Functional caveolae are a prerequisite for CD40 signaling in human renal proximal tubule cells

Hongye Li and Edward P. Nord
Division of Nephrology, Department of Medicine, School of Medicine,
State University of New York at Stony Brook, Stony Brook, New York 11794

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Li, Hongye, and Edward P. Nord. Functional caveolae are a prerequisite for CD40 signaling in human renal proximal tubule cells. Am J Physiol Renal Physiol 286: F711–F719, 2004. First published December 9, 2003; 10.1152/ajprenal.00308.2003.—The role of caveolae in CD40/CD154 activation of proinflammatory chemokines and their potential role in renal inflammatory disease were explored in primary cultures of human renal proximal tubule epithelial cells. With the use of a cell fractionation assay, caveolin-1 (Cav-1), the defining structural protein of caveolae, was detected exclusively in the cell membrane (detergent insoluble) component of resting and CD40-activated cells. In the unstimulated condition, CD40 was associated with Cav-1, and with activation of the receptor by its cognate ligand CD154, CD40 dissociated from Cav-1. Other previously identified components of the CD40 signaling pathway, namely, SAPK/JNK, p38, and ERK1/2 MAPKs, but not tumor necrosis factor receptor-associated factor 6 (TRAF-6), were also present within caveolae and dissociated from this structure with ligation of the CD40 receptor. Disruption of caveolae with fosphin diminished CD40-mediated MAPK activation and blunted downstream monocyte chemoattractant protein-1 (MCP-1) and IL-8 production. Similarly, dislodging of signaling proteins from their scaffolding with a peptide targeted to the Cav-1 scaffolding domain (CSD) resulted in blunted MAPK activation and augmented IL-8 and MCP-1 production. In contrast, epidermal growth factor (EGF)-mediated tyrosine phosphorylation of the EGF receptor and activation of ERK1/2 were not interrupted by the peptide. We conclude that in human renal proximal tubule epithelial cells, CD40 and its downstream MAPK signaling proteins are located in membrane rafts and that disruption of caveolae or dislodgment of signaling proteins from the CSD diminishes MAPK activation and IL-8 and MCP-1 production in these cells.

caveolin-1 scaffolding domain; mitogen-activated protein kinases; monocyte chemoattractant protein-1; interleukin-8; interstitial inflammation

MATERIALS AND METHODS

Cell culture. All experiments were carried out on primary cultures of human PTCs. Normal renal tissue was obtained from nephrectomy specimens during the course of cancer surgery (2), and verification of proximal tubule origin was performed as previously described (13). 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), triodothyronine (5 μM), and sodium selenate (10 nM), and to which 50 μU/ml penicillin and 50 μg/ml streptomycin were added. Cells were usually grown in 25-cm² tissue culture flasks but occasionally in 100-cm² tissue culture flasks (cell fractionation) 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 passages 8-10.

Cell fractionation. In experiments where detergent-soluble and detergent-insoluble fractions were required, vehicle and CD154-stimulated
ulated cells [recombinant human soluble (rhs) CD154 100 ng/ml + 1 μg/ml enhancer] were lysed in 1% Brij 58 (Pierce, Rockford, IL) for 5 min at 4°C as previously described (10). 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 Reagent. Equal aliquots of detergent-soluble and detergent-insoluble material were subjected to SDS-PAGE and immunoblotting as described below.

**Immunoprecipitation and immunoblotting.** Immunoprecipitation and Western blot analysis were performed using protocols previously described by this laboratory (1, 10). Unless otherwise stated, monolayers were challenged with rhs CD154 or vehicle (control) for the time periods indicated, washed extensively with PBS (4°C), and then lysed in a previously described buffer. 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 143 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 three times with TBS containing 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×), antibody-bound proteins were detected using an enhanced chemiluminescence (ECL) system according to the manufacturer’s instructions (Pierce), and 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 Analyist PC software program (Bio-Rad, Richmond, CA). Antibodies were obtained from commercial sources and are detailed in Materials.

