The Fn14 cytoplasmic tail binds tumour-necrosis-factor-receptor-associated factors 1, 2, 3 and 5 and mediates nuclear factor-κB activation

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Fn14 is a growth-factor-inducible immediate-early-response gene encoding a 102-amino-acid type I transmembrane protein. The human Fn14 protein was recently identified as a cell-surface receptor for the tumour necrosis factor (TNF) superfamily member named TWEAK (TNF-like weak inducer of apoptosis). In the present paper, we report that the human TWEAK extracellular domain can also bind the murine Fn14 protein. Furthermore, site-specific mutagenesis and directed yeast two-hybrid interaction assays revealed that the TNFR-associated factor (TRAF) 1, 2, 3 and 5 adaptor molecules bind the murine Fn14 cytoplasmic tail at an overlapping, but non-identical, amino acid sequence motif. We also found that TWEAK treatment of quiescent NIH 3T3 cells stimulates inhibitory κBα phosphorylation and transcriptional activation of a nuclear factor-κB (NF-κB) enhancer/luciferase reporter construct. Fn14 overexpression in transiently transfected NIH 3T3 cells also promotes NF-κB activation, and this cellular response requires an intact TRAF binding site. These results indicate that Fn14 is a functional TWEAK receptor that can associate with four distinct TRAF family members and stimulate the NF-κB transcription factor signalling pathway.

Key words: cytokine, Fn14, signal transduction, tumour necrosis factor (TNF), tumour-necrosis-factor-like weak inducer of apoptosis (TWEAK).

INTRODUCTION

The tumour necrosis factor (TNF) superfamily of structurally related cytokines currently consists of 19 type II transmembrane proteins that are involved in the regulation of cellular survival, proliferation, differentiation and apoptosis [1,2]. These proteins have approx. 20–30% sequence identity in their extracellular, receptor-binding domain, generally function as homotrimers and, in many cases, exist as both membrane-associated and soluble forms. TNF-like weak inducer of apoptosis (TWEAK), also known as Apo3L [3], was first described in 1997 as a broadly expressed TNF-related cytokine with weak apoptotic activity [4]. Subsequent studies have indicated that TWEAK can induce both apoptosis and necrosis, but only in certain human tumour cell lines and under the appropriate experimental conditions [3–7]. In some cell types, for example endothelial cells [7–9] and astrocytes [10], TWEAK promotes cell proliferation, not death. Furthermore, TWEAK displays pro-inflammatory properties when added to human endothelial cells [8,9], astrocytes [11], fibroblasts [12] and synoviocytes [12] cultured *in vitro* and can stimulate blood-vessel formation (angiogenesis) *in vivo* [8].

The biological effects of TNF-like cytokines are mediated via binding to one or more members of the TNF receptor (TNFR) superfamily of type I transmembrane proteins [1,2]. TNFR superfamily members have only approx. 25% overall sequence identity; however, the extracellular, ligand-binding domains of these receptors all contain one or more repeats of an approx. 40-amino acid (aa) cysteine-rich subdomain. One group of TNFR superfamily members, including TNFR-1, Fas, death receptor 3 (DR3), DR4 and DR5, contain an approx. 80-aa region in their cytoplasmic tails termed the death domain (DD). The DD serves as a docking site for DD-containing adaptor proteins, such as Fas-associated DD (FADD) and TNFR-1-associated DD (TRADD), and the recruitment of these proteins promotes caspase activation and programmed cell death [1,13]. Most of the remaining TNFR superfamily members contain short sequence motifs in their cytoplasmic domains that serve as binding sites for another group of cytoplasmic adaptor molecules called TNFR-associated factors (TRAFs). Six structurally related TRAFs have been identified at the present time [14,15]. The recruitment of one or more of these adaptor proteins to TRAF-binding sites result in the activation of signalling pathways that generally function to promote cellular survival and proliferation [14,15].

TWEAK was originally described as a ligand for the DD-containing receptor named Apo3/DR3/TRAMP (TNFR-related apoptosis-mediated protein) [3], but subsequent studies by several groups were unable to detect this interaction [8,16–18]. Recently, an expression-screening strategy employing a human endothelial cell cDNA-expression library was successfully used to isolate a cDNA clone encoding a TWEAK-binding cell-surface molecule [16]. DNA sequence analysis revealed that the predicted protein sequence was identical to that of Fn14, a 102-aa type I trans-

Abbreviations used: aa, amino acid(s); AcNPV, *Autographa californica* nuclear polyhedrosis virus; AP-1, activator protein-1; CRE, cAMP-response element; DD, death domain; DMEM, Dulbecco's modified Eagle's medium; DR, death receptor; ECL®, enhanced chemiluminescence; ERK, extracellular-signal-regulated kinase; FBS, foetal bovine serum; Fn14-tCT, Fn14 construct encoding Fn14 with a truncated cytoplasmic tail; Fn14-WT, Fn14 construct encoding wild-type Fn14; HA, haemagglutinin; HRP, horseradish peroxidase; IκB, inhibitory κB; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; p38 MAPK, p38 mitogen-activated protein kinase; pHLZ, pHybLex/Zeo; SRE, serum-response element; TNF, tumour necrosis factor; TNFR, TNF receptor; TRAF, TNFR-associated factor; TWEAK, TNF-like weak inducer of apoptosis.

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membrane protein originally discovered during a search for growth-factor-inducible genes in NIH 3T3 fibroblasts [19]. Human and murine Fn14 are closely related, with approx. 82% overall sequence identity, and have structural features characteristic of TNFR superfamily members, including an approx. 40-aa cysteine-rich region in the extracellular domain and a putative TRAF-binding site in the cytoplasmic tail [16,19,20].

In the present paper, we report that the human TWEAK extracellular domain can bind murine Fn14. We also demonstrate that four of the six known TRAF proteins interact with the murine Fn14 cytoplasmic tail, and identify the aa residues that are important for TRAF binding. Results are also presented indicating that TWEAK addition or Fn14 overexpression can promote nuclear factor-κB (NF-κB) activation in NIH 3T3 cells.

MATERIALS AND METHODS

Cell culture

Fall armyworm *Spodoptera frugiperda* Sf9 cells [American Type Culture Collection (ATCC), Manassas, VA, U.S.A.] were grown in TNM-FH (Grace's insect medium; Invitrogen, Carlsbad, CA, U.S.A.), supplemented with lactalbumin hydrolysate, L-glutamine, TC-yeastolate and 10% (v/v) foetal bovine serum (FBS; Hyclone, Logan, UT, U.S.A.) at 27 °C and ambient air. NIH 3T3 (ATCC) and NIH 3T3 Fn14–haemagglutinin (HA) #10 fibroblasts [19] were grown in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Herndon, VA, U.S.A.), 10% (v/v) calf serum (Hyclone) and 2 mM glutamine (Mediatech) at 37 °C and 5% CO_2.

