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TRIP: a novel double stranded RNA binding protein which interacts with the leucine rich repeat of Flightless I

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ABSTRACT

A northwestern screen of a CHO-K1 cell line cDNA library with radiolabelled HIV-1 TAR RNA identified a novel TAR RNA interacting protein, TRIP. The human *trip* cDNA was also cloned and its expression is induced by phorbol esters. The N-terminus of TRIP shows high homology to the coiled coil domain of FLAP, a protein which binds the leucine-rich repeat (LRR) of Flightless I (FLI) and the interaction of TRIP with the FLI LRR has been confirmed *in vitro*. TRIP does not bind single stranded DNA or RNA significantly and binds double stranded DNA weakly. In contrast, TRIP binds double stranded RNA with high affinity and two molecules of TRIP bind the TAR stem. The RNA binding domain has been identified and encompasses a lysine-rich motif. A TRIP–GFP fusion is localised in the cytoplasm and excluded from the nucleus. FLI has a C-terminal gelsolin-like domain which binds actin and therefore the association of TRIP with the FLI LRR may provide a link between the actin cytoskeleton and RNA in mammalian cells.

INTRODUCTION

Flightless I (FliI) was originally identified as a mutation in *Drosophila melanogaster* which leads to flightlessness due to disorganisation of the indirect flight muscle myofibrils (1). *Drosophila* embryos with mutations in *fliI* show incomplete cellularisation and this is associated with an abnormal cortical cytoskeleton (2). FliI and the human homologue FLI have two clear domains, an N-terminal leucine-rich repeat (LRR) and a C-terminal gelsolin-like domain which binds actin (3,4). The LRR is a protein–protein interaction domain found in many proteins with diverse functions (5). The crystal structure of the LRR from ribonuclease inhibitor indicates that this motif forms a non-globular horseshoe with RNAse binding to the inner concave face (6). Since FLI contains an actin binding domain and LRR, it has the potential to directly link other proteins to the cytoskeleton. The LRR of FLI has homology with a group of LRRs which interact with RAS-like ligands (5,7) but failed to interact with these proteins in the yeast two-hybrid assay and *in vitro*. A search for proteins which did interact with the FLI LRR identified a partial human cDNA and a full-length murine cDNA for FLAP. FLAP has an N-terminal coiled coil domain which probably mediates the interaction with the FLI LRR (4). The interaction of a LRR with a coiled coil has been observed previously in the interaction between decorin and collagen in which a triple helical collagen molecule fits into the central cavity produced by the decorin LRR (8). FLAP shows no sequence homology with other proteins and its biological role is unclear at present.

The Tat protein of HIV-1 is a potent activator of transcription from the viral promoter within the LTR. Tat binds to the TAR RNA element present at the 5′ end of all viral transcripts. TAR RNA extends from nucleotides +1 to + 57 and folds into a stem–loop structure with a tri-pyrimidine bulge which forms part of the base triple between U23 and A27–U38 in the upper stem, recognised by Tat (9). The lower TAR stem, below the UCU bulge, consists of a 19 bp stem with two mismatched bases. The HIV-1 LTR is responsive to a number of environmental influences such as UV light, and such stresses can lead to activation of the LTR in the absence of the viral transactivator Tat (10,11). This suggests that there may be stress-inducible cellular factors capable of influencing expression from the LTR. A number of cellular TAR binding proteins have been identified previously (12–17), including a series of UV-inducible RNA binding proteins which show a preference for GC-rich double stranded RNA molecules (18). These factors may be involved in the UV activation of the LTR and normally influence RNA metabolism in response to environmental stress.

In order to identify and characterise the constitutive and stress inducible cellular factors which recognise double stranded RNA molecules, we screened UV-induced cDNA expression libraries with radiolabelled TAR. We report here the isolation and characterisation of the human and hamster cDNAs for a novel double stranded TAR RNA interacting protein, TRIP. The N-terminus of TRIP shows extensive homology with the coiled coil domain of the recently identified FLI-binding protein FLAP, and interacts with the FLI LRR.