Immunoblotting was performed as previously described (1). Briefly, following extensive washes with PBS (4°C), cells were lysed in 100 μl of SDS sample buffer (1), 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 match aliquots were subjected to SDS-PAGE in 10% gels as described above. Immunoblotting was performed as outlined earlier.

**Immunocytochemistry and confocal microscopy.** Immunocytochemical staining of cultured proximal tubule cells for CD40 and Cav-1 was carried out on 3.7% parafomaldehyde-fixed cells permeabilized with 0.1% Triton X-100 as previously described by this laboratory (1, 2, 10). Optimal dilution 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 secondary antibody was determined by omitting the primary antibody (vehicle only) from the initial incubate. An irrelevant antibody ensured specificity. In double-labeled experiments, the rabbit anti-human anti-CD40 polyclonal primary antibody was detected by antirabbit IgG-labeled Alexa Fluor 594 (red), and the anti-Cav-1 monoclonal primary antibody was detected by anti-mouse IgG-labeled Alexa Fluor 488 (green). Alexa Fluor 594 and Alexa Fluor 488 (Molecular Probes, Eugene, OR) were detected by fluorescent confocal microscopy (Bio-Rad Radrane 2000), using a Nikon Eclipse E800, 60 × 1.4 numerical aperture oil-immersion objective lens, the appropriate excitation wavelength, a dichroic mirror, and a barrier filter. Images were captured with a digital camera and stored for further processing and evaluation. All studies were blinded.

**Detergent-free purification of caveolin-rich membrane fragments.** Vehicle-treated or rhs CD154 (100 ng/ml + 1 μg/ml enhancer, 10 min)-challenged monolayers were washed (2×) with ice-cold PBS, scraped, and suspended into 0.85 ml of 0.5 M sodium carbonate, pH 11.0 (20). Lysates from five 100-cm² tissue culture flasks were pooled for each experimental point. All subsequent steps were carried out at 4°C. Cell membranes were disrupted with a loose-fitting Dounce homogenizer (10 strokes) followed by three 10 s bursts with a Polytron microtissue grinder and three 20 s bursts with a sonicator to further homogenize the samples. Homogenates were adjusted to 40% sucrose by adding an equal volume of 80% sucrose prepared in Mes-buffered saline (MBS; 25 mM Mes, pH 6.5, and 0.15 M NaCl) and placed on the bottom of an ultracentrifugation tube. A 5 to 35% discontinuous sucrose gradient was formed above the sample by using 1 ml of 5% sucrose and 1.3 ml of 35% sucrose, both in MBS containing 250 mM sodium carbonate. Ultracentrifugation proceeded for 18 h at 50,000 rpm using a model SW65 rotor (Beckman Instruments, Palo Alto, CA). For profiles, 0.3-ml fractions starting at the top of each gradient were collected to yield a total of 13 fractions. Twenty microliters of each fraction were dissolved in equal volumes of 2× SDS sample buffer for Western blot analysis. In separate experiments, the light-scattering band and 35% sucrose-containing band (A5, which contains caveolae but excludes most other cellular structures, was diluted threefold with MBS and centrifuged at 30,000 rpm for 1 h in the above mentioned rotor to pellet caveolae. The caveolae were then solubilized with SDS sample buffer and subjected to Western blot analysis as described.

**ELISA.** MCP-1 and IL-8 production by proximal tubule cells were measured by the quantitative sandwich enzyme immunoassay technique according to the manufacturer’s instructions (Quantikine, R&D Systems, Minneapolis, MN) and as previously reported by this laboratory (10). Proximal tubule cells were grown in 24-well culture plates and the experimental maneuver was performed by adding agonist/vehicle/inhibitor to the supernatant, which was assayed for chemokine production. Optical density of each microtiter plate was read at 450 nm on a Biomed microplate reader. Recombinant MCP-1 and IL-8 standards from 0 to 2,000 pg were used to generate standard curves. Quadruplicate determinations in three to four different series of experiments were performed. Data are presented as means ± SE as the index of dispersion. Analysis of variance was used to compare group means, and the null hypothesis was rejected when P < 0.05.

