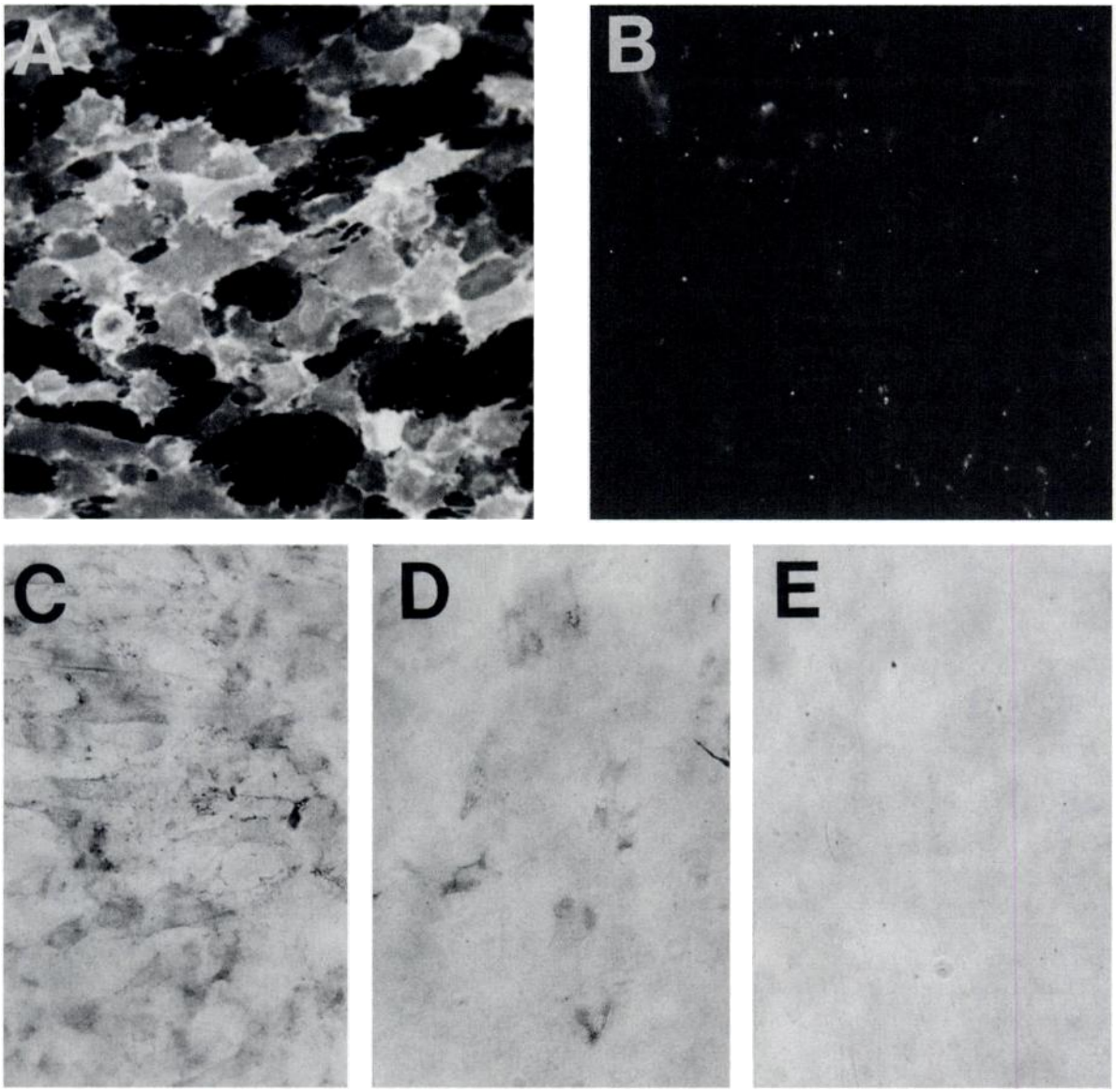

Figure 6. Immunocytochemistry demonstrated reduction of C3 deposition and C5b-9 insertion in Crry-transfected cells. (A) Normal mesangial cells subjected to lytic complement attack stained with anti-C3. Note the bright staining of the cell membranes. (Original magnification, ×200.) (B) Crry-transfected mesangial cells showed faint staining with anti-C3 after lytic complement attack. (Original magnification, ×200.) (C) Normal mesangial cells subjected to lytic complement attack stained with anti-C5b-9. There is marked staining in many cells. (Original magnification, ×200.) (D) Normal mesangial cells subjected to sublytic complement attack stained with anti-C5b-9. There is staining in these cells, which demonstrates there is C5b-9 insertion under this condition of complement attack, but the number of positive cells and the intensity of the staining is less than that in (C). (Original magnification, ×200). (E) Crry-transfected mesangial cells exposed to lytic concentrations of complement stained with anti-C5b-9. C5b-9 insertion is not detected in Crry-transfected cells. (Original magnification, ×200.)