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MATERIALS AND METHODS

DNA/RNA manipulations

DNA subcloning procedures were as described previously (19). 5′ and 3′ deletions of *trip* were generated using the polymerase chain reaction (PCR) and subcloned into the glutathione *S*-transferase (GST) expression vector pGEX4T2. The human FLI gene LRR was cloned and N-terminally epitope tagged with the *myc* peptide sequence EQKLISEEDL, by PCR amplification from a λgt11 human skeletal muscle cDNA library (Clontech) with the following primers: 5′-ggcgggatccccaccatggagcagaaactgatctctgaagaagacctggaggccaccgggtgctgccg and 5′-ccccggatcctctagattaggccagggccttgcagaa. The PCR product which corresponds to amino acids 1–400 of FliI was subcloned on a *Bam*HI fragment into pGEM3Zf+ in both orientations to generate pFLIsense and pFLIantisense. *In vitro* RNA synthesis and purification was carried out using linearised template plasmids and either SP6 or T7 RNA polymerase (Promega) as described previously (19). The templates used for the wild type and mutant TAR RNA syntheses were generated by annealing complementary oligonucleotides for the TAR sequences and subcloned into the *Eco*RI and *Hin*dIII sites of pGEM3Zf+. DNA sequencing was carried out using cycle sequencing with dye-labelled terminators using thermosequenase (Amersham). Samples were analysed using a Perkin Elmer ABI 377 Prism automated sequencer, using synthetic oligonucleotides to sequence both strands of the cDNAs. Prediction of coiled coil regions in protein sequences was done with the Pepcoil program based on the algorithm of Lupas *et al.* (20). DNA fragments for hybridisations were radiolabelled using [^{32}P]dCTP and a Ready To Go DNA labelling kit (Pharmacia). Radioactive gels and blots were dried and exposed overnight using PhosphorImager screens and scanned using a Molecular Dynamics STORM imager which was also used for quantitation of bands.

cDNA library screening

λZAP cDNA expression libraries were constructed using mRNA isolated from the HeLa and CHO-K1 cell lines, which had been irradiated with 30 J/m^2 UV light and harvested 4 h later using mRNA purification and cDNA library construction kits from Stratagene. Expression screening was carried out as described previously (12) using full-length (+1 to +80) radiolabelled TAR RNA generated from plasmid pPE38 (21). Positive plaques coincident on duplicate filters were re-screened twice until single, well-isolated plaques were identified. *In vivo* excision was used to convert phage into pBKCMV plasmids containing the cDNA insert according to the manufacturer's instructions (Stratagene). The full length human *trip* cDNA was isolated by conventional hybridisation screening using an expressed sequence tag (Genome Systems Inc.). Plasmid pSW4 consists of the *in vivo* excised pBKCMV derivative containing the hamster *trip* cDNA, and pSW5 contains the cDNA for the full length human *trip*.

Transfections and fluorescence microscopy

HeLa, 293T and COS-7 cells were grown in DMEM containing 10% FCS and non-essential amino acids. CHOK1 cells were grown in RPMI media containing 10% FCS. Media was removed prior to UV irradiation which was carried out using a Stratalinker. Transient transfections of 293T and COS-7 cells were carried out using calcium phosphate precipitates and cells were harvested 36 h post-transfection. COS-7 cells were fixed using 3% paraformaldehyde prior to examination and photography using an Olympus fluorescence microscope. The following plasmids were used in transfections: pCMVHAhTRIP was generated by PCR amplification of *trip* from pSW5. The 5′ PCR oligonucleotide contained the DNA sequence coding for the HA epitope preceded by a Kozak sequence (22) and initiator methionine and this peptide was in frame with the N-terminal methionine of TRIP in the final construct. The PCR product was subcloned into pCINEO on an *Xba*I fragment. The TRIP GFP construct was generated by subcloning the *trip* gene into pEGFP-N1 (Clontech) on a *Bam*HI fragment generated by PCR.

Protein analysis

GST fusion proteins were expressed and purified from *Escherichia coli* using glutathione agarose according to the manufacturer's instructions (Pharmacia). Protein concentrations were determined using the Bradford assay (23). Expression of the GST–TRIP deletion series fusions was verified by SDS–PAGE and in cases where significant amounts of truncated fusions were produced, the amount of the intact fusion protein to use in EMSA was determined by densitometric scanning of Coomassie stained gels. *In vitro* transcription/translations of *trip* and *fliI* were carried out using pCMVHahTRIP, pFLIsense and pFLIantisense in the TNT coupled rabbit reticulocyte lysate system (Promega) and [^{35}S]methionine according to the manufacturer's instructions. Western blots were prepared and probed with the anti-HA monoclonal antibody 12CA5 (Boehringer), an anti-murine horseradish peroxidase secondary antibody and detected using ECL according to the manufacturer's instructions (Amersham). For GST pull down experiments, 5 µg of GST or GST–hTRIP were bound to glutathione agarose and incubated with 15 µl of *in vitro* transcription-translated FLI for 1 h at 4°C in a total volume of 500 µl RIPA buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS]. Precipitated material was washed five times in RIPA buffer and bound material was eluted with elution buffer [20 mM Tris–HCl (pH 8.0), 20 mM glutathione] and analysed by SDS–PAGE and PhosphorImaging.

RNA analysis

Total RNA was isolated from HeLa cells using RNAeasy columns (Qiagen) and quantitated spectrophotometrically. Phorbol ester induction of HeLa cells was carried out using 100 nM PMA (Sigma) dissolved in dimethyl sulfoxide. The human RNA master blot (Clontech) and multiple tissue northern were probed with a *trip* cDNA probe according to the manufacturer's instructions. The *trip* probe corresponded to a 360 bp *Pst*I–*Xho*I cDNA fragment from the 5′ end of the human *trip* cDNA.