**Peptide synthesis and introduction into cells.** A peptide corresponding to the scaffolding domain of Cav-1 (amino acids 82–101: DGWKAASFTTFVTKYWYFYR; accession number: Q03135) was synthesized as a fusion peptide to the COOH terminus of the Drosophila antennapedia homeodomain-derived carrier peptide (RQIKIWFQNRRMKWKK) (purification >95%) by Biosynthesis (Lewisville, TX). Biotin was linked to the peptide through an aminohexanoic acid spacer at the NH₂ terminus. Confluent monolayers grown on 25-cm² tissue culture flasks or 24-well plates were incubated with 1, 10, or 100 μM Cav-1 peptide for 6 h before stimulation with vehicle, CD154 (100 ng/ml + 1 μg/ml enhancer), or epidermal growth factor (EGF; 100 ng/ml, 10 min). Lysates were subjected to SDS-PAGE and immunoblotted with the phospho or nonphospho antibody as described. MCP-1 and IL-8 production were measured by ELISA. To validate internalization of the peptide by proximal tubule cells, monolayers grown on glass coverslips were incubated with 1, 10, or 100 μM Cav-1 peptide for 6 h. Cell fixation and permeabilization proceeded as described in Immunocytochemistry and confocal microscopy. Cells were subsequently incubated with streptavidin-fluorescein following blocking by 10% goat serum in PBS. Internalization of the peptide was detected by fluorescent microscopy.

**Materials.** DMEM, Ham’s F-12 medium, RPMI 1640, and the penicillin-streptomycin solution were purchased from GIBCO Laboratories (Grand Island, NY) and newborn calf serum was from Sigma.
Expression of Cav-1 by human proximal tubule cells. Cav-1 expression by primary cultures of human proximal tubule cells was first examined in detergent-soluble and detergent-insoluble cell fractions using Western blot analysis. It was deemed important to take the approach of cell fractions because we recently demonstrated that the activated CD40 receptor translocates from the cell membrane (detergent insoluble) to the cytosolic compartment (detergent soluble) (10). Accordingly, quiescent monolayers were either treated with vehicle or challenged with rhs CD154 (100 ng/ml + 1 μg/ml enhancer) for 5 or 10 min as indicated (Fig. 1) and then lysed with a buffer that contained 1% Brij 58 as detailed under MATERIALS AND METHODS. The insoluble pellet was dissolved in 0.5% SDS and then subjected to SDS-PAGE/Western blot analysis using the anti-Cav-1 antibody described above. The soluble pellet was analyzed independently by SDS-PAGE/Western blot analysis. As depicted in Fig. 1, all the Cav-1 was present in the detergent-insoluble fraction both in unstimulated and stimulated cells. In parallel experiments, immunocytochemical staining of intact monolayers detected Cav-1 primarily in the cell membrane of unchallenged cells (Fig. 2B, left), and the membrane-staining pattern persisted through 7 min of CD154 stimulation. Taken together, these observations suggest that Cav-1 is present in a component of the cell membrane of both stimulated and unstimulated proximal tubule cells.

Association between CD40 and Cav-1 in human proximal tubule cells. An early event in CD40 signaling is engagement by the cytoplasmic domain of the receptor of TRAF proteins (7, 9, 10, 15). We recently demonstrated that CD40 and TRAF6 reside in separate detergent-insoluble membrane microdomains, or rafts, and on activation of CD40 by its cognate ligand, the two proteins associate with one another probably via zinc finger domains in the soluble or cytoplasmic compartment (10). To explore whether Cav-1 is involved in CD40 signaling in human proximal tubule cells, coimmunoprecipitation experiments were performed where CD40 immunoprecipitates were subjected to Western blot analysis with an anti-Cav-1 antibody under unstimulated and rhs CD154-activated (100 ng/ml + 1 μg/ml enhancer) conditions. The results are depicted in Fig. 2A. In the unstimulated condition, CD40 coimmunoprecipitated with Cav-1, but at 5 and 10 min following CD154 stimulation, Cav-1 could barely be detected in CD40 immunoprecipitates. In contrast, Cav-1 coimmunoprecipitated weakly with TRAF6 in the unstimulated state, but the association between Cav-1 and TRAF6 was enhanced as a function of time following CD154 stimulation. These data suggest that under resting conditions CD40 is located within caveolae, and with activation the receptor rapidly dissociates from the scaffold protein, Cav-1. Cav-1, in turn, associates more strongly with TRAF6 following CD40 activation. The consequences of these interactions will be analyzed in further detail.