Fn14 expression in Sf9 cells

The plasmid pVL1393/Fn14, which encodes full-length murine Fn14, was constructed as follows. PCR was performed using the plasmid pBluescript/mFn14 as the template [19], a sense primer containing a 5′ *Bam*HI restriction site followed by Fn14 nucleotides 13–35, an antisense primer complementary to a 5′ *Xba*I restriction site followed by Fn14 nucleotides 394–416, and Vent Polymerase (New England Biolabs, Beverly, MA, U.S.A.). The DNA product was isolated, ligated into pPCR-Script using a PCR fragment-cloning kit (Stratagene, La Jolla, CA, U.S.A.), released by *Bam*HI/*Xba*I digestion, and subcloned into the baculovirus transfer vector pVL1393 (BD Pharmingen, San Diego, CA, U.S.A.). This construct was sequenced to confirm that no mutations were introduced during the PCR step. The transfer plasmid was co-transfected with Baculogold DNA (BD Pharmingen) into Sf9 cells using the Baculogold Transfection Kit (BD Pharmingen) according to the manufacturer's instructions. Recombinant viral plaques were detected by Western blot analysis using an anti-Fn14 polyclonal antibody [19]. Recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV) was purified by two rounds of plaque assays and single recombinant plaques were used to produce a large volume, high-titre stock as described in the BD Pharmingen Baculovirus Expression System instruction manual.

Ligand and Western blot analysis of Fn14 expressed in Sf9 cells

Uninfected, AcNPV-infected or AcNPV/Fn14-infected Sf9 cells were harvested at 48 h post-infection and incubated for 5 min at 4 °C in RIPA lysis buffer [20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 10% (v/v) glycerol, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 2 mM EDTA, 1% (v/v) Nonidet P40 and 1× protease inhibitor cocktail (Sigma, St. Louis, MO, U.S.A.)]. Lysates were clarified by centrifugation at 13000 ***g*** for 5 min at 22 °C and equal amounts of each cell lysate were either mixed with 2× gel loading buffer [100 mM Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.2% (w/v) Bromophenol Blue] and heated at 95 °C for 10 min (reduced conditions) or mixed with 2× gel loading buffer prepared without 2-mercaptoethanol and not heated (non-reduced conditions) prior to loading on a 15% polyacrylamide gel. Proteins were transferred to Protran nitrocellulose membranes (Schleicher and Schuell, Keene, NH, U.S.A.) and the membranes were stained with Ponceau S (Sigma) to verify that equal amounts of protein were present in each gel lane. The membrane used to detect Fn14 expression was blocked for 15 h at 4 °C in TBST buffer [50 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.05% (v/v) Tween 20] containing 5% (w/v) non-fat dried milk powder and then incubated for 1 h at room temperature (22 °C) in TBST containing 5% BSA and a 1:500 dilution of rabbit anti-Fn14 polyclonal antiserum [19]. The membrane was then washed three times in TBST and incubated for 1 h at room temperature in TBST containing 5% (w/v) non-fat dried milk powder and a 1:10000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig antibody (Santa Cruz, CA, U.S.A.). The membranes used for ligand blot analysis were first blocked overnight in TBST containing 2.5% (w/v) non-fat dried milk powder and then incubated for 1 h at room temperature in either the absence or presence of 200 ng/ml human recombinant TWEAK (a gift from Dr T. Zheng, Biogen Inc., Cambridge, MA, U.S.A.). The membranes were washed three times in TBST and incubated for 1 h at room temperature in TBST containing 2.5% (w/v) non-fat dried milk powder and 1 μg/ml rabbit anti-TWEAK IgG [4] (also kindly provided by Dr Zheng). The membranes were washed again and then incubated for 1 h at room temperature in TBST containing 2.5% (w/v) non-fat dried milk powder and a 1:2000 dilution of goat anti-rabbit Ig–HRP (Santa Cruz). For all membranes, bound secondary antibodies were detected using the enhanced chemiluminescence (ECL®) system (Amersham Life Science, Piscataway, NJ, U.S.A.).

Construction of the Fn14 and TRAF plasmids for yeast two-hybrid analysis

The PCR overlap extension method [21] was used to clone mutated forms of the murine Fn14 cytoplasmic tail nucleotide sequence (encoding aa residues 102–129) into the vector pPCR-Script (Stratagene). Vent Polymerase was used to minimize the possibility of PCR misincorporation errors and all constructs were verified by DNA sequence analysis. PCR was performed using the plasmid pcDNAIneo/Fn14–HA as the template [19], a sense primer consisting of Fn14 nucleotides 13–27, an antisense primer complementary to Fn14 nucleotides 397–414 and, to generate the mutant plasmids, three additional reactions were performed in the presence of one of the following internal primers: 5′-AAAGTTTACTGCCCCCATAG-3′ (encoding the $Thr^{112} \rightarrow Ala$ substitution), 5′-TACCCCCATAGCGGCGACTGGTGGAG-3′ (encoding the $Glu^{115} \rightarrow Ala$ and $Glu^{116} \rightarrow Ala$ substitutions), or 5′-CATAGAGGAGGCTGGTGGAG-3′ (encoding the $Thr^{117} \rightarrow Ala$ substitution). The DNA products were isolated and ligated into pPCR-Script using a PCR fragment cloning kit (Stratagene). PCR was then performed using each of the four pPCR-Script plasmids as the template, a sense primer consisting of Fn14 nucleotides 328–345, and the antisense primer described above. The four DNA products were then isolated and cloned into the *Pvu*II site of the yeast two-hybrid bait vector pHybLex/Zeo (pHLZ; Invitrogen). The control vector pACT and the prey plasmids pACT/human TRAF1, pACT/human TRAF2 and pACT/human TRAF3 [22] were

kindly provided by Dr R. Arch (Washington University School of Medicine, St. Louis, MO, U.S.A.). pACT/human TRAF4 was constructed by *Eco*RI digestion of the plasmid pcDNA3myc/TRAF4 [23] (a gift from Dr C. Duckett, University of Michigan Medical School, Ann Arbor, MI, U.S.A.), blunt ending of this site using Klenow Polymerase (Roche, Indianapolis, IN, U.S.A.), and release of the insert by *Xho*I digestion. This fragment was isolated and then ligated into the vector pACT that had been first digested with *Bam*HI, blunt-ended, and digested with *Xho*I. pACT/murine TRAF5 was constructed exactly as described above using the plasmid pcDNA3myc/TRAF5 [23] (also kindly provided by Dr Duckett). pACT/human TRAF6 was constructed by *Hin*dIII/*Bgl*II digestion of the plasmid pFLAG/TRAF6 [23] (also from Dr Duckett) and then blunt-ending of the isolated insert. This DNA fragment was ligated into the vector pACT that had been digested with *Bam*HI and blunt-ended using Klenow Polymerase. All three of these prey plasmid constructs were verified by DNA sequence analysis.

