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Factor I is required for the development of membranoproliferative glomerulonephritis in factor H-deficient mice

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The inflammatory kidney disease membranoproliferative glomerulonephritis type II (MPGN2) is associated with dysregulation of the alternative pathway of complement activation. MPGN2 is characterized by the presence of complement C3 along the glomerular basement membrane (GBM). Spontaneous activation of C3 through the alternative pathway is regulated by 2 plasma proteins, factor H and factor I. Deficiency of either of these regulators results in uncontrolled C3 activation, although the breakdown of activated C3 is dependent on factor I. Deficiency of factor H, but not factor I, is associated with MPGN2 in humans, pigs, and mice. To explain this discordance, mice with single or combined deficiencies of these factors were studied. MPGN2 did not develop in mice with combined factor H and I deficiency or in mice deficient in factor I alone. However, administration of a source of factor I to mice with combined factor H and factor I deficiency triggered both activated C3 fragments in plasma and GBM C3 deposition. Mouse renal transplant studies demonstrated that C3 deposited along the GBM was derived from plasma. Together, these findings provide what we believe to be the first evidence that factor I-mediated generation of activated C3 fragments in the circulation is a critical determinant for the development of MPGN2 associated with factor H deficiency.

Introduction

The complement system is an important part of the innate immune system that is composed of a complex group of proteins whose main biological functions include host defense, the physiological clearance of immune complexes and dying cells, and an adjuvant role in the production of immune responses (1). The activation of complement is tightly regulated by a sophisticated group of membrane-bound and fluid-phase proteins that function not only to prevent tissue damage from autologous complement activation but also to prevent depletion of complement proteins (2). In contrast to the classical and lectin pathways, whose activation is triggered principally by immune complexes and bacterial mannose groups, respectively, the alternative pathway of complement activation is in a continuous state of low-level activation, resulting in the continuous generation of activated C3 (C3b) in plasma (3). Spontaneous activation of C3 in plasma occurs through the “tick-over” pathway, which is initiated following the hydrolysis of intact C3 to generate C3i (also known as C3[H₂O]) (4). C3i is able to interact with factors B and D to form an enzyme complex (the C3 convertase, C3iBb), which can cleave intact C3 to produce C3b, releasing the anaphylatoxin C3a. C3b interacts with factors B and D to generate the alternative pathway C3 convertase, C3bBb. This convertase causes further C3 cleavage and thus amplifies the generation of C3bBb. This

enables the production of C3b to rapidly increase and is referred to as the alternative pathway amplification loop (5). Although these C3 convertases will spontaneously decay unless stabilized by properdin (6), active regulation of alternative pathway activation is achieved by 2 plasma proteins, factor H and factor I.

Factor H is an abundant 150-kDa serum glycoprotein that regulates alternative pathway activation. It achieves this by inhibiting the formation of the alternative pathway C3 convertases (C3iBb, C3bBb) (7, 8). It also promotes the dissociation of these C3 convertases once they have formed, an action termed “decay acceleration activity” (8). It is also an essential plasma cofactor in the factor I-mediated proteolytic conversion of C3b to iC3b (9). Its importance in vivo is illustrated by the complement profile described in individuals with complete factor H deficiency. In these individuals, uncontrolled alternative pathway activation occurs with secondary depletion of C3, factor B, and properdin (10).

Factor I is an 88-kDa heterodimeric serine protease with a serum concentration of approximately 39–100 µg/ml (11). It functions, with cofactor, to inactivate C3b and C4b. The factor I-mediated proteolytic inactivation of C3b occurs in 2 steps. Initially, factor I cleaves the α-chain of C3b at 2 sites, releasing a 17-amino acid peptide termed “C3f” and forming iC3b (12). Essential cofactors for this reaction include factor H in the fluid phase (9) and membrane cofactor protein (MCP, CD46) and CR1 on cell surfaces. Further degradation of iC3b occurs following the factor I-mediated cleavage of the Arg954-Glu955 bond to produce C3dg and C3c. Hence, through its actions on C3b, factor I inhibits alternative pathway C3 convertase formation, thus limiting alternative pathway amplification. Similar to factor H deficiency, complete

Nonstandard abbreviations used: AFU, arbitrary fluorescence units; GBM, glomerular basement membrane; MPGN2, membranoproliferative glomerulonephritis type II.

Conflict of interest: The authors have declared that no conflict of interest exists.

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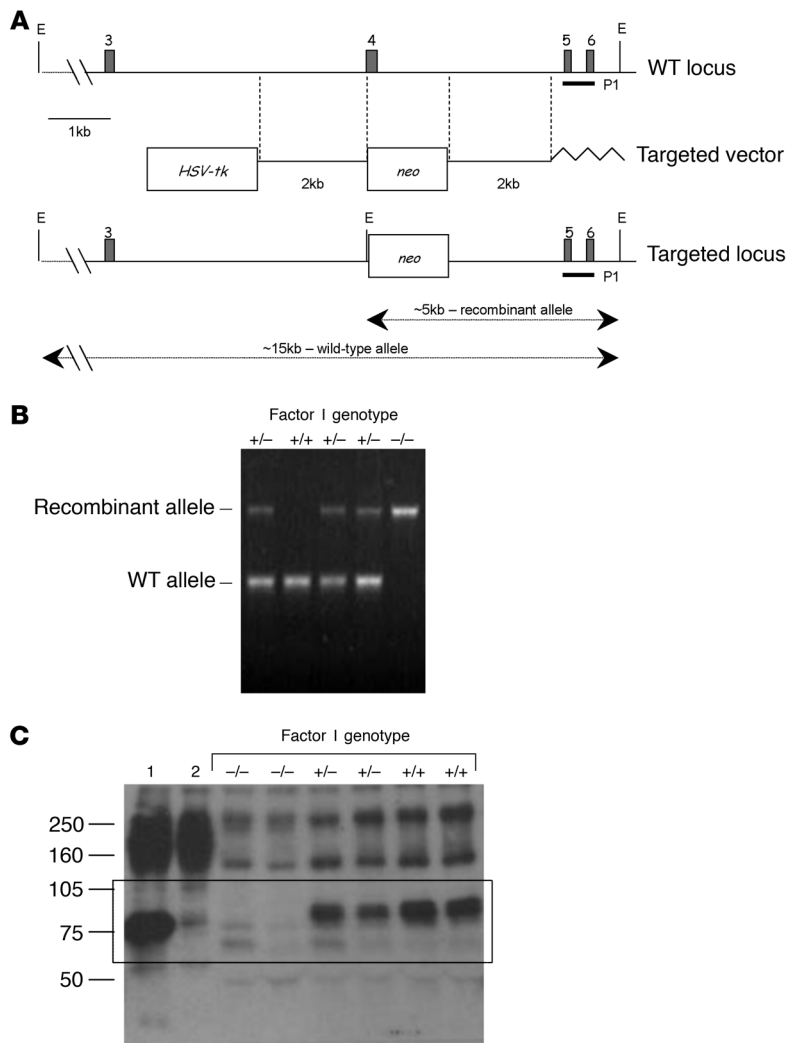


Figure 1

Generation of *Cfi*^{-/-} mice. (A) Targeted *Cfi* locus, targeting vector, and structure of the targeted gene. Exons are represented by filled boxes, and the neomycin resistance gene (*Neo*) and herpes simplex virus thymidine kinase cassette (*HSV-tk*) are indicated. Homologous fragments are indicated by dotted lines. E, *EcoRI*. Double-headed arrows indicate the fragments detected on Southern blot analysis of wild-type and recombinant alleles after *EcoRI* digestion of genomic DNA hybridized with the 3' probe (P1, a cDNA probe containing exons 5 and 6). (B) Amplification of recombinant and wild-type alleles using genomic DNA from *Cfi*^{-/-}, *Cfi*^{+/-}, and wild-type mice. Absence of the wild-type allele is seen in the *Cfi*^{-/-} animal. (C) Western blot using nonreducing conditions for serum factor I using crossreactive polyclonal anti-human factor I anti-sera. Lane 1 indicates normal human sera, lane 2 indicates sera from a factor I-deficient individual. Lanes 3–8 represent mouse sera from homozygous, heterozygous, and wild-type mice, as indicated. Absence of the 88-kDa factor I protein (box) is evident in the factor I-deficient individual (lane 2) and in sera from the *Cfi*^{-/-} mice (lanes 3 and 4).

individuals with normal factor H activity. MPGN2 is associated with C3 nephritic factor, an IgG auto-antibody that binds to and stabilizes the alternative pathway C3 convertase, resulting in enhanced alternative pathway activation. MPGN2 has also been reported in families with inherited dysfunctional C3 molecules, which form C3 convertases resistant to physiological inhibition by factor H (31, 32).