In parallel experiments, quiescent monolayers were incubated with rhs CD154 (100 ng/ml + 1 μg/ml enhancer) for the time periods detailed in Fig. 2B, and the Alexa Fluor-tagged Cav-1 and CD40 antibodies were tracked by confocal microscopy. As indicated earlier, under unstimulated (baseline) conditions the anti-Cav-1 antibody tagged by Alexa Fluor 488 (green) was localized primarily to the cell membrane and cytoplasm immediately adjacent to the cell membrane, and the anti-CD40 antibody tagged by Alexa Fluor 594 (red) was localized primarily to the cell membrane as previously reported by this laboratory (10). Colocalization of these two proteins as depicted in the overlay photomicrograph (colocalization in yellow) is evident in the unstimulated or baseline condition. In rhs CD154-stimulated cells (2 min), the anti-Cav-1-tagged Alexa Fluor 488 (green) antibody could still be detected in the cell membrane with some cytosolic distribution. This pattern persisted through 7 min with increased nonspecific localization to the cell nucleus. The anti-CD40-tagged Alexa Fluor 594 (red) antibody was less evident in the cell membrane and distributed throughout the cell as previously reported (10). Of greater significance is the observation that at 2 min poststimulation, colocalization (yellow) of these two proteins markedly diminished and could barely be detected at 7 min (Fig. 2B, right), in keeping with the coimmunoprecipitation experiments described above. These observations argue that in unstimulated human proximal tubule cells, the CD40 receptor is configured in caveolae.

Evaluation of the role of Cav-1 in CD40 signaling in human proximal tubule cells. To explore whether caveolae form a scaffolding for components of the CD40 signal transduction pathways previously described in proximal tubule cells (10), we examined the subcellular localization of Cav-1, a major structural protein in caveolae, in further detail, using the sucrose gradient method (20). Equal aliquots from each of 13 sucrose gradients were subjected to SDS-PAGE/Western blot analysis using the anti-Cav-1 antibody described above. In the unstimulated condition, Cav-1 was detected in fractions 4 and
Fig. 2. Association between CD40 and Cav-1 in human proximal tubule cells. A: coimmunoprecipitation of CD40 and Cav-1 (top) and Cav-1 and tumor necrosis factor receptor-activated factor 6 (TRAF6) (bottom). Monolayers were either challenged with rhs CD154 (100 ng/ml + 1 μg/ml enhancer) for 5 or 10 min as indicated or left untreated (0 min). Lysates were subjected to immunoprecipitation (IP) using the anti-CD40 or anti-Cav-1 antibody as shown, and following SDS-PAGE they were immunoblotted (IB) with either the anti-Cav-1 or TRAF6 antibody as delineated. B: confocal photomicrographs of rhs CD154-challenged cells. Cells were examined before (baseline) and 2 and 7 min after challenge with rhs CD154 (100 ng/ml + 1 μg/ml enhancer). The rabbit anti-human anti-CD40 polyclonal primary antibody was tagged by an anti-rabbit IgG-labeled Alexa Fluor 594 (red) and the anti-Cav-1 monoclonal primary antibody was tagged by an anti-mouse IgG-labeled Alexa Fluor 488 (green). Colocalization (overlay) is shown in yellow. One of 3 to 4 similar experiments is shown.

