Targeting of Tumor Necrosis Factor Receptor 1 to Low Density Plasma Membrane Domains in Human Endothelial Cells

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TNF-R1 (tumor necrosis factor receptor 1) localizes to caveolae of human endothelial-derived EA.hy926 cells. Transduced TNF-R1 molecules lacking amino acid residues 229–244 (spanning the transmembrane/intercellular boundary) are expressed on the cell surface equivalently to full-length TNF-R1 molecules but incompletely localize to caveolae. A peptide containing this sequence pulls down CAV-1 (caveolin-1) and TNFR1 from cell lysates but fails to do so following disruption of caveolae with methyl-β-cyclodextrin. We previously reported that methyl-β-cyclodextrin eliminates caveolae and blocks tumor necrosis factor (TNF)-induced internalization of TNF-R1 but not TNF-induced activation of NF-κB in EA.hy926 cells. Both CAV-1 and FLOT-2 (flotillin-2), organizing proteins of caveolae and lipid rafts, respectively, associate with caveolae in EA.hy926 cells. Small interfering RNA-mediated knockdown of CAV-1 but not FLOT-2 strikingly reduces caveolae number. Both knockdowns reduce total TNF-R1 protein expression, but neither prevents TNF-R1 localization to low density membrane domains, TNF-induced internalization of TNF-R1, or NF-κB activation by TNF.

Both CAV-1 and FLOT-2 knockdowns reduce TNF-mediated activation of stress-activated protein kinase (SAPK). However, both knockdowns reduce expression of TRAF2 (TNF receptor-associated factor-2) protein, and small interfering RNA targeting of TRAF2 also selectively inhibits SAPK activation. We conclude that TNF-R1 contains a membrane-proximal sequence that targets the receptor to caveolae/lipid rafts. Neither TNF-R1 targeting to nor internalization from these low density membrane domains depends upon CAV-1 or FLOT-2. Furthermore, both NF-κB and SAPK activation appear independent of both TNF-R1 localization to low density membrane domains and to TNF-induced receptor internalization.

Human endothelial cells (EC) are a primary target of tumor necrosis factor (TNF) action (1), and proinflammatory EC responses are predominantly mediated through TNF-R1 (CD120a) (2, 3). Ligand binding to this receptor on the plasma membrane initiates a sequence of responses characterized by early gene transcription and delayed caspase-8 activation (4). TNF-R1 typically initiates transcriptional responses through the rapid activation of nuclear factor-κB (NF-κB) (in 10–15 min) and/or AP-1 (activation protein-1) (in 15–30 min), the latter response being mediated by stress-activated protein kinase (SAPK); also known as c-Jun N-terminal kinase (JNK)-mediated phosphorylation of the c-Jun subunit of AP-1 heterodimers (5, 6). TNF-R1 lacks intrinsic enzymatic activity, and downstream signaling through these pathways depends upon sequential recruitment of cytosolic adaptor proteins, specifically the scaffolding protein TRADD (TNF-associated via death domain protein), the E3 ubiquitin ligase TRAF2 (TNF-associated factor-2), and RIP-1 (serine/threonine kinase receptor interacting protein-1; also known as RIPK-1) (6, 7), forming a “signalosome” complex. Once assembled, the TNF-R1 signalosome initially interacts with and activates certain mitogen-activated kinase kinase kinase (MAP3K) enzymes, such as MEKK3 or TAK1, which, in turn, activate the inhibitor of κB kinase (IKK) complex to initiate the canonical NF-κB pathway (8, 9). Studies with cells derived from RIP-1 knock-out (7) or TRAF-2 knock-out mice (10) have pointed to a primary role for RIP-1 in this response. The subsequent recruitment of AIP-1 (ASK-1 (apoptosis signaling kinase-1)-interacting protein) to the signalosome leads to displacement of NF-κB-activating MAP3K enzymes by another MAP3K, probably ASK-1, that initiates SAPK activation instead of NF-κB (11). The steps controlling the transition from an NF-κB-activating signalosome to a SAPK-activating signalosome are poorly understood, but, based upon studies with cells from knock-out mice, TRAF2 seems particularly important in this switch (10).

In parallel with changes in biochemical composition, TNF-R1-initiated signalosomes traffic among various cellular compartments. TNF-TNF-R1 complexes are initially formed on...
the plasma membrane and are internalized within minutes of ligand binding (12, 13). TRADD and its associated adaptor proteins dissociate from the receptor in a similar time frame. We (14) and others (15) have found that inhibitors of plasma membrane protein internalization can block TNF signaling and target gene transcription. In human EC, most TNFR1 is confined to the Golgi (16), and the small fraction of TNFR1 that is expressed on the plasma membrane in cultured human EC is associated with cholesterol and sphingolipid-rich low density membrane microdomains, commonly called lipid rafts (17). Internalization of proteins from isolated lipid rafts has been linked to the intracellular scaffolding protein flotillin 2 (FLOT-2) (18, 19). In situ, the vast majority of cholesterol and sphingolipid-enriched regions in the EC plasma membrane are concentrated within caveolae, which are flask-shaped invaginations of the plasma membrane organized by the cholesterol-binding cytosolic protein CAV-1 (caveolin-1) (20). CAV-1 has been linked to internalization of membrane proteins from caveolae in other cell types (21). Caveolae, more so than dispersed lipid rafts, may serve to co-localize different receptors, facilitating receptor cross-talk (22). Caveolae are rapidly lost when human EC are placed into primary cell culture despite sustained high expression of CAV-1. Upon dissolution of caveolae, lipid rafts appear to disperse over the rest of the plasma membrane. Thus, cultured human EC may not be an ideal system in which to investigate the role(s) played by caveolae in TNFR1 signaling. We recently showed that the human EC-derived line EA.hy926 retains caveolae in culture, affording an opportunity to study the function of this organelle in a human EC system (17). Treatment of these cells with the cholesterol-extracting agent methyl-β-cyclodextrin (MβCD) both eliminated caveolae and prevented ligand-induced internalization of TNFR1. Experiments using MβCD, however, cannot distinguish responses dependent upon dispersed low density membrane domains (i.e. lipid rafts) from those dependent upon caveolae (23, 24). Moreover, this agent has effects upon other cellular structures (25), further limiting interpretation of such experiments. Therefore, we have turned to retroviral transduction, peptide transfection, and small interfering RNA (siRNA) knockdown approaches to study the structural basis and functional significance of TNFR1 localization to caveolae.