Despite the overwhelming evidence linking alternative pathway dysregulation and MPGN2, it is striking that MPGN2 has never been reported in individuals with factor I deficiency (13). The predominant clinical manifestation in these individuals was of increased risk of pyogenic infection, a feature shared by individuals with factor H or C3 deficiency (13) that is thus likely to be a consequence of the C3 deficiency state common to all 3 conditions. To date, glomerulonephritis and factor I deficiency has been reported in 3 individuals (14, 15, 33). Renal biopsy in one case showed focal segmental glomerulonephritis with glomerular deposition of C4, C3, and immunoglobulins (33). In another individual who also had SLE, diffuse proliferative class IV lupus glomerulonephritis was present (14). Finally, a third case with immune complex glomerulonephritis with glomerular IgG and C3 has been reported (15). Thus these renal lesions with glomerular immunoglobulin deposition and classical pathway activation are pathologically distinct from MPGN2.

To determine why uncontrolled C3 activation in the context of factor I deficiency does not result in MPGN2, we have generated factor I-deficient (*Cfi*^{-/-}) mice. These animals displayed uncontrolled alternative pathway activation as evidenced by reduced C3, factor B, and factor H levels, but did not develop C3 deposition along the GBM or MPGN2. Hence, the renal phenotype of these mice differed significantly from the one reported previously in factor H-deficient (*Cfh*^{-/-}) mice, in which florid C3 deposition along the GBM and MPGN2 occurred (28). Remarkably, GBM C3 deposition did not occur even in mice with combined deficiency of factors H and I (*Cfh*^{-/-}*Cfi*^{-/-}) despite the presence of uncontrolled

deficiency of factor I in humans is associated with uncontrolled alternative pathway activation with secondary depletion of C3, factor B, and properdin (reviewed in ref. 13). Notably, the circulating C3 in factor I-deficient individuals is in the form of C3b (13), indicating an absolute requirement for factor I in the generation of C3b metabolites in vivo. Furthermore, secondary reduction in factor H levels in factor I-deficient individuals (13–18) has also been reported, possibly due to the formation of factor H–C3b complexes (16–18).

Uncontrolled alternative pathway activation is associated with the inflammatory renal condition membranoproliferative glomerulonephritis type II (MPGN2, also termed “dense deposit disease”) (19). MPGN2 is characterized by the presence of intramembranous electron-dense material along the glomerular basement membrane (GBM) (20) together with staining for C3 (21), C5 (22), and C9 (23) along the GBM in the absence of immunoglobulin (24). Complete factor H deficiency in humans (25, 26), pigs (27), and mice (28) results in spontaneous MPGN2. Furthermore, MPGN2 has been reported in an individual with an autoantibody to factor H (29) and recently in an individual with a mutation affecting one of the complement regulatory domains of factor H (30). Uncontrolled alternative pathway regulation and MPGN2 may also occur in

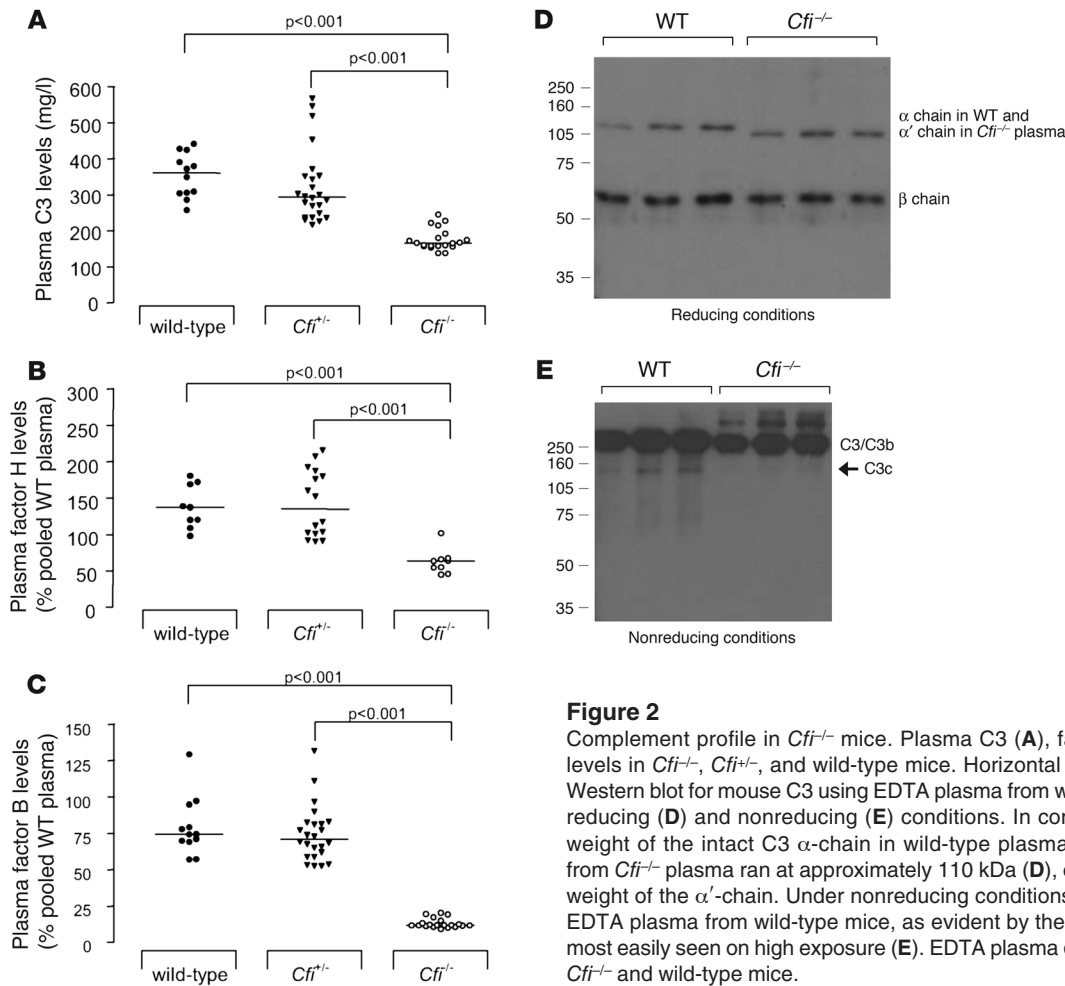


Figure 2 Complement profile in *Cfi*^{-/-} mice. Plasma C3 (A), factor H (B) and factor B (C) levels in *Cfi*^{-/-}, *Cfi*^{+/-}, and wild-type mice. Horizontal bars denote median values. Western blot for mouse C3 using EDTA plasma from wild-type and *Cfi*^{-/-} mice under reducing (D) and nonreducing (E) conditions. In comparison with the molecular weight of the intact C3 α -chain in wild-type plasma, the α -chain of C3 derived from *Cfi*^{-/-} plasma ran at approximately 110 kDa (D), consistent with the molecular weight of the α' -chain. Under nonreducing conditions C3c was detectable only in EDTA plasma from wild-type mice, as evident by the presence of 130-kDa bands most easily seen on high exposure (E). EDTA plasma dilutions were 1/1000 for both *Cfi*^{-/-} and wild-type mice.

alternative pathway activation. Analysis of plasma C3 activation fragments demonstrated that, in the absence of factor I, C3 circulated in the form of C3b. In contrast, in *Cfb*^{-/-} mice, C3b cleavage fragments were detectable in plasma. Reconstitution of factor I in *Cfb*^{-/-}*Cfi*^{-/-} mice precipitated GBM C3 deposition together with the concomitant appearance of C3b cleavage fragments in circulation. Renal transplant experiments confirmed that glomerular C3 deposition derives from the circulation in *Cfb*^{-/-} animals. Taken together, our observations demonstrate that, during uncontrolled alternative pathway activation, factor I-mediated cleavage of C3b using cofactors other than factor H is an absolute requirement for the development of GBM C3 deposition.

