CD40 ligation stimulates MCP-1 and IL-8 production, TRAF6 recruitment, and MAPK activation in proximal tubule cells

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Li, Hongye, and Edward P. Nord. CD40 ligation stimulates MCP-1 and IL-8 production, TRAF6 recruitment, and MAPK activation in proximal tubule cells. Am J Physiol Renal Physiol 282: F1020–F1033, 2002. First published December 18, 2001; 10.1152/ajprenal.00291.2001.—The mechanism of CD40/CD154-induced chemokine production and its potential role in renal inflammatory disease were explored. Human proximal tubule cells maintained in primary culture were used as the experimental model. With the use of immunocytochemistry, confocal microscopy, and a cell fractionation assay, the CD40 receptor was found to be expressed in the cell membrane of the epithelial cell, and, on engagement by CD154, its cognate ligand, translocated to the cytoplasmic compartment. Engagement of CD40 by CD154 stimulated interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) production, which proceeded via receptor activation of the extracellular signal-regulated kinase (ERK)/1,2, stress-activated protein kinase (SAPK/c-Jun NH2-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) pathways. CD40 ligation also engaged tumor necrosis factor receptor-activating factor 6 (TRAF6), as evidenced by colocalization of the activated receptor with TRAF6 in the cytoplasmic compartment, translocation of both proteins from the insoluble to the soluble cell fraction, and immunoprecipitation of the two proteins only under ligand-stimulated conditions. Furthermore, an antisense oligodeoxynucleotide targeted against TRAF6 mRNA blunted p38 and SAPK/JNK but not ERK1/2 MAPK activities, as well as IL-8 and MCP-1 production, arguing that TRAF6 is an upstream activator.

The zinc chelator TPEN, but not the calcium chelator BAPTA, obliterated CD154-evoked MAPK activity and chemokine production, providing indirect evidence for protein-protein interactions playing a critical role in CD40 signaling in these cells. We conclude that in human proximal tubule cells, CD40 and TRAF6 reside in separate low-density, detergent-insoluble membrane microdomains, or rafts, and on activation translocate and associate with one another probably via zinc-finger domains in the soluble or cytoplasmic compartment. TRAF6, in turn, activates SAPK/JNK and p38 MAPK phosphorylation, which in turn stimulates IL-8 and MCP-1 production in these cells.

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nase pathways. Under steady-state conditions, CD40 and TRAF6 reside in separate low-density, detergent-insoluble membrane microdomains, or rafts, and on activation translocate and associate with one another in the soluble or cytoplasmic compartment as a prerequisite for chemokine production.

MATERIALS AND METHODS

Cell culture. Primary cultures of human proximal tubule cells (PTCs) constitute the experimental model. Normal renal tissue from nephrectomy specimens obtained during the course of cancer surgery was used as source material (2). Verification of proximal tubule origin was performed as previously described by this (25) and other laboratories (12). Cultures were maintained in a defined medium composed of 1:1 (vol/vol) mixture of DMEM and Ham’s F-12 medium supplemented with insulin (5 μg/ml), transferrin (5.5 μg/ml), hydrocortisone (50 nM), triiodothyrine (5 μM), and sodium selenate (10 nM) and to which 50 IU/ml penicillin and 50 μg/ml streptomycin were added. Cells were usually grown in 25-cm² tissue culture flasks or in 24-well plates (ELISA) and perpetuated in a humidified incubator at 37°C in 95% air-5% CO₂ (culture medium pH 7.3). Media were exchanged at 48- to 72-h intervals, and cells were propagated through 8–10 passages.

In some experiments, monolayers were coincubated with the human T cell leukemia line Jurkat cells that constitutively express CD154 (D1.1) or control cells (B2.7) that do not. These cells were kindly provided by Dr. Michael Yellin (Columbia University, New York, NY) (46). These cells were maintained in RPMI with 10% newborn calf serum. Aliquots of ~10⁶ cells/ml (in log phase) were used as described by Suttles et al. (38).

Immunocytochemistry and confocal microscopy. Immunocytochemical staining of cultured proximal tubule cells for CD40 and TRAF6 was carried out on acetone-fixed monolayers (unless stated otherwise) according to a procedure adapted from methods described previously by this laboratory (1, 2). Optimal dilutions of the first antibody, determined by checkerboard titration, were incubated with the monolayer at room temperature for 60 min, followed by three rinses in buffered saline at 3–5°C. An Alexa Fluor-coupled secondary antibody was then applied for 30 min under identical conditions. Nonspecific binding of the secondary antibody was determined by omitting the primary antibody (vehicle only) from the initial incubate. An irrelevant antibody ensured specificity. In double-labeling experiments, the rabbit anti-human anti-CD40 polyclonal primary antibody was detected by anti-rabbit IgG-labeled Alexa Fluor 594 (red) and the anti-TRAF6 monoclonal primary antibody by anti-mouse IgG-labeled Alexa Fluor 488 (green). Alexa Fluor 594 and Alexa Fluor 488 (Molecular Probes, Eugene, OR) were detected by confocal microscopy (Bio-Rad Radrince 2000) with a Nikon Eclipse E800 (×60, 1.4-numerical aperture, oil-immersion objective lens), the appropriate excitation wavelength, dichroic mirror, and barrier filters. Images were captured with a digital camera and stored for further processing and evaluation. The rabbit anti-human CD40 polyclonal antibody and the anti-TRAF6 monoclonal antibody used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All studies were blinded.

