Association of a RING finger protein with the cytoplasmic domain of the human type-2 tumour necrosis factor receptor

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A human gene encoding a protein that specifically binds to the intracellular domain of the 75 kDa type-2 tumour necrosis factor (TNF) receptor (TNFR-2IC) has been identified using the yeast-based two-hybrid system. The N-terminal half of the TNF receptor-associated protein (TRAP) contains RING finger and zinc finger motifs often found in DNA-binding proteins including transcription factors. The 2.4 kb TRAP mRNA was barely detectable, if present at all, in lung, and variably expressed in heart, liver, placenta, brain, skeletal muscle, kidney and the pancreas; interestingly, the TRAP was more highly expressed in transformed cell lines than in normal tissues. This observation may be consistent with a role for this TRAP in promoting or regulating cellular proliferation. After *in vitro* transcription/

translation and ³⁵S labelling the TRAP was precipitated using a fusion protein consisting of glutathione S-transferase and the intracellular domain of TNFR-2 (TNFR-2IC), which showed that the two proteins directly interact in a mammalian cell-free system and also that identification of the TRAP was not an artifact of the two-hybrid system. By using truncated TNFR-2ICs for *in vitro* precipitation of ³⁵S-TRAP, it was shown that the C-terminal half of the TNFR-2IC contains the domain necessary for interaction with TRAP. The TRAP identified in the present study shares considerable homology with, and may be the human homologue of, a mouse protein, TNF receptor-associated factor 2 (TRAF2), that binds mouse TNFR-2.

INTRODUCTION

Tumour necrosis factor (TNF) was first identified on the basis of its ability to induce the regression of cancers in animals and by the cytotoxic response that it elicits from transformed cells. Subsequent studies showed that TNF promotes diverse cellular responses such as immunity, antiviral responses, inflammation, shock and, in some chronic diseases, the syndrome of wasting and malnutrition known as cachexia [1–3]. The first step in TNF action is binding specific receptors. Two distinct TNF receptors have been identified (the 55 kDa type-1 TNF receptor, TNFR-1; and the 75 kDa type-2 TNF receptor, TNFR-2) [4–7] and their cDNAs cloned [8–10]. The extracellular domains of TNFR-1 and TNFR-2 share homologies with one another and with a group of cell-surface receptors, all of which are members of the nerve growth factor (NGF)/TNF receptor superfamily [11]. There are no homologies in the intracellular domains of TNFR-1 (TNFR-1IC) and TNFR-2 (TNFR-2IC), suggesting that they couple to different signalling pathways and induce distinct responses. TNFR-2 activation induces T-cell proliferation [12], proliferation of human mononuclear cells [13], granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion [14], is implicated in the inhibition of early haematopoiesis [15] and activates nuclear factor κ B (NF- κ B) [16]. While there is controversy regarding the role of TNFR-2 in cytotoxicity [17,18], it is reported to induce this response in HeLa cells [17], the human rhabdomyosarcoma cell line KYM-1 [19] and U-937 histiocytic lymphoma cells [20,21]. Mice deficient in TNFR-2 show normal T-cell development and activity, but have increased resistance to TNF-induced death [22]. TNFR-1 promotes growth inhibition

and cytotoxicity, antiviral activity, fibroblast proliferation, NF- κ B activity, and plays a decisive role in the host's defence against microorganisms [6,16,23–26].

Neither TNFR contains intrinsic tyrosine kinase activity or any structural feature which suggests how coupling to signalling pathways occurs. We therefore acted on the hypothesis that associated proteins, rather than the intracellular domain of either receptor, mediate signal transmission. To identify such proteins we used the yeast-based two-hybrid system, which is a method that detects proteins capable of interacting with a known protein by transcriptional activation of a reporter gene [27]. The procedure is based on the properties of the yeast GAL4 protein which consists of separable domains that mediate DNA binding and transcriptional activation. Plasmids encoding two-hybrid proteins, one consisting of the GAL4-binding domain (GAL4-BD) fused to protein X and the other consisting of the GAL4 activation domain (GAL4-AD) fused to protein Y, are co-transformed into yeast. Interaction of these proteins permits transcriptional activation of an integrated copy of the GAL4-lacZ reporter gene. The two-hybrid system can also be used to screen cDNA expression libraries for genes encoding proteins that interact with any protein under study. In this manifestation of the method, yeast are co-transformed with a plasmid expressing the GAL4-BD fused to the protein of interest and a pool of plasmids encoding GAL4-AD/cDNA library fusion proteins. In the present study, GAL4-BD fused to TNFR-2IC was used to screen a HeLa S3 cDNA library for encoded proteins capable of binding TNFR-2IC. This has led to identification of a protein which specifically binds TNFR-2IC. The TNF receptor-associated protein (TRAP) contains N-terminal RING finger and zinc finger motifs often found in DNA-binding proteins and