In the present study, we have identified a sequence that spans the boundary of the TNFR1 transmembrane and intracellular domains of TNFR1, which is responsible for receptor localization to low density membrane domains in EA.hy926 cells. Unexpectedly, siRNA experiments fail to establish an indispensable role for either CAV-1 or FLOT-2 in receptor localization to such low density domains, receptor internalization from low density membranes, or receptor-initiated signaling. We do show, however, that CAV-1 and FLOT-2 appear to regulate SAPK signaling through control of TRAF2 protein expression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human-derived EA.hy926 EC line (26) (provided by W. Sessa, Yale University (New Haven, CT)) was maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% (v/v) fetal calf serum, 2% (w/v) hypoxanthine/aminopterin/thymidine (Sigma), 200 μM L-glutamine, 100 units/ml penicillin/streptomycin (Invitrogen) at 37 °C in a 5% CO₂ humidified atmosphere. All experiments were performed on cells grown to confluence. Human umbilical vein EC (HUVEC) were isolated and cultured in accordance with protocols approved by the Yale University Human Investigation Committee. HUVEC were isolated from 3–5 umbilical veins, pooled, and serially cultured as described previously (27). HUVEC cultures were used at passage level 3.

**Reagents and Antibodies**—Recombinant human TNF (TNF-α) and goat anti-TNFFR1 were purchased from R&D Systems Inc. (Minneapolis, MN). Mouse anti-TNFFR1 (H5), rabbit anti-human IkBα, and RIP-1 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-human TNFR1-B1 employed for cytotoxicity analysis, mouse monoclonal anti-CAV-1, rabbit anti-caveolin, and TRAF2, TRADD, LAMP-1, and EEA1 antibodies were from BD Transduction Laboratories (Lexington, KY). Rabbit anti-iNOS antibody was from Cell Signaling (Beverly, MA). Rabbit anti-TNFFR1 and horseradish peroxidase-conjugated mouse anti-FLAG antibodies were from Sigma. Horseradish peroxidase-conjugated secondary antibodies and anti-mouse R-phycocerythrin were from Jackson ImmunoResearch Laboratories (West Grove, PA). Enhanced chemiluminescence used in immunoblotting was from Pierce. Oligofectamine and mouse anti-Golgin 97 were purchased from Invitrogen. MβCD and all other chemicals were purchased from Sigma.

**DNA Constructs and Retroviral Transduction**—The cDNA to mature human TNFR1 (GenBank™ accession number NM_001065) along with an N-terminal eight-amino acid FLAG epitope (DYKDDDK) and signal peptide (obtained from a pRX3 FLAG expression vector generously provided by Dr. P. Yurchenco, Robert Wood Johnson Medical School (Piscataway, NJ)) were first joined by PCR and then inserted into the BamHI and SalI cloning site of the retroviral expression vector pBabe.puro. In order to prevent triggering apoptosis of transduced cells, a previously described inactivating mutational substitution (28) was made inside the TNFR1 death domain (L380A, numbered from the first methionine residue of the TNFR1 open reading frame) using the Stratagene QuikChange II XL mutagenesis kit. The resulting FLAG-tagged pBabe.puro.TNFFR1L380A construct was called full-length (FL) in order to distinguish it from three other FLAG-tagged TNFR1 constructs that were produced by deleting cDNA from nucleotide region 685–732 of the TNFR1. These were named Δ229–244, Δ234–243, and Δ236–239 (UniProtKB/Swiss-Prot accession number P19438), corresponding to amino acid residues deleted from the transmembrane and cytoplasmic regions of TNFR1 (Table 1). The same FLAG-tagged TNFR1 sequences were also inserted into a different retroviral expression vector, pBMN.neo (used in Fig. 1, C and D), generating essentially the same patterns of expression.

**Peptide Synthesis and Detection in Live Cells by Indirect Immunofluorescence**—Peptides used in this work were either synthesized in the Keck Biotechnology Facility (Yale University) or purchased from Anaspec (Anaspec Inc., San Jose, CA). Peptides used in this work consist of three different domains: 1) a peptide corresponding to the transducing sequence (region 47–57) of the HIV-1 protein TAT (YGRKKRRQRRR), allowing...
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intracellular delivery; 2) the TNFR1 domain corresponding to amino acid residues 229–244, FIGLMRYRYQRWKSLKLY (TNFR1(229–244)), or its scrambled version, LIGKYYMKSRWQLRM (TNFR1(229–244SC)); and 3) a spacer domain (GGG) consisting of three glycine residues separating the two domains mentioned above. All peptides were also N terminus-tagged with biotin. For immunofluorescence studies, EA.hy926 cells were incubated with TAT-TNFR1(229–244) peptide or with TAT sequence alone in serum-free medium supplemented with 1% bovine serum albumin for 30 min at 37 °C. Cells were fixed with 2% paraformaldehyde and incubated with AlexaFluor 488 anti-biotin antibody (Invitrogen), and peptide localization was digitally captured with a Zeiss Axiovert 200M fluorescence microscope.