Yeast two-hybrid analysis

Directed yeast two-hybrid protein interaction assays were performed essentially as described in the Hybrid Hunter instruction manual (Invitrogen). Briefly, L40 yeast cells were transformed with the appropriate pHLZ and pACT plasmids, grown for 3 h at 30 °C with shaking, and then cultured on plates either lacking leucine and containing 300 μg/ml zeocin (Invitrogen) or lacking leucine and histidine and containing 300 μg/ml zeocin. After 5 days at 30 °C, colonies on the first set of plates were transferred on to nitrocellulose circles (Schleicher and Schuell) and assayed for β-galactosidase activity. Protein–protein interactions were considered to be positive only if cells grew in the absence of histidine and β-galactosidase expression was apparent within 2 h of adding substrate.

Fn14-WT-myc and Fn14-tCT-myc expression in NIH 3T3 cells

The Fn14 expression plasmids pSecTag2/Fn14-WT-myc, encoding murine Fn14 aa residues 30–129 [19] and pSecTag2/Fn14-tCT-myc, encoding Fn14 aa residues 30–111, were constructed as follows. PCR was performed using pPCR-Script/Fn14-myc–HA as the template, *Taq* DNA polymerase (Roche), a sense primer containing a 5′ *Asc*I restriction site followed by 17 nucleotides encoding part of the c-myc epitope coding sequence [24], and one of two antisense primers. The antisense primer used for PCR amplification of the Fn14 construct encoding Fn14 with the entire Fn14 cytoplasmic tail (Fn14-WT) contained a 5′ sequence complementary to an *Xho*I restriction site followed by sequence complementary to Fn14 nucleotides 397–414. The antisense primer used for PCR amplification of the Fn14 construct encoding Fn14 with a truncated cytoplasmic tail (Fn14-tCT) also contained a 5′ *Xho*I site but this was followed by sequence complementary to Fn14 nucleotides 344–357 and a TGA translation-stop codon. The two DNA products were isolated, ligated into pCR2.1 using a T/A cloning kit (Invitrogen), released by *Asc*I/*Xho*I digestion, and subcloned into pSecTag2A/Hygro (Invitrogen) that had been digested with the same two enzymes. Constructs were verified by DNA sequence analysis. NIH 3T3 cells were transfected with the pSecTag2A vector, the pSecTag2A/Fn14-WT-myc plasmid, or the pSecTag2A/Fn14-tCT-myc plasmid using LIPOFECTAMINE™ Plus (Invitrogen) according to the manufacturer's instructions. At 48 h post-transfection, the cells were harvested and incubated for 10 min at 4 °C in TNEN lysis buffer [50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 % (v/v) Nonidet P40]. Lysates were clarified by centrifugation at 13000 ***g*** for 5 min at 22 °C and protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.). Equal amounts of each protein sample were subjected to SDS/PAGE (15 % gels) and Western blot analysis was performed as described above using a rabbit anti-myc epitope antibody (1:500 dilution; Santa Cruz) as the primary antibody and goat anti-rabbit Ig–HRP (1:10000; Santa Cruz) as the secondary antibody. Bound secondary antibodies were detected using the ECL® system.

Western blot analysis of TWEAK-treated NIH 3T3 cells

Normal NIH 3T3 cells or stably transfected NIH 3T3 cells which overexpress HA-epitope-tagged Fn14 [19] were cultured in normal growth medium and then placed in DMEM, 0.2 % FBS and 2 mM glutamine for 24 h. Serum-starved cells were then either left untreated or treated with 50 ng/ml TWEAK (PeproTech, Rocky Hill, NJ, U.S.A.) for various lengths of time. Cells were harvested and cytoplasmic and nuclear fractions were isolated using the NE-PER® Nuclear and Cytoplasmic Extraction Kit (Pierce), according to the manufacturer's instructions except that the lysis solution was supplemented with various phosphatase inhibitors (Cocktail Kits I and II; Calbiochem, La Jolla, CA, U.S.A.). Protein concentrations in both subcellular fractions were determined as described above. Equal amounts of each cytoplasmic sample were reduced and denatured and then subjected to SDS/PAGE using a 4–12 % Bis-Tris NuPage gel (Invitrogen). Western blot analysis for total inhibitory κBα (IκBα) and phosphorylated IκBα was performed using the PhosphoPlus IκBα (Ser^{32}) Antibody Kit (Cell Signaling, Beverly, MA, U.S.A.), according to the manufacturer's instructions. We also confirmed that equal amounts of protein were present in each gel lane by Western blot analysis using either a 1:1000 dilution of the rat anti-HA epitope monoclonal antibody clone 3F10 (Roche) and a 1:5000 dilution of goat anti-rat Ig–HRP antibody (Santa Cruz) or a 1:500 dilution of the mouse anti-actin monoclonal antibody clone AC-40 (Sigma) and a 1:5000 dilution of goat anti-mouse Ig–HRP antibody (Santa Cruz). Bound secondary antibodies were detected using the ECL® system. Autoradiographic signals were quantified by densitometry and the amount of phosphorylated IκBα protein in each lane was normalized to the amount of Fn14–HA or actin protein to correct for slight differences in protein loading.