If CD40 and Cav-1 are associated in the resting condition and then dissociate from one another following ligation of the receptor, it should be feasible to recapitulate these observations using the sucrose gradient approach. Accordingly, the above-described blots were stripped and reprobed with an anti-CD40 antibody. In the unstimulated condition, CD40 was detected in fractions 3 through 13 with the major bands detected in fractions 3-5 (Fig. 3, third row). After rhs CD154 treatment (100 ng/ml + 1 μg/ml enhancer, 10 min), most of the CD40 protein was detected in fractions 12 and 13, with marked reduction in the amount of protein observed in fractions 3-5 and 9-12, with virtual disappearance from fractions 6-8 (Fig. 3, bottom row). These observations are in keeping with the

5 and 8-13 (Fig. 3, top row). After rhs CD154 treatment (100 ng/ml + 1 μg/ml enhancer, 10 min), increased amounts of Cav-1 were detected in fractions 4 and 5, and also appeared in fractions 6 and 7 (Fig. 3, second row). Differences in Cav-1 content of fractions 8-13 were difficult to quantify because of the large amount of Cav-1 detected in these fractions. The key observations are 1) that Cav-1 was detected in the lower-density fractions (4 and 5) as described by others (20), and 2) that following activation of CD40 by its cognate ligand, increased amounts of Cav-1 were detected in fractions 4 and 5. These findings are indicative of the presence of Cav-1 in lipid-associated membrane fragments (4 and 5), a characteristic feature of caveolae (20).
concept that the CD40 receptor is configured in caveolae in unstimulated proximal tubule cells and dissociates from this structure with ligation.

To examine whether other components of the CD40 signaling pathway previously identified by this laboratory are also present in the lipid-associated membrane fragments, fragments 4 and 5 of the cell fractionation assay described above were pooled and subjected to SDS-PAGE/Western blot analysis using different specific antibodies. As expected, rhs CD154 stimulation (100 ng/ml + 1 μg/ml enhancer, 10 min) increased Cav-1 abundance in the delineated fractions (Fig. 4A). TRAF6 could not be detected in these cell fractions under both unstimulated and stimulated conditions (Fig. 4A). TRAF2 abundance, on the other hand, decreased following CD154 stimulation (Fig. 4A). Under identical experimental conditions, SAPK/JNK, p38, and ERK1/2 MAPK abundance decreased somewhat in the lipid-associated membrane fragments following CD40 ligation (Fig. 4B). In the aggregate, these data argue that in addition to CD40, TRAF2, TRAF6, SAPK/JNK, p38, and ERK1/2 MAPK are all present in caveolae in the unstimulated condition in this cell type. As described previously, TRAF6 resides in a different cell fraction (10).

Functional relevance of caveolae in CD40-associated signaling in human proximal tubule cells. If the association of Cav-1 with CD40 has functional relevance with regard to MAPK activation, then disruption of caveolae should blunt or diminish CD40-mediated SAPK/JNK, p38, and ERK1/2 MAPK activation. A number of different compounds have been used to disrupt caveolae, of which filipin is possibly the most widely used (17). Accordingly, monolayers were challenged with rhs CD154 (100 ng/ml + 1 μg/ml enhancer, 10 min) and MAPK activity was monitored using the appropriate phospho-activated antibody (Fig. 5A). As previously reported (10), CD40 ligation stimulates SAPK/JNK, p38, and ERK1/2 MAPK activity in this cell type (lane 1 vs. 2). Filipin (5 μg/ml, 90 min) had no effect on baseline MAPK activity (lane 3). Filipin totally blocked CD40-mediated SAPK/JNK activity and blunted p38 and to a lesser extent ERK1/2 activity (lane 4). A non-phospho-p38 immunoblot is included as a control, which shows that equal quantities of protein were loaded per lane and that the effect of filipin was specific. Because CD40-mediated MCP-1 and IL-8 production are downstream events of MAPK activation in this cell type (10), disruption of caveolae should also blunt or diminish CD40-mediated chemokine production. CD40 ligation (rhs CD154 100 ng/ml + 1 μg/ml enhancer, 24 h) stimulated MCP-1 production from a baseline value of 8,814 ± 239 to 17,689 ± 360 pg/ml (n = 3, P < 0.001), and filipin (5 μg/ml, 90 min) partially blunted the response to 14,090 ± 244 pg/ml (n = 3, P < 0.05 vs. stimulated value; Fig. 5B). Filipin itself was without effect in vehicle-challenged monolayers; 9,395 ± 536 pg/ml (n = 3, not significant vs. baseline value; Fig. 5B). Under similar experimental conditions (Fig. 5C), rhs CD154 increased IL-8 production from a baseline value of 2,873 to 7,660 ± 416 pg/ml (n = 3, P < 0.001), and filipin blunted the response to 4,981 ± 640 pg/ml (n = 3, P < 0.05 vs. stimulated value). Unchallenged, inhibitor-treated cells had values similar to the baseline value: 3,768 ± 115 pg/ml (n = 3, not significant vs. baseline value). These observations argue that intact, functional caveolae are a prerequisite for CD40-mediated chemokine production that proceeds via SAPK/JNK, p38, and ERK1/2 activation in rhs CD154-stimulated proximal tubule cells.

