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The Novel ATM-Related Protein TRRAP Is an Essential Cofactor for the c-Myc and E2F Oncoproteins

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Summary

The c-Myc and E2F transcription factors are among the most potent regulators of cell cycle progression in higher eukaryotes. This report describes the isolation of a novel, highly conserved 434 kDa protein, designated TRRAP, which interacts specifically with the c-Myc N terminus and has homology to the ATM/PI3-kinase family. TRRAP also interacts specifically with the E2F-1 transactivation domain. Expression of transdominant mutants of the TRRAP protein or antisense RNA blocks c-Myc- and E1A-mediated oncogenic transformation. These data suggest that TRRAP is an essential cofactor for both the c-Myc and E1A/E2F oncogenic transcription factor pathways.

Introduction

Since its initial discovery, considerable evidence has accumulated implicating the c-*myc* oncogene in human cancer. In Burkitt's lymphomas and certain AIDS-related lymphomas, the c-*myc* gene is translocated to one of the immunoglobulin gene loci, and amplification of c-*myc* has been described in approximately 15% of all human tumors (Cole, 1986; Henriksson and Lüscher, 1996). A role for c-*myc* in the control of normal cell cycle progression has also been firmly established by studies of fibroblasts that are genetically deficient for the c-*myc* gene, which have a dramatically prolonged cell cycle (Mateyak et al., 1997).

Both the normal and oncogenic functions of c-*myc* are presumed to rely on the ability of its protein product (c-Myc) to act as a sequence-specific transcription factor, since disruption of the c-Myc B/HLH/LZ domain abolishes its oncogenic capacity (Stone et al., 1987; Amati et al., 1993). However, in addition to the DNA-binding domain, the N-terminal region of c-Myc is also essential for transformation. This region encompasses several discrete blocks of amino acids that are conserved in Myc proteins throughout evolution (Cole, 1986; Henriksson and Lüscher, 1996). Based on this conservation, these regions have been referred to as Myc homology boxes. Among the major conserved regions of the N terminus, the most critical for oncogenic transformation is Myc box II (MbII) (Stone et al., 1987), which is centered at amino acid 135 in human c-Myc. Recent studies have shown that deletions or single point mutations within MbII can eliminate the transformation potential of c-Myc (Brough et al., 1995; MacGregor et al., 1996). In addition to transformation, other functions of c-Myc have been shown to be entirely dependent on MbII. These include the induction of apoptosis (Askew et al., 1991; Evan et al., 1992), the ability of c-Myc to block differentiation (Freytag et al., 1990), and the repression of transcription of several genes (Penn et al., 1990a; Penn et al., 1990b; Freytag and Geddes, 1992; Li et al., 1994). These studies suggest that an understanding of the mechanism by which c-Myc functions would be significantly advanced by determining the function of the essential MbII region. Given the modularity of transcription factors, it initially seemed plausible that MbII might serve as a transactivation domain. However, deletion of MbII fails to eliminate the transactivation potential of c-Myc (Kato et al., 1990; Bello-Fernandez et al., 1993; Brough et al., 1995), suggesting that transformation by c-Myc requires an N-terminal function more specific than recruitment of basal transcription factors.

We formulated the hypothesis that MbII is critical because it facilitates the interaction of c-Myc with an essential cofactor. As an initial test of this hypothesis, we analyzed the ability of the c-Myc N terminus to function as a dominant inhibitor of transformation by full-length c-Myc. These experiments demonstrated the presence of an essential, titratable factor that is sequestered by the addition of excess c-Myc N terminus (in the form of a GAL4 fusion protein) (Brough et al., 1995). Other studies have arrived at similar conclusions, using either a GAL4-B-Myc fusion or a c-Myc mutant lacking the C-terminal dimerization domain (Resar et al., 1993; MacGregor et al., 1996). Most importantly, we found that the dominant inhibitory effect of the c-Myc N terminus was abolished by deletion of MbII (Brough et al., 1995). More direct evidence for the existence of a specific MbII-interacting factor came from an analysis of nuclear extracts in which the dominant inhibitory protein containing the c-Myc N terminus was shown to form a novel complex that was dependent on the presence of MbII (Brough et al., 1995).

The oncogenic and biological functions of c-Myc remarkably parallel those of the adenovirus E1A protein, including an ability to transform and immortalize cells, block differentiation, and both activate and repress transcription (Brockmann and Esche, 1995). However, unlike c-Myc, E1A does not function directly as a DNA-binding transcription factor, but instead binds to several nuclear proteins to indirectly control the cell cycle and transcription factor activity. The control of the cell cycle by E1A is largely mediated by binding to the Rb tumor suppressor, which regulates the activity of the E2F family of transcription factors (Nevins et al., 1997). E1A binding to Rb prevents Rb from inhibiting E2F activity. Like c-Myc, E2F can promote entry into S phase, transform cells, induce apoptosis, and activate transcription (Nevins et al., 1997). Rb binds to the transactivation domain of E2F, which prevents transcriptional activation of E2F target

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genes and converts E2F into a direct repressor (Nevins et al., 1997). While the transactivation domain is essential for most biological activities of E2F (Nevins et al., 1997), positive effectors that are recruited by this domain have not been defined. The connection between the oncogenic functions of E1A/E2F and c-Myc has remained elusive. However the c-Myc N terminus can inhibit oncogenic transformation by E1A, suggesting that some common factor may participate in both pathways (Brough et al., 1995; MacGregor et al., 1996). Consistent with this interpretation, the inhibition of E1A-mediated transformation was dependent on the MbII domain of the c-Myc N terminus (Brough et al., 1995).

