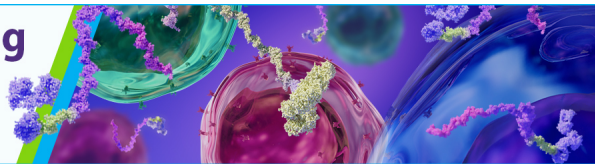


The Power of Sample Multiplexing With TotalSeq™ Hashtags

Read our app note ▶



Renal Expression of the C3a Receptor and Functional Responses of Primary Human Proximal Tubular Epithelial Cells

This information is current as of August 4, 2022.

Michael C. Braun, Rose Y. Reins, Tong-bin Li, Travis J. Hollmann, Ranjan Dutta, Wetsel A. Rick, Ba-Bie Teng and Baozhen Ke

J Immunol 2004; 173:4190-4196; ;
doi: 10.4049/jimmunol.173.6.4190
<http://www.jimmunol.org/content/173/6/4190>

References This article **cites 46 articles**, 14 of which you can access for free at:
<http://www.jimmunol.org/content/173/6/4190.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>



Renal Expression of the C3a Receptor and Functional Responses of Primary Human Proximal Tubular Epithelial Cells¹

Michael C. Braun,^{2*‡} Rose Y. Reins,^{*} Tong-bin Li,[†] Travis J. Hollmann,^{*} Ranjan Dutta,[†] Wetsel A. Rick,^{*} Ba-Bie Teng,[†] and Baozhen Ke^{*}

Although complement activation and deposition have been associated with a variety of glomerulopathies, the pathogenic mechanisms by which complement directly mediates renal injury remain to be fully elucidated. Renal parenchymal tissues express a limited repertoire of receptors that directly bind activated complement proteins. We report the renal expression of the receptor for the C3 cleavage product C3a, a member of the anaphylatoxin family. C3aR is highly expressed in normal human and murine kidney, as demonstrated by immunohistochemistry and in situ hybridization. Its distribution is limited to epithelial cells only, as glomerular endothelial and mesangial cells showed no evidence of C3aR expression. The C3aR is also expressed by primary renal proximal tubular epithelial cells in vitro as demonstrated by FACS, Western blot, and RT-PCR. In vitro C3aR is functional in terms of its capacity to bind ¹²⁵I-labeled C3a and generate inositol triphosphate. Finally, using microarray analysis, four novel genes were identified and confirmed as transcriptionally regulated by C3aR activation in proximal tubular cells. These studies define a new pathway by which complement activation may directly modulate the renal response to immunologic injury. *The Journal of Immunology*, 2004, 173: 4190–4196.

The human receptor for the complement activation product C3a (C3aR) is a 55-kDa protein of the rhodopsin family of G protein-coupled seven-transmembrane receptors (1). A wide range of bone marrow-derived cells including mast cells, basophils, neutrophils, eosinophils, monocytes/macrophages, dendritic cells, T cells, and B cells expresses this receptor (1–5). The C3aR is also expressed in nonlymphoid tissue, including brain, lung, heart, intestine, and liver (6–13). Although it is known that C3aR mRNA is present in whole kidney, as assessed by Northern blot, and that the human proximal tubular epithelial cell line HK-2 expresses functional C3aR, the distribution and function of the receptor in primary renal tissue are unknown (11–14).

Investigation into the functional activity of the C3aR has been focused primarily on leukocyte responses. C3a is chemotactic for leukocytes, and activation of the receptor induces a variety of inflammatory mediators such as IL-1, IL-6, IL-8, and TNF- α (1, 7). C3a promotes the degranulation of granulocytes, mediates histamine release, and induces vasodilatation and smooth muscle contraction (15). In vitro characterization of receptor-mediated responses suggests that this receptor plays a major role in modulating complement-mediated immune responses in a proinflammatory manner. Little is known, however, regarding epithelial cell responses to C3a stimulation save for the induction of IL-8 in pulmonary epithelium and the induction in HK-2 cells of TGF- β ,

a cytokine with many anti-inflammatory activities (7, 14). Recent in vivo studies of C3aR-deficient animals suggest that C3aR may have a dual role in modulating the inflammatory response. Although mice lacking C3aR are protected from the development of Ag-induced airway hyperresponsiveness, C3aR-deficient animals have an increased mortality compared with control mice in an LPS model of septic shock (16, 17). The later study suggests that the C3aR may play a role in attenuating inflammatory responses. Currently, it is unknown what contribution, if any, nonlymphoid C3aR activation plays in modulating host responses to immunologic challenge.

The relationship of complement activation and renal injury is a complex one. Complement activation and/or deposition are associated with both primary immune and nonimmune mediated forms of renal disease (18). Direct complement-mediated injury can be induced via the terminal component cell surface deposition of the membrane attack complex (C5b-C9) or through the direct activation of complement receptors. Renal parenchymal tissue is known to express complement receptors, including CR1, CR3, and CD88, although the functional consequences of activation of these receptors remain ill defined (19–23). To expand our current understanding of how complement activation modulates renal injury, we report the expression of the C3aR in normal human and murine kidney. In addition, microarray analysis was used to identify novel transcriptional responses to C3aR activation in cultured primary renal tubular epithelial cells.

Materials and Methods

Immunohistochemistry

Normal human kidney tissue ($n = 4$) was obtained from the National Diabetes Research Interchange (Philadelphia, PA). The tissue was dehydrated and paraffin embedded using standard techniques. Five-micrometer sections were used in all histologic studies. No evidence of renal pathology was noted in multiple sections stained with both H&E and periodic acid-Schiff (data not shown). Immunohistochemistry was performed using the

Centers for *Immunology and Autoimmune Diseases and [†]Human Genetics, Brown Foundation Institute of Molecular Medicine, and [‡]Division of Pediatric Nephrology and Hypertension, Department of Pediatrics, University of Texas, Houston, TX 77030

Received for publication July 17, 2003. Accepted for publication July 2, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by National Institutes of Health Grant KO8-DK62197 (to M.C.B.).

² Address correspondence and reprint requests to Dr. Michael C. Braun, University of Texas, Brown Foundation Institute of Molecular Medicine, 2121 West Holcombe Boulevard, Room 911, Houston, TX 77030. E-mail address: michael.c.braun@uth.tmc.edu

TSA biotin system (PerkinElmer, Boston, MA), according to the manufacturer's instructions. Nonspecific Ig binding was blocked with 5% normal goat serum in PBS with 5% BSA. Sections were then incubated overnight at 4°C with either: a mouse anti-human C3aR (anti-hC3aR)³ mAb (0.5 µg/ml, clone 8H1; BD Biosciences, San Diego, CA) or an isotype-matched control Ab (0.5 µg/ml; BD Biosciences). Slides were washed and incubated for 1 h at room temperature (RT) with 1/500 dilution of biotin goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS. The slides were again washed, incubated with the TSA biotin amplification reagents, developed with diaminobenzidine substrate, and counterstained with hematoxylin.