In a second approach, a peptide corresponding to the scaffolding domain of Cav-1 (fused to a carrier peptide) was introduced into cells as detailed in MATERIALS AND METHODS. We rationalized that the peptide would dislodge the relevant signaling proteins from caveolae and thereby disrupt their function. Accordingly, monolayers were incubated with vehicle or different concentrations of peptide (1, 10, and 100 μM) for 6 h and subsequently challenged with vehicle or rhs CD154 (100 ng/ml + 1 μg/ml enhancer) for 30 min. Figure 6A confirms that the peptide is indeed internalized by the monolayer with fluorescein visualized in ~80–90% of cells. MAPK activity was monitored using the appropriate phospho-activated antibody (Fig. 6B). A non-phospho-p38 immunoblot is included as a control, which shows that equal quantities of protein were loaded per lane and that the effect of the peptide was specific. As previously demonstrated, CD40 ligation stimulates SAPK/JNK, p38, and ERK1/2 MAPK activity (lane 1 vs. 2), and 10 μM Cav-1 peptide has little effect on baseline MAPK activity (lane 3). One hundred-micromolar Cav-1 peptide totally abrogated CD40-mediated SAPK/JNK activity and blunted p38 and to a lesser extent ERK1/2 activity (lane 6). One-micromolar Cav-1 peptide was essentially without effect (lane 5), whereas 10 μM Cav-1 peptide had a somewhat intermediary effect (lane 4). MCP-1 (Fig. 6C) and IL-8 (Fig. 6D) production were measured under similar experimental conditions. CD40 ligation (rhs CD154 100 ng/ml + 1 μg/ml enhancer, 24 h)
stimulated MCP-1 production from a baseline value of $8,098 \pm 107$ to $16,286 \pm 380$ pg/ml ($n = 3, P < 0.001$), and Cav-1 peptide (100 μM, 6 h) blunted the response to $9,961 \pm 762$ pg/ml ($n = 3, P < 0.05$ vs. stimulated value; Fig. 6C). Cav-1 peptide itself was without effect in vehicle-challenged monolayers: $9,857 \pm 536$ pg/ml ($n = 3$, not significant vs. baseline value). rhs CD154 increased IL-8 production from a baseline value of $7,209 \pm 368$ to $12,460 \pm 122$ pg/ml ($n = 3, P < 0.001$), and Cav-1 peptide blunted the response to $7,004 \pm 325$ pg/ml ($n = 3$, $P < 0.05$ vs. stimulated value).

Fig. 5. Inhibition of CD40-evoked SAPK/JNK, p38, and ERK1/2 activation, and monocyte chemoattractant protein-1 (MCP-1) and IL-8 production by filipin. Monolayers were incubated with vehicle or filipin (5 μg/ml, 90 min) as indicated, before challenge with vehicle or ligand (rhs CD154 100 ng/ml + 1 μg/ml enhancer). A: cell lysates were subjected to SDS-PAGE and immunoblotted with the phospho or nonphospho antibody as shown. B: MCP-1 measured by ELISA. C: IL-8 production measured by ELISA. One of 3 similar experiments for each assay is shown.

