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The Complement Inhibitors Crry and Factor H Are Critical for Preventing Autologous Complement Activation on Renal Tubular Epithelial Cells

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Congenital and acquired deficiencies of complement regulatory proteins are associated with pathologic complement activation in several renal diseases. To elucidate the mechanisms by which renal tubular epithelial cells (TECs) control the complement system, we examined the expression of complement regulatory proteins by the cells. We found that Crry is the only membrane-bound complement regulator expressed by murine TECs, and its expression is concentrated on the basolateral surface. Consistent with the polarized localization of Crry, less complement activation was observed when the basolateral surface of TECs was exposed to serum than when the apical surface was exposed. Furthermore, greater complement activation occurred when the basolateral surface of TECs from Crry<sup>−/−</sup>/B<sup>−/−</sup> mice was exposed to normal serum compared with TECs from wild-type mice. Complement activation on the apical and basolateral surfaces was also greater when factor H, an alternative pathway regulatory protein found in serum, was blocked from interacting with the cells. Finally, we injected Crry<sup>−/−</sup>/B<sup>−/−</sup> and Crry<sup>+/+</sup>/B<sup>−/−</sup> mice with purified factor B (an essential protein of the alternative pathway). Spontaneous complement activation was seen on the tubules of Crry<sup>−/−</sup>/B<sup>−/−</sup> mice after injection with factor B, and the mice developed acute tubular injury. These studies indicate that factor H and Crry regulate complement activation on the basolateral surface of TECs and that factor H regulates complement activation on the apical surface. However, congenital deficiency of Crry or reduced expression of the protein on the basolateral surface of injured cells permits spontaneous complement activation and tubular injury. The Journal of Immunology, 2010, 185: 000–000.

Uncontrolled complement activation causes renal injury in a number of diseases, including immune-complex glomerulonephritis (1–3), ischemic acute kidney injury (4, 5), and acute renal allograft rejection (6). In addition, complement activation contributes to the progression of proteinuric renal diseases (7, 8). Recent reports strongly linked functional deficiencies of the complement regulatory proteins membrane cofactor protein (MCP; CD46) and factor H with the development of atypical hemolytic uremic syndrome (aHUS) (9, 10). Furthermore, inadequate regulation of fluid-phase alternative pathway activation is a primary cause of dense deposit disease (DDD) (11, 12).

All cells in the body express complement regulatory proteins, but the kidney seems to be particularly susceptible to injury in patients with DDD and aHUS caused by defective complement regulation, despite the potential of these systemic defects to damage any organ system (10, 13). The predilection for the kidney as the site of injury suggests that control of the complement system within the kidney by endogenous complement proteins is easily overwhelmed or disrupted and that inadequate control of the complement system predisposes individuals to injury of the kidney.

The alternative pathway of complement seems to mediate renal injury in most of the diseases associated with defective complement regulation (5, 11, 14, 15). The alternative pathway is continually activated in the fluid phase through a process called tickover. Tickover generates C3b, which can bind to amino and hydroxyl groups on the surface of cells. Bound C3b then catalyzes further alternative pathway activation unless actively inhibited by proteins, such as MCP or factor H. Because invasive pathogens typically lack these regulatory proteins, the C3b generated by tickover can bind to the pathogen surface and trigger further complement activation, helping to eliminate the invading organism. Therefore, regulation of the alternative pathway is an important mechanism by which the innate immune system discriminates between host and pathogen.

Because there is continuous autoactivation of the alternative pathway, adequate expression of complement regulatory proteins by host cells is critical for preventing autologous injury by the complement system. The complement regulatory proteins expressed on human cells are MCP, decay accelerating factor (DAF; CD55), and CD59 (16). Factor H is a circulating protein that regulates the alternative pathway in the fluid phase and controls activation on cell surfaces (17, 18). All three membrane-bound complement regulatory proteins are expressed within the kidney, but MCP is the only inhibitor found in abundance on human tubular epithelial cells...
(TECs) (19). MCP is a transmembrane protein that regulates the classical and alternative pathways of complement by acting as a co-factor for factor I-mediated cleavage of C4 and C3 activation fragments (20). Crry is the murine homolog of MCP, and we previously found that Crry is expressed on the basolateral surface of mouse TECs in vivo. Similar to what has been found in human kidneys, DAF and CD59 were not detected by immunofluorescence microscopy of mouse tissue (21).