In situ hybridization

Normal human kidney was obtained, as described above ($n = 4$). Murine C3aR (mC3aR)-deficient and control wild-type age- and sex-matched kidneys were fixed with formalin and embedded ($n = 3$). The C3aR-deficient mice as well as pSP72 plasmids (Promega, Madison, WI) containing hC3aR and mC3aR cDNA inserts have previously been reported (8, 24). Riboprobes correspond to nt 553–885 of the hC3aR cDNA (GenBank NM 004054) and 817–1005 of the mC3aR cDNA (GenBank NM 009779). To generate sense and antisense probes, the hC3aR plasmid was linearized with *Bam*HI and *Hind*III, while the mC3aR plasmid was linearized with *Xho*I and *Bg*III (Roche Molecular Biochemicals, Indianapolis, IN). In vitro transcription was performed using digoxigenin (DIG) RNA labeling kit (SP6/T7; Roche Molecular Biochemicals), according to manufacturer's instructions. Paraffin-embedded sections were dried at 55°C overnight, dehydrated with xylene over 24 h. Sections were then hybridized overnight with DIG-labeled probes diluted 1/50 in hybridization buffer (2× SSC, 10% dextran sulfate, 0.02% SDS, 50% formamide) at 42°C, and washed once at RT with 2× SSC, three times at 42°C with 1× SSC/50% formamide, and twice at room RT with 1× SSC. The sections were blocked in TBS (pH 7.4) containing 10% goat serum for 15 min, incubated with an alkaline phosphatase-conjugated anti-DIG Ab (Roche Molecular Biochemicals) diluted in 5% goat serum/TBS for 1 h, and developed with NBT/5-bromo-4-chloro-3-indolyl phosphate (Roche Molecular Biochemicals). Sections were counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA), dehydrated, mounted, and examined by light microscopy.

Proximal tubular epithelial cell (PTEC) studies

Human PTEC from four separate donor sources were purchased from Cambrex (East Rutherford, NJ) and cultured at 37°C, 5% CO₂ in renal epithelial cell growth medium (BioWhittaker, Walkersville, MD) supplemented with hydrocortisone (500 ng/ml), epithelial growth factor (10 ng/ml), FBS (0.5%), epinephrine (500 ng/ml), triiodothyronine (6.5 ng/ml), penicillin-streptomycin (100 µg/ml), and gentamicin (100 µg/ml). PTEC were studied between passages 3 and 12. At final passage, the cells were recharacterized as von Willebrand factor negative, and positive for cytokeratin, actin, alkaline phosphatase, and γ -glutamyl transferase. U937 cells were obtained from American Type Culture Collection (Bethesda, MD) and cultured in RPMI 1640 supplemented with 10% FBS (BioWhittaker), penicillin-streptomycin (100 µg/ml), and gentamicin (100 µg/ml). To induce C3aR expression, U937 cells were cultured with cAMP (Amersham, Piscataway, NJ) for 72 h before harvesting.

FACS analysis

PTEC ($n = 3$) were grown to confluence and harvested with 0.5 M EDTA in PBS with gentle scraping. Cells were passed through a 70-µm mesh and washed twice with ice-cold PBS, 0.5% BSA, and sodium azide. Following a 30-min incubation with Fc block (E-biosciences, San Diego, CA), the cells were incubated with either anti-hC3aR mAb (0.5 µg/ml) or isotype control Ab (0.5 µg/ml) for 1 h, washed twice, and incubated with PE-conjugated goat anti-mouse F(ab')₂ Ab (1/1000; Jackson ImmunoResearch Laboratories). The cells were again washed, resuspended, and then analyzed using CellQuest software with BD FACSCalibur (BD Biosciences) instrumentation.

Western blot analysis

PTEC ($n = 4$) were grown to confluence in T-75 flasks (Corning-Costar, Corning, NY). For the induction of type I collagen, cells were stimulated for 6, 12, or 18 h with 10 nM rC3a before harvesting. The cells were harvested and lysed with PBS, 1% Nonidet P-40, 0.5% sodium deoxy-

cholate, 0.1% SDS, and Complete Tab protease inhibitor mixture (Roche Molecular Biochemicals). U937 cells, both cAMP treated and untreated, were harvested in a similar manner and served as a positive and negative control, respectively. Protein concentrations were determined using the bicinchoninic acid assay method (Bio-Rad, Hercules, CA). A total of 10 µg of protein was separated on a 6% SDS-PAGE gel by electrophoresis under reducing conditions, and transferred to a nitrocellulose membrane. For C3aR expression, the membrane was blocked for 2 h at RT with 5% nonfat dry milk in PBS with 0.1% Tween 20, and incubated overnight at 4°C with anti-hC3aR mAb (1.0 µg/ml). The membrane was washed and incubated for 1 h at RT with a 1/1000 dilution of goat anti-mouse HRP-labeled IgG (Jackson ImmunoResearch Laboratories) in PBS. For pro- α type 1 collagen induction, the membrane was incubated with rabbit anti-pro- α type 1 collagen (RDI, Flanders, NJ), washed, and incubated for 1 h at RT with a 1/1000 dilution of goat anti-rabbit HRP-labeled IgG (Jackson ImmunoResearch Laboratories) in PBS. The membrane was then stripped and reprobed as above for C3aR expression. The membranes were developed with Lumi-Glo (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and visualized by autoradiography.

RT-PCR

Total RNA was obtained using RNAwiz (Ambion, Austin, TX) from PTEC, whole human kidney, cAMP-treated U937 cells, and untreated U937 cells ($n = 3$). Reverse transcription was performed with Superscript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA) using 5 µg of total RNA as template and random hexamer priming. hC3aR-specific primers (NM_004054, sense, ACATCCAGGT GCTGAAGCCTT; antisense, GCTCATTCCATGGCTGTGAGA) and the endogenous control 18S ribosomal RNA were generated using Primer-Express software (Applied Biosystems, Foster City, CA). SYBR Green I dye system (Applied Biosystems) was then used for quantitative real-time PCR, according to the manufacturer's instructions. PCR was performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems) at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR was performed with triplicate samples. Dissociation curves for each primer pair were obtained and analyzed to confirm a single peak profile for each primer pair studied. At the end of the reaction, data were analyzed using Sequence Detection System Software (version 1.7; ABS Global). Ct data for each sample were then normalized with the threshold cycle (Ct) data of the endogenous control, and mRNA expression was calculated relative to PTEC C3aR mRNA expression. Student's *t* test was used to calculate statistical significance.