Abbreviations used: CD40bp, CD40 binding protein; GST, glutathione-S-transferase; NGF, nerve growth factor; NP-40, Nonidet P-40; NF- κ B, nuclear factor κ B; SV40, simian virus 40; TNF, tumour necrosis factor; TNFR-1, 55 kDa TNF receptor; TNFR-2, 75 kDa TNF receptor; TNFR-1IC, intracellular domain of TNFR-1; TNFR-2IC, intracellular domain of TNFR-2; TRAP, TNF receptor-associated protein.

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transcription factors and a C-terminal domain which contains the binding site for TNFR-2IC.

MATERIALS AND METHODS

Strains and vectors

All yeast strains and shuttle vectors for two-hybrid experiments were obtained from Clontech (Palo Alto, CA, U.S.A.) as components of the MATCHMAKER Two Hybrid System. Yeast strains SFY526 and HF7c, described previously [28], were used to assay for protein-protein interactions and library screening respectively. The *Escherichia coli* strains XL1-blue (Stratagene) and DH5 α were employed for subcloning and overexpression of glutathione S-transferase (GST) fusion proteins respectively. Transformation of yeast was performed according to instructions provided with the MATCHMAKER Two Hybrid System. Plasmids pGBT9 (the GAL4 DNA-binding domain) and pGAD424 (the GAL4 activation domain) as well as the control plasmids pVA3 (the p53 gene), pTD1 [the simian virus 40 (SV40) large T antigen] and pLAM5' (the lamin gene) were from Clontech. Control plasmids pGBT9-TNFR1IC (GAL4 BD-intracellular domain of TNFR-1 fusion) and pGBT9-FASIC (GAL4 BD-intracellular domain of FAS antigen fusion) were constructed as described [29].

Library screening

Constructs for library screening were made by amplifying the necessary inserts by PCR. PCR primers flanked by *Eco*RI and *Bam*HI sites were designed to amplify TNFR-2IC using a cDNA clone of TNFR-2 in pUC19 (a gift from Dr. H. Loetscher, Hoffmann-LaRoche Inc., Geneva, Switzerland) as the template for insertion into pGBT9 digested with *Eco*RI and *Bam*HI (pGBT9-TNFR2IC). HF7c containing pGBT9-TNFR2IC was His⁻ and LacZ⁻, showing that TNFR-2IC alone does not contain latent transcriptional activity. The human HeLa S3 MATCHMAKER cDNA library inserted into pGADGH was purchased from Clontech. Screening of HF7c cells co-transformed with pGBT9-TNFR2IC and the HeLa MATCHMAKER cDNA library was conducted as described [28]. Our modification [28] of the procedure of Miller [30] was used to assay for β -galactosidase activity. Library clones from double positive colonies (His⁺ LacZ⁺) were isolated and retransformed into SFY526, alone, with pGBT9, pGBT9-TNFR2IC, pLAM5', pGBT9-TNFR1IC or with pGBT9-FASIC to test specificity. Clones that activated the *lacZ* reporter gene only in the presence of pGBT9-TNFR2IC were chosen as true positives. Sequencing was conducted using the Sequenase sequencing kit (US Biochemical).