Expression of Crry at the surface of the renal tubules is disrupted by ischemia, and complement activation on the basolateral surface of the tubules correlates with the loss of Crry at this location (21). Furthermore, mice partially deficient in Crry display greater susceptibility to ischemic injury than do wild-type controls (21). In addition, Bao et al. (22) performed cross-transplantation of kidneys from Crry+/− C3−/− mice into wild-type hosts and demonstrated that kidneys lacking Crry are destroyed by uncontrolled complement activation. These studies highlight the important role that Crry plays in protecting TECs from complement-mediated injury after warm ischemia or transplantation.

In the current study, we used in vitro and in vivo experiments to further explore the role of Crry in regulating complement activation in the surface of TECs. We hypothesized that control of the complement system on the surface of TECs is critically dependent upon Crry and that loss of complement regulation by Crry is sufficient to permit alternative pathway activation on the cell surface, even in the absence of cellular injury or hypoxia. We also hypothesized that circulating factor H cannot fully compensate for reduced expression of Crry on the TEC surface. To test these hypotheses, we examined which of the regulatory proteins are expressed on TECs, whether expression of Crry is altered by cellular hypoxia, and whether cells that do not express Crry are susceptible to complement-mediated injury. By examining the mechanisms by which the complement system is regulated on the renal tubules, we hope to gain insight into the pathogenesis of complement-mediated diseases of the kidney.

Materials and Methods

Reagents

Primary Abs to Crry (BD Biosciences, San Jose, CA), DAF (Cedarlane Laboratories, Hornby, ON, Canada), CD59 (Abcam, Cambridge, MA), pan-ectokeratin (Sigma-Aldrich, St. Louis, MO), megalin (Santa Cruz Bio-technology, Santa Cruz, CA), collagen type IV (Millipore, Bedford, MA), and Na+ K+ ATPase (Upstate Biotechnology, Lake Placid, NY) were used in these studies, and species-appropriate secondary Abs were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). To detect C3 activation fragments, an HRP-conjugated goat polyclonal Ab to mouse C3 (MP Biomedicals, Solon, OH) was used for Western blot analysis, and an FITC-conjugated form of the same Ab was used for FACS analysis. CD11b was detected by staining sections with an Alexa Fluor 488-conjugated rat anti-mouse CD11b Ab (Invitrogen, Carlsbad, OH). Complement-activation experiments were performed by adding 10% normal mouse serum to the cell media. To block factor H present within the serum from inhibiting the reaction, as described. Staining of surface proteins was performed by incubating the cells at 37°C for 1 h with 10% normal mouse serum in the cell media. To block factor H present within the serum from inhibiting factor I-mediated cleavage of C4 and C3 activation fragments, an HRP-conjugated goat polyclonal Ab to mouse C3 (MP Biomedicals, Solon, OH) was used for Western blot analysis, and an FITC-conjugated form of the same Ab was used for FACS analysis. CD11b was detected by staining sections with an Alexa Fluor 488-conjugated rat anti-mouse CD11b Ab (Invitrogen, Carlsbad, OH). Complement-activation experiments were performed by adding 10% normal mouse serum to the cell media. To block factor H present within the serum from inhibiting the alternative pathway on the surface of cells, a recombinant murine protein, referred to as recombinant factor H domains 19–20 [rH 19–20, consists of the 19th and 20th short consensus repeats of factor H and the cell media. To block factor H present within the serum from inhibiting the reaction, as described. Staining of surface proteins was performed by incubating the cells at 37°C for 1 h with 10% normal mouse serum in the medium. In some experiments, the cells were grown to confluence on Transwell filters, and serum was added to the apical or basal chamber.

Flow cytometry

Surface expression of Crry, DAF, and CD59 was examined by flow cytometry, and deposition of C3 fragments onto the cell membrane was examined in a similar fashion. TECs were grown to confluence. Some cells were treated with anticytin A, as described in specific experiments. The cells were released from the plates by treatment with Accutase (Innovative Cell Technologies, San Diego, CA) and washed in PBS. For complement-activation experiments, the cells were incubated with streptavidin-coupled Dynabeads (Invitrogen) while rotating for 1 h at 4°C. The mixture was applied to a magnetic column, and the bound supernatant was saved as the basolateral fraction. The bound beads were incubated with an equal volume of radioimmunoprecipitation buffer and heated to 95°C for 5 min. The apical and basolateral proteins were separated by SDS-PAGE and probed for Crry.