Flow cytometry cell analysis. Confluent monolayers were prepared for flow cytometry by brief (2–3 min) trypsinization followed by centrifugation and resuspension of cells in RPMI medium. Cells were further washed and resuspended in RPMI to a final concentration of 10⁶ cells/ml and exposed to a saturating concentration of monoclonal antibodies for 45 min at room temperature (19). Monoclonal antibodies conjugated with FITC or phycoerythrin (PE) were used. Anti-CD40 (FITC) was purchased from Accurate Chemical (Westbury, NY), and IgG1-FITC/IgG2PE molecules specific for keyhole limpet hemocyanin, used as a negative control, were purchased from Becton Dickinson (Mountain View, CA). A FACScan flow cytometer (Becton Dickinson) was used for two-color cell analysis. The level of each cell subset was expressed as the percentage of total lymphocytes. Statistical analysis was done by Student’s t-test and χ-square distribution (Abstat software).

Immunoprecipitation and immunoblotting. Immunoprecipitation and Western blot analysis were performed using protocols previously described by this laboratory (1). Unless otherwise indicated, monolayers were challenged with recombinant human soluble (rhs) CD154 or vehicle (control), washed extensively with PBS (4°C), and then lysed in a previously described buffer. Where indicated, cultures were pretreated with the selective MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor PD-98059 (26) or the selective p38 inhibitor SB-203580 (22, 23). After a 5-min incubation period in lysis buffer, samples were transferred to Eppendorf microcentrifuge tubes, and the insoluble material was removed by centrifugation. Samples were next exposed to the antibody of interest, which had been preincubated with protein A-Sepharose CL-4B beads swollen in 50 mM Tris (pH 7.0). Immunoprecipitation proceeded overnight at 4°C on a rocker. Immunoprecipitates were washed, suspended in sample buffer, and heated to 95°C for 5 min. Matched aliquots were subjected to SDS-PAGE in 10% gels, transferred to membranes, and blocked with 5% nonfat dry milk suspended in 145 mM NaCl, 0.1% Tween 20, and 20 mM Tris base, pH 7.6 (room temperature, 1 h). Filters were probed with the antibody of interest (1:1,000, overnight at 4°C), washed (3 times) with TBS containing 0.1% nonfat dry milk suspended in 145 mM NaCl, 0.1% Tween 20, and subsequently incubated with peroxidase-linked goat anti-rabbit IgG in blocking buffer (1 h, room temperature). After additional washes (3 times), antibody-bound proteins were detected using an enhanced chemiluminescence (ECL) system according to the manufacturer’s instructions (Pierce, Rockford IL), and the membranes were exposed to Hyperfilm ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK). Molecular weight markers were used to determine the size of the detected band. Where required, relative densities of the protein bands were determined with a GS-670 imaging densitometer and Molecular Analyst PC software program (Bio-Rad, Richmond, CA). Antibodies were obtained from commercial sources and are detailed below in Materials.

To determine ERK1/2 expression and phospho-ERK1/2 activity, the immunoprecipitation step was deleted. Accordingly, after extensive washes with PBS (4°C), cells were lysed in 100 μl of SDS sample buffer (1) and scraped, and the extract was transferred to a microcentrifuge tube for sonication (10–15 s) to reduce sample viscosity. Samples were heated to 95°C for 5 min, and matched aliquots were subjected to SDS-PAGE in 10% gels as described above. Immunoblotting was performed as outlined earlier.

Cell fractionation. In experiments in which detergent-soluble and -insoluble fractions were required, CD154-stimulated and unstimulated (vehicle) cells were lysed in 1% Briij 58 (Pierce) for 5 min at 4°C. Lysates were centrifuged at 8,600 g (5 min, 4°C), and after the supernatants were collected, the insoluble pellets were resuspended and sonicated for 15 s in 100 μl of lysis solution that contained 0.5% SDS. The protein concentrations in the supernatants and in the insoluble pellets were detected by BCA Protein Assay Re-
agent. Equal aliquots of detergent-soluble and -insoluble material were subjected to SDS-PAGE and immunoblotting as described earlier. Blots were stripped and reprobed with an anti-tubulin antibody (Sigma, St. Louis, MO) against the cytoplasmic protein to further verify equal loading of proteins.

ELISA. MCP-1 and IL-8 production by proximal tubule cells was measured by the quantitative sandwich-enzyme immunoassay technique according to the manufacturer’s instructions (Quantikine, R&D Systems, Minneapolis, MN). For the purpose of these experiments, proximal tubule cells were grown in 24-well culture plates. The experimental maneuver was performed by adding agonist/vehicle/inhibitor to the supernatant, which was assayed for chemokine production. Preparatory experiments established optimal assay conditions. The optical density of each microtiter plate was read at 405 nm on a Biomed microplate reader. Recombinant

Data are presented as the index of dispersion and are expressed as means ± SE. Analysis of variance was used to compare group means, and the null hypothesis was rejected when P < 0.05.

RNA extraction, RT-PCR, and competitive RT-PCR. Confluent monolayers were challenged with rhsCD154 or vehicle (control) and where indicated were pretreated with the selective MEK inhibitor PD-98059 (26) or the selective p38 inhibitor SB-203580 (22, 23). RNA was extracted as previously described using TRIzol reagent (7, 8). After DNAsel treatment and a phenol/chloroform extraction step, cDNA was made using “downstream” primers (see below). Oligonucleotide PCR primers for amplification of cDNA for CD40, IL-8, MCP-1, and β-actin (housekeeping gene) were custom formulated (Life Technologies, Grand Island, NY), and a list is shown in Table 1. The Tian One Tube RT-PCR System (Boehringer Mannheim, Indianapolis IN) was employed according to the manufacturer’s instructions. Thirty-five cycles of PCR amplification were performed under modified conditions previously described by this laboratory (8): denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 68°C for 2 min. The amplified products were size fractionated on a 1.5% Tris-borate-EDTA-agarose gel containing ethidium bromide for resolution. The agarose gel was analyzed using a Bio-Rad Fluorescent Multi-Imager. To determine the size of the PCR products, a 100-bp DNA molecular ladder was used.