Design and Transfection of CAV-1 siRNA Duplexes—Small interfering RNA duplex oligonucleotides against the coding sequence of human CAV-1 cDNA (NM_001753) synthesized by Integrated DNA Technologies (Coralville, IA) were 5′-ACCAGAAGGACACACAGUdTdT-3′ (sense 538), 5′-ACUGUGACGAUAUCUGUdTdT-3′ (sense 542), and 5′-AUACGUAGACUCGGAGGACdT-3′ (sense 545). The small interfering RNA duplex oligonucleotide employed against the coding sequence of human TNFR1 was 5′-CGAGGAUGAGGAGCAGCUAUGGCAUCA-3′ (TNFR1 sense), and that employed against human TRAF2 was 5′-CCACCGCAGUUCCUGAAUUAC-3′ (TRAF2 sense). Transfection of 80 pmol of siRNA in EA.hy926 was carried out by using Oligofectamine (Invitrogen) (0.3%, v/v), according to the manufacturer’s instructions. Fresh medium was added 5 h after transfection, and experiments were conducted 48–72 h after transfection. Lamin A/C and LFA3 siRNA duplexes were used as control siRNAs (from Dharmaco (Lafayette, CO) and Qiagen (Valencia, CA), respectively). Specificity and effectiveness of knockdowns was confirmed by immunoblotting.

Quantitative RT-PCR Analysis—Total RNA was extracted with a guanidinium isothiocyanate-based RNA isolation kit (RNaseasy minikit, Qiagen), according to the manufacturer’s instructions. Total RNA was extracted with a guanidinium isothiocyanate-based RNA isolation kit (RNeasy minikit, Qiagen), according to the manufacturer’s instructions. Fresh medium was added 5 h after transfection, and experiments were conducted 48–72 h after transfection. Lamin A/C and LFA3 siRNA duplexes were used as control siRNAs (from Dharmaco (Lafayette, CO) and Qiagen (Valencia, CA), respectively). Specificity and effectiveness of knockdowns was confirmed by immunoblotting.

Immunoblotting, Immunoprecipitation, and Pull-down Analysis—For immunoblotting experiments, scrape-harvested EA.hy.926 cells were extracted in 150 μl of lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 2% SDS, and 1 mM Pefabloc) for 15 min on ice. Lysates were passed several times through a 29-gauge needle to shear DNA in the samples. Unbroken cells and debris were spun down by centrifugation at 2000 rpm for 10 min at 4 °C. 15 μg containing supernatant was fractionated by SDS-PAGE, transferred electrophoretically to a nitrocellulose membrane (Trans-Blot transfer medium, Bio-Rad), and subjected to immunoblotting with primary antibody followed by incubation with horseradish peroxidase-conjugated secondary antibody. Detection of the bound antibody by enhanced chemiluminescence (Pierce) was performed according to the manufacturer’s instructions. Results were quantified by densitometric analysis using a laser densitometer scanner (Beckman Coulter) and Scion Image application software (Scion Corp., Frederick, MD) or (in Fig. 1D only) with Quantity One software, version 4.6.5 (Bio-Rad). Protein half-life calculations were made by treating replicate cultures in 6-well cluster plates (35-mm diameter wells) with cycloheximide (2 μg/ml) to stop new protein synthesis and then serially harvesting individual wells at predetermined times followed by immunoblotting as described above. Intensities of the bands for specific proteins of interest were normalized to the intensity of the band for β-actin, and decay curves were analyzed by plotting the log of normalized intensity versus time in hours. Immunoprecipitation of FLAG-tagged TNFR1 constructs was performed on protein lysates of transduced EAhy.926 cells with rabbit anti-FLAG polyclonal antibody linked to magnetic beads (NE Biolabs, Beverly, MA). For peptide pull-down analysis, cells were disrupted in lysis buffer (see above) and incubated 18 h at 4 °C in the presence of the wild type (specific) or the scrambled version of the biotinylated TAT-TNFR1(229–244) peptide, and protein complexes were isolated with anti-biotin antibody linked to magnetic beads onto μ columns (Miltenyi Biotech Inc., Auburn, CA). Eluates were collected in gel sample buffer, separated by 12% SDS-PAGE, and stained with SYPRO Ruby Protein Gel Stain (Invitrogen) following the manufacturer’s instructions.

Evaluation of TNFR1 Internalization and FLAG-TNFR1 Surface Expression by FACS Analysis—Replicate cultures of EA.hy926 cells were subjected to transfection with siRNA oligonucleotides against either CAV-1 or the irrelevant oligonucleotide (against lamin A/C) for 72 h in 6-well plates. Individual wells were treated with 10 ng/ml TNF for 30 min, harvested with trypsin, and split into two separate samples stained either with TNFR1-B1 (1 μg/106 cells) or with isotype control antibody, respectively, for 1 h at 4 °C. The conditions used in our experiments do not compete off the binding of the TNFR1-B1 antibody because there is no reduction of surface staining when internalization is blocked (17). Cells were washed twice in HBSS containing 1% bovine serum albumin and incubated with anti-mouse IgG-phycocerythrin for 30 min at 4 °C, washed three times with HBSS and 1% bovine serum albumin, and analyzed with a Beckman FACSsort. Fluorescence of 1.5 × 104 cells/sam-
ple was acquired and analyzed, using CellQuest software, to quantify the surface expression of TNFR1 by calculating the mean fluorescence intensities and subtracting that of the isotype control. Transduced EAhy.926 cells expressing FLAG-tagged TNFR1 were similarly immunostained with rabbit anti-FLAG, followed by donkey anti-rabbit AlexaFluor 488 secondary antibody (Invitrogen), prior to FACS analysis of surface expression.

Isolation of Caveolae-enriched Membranes and Mbcd Treatments—EAhy.926 were transfected with different siRNA as described above and 72 h later were subjected to sucrose gradient fractionation. Purification of caveolae-enriched membrane fractions was performed as described (29) with minor modifications. In brief, cells were washed twice with ice-cold Dulbecco’s phosphate-buffered saline and scraped into 0.15 ml of 25 mm MES hydrate buffer, pH 6.5, containing 0.15 M NaCl, 5 mM EDTA, and 0.5% Triton X-100 (MBS) with protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM Pefabloc) and left 20 min on ice. Three wells of a 6-well plate were pooled together, and the resultant lysate was subjected to 15 strokes in a Dounce homogenizer and centrifuged at 10,000 g for 10 min at 4 °C to remove nuclei. Clarified postnuclear supernatants were combined with 1 ml of 35% and 0.6 ml of 5% sucrose, respectively. The resultant lysate was subjected to 15 strokes in a Dounce homogenizer and centrifuged for 10 min at 100,000 g at 4 °C to remove the membrane fraction. Clarified postnuclear supernatants were combined with 1 ml of 35% and 0.6 ml of 5% sucrose, respectively. The resulting 5–40% discontinuous sucrose gradients were centrifuged for 18 h at 130,000 rpm to separate the low density lipid rafts/caveolae. After centrifugation, 10 fractions of 200 μl each were collected and subjected to SDS-PAGE and immunoblotting as described above.