Results

Generation of Cfi^{-/-} mice. We disrupted the gene encoding mouse factor I in ES cells by deleting exon 4 of the *Cfi* gene with a standard gene-targeting replacement vector (Figure 1A). Homologous recombination in ES cells was detected by Southern blotting. Mice carrying the disrupted allele were screened by PCR (Figure 1B) and were born at the expected Mendelian ratios. Absence of factor I was confirmed in plasma of *Cfi*^{-/-} mice using western blotting (Figure 1C). *Cfi*^{-/-} mice were viable and fertile under specific pathogen-free conditions.

Complement analysis in Cfi^{-/-} mice. Homozygous factor I deficiency in humans is associated with secondary reduction in C3 levels,

with plasma C3 circulating as C3b (13). Hence, we first measured plasma C3 in wild-type, heterozygous (*Cfi*^{+/-}), and *Cfi*^{-/-} mice (Figure 2A). As expected, the median plasma C3 level in *Cfi*^{-/-} mice (163.3 mg/l, range 134.5–242.9, *n* = 20) was significantly lower than that seen in either *Cfi*^{+/-} (291.3 mg/l, range 214–566.7, *n* = 24) or wild-type (358.3 mg/l, range 254.5–439.1, *n* = 12) animals ($P < 0.001$ for *Cfi*^{-/-} versus wild-type or *Cfi*^{+/-} mice, Bonferroni’s multiple comparison test). Secondary reduction in factor H levels has also been reported in humans with homozygous factor I deficiency (13). In keeping with this, factor H levels were significantly reduced in the *Cfi*^{-/-} mice (median 61.9% pooled normal wild-type sera, range 42.9%–100.2%, *n* = 9; Figure 2B) compared with both wild-type (median 135.1%, range 96.1%–178.4%, *n* = 9) and *Cfi*^{+/-} (median 132.9%, range 88.4%–213.5%, *n* = 16) animals ($P < 0.001$ for *Cfi*^{-/-} versus wild-type or *Cfi*^{+/-} mice, Bonferroni’s multiple comparison test). Finally, we measured factor B levels in *Cfi*^{-/-} mice (Figure 2C), since reduced levels of factor B have been reported in individuals with uncontrolled alternative pathway activation due to either factor I (13) or factor H (10) deficiency. Significant reduction in plasma factor B levels were seen in the *Cfi*^{-/-} mice (median 12% pooled normal wild-type sera, range 9.6%–20.6%, *n* = 20) compared with both wild-type (median 74.6%, range 57.1%–130%, *n* = 12) and *Cfi*^{+/-} (median 71.3%, range 52.8%–132%, *n* = 24) animals ($P < 0.001$ for *Cfi*^{-/-} versus wild-type



Table 1
Renal function and histological analysis in 8-month-old (129/Sv × C57BL/6) *Cfi*^{-/-} mice

Mouse	n	Renal function			Renal histology											
		Plasma urea ^A mmol/l (range)	Albuminuria		Glomerular hypercellularity					Mesangial expansion				MH	GBM DC	
			μg/ml (range)	% Pos (n)	n	0	I	II	III	IV	0	I	II	III	% Pos (n)	% Pos (n)
WT	17	14.9 (11.1–26)	0 (0–746.7)	17.6 (3/17)	17	5	7	4	0	1	12	3	2	0	0	0
<i>Cfi</i> ^{+/-}	29	13.3 (8.2–30.4)	0 (0–684.4)	21.4 (6/28)	29	14	8	4	3	0	22	6	1	0	0	0
<i>Cfi</i> ^{-/-}	22	15.3 (8.1–26.7)	0 (0–99.7)	14.3 (3/21)	22	7	12	2	1	0	3 ^B	8	8	3	45.5% (10)	0

^AValues represent median. ^B*P* < 0.001 for *Cfi*^{-/-} vs. wild-type or *Cfi*^{+/-}, Bonferroni multiple comparisons test. Pos, positive; MH, Mesangial hyalinosis; GBM DC, GBM double contours.

or *Cfi*^{+/-} mice, Bonferroni's multiple comparison test). Thus the plasma complement profile of *Cfi*^{-/-} mice recapitulated that seen in homozygous factor I-deficient humans with reduced plasma C3, factor B, and factor H levels.

We next determined the nature of the plasma C3 in the *Cfi*^{-/-} mice by using western blotting of EDTA plasma samples under reducing conditions, which enabled identification of the intact and cleaved α -chains of C3 (Figure 2D). Murine C3 intact α -chain has a molecular weight of approximately 115 kDa, while the β -chain runs at 55–60 kDa (34). The molecular weight of the C3 α -chain using *Cfi*^{-/-} plasma was lower than that of the C3 α -chain from wild-type plasma and consistent with the molecular weight of the α -chain of C3b (also termed " α' -chain"). Notably, no α' -chain fragments were demonstrable in EDTA plasma from *Cfi*^{-/-} mice, consistent with the inability of factor I-deficient mouse sera to physiologically cleave C3b. Furthermore, while small amounts of C3c (130-kDa bands, Figure 2E) were detectable in EDTA plasma from wild-type animals, C3c was not detected in plasma from *Cfi*^{-/-} animals. High-molecular-weight bands were consistently seen in EDTA plasma from *Cfi*^{-/-} but not wild-type animals, which we postulated were due to aggregates of C3b. We therefore concluded that plasma C3 in the *Cfi*^{-/-} mice circulates in the form of C3b.

Cfi^{-/-} mice do not develop MPGN2. Uncontrolled C3 activation due to complete deficiency of factor H results in spontaneous MPGN2 in mice (28). We wanted to know whether uncontrolled C3 activation secondary to murine factor I deficiency resulted in spontaneous renal disease. To answer this question, we monitored cohorts of wild-type (*n* = 17), *Cfi*^{+/-} (*n* = 29), and *Cfi*^{-/-} (*n* = 22) over an 8-month period, at which point all the animals were sacrificed and renal function and histology assessed (Table 1). We selected an 8 month time period as MPGN2 in *Cfb*^{-/-} mice was fully penetrant by this age (28). Renal function as assessed by plasma urea levels and albuminuria did not significantly differ between the experimental groups (Table 1). Indeed, albuminuria was not detectable in the majority of the experimental animals regardless of genotype. Glomerular histological changes assessed included hypercellularity, mesangial expansion, and the presence of capillary wall thickening. *Cfi*^{-/-} mice had significant mesangial expansion compared

with both wild-type and *Cfi*^{+/-} animals (Table 1). Furthermore, deposition of nodular hyaline material was present within the mesangium in 10 of the *Cfi*^{-/-} mice (45.5%) but not in *Cfi*^{+/-} or wild-type groups (Table 1 and Figure 3). In contrast, glomerular hypercellularity scores did not differ between the experimental cohorts. Notably, light microscopic features of MPGN2, including capillary wall double contours, were not present in any of the *Cfi*^{-/-} mice. In summary, *Cfi*^{-/-} mice did not develop spontaneous glomerulonephritis but, at 8 months, demonstrated increased mesangial expansion and, in some animals, areas of mesangial hyalinosis.