Competitive RT-PCR (27) was performed to quantitate changes in mRNA expression in delineated experiments. Preparatory experiments established the linear range of amplification and the optimal ratio of 18S primer to competitor for each specific primer pair as per the manufacturer’s instructions (Gene Specific Relative RT-PCR Kit, Ambion, Austin, TX). For IL-8, an optimal ratio of 4:6 (18S primer to competitor) with 30 cycles of amplification was determined, and for MCP-1 an optimal ratio of 4:6 (18S primer to competitor) with 29 cycles was found. PCR conditions are detailed above. The agarose gel was resolved using SYBR gold nucleic acid gel stain (1:10,000) for 30 min (40).

Table 1. PCR primers

<table>
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<tr>
<th>mRNA</th>
<th>Size of PCR-Amplified Fragment, bp</th>
<th>Synthesized Primers (Sense and Antisense)</th>
<th>Reference No.</th>
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<tr>
<td>CD40</td>
<td>330</td>
<td>5′-ATG GTC CGT CTG CCT CTG CAG-3′, 5′-CTT CTT CCG ACC GTC ACA TGC-3′</td>
<td>32</td>
</tr>
<tr>
<td>IL-8</td>
<td>253</td>
<td>5′-GGC TCT CTT GGC ACC CTT CCT G-3′, 5′-CTT CTC CAC AAC CTT CTG CAC CCA-3′</td>
<td>33</td>
</tr>
<tr>
<td>MCP-1</td>
<td>290</td>
<td>5′-ATG AAG GTG TGC TCT GCC GCC-3′, 5′-TTG GTT GTG CAG GTG GTC-3′</td>
<td>43</td>
</tr>
<tr>
<td>β-Actin</td>
<td>838</td>
<td>5′-ATC TGC CAC ACC TGC TAC AAT GAG CTG GGC-3′, 5′-GCT CAT ACT CCT GCT GTC TGA TCC ACA ACA TCT GC-3′</td>
<td>24</td>
</tr>
</tbody>
</table>

IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1.
RESULTS

Determination of CD40 expression by human proximal tubule cells. Several lines of approach were taken to determine CD40 expression by primary cultures of human proximal tubule cells. First, confluent monolayers were immunostained with an anti-CD40 antibody and subjected to immunocytochemistry. As depicted in Fig. 1A, CD40 staining was detected in the plasma membrane of the epithelial cells. Second, control and interferon (IFN)-γ-treated cells (500 U/ml, 48 h) were labeled with an FITC-conjugated monoclonal antibody and subjected to flow cytometric analysis. IFN-γ has been shown to upregulate CD40 in other cell systems (32, 38). CD40 is constitutively expressed on the surface of the proximal tubule cell (Fig. 1B), and treatment with IFN-γ increased CD40 expression by about twofold. Although the IFN-γ effect is statistically significant, it is modest. Of greater significance is the detection of CD40 on proximal tubule cells by two independent approaches. As a third approach, and to confirm these findings, confluent monolayers were lysed and cell lysates and CD40 immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with an anti-CD40 antibody. A ~45-kDa band consistent with the CD40 protein was easily detected under immunoprecipitation conditions, whereas the cell lysate yielded only a faint band (Fig. 1C). In aggregate, unstimulated primary cultures of human proximal tubule cells express CD40, and as described in other cell systems, expression may be upregulated by incubation with IFN-γ. CD40 mRNA expression by proximal tubule cells was also examined with RT-PCR. The housekeeping gene β-actin was examined under identical conditions, and a human lung carcinoma cell line (A-549), which expresses CD40, was included as an internal control. Figure 1D summarizes the findings. The annotated number 600 identifies the 600-bp value on the ladder in the far left lane of the ethidium bromide-stained agarose gel. The expected 280-bp PCR product seen in lanes 1 and 4 represents CD40 and is derived from proximal tubule and lung cells, respectively. The β-actin gene product (838 bp) is identified in lane 2, and scrambled CD40 primers were used in lane 3. These observations further confirm CD40 expression in proximal tubule cells, except at the mRNA level, and complement the studies on CD40 protein expression.

Engagement of CD40 by CD154, its cognate ligand, results in translocation of the receptor from the cell membrane to the cytoplasmic compartment. Because in the unstimulated condition the CD40 receptor appears to be located primarily in the cell membrane (Fig. 1A), we sought evidence for possible translocation of the receptor to the cytosolic compartment on engagement by CD154, its cognate ligand. Recent studies have suggested that receptors might be aggregated in detergent-insoluble membrane domains or rafts that are enriched in sphingolipids, cholesterol, and glycosphosphatidylinositol-linked proteins (34). Accordingly, quiescent monolayers were either not treated or challenged with rhsCD154 (100 ng/ml + 1 μg/ml enhancer) for 2 or 7 min as indicated (Fig. 2A) and then lysed with a buffer that contained 1% Brij 58 as detailed in MATERIALS AND METHODS. The insoluble pellet was dissolved in 0.5% SDS and 1% β-mercaptoethanol and then subjected to SDS-PAGE/Western blot analysis using the anti-CD40 antibody. The soluble pellet