Electrophoretic mobility shift assays (EMSA)

Reactions were performed at 0°C for 20 min in reaction buffer [10 mM Tris–HCl (pH 7.9), 2 mM $MgCl_2$, 0.1mM EDTA, 4% glycerol, 5 ng/µl total yeast RNA and 50 mM KCl] using ~0.2 ng [^{32}P]UTP (Amersham) labelled RNA probe (40 000 c.p.m.) and various concentrations of purified TRIP as indicated in the figure legends, in a total volume of 20 µl. Competitor RNAs or DNAs were added to the reaction before the probe and reactions were analysed by electrophoresis on 3.5% non-denaturing polyacrylamide gels run at 10 V/cm at 4°C unless otherwise indicated.

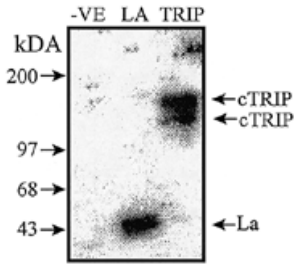


Figure 1. Northwestern analysis of *E.coli* cell-free lysates. Extracts from isopropyl β-D-thiogalactopyranoside (1 mM)-induced *E.coli* containing no plasmid (–VE) or the *in vivo* excised pBKCMV derivatives of the isolated λ ZAP cDNA clones for La (LA) or cTRIP (TRIP) were electrophoresed on a 10% SDS–PAGE, transferred to nitrocellulose and subjected to northwestern analysis using radiolabelled TAR.

RESULTS

Molecular cloning of *trip*

A UV-induced CHO-K1 λcDNA expression library was screened using radiolabelled TAR RNA (+1 to +80). Of the 10^6 plaques screened initially, two consistently bound TAR in three rounds of plaque purification. One of these clones corresponded to La autoantigen which has previously been shown to bind TAR RNA (13). The second clone which appeared to bind TAR equally well in a northwestern assay (Fig. 1) contained a 2063 bp open reading frame (ORF) which was called *trip* (Fig. 2). A database search identified a number of translations of human-expressed sequence tags (ESTs) homologous to the N-terminus of the hamster *trip* ORF (*ctrip*). An EST clone (DDBJ/EMBL/GenBank accession no. N36824) was used to screen a HeLa cDNA library for the full-length human homologue (*htrip*). Several independent clones were identified and the clone with the largest insert was judged to be a full-length cDNA, since an upstream stop codon was identified in the same frame as the major ORF. The cDNA of *htrip* encodes a 784 amino acid protein with a predicted relative molecular mass (RMM) of 86 kDa (Fig. 2). The human and hamster sequences exhibit a high degree of conservation in the N-terminal and C-terminal domains whereas the central domains are less conserved, the overall sequence identity being 61%. The alignment suggests that the cDNA clone for *ctrip* is not full-length.

Database searches indicated that the N-terminal 272 amino acids of hTRIP had 96% sequence identity with the murine FLI associated protein FLAP (4), except murine FLAP has sequence insertions at amino acid positions 51, 83 and 136 of hTRIP (Fig. 2). The splicing pattern reported for a partial cDNA of human FLAP (4) is identical to hTRIP except that hTRIP residues 52–83 are absent in human FLAP. It is not possible to determine whether hTRIP and human FLAP represent alternatively spliced forms of the same gene at present, since the DNA sequence of the partial human FLAP cDNA is not currently available. The C-terminal domain of TRIP has no homology with FLAP or with other proteins in the databases.

A 292 bp (1535–1827) region in the *htrip* cDNA sequence was found to be 100% identical to the sequence encoding the DNA binding domain of the transcriptional repressor GCF (bp 1–292,

Figure 2. Sequence analysis of human and hamster TRIP. Alignment of the predicted amino acid sequences of the human (hTRIP) and hamster (cTRIP) TRIP with murine FLAP. The predicted coiled coil domains are underlined. Identical amino acids in two or more sequences are shown in upper case. The RNA binding domain defined in Figure 6 is shown in bold.

DDBJ/EMBL/GenBank accession no M29204) (24). This highly conserved region of DNA is translated in alternative reading frames in the two genes. The origin of this DNA is unclear though it is unlikely to have arisen from a cloning artefact since we identified a number of independent *htrip* clones containing this sequence together with *ctrip* which has similar sequences in this region. hTRIP has a predicted coiled coil region between residues 127 and 207 (Fig. 2). Residues 561–589 may also form a coiled coil region; however, analysis of the cTRIP sequence only predicted an N-terminal coiled coil domain. This coiled coil domain is conserved in FLAP and corresponds to the domain which interacts with the LRR of FLI (4).