Cfb^{-/-} animals have been shown to have florid deposition of C3 along the GBM, a lesion that precedes the morphological changes of MPGN2 (28). Hence, we next examined glomerular C3 staining in *Cfi*^{-/-} mice. Increased glomerular C3 staining was evident in the 8-month-old *Cfi*^{-/-} mice (Figure 4A), but the staining pattern was mesangial in distribution, in striking contrast to the linear capillary wall staining pattern seen in age-matched *Cfb*^{-/-} animals (Fig-

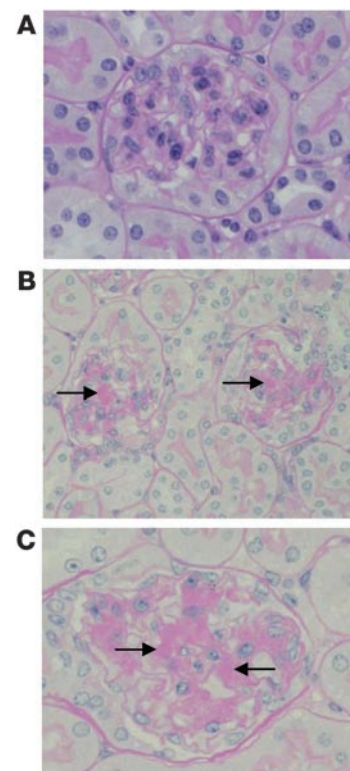


Figure 3
Light microscopic appearances of mesangial hyalinosis in 8-month-old *Cfi*^{-/-} mice. PAS-stained glomerular sections from 8-month-old wild-type (A) and *Cfi*^{+/-} mice (B and C). The glomerulus from the wild-type mouse had normal appearances. In contrast, areas of mesangial expansion with a nodular hyaline appearance were evident in the *Cfi*^{-/-} mice (arrows). Original magnification, $\times 40$ (B); $\times 100$ (A and C).

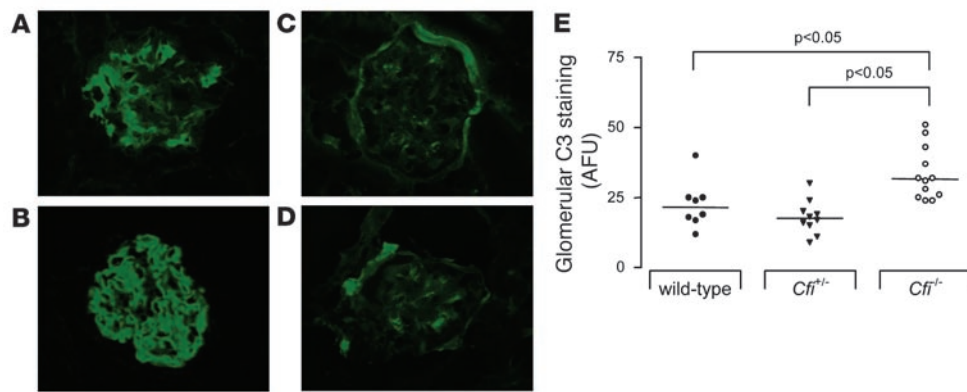


Figure 4

Glomerular C3 staining in 8-month-old *Cfi*^{-/-} mice. (A) Increased glomerular C3 staining was present in *Cfi*^{-/-} mice that was mesangial in distribution, in contrast to the linear capillary wall staining pattern seen in *Cfh*^{-/-} mice (B). Glomerular C3 staining in *Cfi*^{+/+} mice (C) was identical to that seen in age-matched wild-type animals (D). Quantitative immunofluorescence for glomerular C3 from 8-month-old *Cfi*^{-/-}, *Cfi*^{+/+}, and wild-type mice (E). Original magnification, $\times 40$.

ure 4B). Glomerular C3 staining was identical in *Cfi*^{+/+} and wild-type animals (Figure 4, C and D). Quantification of the glomerular C3 staining from a representative number of the 8-month-old cohort animals (Figure 4E) confirmed significantly increased glomerular C3 in the *Cfi*^{-/-} mice (median 31.5 arbitrary fluorescence units [AFU], range 24–51, $n = 12$) compared with wild-type (median 21.5 AFU, range 12–40, $n = 8$) or *Cfh*^{+/+} (median 17.5 AFU, range 9–30, $n = 10$) animals ($P < 0.05$ for *Cfi*^{-/-} versus *Cfi*^{+/+} mice and *Cfi*^{-/-} versus wild-type mice, Bonferroni’s multiple comparison test). In contrast, glomerular IgG staining did not differ between the experimental animals (data not shown). In summary, *Cfi*^{-/-} mice developed increased mesangial C3 staining but not deposition of C3 along the GBM.

Factor I is an absolute requirement for GBM C3 deposition and the development of MPGN2 in Cfh-/- mice. Unlike factor H deficiency, uncontrolled C3 activation due to deficiency of factor I did not result in spontaneous GBM C3 deposition. This led us to hypothesize that during uncontrolled C3 activation, factor H protects the GBM from C3 deposition. To investigate this we examined glomerular C3 staining patterns in *Cfi*^{-/-} mice with either heterozygous (*Cfh*^{+/+}*Cfi*^{-/-}) or homozygous (*Cfh*^{-/-}*Cfi*^{-/-}) deficiency of factor H (Figure 5A). *Cfh*^{+/+}*Cfi*^{-/-} animals had glomerular C3 staining patterns identical to those of mice deficient in factor I alone, indicating that haploinsufficiency of factor H did not influence glomerular C3 staining in *Cfi*^{-/-} mice. Unexpectedly, *Cfh*^{-/-}*Cfi*^{-/-} mice, in marked contrast to the GBM staining pattern seen in factor H-deficient animals (Figure 4B), demonstrated glomerular C3 staining equivalent to that seen in mice deficient in factor I alone (Figure 5A). Thus factor I deficiency completely prevented accumulation of C3 along the GBM in *Cfh*^{-/-} mice.

Since GBM C3 deposition precedes the morphological changes of MPGN2 in *Cfh*^{-/-} mice, we predicted that the lack of GBM C3 deposition in the *Cfh*^{-/-}*Cfi*^{-/-} animals would prevent the development of MPGN2. Hence we examined glomerular histology in 8-month-old *Cfh*^{-/-}*Cfi*^{-/-} mice ($n = 5$). Unlike 8 month old *Cfh*^{-/-} mice, no light microscopic evidence of MPGN2 was present in the *Cfh*^{-/-}*Cfi*^{-/-} animals (data not shown). Hence, factor I deficiency completely prevented the development of MPGN2 in *Cfh*^{-/-} mice.

Nature of circulating C3 activation products in vivo in the absence of factor I and/or factor H. A predicted difference between factor H and factor I deficiency is the nature of the C3 activation products that can be formed. In the absence of factor I, physiological cleavage of the α -chain of C3b is prevented, while in the absence of factor H, factor I may cleave C3b using alternative cofactors. Hence, we next

