Full-length, Membrane-anchored TWEAK Can Function as a Juxtacrine Signaling Molecule and Activate the NF-κB Pathway*

Received for publication, April 8, 2010. Published, JBC Papers in Press, April 12, 2010, DOI 10.1074/jbc.M110.131979

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Tumor necrosis factor (TNF) family members are initially synthesized as type II transmembrane proteins, but some of these proteins are substrates for proteolytic enzymes that generate soluble cytokines with biological activity. TWEAK (TNF-like weak inducer of apoptosis), a member of the TNF family, is a multifunctional cytokine that acts via binding to a cell surface receptor named Fn14 (fibroblast growth factor-inducible 14). Studies conducted to date indicate that TWEAK-producing cells can co-express both membrane-anchored and soluble TWEAK isoforms, but there is little information on TWEAK proteolytic processing. Also, it is presently unclear whether membrane-anchored TWEAK, like soluble TWEAK, is biologically active. Here we show that full-length human TWEAK is processed intracellularly by the serine protease furin and identify TWEAK amino acid residues 90–93 as the predominant furin recognition site. In addition, we report that TWEAK-producing cells can bind the Fn14 receptor on neighboring cells and activate the NF-κB signaling pathway. Thus, TWEAK can act in a juxtacrine manner to initiate cellular responses, and this property may be important for TWEAK function during physiological wound repair and disease pathogenesis.

TWEAK (tumor necrosis factor-like weak inducer of apoptosis) is a member of the TNF superfamily of structurally related cytokines that together are involved in many critical biological processes, including development, organogenesis, tissue repair, and the innate and adaptive immune responses (1–3). TWEAK was initially described as a proapoptotic factor for certain tumor cell lines (4), but subsequent studies revealed that it can stimulate many other cellular responses, including cell proliferation, survival, and differentiation (5, 6). This cytokine acts on responsive cells via binding to the Fn14 (fibroblast growth factor-inducible 14) cell surface receptor, a 102-amino acid type I transmembrane protein originally described as a serum- and growth factor-inducible gene product in fibroblasts and smooth muscle cells (7–9). The TWEAK/Fn14 axis has been implicated in physiological wound repair and in the pathogenesis of ischemic stroke and several chronic inflammatory diseases (5, 6), including rheumatoid arthritis (10, 11) and inflammatory bowel disease (12).

The human TWEAK gene encodes a 249-amino acid type II transmembrane protein, and when this full-length form of TWEAK was overexpressed in transfected HEK293-EBNA cells it was detected on the cell surface by FACS analysis, as expected (4). However, when a metabolic labeling and immunoprecipitation experiment was performed using the transfected cells, a smaller TWEAK form was found in conditioned medium (4). These results indicated that HEK293-EBNA cells could produce two TWEAK isoforms: a full-length, membrane-anchored form and a smaller secreted form that is probably generated by TWEAK proteolytic processing. Subsequent studies have shown that other cell types can also co-express membrane-anchored and soluble TWEAK, indicating that, in general, full-length TWEAK is not cleaved with 100% efficiency (13–15). Membrane TWEAK activity has not yet been conclusively demonstrated, but the secreted TWEAK form, which contains the TNF homology domain that binds to the Fn14 receptor, is biologically active (5, 6).

The TWEAK processing mechanism in mammalian cells has not been described to date. However, N-terminal sequence analysis of the secreted TWEAK form produced in an insect cell overexpression system revealed that TWEAK was cleaved in the stalk region following arginine 93 (4). This arginine is the C-terminal residue in an RPRR consensus cleavage motif for furin, a member of the proprotein convertase family of serine proteases (16–18). Therefore, many investigators in the TWEAK/Fn14 field have assumed that furin is in fact the TWEAK-processing enzyme and that arginine 93 is the sole furin cleavage site. However, furin is but one member of a family of seven proprotein convertases that can cleave after basic RX(R/K/X)R motifs (16–18), and there are actually two putative furin consensus cleavage sites in the human TWEAK stalk region. One group of investigators has even stated in a published paper that “TWEAK is cleaved at the cell surface by furin” (19), although neither the TWEAK cleavage enzyme nor the

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*This work was supported, in whole or in part, by National Institutes of Health Grants NS55126 and CA130967. This work was also supported by Susan G. Komen for the Cure Grant KG081095 (to J. A. W.).

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2 The abbreviations used are: TNF, tumor necrosis factor; CMK, decanoyl-RVKR-chloromethylketone; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; HA, hemagglutinin; mAb, monoclonal antibody; WT, wild type; TAPI-O, tumor necrosis factor-α protease inhibitor.
cellular location of TWEAK processing has been reported in the literature.