Ultrastructural Studies—Cells were fixed in 4% formaldehyde in 0.1 M PIPES buffer, pH 7.4, for 1.5 h at 4 °C; washed in three changes of 0.1 M PIPES buffer, pH 7.4; and postfixed in 1% osmium tetroxide in 0.1 M PIPES at pH 7.4 containing 1.5% potassium ferricyanide and 2 mM/L calcium chloride for 1 h at room temperature. Tissues were then washed in 0.1 M PIPES buffer, pH 7.4, for 2 min, followed by 0.05 M sodium maleate buffer, pH 5.2, for 10 min at 20 °C, and then immersed in 2% uranyl acetate in 0.05 M sodium hydrogen maleate buffer for 1 h. After rinsing in deionized water, tissues were dehydrated in an ascending series of ethanol solutions, treated with propylene oxide (two changes of 15 min each), and transferred to a 50:50 mixture of propylene oxide and Spurr’s resin (TAAB Laboratories Ltd., Aldermaston, UK). After an overnight agitation, tissues were treated for 8 h in 25:75 propylene oxide and Spurr’s resin and four changes of 100% Spurr’s resin over 36 h. They were next transferred to latex molds filled with resin and thermally cured at 60 °C for 48 h. Ultrathin sections of about 40–60 nm (showing silver to silver-gray interference colors) were cut using a Reichert Ultracut S and mounted on copper grids. Sections were double-stained with uranyl acetate and lead citrate for 30 s each and examined in a Philips CM100 electron microscope at an accelerating voltage of 80 kV.

Statistical Analysis—Differences between treatment and control groups were analyzed using a two-sample, two-tailed Student’s t test.

RESULTS

A TNFR1 Membrane-proximal Sequence Localizes TNFR1 to Low Density Membrane Domains—We tested the hypothesis that TNFR1 contains a specific amino acid sequence that is responsible for targeting this receptor to low density membrane regions, such as lipid rafts or caveolae. We noted that the transmembrane/intercytoplasmic membrane-proximal region of the receptor, extending from residue 229 to 244, resembles sequences in other proteins that interact with CAV-1 (30). We generated a series of FLAG-tagged constructs of TNFR1 that each included a mutation in the death domain to prevent induction of apoptosis. These constructs consisted of a FL TNFR1 sequence as well as three different deletion mutants removing all or part of the transmembrane/intercytoplasmic membrane-proximal region (Table 1). Each was inserted into a retroviral expression vector and transduced into EA.hy926 cells (described under “Experimental Procedures”). As detected by flow cytometric analysis (Fig. 1A), all four constructs were expressed on the cell surface. The deletion constructs appear to show slightly greater surface expression than the full-length construct, although these surface expression levels are not normalized for total expression. We then fractionated lysates of each transduced cell line on a sucrose density gradient to isolate the low density fractions enriched in CAV-1. Immunoprecipitating pooled low density fractions with anti-FLAG antibody followed by immunoblotting for TNFR1 showed that transduced full-length TNFR1, like the endogenous receptor, localized to low density membranes (Fig. 1B) and to high density Golgi-containing membranes (data not shown). Interestingly, only deletion of the entire membrane-proximal sequence of TNFR1 (residues 229–244) appeared to reduce targeting to low density membranes relative to total expression. To quantify this effect, we performed immunoblot and densitometric analysis on whole cell lysates and individual fractions of a sucrose gradient. The expression of FLAG-TNFR1 Δ229–244 was about

| Construct | No. of residues deleted | Residues deleted | FLAG-TNFR1 sequence (from Val212 to Lys250) |
|-----------|-------------------------|-----------------|---------------------------------------------|
| Full-length | 0 | | VLLPLVFFGLCLLLSLIFGLMYRQWRKWSKLYSIVCGK |
| Δ236–239 | 4 | YQRW | VLLPLVFFGLCLLLSLIFGLMYRQWRKWSKLYSIVCGK |
| Δ234–243 | 10 | YRYQRWKSL | VLLPLVFFGLCLLLSLIFGLMYRQWRKWSKLYSIVCGK |
| Δ229–244 | 16 | FIGLMYRQWRKWSKLY | VLLPLVFFGLCLLLSLIFGLMYRQWRKWSKLYSIVCGK |

TABLE 1
TNFR1 sequences used for designing constructs
Shown is the 16-residue TNFR1 sequence found to be homologous to caveolin-binding domains in other proteins that formed a basis for synthesizing the retroviral vector constructs and Tat-linked peptides used in this study. TNFR1 transmembrane domain amino acid residues are in italic type, cytoplasmic domain residues are in normal type, and the 16-residue sequence spanning the boundary of the TNFR1 transmembrane and cytoplasmic domains is in boldface type.
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FIGURE 1. Expression and localization of transduced TNFR1 proteins. A, FLAG-TNFR1 surface protein expression in EAhy.926 transductants was measured by FACS analysis as described under “Experimental Procedures,” comparing pBabe.puro vector control, full-length FLAG-TNFR1 or three FLAG-tagged TNFR1 constructs with deletions in the putative CAV-1 binding domain (Δ236–239, Δ234–243, and Δ229–244). Indirect immunostaining was with polyclonal rabbit anti-FLAG IgG (gray shading) or with negative control nonspecific rabbit IgG (black line) followed by AlexaFluor 488-conjugated donkey anti-rabbit secondary antibody (Invitrogen). One of two experiments with similar results is shown. B, expression of transduced TNFR1 proteins was measured by immunoprecipitating FLAG-containing proteins (with rabbit anti-FLAG), followed by immunoblotting for TNFR1 (with mouse anti-TNFR1). Top, pooled low density membrane fractions positive for CAV-1 protein obtained from sucrose gradient ultracentrifugation of EAhy.926 protein lysates. Bottom, whole cell lysates. One of two experiments with similar results is shown. FL, full-length TNFR1; V, vector. C, EAhy.926 whole cell lysates of full-length and Δ229–244 FLAG-TNFR1 (left) as well as sucrose gradient separations (middle and right) were analyzed by Western blot with anti-FLAG-horseradish peroxidase and anti-CAV-1 primary antibodies. One of two experiments with similar results is shown. D, densitometric quantification comparing full-length and Δ229–244 TNFR1 localization with sucrose gradient light fractions of the experiment depicted in C. The graph shows the expression of FLAG-TNFR1 in fractions 3, 4, and 5 normalized to CAV-1 after correcting for different expression levels measured in whole cell lysates.