Since the dominant inhibition assays and MbII-specific nuclear complexes provided strong evidence for the existence of an essential effector of c-Myc function, we initiated the biochemical purification of this cofactor and isolated the corresponding cDNA, which we have designated the *TRRAP* gene, for transformation/transcription domain–associated protein. TRRAP is conserved in evolution and is essential for oncogenic transformation of mammalian cells. Identification of the TRRAP protein provides a novel connection between the c-Myc and E2F transcription factors and a new avenue to define their oncogenic functions.

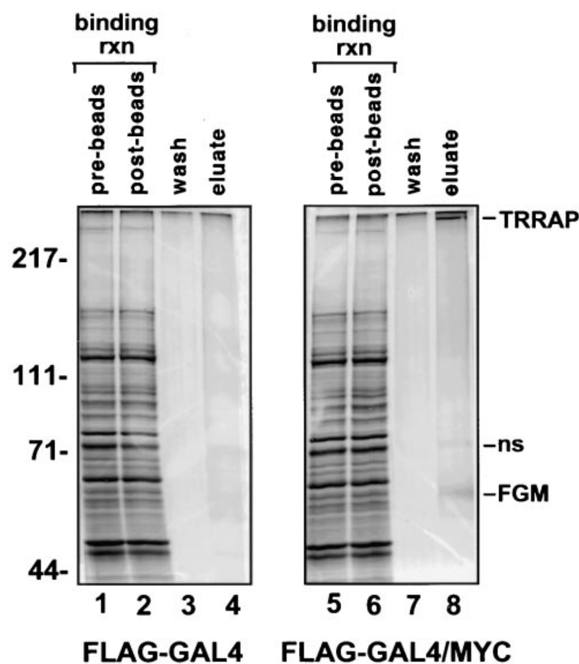

Figure 1. Affinity Purification of a Nuclear Protein Interacting with the c-Myc Amino Terminus

The FLAG-GAL4/Myc and FLAG-GAL4 proteins were produced as nuclear extracts from Sf9 cells and mixed with heat-treated HeLa nuclear extract and anti-FLAG monoclonal antibody. After capture on Protein A/G beads, beads were washed and the bound proteins eluted by the addition of FLAG peptide. Aliquots from indicated stages of a large-scale binding reaction were run on an 8% SDS/polyacrylamide gel, and proteins were visualized by silver staining. Lanes 1–3, aliquots of the binding reaction, postbead supernatant, and bead wash for a reaction containing FLAG-GAL4, HeLa cell nuclear extract, and anti-FLAG antibody. Lane 4, FLAG peptide eluate from this reaction. No c-Myc interacting nuclear factor (TRRAP) or FLAG-GAL4/Myc are visible. Lane 5–7, aliquots of the binding reaction, postbead supernatant, and bead wash for a reaction containing FLAG-GAL4/Myc, HeLa cell nuclear extract, and anti-FLAG antibody. Lane 8, eluate from this reaction. The eluate contains a protein of 60 kDa corresponding to the FLAG-GAL4/Myc fusion protein (labeled FGM) and a large protein, designated TRRAP, that migrates near the top of the resolving gel. The identity of the FLAG-GAL4/Myc band was confirmed by Western blotting (data not shown). A nonspecific protein associated with the Protein A/G beads in both binding reactions (ns in lanes 4 and 8). Molecular weight (indicated in kilodaltons at left) was determined by coelectrophoresis of protein MW marker (Novex).

Results

Affinity Purification of a c-Myc-Interacting Nuclear Protein

We have previously demonstrated that one or more unknown nuclear proteins associate with the c-Myc N terminus and that this interaction is dependent on the presence of MbII (Brough et al., 1995). To affinity purify this MbII-dependent, c-Myc-interacting protein(s), a fusion protein containing c-Myc amino acids 1–262 and the GAL4 DNA-binding domain was tagged with the FLAG epitope (Hopp et al., 1988). As a control, an independent fusion protein containing only the FLAG-GAL4 portion was produced. The FLAG-GAL4 and FLAG-GAL4/Myc proteins were produced as nuclear extracts from baculovirus-infected insect cells. These extracts were mixed with nuclear extract from HeLa cells, which was previously shown to contain the N-terminal interacting factor(s). It was determined that the factor was stable to heating of HeLa cell nuclear extracts to 55°C for 10 min (data not shown). Since this treatment resulted in the precipitation of approximately 80% of the nuclear proteins, it was used as an initial purification.

Affinity purification of the c-Myc interacting protein was achieved by large-scale capture and elution of the multiprotein complex with FLAG peptide (Figure 1). Following separation by SDS-PAGE and silver staining, the initial binding reaction contains a wide range of polypeptides, reflecting the complexity of the nuclear extract (Figure 1, lane 5). Surprisingly, the eluate from the FLAG-GAL4/Myc extracts contained only two specific polypeptides, one of which is the 60 kDa FLAG-GAL4/Myc protein (labeled FGM, lane 8). The only other prominent polypeptide in the eluate migrated well above the 217 kDa marker (Figure 1, lane 8). No other specific polypeptides were visible even when eluates were resolved by higher percentage SDS-PAGE (data not shown). While it remains possible that a third, unresolved polypeptide links c-Myc to the large polypeptide visible in Figure 1, the most straightforward interpretation is that the interaction is direct. The large protein identified here has been designated TRRAP based on the characterization presented in the remainder of this study. The binding specificity of this large unknown polypeptide for the c-Myc N terminus was demonstrated by its absence in eluates from a parallel reaction in which FLAG-GAL4 was used in place of FLAG-GAL4/Myc (Figure 1, lanes 1–4).