C3a-binding studies

Purified hC3a (Advanced Research Technologies, San Diego, CA) was labeled with ¹²⁵I using IODO-GEN-precoated iodination tubes (Pierce, Rockford, IL). Briefly, 5 µl of 0.2 M NaPO₄, followed by 5 µl of Na¹²⁵I (0.5 mCi; Amersham), was added to an IODO-GEN tube, followed by 25 µg of hC3a, mixed and incubated at RT for 10 min. The reaction mixture was transferred to a microfuge tube containing 20 µl of saturated tyrosine solution. Following a 1-min incubation, 380 µl of 10% BSA (borate saline containing 10 mM KI, pH 8.0) was added. Solution was then dialyzed for 2 h at RT and then overnight at 4°C. The determined sp. act. of the iodinated hC3a was 1.76 × 10⁷ cpm/µg. PTEC cells were grown to confluence in 24-well plates (Corning-Costar). Medium was removed from cells, and 10 nM iodinated hC3a in 500 µl of binding/wash buffer (DMEM/1% BSA/0.05% Tween 20) was added to each well. Cold C3a or fMLP (Sigma-Aldrich, St. Louis, MO) was added to compete with binding at concentrations of 0, 1, 10, 100, 500, and 750 nM, and incubated with cells for 30 min at RT. Cells were washed four times with buffer and lysed with 1 M NaOH. Binding of ¹²⁵I-C3a was quantitated using a Cobra II auto gamma counter (Packard Instrument, Meriden, CT).

Inositol incorporation

PTEC ($n = 3$) were grown to confluence in 24-well plates (Corning-Costar), and washed once with PBS. Fresh epithelial cell growth medium supplemented with 0.5% FBS and 10 mCi/ml myo-(2-[³H])inositol (Amersham) was added. Twenty-four hours later, the cells were washed with PBS, and the cells were incubated for 30 min at 37°C with fresh medium containing 0.5% FCS and 10 mM LiCl. The medium was then removed, and fresh medium containing 0.5% FCS, 10 mM LiCl, and 10 nM purified hC3a was added. After 30 min of stimulation, the medium was removed, and the reaction was stopped by adding 0.25 ml of ice-cold 10 mM formic acid. Sample pH was adjusted with 15 mM NH₄OH, and then transferred to an AG1X8 anion exchange resin (Bio-Rad) column equilibrated with 5 mM borax and 60 mM sodium formate. Incorporated inositol was then eluted from the columns with 1 M ammonium formate and 0.1 M formic

³ Abbreviations used in this paper: hC3a, human C3a; Ct, threshold cycle; DIG, digoxigenin; mC3a, murine C3a; PTEC, proximal tubular epithelial cell; RT, room temperature; EST, expressed sequence tag.

acid and quantitated using a LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA). Studies were performed in triplicate.

Microarray experiments and analysis

PTEC were grown to confluence in T-75 flasks. The cells were washed twice with HBSS, and fresh medium was supplemented with 0.5% FBS added. After 30 min, the medium was again changed and 10 nM hC3a was added. The cells were harvested after 6 h of stimulation, and total RNA was extracted, as described above, followed by purification using RNeasy spin columns (Qiagen, Valencia, CA). Total RNA was obtained from three independent experiments and consisted of cells stimulated with hC3a and untreated controls. A total of 5 μ g of RNA from each sample was used to synthesize cDNA, according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA was then generated using 1 μ g of cDNA with BioArray high yield RNA transcript labeling kit (Enzo Biochem, Farmingdale, NY). The labeled RNA transcripts were purified, fragmented, and hybridized to a Test 3 array (Affymetrix) to verify the quality and quantity of the sample. The fragmented cRNA was then used to hybridize human genome U133A GeneChips (Affymetrix). After hybridization, each array was washed, stained with goat IgG (10 mg/ml) and streptavidin-PE (1 mg/ml) containing 50 mg/ml acetylated BSA, and then scanned using an Affymetrix GeneArray scanner. The fluorescence results were analyzed using Affymetrix microarray suite version 5.0 software. A global scaling algorithm was applied to each array, which allows comparison of all samples. Arrays were analyzed both independently (absolute analysis) and in pairwise comparison between stimulated and unstimulated samples (comparison analysis). In absolute analysis, the signal intensity value that represents the level of expression of a transcript from each array was obtained. In comparison analysis, the mean intensity from three experiments of stimulated and unstimulated samples was calculated. Fold changes were obtained as the ratio of stimulated to unstimulated samples, and *p* values using *t* test were calculated. A transcript was considered differentially expressed if it met the following criteria: 1) a *p* value <0.025

by *t* testing, 2) an absolute difference in signal intensity greater than 100, and 3) a greater than 3-fold change in expression between samples.

Quantitative PCR

Verification of observational data from the microarray experiments was obtained by RT-PCR. Three independent RNA samples were obtained from three separate PTEC donors stimulated for 6 h with 10 nM C3a or unstimulated control samples. Semiquantitative PCR was performed, as described above. Sequence-specific primers for the differentially expressed transcripts identified by microarray analysis were generated using Primer-Express software (Applied Biosystems). The GenBank database was screened to confirm the uniqueness of each primer sequence. The primer sequences were as follows: Gulp (AK023668), sense, CCTCCAGCAGGCAGTATGACA, and antisense, TTGCGAGTAGGCATCGAAGAC; pyrin (NM_000243.1), sense, CGCTCAGGCACATGCTGTTA, and antisense, GGGTAAGCGGTTCTGCATC; DYKR2 (NM_006482.1), sense, ACAGTGCTCACGACAA CCA, and antisense, CCCTGCCGTCTATGAATGCT; Pro-1 α type 1 collagen (NM_000088.1), sense, CCACCAATCACCTGCCTACA, and antisense CACGTCTCGGTCATGGTACCT. Ct data for each sample were then normalized with the Ct data of the endogenous control, and changes in C3a-stimulated mRNA expression were calculated relative to unstimulated mRNA expression. Student's *t* test was used to calculate statistical significance.

Results

C3aR expression in normal human renal tissue

Immunohistochemical analysis using a hC3aR-specific mAb revealed prominent expression in the tubular segments of normal human kidney with high levels of expression in the renal medulla (Fig. 1, A and C). Glomerular expression was limited. Staining was noted in occasional glomerular epithelial cells and epithelial cells lining Bowman's capsule (Fig. 1A). No staining was detected on either mesangial cells or glomerular endothelial cells. Cortical staining was also demonstrated in proximal tubule epithelium. The C3aR was expressed on both the luminal brush border and the basolateral aspects of the proximal tubule (Fig. 1E). No staining was detected in the thick ascending limb of the loop of Henle (Fig. 1E). Ab specificity was confirmed by the lack of staining with isotype control Ab (Fig. 1, B and D). In situ hybridization studies demonstrated a similar expression pattern. The highest intensity of staining for the C3aR mRNA was noted in the medulla and tubular segment within the renal cortex (Fig. 2, C and D). The glomerular C3aR mRNA expression mirrored the Ab staining with mRNA detected only in glomerular epithelium and in visceral epithelium