determined C3 levels and C3 activation products present in the plasma of the *Cfh*^{-/-} and *Cfi*^{-/-} mice (Figure 5, B, D, and E). Median levels of plasma C3 levels in both *Cfi*^{-/-} and *Cfh*^{-/-} mice were significantly reduced compared with wild-type mice (Figure 5B). However, the median C3 level in the *Cfi*^{-/-} mice was significantly higher than that seen in *Cfh*^{-/-} animals. Moreover, mice with combined deficiency of factors H and I also had a median C3 level that was significantly higher than that seen in mice with deficiency of factor H alone but was not different from that seen in the *Cfi*^{-/-} animals. Importantly, in mice with single or combined deficiency of factors H and I, factor B levels always remained low, indicating that factor B consumption occurred irrespective of whether uncontrolled alternative pathway was due to factor H or factor I deficiency (Figure 5C). We next assessed the nature of the circulating C3 by western blotting under reducing conditions using EDTA plasma from *Cfh*^{-/-}, *Cfi*^{-/-}, and *Cfh*^{-/-}*Cfi*^{-/-} mice (Figure 5D). This analysis showed that, even on prolonged exposure of the blot, cleavage of the C3 α' -chain to produce α' -chain fragments was only evident in EDTA plasma from *Cfh*^{-/-} mice (Figure 5D). Notably, in mice with factor I deficiency either alone or in combination with factor H deficiency, the molecular weight of the α -chain was reduced compared with wild-type α -chain, consistent with the molecular weight of the C3 α' -chain of C3b. High-molecular-weight bands were detected in the *Cfi*^{-/-} and *Cfh*^{-/-}*Cfi*^{-/-} EDTA plasma, which we postulated were due to the formation of C3b oligomers, since these were not seen in the wild-type animals (excluding oligomers of intact C3) and were not seen in *C3*^{-/-} EDTA plasma control (excluding nonspecific antibody binding). Cleavage of the C3b α' -chain from sera from either the *Cfi*^{-/-} or *Cfh*^{-/-}*Cfi*^{-/-} mice was readily achieved by incubating the respective sera with C3-deficient mouse sera (as a source of autologous factors I and H), generating an identically sized α -chain cleavage fragment (Figure 5D) that could be seen using EDTA plasma from a *Cfh*^{-/-} animal (Figure 5D). To determine whether C3c in addition to iC3b was present in EDTA plasma from *Cfh*^{-/-} mice, we performed western blotting under nonreducing conditions. Under these conditions, C3c was demonstrable in the EDTA plasma from the *Cfh*^{-/-} animals (Figure 5E). Hence, together these findings demonstrated that the critical difference between *Cfh*^{-/-} and *Cfh*^{-/-}*Cfi*^{-/-} animals was the nature of the circulating C3 activation fragment: in *Cfh*^{-/-} mice, formation of iC3b and C3c occurred, while in both *Cfi*^{-/-} and *Cfh*^{-/-}*Cfi*^{-/-} animals, C3 circulated in the form of C3b.

Infusion of serum containing factor I to mice with combined deficiency of factor H and factor I triggers GBM C3 deposition in vivo. To further dem-

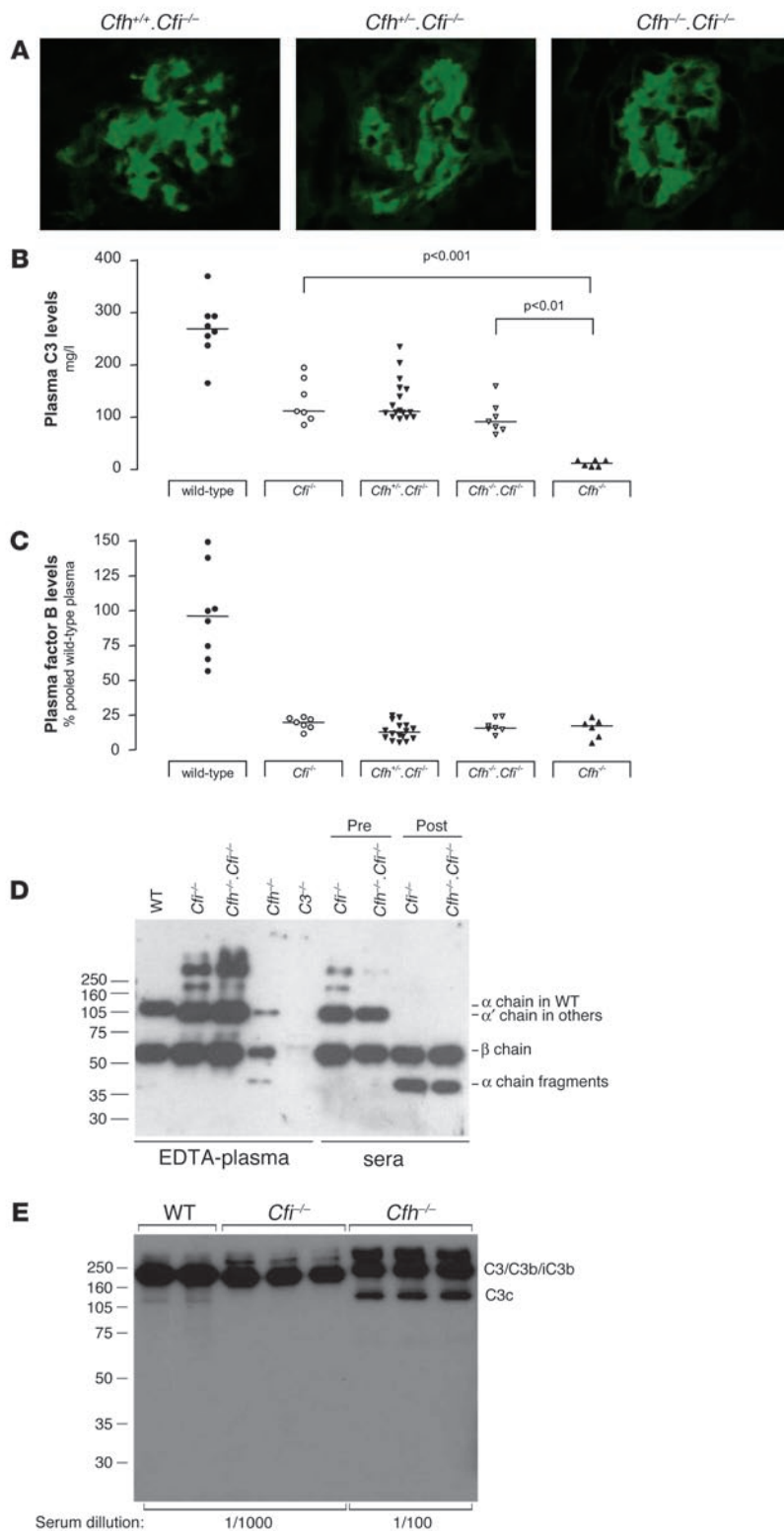


Figure 5

Glomerular C3 staining and complement profile in mice with deficiency of both factor H and factor I. (A) Glomerular C3 staining in *Cfi*^{-/-} mice with normal (*Cfh*^{+/+}), heterozygous (*Cfh*^{+/-}), and homozygous (*Cfh*^{-/-}) factor H genotypes. A mesangial staining pattern was evident in the glomeruli of the *Cfi*^{-/-} mice regardless of factor H genotype. Note the marked contrast in glomerular C3 staining pattern between the *Cfi*^{-/-}*Cfh*^{+/-} mice and that seen in mice with factor H deficiency alone (Figure 4B). Original magnification, ×40. Plasma C3 (B) and factor B (C) levels in *Cfi*^{-/-} mice with heterozygous and homozygous factor H deficiency. Horizontal bars denote median values. (D) Western blot for C3 from *Cfi*^{-/-} and *Cfi*^{-/-}*Cfh*^{+/-} mice under reducing conditions. On this high-exposure α-chain, fragments were only present in the *Cfh*^{-/-} EDTA plasma (lane 4). The lower molecular weight of the α'-chain (lanes 2, 3, and 4) compared with the intact α-chain (lane 1) was also evident. Also shown are sera from *Cfi*^{-/-} and *Cfi*^{-/-}*Cfh*^{+/-} mice before and after incubation with murine sera deficient in C3 (as a source of autologous factors I and H). Note that in both *Cfi*^{-/-} and *Cfi*^{-/-}*Cfh*^{+/-} mice, complete cleavage of the α'-chain occurred with the concomitant appearance of the 43-kDa α'-chain fragment that was evident in unmanipulated EDTA plasma taken from a *Cfh*^{-/-} animal (lane 4). (E) Under nonreducing conditions C3c was detectable in EDTA plasma from *Cfh*^{-/-} and wild-type mice, but not from *Cfi*^{-/-} mice. Note that EDTA plasma dilutions were 1/1000 for wild-type and *Cfi*^{-/-} mice and 1/100 for *Cfh*^{-/-} mice.

of autologous factor I to *Cfh*^{-/-}*Cfi*^{-/-} mice. Following serial injections of *Cfh*^{-/-}*C3*^{-/-} sera, we observed first that there was a rapid decrease in plasma C3 levels (Figure 6A) together with the appearance of C3 α-chain fragments in the circulation (Figure 6B). Importantly, 72 hours after serial injections of *Cfh*^{-/-}*C3*^{-/-} sera to *Cfh*^{-/-}*Cfi*^{-/-} animals, florid glomerular C3 staining was evident in a capillary wall distribution consistent with GBM C3 deposition (Figure 6C) and identical in pattern to that seen in unmanipulated *Cfh*^{-/-} mice (Figure 4B).