GST-TNFR2 fusion preparation

To prepare GST-TNFR2 fusion constructs, four PCR primers were used to amplify the full-length TNFR-2IC (GST-TNFR2IC), the N-terminal half of TNFR-2IC (amino acids 262–357, GST-TNFR2NH2) and the C-terminal half of TNFR-2IC (amino acids 358–439, GST-TNFR2COOH). The PCR products were subcloned into the *Bam*HI and *Eco*RI sites of pGEX-2T (Pharmacia). The GST fusion proteins were purified using our modification of the procedure of Smith and Johnson [31]. After induction, cells were grown at 30 °C for 3 h, suspended in lysis buffer [20 mM Tris, pH 8.0, 200 mM NaCl, 10 % glycerol, 0.5 % Nonidet P-40 (NP-40), 1 mM PMSF, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 0.1 % 2-mercaptoethanol] and sonicated. The lysate was centrifuged (12000 *g*, 30 min) to remove debris. One-tenth volume of 50 % GST-agarose slurry (Sigma) was

added to the supernatant which was incubated at 4 °C for 1 h. The beads were washed three times with lysis buffer and the purity of the GST fusion proteins was confirmed by SDS/PAGE.

In vitro translation

The TnT-coupled rabbit reticulocyte lysate system (Promega) was used for one tube transcription/translation of TRAP according to the instructions of the manufacturer. Template was prepared by inserting the 2.4 kb TRAP cDNA into pGEM7 (pGEM7-TRAP) under control of the SP6 promoter. Rabbit reticulocyte was mixed with 1 μ g of pGEM7-TRAP along with SP6 RNA polymerase, after which an amino acid mixture without methionine plus [³⁵S]methionine was added. After 2 h at 30 °C, the purity and level of expression of TRAP was analysed by SDS/PAGE and phosphoimage analysis (Bio-Rad).

In vitro binding

Approximately 10 μ g of each agarose-bound GST-TNFR2 fusion protein was added into binding buffer (20 mM Tris, pH 7.7, 0.5 % NP-40, 200 mM NaCl, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mM PMSF, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 0.1 % 2-mercaptoethanol) and 40 μ l of reticulocyte lysate that contained [³⁵S]methionine-labelled TRAP. After 2 h at 4 °C, the agarose beads were washed three times with binding buffer before addition of SDS sample buffer, electrophoresis and phosphoimage analysis.

Northern blotting

Membranes preblotted with 10 μ g of poly(A) RNA from human tissues and cancer cell lines were obtained from Clontech. The N-terminal 0.9 kb *Eco*RI/*Bgl*II fragment of TRAP cDNA was used as a probe after labelling with the Prime A gene labelling kit (Promega). Hybridization and washing were performed as suggested by the manufacturer.

RESULTS AND DISCUSSION

To identify TRAPs, human TNFR-2IC fused with the GAL4-BD (pGBT9-TNFR2IC) was used to screen a HeLa S3 MATCHMAKER cDNA library (constructed in the activation domain vector pGADGH). A total of 700000 double transformants in HF7c were screened for histidine prototrophy and *lacZ* activation; among 36 His⁺ colonies three His⁺ LacZ⁺ double positive colonies were identified. Two colonies encoded ribosomal proteins known to bind non-specifically in two-hybrid assays [32] and were false positives. To test for the specificity of interaction between the third clone, II-36, and TNFR-2IC, we transformed the SFY526 yeast strain, which has a different GAL4 recognition site than HF7c, with II-36 alone, pGBT9 and II-36, pGBT9-TNFR2IC and II-36, pLAM5' and II-36, pGBT9-TNFR1IC and II-36, and pGBT9-FASIC and II-36. As shown in Table 1, β -galactosidase activity was detected only in yeast co-transformed with p53 and SV40 antigen, which are known to interact and constitute a positive control, and with pGBT9-TNFR2IC and II-36. Other members of the TNFR superfamily, TNFR-1IC and FAS-IC, failed to interact with the protein encoded by II-36. This showed that interaction between TRAP and TNFR-2 is specific.

We used the N-terminal 0.9 kb *Eco*RI/*Bgl*II fragment of the 2.2 kb TRAP cDNA insert from clone II-36 to probe Northern blots of mRNA from normal tissues and transformed cell lines. This identified a single 2.4 kb TRAP transcript, which showed that the two-hybrid screen had not yielded the full-length TRAP

Table 1 β -Galactosidase activity and filter colour of TRAP co-transformants

Yeast strain SFY526 was transformed with various combinations of plasmids and β -galactosidase activity was assayed.