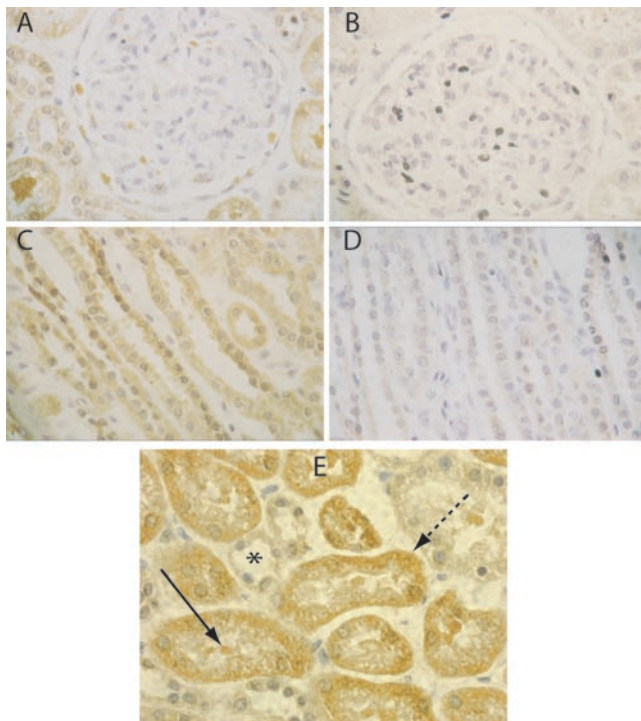


FIGURE 1. C3aR expression and distribution in normal human kidney as determined by immunohistochemistry using a C3aR mAb (clone 8H1). Expression in the renal cortex is limited to tubular epithelial, glomerular visceral, and parietal epithelial cells (A). The C3aR is highly expressed in the renal medulla (C). C3aR is expressed by both the brush border (solid arrow) and the basolateral membrane (hatched arrow) of proximal tubular epithelium within the renal cortex (E), but not in the thick ascending limb of the loop of Henle (*). Minimal background staining is detected with isotype control Ab: renal cortex (B) and renal medulla (D). Magnification for all photomicrographs is $\times 400$.

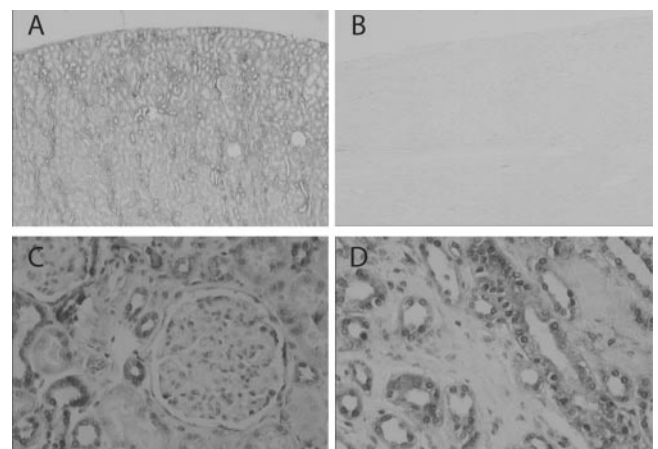


FIGURE 2. C3aR mRNA expression in normal human kidney as determined by in situ hybridization using a digoxin-labeled riboprobe: $\times 100$ magnification using antisense probe (A) and sense probe (B). Cortical expression of C3aR mRNA using antisense is limited to epithelial cells (C, $\times 400$ magnification); C3aR mRNA is highly expressed within the renal medulla (D, antisense probe magnification $\times 400$).

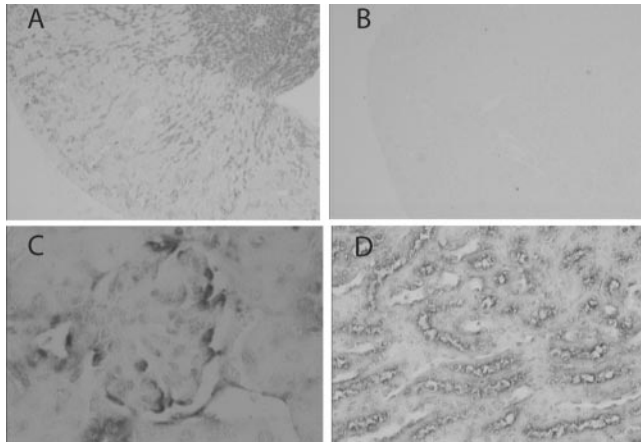


FIGURE 3. In situ hybridization using antisense riboprobes demonstrates renal expression of mC3aR mRNA in wild-type mice (A, $\times 200$) and mice with targeted deletion of mC3aR (B, $\times 200$). The distribution of mC3aR mRNA in wild-type mice is similar to that seen in normal human kidney; mC3aR mRNA is expressed in cortical and glomerular epithelial only (C, $\times 400$) and highly expressed in the renal medulla of wild-type mice (D, $\times 400$).

of Bowman's capsule (Fig. 2C). Glomerular mesangial and endothelial cells were uniformly negative for C3aR mRNA. Confirmation of the receptor distribution was obtained using in situ hybrid-

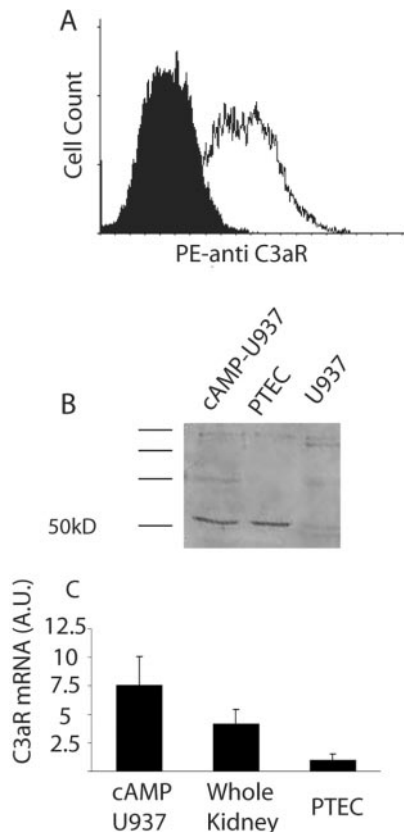


FIGURE 4. Cultured human primary proximal tubular epithelial cells express the C3aR: FACS histogram using either an hC3aR-specific mAb (clone 8H1) or an isotype control mAb (A). Western blot of whole cell lysates probed with mAb (clone 8H1) to hC3aR (B); semiquantitative RT-PCR of total mRNA isolated from U937 cells, whole human kidney, and PTEC (C). Figures are representative data from three separate experiments using PTEC from separate donor sources.