Secondly, we examined glomerular C3 staining in *Cfh*^{-/-} mice with heterozygous deficiency of factor I (Figure 6D). In these animals, glomerular C3 staining was identical to that seen in mice with factor H deficiency alone. Furthermore, unlike *Cfh*^{-/-}*Cfi*^{-/-} mice that had C3 levels comparable with those seen in the *Cfi*^{-/-} mice, the *Cfh*^{-/-}*Cfi*^{+/-} animals had markedly reduced C3 levels (median 17.8 mg/l, range 11.5–29.2, n = 10) comparable with mice deficient in *Cfh*^{-/-} alone.

Hence, analysis of glomerular C3 staining patterns in *Cfh*^{-/-}*Cfi*^{-/-} mice reconstituted with factor I and in *Cfh*^{-/-} mice with heterozygous factor I deficiency confirmed that factor I is critically required for GBM C3 deposition in *Cfh*^{-/-} mice.

onstrate in vivo that deficiency of factor I abolished accumulation of C3 along the GBM in *Cfh*^{-/-} mice, we examined glomerular C3 staining in *Cfh*^{-/-}*Cfi*^{-/-} animals reconstituted with mouse factor I. To achieve this, we administered injections of sera deficient in both factor H and C3 (obtained from *Cfh*^{-/-}*C3*^{-/-} mice) as a source

The C3 deposited along the GBM in factor H-deficient mice derives from the circulation. The absolute requirement of factor I for the development of GBM C3 deposition in the *Cfh*^{-/-} mice suggested that the

deficiency confirmed that factor I is critically required for GBM C3 deposition in *Cfh*^{-/-} mice.

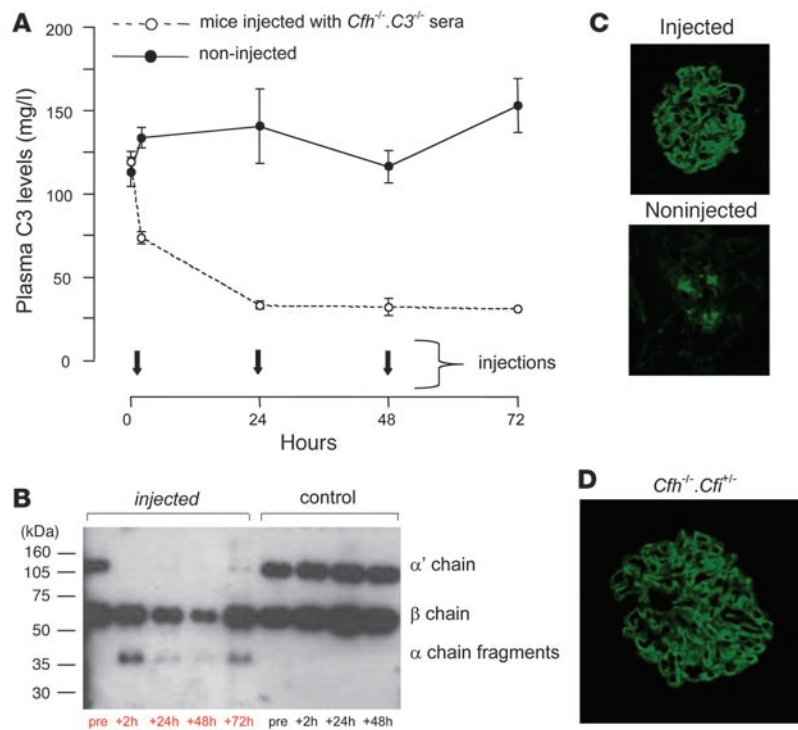


Figure 6

Administration of sera containing factor I to *Cfh*^{-/-}·*Cfi*^{-/-} mice. Following injections of sera from *Cfh*^{-/-}·*C3*^{-/-} mice (used as a source of autologous factor I), plasma C3 levels fell rapidly to levels comparable with those seen in mice with factor H deficiency alone (A). Furthermore, α'-chain fragments became evident in plasma (B). Finally, at the end of the experiment, florid glomerular C3 staining identical to that seen in unmanipulated *Cfh*^{-/-} mice was present in the reconstituted animals, in marked contrast to the mesangial C3 staining present in the noninjected *Cfh*^{-/-}·*Cfi*^{-/-} control mice (C). Consistent with these data was the observation that *Cfh*^{-/-} mice with heterozygous factor I deficiency had identical glomerular C3 staining to that seen in *Cfh*^{-/-} animals (D). Original magnification, ×40.

source of the GBM C3 was from the circulation and not derived from local renal C3 synthesis. To test this hypothesis, we conducted renal transplants in the *Cfh*^{-/-} animals (Figure 7). We first transplanted kidneys from wild-type mice into *Cfh*^{-/-} recipients and then examined glomerular C3 staining 6 weeks later. C3 staining along the GBM was seen in the wild-type kidneys transplanted into *Cfh*^{-/-} recipients although the intensity of staining was not as marked as that seen in a *Cfh*^{-/-} kidney transplanted into a *Cfh*^{-/-} recipient. We hypothesized that this could be due to partial glomerular C3 regulation by local factor H synthesis within the wild-type donor kidney, which has been previously demonstrated (35). Hence, we also examined glomerular C3 staining in a kidney from a donor with combined deficiency of C3 and factor H (*Cfh*^{-/-}·*C3*^{-/-}) that had been transplanted into a *Cfh*^{-/-} host. Glomerular C3 staining in this kidney demonstrated florid capillary wall deposition of C3, equivalent to that seen in unmanipulated *Cfh*^{-/-} kidneys. Notably, no abnormal glomerular C3 staining was demonstrable in a wild-type donor kidney transplanted into a wild-type recipient. Hence, we conclude that GBM C3 deposition in *Cfh*^{-/-} mice is entirely derived from the circulation.

Discussion

We have generated factor I-deficient mice and demonstrated that these animals have reduced plasma levels of C3, together with reduced plasma levels of the alternative pathway activation protein, factor B. In addition, C3 circulated as C3b in these animals with no in vivo evidence of formation of C3 metabolites (iC3b, C3c, C3dg). These features are characteristic of the complement abnormalities reported in humans with homozygous factor I deficiency (13). An important difference between factor I and factor H deficiency is the fate of plasma C3b: factor I-mediated cleavage of C3b to form iC3b, C3c, and C3dg may occur in factor H-deficient but not factor I-deficient individuals. In factor I-deficient

individuals, plasma C3 circulates predominantly as C3b, while C3b metabolites such as C3dg are not detectable in plasma (13). In contrast, studies of humans with complete absence of factor H have demonstrated that iC3b (36), C3c (37), and C3dg (37, 38) are present in the circulation in addition to C3dg present on erythrocytes (25). In both humans (9) and mice (39), the factor I-mediated cleavage of fluid-phase C3b in vitro does not occur in the absence of a cofactor such as factor H. Hence, the presence of C3b metabolites in factor H-deficient human sera is most likely a consequence of alternative factor I cofactors in circulation. These include CR1 on erythrocytes (36, 37), plasma factor H-like protein 1 (38), and plasma factor H-related protein 5 (40). Our data demonstrated that there was no evidence of C3b fragments in EDTA plasma in mice with factor I deficiency, irrespective of the factor H genotype. In contrast, in the *Cfh*^{-/-} mice, iC3b and C3c could be detected in EDTA plasma, indicating that murine C3b can be cleaved by factor I in the absence of factor H. In this regard it is notable that in factor H-deficient pigs, which completely lack circulating plasma factor H due to a point mutation that prevented protein secretion (41), evidence of C3c formation was readily demonstrable in plasma (27). In fact, this observation was presented as indirect evidence to exclude factor I dysfunction as a cause of the uncontrolled C3 activation in these animals (27). Hence, analogous to human factor H deficiency, in both pigs and mice, factor I-mediated cleavage of C3b in plasma can proceed in the absence of factor H through the use of alternative cofactors.