Luciferase reporter assays

NIH 3T3 cells were plated in 12-well cluster dishes and then incubated in normal growth medium for 24–48 h until they reached approx. 70 % confluence. Cells were transfected with 0.4 μg or 2.0 μg of either the pSecTag2A/Hygro (control vector), pSecTag2A/Fn14-WT-myc, or pSecTag2A/Fn14-tCT-myc plasmids, 0.3 μg of either the pTAL-Luc (enhancerless control plasmid), pAP1-Luc, pCRE-Luc, pNFκB-Luc or pSRE-Luc reporter plasmids included in the Mercury Pathway Profiling Luciferase System (BD Biosciences Clontech, Palo Alto, CA, U.S.A.) and 0.1 μg of pCMV/β-gal plasmid (Tropix, Bedford, MA, U.S.A.) using LIPOFECTAMINE™ Plus (Invitrogen) according to the manufacturer's instructions. At 3 h post-transfection, the medium was replaced with DMEM supplemented with 0.5 % calf serum. In some cases, the cells were treated with 50 ng/ml TWEAK (PeproTech), 50 ng/ml PMA (Calbiochem), 25 μM forskolin (Biomol, Plymouth Meeting, PA, U.S.A.) or 10 % calf serum. All cells were harvested at 48 h after transfection,

washed with PBS, and lysed in Galacto-Star lysis buffer (Tropix). An aliquot of each lysate was analysed in a Dynatech luminometer (Chantilly, VA, U.S.A.). Luciferase activity was measured using the Luciferase Assay Kit (Applied Biosystems, Foster City, CA, U.S.A.) and β-galactosidase activity was measured using the Galacto-Star Chemiluminescent Reporter Gene Assay System (Tropix). Light production was measured for 1 s and the results were expressed as relative light units. Luciferase activity was normalized to β-galactosidase activity to control for differences in transfection efficiency.

RESULTS

Human TWEAK can bind the murine Fn14 protein

Human TWEAK has been shown to bind a human Fn14 extracellular domain–Fc chimaeric protein with an interaction affinity constant of approx. 2.3 nM [16]. Since the human and murine Fn14 extracellular domains are highly homologous [20], we predicted that human TWEAK would also bind the murine Fn14 receptor. This was tested in a qualitative manner by determining whether a soluble human TWEAK extracellular domain fragment could interact with murine Fn14 immobilized on a membrane (i.e. ligand blot analysis). A baculovirus expression vector encoding full-length murine Fn14 was constructed and insect cells were either left uninfected, infected with wild-type virus or infected with the Fn14 virus. Cell lysates were prepared at 48 h post-infection and equal amounts of protein were analysed by SDS/PAGE. The three protein samples were either left untreated (non-reduced conditions) or treated with 2-mercaptoethanol and heat-denatured (reduced conditions) prior to gel loading. We found by Western blot analysis that reduced and denatured Fn14 migrated as a single immunoreactive protein of approx. 23 kDa, but non-reduced, undenatured Fn14 migrated at apparent molecular masses of approx. 39, 28.5, 20, and 18 kDa (Figure 1A). When a duplicate blot was incubated sequentially with recombinant TWEAK and then anti-TWEAK antibodies, proteins of the same apparent molecular mass were detected (Figure 1B). These proteins were not detected when a third blot was incubated with the anti-TWEAK antibodies alone (Figure 1C).

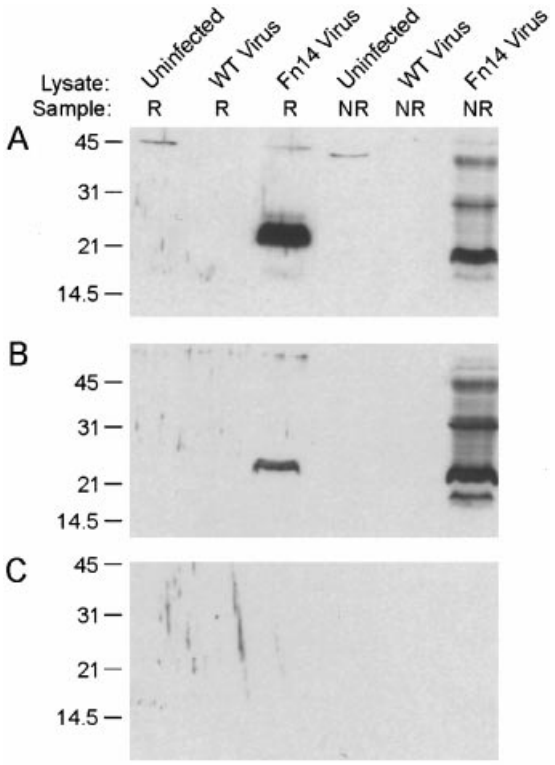

Figure 1 Ligand blot analysis using human recombinant TWEAK (extracellular domain)

Cell lysates were prepared from uninfected insect cells, wild-type (WT) baculovirus-infected cells and Fn14 baculovirus-infected cells and equal amounts of protein were analysed by SDS/PAGE under either reducing (R) or non-reducing (NR) conditions. Three identical gels were transferred on to nitrocellulose membranes and then subjected to either Western blot analysis using anti-Fn14 antibodies (**A**), ligand blot analysis using recombinant TWEAK followed by anti-TWEAK antibodies (**B**) or, as a control for (**B**), Western blot analysis using anti-TWEAK antibodies (**C**).

TRAFs 1, 2, 3 and 5 associate with the murine Fn14 cytoplasmic tail

The majority of the TNFR superfamily members that have been identified at the present time associate with TRAFs, a family of cytoplasmic adaptor proteins that activate downstream signal transduction molecules [14,15]. TRAFs 1, 2 and 3 have been previously shown to bind the human Fn14 cytoplasmic tail [16]; however, in this earlier study, an *in vitro* binding assay was utilized, the ability of TRAF5 to bind Fn14 was not evaluated, and the TRAF-binding site(s) was not identified. We have combined a site-directed mutagenesis approach with directed yeast two-hybrid analysis to investigate TRAF binding to the murine Fn14 cytoplasmic tail. A major aa consensus sequence motif for TRAF2 binding, (Pro/Ser/Ala/Thr)-Xaa-(Gln/Glu)-Glu, has been identified by Ye et al. [25] and the key residues of TRAF2 that recognize this motif are also conserved in TRAFs 1, 3 and 5 [26]. The human and murine Fn14 cytoplasmic tail contains the sequence Pro-Ile-Glu-Glu, a perfect match with this consensus sequence (Figure 2A). Therefore, to test whether this was the Fn14 TRAF-binding site, we constructed bait plasmids encoding either the wild-type Fn14 cytoplasmic tail or a mutant Fn14 tail, designated $Glu^{115/116} \rightarrow Ala$, in which the two most highly conserved positions in the consensus motif shown above, the glutamic acid residues, were replaced with alanine residues. We also made two additional bait plasmids, designated $Thr^{112} \rightarrow Ala$ and $Thr^{117} \rightarrow Ala$, to determine whether one or both of the threonine residues flanking the putative TRAF-binding site are also involved in Fn14–TRAF association. We found that TRAFs 1, 2, 3 and 5 could bind to the wild-type Fn14 cytoplasmic tail, and all of these interactions were completely dependent on the presence of the Glu-Glu motif (Figure 2B). These same four TRAF family members were also able to bind the Fn14 $Thr^{112} \rightarrow Ala$ mutant tail; in contrast, only TRAF3 could bind the Fn14 $Thr^{117} \rightarrow Ala$ mutant tail.