Expression and mitogen induction of *trip*

Northern blot studies using a DNA probe from the 5′ end of the *gcf* gene which encompassed the 292 bp region homologous to *htrip* identified a 3.0 kb *gcf* transcript and two transcripts of unknown origin with sizes of 1.2 and 4.5 kb which were all induced by phorbol esters (25). Northern analysis with a probe to the 5′ end of the *htrip* gene identified a single transcript of 4.5 kb (Fig. 3A), and there was some variation in the levels of *htrip* expression between different cell lines. Despite the protein sequence homology between TRIP and FLAP in the region corresponding to the DNA probe used in northern analysis, no other transcripts were observed even after an extended exposure, raising the possibility that TRIP and FLAP may be encoded by separate genes. The tissue specific expression of *htrip* was investigated using a multiple tissue RNA dot blot (Fig. 3B) and showed significant variations between different tissues with the highest expression in foetal heart. Northern analysis indicated that *htrip* was rapidly induced by phorbol esters, with expression peaking at 4 h after induction and returning to near basal levels at 6 h (Fig. 3C), as observed previously for the 4.5 kb *gcf* related transcript (25). Despite being rescued from a UV-induced cDNA expression library, *htrip* shows no significant induction following UV irradiation of cells (not shown).

Purification and RNA binding specificity of TRIP

To investigate the RNA binding specificity of TRIP, we expressed hTRIP and cTRIP in *E.coli* as GST fusion proteins. Purification of hTRIP produced a significant amount of truncated protein; consequently cTRIP was used in EMSA. The purified recombinant cTRIP protein migrated anomalously on SDS–PAGE with an RMM of 174 kDa, compared to the actual RMM of 113 kDa, and contained a low level of degradation products (Fig. 4A). hTRIP was detected *in vivo* by transient transfection of a HA-tagged *htrip* cDNA. (Fig. 4B). The epitope tagged hTRIP consistently migrated as a doublet with RMMs of 155 and 160 kDa with some minor high mobility bands. This suggests there may be some post-translational processing of TRIP *in vivo*.

The overall RNA binding specificity of cTRIP was investigated by EMSA using single and double stranded RNA and DNA as competitors for TAR (+1 to +57) binding (Fig. 4C). None of the single stranded RNAs was an efficient competitor even when present at a 1000-fold excess (w/w) except polyG (Fig. 4C and D). However, polyG can form higher order structures and has previously been shown to be an efficient competitor for two other TAR stem binding factors, La and TRBP (13,26). Comparison of the competition efficiencies (Fig. 4D) indicates that polyGC has the highest affinity, followed by polyG and then polyAU. Single stranded DNA did not appear to compete for the cTRIP/TAR interaction, but double stranded DNA showed some competition, though only at the higher concentrations (Fig. 4D). These data

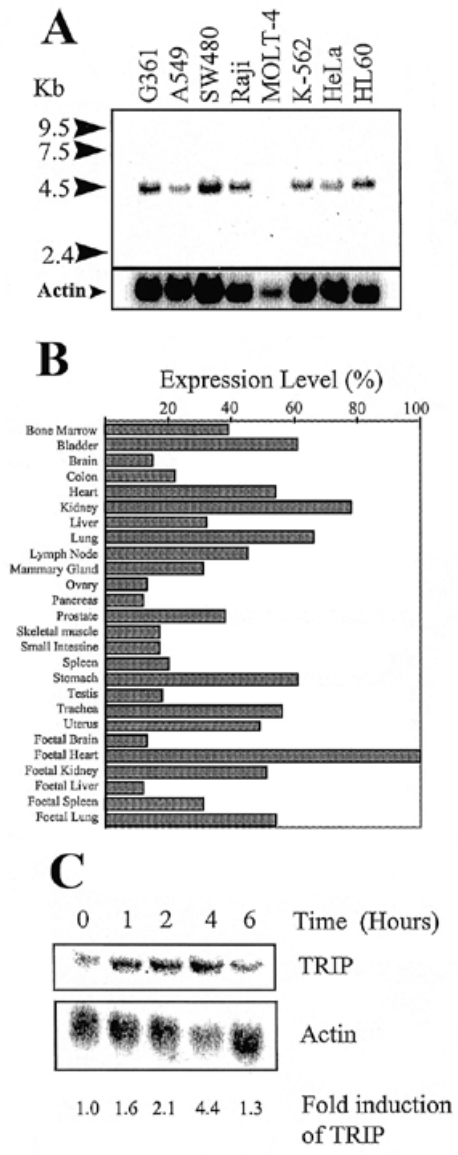


Figure 3. Analysis of *trip* gene expression. (**A**) Northern blot analysis using a fragment from the 5′ end of the *htrip* gene with poly(A)+ RNA isolated from a variety of cancer cell lines. A single transcript of ~4.5 kb is observed. The lower panel shows the signal obtained when the blot was re-hybridised with an actin probe. (**B**) Expression of *htrip* in a variety of human tissues. An RNA dot blot with normalised levels of poly(A)+ RNA (Clontech) was hybridised with an *htrip* probe and the signal quantitated using a PhosphorImager. Expression level is shown as a percentage of the signal seen in foetal heart. (**C**) Phorbol ester induction of *htrip*. HeLa cells were treated with 100 nM PMA, total RNA was isolated at the indicated times and 10 µg was used in northern analysis with an *htrip* probe. The expression of *htrip* was quantitated using a PhosphorImager and normalised with respect to the actin signal.