TRRAP Polypeptide Sequence and cDNA Isolation

Approximately 200 pmol (80 μg) of the c-Myc binding protein was isolated and processed for automated microsequencing. Reliable amino acid sequence was obtained from ten peptides. Degenerate oligonucleotides

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MAFVATQGATVVDQTTLMKKYLQFVAALTDVNTPDETKLKMMQEVSENFENVTSSPQYSTFLEHIIPRFLTFLQDGEVQFLQEKPAQQLRKLVLEIIHRI  100
PTNEHLRPHTKNVLSVMFRFLETENEENVLICLRIIIELHKQFRPPITQEIHHFLDFVKQIYKELPKVVNRYFENPQVIPENTVPPPEMVGMITTIAVKV  200
NPEREDSETRTHSIIPRGSLSLKVLAELPIIVVLMYQLYKLNIHNVVAEFVPLIMNTIAIQVSAQARQHKLYNKELYADFIAAQIKTLSFLAYIIRIYQE  300
LVTKYSQQMVKGMLQLLSNCPAETAHLRKELLIAAKHILTTELRNQFIPCMDKLFDESILIGSGYTARETLRPLAYSTLADLVHHVRQHLPLSDLSLAVQ  400
LFAKNIDDESLPSSIQTMSCKLLLNLVDCIRSKSEQESGNGRDVLMRMLEVFVLKFHTIARYQLSAIFKKCKPQSELGAVEAALPGVPTAPAAPGPAPSP  500
APVPAPPPPPPPPPPATPVTPAPVPPFEKQGEKDKEDKQTFQVTDCRSLVKTLVCGVKTITWGITSCKAPGEAQFIPNKQLQPKETQIYIKLVKYAMQAL  600
DIYQVQIAGNGQTYIRVANCQTVRMKEEKEVLEHFAGVFTMMNPLTFKEIFQTTVPYMVERISKNYALQIVANSFLANPTTSALFATILVEYLLDRLPEM  700
GSNVELSNLYLKLFKLVFGSVSLFAAENEQMLKPHLHKIVNSSMELAQTAKEPYNYFLLLRALFRSIGGGSHDLLYQEFLPLLPNLLQGLNMLQSGLHKQ  800
HMKDLFVELCLTVPVRLSSLLPYLPMLMDPLVSALNGSQTLVSQGLRTLELCVDNLQPDFLYDHIQPVRAELMQALWRTLRNPADSISHVAYRVLGKFGG  900
SNRKMLKESQKLHYVVTEVQGPSITVEFSDCKASLQLPMEKAIETALDCLKSANTEPYYRRQAWEVIKCFLVAMMSLEDNKHALYQLLAHPNFTEKTIPN 1000
VIISHRYKAQDTPARKTFEQALTGAFMSAVIKDLRPSALPFVASLIRHYTMVAVAQQCGPFLLPCYQVGSQPSTAMFHSEENGSKGMDPLVLIDAIAICM 1100
AYEEKELCKIGEVALAVIFDVASIILGSKERACQLPLFSYIVERLCACCYEQAWYAKLGGVVSIKFLMERLPLTWVLQNQQTFLKALLFVMMDLTGEVSN 1200
GAVAMAKTTLEQLLMRCATPLKDEERAEEIVAAQEKSFHHVTHDLVREVTSPNSTVRKQAMHSLQVLAQVTGKSVTVIMEPHKEVLQDMVPPKKHLLRHQ 1300
PANAQIGLMEGNTFCTTLQPRLFTMDLNVVEHKVFYTELLNLCEAEDSALTKLPCYKSLPSLVPLRIAALNALAACNYLPQSREKIIAALFKALNSTNSE 1400
LQEAGEACMRKFLEGATIEVDQIHTHMRPLLMMLGDYRSLTLNVVNRLTSVTRLFPNSFNDKFCDQMMQHLRKWMEVVVITHKGGQRSDGNEMKICSAII 1500
NLFHLIPAAPQTLVKPLLEVVMKTERAMLIEAGSPFREPLIKFLTRHPSQTVELFMMEATLNDPQWSRMFMSFLKHKDARPLRDVLAANPNRFITLLLPG 1600
GAQTAVRPGSPSTSTMRLDLQFQAIKIISIIVKNDDSWLASQHSLVSQLRRVWVSENFQERHRKENMAATNWKEPKLLAYCLLNYCKRNYGDIELLFQLL 1700
RAFTGRFLCNMTFLKEYMEEEIPKNYSIAQKRALFFRFVDFNDPNFGDELKAKVLQHILNPAFLYSFEKGEGEQLLGPPNPEGDNPESITSVFITKVLDP 1800
EKQADMLDSLRIYLLQYATLLVEHAPHHIHDNNKNRNSKLRRLMTFAWPCLLSKACVDPACKYSGHLLLAHIIAKFAIHKKIVLQVFHSLLKAHAMEARA 1900
IVRQAMAILTPAVPARMEDGHQMLTHWTRKIIVEEGHTVPQLVHILHLIVQHFKVYYPVRHHLVQHMVSAMQRLGFTPSVTIEQRRLAVDLSEVVIKWEL 2000
QRIKDQQPDSDMDPNSSGEGVNSVSSSIKRGLSVDSAQEVKRFRTATGAISAVFGRSQSLPGADSLLAKPIDKQHTDTVVNFLIRVACQVNDNTNTAGSP 2100
GEVLSRRCVNLLKTALRPDMWPKSELKLQWFDKLLMTVEQPNQVNYGNICTGLEVLSFLLTVLQSPAILSSFKPLQRGIAACMTCGNTKVLRAVHSLLSR 2200
LMSIFPTEPSTSSVASKYEELECLYAAVGKVIYEGLTNYEKATNANPSQLFGTLMILKSACSNNPSYIDRLISVFMRSLQKMVREHLNPQAASGSTEATS 2300
GTSELVMLSLELVKTRLAVMSMEMRKNFIQAILTSLIEKSPDAKILRAVVKIVEEWVKNNSPMAANQTPTLREKSILLVKMMTYIEKRFPEDLELNAQFL 2400
DLVNYVYRDETLSGSELTAKLEPAFLSGLRCAQPLIRAKFFEVFDNSMKRRVYERLLYVTCSQNWEAMGNHFWIKQCIELLLAVCEKSTPIGTSCQGAML 2500
PSITNVINLADSHDRAAFAMVTHVKQEPRERENSESKEEDVEIDIELAPGDQTSTPKTKELSEKDIGNQLHMLTNRHDKFLDTLREVKTGALLSAFVQLC 2600
HISTTLAEKTWVQLFPRLWKILSDRQQHALAGEISPFLCSGSHQVQRDCQPSALNCFVEAMSQCVPPIPIRPCVLKYLGKTHNLWFRSTLMLEHQAFEKG 2700
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LWEDHWIRCSKELNQWEALTEYGQSKGHINPYLVLECAWRVSNWTAMKEALVQVEVSCPKEMAWKVNMYRGYLAICHPEEQQLSFIERLVEMASSLAIRE 2900
WRRLPHVVSHVHTPLLQAAQQIIELQEAAQINAGLQPTNLGRNNSLHDMKTVVKTWRNRLPIVSDDLSHWSSIFMWRQHHYQAIVTAYENSSQHDRSSIT 3000
LSWVHASAQRSSSMEKSRKQGLVNVALDILSRIHTIPTVPIVDCFQKIRQQVKCYLQLAGVMGKNECMQGLEVIESTNLKYFTKEMTAEFYALKGMFLAQ 3100
INKSEEANKAFSAAVQMHDVLVKAWAMWGDYLENIFVKERQLHLGVSAITCYLHACRHQNESKSRKYLAKVLWLLSFDDDKNTLADAVDKYCIGVPPIQW 3200
LAWIPQLLTCLVGSEGKLLLNLISQVGRVYPQAVYFPIRTLYLTLKIEQRERYKSDPGPIRATAPMWRCSRIMHMQRELHPTLLSSLEGIVDQMVWFREN 3300
WHEEVLRQLQQGLAKCYSVAFEKSGAVSDAKITPHTLNFVKKLVSTFGVGLENVSNVSTMFSSAASESLARRAQATAQDPVFQKLKGQFTTDFDFSVPGS 3400
MKLHNLISKLKKWIKILEAKTKQLPKFFLIEEKCRFLSNFSAQTAEVEIPGEFLMPKPTHYYIKIARFMPRVEIVQKHNTAARRLYIRGHNGKIYPYLVM 3500
NDACLTESRREERVLQLLRLLNPCLEKRKETTKRHLFFTVPRVVAVSPQMRLVEDNPSSLSLVEIYKQRCAKKGIEHDNPISRYYDRLATVQARGTQASH 3600
QVLRDILKEVQSNMVPRSMLKEWALHTFPNATDYWTFRKMFTIQLALIGFAEFVLHLNRLNPEMLQIAQDTGKLNVAYFRFDINDATGDLDANRPVPFRL 3700
TPNISEFLTTIGVSGPLTASMIAVARCFAQPNFKVDGILKTVLRDEIIAWHKKTQEDTSSPLSAAGQPENMDSQQLVSLVQKAVTAIMTRLHNLAQFEGG 3800
ESKVNTLVAAANSLDNLCRMDPAWHPWL
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Figure 2. Sequence of the Human TRRAP Protein