Fig. 1. CD40 expression by human proximal tubule cells (PTCs). A: confocal photomicrograph of an immunostained proximal tubule cell using an anti-CD40 polyclonal primary antibody and an Alexa Fluor 594-labeled secondary antibody. The monolayer was fixed with acetone after application of the primary antibody. B: flow cytometric analysis using an FITC-conjugated anti-CD40 monoclonal antibody in the absence of (control) or after treatment with interferon (IFN)-γ (500 U/ml, 48 h). C: immunoblot of cell lysate (left) and CD40 immunoprecipitate (right). D: CD40 mRNA expression examined by RT-PCR. Oligonucleotide primers (CD40) detailed in Table 1 were used with cultures derived from proximal tubule (lane 1) and lung (lane 4) cells. Scrambled primers (CD40) were used in lane 3, and primers for β-actin were used in lane 2. One of 4–5 similar experiments for each parameter is shown.
was analyzed in parallel by SDS-PAGE/Western blot analysis. Most of the CD40 from unstimulated cells was present in the detergent-insoluble fraction with only small amounts present in the soluble fraction (Fig. 2A). In rhsCD154-challenged cells, much of the CD40 became associated with the soluble fraction as a function of time, with lesser amounts evident in the insoluble fraction. Immunocytochemical staining was performed using an anti-CD40 polyclonal primary antibody and an Alexa Fluor 594-labeled secondary antibody. A phase contrast photomicrograph (I) of the unstimulated cells (III) and a photomicrograph where the primary antibody (anti-CD40) had been deleted (II) are included as controls. One of 3 similar experiments is shown.

**CD40 activation stimulates chemokine production by human proximal tubule cells.** For reasons outlined in the **DISCUSSION**, we focused our efforts on two chemotactic agents, namely, IL-8 and MCP-1. To confirm that our cultures produce IL-8, quiescent monolayers were incubated with IFN-γ (500 U/ml, 24–48 h) and subsequently cocultured with the human T cell leukemia line Jurkat cells that constitutively express CD154 (D1.1) or control cells (B2.7) that do not express the ligand, and IL-8 expression was determined by ELISA. Figure 3A depicts the results. Preincubation with IFN-γ decreased IL-8 production from a control value of 936 ± 73 to 171 ± 18 pg/ml (n = 3, P < 0.001), and CD154-expressing Jurkat cells increased this value to 3,165 ± 252 vs. 265 ± 25 pg/ml (n = 3, P < 0.001) by coincubation with control Jurkat cells. In the absence of proximal tubule cells, IL-8 was not detected (data not shown). Importantly, when cells were not preincubated with IFN-γ, rhsCD154 (5 µg, 24 h) itself significantly increased IL-8 production to 3,786 ± 180 vs. 936 ± 73 pg/ml (n = 3, P < 0.001) at baseline.
separate experiments, CD40/CD154 stimulation of MCP-1 production was examined. Quiescent monolayers produced 952 ± 118 vs. 3,528 ± 297 pg/ml MCP-1 (n = 3, P < 0.001) after challenge with rhsCD154 (5 μg, 24 h) in the absence of IFN-γ pretreatment (Fig. 3B), and pretreatment with an anti-CD40 antibody (100 ng/ml, 2 h) blunted CD154-induced MCP-1 production to 2,132 ± 204 pg/ml (n = 3, P < 0.05). These data provide functional evidence for CD40 activation of IL-8 and MCP-1 in proximal tubule cells and that these cells respond briskly to the recombinant product in the absence of IFN-γ “priming” of CD40.

mRNA expression of IL-8 and MCP-1 by human proximal tubule cells was explored in subsequent experiments. With the use of oligonucleotide primers for IL-8 and MCP-1 detailed in Table 1, monolayers were subjected to RT-PCR, and the amplified product sizes were fractionated on a 2% agarose gel containing ethidium bromide. The expected 253-bp IL-8 PCR product and the 290-bp MCP-1 PCR product from 3 different monolayers are depicted in lanes 4–6 and lanes 1–3, respectively (Fig. 3C). The 838-bp β-actin PCR product was included as a control (lane 7). Hence, IL-8 and MCP-1 are detected at both the mRNA and protein level in this cell type.

Delineation of signal pathways for CD40-evoked stimulation of IL-8 and MCP-1 production by human proximal tubule cells. The role of MAPK signaling pathways in CD40-mediated cytokine production was examined using three different approaches, namely, ELISA, Western blot analysis, and competitive RT-PCR. In all subsequent experiments, and on the basis of the data shown in Fig. 3, we used only rhsCD154 in the presence of an enhancer according to the manufacturer’s instructions. Preparatory experiments (not shown) determined that ligand-induced IL-8 and MCP-1 production, as measured by ELISA, was detected as early as 1 h postchallenge, with maximal effect of rhsCD154 (100 ng/ml + 1 μg/ml enhancer) observed at 12–24 h poststimulation. Under these conditions, rhsCD154 increased IL-8 production from a baseline value of 8,020 ± 510 to 13,020 ± 1,032 pg/ml (n = 4, P < 0.001), and the selective MEK inhibitor PD-98059 (50 μM, overnight) blocked the response (9,644 ± 102 pg/ml [n = 4, not significant (NS) vs. baseline value]) (Fig. 4A). PD-98059 itself was without effect in vehicle-challenged monolayers: 8,335 ± 367 pg/ml (n = 4, NS vs. baseline value) (Fig. 4A). In parallel experiments, monolayers were preincubated with the p38 inhibitor SB-203580 (2.5 μM, overnight) before challenge with rhsCD154. SB-203580 blocked IL-8 production [6,170 ± 217 vs. 13,020 ± 1,032 pg/ml (n = 4, P < 0.001)] under rhsCD154-challenge conditions but was without effect in vehicle-challenged cells [6,984 ± 465 pg/ml (n = 4, NS vs. baseline value)] (Fig. 4A). Preparatory experiments established the optimal conditions for both PD-98059 and SB-203580 pretreatment. Under similar experimental conditions (Fig. 4B), rhsCD154 increased MCP-1 production from a baseline value of 9,215 ± 975 to 14,455 ± 1,214 pg/ml (n = 4, P < 0.001), and the selective MEK inhibitor (PD-980590) and p38 inhibitor (SB-203580) blocked the response [10,876 ± 607 and 7,455 ± 756 pg/ml, respectively; both values n = 4, NS vs. baseline value]. Unchallenged, inhibitor-treated cells had values similar to the baseline value: 10,171 ± 723 pg/ml for PD-
9,215 ± 978 pg/ml (baseline value) (Fig. 4B). These findings are in keeping with the proposal that CD40 activation by its cognate ligand stimulates IL-8 and MCP-1 production in human proximal tubule cells via the ERK1/2 and p38 MAPK pathways. In the absence of a commercially available specific SAPK/JNK MAPK inhibitor, the latter signaling pathway could not be explored in isolation.