Protein fused to GAL4 domain		β -Galactosidase activity*	Filter colour
DNA binding	Activating		
pVA3**	pTD1**	494 $\pm$ 70	Blue
None	II-36	< 0.1	White
pGBT9	II-36	< 0.1	White
pGBT9-TNFR2IC	II-36	269 $\pm$ 34	Blue
pLAM5'	II-36	< 0.1	White
pGBT9-TNFR1IC	II-36	< 0.1	White
pGBT9-FASIC	II-36	< 0.1	White

* β -Galactosidase activity was assayed by modification [28] of the procedure of Miller [30]. In the method, chlorophenol red- β -D-galactopyranoside at a final concentration of 5 mM was used as the substrate. Units were calculated as $1000 \times A_{574}$ divided by the product of the volume (ml) $\times$ time (min) $\times A_{600}$. The activity represents the mean of triplicate determinations $\pm$ S.D. **The plasmids pVA3 and pTD1 encode murine p53 and SV40 large T-antigen, respectively. pLAM5' encodes lamin. Clone II-36 encodes the TRAP gene.

Figure 1 Northern-blot analysis of TRAP expression in normal tissues and cancer cell lines

Preblotted membranes were hybridized using a TRAP cDNA probe. The arrow to the right indicates the size of TRAP. For lung tissue, a spot below 2.4 kb is an artifact.

(Figure 1). To obtain the full-length sequence of the TRAP gene, we screened a 5' stretch plus HeLa S3 cDNA library in λ gt11 (Clontech). Sequence analysis of a phage clone with a 2.4 kb insert revealed an open reading frame which encoded a protein of 502 amino acids (Figure 2). The start codon (nucleotides 55–57) of the TRAP is surrounded by a consensus Kozak sequence [33] and the open reading frame spans through nucleotide 1560. The TRAP cDNA has a relatively long 3' untranslated region (700 bp) that contains a potential polyadenylation signal (ATTAAG) 20 bases upstream from the C-terminal poly(A) attachment site.

An *in vitro* binding assay was developed to establish that identification of the TRAP as a human TNFR-2 binding protein was not an artifact of the two-hybrid system, to demonstrate direct interaction between the TRAP and TNFR-2IC, and to identify the domain in TNFR-2IC to which the TRAP binds. GST fusion proteins containing full-length TNFR-2IC (amino acids 267–439, GST-TNFR2IC), the N-terminal half of TNFR-2IC (amino acids 267–357, GST-TNFR2NH2) and the C-terminal half of TNFR-2IC (amino acids 358–439, GST-TNFR2COOH) were prepared and purified to homogeneity