To induce chemical hypoxia in the cells, the media was removed and replaced with 1 μM antimycin A (Sigma-Aldrich) in DMEM without glucose. ATP measurements of cell extracts were performed by washing cell monolayers in PBS and harvesting the cells into buffer containing 0.220 M mannitol, 0.070 M sucrose, 0.5 mM EGTA, 2 mM HEPES, and 0.1% fatty acid-free BSA. The extracts were incubated on ice for 30 min and then centrifuged at 750 g for 5 min. The ATP in the supernatant was determined using a bioluminescence assay (Molecular Probes, Eugene, OR). ATP values were expressed as a percentage of control samples. Complement-activation experiments were performed by incubating the cells at 37°C for 1 h with 10% normal mouse serum in the medium. In some experiments, the cells were grown to confluence on Transwell filters, and serum was added to the apical or basal chamber.

Purification of factor B

Mouse complement factor B was purified from normal mouse plasma by affinity purification and size-exclusion chromatography. The affinity column was created by coupling a mAb to mouse factor B [mAb 1379 (26)] to CNBr-activated Sepharose (GE Healthcare, Piscataway, NJ), as per the manufacturer’s instructions. The eluate from the column was lyophilized and resuspended in 50 μl 500 mM EDTA to prevent alternative pathway activation. The blood was centrifuged at 10,000 rpm for 15 min, and the plasma was collected. The plasma was diluted 1:1 with buffer (aminocaproic acid 50 mM, EDTA 0.5 M). The antigenicinity of the plasma was determined by ELISA and Western blotting.
10 mM, benzamidine 2 mM in PBS [pH 7.4]), passed through a 0.22-μm filter (Corning Glass, Corning, NY), and added to the affinity column. The column was washed with 10 column volumes of buffer, and factor B was eluted using 5 M LiCl. The buffer in the eluent was exchanged with PBS, and the eluted proteins were separated on a HiLoad 26/60 Superdex column (GE Healthcare) using an AKTAfplc (GE Healthcare). The purified factor B was subjected to electrophoresis on a 10% Bis-Tris gel (Invitrogen) and stained with Coomassie. The protein appeared ~95% pure by visual inspection, and we previously found that protein purified by this method restores alternative pathway activity when added to serum from B−/− mice.

Immunofluorescence microscopy

For immunofluorescence microscopy, sagittal sections of the kidneys were snap-frozen in OCT compound (Sakura Finetek U.S.A., Torrance, CA). Five-micrometer sections were cut with a cryostat and stored at −80°C. The slides were later fixed with acetone and stained with Ab to mouse C3, CD59, collagen type IV, or mouse CD11b. The slides were counterstained with hematoxylin (Vector Laboratories, Burlingame, CA). To assess the extent of C3 deposition, 10 high-powered fields (>400) were examined in the outer medulla of each section. The number of fields with C3 staining was averaged for each experimental group. To assess neutrophil infiltration, 10 high-powered fields in the outer medulla of each section were examined. The number of CD11b+ cells was expressed as an average per high-power field. To confirm that isolated TECs retained an epithelial cell phenotype, the cells were stained with anti-pan cytokeratin or anti-megalin, and nuclei were visualized with DAPI. Slides were imaged using a Nikon T-2000 inverted microscope and analyzed with Slidebook 4.2 software (Intelligent Imaging Innovations, Denver, CO). Cells on coverslips were fixed and permeabilized in cold methanol/acetic acid (1:1) for 5 min and then stained for Crry. Images of the cells were acquired on a Zeiss LSM 5 META confocal microscope.

Measurement of C3a

C3a in cell supernatants was measured by ELISA using mAbs and standards from BD Biosciences, according to the manufacturer’s instructions. To ensure that variations among serum batches did not affect C3a generation, values were only compared for cells treated simultaneously with the same batch of pooled serum. To determine the amount of C3a generated by contact with the TEC surface, the values were corrected for the amount of C3a generated in serum incubated with media at 37°C without cells present. To determine whether the pH 19–20 affects complement activation in the fluid phase, 10% serum was incubated with or without pH 19–20. The reaction was sampled at 10, 20, and 30 min, and C3a levels were measured.

Measurement of serum urea nitrogen

Serum urea nitrogen levels were measured using a Quantichrom Urea Assay Kit (BioAssay Systems, Hayward, CA), according to the manufacturer’s instructions.

Statistics

Statistical comparisons were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA). A t test was used for comparison of two groups, and multiple groups were compared using one-way ANOVA, with a Tukey posttest. A p value <0.05 was considered statistically significant. Results are reported as mean ± SEM.