2-fold that of FL FLAG-TNFR1 as normalized to CAV-1 levels (Fig. 1C, left). Densitometric analysis of the immunoblots of the sucrose gradients (Fig. 1C, middle and right), normalized to CAV-1 and corrected for expression levels, suggests that the full-length construct is about 5 times more likely to localize to low density membrane fractions 3–5 (Fig. 1D). These data suggest that the full proximal region extending from residue 229 to 244 contains information for targeting TNFR1 to caveolin-enriched low density membranes and that residues 234–244 do not appear to be necessary for this localization.
In order to evaluate whether the 229–244 region of the TNFR1 directly interacts with CAV-1, a biotinylated Tat-linked cell-permeable peptide containing this sequence was synthesized (Fig. 2A). Indirect immunofluorescence studies showed localization of the biotinylated TAT-TNFR1(229–244) peptide on the cell surface and to cytoplasmic organelles, whereas biotinylated-TAT, used as a control peptide, localized to the nucleus (Fig. 2B). However, immunofluorescence microscopy was insufficient to identify whether the peptide was actually localizing to caveolae. In order to more directly evaluate an interaction between TNFR1(229–244) and CAV-1, we performed pull-down analyses using TAT-TNFR1(229–244) or a scrambled TAT-TNFR1 peptide sequence (TNFR1(229–244SCR)) as bait. The total pull-down by biotinylated specific or scrambled peptide was analyzed by SDS-PAGE (Fig. 3A). A number of bands were markedly enriched in the specific peptide pull-down compared with the control scrambled peptide (e.g. in the region above 75 kDa, at around 55 kDa (possibly TNFR1 or TRAF2), and at 23 kDa (possibly CAV-1)). To identify specific caveolar proteins in the pull-downs, we then subjected pull-downs to immunoblotting. The biotinylated TAT-TNFR1(229–244) peptide pulled down several proteins, including CAV-1, TNFR1, and TRAF2, whereas the peptide containing the scrambled sequence, TAT-TNFR1(229–244SCR), as well as the TAT sequence alone both failed to do so (Fig. 3B). We expected that our sequence would interact with CAV-1 because it was identified as a putative CAV-1-interacting region. CAV-1 is known to interact with TRAF2 (31, 32), and it is clearly possible that CAV-1 and TNFR1 directly interact. Additionally, TNFR1 is known to interact with itself, forming trimers in the membrane even in the absence of ligand (33). However, it is also possible that the interactions we have observed involve binding of the peptide to some other protein or proteins present in low density membranes, allowing pull-down of multiprotein lipid-containing complexes. In order to first evaluate whether the interaction between TNFR1(229–244) and CAV-1 was restricted to low density membranes, we performed sucrose gradient fractionation of cell homogenates. After centrifugation, individual fractions were pooled into three separate groups (indicated in Fig. 3C), and the pooled fractions were subjected to pull-down with the biotinylated TAT-TNFR1(229–244) peptide. Interestingly, TAT-TNFR1(229–244) could only interact with CAV-1 in low density membrane fractions (pool 2) despite the presence of CAV-1 in the high density fractions (pool 3) of this gradient (Fig. 3D), suggesting either that the interaction was not occurring directly with CAV-1 or that it only occurred when CAV-1 was associated with cholesterol/sphingomyelin-rich membranes (where it may assume a different conformation). Consistent with an interaction with low density membranes, rather than with a specific protein, pretreatment of cells with MβCD, which disrupts low density mem-

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**FIGURE 2. Localization of a biotinylated TAT-TNFR1 sequence-containing peptide in EA.hy926 cells.** A, schematic model of a biotinylated TAT-TNFR1 sequence-based peptide (TAT-TNFR1(229–244)). B, immunofluorescence microscopy of cells treated with the biotinylated TAT-TNFR1(229–244) peptide versus biotinylated Tat peptide alone. Note that the peptide containing a TNFR1 sequence localizes to cytoplasmic organelles and plasma membrane, whereas Tat peptide alone goes to the nucleus. Data are representative of results of two independent experiments.
branes by extracting cholesterol, decreased pull-down of CAV-1 and TNFR1 by the TAT-TNFR1(229–244) peptide (Fig. 3E). This interpretation is supported by our previous finding that immunoprecipitation of TNFR1 from EA.hy926 cells efficiently co-immunoprecipitates CAV-1, detected by immunoblotting, and that this interaction is largely disrupted by pretreatment of the cells with MβCD (17).