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gaattccggcgccgtgcgacccgttggggcttctgttcgccccgggtcacagctctcatggct 60
M A
gcagctagcgtgaccccccttggtcccttgaggtgtgtacagcccggtcttccaaagacc 120
A A S V T P P G S L E L L Q P G F S K T
ctcttggggaccaagcttgaagccaaagtaactgtgtccgctgcagaaagctctccgc 180
L L G T K L E A K Y L C S A C R N V L R
aggcccttcagggcagctgttgccacgggtactgtctctctgtctggccagcatctc 240
R P F Q A Q C G H R Y C S F C L A S I L
agctctgggctcagaactgtgtgtgtgttccaggggcatatagaagaagccatt 300
S S G P Q N C A A C V H E G I Y E E G I
tctattttgaaagcagcttcggccttccagataaactgtgcgcgcagggaggtggagagc 360
S I L E S S S A F P D N A A R R E V E S
ctgcgcgcgtctgtccagtgatggatgcacctggaggggacccctgaaagaatacagag 420
L P A V C P S D G C T W K G T L K E Y E
agctgcacgaagggcgtgcgcgcctcatgtgcacgaagtgccgcgtgttaaaggcctg 480
S C H E G R C P L M L T E C P A C K G L
gtccgccttggtgaaagagcgcacacctggagcagcagtgccgcgcagagaagcctgagc 540
V R L G E K E R H L E H E C P E R S L S
tgccgccttgccgggcacacctgtgtgcggagcagcagtggaaggcgaccacagagctgc 600
C R H C R A P C C G A D V K A H H E V C
cccaagttcccttaactgtgtgcgcgtgcggcgaagaagatcccccgaggagagttt 660
P K F P L T C D G C G K K K I P R E K F
caggacacagctcaagactgtgtgcaagtggtgcaccttgcaagatcccccagccatggc 720
Q D H V K T C G K C R V P C R F H A I G
tgcttcagagcgttagaggttgagaacacagcagcagcagcagtggtgagtggtgcgggag 780
C L E T V E G E K Q Q E H E V Q W L R E
caactggccatgtacttagctcgtgtgtggagcaaacccctcttgggagdcagagc 840
H L A M L L S S V L E A K P C L T G D C Q S
cagcgggggtcagagctctctgcagaggtgcgcagagcctggagaaagagcagccatttt 900
H A G S E L L Q R C E S L E K K T A T F
gagaacattgtctgtctgtgaacgggaggttgagaggttggtccatgactgcgcagggcc 960
E N I V C V L N R E V E R V A M T A E A
tgccgcgcgcgcgcgcgcgtggacacagacaagattgaagccctgagtagcaaggtgcag 1020
C S R Q H R L D Q D K I E A L S S K V Q
cagcttgagagcagcatttgccctcaaggacctggcgatggctgacttgagcagagagtc 1080
Q L E R S I G L K D L A M A D L E Q K V
ttggagatggagcattccacctacgatggggcttcttcagagatctcagactttccc 1140
L E M E A S T Y D G V F I W K I S D F P
aggagctccaggaagctgtgtgtgtgcgcgcatacccccattcttctccagcccttctac 1200
R K L Q E A V A G R I P A I F S P A F Y
accagcaggtacgggtacagatgtgtctgtctgtcttactgaaacggcgagcgcgcgcgg 1260
T S R Y G Y K M C L R I Y L N G D G T G
cgaggaacacacctgtccctcttcttgggtgtgtgaaagggcccgaaatgagcgcgcgtgtg 1320
R G T H L S L F F V V M K G P N D A L L
cgggtggcccttcaaccagaagtgaccttaatgtgtgtgtgcgcagagaataacccggagcac 1380
R W P F N Q K V T L M L L D Q N N R E H
gtgattgagccttcaggcccgagctgacttcatctcttcttcagagggccagtcacagac 1440
V I D A F R P D V T S S S F Q R P V N D
atgaacatgcagcgcgtgcgcgccttcttctgcgcgccttccaaagatggagcgaagaat 1500
M N I A S G C P L F C P V S K M E A K N
tcttaactgtggggagcagatgccattcttcaaggccattgtggacctgacagggcctctaa 1560
S Y V R D D A I F I K A I V D L T G L *
ctgcgcgcctactgtgtctgggggttgggggacagcagcagcagcgcgcgcgcgcgggg 1620
ccacacgccttggggcaggggttctactgtacaaagtgaggggcccgccgtctggggcgttg 1680
ggaggggtgtgcgcctgcagccaaagttcactgtgcagcggggagagggcagccagcagctc 1740
tcagatttcagagactgcgggggggttggcagagcgtcttagcgaagggtgtgtgtggtgc 1800
attggcgggggtcttctgggtgtctccagcacaagctgcccctgtgtgtctgtgtgagtg 1860
aaggagagggccctgggtgggggacactcagagtgaggagacatccagcagtgcccatgg 1920
tagcagggagcagctggatggccttgtgtcccttcgggcatgacagggcagaaacaggggtc 1980
gctccagagagaagggcctctctgtgtggcagagcagagcagcagcagcagctgtgtctcc 2040
cctctggcccttggagagaagggagcattctagacccctgggtgtgtgtgtgtgtgtgtgt 2100
ctgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgt 2160
tgtctcagacactgtgtgtgggggggcagcagcagcagcagcagcagcagcagcagcagc 2220
tcagctcaggaagacagaggttattaaaccattacaaatctc

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Figure 2 Nucleotide sequence of TRAP cDNA

The TRAP open reading frame is indicated by the translated amino acid sequence underneath the coding sequence. This sequence has been deposited in the Genbank database (TRAP accession number U12597).