Sequences of *TRRAP* cDNA clones identified as described in the text were used to determine the predicted 3828–amino acid sequence of the human TRRAP protein. A bipartite nuclear localization signal at 2029–2046, a potential leucine zipper at 3403–3424, and two TPR regions at 2752–2785 and 3088–3121 are underlined. The region of TRRAP with homology to the ATM/PI-3 kinase family is boxed.

were designed based on the sequence of two of the peptides and then used to probe a random-primed cDNA library from HeLa cells. From 5 $\times$ 10^5 phage, a single plaque hybridized to both degenerate oligonucleotides. Translation of the nucleotide sequence demonstrated that the polypeptide it encoded contained both of the peptides on which the screen was based, as well as another of the ten peptides derived from the affinity-purified protein. Probes were derived from either end of the original cDNA insert and used to rescreen the same library for clones that extended beyond the original isolate. A complete 12.4 kb cDNA can be reconstructed by aligning a minimum of seven of these partial cDNAs (data not shown). This assembled cDNA encodes a novel protein of 3828 amino acids (Figure 2), which contains all ten of the peptides obtained by microsequencing the original polypeptide. When used to search the EST database, the extreme 3′ end of this cDNA matched a collection of approximately 60 human and mouse ESTs. Fortuitously, one of the human ESTs corresponding to the *TRRAP* cDNA had been included in a study that randomly mapped the chromosomal location of several hundred unknown cDNAs (Berry et al., 1995). The *TRRAP* gene corresponds to the EST NIB217, and this gene is located on human chromosome 7 at q21.3-22.1.