Results

Crry is the only membrane-bound complement regulator expressed by TECs under in vitro culture conditions

Immunofluorescence microscopy of mouse (21) and human (19) tissue suggests that Crry (MCP in humans) is expressed on the tubular epithelium and that there is no detectable DAF or CD59 expression in vivo (19). TECs were grown in primary culture to determine whether in vitro growth alters the expression of the complement-regulatory proteins. Freshly isolated TECs were grown in medium selective for epithelial cells for ≥2 wk. The cells were stained for cytokeratin, confirming that >95% of the cells were epithelial (Fig. 1A), and staining for megalin indicated that they were primarily proximal tubular epithelial phenotype (Fig. 1B). FACS analysis was performed using mAbs specific for Crry, DAF, and CD59 (Fig. 1C–E). Consistent with staining of the tissue sections, the Crry was abundantly expressed on the surface of the cells, but DAF and CD59 were not detected. Therefore, cultured TECs retain their original phenotype with regard to expression of complement-regulatory proteins.

Complement is spontaneously activated on the apical surface of TECs

It was reported that the alternative pathway of complement is activated on TECs exposed to fresh serum (27, 28). We grew TECs on Transwell filters until a stable TER was obtained. We biotinylated proteins on the apical or basal surface, isolated the biotinylated proteins using streptavidin beads, and performed a Western blot for Crry (Fig. 2A). More Crry was isolated from the basolateral surface than the apical surface, supporting the polarized expression of this protein. Cells on Transwell filters were exposed to 10% complement-sufficient mouse serum on the apical or the basal surface for 1 h. Cells lysates were blotted for C3-activation fragments (Fig. 2B). Abundant fixation of C3 was seen in samples exposed to serum on their apical surface, whereas much less C3 was seen in cells exposed on the basal surface.

To determine whether complement activation on the TEC cell surface is controlled, in part, by factor H in the serum, the interaction of factor H with the cell surface was blocked using a recombinant–dominant negative inhibitor containing the carboxy terminus of factor H (23). C3a was measured in the supernatants of the cell cultures (Fig. 2C), confirming that more C3a is generated when the serum is exposed to the apical surface than the basolateral surface. The difference in C3a generation on the apical and
FIGURE 2. Complement is activated on the apical surface of TECs exposed to serum. TECs from C57BL/6J mice were grown on Transwell filters until confluent monolayers were formed. A, Apical proteins were biotinylated and isolated using streptavidin beads. Western blot analysis demonstrated that most of the Crry was purified from the basolateral fraction. B, Cells were incubated with 10% serum in the basal or apical chamber for 1 h. Western blot analysis of reduced cell lysates demonstrated that C3-activation fragments were spontaneously deposited on the cells when the apical surface was exposed to serum. C, Measurement of C3a in the cell supernatants confirmed that less activation occurred when the basolateral surface of the cells was exposed to serum than when the apical surface was exposed. The addition of rH 19–20 caused greater C3a generation when added to the apical or basolateral surface. D, Incubation of 10% serum with the same concentration of rH 19–20 in the absence of cells generated slightly more C3a than incubation of serum alone. However, this quantity of C3a is less than the increase of C3a generated on the cell surfaces in the presence of rH 19–20.

basal surfaces was not as great as the difference in C3 fixation to these surfaces (Fig. 2B). This may be due to fluid-phase C3a generation in the apical and basolateral compartment, which obscures the difference in C3a generation on the cell surfaces. When cells were grown on Transwell filters and exposed to 10% mouse serum in the apical or basolateral compartments, the addition of rH 19–20 (50 μg/ml) caused greater generation of C3a (Fig. 2C); the amount of C3a generated on either surface was greater with the addition of rH 19–20. When the same concentration of rH 19–20 was incubated with serum in the absence of cells, there was a slight increase in the amount of C3a generated (Fig. 2D), suggesting that the rH 19–20 may have a modest effect on fluid-phase activation. However, the quantity of C3a generated was not sufficient to account for the increase in C3a seen when rH 19–20 was applied to cells. These results indicate that complement activation is regulated more effectively on the basolateral surface of TECs than on the apical surface and that factor H present in the serum contributes to the regulation of complement activation on both surfaces. Factor H is found in the urine of some patients with renal disease, indicating that this interaction may be functionally important in proteinuric patients (29, 30).