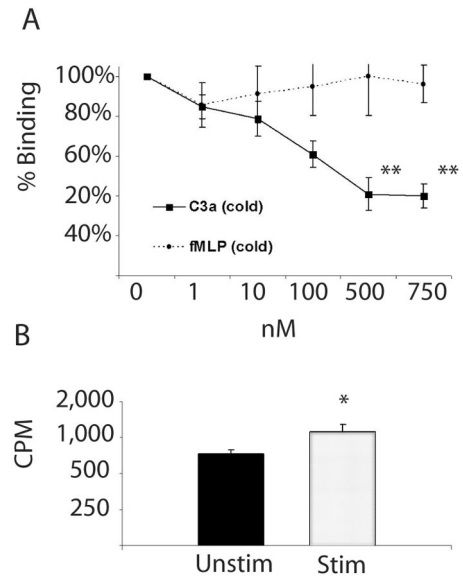


FIGURE 5. The C3aR expressed on PTEC is functional. ^{125}I -labeled C3aR binding to cultured PTEC in the presence of increasing concentrations of either unlabeled C3a or nonspecific unlabeled peptide fMLP (A). Data are mean values \pm 1 SD of triplicate samples from one of three independent experiments (**, $p < 0.01$). C3a stimulation of PTEC increases radiolabeled inositol incorporation (B). Data are mean values \pm 1 SD of triplicate samples from one of three independent experiments (*, $p < 0.05$).

ization studies in wild-type mice and mice with targeted deletions of the mC3aR (Fig. 3). The highest intensity of mRNA staining was again seen in the medulla (Fig. 3, A and D), with staining in the cortex limited to tubular and glomerular epithelia (Fig. 3C).

Expression and functional analysis of the C3aR in primary renal proximal tubular epithelial cells

FACS analysis of cultured PTEC demonstrated expression of the C3aR (Fig. 4A). C3aR protein expression was confirmed by Western blot (Fig. 4B). C3aR mRNA was detected in PTEC in lower amounts than that seen in either whole kidney RNA or RNA obtained from cAMP-treated U937 cells: 8- and 4-fold less, respectively (Fig. 4C). Binding studies using ^{125}I -labeled purified hC3a confirmed cell surface expression of the C3aR (Fig. 5A). The absolute amount of labeled C3a binding was low (absolute count 1200 ± 300 cpm), suggesting that the density of receptor expression on these cells is lower than that reported in human monocytes (1). The specificity of binding was demonstrated by inability of unlabeled fMLP to displace bound C3a. Similar to the data reported by Peake et al. (14), primary PTEC are able to functionally respond to C3a stimulation as measured by inositol incorporation (Fig. 5B). The relatively modest increase in inositol incorporation compared with unstimulated PTEC may reflect the basal activity of growth factors required to maintain these cells in culture.

We then used microarray gene expression analysis to identify genes induced by C3a stimulation. We used Affymetrix U133A human microarray chips to analyze 16,000 genes simultaneously. Cells were stimulated for 6 h to identify early transcriptional responses to C3a stimulation; furthermore, the short stimulation time would avoid confounding paracrine effects. Using the criteria described in *Materials and Methods*, we identified 4 known genes and 2 expressed sequence tag (EST) sequences that were up-regulated and 6 known genes and 1 EST sequence that were down-regulated by C3a stimulation (Table I). Using 3 independent sets of RNA, we conducted quantitative RT-PCR on all 10 known genes.

Table I. C3aR mediated differentially regulated gene expression identified by Genechip analysis^a

Gene	Ascension No.	Fold Change (mean)	<i>p</i>	Gene	Ascension No.	Fold Change (mean)	<i>p</i>
Gulp	AK023668	-7.27	0.001	PECAM-1	NM_000442.1	+4.96	0.023
Branched chain acetyltransferase	NM_001918.1	-6.25	0.023	Cytochrome P450-XIX	NM_000103.1	+4.81	0.007
THR ^b coactivator	AL136823.1	-5.08	0.001	Unknown EST	AI192838	+4.31	0.009
Unknown EST	AK022061.1	-5.02	0.023	DYKR2	NM_006482.1	+4.27	0.012
CHRM-4	NM_000741.1	-4.06	0.005	Pro-1 α type 1 collagen	NM_000088.1	+3.82	0.025
ARF-1	AL117549.1	-3.74	0.008	Unknown EST	GB2531119	+3.71	0.002
Pyrin	NM_000243.1	-3.4	0.001				

^a Data represents mean values obtained from three independent experiments.

^b THR, thyroid hormone receptor; CHRM, cholinergic receptor, muscarinic; ARF, AD ribosylation factor.

Results of these experiments are shown in Table II. Using this method, 4 genes, pro-1 α type 1 collagen, DYKR2, pyrin, and Gulp, were independently confirmed as significantly differentially regulated by C3a stimulation. The remaining 6 genes also appeared to be differentially regulated, although they failed to meet criteria for statistical significance (data not shown). None of these genes have been previously associated with C3aR activation. The induction of pro-1 α type 1 collagen was confirmed by Western blot (Fig. 6).

Discussion

These studies demonstrate for the first time in humans and in mice that the C3aR is expressed by normal kidney epithelium. The receptor distribution in human kidney appears to be similar to that seen in the mouse. Renal parenchymal expression in normal tissue appears to be restricted to glomerular and tubular epithelial cells only. Neither immunohistochemistry nor in situ hybridization suggested C3aR expression in normal glomerular mesangial or glomerular endothelial cells. Although the C3aR is expressed within the renal cortex, the highest level of mRNA expression appears to be found in tubules of the renal medulla. Staining for both C3aR protein and mRNA suggests that expression of the receptor is limited to epithelial cells. In distinction to other complement receptors, the C3aR is expressed on both the luminal and antiluminal surfaces of the proximal tubule. This suggests that the C3aR may mediate cellular responses to both luminal complement activation as well as tubular response to interstitial injury. Although these studies demonstrate that the C3aR is expressed in multiple tubular segments, further work will be required to define the precise tubular expression of the C3aR. The renal distribution of the C3aR is similar to that reported for C5aR (22, 23, 25). In regard to C5aR, published reports indicate that the pattern of expression of this receptor is altered in disease states with up-regulation in glomerular mesangial cells (26). This finding suggests that further investigation into the distribution of the C3aR in pathologic conditions is warranted.