Competitive RT-PCR for IL-8 and MCP-1 mRNA expression was performed under similar conditions described above for IL-8 and MCP-1 protein expression, in which the MEK and p38 inhibitors were used. The data are summarized in Fig. 5. rhsCD154 (100 ng/ml + 1 μg/ml enhancer, 1 h) increased MCP-1 mRNA expression (Fig. 5B) by 2.3-fold (ratio depicted in Fig. 5C), and this increment was reduced by preincubation of the monolayers with either the MEK inhibitor PD-98059 or the p38 inhibitor SB-203580. rhsCD154 also increased IL-8 mRNA expression (Fig. 5A) but by 1.7-fold, and the p38 inhibitor SB-203580, but not the MEK inhibitor PD-98059, blunted this increment (Fig. 5C). These data, at the mRNA level, concur with the observation at the protein level demonstrating CD154 stimulation of IL-8 and MCP-1. It is noteworthy that SB-203580 blunted both MCP-1 and IL-8 mRNA abundance consequent to rhsCD154 treatment, whereas PD-98059 blunted only MCP-1 but not IL-8 abundance.

**Determination of CD40-mediated MAPK activation in human proximal tubule cells.** CD40-mediated activation of the above-delineated MAPK signaling pathways was next investigated using Western blot analysis and specific phospho-activated antibodies. rhsCD154 (100 ng/ml + 1 μg/ml enhancer, 30 min) increased ERK1/2 (Fig. 6A), p38 (Fig. 6B), and, most impressively, SAPK/JNK activity (Fig. 6C) in proximal tubule cells by 3- to 10-fold (lane 1 vs. lane 2). In control experiments, no differences were seen in the nature or...
size of the protein band in stimulated vs. unstimulated monolayers, confirming that similar aliquots were loaded on each lane (Fig. 6, bottom bands). The MEK inhibitor PD-98059 blocked ligand-induced ERK1/2 activity (Fig. 6A), and the p38 inhibitor SB-203580 blocked ligand-induced p38 activity (Fig. 6B). Neither PD-98059 nor SB-203580 had any effect on SAPK/JNK activity (Fig. 6C). Note, however, that whereas PD-98059 does not hinder p38 activity (Fig. 6B), SB-203580 unexpectedly did blunt ERK1/2 activity (Fig. 6A). In the aggregate, it is evident that CD154/CD40 ligation activates the ERK1/2, p38, and SAPK/JNK signaling pathways in human proximal tubule cells.

**Engagement of TRAF6 in CD40 signaling in human proximal tubule cells.** A role for TRAFs in CD40-mediated MAPK activation of MCP-1 and IL-8 was next explored. For reasons delineated below, we focused our efforts on TRAF6. To demonstrate that after ligation CD40 engages TRAF6, two separate approaches were taken. First, quiescent monolayers were incubated with rhsCD154 (100 ng/ml + 1 μg/ml enhancer) for the time periods detailed in Fig. 7, and the Alexa Fluor-tagged CD40 and TRAF6 antibodies were tracked by confocal microscopy. Under unstimulated (baseline) conditions the anti-CD40 antibody tagged by Alexa Fluor 594 (red) was localized primarily to the cell membrane, whereas the anti-TRAF6 antibody tagged by Alexa Fluor 488 (green) was found throughout the cell with some perinuclear distribution. In rhsCD154-stimulated cells (2 min), the anti-CD40-tagged Alexa Fluor 594 (red) antibody was now distributed throughout the cell and colocalized with TRAF6 (colocalization in yellow). Colocalization persisted for 7 min. In parallel experiments the insoluble and soluble pellets from untreated and challenged (rhsCD154 100 ng/ml + 1 μg/ml enhancer) cells were subjected to SDS-PAGE/Western blot analysis as described earlier. At the 2- and 7-min time points, there was a progressive increase in TRAF6 association with the soluble fraction, with a concomitant decreased association with the insoluble fraction (Fig. 7B). These observations persisted for 30 min, the longest time period explored (data not shown). Note, also that in the unchallenged state TRAF6 was not detected in the cell membrane by immunocytochemistry (Fig. 7A) and in additional experiments (not shown) was undetectable in nonpermeabilized immunostained cells. The significance of these observations will be alluded to in the DISCUSSION.