Chemical hypoxia decreases surface expression of Crry by TECs and increases complement activation on the surface of the cells

We used antimycin A to induce chemical hypoxia in the TECs. After treatment of the cells with 1 μM antimycin A for 1 h, levels of ATP in the cells were reduced by 60 ± 15% (p < 0.001; Fig. 3A). The integrity of the monolayer, as assessed by the TER, was also disrupted by this treatment protocol (Fig. 3B). Flow cytometry of surface Crry demonstrated that the surface levels of this protein were decreased on hypoxic cells relative to unmanipulated controls (Fig. 3C). Flow cytometry was performed to measure the deposition of C3 on the surface of the cells after exposure to 10% mouse serum. When gated on live cells, the amount of C3 deposited on hypoxic cells was greater than that deposited on unmanipulated cells (Fig. 3D), although the effect was modest. The overall level of Crry in the cells was not changed (Fig. 3E), indicating that the protein may be internalized in response to hypoxia. When 10% serum was applied to the cells in the absence of antimycin, no change in the levels of surface Crry was seen (data not shown).

Cells were grown on coverslips until confluent and then were subjected to the same protocol of treatment with antimycin A. At baseline, confocal microscopy of the cells for the Na+K+ ATPase and for Crry demonstrated that both proteins are expressed on the basolateral aspect of the cells (Fig. 3F). After treatment of the cells with antimycin A, the basolateral concentration of both proteins was reduced, and accumulated regions of Crry were redistributed toward the apical portion of the cells (Fig. 3G).

DAF and CD59 expression can be induced on some renal cell types in response to complement activation (31, 32). We sought to determine whether chemical hypoxia or complement activation on the TECs induces DAF or CD59 expression. TECs were treated with 1 μM antimycin A for 1 h, and flow cytometry was performed. Neither DAF nor CD59 could be detected on the surface of the TECs after treatment with antimycin A (data not shown). Confluent cells were also exposed to 10% mouse serum to activate complement on the cell surface and were examined by flow cytometry. Surface DAF and CD59 were not detected on TECs after they were exposed to mouse serum (data not shown).

Increased complement activation is seen on TECs that do not express Crry

To determine whether the absence of Crry renders TECs susceptible to complement activation, even without concomitant hypoxia, we generated mice with homozygous deficiency of Crry. Homozygous deficiency of Crry is lethal in utero as a result of uncontrolled complement activation in the placenta (24), but deficiency of complement factor B (a protein necessary for alternative pathway activation) rescues the phenotype and allows the appropriate number of Crry−/−fB−/− births (33). Therefore, we bred mice doubly deficient in Crry and in complement factor B (Crry−/−fB−/− mice) and confirmed the genotype by PCR (Fig. 4A). The absence of Crry on TECs cultured from these mice was confirmed with flow cytometry (Fig. 4B). When TECs from
FIGURE 3. ATP depletion with antimycin A reduces surface Crry on TECs and permits increased complement activation on the cell surface. TECs were grown in culture and exposed to 1 μM antimycin A for 2 h. A. Measurement of ATP in the cells confirmed that this treatment significantly reduced the ATP levels. B. Transepithelial resistance also decreased after treatment with antimycin A, demonstrating disruption of the tight junctions. C. Crry expression was assessed by FACS analysis. Crry expression was reduced on the antimycin A-treated cells (filled graph) compared with unmanipulated controls (solid line), although the levels were still higher than the background levels seen with an isotype control (light gray line). D. Cells were exposed to 10% mouse serum for 1 h, and deposition of C3 on the surface of the cells was examined by FACS analysis. C3 deposition was greater on cells treated with antimycin A (filled graph) than on unmanipulated controls (solid line). E. Overall levels of Crry did not change notably after treatment with antimycin A when assessed by Western blot analysis. F. Confocal microscopy was performed for Na⁺-K⁺ ATPase (red) and for Crry (green), and lateral views were obtained. Both proteins were concentrated on the basolateral surface of the cells. G. Treatment of the cells for 2 h with antimycin A reduced the basolateral concentration of both proteins, and accumulations of Crry were seen throughout the cells and toward the apical surface. F and G, Original magnification ×400.

Crry⁻/⁻fB⁻/⁻ or from wild-type control mice were exposed to normal mouse serum, more C3 deposition was seen on Crry-deficient cells (Fig. 4C). Cells were grown on Transwell filters and exposed to 10% mouse serum on their apical or basal surface (Fig. 4D). The amount of C3a generated after Crry⁻/⁻fB⁻/⁻ TECs were exposed to serum on their basal surface was greater than that generated when TECs from wild-type mice received the same treatment (Fig. 4D), and the levels were similar to those produced when rH 19–20 was added to the reaction (Fig. 2C). Furthermore, the difference in C3a generated on the apical and basal surfaces of TECs from wild-type mice was no longer seen when Crry⁻/⁻fB⁻/⁻ TECs were used, indicating that the basolateral expression of Crry accounts for the greater control of complement activation on the basolateral surface. More C3a was generated when the apical surface of the Crry⁻/⁻fB⁻/⁻ TECs was exposed to serum in the presence of rH 19–20 than when it was exposed to serum alone (Fig. 4E), indicating that factor H still regulates complement on this surface in the absence of Crry.