TWEAK treatment of either Fn14-overexpressing NIH 3T3 cells or normal, untransfected NIH 3T3 cells promotes IκBα phosphorylation

Ligand binding to TNFR superfamily members that associate with TRAF adaptor molecules has been shown to promote the activation of numerous intracellular-signal-transduction cascades, including those involving the IκB kinase that is responsible for NF-κB activation and the protein kinases c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK) and extracellular-signal-regulated kinase (ERK) that are responsible for c-Jun, ATF-2 and Elk-1 phosphorylation [1,14,15,27]. We first evaluated whether TWEAK binding to the Fn14 receptor can activate the NF-κB signalling pathway using several

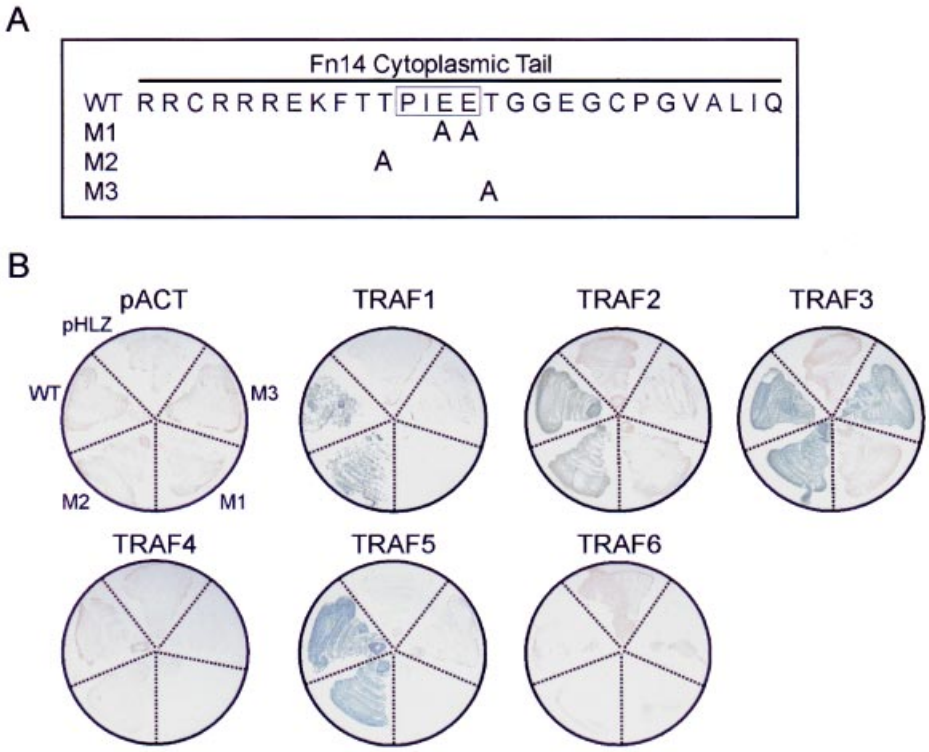

Figure 2 Binding of TRAFs to the murine Fn14 cytoplasmic tail in yeast cells

(**A**) The wild-type (WT) murine Fn14 cytoplasmic tail aa sequence is shown and a motif corresponding to a major consensus sequence for TRAF binding to TNFR family members is boxed. The positions of aa substitutions in the Fn14 cytoplasmic tail mutants M1, M2 and M3 are indicated. (**B**) Representative filters from yeast two-hybrid β-galactosidase overlay assays are shown, where the blue colonies indicate *lacZ* activation. The yeast were co-transformed with plasmids that expressed the LexA DNA-binding domain alone (pHLZ) or were fused to the wild-type (WT) or mutated (M) Fn14 cytoplasmic tail, in combination with plasmids that expressed the B42 activation domain alone (pACT) or were fused to the TRAF1, TRAF2, TRAF3, TRAF4, TRAF5 or TRAF6 proteins. Cells were then screened as described in the Materials and methods section.

different experimental approaches. NF-κB/Rel transcription factor family members are present in the cytoplasm of most cell types in an inactive form associated with IκBα proteins [28,29]. NF-κB activation occurs via signal-induced phosphorylation of IκBα at serine residues 32 and 36, resulting in proteosome-mediated IκBα degradation and nuclear translocation of NF-κB [28,29]. Therefore, we initially determined whether TWEAK could stimulate IκBα phosphorylation when added to either transfected NIH 3T3 Fn14–HA #10 cells that constitutively overexpress the Fn14 receptor [19] or untransfected NIH 3T3 cells that express normal levels of Fn14. Serum-starved cells were treated with TWEAK for various lengths of time and then harvested. Since IκBα phosphorylation occurs in the cytoplasm, a subcellular fractionation protocol was utilized to separate cytoplasmic (and membrane) proteins from nuclear proteins, and then the former fraction was analysed by SDS/PAGE and Western blot analysis using anti-(phospho-IκBα) antibodies. Some phosphorylated IκBα protein was detected in unstimulated Fn14–HA #10 cells, suggesting that Fn14 overexpression in this cell line was promoting a low level of ligand-independent NF-κB pathway activation (Figure 3A). Nevertheless, an increased amount of phosphorylated IκBα (approx. 3.1-fold induction over the baseline level) was detected at 10 min after TWEAK addition. The amount of phosphorylated IκBα protein then decreased, as expected, but complete degradation was not observed since there is a basal level of IκBα phosphorylation in this transfected cell line.

TWEAK addition to normal, untransfected NIH 3T3 cells also resulted in a rapid and transient increase in the level of phosphorylated IκBα protein (Figure 3B). An increased amount of phosphorylated IκBα was detected as early as 5 min after TWEAK addition, with maximal levels (approx. 2.2-fold induction over the baseline level) present at the 10 min time-point. Phosphorylated IκBα levels then returned to baseline by 30 min after TWEAK addition.