Activation of the complement cascade by either the classical, alternative, or lectin pathways results in the formation of the C3 convertase, a proteolytic enzyme that cleaves C3-producing C3b and C3a. C3a is a small 9-kDa peptide that is freely filtered by the

glomerular basement membrane, and thus, systemic complement activation may result in tubular responses to filtered intraluminal C3a. In addition to the production of C3a via systemic complement activation, it is clear that complement can be activated locally within the kidney. Complement components including C3, C4, factor B, and C5 can be synthesized by PTEC (27). Prior studies have demonstrated that alternative pathway complement activation can occur in tubular epithelium, and that C3 can be cleaved intraluminally, and thus generate C3a (28, 29). This suggests that the C3aR expressed in tubular epithelial cells has the capacity to respond in both a paracrine and an autocrine manner to complement activation. The relevance to human disease is clearly demonstrable in numerous pathologic studies. C3 deposition is a consistent feature in a range of both primary immune and nonimmune mediated renal disease, including lupus nephritis, membranoproliferative glomerulonephritis, acute postinfectious glomerulonephritis, renal allograft rejections, and chronic tubulointerstitial nephritis. Historically, it has been presumed that the generation of C3a concurrent with the deposition of C3 promotes renal injury via the recruitment of leukocytes and through the induction of proinflammatory mediators. The finding that renal tubular and glomerular epithelial cells express functional C3aR supports the hypothesis that activation of complement and the production of C3a have direct effects on renal parenchymal tissue.

Experimental data from cultured primary human proximal tubular epithelial cells confirmed C3aR expression by FACS, Western blot, and PCR. These findings are consistent with prior reports by Peake et al. (14) regarding C3aR expression in the human proximal tubular cell line HK-2. Functionality of the receptor was confirmed by radiolabeled C3a-binding studies as well as increased inositol incorporation in response to C3a stimulation. These findings combined with the immunohistochemistry and in situ hybridization data indicate that the C3aR is functionally expressed by normal human tubular epithelial cells.

To further define renal responses to C3aR activation, primary PTEC were stimulated in vitro with purified hC3a. Given the paucity of experimental data regarding epithelial cell responses to C3a stimulation, a genomic approach to the identification of transcription responses was taken. Ten known genes were identified by

Table II. PCR confirmed differentially regulated genes/ESTs from Table I^a

Gene	Average Ct	$\Delta\Delta Ct$	Fold Change	Range	<i>p</i>
Pro-1 α type 1 collagen	17.03 \pm 0.82	-4.27 \pm 0.82	+19.29	34.1-10.9	0.02
DYKR2	25.01 \pm 0.16	-1.56 \pm 0.13	+2.95	3.24-2.86	0.04
Pyrin	11.87 \pm 0.02	+2.68 \pm 0.11	-6.43	6.58-6.32	0.006
Gulp	23.36 \pm 0.38	+1.35 \pm 0.13	-2.56	2.59-2.37	0.03

^a Quantitative PCR data were obtained from triplicate samples generated in three independent experiments and normalized to 18S mRNA expression.

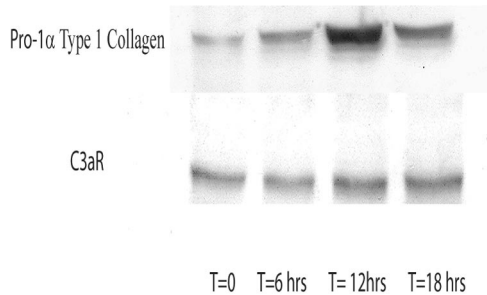


FIGURE 6. C3a stimulation of PTEC induces increased pro-1 α type 1 collagen protein production. Cultured PTEC were stimulated at the indicated time points with 10 nM C3a. The blot was stained with anti-pro-1 α type 1 collagen (*upper panel*), stripped, and then reprobed with anti-C3aR Ab (*lower panel*) for equivalency of loading. Western blot data are representative data from one of four separate experiments using PTEC derived from four separate donors.

GeneChip analysis as being differentially regulated; of these, four were independently confirmed by quantitative PCR. Pro-1 α type 1 collagen was strongly induced by C3aR activation, with a mean 19.29-fold increase in mRNA expression. The induction of type I collagen was confirmed by Western blot. Prior *in vitro* studies have demonstrated that type I collagen is produced by PTEC and alterations in synthesis of both type I collagen and type IV collagen occur in response to PTEC activation (30, 31). Although data regarding the expression of type 1 collagen in renal pathology are limited, the production of basement membrane components by PTECs clearly contributes to the development of chronic tubular injury and the development of interstitial fibrosis (32, 33). Data reported in the early 1980s suggested that collagen in general and type I collagen specifically may act as an autoantigen in some forms of autoimmune renal disease (34). The finding that C3a induces the production of type 1 collagen provides a potential direct link between chronic complement activation and the development of progressive interstitial fibrosis.

The signaling protein DYRK2 was also induced by C3aR activation, although to a lesser extent than type I collagen. This protein is a dual-specific tyrosine (Y) phosphorylation-regulated kinase. Members of this family of kinases are characterized by their ability to autophosphorylate tyrosine residues as well as phosphorylate serin/threonine residues on exogenous substrate (35, 36). DYRK2 has a cytosolic pattern of expression, while other family members are localized to the nucleus (36). DYRK2 has been shown to activate the transcriptional initiator eIF2B and the microtubule-associated protein τ (37). As a class, DYRK family members appear to be involved in the negative regulation of both cellular proliferation and differentiation (35). Although the renal expression and function of the DYRK2 are unknown, the reduction in DYRK2 mRNA mediated by C3a could potentially enhance the proliferation of PTEC in response to tubular injury.

Pyrin, also known as marenostatin, was down-regulated by C3aR activation. Pyrin, the gene responsible for familial Mediterranean fever, is expressed primarily by granulocytes (38, 39). Initial characterization of pyrin tissue expression indicated only low level of mRNA expression in Northern blots of whole kidney tissue, which was attributed to leukocyte contamination (40). However, the presence of mRNA for pyrin in proximal tubular cells in culture suggests that it is in fact expressed in renal tissue. The function of this newly characterized gene and its other family members is currently an intense area of investigation. It appears that it functions to attenuate inflammatory response through modulation of cytoskeletal organization and the regulation of NF- κ B and IL-1 β induction (41,

42). There are also reports that pyrin regulates the expression of C5a-inhibitor activity (43). Gulp, the remaining gene identified as negatively regulated by the C3aR, is perhaps the most intriguing. A human homologue for CED-6, Gulp is a member of a highly conserved family of *Caenorhabditis elegans* genes that regulate apoptosis. Although characterization of the function of this gene in the human system is ongoing, early data suggest that Gulp encodes an adapter protein that interacts with CED-1 and CD91, mediating the engulfment of apoptotic bodies (44). Down-regulation of Gulp would act to decrease the ability of PTEC to clear apoptotic bodies present in the tubular lumen. Recently, the murine homologue of CED-6 has been cloned from a murine kidney cDNA library; supporting the finding that CED-6 may regulate the processing of apoptotic bodies by renal epithelium (45). Although the ability of nonprofessional phagocytic cells to clear apoptotic bodies has been demonstrated in other systems, including intestinal epithelia, the role of renal epithelia in this process is uncertain (46).