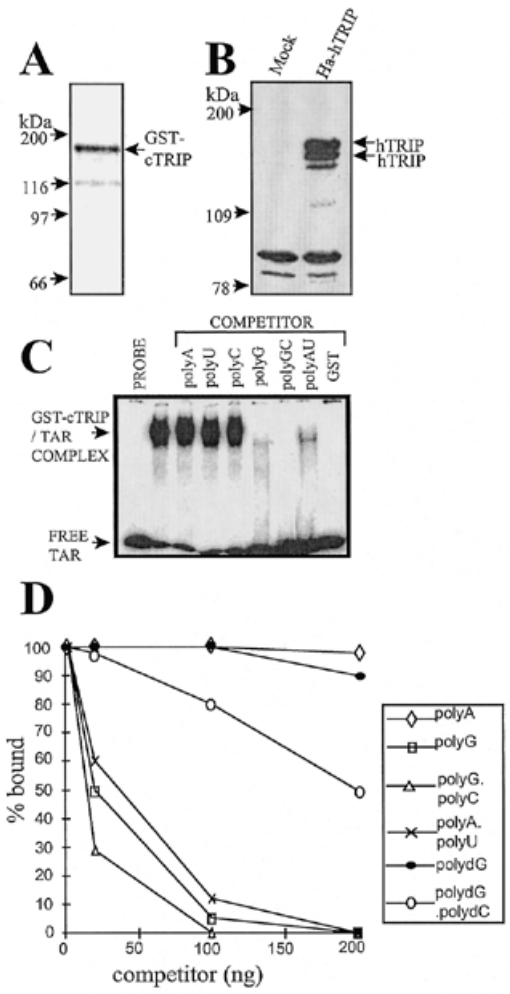


Figure 4. Purification and nucleic acid binding specificity of TRIP. (**A**) A Coomassie-stained 10% SDS–PAGE of the purified GST–cTRIP fusion protein. The faint band seen below the major band represents a minor degradation product of the fusion protein. (**B**) Western blot analysis of 293T cell extracts prepared from mock-transfected and pCMVHAhTRIP-transfected cells, probed with the anti-HA monoclonal antibody 12CA5. (**C**) EMSA were carried out with with TAR RNA (+1 to +57), recombinant purified GST–cTRIP (100 nM) and various ribonucleic acid competitors (100 ng). (**D**) Graphical representation of EMSA competition assays using the indicated competitors at varying concentrations and 100 nM cTRIP protein. The percentage bound was quantitated using a STORM imager and represents the amount of protein–RNA complex formed, relative to the amount of protein–RNA complex formed in the absence of competitor, in each experiment. Experiments were carried out on at least three separate occasions and a representative dataset is shown.

indicate that TRIP preferentially binds to double stranded GC-rich RNA.

There are two TRIP binding sites on the TAR stem

To identify the cTRIP binding site on TAR, a number of mutant TAR RNAs were synthesised and the relative affinities of cTRIP for these RNAs were determined by EMSA. Experiments with wild type TAR showed the formation of two discrete protein–RNA complexes (Fig. 5A). The apparent equilibrium K_d for binding wild type TAR was 14 nM (Fig. 5B) which is comparable with the binding affinity of La for TAR (17 nM) (13). Alteration of the loop sequences to the antisense (mutant LS) did not influence the cTRIP/TAR interaction significantly, nor did deletion of the UCU bulge sequence (TM29) (Fig. 5B). Similarly, alterations to the primary sequence of the lower stem (TM22, TM23) which maintained the structure, did not influence cTRIP binding. A point mutation in the lower stem (TM37) which disrupted the structure of the stem showed a 2-fold reduction in binding affinity for TRIP (Fig. 5B). These data indicate that TRIP binds the TAR stem. The TAR construct lacking much of the lower stem (TARLET) appeared to contain a single cTRIP binding site since the higher mobility complex predominated (Fig. 5A), presumably because there is insufficient RNA duplex for a second TRIP binding site. The binding affinity for this mutant was nearly 3-fold lower than that for wild type TAR, suggesting that cTRIP may bind co-operatively to the two sites. The failure to observe significant amounts of the low mobility complex with TARLET RNA also indicates that the two protein–RNA complexes formed with wild type TAR are not simply due to oligomerisation of TRIP at high concentrations.