TRRAP Is Highly Conserved in Evolution

The predicted amino acid sequence of human TRRAP was compared to protein databases, revealing unique homologs in four other eukaryotes (Figure 3). The TRRAP homologs are all defined as predicted open reading frames found by genome sequencing projects in *S. cerevisiae*, *S. pombe*, *C. elegans*, and *A. thaliana*. Consequently, no functional data have been reported for any of the TRRAP homologs. Remarkably, the ORFs from all five species predict very large proteins that are nearly

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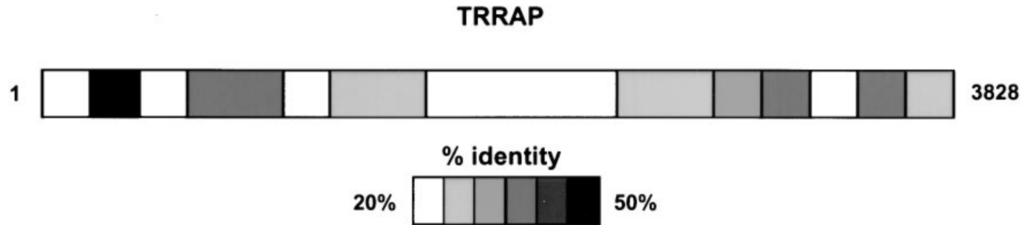

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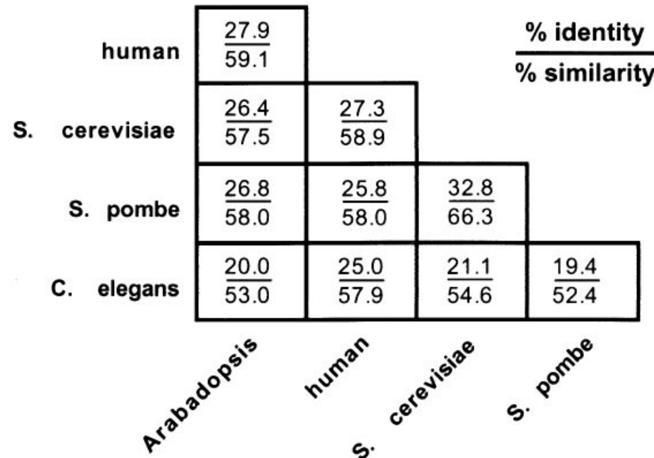

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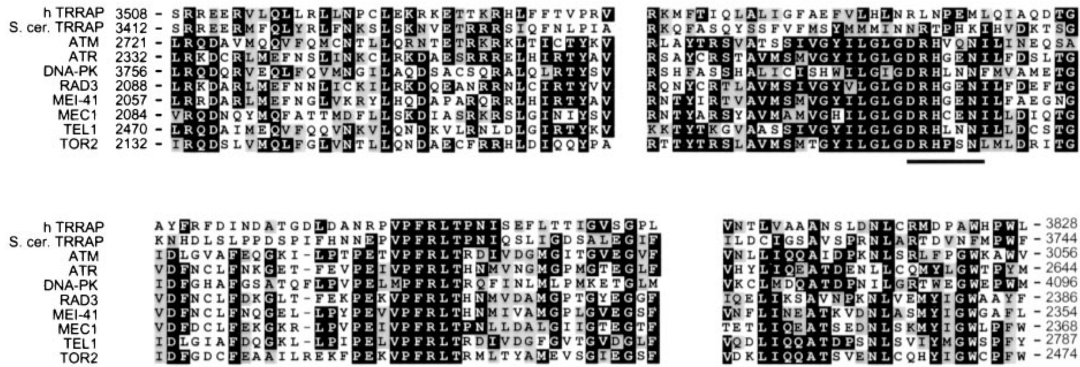

Figure 3. TRRAP Is Conserved in Evolution and Related to Proteins of the ATM/PI-3 Kinase Family

(A) The human TRRAP protein was compared to the *S. cerevisiae* homolog in 200–amino acid blocks using the LALIGN algorithm (Huang and Miller, 1991). Shading indicates the percentage of identical amino acids between the two proteins within each block.

(B) The TRRAP homologs from human, *S. cerevisiae*, *S. pombe*, *A. thaliana*, and *C. elegans* (accession numbers P38811, O10064, AC003952, and Z69902, respectively) were aligned using the LALIGN algorithm (Huang and Miller, 1991). The percent of identical and similar amino acids between each pair of TRRAP homologs is indicated.