**siRNA Silencing of CAV-1 Inhibits Formation of Caveolae**—The data reported so far are consistent with a role for CAV-1 interactions in the targeting of TNFR1 to low density membrane regions. We next employed transfection of siRNAs in the human EC-like line EA.hy926 to more directly examine the role of CAV-1 on TNFR1 targeting to low density membranes. For this purpose, three different small siRNA sequences against CAV-1 were designed, and cells were transfected as described under “Experimental Procedures.” Controls included transfection with siRNAs *versus* irrelevant targets (lamin A/C or LFA-3) or mock transfection. In order to optimize CAV-1 knockdown, we performed a series of preliminary experiments to evaluate the most effective and target-specific siRNA concentration as well as the most appropriate time point for the silencing experiment using EA.hy926 cells. We found that three different siRNAs targeting CAV-1 significantly decreased CAV-1 protein expression in EA.hy926 cells at 48 h, and at 72 h after transfection, we were routinely able to achieve a reduction of more than 90% of CAV-1 protein expression (supplemental Fig. S1A). It has been previously shown that CAV-1 silencing can reduce the number of caveolae in rat lung EC (34). To determine the effect of CAV-1 siRNA transfection in our system, we performed electron microscopy to quantify the reduction in the number of caveolae in EA.hy926 cells. Both non-transfected cells and cells transfected with an irrelevant (control) siRNA sequence (targeting LFA-3) contained abundant cell surface caveolae, which appear as 50–100-nm invaginations of the plasma membrane (supplemental Fig. S1B, upper panels). However, both siRNA-538 and -545 targeting of CAV-1 highly reduced the number of caveolae (supplemental Fig. S1B, lower panels). Quantitative analysis of the number of caveolae per cell showed a statistically significant difference between control samples (both non-transfected and LFA-3-transfected cells) and CAV-1 siRNA-treated cells (supplemental Fig. S1C). Despite substantial down-regulation of CAV-1 protein seen by immunoblotting, we could still detect a few caveolae on the cell surface.

**siRNA Silencing of CAV-1 or FLOT-2 Does Not Inhibit TNFR1 Targeting to Low Density Membrane Domains or Ligand-induced Receptor Internalization**—We have previously shown that treatment of EA.hy926 cells with the cholesterol-extracting agent MβCD eliminated TNFR1 from low density plasma membrane fractions (caveolae plus isolated lipid rafts) without reducing total plasma membrane TNFR1 expression levels. However, ligand-induced internalization was markedly inhibited (17). To address the effect of CAV-1 knockdown on TNFR1 surface expression levels and internalization, we first assessed the effect of this treatment on TNFR1 cell surface expression. Based on FACS analysis, plasma membrane TNFR1 levels were inconsistently and minimally affected, with no statistically significant effect compared with control-treated cells over five independent experiments (data not shown). Interestingly, total levels of TNFR1 were reduced in CAV-1 siRNA-treated cells, but the loss, as judged by immunofluorescence, was primarily observed in the Golgi (data not shown). Despite loss of caveolae, CAV-1 silencing did not appear to reduce the formation of low density plasma membrane domains as detected by sucrose gradient fractionation, presumably now in the form of dispersed lipid rafts, and TNFR1 was still localized to these low density membrane fractions, as judged by immunoblotting of the sucrose gradient fractions (supplemental Fig. S2A). Second, previous studies analyzing receptor localization following ligand interaction have shown that TNF-induced loss of surface TNFR1 is caused by receptor internalization and unrelated to shedding (17). Serial FACS analyses of plasma membrane TNFR1 levels in TNF-treated cells after siRNA knockdown of CAV-1 revealed that relocation of TNFR1 from caveolae to dispersed lipid rafts did not affect ligand-induced receptor internalization (supplemental Fig. S2B). Because CAV-1 does not appear to be necessary for localization to or internalization of TNFR1 from low density membrane domains, we considered an additional candidate, namely FLOT-2. FLOT-2 belongs to the SPFH domain-containing protein family, consisting of proteins found in lipid raft domains (19). FLOT-2 has specifically been implicated in the internalization of plasma membrane proteins from the lipid rafts of epithelial cells (19). To examine the role of FLOT-2 in TNFR1 localization to and internalization from low density membranes in EA.hy926 cells, we again turned to siRNA silencing using conditions similar to those optimized for CAV-1 silencing (Fig. 4A). In contrast to our studies with CAV-1 silencing, FLOT-2 silencing did not produce a statistically significant reduction of caveolae (Fig. 4, B–D). FLOT-2 silencing, like CAV-1 silencing, reduced the expression of TNFR1 detected by immunoblotting (Fig. 4A) but, unlike CAV-1 silencing, produced a consistent and statistically significant reduction in TNFR1 surface expression (Fig. 5A). However, FLOT-2 silencing did not differentially reduce the localization of TNFR1 to low density membranes (Fig. 5B), and similarly to CAV-1 knockdown (see supplemental Fig. S2), ligand-induced internalization of TNFR1 was not inhibited by FLOT-2 knockdown (Fig. 5C).