In this report, we demonstrate that full-length TWEAK is processed intracellularly by furin convertase in mammalian cells, and we use a deletion mutagenesis approach to identify TWEAK amino acid residues 90–93 as the predominant furin recognition site. In addition, we report that full-length, membrane-anchored TWEAK can bind the Fn14 receptor on neighboring cells and activate the NF-κB signaling pathway. Our observation that membrane-anchored TWEAK can act in a juxtacrine manner to initiate cellular responses has implications for the development of TWEAK antagonists for clinical application.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HEK293 cells (American Type Culture Collection) were grown in Earle’s modified Eagle’s medium supplemented with 10% FBS, 1 mM sodium pyruvate, and 1 x non-essential amino acids. Human LoVo cells (American Type Culture Collection) were grown in Ham’s F-12 medium supplemented with 10% FBS, 0.003% sodium bicarbonate, and 2 mM glutamine. HEK293/NFκB-luc cells (Panomics) were grown in Dulbecco’s modified Earle’s medium supplemented with 10% FBS and 100 μg/ml hygromycin.

**Human TWEAK and Fn14 Expression Plasmids**—The expression plasmid encoding full-length human TWEAK (TWK) with an N-terminal Myc epitope tag was constructed by PCR overlap extension using Taq polymerase (Roche Applied Science) and appropriately designed primer pairs. Briefly, the pBluescript/TWK plasmid that we described previously (20) was used as the PCR template, and the DNA sequence encoding the Myc epitope peptide (EQKLQSEEDL) was inserted immediately following the ATG start codon using overlapping primers in a two-step process. The final PCR product was isolated and then ligated into the pcDNA3.1 expression vector (Invitrogen) according to the manufacturer’s instructions. Expression plasmids encoding Myc-tagged TWEAK proteins with a deletion of amino acids 90–93 (the TWK-dF1 plasmid), 102–105 (the TWK-dF2 plasmid), or both 90–93 and 102–105 (the TWK-dF1/F2 plasmid) were also constructed using the PCR overlap extension method and appropriately designed primer pairs. The wild type, full-length TWEAK expression plasmid described above was used as the template to construct the TWK-dF1 and TWK-dF2 plasmids. The TWK-dF1 plasmid was used as the template to construct the TWEAK-dF1/F2 plasmid. PCR was performed using template DNA, appropriate TWEAK primers, and Taq polymerase. The PCR products were isolated by agarose gel electrophoresis and ligated into pcDNA3.1 as above. The expression plasmid encoding full-length human Fn14 with an N-terminal HA epitope tag was constructed by the PCR overlap extension method with Vent polymerase. The final pCMVScript/Fn14-HA plasmid was then digested with NotI, and the released DNA fragment was ligated into NotI-digested pcDNA6 plasmid (Invitrogen). All final expression constructs were verified by DNA sequence analysis using appropriate primers and an Applied Biosystems automated sequencer.

**Transient Transfections and Cell Treatments**—HEK293 or LoVo cells were transiently transfected with the plasmids pcDNA3, pcDNA3/TWK-WT, pcDNA3/TWK-dF1, pcDNA3/TWK-dF2, pcDNA3/TWK-dF1/F2, or pCMV/Furin (provided by Dr. J. Evan Sadler, Washington University School of Medicine) using Lipofectamine PLUS (Invitrogen) according to the manufacturer’s recommendations. Cells were harvested 48 h post-transfection; in some HEK293 cell experiments, the cells were treated with either 20 or 100 μM CMK (Axonora), 20 μM GM6001 (Calbiochem), 20 μM TAPI-0 (Calbiochem), or 1 μM brefeldin A (Sigma) for 12 h prior to harvest.

**Isolation of Stably Transfected Cell Lines and Cell Treatments**—To obtain stably transfected HEK293 cell lines, the pcDNA3 vector, the pcDNA3/TWK-WT plasmid and the pcDNA3/TWK-dF1/F2 plasmid were transfected into parental cells using Lipofectamine PLUS (Invitrogen). Cells were cultured in standard growth medium containing 400 μg/ml G418, and individual drug-resistant colonies were recovered. The cell lines were screened for TWEAK expression levels by Western blot analysis using an anti-Myc antibody (see below) and one vector control, one TWEAK-WT-expressing, and two TWEAK-dF1/F2-expressing cell lines (lines 8 and 9) were chosen for further characterization. The TWEAK-WT-expressing cells were treated with either 100 μM CMK, 15 μg/ml human recombinant α1-PDX (EMD Biosciences), or 60 μM hexa-D-arginine (EMD Biosciences) for 12 h and then harvested. To obtain a HEK293/NFκB-luc cell line that overexpressed the Fn14 receptor, the parental cells were transfected with the pcDNA6/Fn14-HA plasmid using Effectene (Qiagen) according to the manufacturer’s recommendations. The cells were cultured in standard growth medium containing 4 μg/ml blasticidin, drug-resistant cells were pooled, and Fn14 expression was confirmed by Western blot analysis using an anti-HA antibody (see below).