(C) Selected blocks of conservation from the C-terminal 300 amino acids of the human and *S. cerevisiae* TRRAP homologs are aligned with similar regions from the ATM/PI-3 kinase family using the CLUSTALW v.1.6 algorithm (Myers and Miller, 1988) and conserved amino acids highlighted with BOXSHADE. The DXXXXN and DFG motifs that constitute part of the catalytic site in the ATM/PI-3 kinase–related proteins are underlined, and these motifs are missing from TRRAP.

identical in size. The conservation among TRRAP homologs is not due to a small protein domain containing a common structural motif, since homology can be identified in numerous colinear blocks throughout the length of the protein (Figure 3A). Human TRRAP is ~25% identical to its homologs in pairwise comparisons using the LALIGN algorithm (Figure 3B). When conservative substitutions are allowed, the total similarity between TRRAP

proteins in different species is 55%–60%. Our studies of the *S. cerevisiae* TRRAP protein (designated TRA1p) indicate that it is essential for vegetative growth (data not shown).

TRRAP Is Related to the ATM/PI-3 Kinase Family

Beyond the identification of closely related TRRAP homologs in different species, database searches also revealed that the C-terminal portion of TRRAP exhibits a significant homology to the PI-3 kinase domain of the ataxia telangiectasia mutated (ATM) protein family (Figure 3C). In TRRAP, as in all of the diverse members of the ATM family, the homology is confined to the extreme C terminus of these very large proteins (Enoch and Norbury, 1995; Zakian, 1995; Lavin and Shiloh, 1996). The homology extends over ∼300 amino acids, and there are numerous residues that are conserved among all members of the family, including TRRAP. Curiously, the amino acids that map to the catalytic site of the ATM/PI-3 kinase family proteins are not found in TRRAP, although flanking residues are conserved (Figure 3C; see Discussion for further detail).

As predicted by our previous studies (Brough et al., 1995), TRRAP is almost exclusively nuclear in localization (data not shown) and contains a bipartite nuclear localization signal consensus at amino acids 2029–2046. Beyond the ATM/PI-3 kinase domain, TRRAP contains two other motifs that could prove functionally relevant. There are eight LXXLL sequences dispersed throughout the protein, a motif associated with several transcriptional coactivators (Heery et al., 1997; Torchia et al., 1997). TRRAP also includes two TPR motifs located near the C terminus. In general, the TPR motif is found in proteins that are components of large multisubunit complexes (Sikorski et al., 1990).

TRRAP Binding Requires Domains of c-Myc that Are Critical for Transformation

The experiments presented above demonstrate an association between TRRAP and the c-Myc N terminus as a GAL4 fusion protein. To confirm that an interaction with endogenous TRRAP requires the critical MbII domain of c-Myc, duplicate plates of 293 cells were transiently transfected with an expression vector directing production of a c-Myc protein that included an N-terminal FLAG epitope. A similar construct encoding a FLAG epitope–tagged version of the transformation-defective c-Myc mutant lacking MbII (Δ129–145) was also transfected. Transfection of vector alone served as a negative control. Cells were lysed using native conditions at 24 hr posttransfection (Harlow et al., 1986), and the equivalent expression of the FLAG-tagged c-Myc proteins was demonstrated by probing Western blots of the lysates with anti-FLAG antibodies (Figure 4B, lower panel, lanes 2 and 3). The remaining lysate was immunoprecipitated using anti-FLAG antibodies, resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with antisera against the TRRAP protein. The results showed that the endogenous TRRAP protein was specifically precipitated from cells expressing FLAG-tagged c-Myc, but not from cells expressing the control vector that only encodes the FLAG epitope (Figure 4B, upper panel, lanes 3 and 4). Strikingly, the in vivo interaction of c-Myc and TRRAP was abrogated by deletion of MbII (Figure 4B, upper panel, lanes 5 and 6). Equivalent immunoprecipitation of the FLAG-tagged proteins was confirmed by probing a fraction of each sample with anti-FLAG (data not shown). A weak interaction between TRRAP and c-Myc Δ129–145 has been observed upon longer exposures. Thus, the interaction of the c-Myc protein with TRRAP requires a domain that is essential for cell transformation, and this in vivo binding parallels the MbII-dependent dominant inhibitory activity of the c-Myc N-terminal fusion protein that initially provided an assay for the presence of TRRAP (Brough et al., 1995).

Deletion mutants of the FLAG-GAL4/MYC protein indicated that a region N-terminal of amino acid 110 in c-Myc was also required for TRRAP interaction (data not shown). To define this second critical region, a series of deletion mutants was created in the FLAG-tagged c-Myc protein. These deletions spanned amino acids 1–110, 20–48, 24–31, 33–38, or 38–48 in the c-Myc protein. As a test of the biological activity of these c-Myc mutants, c-*myc*/H-*ras* cotransformation assays were performed using primary rat embryo fibroblasts (REFs) (Figure 4A). In addition to the deletion mutants in the c-Myc 1–110 region, the MbII deletion mutant (Δ129–145) was also tested. As expected, deletion of MbII eliminated the transformation potential of c-Myc, and four of the other N-terminal deletion mutants (Δ1–110, Δ20–48, Δ24–31, and Δ38–48) either eliminated or severely reduced transformation (Figure 4A). In contrast, one of the smaller deletion mutants, Δ33–38, retained transformation potential that was equal to that observed with wild-type c-Myc. These mutants were then tested in 293 cells for their ability to interact with endogenous TRRAP. Following transient transfection of 293 cells, the various c-Myc proteins were immunoprecipitated, and the precipitates were probed for either the FLAG epitope–tagged c-Myc proteins (Figure 4C, bottom) or for TRRAP (Figure 4C, top). In support of a role for TRRAP in cellular transformation by c-Myc, only the single c-Myc mutant capable of transformation, Δ33–38, was competent for TRRAP interaction. These results suggest that c-Myc has an extended domain of interaction with TRRAP, requiring both MbII and a more N-terminal domain. An alternative explanation is that many different N-terminal c-Myc mutations can induce a global change in conformation that is unfavorable for TRRAP binding. The bipartite nature of the TRRAP interaction domain on c-Myc is similar to that seen for the interaction of c-Myc with other proteins, including Rb, TBP, and Bin1 (Hateboer et al., 1993; Sakamuro et al., 1996).