As a second approach, coimmunoprecipitation experiments were performed using CD40 and TRAF6 immunoprecipitates. As illustrated in Fig. 7C, in the unchallenged state CD40 immunoprecipitates were not detected by Western blot analysis using the anti-TRAF6 antibody (lane 1). Exposure of monolayers to rhsCD154 (100 ng/ml + 1 μg/ml enhancer, 30 min) resulted in obvious detection of CD40 immunoprecipitates by the anti-TRAF6 antibody (lane 2). Control experiments validated the nature of the TRAF6 and CD40 immunoprecipitates (lane 3 and lane 4, respectively). Taken together, these observations provide strong evidence for CD40 engagement of TRAF6 on activation of the receptor by its cognate ligand.

To evaluate whether TRAF6 mediates MAPK activation of MCP-1 and IL-8, antisense and sense ODNs targeted to the 3′-untranslated region of human TRAF6 mRNA were introduced into subconfluent monolayers (as detailed in MATERIALS AND METHODS), and MAPK and chemokine production was measured. Western blot analysis for TRAF6 confirmed that both 5 and 27 μM antisense ODN largely obliterated TRAF6 expression in these cells (Fig. 8A). The sense ODN (10 μM) had no effect (Fig. 8A). In parallel experiments monolayers were challenged with rhsCD154 (100 ng/ml + 1 μg/ml enhancer) for 30 min, and MAPK activity was determined with the specific phospho-antibody as indicated. Both SAPK/JNK and p38 activity were significantly blunted in TRAF6-antisense ODN-treated cells challenged with rhsCD154, but ERK1/2 activity was essentially unchanged (Fig. 8B). The sense ODN did not affect MAPK activity (data not shown). Under identical conditions, MCP-1 and IL-8 levels were blunted in TRAF6-antisense ODN-treated cells challenged with rhsCD154, but ERK1/2 activity was essentially unchanged (Fig. 8C). rhsCD154 increased IL-8 production from a baseline value of 5,557 ± 891 pg/ml (n = 4, P < 0.001) in untreated monolayers, but the response was blunted in TRAF6-antisense ODN-treated cells exposed to rhsCD154 (Fig. 8C). rhsCD154 increased IL-8 production from a baseline value of 5,557 ± 891 pg/ml (n = 4, P < 0.001) in untreated monolayers, but the response was blunted in TRAF6-antisense ODN-treated cells exposed to rhsCD154 (Fig. 8C). rhsCD154 increased MCP-1 production from a baseline value of 5,557 ±
1,017 to 9,348 pg/ml (n = 4, P < 0.001) in untreated monolayers, and in TRAF6-antisense ODN-treated cells exposed to rhsCD154 the response was blunted to 4,528 ± 621 pg/ml (n = 4, NS vs. control). The sense ODN did not affect chemokine production (data not shown). These observations argue that CD40 activation proceeds through TRAF6, which in turn stimulates MCP-1 and IL-8 activity via the p38 (and possibly the SAPK/JNK) pathways and that the ERK1/2 pathway is not engaged by TRAF6.

Involvement of zinc fingers in TRAF6 signaling in human proximal tubule cells. TRAF6, like other TRAF family proteins, has a RING finger domain, a cluster of zinc fingers, which are thought to mediate protein-protein interactions and hence play a critical role in the intracellular transduction of signals (16). Hence a che-
A cell membrane-permeable chelator of zinc (10 μM, 30 min) was used as a control. The results are depicted in Fig. 9A. As demonstrated earlier, stimulation of cells with rhsCD154 markedly increased phospho-SAPK/JNK, phospho-p38, and phospho-ERK1/2 activity. Pretreatment of monolayers with BAPTA-AM did not dampen the response. In contrast, TPEN pretreatment obliterated all three phospho-responses. Under comparable experimental conditions, TPEN treatment of monolayers (2.5 μM, 30 min) blunted rhsCD154 (100 ng/ml + 1 μg/ml enhancer, 30 min) increased production of MCP-1 and IL-8 (Fig. 9B). rhsCD154 increased IL-8 production from a baseline value of 5,540 ± 554 to 8,155 ± 628 pg/ml (n = 4, P < 0.001), and pretreatment with TPEN blocked the response to 4,912 ± 307 pg/ml (n = 4, NS vs. control). rhsCD154 increased MCP-1 production from a baseline value of 9,807 ± 958 to 14,590 ± 2,427 pg/ml (n = 4, P < 0.001) and TPEN pretreatment of monolayers blocked the response to 7,276 ± 344 pg/ml (n = 4, NS vs. control). TPEN itself was without effect in vehicle-challenged monolayers.
6,885 ± 1,032 pg/ml (MCP-1) and 5,001 ± 793 pg/ml (IL-8). These data provide supportive evidence for TRAF protein involvement in the downstream recruitment of SAPK/JNK, p38, and ERK1/2 in CD40 signaling and TRAF involvement in MCP-1 and IL-8 production. Clearly, the nature of the specific TRAF protein involved cannot be identified in this manner nor is there assurance that the RING finger domain is necessarily on a TRAF protein. The data also provide indirect evidence for CD40 activation of ERK1/2 possibly via some other member of the TRAF family.