**Western Blot Analysis**—Cells were harvested and then lysed in 20 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% glycerol, and 1.5 mM MgCl₂. Protein concentrations were determined using the BCA protein assay (Pierce). Equal amounts of protein from each cell lysate were subjected to SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were blocked, sequentially incubated with the appropriate primary antibody (see below) and horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences), and washed, and then immunoreactive proteins were detected using the Amersham Biosciences Enhanced Chemiluminescence Plus kit as described previously (20). Primary antibodies were (a) anti-Myc epitope mAb clone 9E10 (provided by...
Full-length TWEAK Processing and Activity

Sue Robinson, University of Maryland School of Medicine), (b) anti-furin mAb clone MON-152 (Alexis Biochemicals), (c) anti-actin mAb clone AC-40 (Sigma), (d) anti-TWEAK polyclonal antibody R1271 (provided by Dr. Linda Burkly, Biogen Idec), (e) anti-HA epitope polyclonal antibody (BD Biosciences), and (f) anti-tubulin mAb clone DM1A (Millipore).

FACS Analysis—Flow cytometry was conducted using phycoerythrin-labeled anti-TWEAK mAb CARL-1 and IgG3 isotype control protein (both from eBioscience) as described previously (20).

ELISA—Immulon 4HBX 96-well microtiter plates (Thermo) were coated with either recombinant Fn14-Fc decoy receptor or Fc control protein (21, 22) in phosphate-buffered saline at 0.1 μg/well for 1 h at room temperature. This solution was removed, and HEK293 cell growth medium (prepared using low IgG FBS) was added to each well (blocking step). After overnight incubation at 4 °C, the medium was removed, and the plates were washed five times with phosphate-buffered saline, 0.05% Tween 20. HEK293 vector control cells, the two HEK293 TWEAK-dF1/F2 cell lines, and a stably transfected HEK293 cell line engineered to overproduce the secreted TWEAK isoform (20) were plated in the growth medium described above, and 48 h later the cells were harvested and counted, and the conditioned medium was collected. The medium was centrifuged to remove any cells and membrane fragments, and equal volumes of each sample were added to triplicate wells. Recombinant TWEAK (Alexis Biochemicals) diluted in the growth medium described above was added at increasing concentrations in triplicate to additional wells in order to establish a standard curve. The plates were incubated for 1 h at room temperature and then washed as above. A goat anti-TWEAK polyclonal antibody (Cell Sciences) diluted to 1 μg/ml in the growth medium described above was added, and the plates were incubated for 1 h at room temperature and then washed. A horseradish peroxidase-conjugated donkey anti-goat secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added at a 1:2000 dilution, and the plates were incubated as above. Immunoreactivity was detected using Sigma FAST o-phenylenediamine dihydrochloride tablets (Sigma) according to the manufacturer’s instructions. Color development was read at 450 nm using a microtiter plate reader (Molecular Devices).

Background binding to the control Fc protein was negligible, and these values were subtracted from binding to the Fn14-Fc protein. The detection limit of the ELISA was 200 pg/ml.

NF-κB Pathway Luciferase Reporter Assays—In one set of experiments, parental HEK293/NFκB-luc cells and the HEK293/NFκB-luc/Fn14 stably transfected cell line were plated in normal growth medium at 5 × 10^5 cells/well in quadruplicate wells of a 6-well plate. The cells were either left untreated or transiently transfected with either the pcDNA3 vector or the pcDNA3/TWK-dF1/F2 plasmid using Effectene (Qiagen) according to the manufacturer’s recommendations. Cells were harvested 24 h post-transfection, one well was lysed for Western blot analysis, and the remaining wells were lysed and analyzed for luciferase activity as above.