To determine whether the association between TRRAP and c-Myc can be observed with endogenous c-Myc, coprecipitation studies were conducted using the human colon carcinoma line COLO 320, which produces high levels of c-Myc protein (Alitalo et al., 1983). COLO 320 cells were lysed using a protocol designed for the efficient extraction of endogenous c-Myc (Sommer et al., 1998), and the c-Myc protein was immunoprecipitated using antibodies directed against its obligate partner, Max. Antibodies to Max were utilized because antibodies directed against c-Myc itself might block the interaction between c-Myc and TRRAP. The anti-Max precipitate contained the c-Myc protein (Figure 4D, lower panel), and the same precipitate also contained

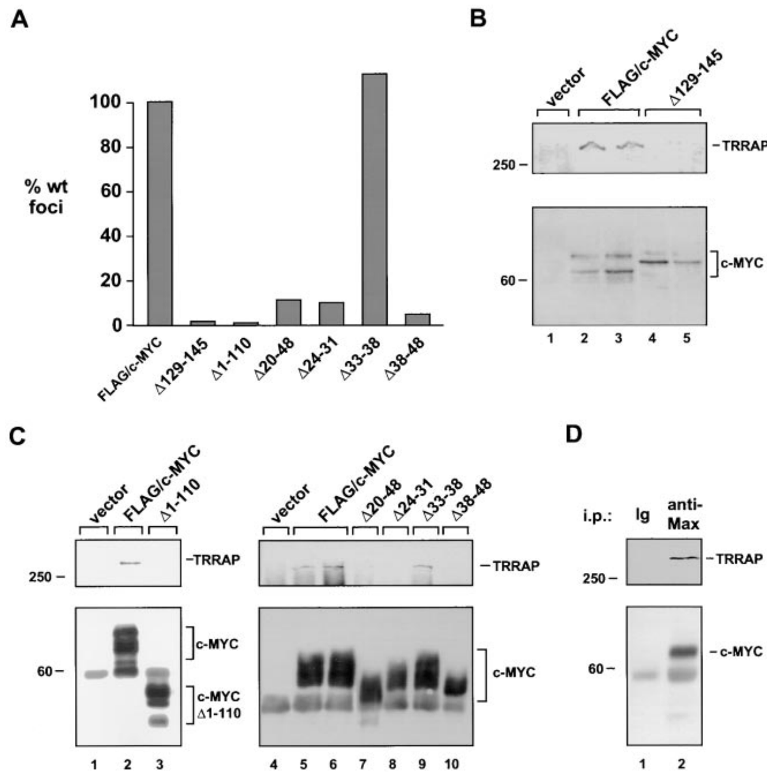

Figure 4. TRRAP Interaction Correlates with Cellular Transformation by c-Myc

(A) Primary rat embryo fibroblasts (REFs) were transfected with expression vectors for H-*ras* and one of various FLAG-tagged c-Myc proteins. The number of transformed foci are expressed as a percentage of the number of foci obtained in the same assay for wild-type c-Myc. The total number of plates assayed with each c-Myc protein was as follows: 8 for FLAG/c-Myc, 4 for Δ129–145 and Δ1–110, and 6 for Δ20–48, Δ24–31, Δ33–38, and Δ38–48.

(B) 293 cells were transiently transfected with expression vectors for either FLAG epitope–tagged mouse c-Myc protein or an MbII deletion mutant (Δ129–145). Lysates from duplicate transfections were prepared, and a fraction of each lysate (5%) was resolved in 8% SDS-PAGE and Western blotted with anti-FLAG antibody (bottom). The remainder of each lysate was immunoprecipitated with anti-FLAG antibody, resolved on 4% SDS-PAGE, and Western blotted with anti-TRRAP antibodies (top). Lane 1, lysates from cells transfected with a vector expressing the FLAG epitope alone. Lanes 2 and 3, lysates from cells expressing FLAG-c-Myc. Lanes 4 and 5, lysates from cells expressing FLAG-c-Myc(Δ129–145). Size (in kilodaltons) at the left was determined by coelectrophoeresis of protein MW markers (Novex).

(C) 293 cells were transiently transfected with CMV-driven expression vectors for FLAG-tagged full-length mouse c-Myc or N-terminal deletion mutants of this construct, as indicated. Lysates were prepared and c-Myc proteins immunoprecipitated with anti-FLAG antibody. Precipitated proteins were resolved by SDS-PAGE and Western blotted for either TRRAP (top) or the FLAG epitope of the transfected c-Myc proteins (bottom).

(D) Cells from the human colon carcinoma line COLO 320 were lysed and subjected to immunoprecipitation using either rabbit anti-Max antisera or normal rabbit immunoglobulin as a control. Precipitates were resolved by 8% SDS-PAGE and blotted for either c-Myc (using the anti-human c-Myc monoclonal 9E10) or for TRRAP as indicated.

the endogenous TRRAP protein (Figure 4D, upper panel). This observation indicates that the association observed with transfected c-Myc also occurs with endogenous c-Myc. Neither c-Myc nor TRRAP were detectable in control immunoprecipitates for COLO 320 cells (Figure 4D).