**Comparison of the Effects of MβCD with CAV-1 and FLOT-2 Silencing on TNF Signaling**—In the next series of experiments, we evaluated the contribution of caveolae or lipid rafts to the TNF signaling response in EC, comparing CAV-1 and FLOT-2 silencing with MβCD extraction. Degradation of IκBα following its TNF-induced phosphorylation by IKK is the key step in the activation of NF-κB by the canonical activation pathway. We first confirmed our previous findings that extraction of cholesterol with MβCD did not inhibit NF-κB signaling (Fig. 6A). siRNA-induced silencing of CAV-1 also did not show any effect on TNF-induced degradation of IκBα (Fig. 6B). Similarly, no effect of NF-κB activation was observed in FLOT-2-silenced cells (Fig. 6C). Densitometric analysis performed on both control and CAV-1 siRNA (siRNA-538)-transfected cells confirmed a similar pattern of IκBα degradation in response to different doses of TNF (Fig. 6H). In contrast to our findings for NF-κB signaling, we did observe changes in SAPK signaling. Treatment with MβCD did not reduce and may have even
increased SAPK activation (Fig. 6D), whereas treatment with either CAV-1 (Fig. 6E) or FLOT-2 siRNA (Fig. 6F) appeared to be inhibitory. The effect on SAPK consistently appeared more pronounced than that on p38 MAPK (data not shown). To further analyze the role of CAV-1 in SAPK activation, we examined the effects of CAV-1 siRNA treatment on components of the TNFR1 signalosome. Despite an inconsistent effect of CAV-1 knockdown on levels of TNFR1 on the cell surface, we saw a highly reproducible reduction in total TNFR1 levels by around 50% (data not shown), which appeared to occur largely at the expense of the Golgi TNFR1 pool. Interestingly, in addition to reducing colocalization of caveolin proteins with TNFR1 (Fig. 7E) and the Golgi compartments (Fig. 7F), CAV-1 silencing resulted in an increase in endosomal/lysosomal association of TNFR1 (Fig. 7G, H). Parallel studies also indicated that FLOT-2 knockdown induced a greater reduction in total TNFR1 levels (that somewhat reduced cell surface expression), a greater loss of TNFR1 localization to the Golgi, and a greater redistribution to endosomal/lysosomal compartments than that seen with CAV-1 silencing (data not shown). In other words, perturbation of the lipid raft compartments by knockdown of scaffolding proteins has effects on both TNFR1 expression levels and distribution among various cellular compartments. CAV-1 silencing also significantly diminished the level of TRAF2 protein (Fig. 8A). In contrast, levels of TRADD and RIP-1 proteins were not affected (Fig. 8A), indicating that the effect of CAV-1 knockdown appears selective for specific components of the signalosome. TRAF2 protein levels were also reduced by FLOT-2 silencing (Fig. 8B). However, quantitative real-time PCR analysis revealed that TNFR1 as well as TRAF2 transcript expression was largely unaffected by CAV-1 knockdown (data not shown). Similarly, the expression of transcripts encoding TRADD, FADD, cFLIP, and caveolin-2 also did not change after CAV-1 knockdown (data not shown). In other words, the effects of CAV-1 (and presumably FLOT-2) knockdown appear to be mediated at the level of protein expression.

To examine if TNFR1 reduction could explain the selective inhibition of SAPK signaling, we used siRNA knockdown of TNFR1. This treatment reduced both NF-κB and SAPK activation (Fig. 8B). However, quantitative real-time PCR analysis revealed that TNFR1 as well as TRAF2 transcript expression was largely unaffected by CAV-1 knockdown (data not shown). Similarly, the expression of transcripts encoding TRADD, FADD, cFLIP, and caveolin-2 also did not change after CAV-1 knockdown (data not shown). In other words, the effects of CAV-1 (and presumably FLOT-2) knockdown appear to be mediated at the level of protein expression.

The basis of this reduction of TRAF2 (or TNFR1) protein levels in cells with reduced levels of CAV-1 or FLOT-2 is unknown. Attempts to examine protein stability by treating cells with cycloheximide for specified periods of time followed
FIGURE 6. Effects of cholesterol extraction versus CAV-1 or FLOT-2 silencing on TNF signaling. Modulation of TNF signaling by knockdown of CAV-1 or FLOT-2 or by MβCD treatment. A, effect of MβCD treatment on NF-κB signaling. B, effect of CAV-1 knockdown on NF-κB signaling. Cells were subjected to CAV-1 silencing with siRNA-538 and subsequently stimulated for 30 min with the indicated doses of TNF prior to SDS-PAGE and Western blotting analysis of IκBa. C, effect of FLOT-2 silencing on IκBa degradation. D, effect of MβCD on SAPK/JNK activation. E, effect of CAV-1 knockdown on SAPK/JNK activation. F, effect of FLOT-2 siRNA on SAPK/JNK activation. G–N, densitometric analysis of immunoblot data. Specific protein levels are normalized to that of β-actin. Data are representative of results from three independent experiments.
by immunoblotting showed that TNFR1 has a very short half-life (less than 3 h), whereas TRAF2 appeared very stable (showing no decay at 6 h, the longest time that the cells could tolerate cycloheximide treatment), and neither protein half-life appeared to be changed by CAV-1 knockdown. These data support the idea that the reduction in TRAF2 underlies the selective inhibition of SAPK activation. Because it appears that the effect of CAV-1 silencing is unrelated to its role in formation of caveolae, in a final set of experiments, we investigated if the effects of CAV-1 siRNA knockdown we had observed in EA.hy926 (an immortalized cell line) could also be seen in primary cultures of passaged HUVEC, which is the most widely used model for the study of endothelium and which essentially lack caveolae. Treatment of HUVEC with CAV-1 siRNA effectively reduced the level of CAV-1 protein and also the level of TRAF2 protein in these primary cells, albeit the reduction of TRAF2 was not as pronounced as that observed in EA.hy926 cells (Fig. 9A). Similar to what had been observed in EA.hy926 cells, in HUVEC, TNF-induced activation of SAPK (Fig. 9B) but not degradation of IκBα was also diminished as a result of CAV-1 knockdown (Fig. 9C). These results in an untransformed EC system support our interpretation that the effects of CAV-1 on TNF signaling are independent of the role of this protein in organizing caveolae.

**DISCUSSION**

We previously reported that TNFR1 on the plasma membrane of the human EA.hy926 EC line is localized in caveolae and that disrupting the caveolar network by means of the cholesterol-extracting agent MβCD both displaced TNFR1 from this organelle to other regions of the plasma membrane and inhibited TNF-induced internalization of this receptor. However, treatment with MβCD did not change TNF-induced degradation of IκBα (17). In the present study, we further investigated the connections between caveolae and TNFR1 in the EAhy.926 endothelial-like cell line (35, 36). We report several new findings. First, based on stable transduction approaches, we have identified a transmembrane/intracytoplasmic sequence in TNFR1 responsible for receptor localization to low density membrane regions. A peptide containing this 16-residue sequence can be used to pull down CAV-1 and TNFR1 from sucrose gradient fractions that contain low density membrane domains, but the same sequence does not work to pull down these proteins from other fractions or from cell preparations in which low density membranes have been disrupted by...
Targeting TNFR1 to Lipid Rafts

A

B

C

FIGURE 9. Analysis of the effects of CAV-1 silencing by siRNA in HUVEC. HUVEC were prepared and incubated with siRNA against CAV-1 or with lamin A/C as an irrelevant control. A, effect of siRNA knockdown of CAV-1 on expression of CAV-1 and TRAF2 protein, respectively. Dark bars, lamin A/C; stippled bars, CAV-1 siRNA. B, effect of CAV-1 knockdown on TNF-induced SAPK activation. C, effect of CAV-1 knockdown on TNF-induced NF-κB activation. In B and C, cells were treated with the indicated doses of TNF for 30 min prior to SDS-PAGE and immunoblotting. Results are from one of two independent experiments with similar results.