Co-culture Experiments—The HEK293/NFκB-luc/Fn14 target cells were plated in normal growth medium (without selection antibiotics) at 2 × 10^5 cells/well in 6-well plates. One day later, the HEK293 effector cell lines (vector control, TWEAK-dF1/F2 line 8, TWEAK-dF1/F2 line 9) were harvested using Enzyme Free Cell Dissociation Solution (Millipore) and counted. The effector cells were centrifuged to remove dissociation solution, resuspended in normal growth medium (without G418), and then plated in triplicate wells of target cells at a concentration of either 0.5 or 1.0 × 10^6 cells/well. After 6 h of incubation, the cells were harvested and lysed, and NF-κB-luciferase reporter expression was assayed as described above. In the blocking experiments, HEK293 vector or TWEAK-dF1/F2 line 9 effector cells were plated onto target cells at 1.0 × 10^6 cells/well as described above. The TWEAK-dF1/F2 line 9 cells were added alone or in combination with either 5 μg/ml soluble Fn14-Fc decoy receptor or Fc control protein (purified as described (21, 22)) or 10 μg/ml anti-TWEAK mAb (CARL-1) or IgG3 isotype control protein (both from eBioscience). After 6 h of incubation, the cells were harvested and lysed, and NF-κB-luciferase reporter expression was assayed as described above.

RESULTS

Human TWEAK Is Processed by Furin—In a previous report, Chicheportiche et al. (4) constructed a recombinant baculovirus encoding the human TWEAK extracellular domain and found that when this protein was overproduced in insect cells, it was cleaved after arginine 93 to generate a secreted TWEAK isoform. Although not noted in this report, this is the C-terminal residue of a proprotein convertase consensus recognition motif located at TWEAK residues 90–93 (RPRR). A second putative recognition motif, RARR, is also present in the predicted human TWEAK sequence at residues 102–105. To determine whether a proprotein convertase family member could cleave full-length TWEAK, we transiently transfected HEK293 cells with a TWEAK-Myc expression plasmid and then treated the cells with CMK, a proprotein convertase inhibitor (23–25). Cells were also treated with GM6001, a broad spectrum matrix metalloproteinase inhibitor (26), and TAPI, a TACE (tumor necrosis factor-α convertase)/ADAM17 inhibitor (27). Western blot analysis indicated that CMK treatment, but not GM6001 or TAPI treatment, effectively blocked TWEAK cleavage (Fig. 1A). This result demonstrates that TWEAK is processed by one or more members of the proprotein convertase family.

Furin, a ubiquitously expressed proprotein convertase, has been shown to cleave the TNF superfamily members APRIL (a proliferation-inducing ligand) (28), EDA (ectodysplasin-A) (29–31), and BAFF (B cell-activating factor) (32–34); therefore,
ment are indicated with arrowheads and the positions of full-length (FL) TWEAK and the N-terminal TWEAK fragment are indicated with arrowheads on the right.

we tested whether this specific protease could also cleave TWEAK. These experiments were conducted using human LoVo colon carcinoma cells, which express enzymatically inactive furin due to mutations in both alleles of the furin gene (35, 36). When LoVo cells were transiently transfected with the TWEAK-Myc expression plasmid and Western blot analysis was performed, TWEAK processing was not detected (Fig. 1B). However, co-transfection of a furin expression plasmid restored TWEAK processing; therefore, TWEAK is a substrate for furin convertase.

The TWEAK Sequence Motif RPRR Is the Predominant Furin Recognition Site—The TWEAK stalk region contains two putative furin recognition motifs, with cleavage predicted after arginine residues 93 and 105 (Fig. 2A). We determined whether one or both of these motifs were TWEAK processing sites by constructing expression plasmids encoding TWEAK-Myc proteins missing either furin motif 1 (RPRR), motif 2 (RARR), or both motifs and then transfecting these plasmids into HEK293 cells. TWEAK cleavage was monitored by Western blot analysis as before. We found that deletion of furin motif 1, but not furin motif 2, prevented TWEAK cleavage (Fig. 2B).