Previous reports have indicated IL-8 and TGF- β induction in cell supernatants of C3a-stimulated epithelial cells; however, neither of these genes was identified in our microarray studies as being transcriptionally regulated at 6 h (7, 14). RT-PCR was also performed for TGF- β and IL-8, but failed to detect significant induction (data not shown). The inability to induce TGF- β and the limited number of genes identified as regulated by C3aR activation is most likely due to either the short period of stimulation used in our study compared with the previous report (6-h vs overnight stimulations), the differences in growth factors required to maintain primary vs transformed cells in culture, or the stringency of the statistical conditions of our microarray studies. However, our data regarding the induction of type I collagen support the hypothesis that C3a may play a role in the development of interstitial fibrosis, which has been shown to be dependent on both TGF- β and the production of extracellular matrix components such as type I collagen.

In conclusion, the data presented in this work demonstrate the receptor for the complement activation product C3a is highly expressed by normal murine and human kidney in an epithelial cell distribution. In primary human proximal tubular epithelial cells, the C3aR is both expressed and functional. Four genes not previously reported to be regulated by complement activation were identified as being differentially regulated by C3aR activation and provide insights into possible roles for C3a in novel signaling pathways, cytoskeletal organization, the induction of extracellular matrix components, and the clearance of apoptotic bodies. These findings provide clear evidence for a direct role for C3a in mediating parenchymal responses to immunologic renal injury.

Acknowledgment

We thank Dr. Irma Gigli for her insightful discussions and critique of the manuscript.

References

- Wetsel, R. A. 1995. Structure, function and cellular expression of complement anaphylatoxin receptors. *Curr. Opin. Immunol.* 7:48.
- Zwirner, J., O. Gotze, G. Begemann, A. Kapp, K. Kirchhoff, and T. Werfel. 1999. Evaluation of C3a receptor expression on human leucocytes by the use of novel monoclonal antibodies. *Immunology* 97:166.
- Werfel, T., K. Kirchhoff, M. Wittmann, G. Begemann, A. Kapp, F. Heidenreich, O. Gotze, and J. Zwirner. 2000. Activated human T lymphocytes express a functional C3a receptor. *J. Immunol.* 165:6599.
- Kirchhoff, K., O. Weinmann, J. Zwirner, G. Begemann, O. Gotze, A. Kapp, and T. Werfel. 2001. Detection of anaphylatoxin receptors on CD83⁺ dendritic cells derived from human skin. *Immunology* 103:210.
- Fischer, W. H., and T. E. Hugli. 1997. Regulation of B cell functions by C3a and C3a(desArg): suppression of TNF- α , IL-6, and the polyclonal immune response. *J. Immunol.* 159:4279.
- Schraufstatter, I. U., K. Trieu, L. Sikora, P. Sriramarao, and R. DiScipio. 2002. Complement C3a and C5a induce different signal transduction cascades in endothelial cells. *J. Immunol.* 169:2102.