Crry⁻/⁻fB⁻/⁻ mice reconstituted with purified factor B develop tubular complement activation and renal injury

We purified factor B from mouse plasma by affinity chromatography followed by size-exclusion chromatography (Fig. 5A). We injected Crry⁻/⁻fB⁻/⁻ mice and Crry⁺/⁺fB⁻/⁻ control mice with 50 μg of the purified factor B via the tail vein to restore alternative pathway activity. Immunofluorescence microscopy demonstrated that all of the Crry⁻/⁻fB⁻/⁻ mice had detectable C3 deposits after reconstitution with factor B. Only one of the Crry⁻/⁻fB⁻/⁻ control mice had detectable C3 after injection with factor B. When the percentage of high-powered fields containing C3 deposits was assessed, a greater degree of C3 deposition was seen in the kidneys of Crry⁻/⁻fB⁻/⁻ mice than in Crry⁺/⁺fB⁻/⁻ mice (Fig. 5B).

C3 deposition was seen along the proximal tubule at the tubular pole of the glomerulus and faintly within the mesangium (Fig. 5C). Patches of tubular staining were also seen in the outer medulla (Fig. 5D). It is not clear why the deposition was patchy, but the tubulointerstitial deposition that occurs in wild-type mice is similarly patchy, suggesting regional heterogeneities that may foster complement activation.

C3 deposition on the tubules of wild-type mice is typically seen along the tubular basement membrane. Reconstitution of the Crry⁺/⁺fB⁻/⁻ mice did not restore the wild-type pattern of patchy tubular C3 deposition. It is possible that the quantity of factor B that we used was too small or too short-lived to support complement activation in the tubulointerstitium of Crry⁺/⁺fB⁻/⁻ mice. In the reconstituted Crry⁻/⁻fB⁻/⁻ mice, some of the C3 staining in the proximal tubules appeared to be cytosolic (unlike what is seen in wild-type mice), and it was located within the tubular basement membrane (Fig. 5G).

Serum urea nitrogen values in unmanipulated Crry⁺/⁺fB⁻/⁻ and Crry⁻/⁻fB⁻/⁻ mice were 15 ± 2 mg/dl and 10 ± 1 mg/dl, respectively. By 24 h after reconstitution with factor B, serum urea nitrogen values in Crry⁻/⁻fB⁻/⁻ mice were significantly elevated (85 ± 24 mg/dl in Crry⁻/⁻fB⁻/⁻ mice versus 16 ± 7 mg/dl in Crry⁺/⁺fB⁻/⁻ mice; p < 0.01; Fig. 6A). Immunofluorescence staining demonstrated infiltration of neutrophils in the kidneys of Crry⁻/⁻fB⁻/⁻ mice reconstituted with factor B (Fig. 6B, 6C). No evidence of neutrophil infiltration was seen in the kidneys of Crry⁺/⁺fB⁻/⁻ mice reconstituted with factor B.

Finally, we sought to determine whether the tubulointerstitial complement activation would induce expression of other complement-regulatory proteins. We examined tissue sections from Crry⁻/⁻fB⁻/⁻ mice for expression of CD59. CD59 was detected in the mesangium of unreconstituted mice (Fig. 7A) and mice...
Crry demonstrated greater C3 on the TECs from and stained for surface C3. FACS analysis were then incubated with 10% serum for 1 h from Crry wild-type controls (solid dark line). Mice from which the wild-type gene was amplified were designated as Crry+/+. Mice from which the Neo insert was amplified but the wild-type gene was not were designated as Crry+/-.

TECs and preventing autologous injury. Polymorphisms in factor H and MCP (9, 10) are associated with the development of aHUS. The kidney is particularly susceptible to complement-mediated injury. Our findings demonstrate that control of the complement system on the TEC requires proper functioning of both of these proteins and proper localization of the membrane inhibitor on the basolateral surface when it is exposed to serum complement proteins. Hypoxia of the cells disrupts the organization of the cell membrane and renders the cell susceptible to complement activation. This may be due to the overall reduction in surface Crry. It may also be due to increased access of serum complement proteins to the apical surface as the integrity of the monolayer is lost.