**TWEAK Is Processed inside the Cell—**Furin is a transmembrane protein that cycles between the plasma membrane, endosomes, and trans-Golgi network (16, 37). Many furin substrates are cleaved in the latter cellular compartment (16, 17). The previous Western blot experiments to monitor TWEAK cleavage utilized an anti-Myc epitope antibody to detect full-length TWEAK and the N-terminal TWEAK cleavage product. This latter product is present in cell lysates, but one would predict that this would be the case regardless of whether TWEAK was processed intracellularly during its synthesis or when anchored on the cell surface. To determine the site of TWEAK processing, HEK293 cells were transfected with plasmids encoding either TWEAK-WT or the TWEAK mutant missing both motifs 1 and 2 (as a negative control), and Western blot analysis was conducted using an anti-TWEAK polyclonal antibody. This antibody was raised in rabbits using recombinant TWEAK extracellular domain as the antigen (4); therefore, it is predicted to detect full-length TWEAK in cell lysates and, if TWEAK is processed intracellularly, the C-terminal TWEAK cleavage product. We were able to detect the C-terminal TWEAK fragment in the TWEAK-WT plasmid-transfected cells but not the TWEAK-dF1/F2 plasmid-transfected cells, consistent with an intracellular cleavage location (Fig. 2C). Two additional findings also support an intracellular processing mechanism. First, when TWEAK-WT plasmid-transfected cells were treated with brefeldin A, which blocks protein trafficking from the ER to the Golgi apparatus (38), TWEAK cleavage did not occur (Fig. 2D). Second, when stably transfected HEK293 cells that overexpress Myc-tagged TWEAK-WT protein were treated with CMK, a cell-permeable furin inhibitor, TWEAK cleavage was abrogated, as expected (see Fig. 1A), but treatment with two different cell-impermeable furin inhibitors, α1-PDX (25, 39, 40) and hexa-D-arginine (41), did not prevent TWEAK processing (Fig. 2E).

**Full-length, Membrane-anchored TWEAK Is Biologically Active**—Previous studies using FACS analysis have shown that numerous cell types express full-length, membrane-bound TWEAK (13–15, 20, 42–47); however, it has not yet been conclusively demonstrated that membrane TWEAK can bind and activate the Fn14 receptor. We initially investigated whether membrane TWEAK was biologically active using a transient transfection assay. Because soluble TWEAK treatment of Fn14-positive cells is known to activate the NF-κB signaling pathway (6) (see below), we used NF-κB activation in the transfected cells as our read-out for membrane TWEAK function. First, we generated a target cell line for the transfection experiments. HEK293/NF-κB-luc reporter cells were transfected with an Fn14-HA expression plasmid, drug-resistant cells were pooled, and Fn14 expression was confirmed by Western blot analysis (Fig. 3A). TWEAK treatment of the parental cells and the Fn14-overexpressing cells resulted in a 3.8- and 7.3-fold induction of the NF-κB pathway reporter gene, respectively, demonstrating that Fn14 overexpression in these cells increased their sensitivity to soluble TWEAK stimulation (Fig. 3B). Second, the
HEK293/NF/H9260/B-luc/Fn14 cells were either left untransfected or transfected with either the pcDNA3 vector or the pcDNA3/TWK-dF1/F2 plasmid encoding non-cleavable TWEAK. Cells were harvested 24 h later and processed for Western blot analysis and luciferase activity assays. TWEAK expression was detected in the transfected cells (Fig. 4A) and resulted in a 7.1-fold induction of the NF-κB pathway reporter gene (Fig. 4B).

Membrane TWEAK-mediated NF-κB Pathway Activation Can Occur via a Juxtacrine Mechanism—The transient transfection results described above could reflect the ability of membrane TWEAK to act as an autocrine and/or juxtacrine signaling molecule. In autocrine signaling, the cytokine and its receptor are expressed by the same cell, and ligand-receptor binding can occur. In comparison, juxtacrine signaling requires cell-cell contact between an effector cell that produces a membrane-anchored ligand and a target cell that produces the appropriate membrane-anchored receptor. We next investigated whether TWEAK can signal in a juxtacrine manner by performing cell co-culture assays using engineered effector cell lines.

HEK293/NFκB-luc/Fn14 cells were either left untransfected or transfected with either the pcDNA3 vector or the pcDNA3/TWK-dF1/F2 plasmid encoding non-cleavable TWEAK. Cells were harvested 24 h later and processed for Western blot analysis and luciferase activity assays. TWEAK expression was detected in the transfected cells (Fig. 4A) and resulted in a 7.1-fold induction of the NF-κB pathway reporter gene (Fig. 4B).

Equal amounts of protein were used for Western blot analysis using either an anti-Myc epitope (top) or anti-actin antibody. E, stably transfected HEK293 cells expressing TWK-WT were either left untreated (no treatment (NT)) or treated with CMK, human recombinant α1-PDX, or hexa-D-arginine peptide. Cells were harvested 12 h later and lysed. Equal amounts of protein were used for Western blot analysis using either an anti-Myc epitope (top) or anti-tubulin antibody. In B–E, the positions of molecular size markers are shown on the left (in kDa), and the positions of full-length (FL) TWEAK and the N- or C-terminal TWEAK proteolytic products are indicated with arrowheads on the right.
Full-length TWEAK Processing and Activity