TWEAK addition or Fn14 overexpression can promote transcription from an NF-κB enhancer/luciferase reporter plasmid

The above results were consistent with the possibility that TWEAK binding to the Fn14 receptor results in NF-κB activation. Since previous studies have shown that many TNFR superfamily members can activate NF-κB when overexpressed in mammalian cells, an Fn14 overexpression strategy was employed to investigate further Fn14-mediated signal transduction. We used serum-starved NIH 3T3 cells for these experiments since they express a relatively low level of Fn14 [19]. Expression plasmids encoding either myc epitope-tagged wild-type Fn14 (Fn14-WT) or a myc epitope-tagged truncated Fn14 protein missing aa residues 112–129 (Fn14-tCT), a region including the

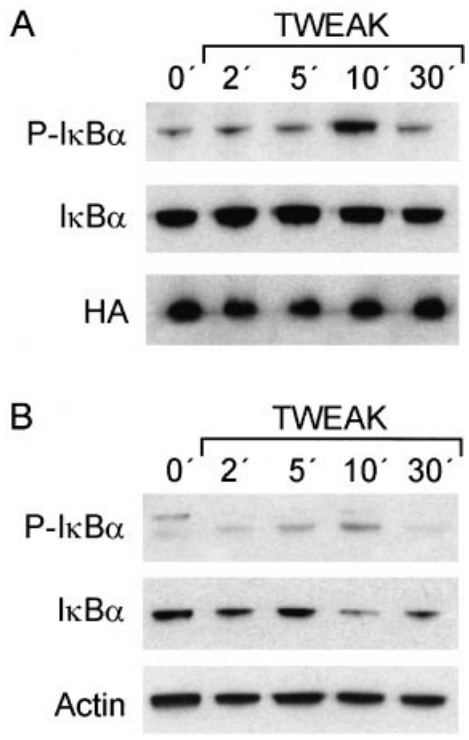

Figure 3 Effect of TWEAK addition on IκBα phosphorylation in Fn14-overexpressing and normal NIH 3T3 cells

(**A**) Serum-starved, stably transfected NIH 3T3 Fn14–HA #10 cells were either left untreated (0′) or treated with TWEAK for the indicated periods of time. The cells were harvested and equal amounts of the cytoplasmic protein subcellular fraction were analysed by SDS/PAGE and Western blot analysis using antibodies that detect either only the phosphorylated form of IκBα (P-IκBα), both unphosphorylated and phosphorylated forms of IκBα (IκBα) or the HA epitope (HA). (**B**) Serum-starved, untransfected NIH 3T3 cells were treated as described above and Western blot analysis conducted using the indicated antibodies.

TRAF-binding sequence motif described earlier (see Figure 2) were constructed (Figure 4A). When these plasmids were transfected into NIH 3T3 cells, Fn14 proteins of the appropriate apparent molecular mass were detected by Western blot analysis, and the wild-type and mutant proteins were expressed at similar levels (Figure 4B). To determine whether Fn14 overexpression could promote NF-κB transcriptional activity, NIH 3T3 cells were co-transfected with either a control vector or the Fn14-WT expression plasmid together with an NF-κB enhancer/luciferase reporter plasmid. Luciferase assays revealed that overexpression of Fn14 induced an approx. 2.5-fold increase in NF-κB transcriptional activity over the activation level detected in the vector-transfected cells; furthermore, when TWEAK was included in the culture medium of either the vector- or Fn14-WT plasmid-transfected cells, NF-κB activity was potentiated (Figure 4C). Fn14-mediated NF-κB activation was even more apparent when a greater dose of Fn14-WT expression plasmid was employed (approx. 4-fold activation over the level detected in vector-transfected cells), and this activity was totally dependent on co-transfection with the NF-κB enhancer/luciferase reporter plasmid (Figure 4D). Finally, transfection of NIH 3T3 cells with the Fn14-tCT expression plasmid failed to activate NF-κB, indicating that the region of the cytoplasmic tail containing the TRAF-binding site is essential for Fn14-mediated NF-κB activation (Figure 4D).

Fn14 overexpression does not activate the JNK, p38 MAPK, and/or ERK signalling pathways in NIH 3T3 cells

We performed additional transient transfection experiments using the Fn14-WT plasmid in combination with activator protein-1 (AP-1), cAMP-response element (CRE) or serum-response element (SRE) enhancer/luciferase reporter plasmids to investigate whether the JNK, p38 MAPK, and/or ERK signalling pathways were stimulated in response to Fn14 overexpression. Activation of these pathways was not detected; however, control experiments demonstrated that the reporter plasmids could be activated when the cells were treated with the appropriate pathway agonists (Figure 5). These results are consistent with Western blot data indicating that TWEAK stimulation of NIH 3T3 Fn14–HA #10 cells does not promote JNK, p38 MAPK, ERK or Elk-1 phosphorylation (H. N. Hanscom and J. A. Winkles, unpublished work).

DISCUSSION

Fn14 is a serum- and growth-factor-regulated gene that is expressed at elevated levels during liver regeneration and vessel wall repair *in vivo* [16,19,20]. It encodes a 102-aa plasma-membrane-anchored transmembrane protein and initial ectopic overexpression experiments suggested it could play a role in cell–extracellular matrix interactions [19]. A recent study demonstrated that human Fn14 is, in fact, a cell-surface receptor for the TNF superfamily member named TWEAK [16]. Indeed, subsequent analysis of the Fn14 aa sequence revealed that the predicted protein had features that are characteristic of TNFR superfamily members, including a cysteine-rich region in the extracellular domain and a putative TRAF-interaction motif in the cytoplasmic tail [16]. Here, we present results indicating that (i) human TWEAK can bind to murine Fn14, (ii) TRAFs 1, 2, 3 and 5 bind to an overlapping, but non-identical, aa sequence motif in the murine Fn14 cytoplasmic tail, and (iii) TWEAK treatment or Fn14 overexpression activates the NF-κB signalling pathway in NIH 3T3 cells.

The human TWEAK extracellular domain has been shown to bind human Fn14 using multiple experimental approaches [16]. The human and murine Fn14 extracellular domains are each 53 aa in length and have only four aa differences [20]; therefore, we predicted that human TWEAK would also bind the murine Fn14 receptor, and this was tested using a ligand blot assay. These experiments utilized murine Fn14 that was expressed in insect cells. Reduced and denatured Fn14 migrated at an apparent molecular mass significantly greater than its predicted mass (approx. 23 kDa compared with approx. 11 kDa), consistent with our Western blot results examining endogenous Fn14 or Fn14 overexpressed in transfected NIH 3T3 cells [19]. In contrast, 'native' Fn14 migrated as several distinct molecular-mass forms. Some TNFR superfamily members undergo oligomerization [1,2]; thus it is possible that the three major forms observed, approx. 20, 28.5, and 39 kDa in size, correspond to Fn14 monomers, dimers and trimers respectively. In any case, we found that TWEAK could bind to all of these native forms. Interestingly, some TWEAK binding to reduced and denatured Fn14 was also noted using the ligand blot assay, suggesting that TWEAK binding may not be entirely dependent on Fn14 disulphide bond formation.