7. Monsinjon, T., P. Gasque, A. Ischenko, and M. Fontaine. 2001. C3a binds to the seven transmembrane anaphylatoxin receptor expressed by epithelial cells and triggers the production of IL-8. *FEBS Lett.* 487:339.
8. Drouin, S. M., J. Kildsgaard, J. Haviland, J. Zabner, H. P. Jia, P. B. McCray, Jr., B. F. Tack, and R. A. Wetsel. 2001. Expression of the complement anaphylatoxin C3a and C5a receptors on bronchial epithelial and smooth muscle cells in models of sepsis and asthma. *J. Immunol.* 166:2025.
9. Van Beek, J., M. Bernaudin, E. Petit, P. Gasque, A. Nouvelot, E. T. MacKenzie, and M. Fontaine. 2000. Expression of receptors for complement anaphylatoxins C3a and C5a following permanent focal cerebral ischemia in the mouse. *Exp. Neurol.* 161:373.
10. Davoust, N., J. Jones, P. F. Stahel, R. S. Ames, and S. R. Barnum. 1999. Receptor for the C3a anaphylatoxin is expressed by neurons and glial cells. *Glia* 26:201.
11. Tornetta, M. A., J. J. Foley, H. M. Sarau, and R. S. Ames. 1997. The mouse anaphylatoxin C3a receptor: molecular cloning, genomic organization, and functional expression. *J. Immunol.* 158:5277.
12. Ames, R. S., Y. Li, H. M. Sarau, P. Nuthulaganti, J. J. Foley, C. Ellis, Z. Zeng, K. Su, A. J. Jurewicz, R. P. Hertzberg, et al. 1996. Molecular cloning and characterization of the human anaphylatoxin C3a receptor. *J. Biol. Chem.* 271:20231.
13. Crass, T., U. Raffetseder, U. Martin, M. Grove, A. Klos, J. Kohl, and W. Bautsch. 1996. Expression cloning of the human C3a anaphylatoxin receptor (C3aR) from differentiated U-937 cells. *Eur. J. Immunol.* 26:1944.
14. Peake, P. W., S. O'Grady, B. A. Pussell, and J. A. Charlesworth. 1999. C3a is made by proximal tubular HK-2 cells and activates them via the C3a receptor. *Kidney Int.* 56:1729.
15. Hugli, T. E., and H. J. Muller-Eberhard. 1978. Anaphylatoxins: C3a and C5a. *Adv. Immunol.* 26:1.
16. Humbles, A. A., B. Lu, C. A. Nilsson, C. Lilly, E. Israel, Y. Fujiwara, N. P. Gerard, and C. Gerard. 2000. A role for the C3a anaphylatoxin receptor in the effector phase of asthma. *Nature* 406:998.
17. Kildsgaard, J., T. J. Hollmann, K. W. Matthews, K. Bian, F. Murad, and R. A. Wetsel. 2000. Cutting edge: targeted disruption of the C3a receptor gene demonstrates a novel protective anti-inflammatory role for C3a in endotoxin-shock. *J. Immunol.* 165:5406.
18. Hebert, L. A., F. G. Cosio, and D. J. Birmingham. 1992. The role of the complement system in renal injury. *Semin. Nephrol.* 12:408.
19. Appay, M. D., M. D. Kazatchkine, M. Levi-Strauss, N. Hinglais, and J. Bariety. 1990. Expression of CR1 (CD35) mRNA in podocytes from adult and fetal human kidneys. *Kidney Int.* 38:289.
20. Rui-Mei, L., A. U. Kara, and R. Sinniah. 1998. In situ analysis of adhesion molecule expression in kidneys infected with murine malaria. *J. Pathol.* 185:219.
21. Honkanen, E., E. von Willebrand, A. M. Teppo, T. Tornroth, and C. Gronhagen-Riska. 1998. Adhesion molecules and urinary tumor necrosis factor- α in idiopathic membranous glomerulonephritis. *Kidney Int.* 53:909.
22. Zahedi, R., M. Braun, R. A. Wetsel, B. H. Ault, A. Khan, T. R. Welch, M. Frenzke, and A. E. Davis. 2000. The C5a receptor is expressed by human renal proximal tubular epithelial cells. *Clin. Exp. Immunol.* 121:226.
23. Braun, M., and A. E. Davis. 3rd. 1998. Cultured human glomerular mesangial cells express the C5a receptor. *Kidney Int.* 54:1542.
24. Hollmann, T. J., D. L. Haviland, J. Kildsgaard, K. Watts, and R. A. Wetsel. 1998. Cloning, expression, sequence determination, and chromosome localization of the mouse complement C3a anaphylatoxin receptor gene. *Mol. Immunol.* 35:137.
25. Fayyazi, A., O. Scheel, T. Werfel, S. Schweyer, M. Oppermann, H. J. Radzun, and J. Zwirner. 2000. The C5a receptor is expressed in normal renal proximal tubular but not in normal pulmonary or hepatic epithelial cells. *Immunology* 99:38.
26. Abe, K., M. Miyazaki, T. Koji, A. Furusu, T. Nakamura-Kurashige, T. Nishino, Y. Ozono, T. Harada, H. Sakai, and S. Kohno. 2001. Enhanced expression of complement C5a receptor mRNA in human diseased kidney assessed by in situ hybridization. *Kidney Int.* 60:137.
27. Zhou, W., J. E. Marsh, and S. H. Sacks. 2001. Intrarenal synthesis of complement. *Kidney Int.* 59:1227.
28. Camussi, G., M. Rotunno, G. Segoloni, J. R. Brentjens, and G. A. Andres. 1982. In vitro alternative pathway activation of complement by the brush border of proximal tubules of normal rat kidney. *J. Immunol.* 128:1659.
29. David, S., L. Biancone, C. Caserta, B. Bussolati, V. Cambi, and G. Camussi. 1997. Alternative pathway complement activation induces proinflammatory activity in human proximal tubular epithelial cells. *Nephrol. Dial. Transplant.* 12:51.
30. Han, D. C., M. Isono, B. B. Hoffman, and F. N. Ziyadeh. 1999. High glucose stimulates proliferation and collagen type I synthesis in renal cortical fibroblasts: mediation by autocrine activation of TGF- β . *J. Am. Soc. Nephrol.* 10:1891.
31. Zeisberg, M., G. Bonner, Y. Maeshima, P. Colorado, G. A. Muller, F. Strutz, and R. Kalluri. 2001. Renal fibrosis: collagen composition and assembly regulates epithelial-mesenchymal transdifferentiation. *Am. J. Pathol.* 159:1313.
32. Peleg, I., Z. Greenfeld, H. Cooperman, and S. Shoshan. 1993. Type I and type III collagen mRNA levels in kidney regions of old and young rats. *Matrix* 13:281.
33. Kemeny, E., T. Nadasdy, P. Szenohradszky, E. Csajbok, and J. Ormos. 1991. Comparative distribution of laminin and collagen types I, III and IV in transplant vasculopathy. *Acta Morphol. Hung.* 39:177.
34. Phadke, K., R. Fouts, J. Parrish, and R. S. Baker. 1984. Autoreactivity to collagen in a murine lupus model. *Arthritis Rheum.* 27:313.
35. Becker, W., and H. G. Joost. 1999. Structural and functional characteristics of Dyrk, a novel subfamily of protein kinases with dual specificity. *Prog. Nucleic Acid Res. Mol. Biol.* 62:1.
36. Becker, W., Y. Weber, K. Wetzel, K. Eirnbter, F. J. Tejedor, and H. G. Joost. 1998. Sequence characteristics, subcellular localization, and substrate specificity of DYRK-related kinases, a novel family of dual specificity protein kinases. *J. Biol. Chem.* 273:25893.
37. Woods, Y. L., P. Cohen, W. Becker, R. Jakes, M. Goedert, X. Wang, and C. G. Proud. 2001. The kinase DYRK phosphorylates protein-synthesis initiation factor eIF2B ϵ at Ser⁵³⁹ and the microtubule-associated protein tau at Thr²¹²; potential role for DYRK as a glycogen synthase kinase 3-priming kinase. *Biochem. J.* 355:609.
38. Booth, D. R., J. D. Gillmore, H. J. Lachmann, S. E. Booth, A. Bybee, M. Soyuturk, S. Akar, M. B. Pepys, M. Tunca, and P. N. Hawkins. 2000. The genetic basis of autosomal dominant familial Mediterranean fever. *Q. J. Med.* 93:217.
39. Booth, D. R., J. D. Gillmore, S. E. Booth, M. B. Pepys, and P. N. Hawkins. 1998. Pylrin/Marenostrin mutations in familial Mediterranean fever. *Q. J. Med.* 91:603.
40. Centola, M., G. Wood, D. M. Frucht, J. Galon, M. Aringer, C. Farrell, D. W. Kingma, M. E. Horwitz, E. Mansfield, S. M. Holland, et al. 2000. The gene for familial Mediterranean fever, MEFV, is expressed in early leukocyte development and is regulated in response to inflammatory mediators. *Blood* 95:3223.
41. Chae, J. J., H. D. Komarow, J. Cheng, G. Wood, N. Raben, P. P. Liu, and D. L. Kastner. 2003. Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. *Mol. Cell* 11:591.
42. Mansfield, E., J. J. Chae, H. D. Komarow, T. M. Brotz, D. M. Frucht, I. Aksentijevich, and D. L. Kastner. 2001. The familial Mediterranean fever protein, pyrin, associates with microtubules and colocalizes with actin filaments. *Blood* 98:851.
43. Matzner, Y., S. Abedat, E. Shapiro, S. Eisenberg, A. Bar-Gil-Shitrit, P. Stepensky, S. Calco, Y. Azar, and S. Urieli-Shoval. 2000. Expression of the familial Mediterranean fever gene and activity of the C5a inhibitor in human primary fibroblast cultures. *Blood* 96:727.
44. Su, H. P., K. Nakada-Tsukui, A. C. Tosello-Tramont, Y. Li, G. Bu, P. M. Henson, and K. S. Ravichandran. 2002. Interaction of CED-6/GULP, an adapter protein involved in engulfment of apoptotic cells with CED-1 and CD91/low density lipoprotein receptor-related protein (LRP). *J. Biol. Chem.* 277:11772.
45. Banerjee, H., Z. Hawkins, T. Johnson, S. Eley, A. Alikhan, M. McDaniel, I. Singh, and J. Raymond. 2003. Identification of a mouse orthologue of the CED-6 gene of *Caenorhabditis elegans*. *Plasmid* 49:30.
46. Parnai, R., M. C. Raff, and J. Scholes. 2000. Differences between the clearance of apoptotic cells by professional and non-professional phagocytes. *Curr. Biol.* 10:857.