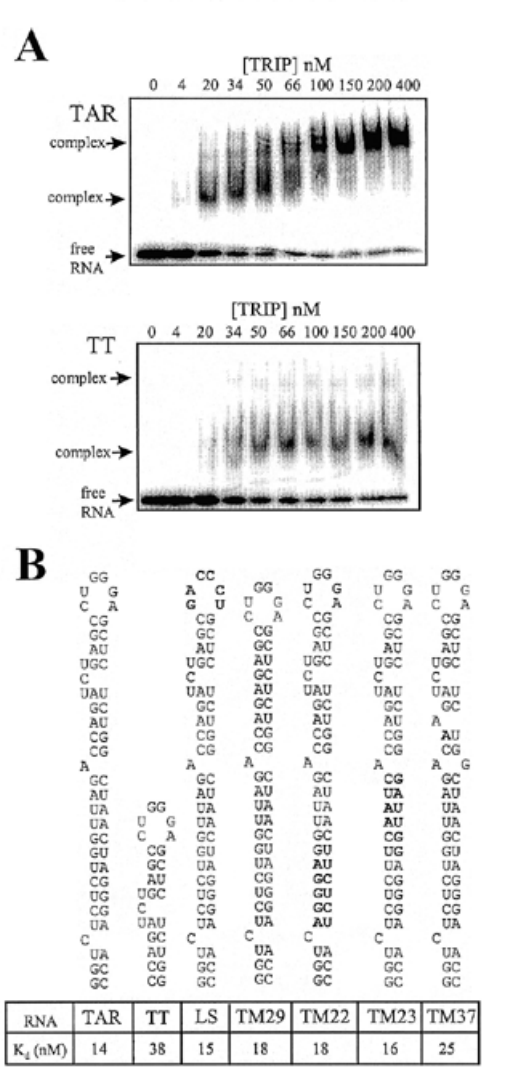
Identification of the TRIP RNA binding domain

To define the RNA binding domain of TRIP, a series of N- and C-terminal deletions of cTRIP were expressed and purified from *E.coli* (Fig. 6A). Constructs which lacked the N-terminal coiled coil domain (cTRIP1, cTRIP2) still migrated anomalously on SDS–PAGE (not shown), suggesting that this was due to aberrant SDS binding rather than the formation of TRIP dimers resistant to boiling in SDS–PAGE loading buffer. The cTRIP5 protein (amino acids 1–440) failed to bind TAR, whereas the cTRIP4 protein (amino acids 1–510) did bind TAR; in addition the cTRIP2 protein (amino acids 388–712) bound TAR (Fig. 6B), thus defining approximate boundaries for the RNA binding domain between amino acids 388 and 510.

A basic region rich in lysines was identified within the RNA binding domain (Fig. 6A) and a further series of GST fusion proteins were expressed and purified to confirm this region was responsible for the RNA binding activity. Firstly, amino acids 459–511 from cTRIP (cTRIP7) and the equivalent residues (amino acids 516–569) based on the sequence alignment (Fig. 2) from hTRIP (hTRIP1) were tested for RNA binding activity (Fig. 6C). Both fusion proteins bound TAR, though increased amounts of fusion protein were required compared with full-length cTRIP to achieve the same amount of complex formation. This may be due to steric hindrance effects caused by the relatively large GST additions or poor folding of the peptides in the context of a GST fusion. The RNA binding domain was further delineated using proteins cTRIP8–11 (Fig. 6A and D). cTRIP8, 10 and 11 all bound TAR, indicating that the lysine-rich domain from residues 482 to 501 was sufficient for binding TAR. Consistent with this, the cTRIP8 protein which only contained half of the lysine-rich domain failed to bind TAR. These data indicate that the RNA binding domain of TRIP encompasses a lysine-rich sequence of 20 amino acids. However, since these peptides did not bind as efficiently as the full-length protein, we cannot exclude the possibility of a contribution from flanking sequences at present.

TRIP binds the LRR of FLI

The almost complete sequence identity between TRIP and the FLI interacting domain of murine and human FLAP suggested that TRIP might also bind the LRR of FLI. To test this, we



RNA	TAR	TT	LS	TM29	TM22	TM23	TM37
K_d (nM)	14	38	15	18	18	16	25

Figure 5. TRIP binds to the TAR stem. (**A**) EMSA were carried out with TAR (+1 to +57) and mutant RNA molecules with varying concentrations of recombinant cTRIP as indicated. Protein–RNA complexes are indicated by arrows. A representative selection of mutant TAR EMSA are shown. (**B**) Sequence of TAR and mutant TAR RNA molecules used in EMSA and cTRIP binding affinities. Sequences shown in bold represent mutations from wild type TAR. The apparent equilibrium K_ds for binding the mutant RNAs were taken as the concentration of cTRIP at which 50% of the input RNA was bound, where the percentage bound = [bound/(bound + free)] × 100. The values shown represent the averages of two independent determinations.