TNFR superfamily members such as Fn14, which do not contain a DD in their cytoplasmic tail, signal via the recruitment of adaptor molecules known as TRAFs [14,15]. A major consensus sequence motif for TRAF2 binding, (Pro/Ser/Ala/Thr)-Xaa-(Gln/Glu)-Glu, was identified by Ye et al. [25] and the key

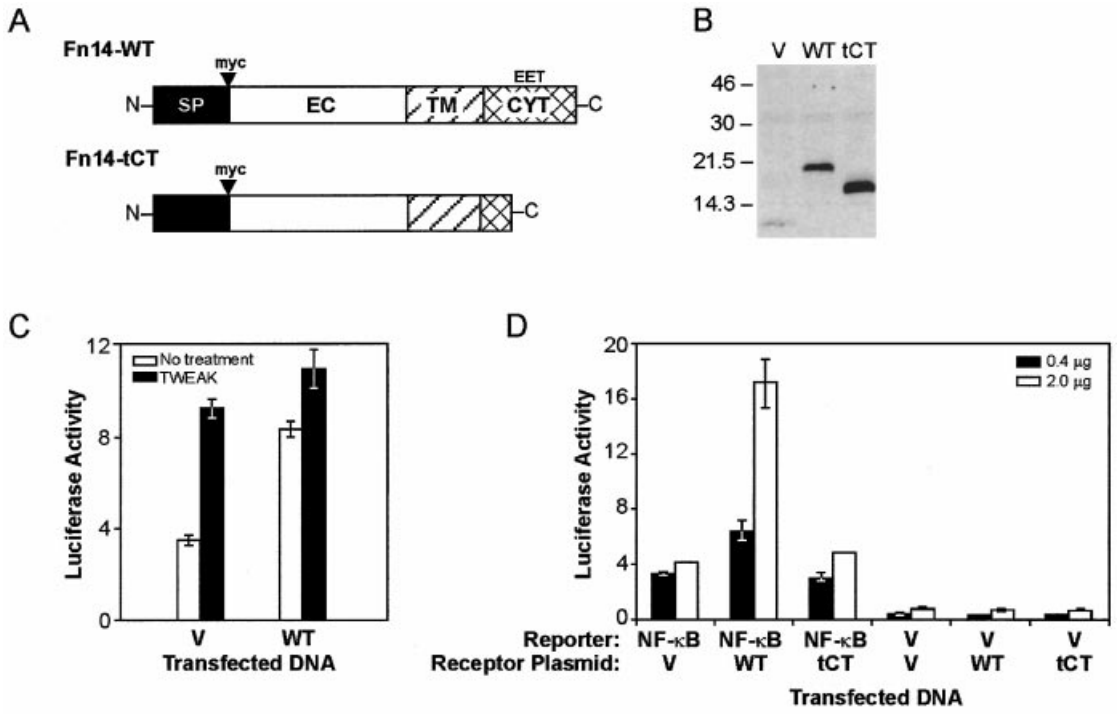

Figure 4 Effect of Fn14 overexpression on transcriptional activation of an NF-κB enhancer/luciferase reporter plasmid

(**A**) Schematic representation of the expression constructs encoding myc-epitope-tagged wild-type Fn14 (Fn14-WT) or an Fn14 protein with a truncated cytoplasmic tail (Fn14-tCT). The Fn14 signal peptide (SP), extracellular domain (EC), transmembrane domain (TM) and cytoplasmic tail (CYT) are indicated and the positions of the myc epitope and the three aa residues that are critical for TRAF-binding (EET) are shown. (**B**) Cell lysates were prepared from pSecTag2A vector (V)-transfected, Fn14-WT-transfected (WT), and Fn14-tCT-transfected (tCT) NIH 3T3 cells and equal amounts of protein were analysed by SDS/PAGE and Western blot analysis using an anti-myc antibody. (**C**) Cells were transfected with the NF-κB enhancer/luciferase reporter plasmid in combination with either the pSecTag2A vector (V) or the Fn14-WT (WT) plasmid. Cells were cultured for 48 h in either the absence or presence of TWEAK and then analysed for luciferase activity. (**D**) Cells were transfected with either the NF-κB enhancer/luciferase reporter plasmid or the enhancerless vector control in combination with two different concentrations of either the pSecTag2A vector (V), the Fn14-WT plasmid (WT) or the Fn14-tCT plasmid (tCT). Cells were cultured for 48 h and then analysed for luciferase activity. In all of the above transient transfection experiments, a plasmid encoding β-galactosidase was also transfected into the cells and luciferase values were normalized to β-galactosidase activity. Relative light units are expressed as means ± S.D. for triplicate wells from one representative experiment of three independent experiments.

residues of TRAF2 that recognize this motif are also conserved in TRAFs 1, 3 and 5 [26]. Both the human and murine Fn14 cytoplasmic tail sequences contain a matching motif, Pro-Ile-Glu-Glu, indicating that Fn14 could associate with TRAF1, TRAF2, TRAF3 and/or TRAF5. We used directed yeast two-hybrid analysis to demonstrate that all four of these TRAFs, but not TRAF4 nor TRAF6, bind to the murine Fn14 cytoplasmic tail. This finding is consistent with a previous report demonstrating that TRAFs 1, 2 and 3 can bind a glutathione S-transferase–human Fn14 cytoplasmic tail fusion protein *in vitro* [16]. Our inability to detect an interaction between the Fn14 tail and TRAF4 or TRAF6 was not unexpected as TRAF4 has been shown to interact with only two TNFR superfamily members [30,31] and the Fn14 cytoplasmic tail does not contain the consensus sequence motif described for effective TRAF6 binding [32,33].