Fig. 6. Peptide (pep) targeting of Cav-1. Confluent monolayers were incubated for 6 h with vehicle or 1, 10, or 100 μM peptide corresponding to the scaffolding domain of Cav-1 detailed in MATERIALS AND METHODS. Monolayers were subsequently challenged with vehicle or rhs CD154 (100 ng/ml + 1 μg/ml enhancer). A: photomicrograph of internalized Cav-1 peptide (10 μM) detected with streptavidin-fluorescein as detailed in MATERIALS AND METHODS. B: cell lysates were subjected to SDS-PAGE and immunoblotted with the phospho or nonphospho antibody as indicated. C: MCP-1 measured by ELISA. D: IL-8 production measured by ELISA. One of 3 similar experiments for each assay is shown.
CD40 signaling in human proximal tubule cells. Evidence that functional caveolae are a prerequisite for normal cell function.

The effect of peptide introduction might have on any nonspecific or an anti-phospho-ERK1/2 antibody (A). p38 Immunoblots were included for SDS-PAGE and immunoblotted with an anti-phospho-EGF receptor antibody (B). EGF was examined in parallel experiments. The results are shown in Fig. 7A. EGF (100 ng/ml, 10 min) markedly increased EGFR tyrosine phosphorylation (lane 1 vs. 2), and Cav-1 peptide (1, 10, and 100 μM for 6 h) had no effect on this response. A non-phospho-p38 immunoblot is included as control, which confirms that equal quantities of protein were loaded per lane. Furthermore, EGF-mediated ERK1/2 activation was not blunted by the presence of the peptide (Fig. 7B). These data provide specificity to the effect of the peptide on CD40-mediated MAPK signaling (see DISCUSSION) and negate any nonspecific effect that peptide introduction might have on cell function.

DISCUSSION

The results presented demonstrate that Cav-1 can be detected exclusively in the cell membrane (detergent insoluble) component of resting and CD40-activated human proximal tubule cells (Fig. 1) and that under unstimulated conditions CD40 is located within caveolae (Figs. 2–4). With stimulation by its cognate ligand CD154, CD40 dissociates from Cav-1 (Figs. 2 and 3). In addition, CD40, together with other key components of the CD40 signaling pathway (SAPK/JNK, p38, ERK1/2) but not TRAF6, is present in caveolae (Fig. 4). Disruption of caveolae by filipin (Fig. 5) or dislodgment of signaling proteins from their scaffolding (Fig. 6) results in diminished CD40-mediated MAPK activation and MCP-1 and IL-8 production, arguing that there is functional relevance to intact caveolae with regard to CD40-associated downstream events. Finally, dislodgment of CD40-mediated signaling proteins from their scaffolding is specific because EGF-mediated EGFR phosphorylation and ERK1/2 activation proceed uninterrupted in the presence of the peptide (Fig. 7).

The detection of Cav-1 in lipid-associated low-density membrane fragments is indicative of the presence of caveolae (20). With the use of the sucrose gradient approach described earlier, Cav-1 was detected in fractions 4 and 5 in the unstimulated condition with increased amounts detected following CD40 ligation (Fig. 3). Interestingly, Cav-1 was also detected in large amounts in high-density lipid-insoluble fragments under both conditions. This observation indicates that human PTCs possess large amounts of Cav-1 and that not all Cav-1 is associated with lipid-rich microdomains or rafts. The relevance of this observation will require further work in the future.

To the best of our knowledge, this is the first report demonstrating involvement of caveolae in CD40 signaling in human renal epithelial cells. Indeed, there is a paucity of and somewhat conflicting data implicating lipid rafts in CD40 signaling in other cell types. For example, membrane rafts have been shown to play an integral role in the proximal events of CD40 signaling in human monocyte-derived dendritic cells (24). On the other hand, CD40 is excluded from the lipid rafts of both mature and immature B cells (11). Of much interest is a recent publication implicating the transmembranous domain of the CD40 receptor as being crucial both for clustering into lipid rafts and effective CD40 signaling (3). Because CD45 is known to be excluded from lipid rafts, the investigators constructed a CD40/CD45 chimera. Normal ligand binding was confirmed by flow cytometry. Chimeric CD40/CD45 receptors transfected into T cells failed to cluster and were unable to activate MAPK signaling pathways in contradistinction to the

![Fig. 7. Specificity of effect of peptide targeting of Cav-1.](Image)