**DISCUSSION**

The results presented demonstrate that human proximal tubule cells maintained in primary culture express the CD40 receptor (Fig. 1) and on activation of this receptor by CD154, its cognate ligand, translocates from the cell membrane to the cytoplasmic compartment (Fig. 2). Engagement of CD40 by CD154 stimulates IL-8 and MCP-1 production (Fig. 3) and also increases the activities of ERK1/2, p38, and SAPK/JNK (Fig. 6). IL-8 and MCP-1 production proceeds via ligand activation of ERK1/2 and p38 (Figs. 4–6), but whether SAPK/JNK is also involved in CD40-activated chemokine production could not be definitively determined. CD40 ligation engages TRAF6, as evidenced by colocalization of the activated receptor with TRAF6 in the cytoplasm, translocation of both proteins from the insoluble to soluble cell fraction, and coimmunoprecipitation of the two proteins (Fig. 7). In addition, an antisense ODN targeted against TRAF6 mRNA blunts p38 and SAPK/JNK, but not ERK1/2, activity as well as IL-8 and MCP-1 production (Fig. 8), arguing that TRAF6 is an upstream activator. Finally, the zinc chelator TPEN, but not the calcium chelator BAPTA-AM, obliterates CD154-evoked p38, SAPK/JNK, and ERK1/2 activation and IL-8 and MCP-1 production (Fig. 9), providing indirect evidence that protein-protein interactions play a critical role in CD40 signaling in human proximal tubule cells.

CD40 was initially described as a B cell-specific receptor that on engagement by CD154, its ligand (also termed CD40L or gp39), resulted in B lymphocyte activation and proliferation and immunoglobulin production (9). It is now recognized that CD40 is fairly widely distributed and is expressed on a variety of different cell types, including macrophages, endothelial cells, keratinocytes, certain fibroblasts, and dendritic cells (36, 46). Its role in non-B lymphocyte function is poorly understood. With regard to the kidney, CD40 is expressed in normal renal tissue, but its precise cellular expression is somewhat controversial. Yellin and co-workers (45) found CD40 expressed on glomerular endothelial, parietal epithelial, and mesangial cells within the glomerulus by immunocytochemistry and on interstitial capillaries and especially distal tubule epithelial cells. Importantly, no immunoreactivity was detected on proximal tubule epithelial cells. In a second study (42), weak immunostaining was detected “on some tubuli and other renal structures,” a rather imprecise description. Using immunocytochemistry (Fig. 1A) and flow cytometric analysis (Fig. 1B), we definitively demonstrate CD40 expression on primary cultures of human proximal tubule cells and confirm this observation by Western blot analysis (Fig. 1C). CD40 mRNA expression detected by RT-PCR (Fig. 1D) is in keeping with such a conclusion.

Precedent exists for CD40 signaling through MAPK pathways, i.e., ERK1/2, SAPK/JNK and p38, but the precise pathway(s) involved appears to some extent to be cell type dependent. ERK1/2 is engaged in human monocytes, whereas SAPK/JNK and p38 do not appear to be involved (38). In human B cell lymphoma lines (Burkitt’s, Daudi), CD40 signals through SAPK/JNK but not through ERK1 and ERK2 (3, 31); p38 was not examined in these studies but did appear to be involved in a separate study (10). In the WEHI-231 B lymphoma cell line, CD40 is a potent activator of SAPK/JNK and p38 activity but has no effect on ERK1 or ERK2 (37). In our epithelial cell model, all three signaling pathways appear to be involved. Perhaps the most robust signal was that of SAPK/JNK phosphorylation (Fig. 6C), but unequivocal phosphorylation of p38 (Fig. 6B) and ERK1/2 (Fig. 6A) was also observed. Furthermore, pretreatment of cells with the specific MEK or p38 inhibitor completely inhibited CD154-induced phosphorylation of the respective proteins (Fig. 6, A and B). In the absence of a commercially available specific SAPK/JNK inhibitor, the latter pathway could not be probed in further detail. Because in proximal tubule cells all three signaling pathways appear to be activated in response to CD154 ligation, analysis of downstream events is rendered more complex.

Previous reports by other workers have shown that renal proximal tubule cells, in response to CD154 ligation, produce a number of chemokines, as exemplified by IL-8 and MCP-1 production (14, 28). The present study confirms these observations but extends them in two important dimensions. First, using the approach taken by others, coculture experiments with cells that constitutively express CD154 and, in the presence of IFN-γ, yielded an approximately threefold increase in IL-8 production (Fig. 3A). Under similar experimental conditions, rhsCD154 in the absence of IFN-γ preconditioning resulted in an approximately fourfold increment in IL-8 production (Fig. 3A) and an approximately fourfold increment in MCP-1 production (Fig. 3B). It is evident from these observations that IFN-γ preconditioning is not a prerequisite for ligand binding to its receptor in renal proximal tubule epithelial cells and that the recombinant ligand is functionally active in this setting. Second, production of IL-8 and MCP-1 is mediated via the ERK1/2, p38, and probably SAPK/JNK MAPK pathways, because inhibition of the former two pathways by their respective inhibitor blunted ligand-evoked chemokine production. The role of SAPK/JNK activation can only be implied from independent observations. It might be argued that blockade of one MAPK pathway should not influence ligand-induced chemokine production via alternate MAPK
pathways (Fig. 4). The fact that PD-98059, the selective MEK inhibitor, did not influence p38 (Fig. 6B) and SAPK/JNK (Fig. 6C) phosphorylation and that SB-203580, the selective p38 inhibitor, had no effect on SAPK/JNK activation (Fig. 6C) argues against a trivial explanation. Rather, selective inhibition of one MAPK pathway blunts chemokine production at the mRNA level (Fig. 5) independently of the fact that alternate MAPK pathways are left intact. An alternative explanation is that each MAPK pathway does not function in isolation and interactive signals or cross talk might be interrupted. In this regard, inactivation of p38 by SB-202190 treatment results in delayed activation of ERK 1/2 in the human hepatoma cell line HepG2 (35). Phosphorylated p38 is also capable of activation of ERK 1/2 in the human hepatoma cell line HepG2 (35). Phosphorylated p38 is also capable of forming a complex with ERK1/2 and prevents their phosphorylation by the upstream activator (47). In the human embryonic kidney cell line HEK-293, ERK activation is strongly enhanced by overexpression of p38 and is blocked by the dominant-negative kinase version of p38 or the specific p38 inhibitor SB-203580 (21), as observed in this study (Fig. 6A). All of these concepts would explain why, for example, selective inhibition of either ERK1/2 or p38 inhibits CD154/CD40-mediated MCP-1 and IL-8 protein production.