FIGURE 4. Membrane TWEAK expression in HEK293/NFκB-luc/Fn14 cells can activate the NF-κB signaling pathway. A, stably transfected HEK293/NFκB-luc/Fn14 cells were either left untransfected (No DNA) or transfected with either the pcDNA3 vector or the pcDNA3/TWK-dF1/F2 plasmid encoding Myc-tagged non-cleavable TWEAK. The cells were harvested 1 day later and lysed. Equal amounts of protein were used for Western blot analysis using either an anti-Myc epitope (top) or anti-tubulin antibody. B, NF-κB reporter activation in the control and transiently transfected cells was measured using a luminometer. The values shown are mean ± S.D. (error bars) of triplicate wells from one representative experiment of three independent experiments.

Full-length TWEAK Processing and Activity

FIGURE 5. Characterization of stably transfected HEK293 cell lines over-expressing non-cleavable, membrane-anchored TWEAK. A, the TWK-dF1/F2 line 8 (TWK-dF1/F2 #8), TWK-dF1/F2 line 9 (TWK-dF1/F2 #9), and vector control HEK293 (Vector) cell lines were plated and then harvested at subconfluence. The cells were lysed, and equal amounts of protein were used for Western blot analysis using either an anti-Myc epitope (top) or anti-actin antibody. The positions of molecular size markers are shown on the left (in kDa), and the position of full-length (FL) TWEAK is indicated with an arrowhead on the right. B, the same three HEK293 cell lines described above were stained with phycoerythrin-labeled anti-TWEAK antibody (solid line) or control IgG (dotted line) and then analyzed by flow cytometry. C, the same three lines described above and an HEK293 cell line engineered to produce the secreted TWEAK form (RBD #4) were plated, and 2 days later, the cells were harvested and counted, and the conditioned medium was collected. The amount of TWEAK present in the conditioned medium samples was determined by ELISA. The values shown are mean ± S.D. (error bars) of triplicate wells from one representative experiment of two independent experiments.

lines and the HEK293/NFκB-luc/Fn14 target cell line described above. First, to obtain the effector cells, HEK293 cells were transfected with either the pcDNA3 vector or the pcDNA-TWK dF1/F2 plasmid encoding non-cleavable TWEAK. Stable cell lines were isolated by drug selection and then screened for TWEAK expression levels by Western blot analysis. Two cell lines expressing similar levels of non-cleavable TWEAK were chosen for the subsequent experiments (Fig. 5A). These two TWEAK-producing cell lines had more TWEAK on the cell surface than the vector control cell line, as determined by FACS analysis using an anti-TWEAK antibody that would recognize both endogenous and ectopically expressed TWEAK (Fig. 5B). Immunoreactive TWEAK was present in both pcDNA3/TWK-dF1/F2 line 8 and pcDNA3/TWK-dF1/F2 line 9 cell conditioned medium as determined by ELISA, but the amount detected was quite low, at 4.9 and 3.2 ng of TWEAK protein/10⁶ cells, respectively (Fig. 5C). Second, co-culture assays were conducted as follows. The HEK293/NFκB-luc/Fn14 target cells were plated in 6-well cluster dishes, and 1 day later, either they were left untreated or the three HEK293 effector cell lines were added. Cells were harvested 6 h later, and NF-κB-luciferase reporter expression was assayed. The vector-transfected cell line, which expresses some endogenous membrane-anchored TWEAK (Fig. 5B), had a slight stimulatory effect on the target cells, but this effect was not statistically significant (p = 0.32; no cell addition versus vector cell addition) (Fig. 6A). In contrast, both of the engineered non-cleavable TWEAK-overexpressing cell lines significantly increased NF-κB-luciferase reporter expression when added to the target cells. Specifically, the addition of the pcDNA3/TWK-dF1/F2 line 8 cell line increased reporter expression in a cell dose-dependent manner, with 5 × 10⁵ cells causing a 2.0-fold induction and 1 × 10⁶ cells causing a 3.6-fold induction over the value obtained without effector cell addition. Similarly, when the pcDNA3/TWK-dF1/F2 line 9 cell line was used at the 1 × 10⁶ cell concentration, a 4.3-fold induction of the NF-κB-luciferase reporter gene was detected. We next investigated whether membrane-anchored TWEAK was in fact the molecular entity triggering NF-κB activation in the engineered target cells by examining the effect of adding either an Fn14-Fc decoy receptor or a TWEAK-specific mAb to the cell co-culture medium. In this set of experiments, there was again a slight stimulatory effect when the vector cells were added (p = 0.05; no cell addition versus vector cell addition) (Fig. 6B). The TWK-dF1/F2 line 9 effector cells triggered a 2.8-fold induction of reporter gene expression over the value obtained without effector cell addition. Co-treatment with the Fn14-Fc protein or TWEAK mAb inhibited effector cell stimulation of the NF-κB pathway by 92 and 80%, respectively. In contrast, co-treatment with either the Fc or IgG control proteins reduced effector cell activity by 13 and 16%, respectively. These results indicate that membrane-anchored TWEAK can act in a juxtacrine manner to activate Fn14-positive cells in vitro.
Full-length TWEAK Processing and Activity