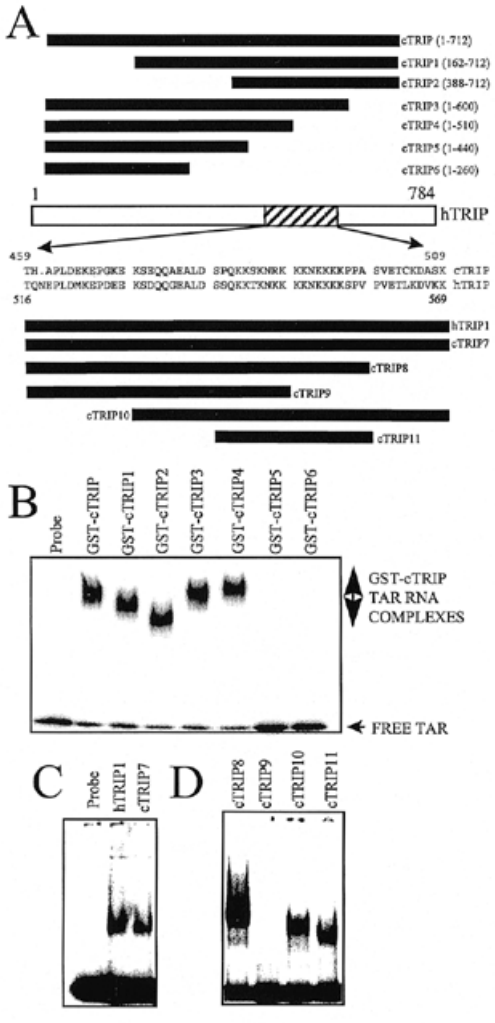


Figure 6. The TRIP RNA binding domain contains a lysine rich motif. (**A**) The extent of 5′ and 3′ end deletions in the *trip* genes used to define the RNA binding domain. The numbering refers to the amino acids incorporated into each fusion. (**B**) EMSA using the purified cTRIP proteins described in (A). An aliquot of 100 nM of the intact purified fusion protein was used in each reaction. (**C**) EMSA using 200 nM of the hTRIP1 and cTRIP7 proteins resolved on a 5% polyacrylamide gel. (**D**) EMSA using 200 nM of the cTRIP8–11 proteins described in (A) resolved on a 5% polyacrylamide gel.

generated radiolabelled FLI LRR and tested its ability to bind hTRIP *in vitro* (Fig. 7). The FLI cDNA used corresponds to amino acids 1–400 which encompasses all of the LRR (amino acids 1–380) and produced a protein of 48 kDa on SDS–PAGE, which is slightly higher that the predicted molecular weight of 46 kDa (Fig. 7, lane 2) whereas a reticulocyte lysate programmed with an antisense cDNA produced no protein (lane 1). Glutathione agarose, either alone or charged with GST (lanes 3 and 4), failed to precipitate FLI LRR. However, GST–hTRIP specifically precipitated FLI LRR (lane 5), demonstrating that TRIP binds the LRR of FLI.

TRIP is localised in the cytoplasm

To determine the intracellular localisation of TRIP, the human cDNA was fused to the green fluorescent protein (GFP) to generate a TRIP–GFP fusion mammalian expression vector and this was transfected into COS-7 cells (Fig. 8). The GFP control is localised throughout the cell whereas a TRIP–GFP fusion is localised in the cytoplasm and appears to be excluded from the nucleus. The basic RNA binding domain of TRIP might have been expected to function as a nuclear localisation signal, but this does not seem to be the case, perhaps because TRIP is anchored in the cytoplasm through its interaction with FLI.

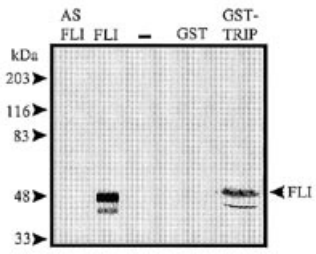


Figure 7. TRIP binds the LRR of FLI. FLI was generated by *in vitro* transcription/translation with reticulocyte lysate (RT) and [^{35}S]methionine and tested for an interaction with GST–hTRIP. Lane 1 (AS FLI), 2 µl RT programmed with pFLIantisense; lane 2 (FLI), 2 µl RT programmed with a sense FLI cDNA; lane 3, proteins from 15 µl RT programmed with pFLIsense bound to glutathione agarose; lane 4, the same as lane 3 except the glutathione agarose was charged with 5 µg GST; lane 5, the same as lane 3 except the glutathione agarose was charged with 5 µg GST–hTRIP. Protein samples were examined on a 10% SDS–PAGE followed by PhosphorImaging.

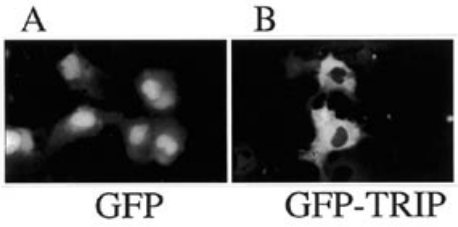


Figure 8. Fluorescence microscopy of COS-7 cells transiently transfected with GFP fusions. (**A**) Cells transfected with pEGFPN1 which expresses a human codon optimised GFP protein. The protein is localised throughout the cell. (**B** Cells transfected with a derivative of pEGFPN1 which expresses a hTRIP–GFP fusion protein which is exclusively localised in the cytoplasm.