(Figure 3a). The TRAP was expressed in a TnT rabbit reticulocyte transcription/translation system using pGEM7-TRAP as a template and labelled with [^{35} S]methionine. *In vitro* translated TRAP protein ran as a 56 kDa protein on a gel, which is the expected size based on the amino acid sequence of the protein (data not shown). The ^{35} S-TRAP was incubated with agarose-immobilized GST, GST-TNFR2IC, GST-TNFR2NH2 or GST-TNFR2COOH. GST-TNFR2IC and GST-TNFR2COOH precipitated TRAP and a number of lower-molecular-mass species which may be early-terminated *in vitro* translation products or degradation products (Figure 3b). In contrast, GST or GST-TNFR2NH2 did not precipitate TRAP. These observations demonstrate that the interaction between the TRAP and TNFR-

Figure 3 *In vitro* precipitation of TRAP

(a) Full-length and truncated TNFR-2IC were overexpressed and purified as GST fusions. Lane 1, GST-TNFR2NH₂; lane 2, GST-TNFR2IC; lane 3, GST-TNFR2COOH. (b) *In vitro* precipitation of TRAP using GST fusion proteins: lane 1, GST alone, control; lane 2, GST-TNFR2IC; lane 3, GST-TNFR2NH₂; lane 4, GST-TNFR2COOH. The arrow indicates TRAP precipitated by GST-TNFR2IC and GST-TNFR2COOH.

Figure 4 Amino acid sequence comparison of human TRAP and mouse TRAF2

The TRAP (upper) and TRAF2 (lower) amino acid sequences were aligned. For TRAF2, only the residues that are different from those in TRAP are shown. The RING finger motif is boxed. The cysteine/histidine-rich domain between amino acids 110 and 235 is bracketed. Lines over the sequence delineate the type TFIIIA zinc finger motifs and the TRAF domain.

2 is specific in a mammalian cell-free system as well as in yeast, and that the C-terminal half of TNFR-2IC binds the TRAP.

Structural analysis of TRAP reveals an N-terminal cysteine-rich RING finger, which is a modified zinc finger that contains two zinc-binding sites [34]. The RING finger in the TRAP spans amino acid residues 34 to 72 and contains a Cys-Xaa₂-Cys-Xaa₁₁-Cys-Xaa-His-Xaa₂-Cys-Xaa₂-Cys-Xaa₁₁-Cys-Xaa₂-Cys motif, where Xaa can be any amino acid (Figure 4). Downstream from the RING finger is a cysteine/histidine-rich sequence between amino acids 110 and 235 (17 cysteines, nine histidines) within which are two type TFIIIA zinc fingers which contain the motif Cys/His-Xaa₍₂₋₄₎-Cys/His-Xaa₍₂₋₁₅₎-Cys/His-Xaa₍₂₋₄₎-Cys/His. The domain between amino acids 110 and 235 also bears some resemblance to another cysteine-rich motif called a B-box, which has been identified C-terminal to the RING finger in a discrete number of RING-family proteins [34]. The present

TRAP	C	S	A	C	R	N	L	R	R	F	F	Q	A	C	G	H	R	Y	C	S	F	C	L	A	S	S	S	G	F	N	C	A	A	C			
TRAF2	C	S	A	C	R	N	L	R	R	F	F	Q	A	C	G	H	R	Y	C	S	F	C	L	T	S	S	S	G	F	N	C	A	A	C			
CD40BP	C	E	K	C	H	L	V	C	S	P	K	Q	T	E	C	G	H	R	F	C	E	S	C	M	A	L	L	S	S	S	P	K	C	T	A	C	
RAP	C	Q	V	C	D	H	L	S	E	P	V	Q	S	P	C	R	H	L	F	C	R	S	C	I	A	K	T	Y	S	L	G	H	C	P	A	C	
RO	C	S	I	C	L	D	M	V	E	P	M	S	T	E	C	G	H	C	F	C	K	C	I	F	E	V	G	R	N	G	S	S	C	P	E	C	
BRCA-1	C	P	I	C	L	E	L	I	K	E	P	A	S	T	K	C	D	H	I	F	C	K	F	C	M	L	K	L	N	K	G	P	S	C	P	L	C

Figure 5 Sequence comparison of RING finger motifs

Motifs from four proteins with RING fingers most closely related to that in TRAP are shown. TRAF2, mouse TNFR-2-associated factor-2; CD40bp, CD40-binding protein; RAP (RAG-1), recombination activating protein from *Oncorhynchus mykiss*; RO, *Mus musculus* homologue of human SS-A/Ro autoantigen. Also shown is the related BRCA-1 RING finger motif.

consensus for the B-box motif can be defined as Cys-Xaa₂-His-Xaa₂-Cys-Xaa₂-Cys-Xaa₂-His-Xaa₂-His and it is of interest as more than half the proteins that contain this recently described feature have transforming potential [35]. Proteins containing the RING finger motif are often DNA-binding proteins and transcriptional regulators that may be involved in development, cellular transformation, transcriptional activation and DNA repair, and sometimes tumour suppression. The possibility that a transcriptional activator may be a proximal component of the TNF signal transduction cascade is reminiscent of the JAK/STAT pathway in which interferons activate latent, cytoplasmic transcription factors which translocate into the nucleus and turn on interferon-responsive elements [36]. Thus, the TRAP contains structural elements which suggest a mechanism through which it may mediate cellular actions initiated by activation of TNFR-2.