Although endothelial injury is regarded as the trigger of aHUS, tubular injury and cortical necrosis are also well-described findings (34), reflecting the vulnerability of the TEC to complement-mediated injury. Our findings demonstrate that control of the complement system on the TEC requires proper functioning of both of these proteins and proper localization of the membrane inhibitor on the basolateral surface when it is exposed to serum complement proteins. Hypoxia of the cells disrupts the organization of the cell membrane and renders the cell susceptible to complement activation. This may be due to the overall reduction in surface Crry. It may also be due to increased access of serum complement proteins to the apical surface as the integrity of the monolayer is lost.

Tickover of the alternative pathway in the fluid phase deposits C3b on nearby surfaces, and the deposited C3b causes autoactivation unless effectively inhibited by complement-regulatory proteins (35). Several studies indicated that the absence or functional loss of Crry renders the kidney susceptible to tubular injury (21, 22, 36). The studies presented in this article demonstrate that the loss of surface regulation by Crry expression at this site. As with renal ischemia/reperfusion (I/R) (21), chemical hypoxia of the TECs causes a reduction in surface Crry levels, and the distribution within the cell is also altered.

Spontaneous complement activation on the surface of TECs is also controlled by endogenous factor H. When rH 19–20 was added to TECs, it permitted increased spontaneous deposition of C3 on the apical and basal surfaces. Thus, factor H and Crry are important for regulating the complement system on the surface of TECs and preventing autologous injury. Polymorphisms in factor H and MCP (9, 10) are associated with the development of aHUS.

**FIGURE 4.** Complement is activated on TECs from Crry+/B+- cells exposed to wild-type serum. Crry+/B+- mice were bred as described. A, PCR was performed using primers for the wild-type Crry gene and for the Neo insert used to disrupt the gene. Mice from which the wild-type gene was amplified were designated as Crry+/+.

**A** Wild-type gene

**B** Neo insert

**C** Cells

**D** FACS analysis demonstrated greater C3 on the TECs from Crry+/B+- mice (filled graph) than from wild-type controls (solid dark line). D, TECs from Crry+/B+- and wild-type mice were grown on Transwell filters and treated with serum on their apical or basolateral surface. After 1 h, the supernatants were collected, and C3a was measured by ELISA as a marker of complement activation. Activation on the basolateral surface of Crry+/B+- TECs was greater than that on the basolateral surface of wild-type TECs, and there was not a significant difference between activation on the apical and basal surfaces of Crry+/B+- TECs. E, TECs from Crry+/B+- mice were grown on Transwell filters and exposed to serum on their apical surface with or without the addition of rH 19–20. More C3a was generated when the cells were exposed to serum with rH 19–20.

reconstituted with factor B (Fig. 7B). Tubulointerstitial expression was not detected in either group of mice.

**Discussion**

The kidney is particularly susceptible to complement-mediated injury in a number of clinical settings, and congenital deficiency or defects in the complement-regulatory proteins MCP and factor H are strongly associated with the development of renal disease. In the current study, we demonstrated that Crry (the murine homolog of MCP in the kidney) is the only membrane-bound regulator of complement expressed by murine TECs. Crry is expressed on the cell membrane, and its expression is concentrated in the basolateral portion of the cell. Polarized TECs regulate complement more efficiently on the basolateral surface of the cells than on the apical surface, in part because of Crry expression at this site. As with renal ischemia/reperfusion (I/R) (21), chemical hypoxia of the TECs causes a reduction in surface Crry levels, and the distribution within the cell is also altered.

Spontaneous complement activation on the surface of TECs is also controlled by endogenous factor H. When rH 19–20 was added to TECs, it permitted increased spontaneous deposition of C3 on the apical and basal surfaces. Thus, factor H and Crry are important for regulating the complement system on the surface of TECs and preventing autologous injury. Polymorphisms in factor H and MCP (9, 10) are associated with the development of aHUS.
The susceptibility of the kidney to complement-mediated injury is likely influenced by the fact that expression of the membrane-bound inhibitor by the TECs is restricted to the basal surface of the cells, and levels are decreased in response to cellular stress and injury. It was also demonstrated recently that properdin binds to the apical surface of TECs and may catalyze alternative pathway activation on this surface (28). Factor H regulates the alternative pathway on the apical and basal surface of TECs, but regulation of complement by factor H is inadequate to prevent spontaneous complement activation on the apical surface of the cells, and it cannot compensate for the loss of regulation by Crry on the basolateral surface of the cells. Likewise, expression of Crry on the basolateral surface of TECs does not fully prevent complement activation when the C-terminal membrane-interacting portion of factor H was blocked by rH 19–20. Although DAF and CD59 are not expressed in the interstitium of normal kidneys, they can be detected in the tubulointerstitium of some diseased kidneys (31, 32). Therefore, the in vivo dependence of TECs on protection by MCP may not be as stringent as our current results would suggest.