c-myc fusion protein by immunohistochemical and Western blot analysis. The c-myc tag permitted transfected protein to be distinguished from constitutively expressed Crry, which is made by normal mesangial cells (31). Crry-transfected cells exhibited normal morphologic characteristics in cell culture and displayed standard mesangial phenotypic markers when tested. Moreover, when exposed to a non-complement derived stimulus (PMA), the response of the transfected cells was indistinguishable from that of normal mesangial cells.

We next examined the functional consequences of rat Crry overexpression in mesangial cells by quantitating their susceptibility to lysis induced by various concentrations of homologous complement. To make the experiment analogous to a mechanism of experimental glomerular disease *in vivo*, complement activation was induced by first sensitizing normal or transfected mesangial cells with ATS IgG, which recognizes antigens on the membrane of mesangial cells (55). When injected *in vivo*, this antibody induces an initial complement-mediated lysis of mesangial cells followed by a phase of mesangial cell proliferation that is C5b-9–mediated (ATS model) (56–58). Sensitized mesangial cells transfected with Crry exhibited a total resistance to complement-mediated lysis at serum concentrations that produced over 25% lysis in sensitized normal mesangial cells. This protection was the result

of Crry overexpression, because it was reversed by prior exposure to neutralizing antibody to Crry.

In the glomerulus, complement-mediated cell lysis (although it probably occurs) has not been well established as a major mechanism of clinical renal disease (2). The principal nephritogenic effect of complement-mediated injury *in vivo* is attributed to the activation of glomerular cells induced by sublytic complement attack (39,59–63). Thus nucleated cells, relatively resistant to cell lysis because of their ability to shed C5b-9 complexes, may become activated to produce a host of potential inflammatory mediators. Of these, one that has been well studied is the production of reactive oxygen species, which occurs after sublytic complement attack on mesangial cells (39). We therefore chose to measure the ability of Crry-transfected cells to respond to sublytic complement attack by increasing oxidant production. Complement attack induced an increase in superoxide production by sensitized normal mesangial cells. In contrast, Crry-transfected cells showed no significant increase in oxidant production in response to a similar sublytic stimulus, although these cells responded normally to a non-complement–derived stimulus.

In addition to these two functional analyses, resistance to complement attack in Crry-transfected cells was also demonstrated by immunocytochemical methods. Crry-transfected cells showed a marked decrease in C3 deposition and C5b-9 insertion, compared with that seen in normal mesangial cells.

Although a role for complement in mediating tissue injury in diverse clinical situations is now widely accepted, no suitable therapeutic strategies for inhibiting complement have been available until recently. The potency and low toxicity of naturally occurring complement regulatory proteins confer significant potential on these molecules as effective therapeutic agents. Soluble CR1 has now been shown to dramatically improve three different forms of complement-mediated injury in experimental glomerulonephritis (64). Expression of CD59 also protects glomerular cells *in vitro* (38). The study presented here confirms an even greater potency of Crry in protecting glomerular cells from antibody/complement attack and suggests that this molecule too may be of potential therapeutic efficacy.

Acknowledgments

This work was supported in part by research grants from the United States Public Health Service (DK34198 and DK43422), a George M. O'Brien Kidney Research Center award (DK47659), and research and fellowship awards for the Northwest Kidney Foundation. Dr. Johnson is the recipient of a Research Career Development Award (DK02142) from the National Institutes of Health. Dr. Quigg is the recipient of NIH Grant DK41873. Dr. Nangaku is the recipient of an Eli Lilly international fellowship award. We thank Dr. Hidechika Okada (Department of Molecular Biology, Nagoya City University School of Medicine, Nagoya, Japan) and Dr. Seiichi Matsuo (The Third Department of Internal Medicine, Nagoya University School of Medicine, Nagoya, Japan) for their useful advice. We also thank Dr. Rick Meek (Division of Nephrology, University of Washington, Seattle) for his technical advice and Dr. Kiyoshi Kurokawa (1st Department of Internal Medicine, University of Tokyo, Tokyo, Japan) for his kind support.

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