While we were preparing this manuscript, Rothe et al. [37] identified two mouse genes encoding proteins called TRAF1 and TRAF2 that interact with TNFR-2IC, and Hu et al. [38] identified a human CD40-binding protein (CD40bp), which interacts with the intracellular domain of CD40 (CD40-IC), another member of the TNF/NGF receptor superfamily. The C-termini of both TRAP and CD40bp [38] contain a conserved domain first identified in TRAF1 and TRAF2 (the TRAF domain) which mediates receptor binding [37]. Identification of a TRAF domain in the CD40bp is rather surprising as there is no apparent sequence homology between CD40-IC and TNFR2-IC; however, conformational similarities may relate the proteins. These observations show that more than one member of the TNF/NGF receptor superfamily may interact with and use proteins that contain TRAF domains in signal transduction.

TRAF2 and CD40bp contain N-terminal RING finger and cysteine/histidine-rich motifs similar to those in the TRAP identified in our study (Figure 5). A search of the Genebank database identified two additional proteins with a RING finger motif closely related to that in TRAP, TRAF2 and CD40bp: RAP (RAG-1, recombination activating gene-1) which activates V(D)J recombination when introduced into NIH 3T3 fibroblasts [39] and the 52 kDa protein component (RO) of the SS-A/Ro autoantigen, an RNA-protein complex that is the target of autoantibodies present in the sera of some patients with Sjogren's syndrome [40]. The recently discovered BRCA-1 gene also contains a RING finger with similarity to the motif identified in the proteins enumerated above. BRCA-1 may be a tumour suppressor gene [41]; mutations in the RING finger in this gene are implicated in the development of breast cancer [42].

TRAF2 heterodimerizes with TRAF1 and mediates its interaction with TNFR-2IC [37]. Expression of TRAF1 mRNA is tissue-specific whereas TRAF2 mRNA is constitutively expressed

in all tissues thus far tested. Thus, heterodimerization of TRAF1 and TRAF2 can occur in only a select group of tissues, raising the possibility that other TRAFs exist as tissue-specific dimerization partners [37]. Such tissue-specific heterocomplexes could induce different intracellular signals thereby accounting for the distinct responses mediated by TNFR-2 in various cell types. Northern-blot analysis showed that TRAP mRNA was variably expressed in normal tissues; it was barely detectable, if present at all, in lung and more highly expressed in heart, brain, placenta, liver, skeletal muscle, kidney or pancreas. These observations suggest that if the TRAP is present to act as a dimerization or heterodimerization partner, the levels of such complexes in different tissues must be highly variable. The significance of such variability to TNF action is yet to be determined. Additionally, the TRAP, which contains a RING finger often found to be involved in transcriptional regulation and a B-box-like motif found in proteins with transforming potential, is more highly expressed in transformed cells than in normal tissues. This is worthy of comment, as it may suggest that this protein plays a role in promoting cell proliferation or in the process of transformation.

TRAP is most closely related to TRAF2 [37] (Figure 4), the mouse TNFR-2-binding protein with which it shares 87% identity and 91% overall sequence similarity. This suggests that TRAP may be the human homologue of TRAF2 (Figure 4). Comparison of the amino acid sequences of TRAP and TRAF2 shows that the TRAF domain and RING finger are highly conserved, suggesting their importance to the potential function of the TRAP. Mismatches are most frequent within the first TFIIIA-type zinc finger and between the second zinc finger and the TRAF domain; however, the cysteine and histidine residues that comprise the zinc finger motifs are completely conserved (Figure 4). The retention of the significant homologies that relate human TRAP and mouse TRAF2 strongly suggests that this protein will be found to play an important role in mediating some of the cellular actions of TNF.

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