![Graph]

**DISCUSSION**

TNF superfamily members are synthesized as type II transmembrane proteins, but some family members are proteolytically cleaved in the stalk region between the transmembrane and receptor-binding domains, leading to the release of soluble, biologically active ligands (2). For example, the full-length TNF-α (48–51), FasL (52–55), CD40 (56), and RANKL (57–61) proteins are substrates for metalloproteases that release the extracellular domain from the plasma membrane, a process called ectodomain shedding. In some cases, the metalloprotease responsible for cleavage is known; for example, TACE, a member of the ADAM (a metalloprotease and disintegrin) family (62), is considered to be the major TNF-α sheddase (49–51). Other TNF family members, including APRIL (28), EDA (29), and BAFF (32–34), are proteolytically processed in the stalk region by furin, a member of the proprotein convertase family of membrane-associated serine proteases that cleave after basic RX(R/K/X)R motifs (16–18). Furin processing of these three proteins can occur intracellularly (28, 29, 34) or on the cell surface (31–34).

TWEAK was first described by Chicheportiche et al. (4) in 1997 as a new member of the TNF superfamily with weak apoptotic activity on certain tumor cell lines. These authors overexpressed full-length TWEAK in HEK293-EBNA cells and detected both a membrane-anchored form and a smaller, secreted form, suggesting that TWEAK, like the other TNF-like cytokines mentioned above, was a substrate for proteolytic processing. It is now recognized that TWEAK is expressed in both membrane-bound and soluble forms in diverse cell types and that the soluble form is biologically active (4, 13), but there has been no published information regarding the TWEAK processing mechanism in mammalian cells and only one prior report investigating the functional capacity of membrane TWEAK (13). We have addressed these important issues in the present study.

The human TWEAK stalk region contains putative proprotein convertase recognition motifs at residues 90–93 (RPRR) and 102–105 (RARR), with cleavage predicted to occur at each motif after the terminal arginine residue. These same two sequence motifs are found in the predicted rhesus monkey, horse, pig, and cow TWEAK sequences (63) (BLAST analysis); in contrast, the predicted mouse and rat TWEAK sequences have the motif 1 sequence duplicated in motif 2 (4) (BLAST analysis), and the predicted zebrafish TWEAK stalk region has a single proprotein convertase-processing motif (RFRR) (64). We tested whether TWEAK was indeed a proprotein convertase substrate by transfecting HEK293 cells with a full-length TWEAK-Myc expression plasmid and then treating the transfected cells with CMK, a proprotein convertase inhibitor (23–25). TWEAK processing was abrogated by CMK but not by the metalloprotease inhibitors GM6001 (26) or TAPI (27). We also demonstrated that full-length TWEAK was not processed in a cell line that produces enzymatically inactive furin, but processing could be reconstituted in these cells by ectopic expression of active furin. Taken together, these results indicate that the proprotein convertase family member furin can cleave full-length TWEAK, but they do not rule out the possibility that one or more of the other six proprotein convertase family members could also be effective processing enzymes. Because LoVo cells express PACE4 and PC7 (65, 66), TWEAK does not appear to be a substrate for these two proprotein convertases.

We constructed expression plasmids encoding TWEAK mutants missing one or both of the putative furin recognition motifs and transfected them into HEK293 cells in order to identify the TWEAK processing site(s). We found that deletion of motif 1 (RPRR) but not motif 2 (RARR) prevented TWEAK processing, indicating that arginine 93, not arginine 105, was the predominant cleavage site in these cells. The precise reason for preferential processing at this site is not known, but motif 1 does have more favorable flanking residues for effective proprotein convertase cleavage (18). Our finding that the RPRR motif is required for full-length TWEAK cleavage in mammalian cells is consistent with earlier data reported by Chicheportiche et al. (4). These authors overexpressed the TWEAK extracellular domain in insect cells, purified the secreted TWEAK isoform, and performed N-terminal sequence analysis. This analysis revealed that TWEAK was cleaved in the stalk region following arginine 93.
Furin undergoes autoactivation in the trans-Golgi network, and although many substrates are cleaved in this same compartment, furin cycles between the trans-Golgi network, the cell surface, and early endosomes (16, 17, 37). We found using several experimental approaches that TWEAK is likely to be processed during its biosynthesis. This finding, in combination with those described earlier by Chicheportiche et al. (4), indicate that although some proportion of the full-length TWEAK synthesized in a cell is inserted into the plasma membrane, some is processed by a furin convertase inside the cell, most likely in the trans-Golgi network, and then the C-terminal fragment containing the TNF homology domain is secreted into the extracellular milieu.