The reduction of surface Crry on hypoxic cells may be an active response to stress. Cervical epithelial cells were demonstrated to downregulate surface MCP postinfection with piliated Neisseria gonorrhoea (37), and downregulation of surface regulatory proteins may be a mechanism by which epithelial cells foster the immune response to invasive pathogens. Renal TECs form a barrier epithelium, and they may play an important role in the antimicrobial response to pathogens (38). However, in aseptic diseases, such as I/R, such a response to stress could be maladaptive. Our findings help to explain why TECs are a target for uncontrolled complement activation in several clinical settings, including proteinuric states.
and ischemic injury. The mechanisms we described may also apply to other diseases in which TECs sustain cellular stress or injury.

Recent studies examined complement regulation in Crry−/− mice (39, 40). Although maternal complement proteins injure the placenta of Crry−/− embryos (24), if the mother is deficient in alternative pathway proteins (C3 or factor B) Crry−/− pups can be generated. Two groups bred Crry−/− mice that do not have genetic deletion of the C3 or factor B genes. The Crry−/− mice displayed increased consumption of factor B and C3, and levels of these two proteins were decreased in plasma (39, 40). Although the alternative pathway activation was presumably occurring on tissue surfaces, no injury phenotype was described, and pathologic complement activation in the glomeruli was not seen. Renal function was not reported in the studies, but the investigators stated that the mice were followed for >1 y without evidence of renal damage (39). Just as the chronic, diffuse consumption of the complement proteins allows successful parturition in these mice, the extrarenal consumption of factor B and C3 may prevent complement activation on the tubules from reaching a level sufficient to cause renal injury.

Several studies examined the role of Crry in protecting the kidney from complement-mediated injury of the tubules (21, 22, 36). One of these studies demonstrated that basolateral expression of Crry in mice is disrupted by renal ischemia (21). Another study demonstrated progressive complement-mediated injury of Crry−/− kidneys transplanted into wild-type hosts (22). In the current study, the acute infusion of factor B restored sufficient activity to cause foci of complement deposition and tissue injury within the kidney. Thus, in a setting that is not potentially complicated by effects of warm ischemia or transplantation-related I/R, our results clearly show that the absence of Crry renders TECs susceptible to spontaneous complement-mediated injury.

Although several different renal diseases are closely linked to uncontrolled activation of the alternative pathway, the molecular mechanisms seem to be distinct in several of the models. In DDD, for example, activation in the absence of adequate factor H function seems to occur primarily in the fluid phase (11, 41). The tropism of systemically generated C3 fragments for the glomerular basement membrane has not been explained. aHUS involves defects in alternative pathway regulation that could potentially cause injury on many host cells, but it usually manifests as renal injury (10). The mechanisms of complement regulation on the TECs are distinct from those that protect the glomerular endothelial cells and the glomerular basement membrane; yet, it is striking that the kidney is such a common target of injury in patients with defects in complement-regulatory proteins. Other factors, such as the large
percentage of cardiac output that the kidneys receive, might contribute to the development of renal injury as a consequence of global defects in complement regulation.

In conclusion, we found that Cryr is the only cell surface complement-regulatory protein expressed by murine TECs, but factor H in the serum also contributes to complement regulation on the TEC surface. At baseline, Cryr is concentrated on the basolateral surface, and complement regulation is more efficient on this surface than on the apical surface. Cellular hypoxygen disrupts the TEC tight junctions, reduces surface levels of Cryr, and potentiates complement activation on the TEC membrane. Finally, we performed studies demonstrating that complement is spontaneously activated on TECs, with genetic deletion of Cryr when they are exposed to an intact alternative pathway, and reconstitution of Cryr−/−/fB−/− mice with purified factor B protein induced acute kidney injury. These results underscore the importance of Cryr for protecting TECs from autologous injury. These findings also help to explain the particular vulnerability of TECs to complement-mediated injury in several clinical settings and indicate that therapies capable of restoring control of the complement system at the TEC surface may be beneficial in renal tubulointerstitial disease.

Disclosures
J.M.T. is a stockholder in and a consultant for Taligen Therapeutics, Inc. V.M.H. is a stockholder in and is chief scientific officer for Taligen Therapeutics, Inc. M.K.P. is an officer of and has a financial interest in Complement Therapeutics, Inc., a supplier of complement reagents.

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