The cytoplasmic domain of human CD40 interacts directly with TRAF1, TRAF2, TRAF3, and TRAF6 (16, 17, 30). TRAF1, TRAF2, and TRAF3 appear to interact within the same region of the cytoplasmic tail, whereas the recognition site for TRAF6 appears to be a nonoverlapping proximal region (30). For this reason, we focused our efforts on TRAF6. We demonstrate that in human proximal tubule cells TRAF6 is the upstream activator of p38 and SAPK/JNK but not of ERK1/2 and that downregulation of TRAF6 blunts CD154/CD40-evoked MCP-1 and IL-8 production (Fig. 8). Furthermore, TPEN, a chelator of zinc, but not BAPTA, a chelator of calcium, blunted rhsCD154-evoked activity of all three MAPK pathways (Fig. 9), arguing that interruption of protein-protein interactions at the level of zinc-finger domains, as exemplified by those exhibited by TRAFs (13, 16, 39), plays a key role in CD40-mediated MCP-1 and IL-8 production. It is recognized that the effect of zinc chelation is somewhat nonspecific but nonetheless supports the role for TRAF6 involvement in CD40 signaling. It is also of interest that whereas TPEN obviates ERK1/2 phosphorylation in this setting, TRAF6 downregulation is without effect on phospho-ERK1/2 activity, arguing that other TRAF family members may play a role in CD40 stimulation of MCP-1 and IL-8 production in human proximal tubule cells. In addition, CD40 signaling in human proximal tubule cells appears to differ somewhat from that described in other cell types. For example, in the human embryonic kidney cell line HEK-293, TRAF6 was shown to be a major transducer of ERK activation by CD40 (18) whereas SAPK/JNK activation required both TRAF6 and TRAF1/TRAFF2/TRAFF3 (29). Akin to HEK-293 cells, TRAF6 binding appears critical for p38 activation through CD40 in proximal tubule cells (29).

Our findings indicate that TRAF6 in the proximal tubule cell is not bound to CD40 in the unstimulated state. This can be concluded primarily from intact cell studies in which confocal microscopy and fluorescent tags of different colors (red, green) were used and that no colocalization (yellow) of these two proteins was visualized in the unstimulated state (Fig. 7A). Coimmunoprecipitation experiments (Fig. 7C) are in keeping with such a conclusion. Conflicting evidence exists in this regard at least in the context of CD40 and TRAF6. Some observers have concluded that CD40 and TRAF2 are constitutively associated with each other under unstimulated conditions (6), whereas others have refuted this possibility (15). The present observation with regard to TRAF6 and CD40 is in keeping with the latter concept.

On stimulation by its cognate ligand, CD40 translocated from the cell membrane to the cytosolic compartment as determined by confocal microscopy (Figs. 2B and 7A), and most of the CD40 that was present in the detergent-insoluble fraction became associated with the soluble fraction (Fig. 2A). In parallel, little change in TRAF6 distribution could be detected by confocal microscopy (Fig. 7A), yet the largely detergent-insoluble fraction also became associated with the soluble fraction (Fig. 7B). The simplest explanation for these observations is that under unchallenged conditions the CD40 receptor and TRAF6 proteins reside in different membrane fragments that are insoluble in nonionic detergents and that on activation associate with one another and translocate to the soluble component or cytoplasm. Confocal microscopy would argue that in the unstimulated state CD40 resides in the cell membrane, whereas TRAF6 would be present in some intracellular structure such as the trans-Golgi network or elsewhere. Indeed, it has been suggested that the low solubility of certain TRAFs may be a result of their association with cytoskeletal scaffolding components with roles in signaling (11). Such a finding would be in

Fig. 10. Schema of CD40 signaling in human proximal tubule cells. Proposed signaling pathways for CD40 mediated MCP-1 and IL-8 production. MCP-1 and IL-8 production is mediated via the p38, ERK 1/2, and probably the SAPK/JNK pathways. SAPK/JNK and p38 activation is mediated via TRAF6 whereas ERK 1/2 activity is mediated via a yet to be determined intermediate, possibly a member of the TRAF family.
keeping with the concept that under unchallenged conditions, CD40 and TRAF6 reside in low-density, detergent-insoluble membrane microdomains, or rafts (4, 34), that are rich in sphingolipids and cholesterol and on activation translocate and associate with one another in the soluble or cytosolic compartment, where the resultant downstream chain of events is put into motion. Although both TRAF2 and TRAF3 have been shown to be recruited from the cytoplasm to the cell membrane by CD40, this was not the case for TRAF6 recruitment (15). We suggest an alternate mechanism for TRAF6 recruitment by activated CD40.

Figure 10 is a proposed schema for CD40 signaling in human proximal tubule cells and summarizes our observations. CD40 ligation results in increased phosphorylation of SAPK/JNK, p38, and ERK1/2 MAP kinases. Activation of SAPK/JNK and p38 is mediated via TRAF6 whereas ERK1/2 activity is potentially mediated via other TRAF member(s). Stimulation of all three MAPK pathways appears to be required for MCP-1 and IL-8 production.

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