DISCUSSION

In this study we have identified a novel mammalian RNA binding protein, TRIP, which is localised in the cytoplasm. TRIP binds to double stranded RNAs with high affinity and shows little binding to single stranded RNA or DNA. Double stranded DNA only acts as a competitor at high concentrations, suggesting that TRIP preferentially recognises an A form RNA helix rather than a B form DNA helix. There is no obvious sequence specificity to the TRIP RNA interaction, and this is a property shared with a number of other double stranded RNA binding proteins. This probably reflects the geometry of an A form helix in which the accessible minor groove provides few opportunities for sequence specific recognition (27,28). Whether TRIP binds to a specific subset of RNAs such as HIV-1 TAR *in vivo* remains to be determined. The RNA binding domain of TRIP does not conform to the well-characterised double stranded RNA binding motif seen in proteins such as TRBP (26), though this motif does have lysine rich elements (29). Instead, the highly basic domain is more reminiscent of those seen in viral proteins such as Tat (30,31), which interact with bulges within regions of double stranded RNA, and cellular proteins such as bicoid whose homeodomain contains a lysine/arginine-rich helix which binds DNA and RNA and regulates translation in *Drosophila* (32).

The DNA sequence of *htrip* has a region with complete sequence identity to the transcriptional repressor *gcf* and previous studies identified two *gcf* related transcripts (25). It seems likely that *htrip* corresponds to one of those transcripts identified previously though the relationship between *trip* and *gcf* remains unclear. It is unlikely that the two transcripts arise by differential splicing since there are no other regions of homology within the genes. It is interesting to note that despite being translated in alternative reading frames, this region of DNA encodes basic amino acids in both proteins, and in each protein this region is responsible for nucleic acid binding. Both *gcf* and *htrip* are induced by phorbol esters, suggesting that *htrip* may play some role in a cell proliferation or growth response.

The N-terminus of TRIP shows almost complete sequence identity with the coiled coil domain of the FLI binding protein, FLAP. Human FLAP was isolated on the basis of its interaction with the FLI LRR by a yeast two-hybrid screen, though its biological function is unclear at present. Northern analysis with human FLAP indicates there are multiple splice variants which are expressed in a tissue-specific manner, and human and murine FLAP show a different splicing pattern, but both proteins interact with the FLI LRR as demonstrated by co-immunoprecipitation (4). Whether hTRIP corresponds to a splice variant of FLAP or arises from a separate gene is not clear at present. We have confirmed that hTRIP interacts with the FLI LRR *in vitro*; therefore the FLI LRR probably has multiple binding partners *in vivo*. Coiled coil domains frequently homo- and hetero-dimerise (33) and TRIP and FLAP or combinations with other coiled coil proteins might dimerise and bind the FLI LRR *in vivo*.

As a cytoplasmic RNA binding protein, TRIP may play a role in a number of different processes including translation, regulation of RNA stability or RNA localisation. Two other TAR stem binding factors identified previously, La and TRBP, have both been implicated in translational regulation. TRBP forms heterodimers with the double stranded RNA activated protein kinase, PKR, preventing its autophosphorylation, and this blocks PKR mediated inhibition of viral protein synthesis (34). In the cytoplasm, La is proposed to render translation cap-dependent in conjunction with other general RNA binding proteins, by binding mRNA and preventing spurious initiations at internal methionines (35). However, since FLI binds actin it is possible that TRIP and FLAP are associated with the actin cytoskeleton *in vivo* and TRIP may play a role in the localisation of RNA to the cytoskeleton. The actin binding domain of FLI shows homology with gelsolin and therefore might be expected to have actin filament severing and capping functions and be involved in reorganisation of the actin cytoskeleton; this is consistent with the observations that FLI mutations in *Drosophila* have a disorganised actin cytoskeleton (2). Therefore TRIP may be involved in the recruitment of RNA to sites of cytoskeletal reorganisation through its interaction with FLI. The TRIP–GFP fusion which would be expected to colocalise with FLI, and therefore actin, does not show a strikingly filamentous distribution; this is consistent with the intracellular distribution seen for a number of actin cytoskeleton modifying proteins such as gelsolin and CapG which show either a diffuse or punctate cytoplasmic distribution (36,37).

For some time it has been known that poly(A)+ RNA co-localises with actin filaments (38) and a number of recent studies have implicated the cytoskeleton in the movement of RNA to specific cellular subcompartments (39). In particular, *ashI* mRNA in *Saccharomyces cerevisiae* is specifically localised

to the presumptive daughter cell nucleus, and this process requires the actin cytoskeleton (40,41). In mammalian cells, the binding of extracellular matrix components to integrin receptors stimulates the formation of focal adhesion complexes (FACs). Recently it has been demonstrated that mRNA and ribosomes rapidly localise to FACs formed following integrin binding (42). This process depends on the ability of integrins to mechanically couple the extracellular matrix to the cytoskeleton, and is associated with tension-dependent restructuring of the actin lattice. Disruption of the actin lattice with cytocholasin D blocks the RNA and ribosome recruitment process, whereas microtubule modifying drugs have only small effects, thus illustrating the importance of the actin cytoskeleton in RNA localisation. It will be interesting to determine whether FLI has gelsolin-like actin filament severing and capping properties and whether an actin–FLI–TRIP interaction causes localisation of RNA to sites of cytoskeleton reorganisation, perhaps in response to mitogenic signals which induce TRIP expression.

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