In both *Cfh*^{-/-} mice (28) and factor H-deficient pigs (42), C3 deposition along the GBM in the absence of immunoglobulin was the first glomerular abnormality, preceding both the appearance of electron-dense changes along the GBM and morphological changes of MPGN2. In contrast, mice deficient in factor I did not develop GBM C3 deposition. In these animals glomerular C3 staining was abnormal but clearly localized to the mesangium. By 8 months of

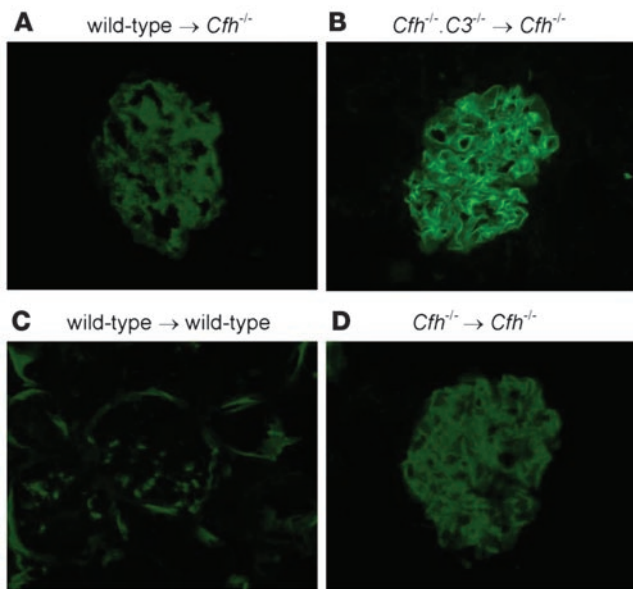


Figure 7

Glomerular C3 staining in renal transplant studies in *Cfh*^{-/-} mice. Kidneys from wild-type (A), *Cfh*^{-/-}*C3*^{-/-} (B), and *Cfh*^{-/-} (D) mice were transplanted into *Cfh*^{-/-} animals. Linear capillary wall C3 staining developed in both wild-type (A) and *Cfh*^{-/-}*C3*^{-/-} (B) kidneys transplanted into *Cfh*^{-/-} animals. Transplantation of a wild-type kidney into a wild-type recipient did not result in abnormal glomerular C3 staining (C), and transplantation of a *Cfh*^{-/-} kidney into a *Cfh*^{-/-} recipient did not alter the abnormal glomerular C3 staining seen in unmanipulated *Cfh*^{-/-} kidneys (D). Original magnification, $\times 40$.

age, significantly increased mesangial expansion was evident on light microscopy in these animals, together with, in some mice, nodular hyaline deposits in the mesangium. However, there was no evidence of renal impairment or glomerulonephritis, including MPGN2. In this respect, it is notable that mesangial deposits of C3 in the absence of mesangial proliferation has recently been noted in 6 individuals, 4 of whom harbored heterozygous mutations affecting either factor H or factor I (43). We have not detected any evidence of spontaneous renal pathology in mice with heterozygous deficiency of either factor I (as discussed in this study) or factor H (28). However, it is clearly possible that, in the presence of other predisposing factors to glomerular inflammation, the presence of such mutations could influence the pattern of glomerular injury.

We hypothesized that circulating or locally produced factor H in the *Cfi*^{-/-} mice could function to protect the GBM from C3b deposition and hence the development of MPGN2. Hence, we generated mice with combined deficiency of these regulators. Homozygous combined deficiency of factor H and I has never been reported in humans, and hence the generation of the *Cfb*^{-/-}*Cfi*^{-/-} mice represented a unique in vivo scenario in which we could test this hypothesis. Mesangial C3 staining developed in *Cfb*^{-/-}*Cfi*^{-/-} mice, equivalent to that seen in mice deficient in factor I alone, but there was no evidence of GBM C3 deposition. Furthermore, in 8-month-old *Cfb*^{-/-}*Cfi*^{-/-} mice there was no light microscopic evidence of MPGN2. This clearly demonstrated both that in *Cfi*^{-/-} mice factor H was not responsible for the lack of GBM C3 deposition and that factor I was a critical requirement for GBM C3 deposition and subsequent MPGN2 in *Cfb*^{-/-} mice.

To test the hypothesis that factor I was essential for the development of GBM C3 deposition in *Cfb*^{-/-} mice, we administered injections of sera deficient in both C3 and factor H, as a source of autologous factor I, to *Cfb*^{-/-}*Cfi*^{-/-} mice. This resulted in further reduction in plasma C3 levels (equivalent to those seen in *Cfb*^{-/-} mice), and critically, the development of florid linear capillary wall C3 deposition. Consistent with this data, unmanipulated *Cfb*^{-/-} mice with heterozygous factor I deficiency developed glomerular GBM C3 deposition identical to that seen in *Cfb*^{-/-} mice. To determine whether the GBM C3 deposition in *Cfb*^{-/-} mice derived from the circulation and not from local renal synthesis of C3, we performed renal transplantation experiments in *Cfb*^{-/-} mice. These experiments demonstrated that both wild-type donor kidneys and donor kidneys from mice with combined deficiency of C3 and factor H developed linear capillary wall C3 staining when transplanted into the *Cfb*^{-/-} mice, confirming that the glomerular C3 in the *Cfb*^{-/-} mice is derived from the circulation. These data demonstrated that not only is factor I an essential requirement for the development of GBM C3 deposition in *Cfb*^{-/-} mice but also that the GBM C3 deposition in *Cfb*^{-/-} mice derives from the circulation.

It is striking that the median C3 levels in the *Cfi*^{-/-} mice were significantly higher (approximately 10-fold; Figure 5) than those seen in the *Cfb*^{-/-} mice. Factor H deficiency in humans is associated with markedly reduced C3 levels. For example, in cases in which factor H is completely undetectable in plasma, C3 levels are often below the lower limit of assay detection (36, 37, 44), equating to levels of less than 10 mg/l (36). In contrast, in patients with homozygous factor I deficiency, C3 levels, although invariably reduced, are typically in the range of 15%–30% (13) and may reach 50% of normal values (15, 18). Thus the greater reduction in C3 levels seen in *Cfb*^{-/-} mice compared with the reduction seen in *Cfi*^{-/-} mice parallels that seen in the respective human deficiencies.

The presence of secondary reduction in factor H levels, a consistent feature of human factor I deficiency, has been thought to be a consequence of increased binding of C3b to circulating factor H to form C3b–factor H complexes (16–18), which are then presumably removed from the circulation. We hypothesized that this may be one mechanism that limits the production of C3b in the setting of factor I deficiency. However, our data demonstrated that C3 levels were reduced to similar levels in *Cfi*^{-/-} and *Cfb*^{-/-}*Cfi*^{-/-} mice. Furthermore, the degree of factor B depletion in these animals was also identical, indicating that the presence of factor H was not limiting alternative pathway activation in the *Cfi*^{-/-} mice. This observation would also suggest that the formation of the alternative pathway convertase C3bBb, with consequent consumption of factor B, was occurring to a similar extent in both deficiency states. Plasma C3 levels are clearly influenced by both C3 synthetic rate and the degree of C3 activation. Notably, depression of C3 synthesis during hypocomplementemia has been reported in humans and was most pronounced in patients with circulating C3dg (45). In contrast, C3 synthesis was not altered in a patient with hypocomplementemia due to factor I deficiency, which also prevented the generation of plasma C3dg (45). Hence, the difference in the magnitude of depression of plasma C3 between factor H and factor I deficiency may be a consequence of differential C3 synthetic rates.