![Fig. 8. Schema of proposed involvement of caveolae in CD40 signaling in human renal proximal tubule epithelial cells.](Image)
wild-type CD40 receptor. Implicit in this observation, although not stated, is that caveolae were involved. Our approach differed somewhat in that we introduced a peptide corresponding to the scaffolding domain of Cav-1 (5, 14) known to recognize a diverse group of signal transducer and demonstrate that MAPK activation and downstream IL-8 and MCP-1 production were either obviated or blunted (Fig. 6). Based on these observations, it seems reasonable to conclude that lipid rafts of the caveolae variety are a prerequisite for normal CD40 signaling in human PTCs.

It might be argued that introduction of the peptide targeting the scaffolding domain of Cav-1 may have a nonspecific effect on cell function. Several lines of evidence would suggest that this indeed is not the case. First, identical amounts of p38 MAPK were detected in all specimens (Figs. 6B and 7). Second, MAPK activities were blunted in a concentration-dependent manner (Fig. 6B). Third, EGF-mediated tyrosine phosphorylation of the EGFR and ERK1/2 proceeded independently of the concentration of peptide introduced into the cell (Fig. 7, A and B). In this regard, it has been demonstrated that the EGFR is localized within caveolae along with its downstream signaling molecules (5, 12, 19). Interestingly, in mesangial cells, endothelin-1 (ET-1)-mediated ERK1/2 transactivation via the EGFR was disrupted by cholesterol depletion using filipin and β-cyclodextrin yet EGF-mediated tyrosine phosphorylation of the EGFR was not impacted (8). In addition, the peptide targeting the Cav-1 scaffolding domain diminished ET-1 transactivation of ERK1/2. EGF-induced tyrosine phosphorylation of the EGFR in this setting was not reported. Along similar lines, in vascular smooth muscle cells, cholesterol depletion significantly inhibited ANG II-mediated EGFR tyrosine phosphorylation and protein kinase B (PKB)/Akt transactivation via the EGFR, yet the identical maneuver had no impact on EGF-mediated EGFR tyrosine phosphorylation and PKB/Akt transactivation, leading the authors to conclude that transactivated EGFRs localize in focal adhesions (22). Whether EGFRs are located in focal adhesions in PTCs is currently not known, but it was beyond the scope of this investigation.

Although key CD40 signaling proteins including SAPK/JNK, p38, and ERK1/2 MAPK appear to be present in caveolae in this cell type and dissociate from this structure with ligation of the receptor (Fig. 4), TRAF6 does not appear to be present in this membrane component in both the unchallenged and CD40-stimulated condition. As a control, we examined another protein of this group, namely, TRAF2, which has been shown by others to be involved in CD40 signaling (6, 15). Similar to the signaling proteins detailed above, and unlike TRAF6, TRAF2 was detected in unchallenged cells in the membrane fragments containing caveolae and dissociated from this fragment with ligation of the receptor (Fig. 4). We have not yet examined in further detail the consequence of CD40 engagement of TRAF2 in human proximal tubule cells. However, the observation that TRAF6 could not be identified in the cell fraction containing caveolae, yet is a necessary prerequisite for the activation of SAPK/JNK and p38 MAPK in this cell model (10), is in keeping with our previous observation, namely, that CD40 and TRAF6 reside in separate membrane microdomains and on activation translocate and associate with one another in the cytoplasmic compartment (10).

Figure 8 is a proposed schema for the involvement of caveolae in CD40 signaling in human renal PTCs and summarizes our observations in this regard. CD40 together with SAPK/JNK, p38, and ERK1/2 MAPKs are anchored to Cav-1 in the unstimulated condition. TRAF6 is not located in caveolae but as previously shown (10) resides in a separate membrane microdomain. With ligation of the receptor, CD40 engages TRAF6, SAPK/JNK, p38, and ERK1/2 MAPKs dissociate from Cav-1. TRAF6 stimulates SAPK/JNK and p38 MAPK activities but is not involved in ERK1/2 MAPK activation (previously shown in Ref. 10). Cav-1 and TRAF6 associate with one another, presumably for recycling to their respective membrane microdomains.

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