We utilized an alanine substitution mutagenesis strategy to identify those aa residues in the murine Fn14 cytoplasmic tail required for TRAF association. Substitution of the two glutamic acid residues located at the putative TRAF-binding site prevented TRAF binding, indicating that either one or both of these residues is critical for Fn14–TRAF interactions. Similar mutagenesis studies that disrupt glutamic acid residues within the TRAF-binding sites of other TNFR superfamily members have also demonstrated the importance of acidic residues at these sites [22,34–36]. We also mutated each of the two threonine residues (Thr^{112} and Thr^{117}) that flank the Pro-Ile-Glu-Glu motif, since threonine residues have a hydroxyl group that could participate in hydrogen-bond interactions. Alanine substitution of Thr^{112} had no effect on TRAF binding, but alanine substitution of Thr^{117} prevented TRAF1, TRAF2 and TRAF5 binding. TRAF3 could still associate with the $Thr^{117} \rightarrow Ala$ mutated cytoplasmic tail, demonstrating that TRAFs 1, 2, 3 and 5 bind an overlapping, but non-identical, sequence motif. Several other TNFR superfamily members besides Fn14 also contain a threonine residue C-terminal to the TRAF2-binding consensus sequence motif. In CD30, this residue has been shown to be critical for both TRAF2 and TRAF3 association *in vitro* [37]. In contrast, alanine substitution of this residue in the herpesvirus entry mediator (HVEM) cytoplasmic tail does not prevent TRAF2 (nor TRAF5) binding in yeast cells [34]. In CD40, this threonine residue is required for TRAF2, TRAF3, and TRAF5 binding [33,38–42]. Crystallographic analysis of CD40 cytoplasmic domain peptides in complex with the TRAF domain of TRAF2 [43] or TRAF3 [44] has revealed that this threonine is involved in intermolecular hydrogen bonding in the case of TRAF2 association and intramolecular hydrogen bonding in the case of TRAF3 association. Our findings, together with previous results [33,34,37–44], suggest that the precise role of this flanking threonine residue can vary depending on the structural characteristics of both the TNFR superfamily member and its associated TRAF molecule.

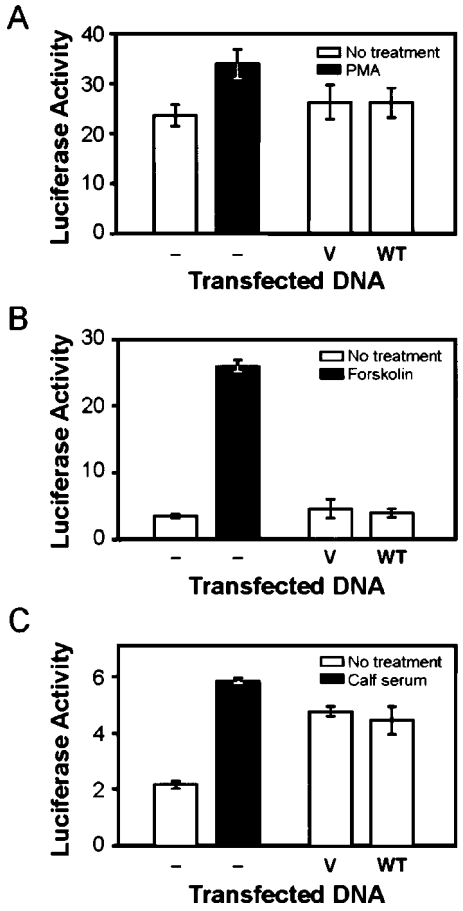

Figure 5 Effect of Fn14 overexpression on transcriptional activation of an AP-1, CRE or SRE enhancer/luciferase reporter plasmid

(**A**) NIH 3T3 cells were transfected with the AP-1 enhancer/luciferase reporter plasmid alone, cultured for 48 h in either the absence or presence of PMA, and then harvested or co-transfected with this same plasmid in combination with either the pSecTag2A vector (V) or the Fn14-WT (WT) plasmid and harvested after 48 h. Cells were then analysed for luciferase activity. (**B**) Cells were transfected with the CRE enhancer/luciferase reporter plasmid alone, cultured for 48 h in either the absence or presence of forskolin, and then harvested or co-transfected with this same plasmid in combination with either the pSecTag2A vector (V) or the Fn14-WT (WT) plasmid and harvested after 48 h. Cells were then analysed for luciferase activity. (**C**) Cells were transfected with the SRE enhancer/luciferase reporter plasmid alone, cultured for 48 h in either the absence or presence of 10% (v/v) calf serum, and then harvested or co-transfected with this same plasmid in combination with either the pSecTag2A vector (V) or the Fn14-WT (WT) plasmid and harvested after 48 h. Cells were then analysed for luciferase activity. In all of the above transient transfection experiments, a plasmid encoding β-galactosidase was also transfected into the cells and luciferase values were normalized to β-galactosidase activity. Relative light units are expressed as means $\pm$ S.D. for triplicate wells from one representative experiment of three independent experiments.

Many TNF superfamily members promote cell growth or survival by activation of the multisubunit IκB kinase complex (i.e. via the NF-κB pathway) and/or by activation of one or more members of the MAPK family (i.e. via the JNK, p38 MAPK, and/or ERK pathways) [1,14,15,27]. We have found that TWEAK can stimulate the NF-κB signal transduction pathway in NIH 3T3 cells using three different experimental approaches. Specifically, (i) TWEAK promotes IκBα phosphorylation in both normal and Fn14-overexpressing NIH 3T3 cells, a critical event required for NF-κB translocation to the nucleus and gene activation [28,29], (ii) TWEAK promotes enhanced expression of a transfected NF-κB enhancer/luciferase reporter plasmid, indicative of increased NF-κB transcriptional activity, and (iii) ectopic overexpression of wild-type Fn14, but not an Fn14 mutant with a truncated cytoplasmic tail, also promotes expression of an NF-κB enhancer/luciferase reporter plasmid. Three previous studies utilized electrophoretic mobility shift assays to investigate whether TWEAK could activate the NF-κB pathway: TWEAK addition to human embryonic kidney HEK-293 cells [3] or endothelial cells [9] has been shown to induce NF-κB DNA-binding activity; however, a similar response was not observed in TWEAK-treated rhabdomyosarcoma cells [5]. Our results, using murine NIH 3T3 fibroblasts, are in agreement with the first two studies and are consistent with other reports indicating that TWEAK treatment of various cell types can increase the expression of several NF-κB-regulated genes, including interleukin 6 [11,12], interleukin 8 [4,8,9,11,12,17], the chemotactic cytokine RANTES (regulated upon activation, normal T-cell expressed and secreted) [12] and intercellular cell-adhesion molecule 1 (ICAM-1) [9,11].

In summary, we have shown that human TWEAK can bind the murine Fn14 protein. Furthermore, TRAFs 1, 2, 3 and 5 associate with the Fn14 cytoplasmic tail by binding to a single overlapping, but non-identical, TRAF-binding site. Finally, TWEAK addition or Fn14 overexpression can activate the NF-κB pathway in NIH 3T3 cells. TWEAK is an angiogenic factor [8]; therefore, it may have a role in the pathological neovascularization associated with various human diseases, including rheumatoid arthritis and cancer [45]. Our findings indicate that the NF-κB signalling pathway may be a good target for therapeutic inhibition of TWEAK biological activity *in vivo*.

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