Taken together, our data suggest that during uncontrolled alternative pathway activation it is the nature of the plasma C3 activation product that critically determines whether GBM C3 deposition develops. In factor I deficiency, in which only C3b is



produced, no GBM C3 deposition occurs, but increased mesangial C3 staining is present. In contrast, factor H deficiency is accompanied by florid GBM C3 deposition together with the presence of circulating C3b metabolites. One could postulate that C3b metabolites (iC3b, C3c, C3dg) might have an increased affinity for the GBM compared with C3b. In human MPGN2 associated with C3 nephritic factor, complete cleavage of C3 is typical with the formation of C3c and C3dg in circulation (46). Lack of murine reagents that can unequivocally differentiate between C3b, iC3b, C3c, and C3dg has impeded the definitive characterization of the nature of the C3 activation fragments present along the GBM in *Cfhr*^{-/-} mice. However, our present data would strongly suggest that the GBM C3 in these animals, at least initially, is comprised of iC3b and/or its metabolites, C3c and C3dg. Additional support for this derives from the observation that iC3b was identifiable in glomeruli isolated from *Cfhr*^{-/-} mice by laser capture microdissection (47).

In contrast to C3b, iC3b and its metabolites cannot interact with factor B or properdin to form a C3 convertase. In this respect it is notable that in the factor H-deficient pigs the GBM C3 did not stain for either factor B or properdin (42). Furthermore, C3 deposits in the paramesangial areas in human MPGN2 appear to consist only of C3c (22), and properdin is usually absent in the GBM deposits (22, 48). Glomerular deposits consisting of iC3b, C3c, or C3dg would not allow local formation of C5 convertase, yet we have previously reported that GBM C9 staining is present in *Cfhr*^{-/-} mice (28). Terminal complement complex (TCC), C5, and C6 were present in the factor H-deficient pigs (42). Furthermore, in some renal biopsy specimens from humans with factor H deficiency, staining along the GBM for TCC has been demonstrated (25). Moreover, in some human MPGN2 renal biopsies, both C9 (23) and, in rapidly progressing disease, C5 have been detected (22). We reasoned that this may be explained by the fact that the TCC is formed in the circulation and then deposited along the GBM. Indirect support for this hypothesis derives from the observation that in the factor H-deficient pigs, glomerular TCC invariably costained for vitronectin (42). In addition, these animals had increased plasma levels of TCC (42), and plasma C5 activation has also been demonstrated in *Cfhr*^{-/-} mice (28).

There remains no definitive therapy for MPGN2 (19). We have previously shown that the inability to inhibit C5 activation ameliorated but did not prevent the development of MPGN2 in *Cfhr*^{-/-} mice (49). In the present study, we have shown that the metabolism of C3b in the circulation is an absolute requirement for the initiation of GBM C3 deposition in the *Cfhr*^{-/-} mice. This raises the intriguing and novel possibility that GBM C3 deposition secondary to alternative pathway dysregulation could be prevented, regardless of etiology, by strategies that either prevent proteolytic cleavage of C3b or sequester its metabolites in the circulation.

Methods

Animals. *Cfhr*^{-/-} mice were developed as previously reported (28). Generation of mice with combined deficiency of factors H and I were generated by intercrossing *Cfhr*^{-/-} and *Cfi*^{-/-} mice. The genotyping of the *Cfhr*^{-/-} mice has been previously described (28). All procedures were performed in accordance with institutional guidelines. Animal studies were performed under a Project Licence issued by the United Kingdom Home Office.

Generation of *Cfi*^{-/-} mice. We generated mice with a disrupted *Cfi* locus by homologous recombination in ES cells. In the targeting gene locus, exon 4 of the murine factor I gene was replaced by a positive selectable marker, the neomycin-resistant gene (pMC1NeoPolyA; Stratagene). The neomy-

cin-resistance cassette was flanked by two 2-kb regions of target homology, and a negative selectable marker (*HSV-tk*) was inserted outside the region of homology. We screened ES cells by Southern blotting analysis of *EcoRI*-digested genomic DNA using a 3' external cDNA probe. Germline transmission of the recombinant ES cells was achieved. Heterozygotes were interbred to obtain viable (129/Sv × C57BL/6) mice that were homozygous for the disrupted allele. Genotyping of the *Cfi*^{-/-} mice was performed using the following primers on genomic DNA isolated from tail digests: 5'-GTATGTCACGAACACCCCTGC-3' (FI4D-), 5'-GAGGGGATCAATCTCTAGAGCT-3' (FINeo1-), and 5'-GAGATCAACCACGCGAGGCTT-3' (KFI+). For the spontaneous phenotypic analysis (129 × C57BL/6), homozygous factor I-deficient mice together with littermate wild-type and heterozygous factor I-deficient animals were used.

Measurement of C3, factor H, and factor B levels and western blotting of plasma factor I and C3. Factor H levels were measured by ELISA using a goat anti-rat FH antibody (a gift from M. Daha, Leiden University Medical Center, Leiden, The Netherlands) and a rabbit anti-mouse FH antibody (a gift from S. Rodriguez de Cordoba, Centro de Investigaciones Biológicas, Madrid, Spain). Factor B levels were measured by ELISA using a goat anti-human factor B (Diasorin) and a monoclonal anti-mouse factor B antibody (a gift from J. Thurman, University of Colorado Health Sciences Center, Denver, Colorado, USA). Samples for factor H and factor B were quantified by reference to a standard curve generated using normal wild-type mouse serum. C3 levels were measured by ELISA using a goat anti-mouse C3 antibody (MP Biomedicals). Results were quantified by reference to a standard curve generated from acute-phase sera containing a known quantity of C3 (Calbiochem). For western blotting of serum, murine factor I was detected using a crossreactive polyclonal goat antibody against human factor I (Quidel), and C3 was detected using goat anti-mouse C3 antibody (MP Biomedicals).

Histological studies. For light microscopy, kidneys were fixed in Bouin's solution and sections stained with PAS reagent. For immunofluorescence studies, kidneys were snap-frozen. Glomerular histology was graded as follows: grade 0, normal; grade I, hypercellularity in 10%–25% of glomeruli; grade II, hypercellularity in 25%–50% of glomeruli; grade III, hypercellularity in 50%–75% of glomeruli; grade IV, glomerular hypercellularity in >75% or crescents in >25% of glomeruli. Specific changes of MPGN were the presence of GBM double contours and capillary wall thickening. Histological analysis was performed in a blinded fashion, and 50 glomeruli per section were analyzed. FITC-conjugated goat anti-mouse C3 (ICN Pharmaceuticals) and FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) were used at dilutions of 1/100 and 1/200, respectively, in phosphate-buffered saline. FITC-conjugated goat immunoglobulins were used as a control for these 2 antibodies. Quantitative immunofluorescence studies were performed as previously described (50), and results are expressed as AFU.

Assessment of renal function. Urinary albumin was measured by radial immunodiffusion using a rabbit anti-mouse albumin antibody (Biogenesis) and purified mouse albumin (Sigma-Aldrich) as standards, as previously described (50). Serum urea was measured using a UV method kit (R-Biopharm Rhone) according to the manufacturer's instructions.

Administration of sera containing factor I. For these experiments serum was obtained from mice with combined deficiency of factor H and C3 (*Cfhr*^{-/-}*C3*^{-/-}). An initial intravenous injection of 200 µl of sera was administered to *Cfhr*^{-/-}*Cfi*^{-/-} animals, and then a further 400 µl of sera was injected intraperitoneally at 24 and 48 hours.

Renal transplant studies. The renal transplantation procedure has been described previously (51). Briefly, the left kidney was removed from 6- to 8-week-old donor mice and preserved in cold saline. The right kidney was removed from recipient mice and the donor kidney transplanted with end-to-side anastomoses of the donor renal vein to the inferior vena cava and



the donor aortic cuff to the aorta. Ureter-to-bladder anastomosis was performed to reconstruct the urinary tract. Mice were sacrificed 4–6 weeks after transplant procedure, and renal histology was assessed.

Statistics. The Mann-Whitney *U* test was used for comparison of 2 groups, while for analysis of 3 or more groups, Bonferroni's multiple comparisons test was used. Data were analyzed using GraphPad Prism version 3.0 for Windows (GraphPad Software).

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