Cholesterol extraction with MβCD. Second, we demonstrate that knockdown of CAV-1 but not FLOT-2 is sufficient to reduce the number of caveolae in EA.hy926 cells. Knockdown of CAV-1 slightly reduces overall expression levels of TNFR1 but does not consistently affect surface expression, does not disrupt lipid rafts, does not interfere with the localization of TNFR1 to lipid rafts, and does not interfere with TNF-induced internalization of TNFR1 from lipid rafts. Knockdown of FLOT-2, a protein linked to internalization of other plasma membrane proteins from lipid rafts (see Ref. 19 for a review), does not reduce the number of caveolae but does reduce surface expression of TNFR1. However, the reduction in surface TNFR1 appears to equally affect the levels of protein in low and high density membrane fractions, arguing against a role for FLOT-2 in localization to low density regions. A preliminary analysis of FLOT-2 knockdown cells by confocal immunofluorescence microscopy suggests that loss of this protein may lead to an increase in TNFR1 associated with endosomes and lysosomes concomitant with loss of plasma membrane and Golgi expression. A similar finding is observed in CAV-1 knockdown cells (Fig. 7), but FLOT-2 knockdown appears to have a more pronounced effect both on this redistribution and on total TNFR1 levels. The bases of these effects are unknown. Despite these changes, the residual TNFR1 on the surface is still subject to a comparable degree of ligand-induced internalization in the absence of FLOT-2. Finally, we show that TNFR1 signaling, assessed by activation of NF-κB or SAPK, is not dependent upon caveolae. Although knockdown of CAV-1 and FLOT-2 does reduce TNF-mediated activation of SAPK, this effect correlates with a reduction in the expression of TRAF-2 rather than a reduction of caveolae and is phenocopied by siRNA-mediated knockdown of TRAF-2.

The sequence spanning the boundary of the TNFR1 transmembrane and cytoplasmic domains that we studied in this report was selected for investigation because of its resemblance to sequences in other proteins that interact with CAV-1 (30, 37). This region of the molecule had been previously identified as a sequence involved in receptor internalization (38). In a human EC line, we show by comparing transfection of full-length and deletion mutants that this sequence does not mediate targeting to low density membrane domains. However, only the tryptophan residue of this sequence, which in our hands did not appear to mediate localization to low density membranes, was previously shown to be necessary for internalization. A simple explanation for this discrepancy is that internalization and localization to low density membrane domains involve different interactive proteins and that when expressed in EAhy.926 cells, such proteins bind to overlapping but distinct sites of the TNFR1 molecule. Our peptide pull-down experiments further support the idea that the sequence we have identified can interact with CAV-1-containing low density membranes. It is unclear if this interaction directly involves CAV-1, which could assume a different structure when associated with cholesterol-rich membranes, or some other protein or component of low density membranes. However, our findings that TNFR1 still localizes to low density membranes after CAV-1 has been silenced suggest that a direct interaction, if it exists, is redundant. Identification of protein(s) that directly associate with TNFR1 in low density membranes is an area for future investigation, perhaps through siRNA library screening.

TNF binding to TNFR1 induces rapid internalization of the receptor. We and others have shown that several agents that prevent internalization of plasma membrane proteins appear to block signaling (15, 17). Our MβCD experiments, in which we blocked internalization from low density membranes by disrupting these structures, seemingly contradict this conclusion because this agent can block internalization without inhibiting signaling, as assessed by activation of NF-κB or SAPK. TRADD is rapidly recruited to TNFR1 upon ligand binding and then rapidly dissociates from the receptor upon internalization. It is possible that it is internalization of TRADD rather than of TNFR1 that is crucial for signaling. Because MβCD did block ligand-induced internalization of TNFR1, we hypothesized that TNFR1 is internalized via CAV-1 (from caveolae) and/or...
FLOT-2 (via dispersed lipid rafts). However, our new siRNA experiments suggest that neither CAV-1 nor FLOT-2 is responsible for this process. Nevertheless, the fact remains that TNFR1 is internalized from within caveolae. A second future direction for this work may be siRNA library screening to identify proteins mediating TNFR1 internalization. The present study significantly extends our investigation of the role of caveolae or lipid rafts on TNFR1 signaling. It would initially seem that it should be possible to test the role of caveolae/lipid rafts on TNFR1 signaling using our expression constructs. However, previous experience in our group (39) and others (28) had shown that overexpression of TNFR1 leads to cell death even in the absence of ligand. This problem has been overcome by introducing a mutation into the death domain of the receptor that was based on the spontaneous mutation in Fas (known as lpr) that prevents interaction with FADD (40). It is likely that this mutation in TNFR1 allows cells to survive by preventing recruitment of TRADD, a result that prevents cell death but also blocks canonical TNFR1 signaling. We therefore have used other approaches, such as MβCD treatment or siRNA silencing, and assessed signaling from the endogenous receptor. The striking conclusion of these studies is that low density membrane domains are not required for either NF-κB or SAPK signaling. Although we did see inhibition of SAPK activation in cells in which either CAV-1 or FLOT-2 were silenced, this effect is best explained by reductions in TRAF-2 expression. This raises the question of what purpose, if any, could be served by localization of TNFR1 to these specialized membrane regions. A likely possibility is that caveolar localization favors receptor cross-talk rather than intrinsic receptor signaling. For TNF, an example of this cross-talk could be the activation of sphingosine kinase (41), an enzyme that generates sphingosine 1 phosphate, suggesting that the effects of this reaction may be linked to the localization of sphingosine 1 phosphate receptors to caveolae (42). The studies reported here can form the basis for such analyses.

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