Membrane-bound TWEAK has been detected on a variety of cell types by FACS analysis (13–15, 20, 42–47), and it has generally been assumed that this protein can trigger Fn14-mediated signaling, but there has only been one prior study investigating the functional activity of membrane TWEAK. In this previous report, Nakayama et al. (13) transfected a mouse B lymphoma cell line with an expression plasmid encoding full-length, wild type TWEAK and isolated a stable cell line to serve as the effector cells in co-culture experiments. The target cells were a human tumor cell line sensitive to soluble TWEAK-triggered cell death. Although these authors found that the full-length TWEAK-producing effector cells could kill the target cells in this co-culture assay, they also reported that the cells released soluble TWEAK with cytotoxic activity; therefore, with this experimental design, one cannot definitively conclude that membrane-bound TWEAK was biologically active. Here, we report that this TWEAK isoform is indeed functional using two different experimental approaches. In the first assay, we transiently transfected an expression plasmid encoding a non-cleavable TWEAK mutant into Fn14-positive HEK293 NF-κB-luciferase reporter cells and found that membrane TWEAK expression stimulated NF-κB activity. In the second assay, we conducted co-culture experiments using stably transfected cells that expressed this same TWEAK mutant as the effector cells and the NF-κB-luciferase reporter cell line as the target cells. We found that membrane-anchored TWEAK could act on neighboring Fn14-positive cells in our co-culture assays, indicating that this TWEAK isoform can bind Fn14 and act as a juxtacrine signaling molecule. It should be mentioned that although we did not detect full-length TWEAK processing in these effector cells by Western blot analysis, we could detect a low level of immunoreactive TWEAK protein in effector cell conditioned medium by ELISA. This suggests that there may be a low level of TWEAK shedding from the plasma membrane; alternatively, intracellular or membrane-associated TWEAK may have been released into the effector cell medium due to cell injury or death occurring during the 48-h incubation period. In any case, even if one assumes that all of the TWEAK detected by our ELISA is in a biologically active form, the TWEAK concentration in the medium was 1.9 ng/ml (line 9) and 3.3 ng/ml (line 8), which is too low for effective stimulation of the engineered NF-κB reporter cells (data not shown). Also, it should be noted that for the effector cell ELISA, we used 48-h conditioned medium samples, but in the juxtacrine signaling assays the effector and target cells are only together for a 6-h incubation period.

We do not know at this time whether the cell surface-bound and secreted TWEAK isoforms have comparable affinities for the Fn14 receptor or comparable biological activity. Studies investigating this issue with respect to other TNF family members have indicated that this depends on the specific family member. For example, both membrane-bound and soluble BAFF are effective costimulators of B cell growth (33), but the membrane form of FasL is a more potent pro-apoptotic factor than the soluble form (67–69). Also, the membrane-bound and secreted forms of TNF-α can trigger different cellular responses (70–75).

The TWEAK/Fn14 axis has been implicated in the pathogenesis of several diseases, including acute ischemic stroke (22, 76, 77) and rheumatoid arthritis (10, 11); accordingly, there are ongoing efforts to develop effective TWEAK antagonists for potential clinical application. Our finding that TWEAK can act in a juxtacrine manner may be important for TWEAK function in these pathological settings. Thus, we propose that the most effective antagonists will be those that can block the functional activity of both the membrane-anchored and soluble TWEAK forms.

Acknowledgments—We thank Jana Slovic, Emily Cheng, Kausal Asrani, Nichole Charlton, and Heather Hanscom for excellent technical assistance and Dr. Mark Williams for help with the FACS analysis. We also thank Sue Robinson for providing the anti-Myc tag antibody, Dr. Andrew Kung for providing the U87 cell line, Dr. Linda Burcky for providing the anti-TWEAK antibody, and Dr. J. Evan Sadler for providing the furin expression plasmid.

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Full-length TWEAK Processing and Activity

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