Transdominant Mutants and Antisense TRRAP Inhibit Transformation by c-Myc

Since the MbII-dependent binding of TRRAP paralleled the dominant inhibitory activity of the c-Myc N terminus (Brough et al., 1995), it was important to test directly the link between TRRAP and the biological activity of c-Myc. This same strategy was employed to identify dominant inhibitory fragments of TRRAP capable of blocking c-Myc function. The c-*myc*/H-*ras* transformation assay with primary REFs was supplemented with expression vectors directing the production of various TRRAP protein fragments. Partial *TRRAP* cDNAs encoding amino acids 270–599, 856–1134, 1261–1579, or 3402–3828 were cloned into a CMV promoter–driven mammalian expression vector downstream of sequences for the FLAG epitope. The N-terminal FLAG epitope provided the initiation codon for these proteins, as well as a method for assessing protein production, and the proteins were expressed at relatively similar levels (Figure 5A). The CMV vector containing the FLAG sequences alone (labeled CβF) was used as a control. At 12–14 days posttransfection, the number of transformed foci per plate was determined and expressed as a percentage of the foci obtained on plates transfected with equivalent amounts of the vector control. Expression of TRRAP amino acids 270–599 or 856–1134 had no significant effect on transformation of REFs by c-*myc* and H-*ras*. In marked contrast, expression of TRRAP amino acids 1261–1579 or 3402–3828 inhibited transformation by 92% and 52%, respectively. Interestingly, the most inhibitory domain of TRRAP (amino acids 1261–1579) contains a leucine-rich motif similar in position to a leucine-rich domain of ATM that also functions as a dominant inhibitor (Morgan et al., 1997). Whether the inhibitory mutants of TRRAP function by directly binding c-Myc or by sequestration of a downstream component in the c-Myc/TRRAP pathway remains to be determined.

As a second method of interfering with endogenous cellular TRRAP function, antisense RNA was used to block the synthesis of TRRAP protein. A fragment of the *TRRAP* cDNA was cloned into a CMV expression vector in antisense orientation and cotransfected with c-*myc* and H-*ras* in a transformation assay. The empty vector served as a control. Expression of *TRRAP* antisense inhibited focus formation by 90%, relative to the control level (Figures 5C and 5D). In addition to the results obtained with transdominant mutants of TRRAP, this anti-

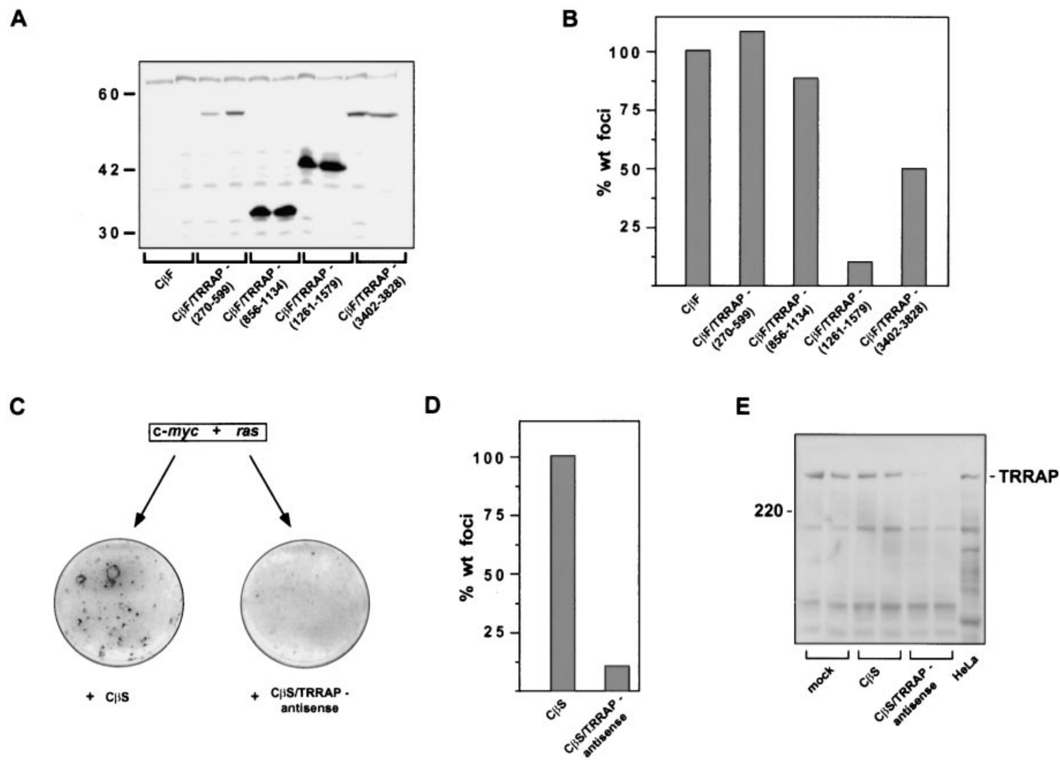

Figure 5. Expression of Transdominant TRRAP Mutants or TRRAP Antisense Blocks Transformation of Rat Embryo Fibroblasts by c-*myc* and H-*ras*

(A) Expression of TRRAP protein fragments in 293 cells was assayed by Western blotting for the FLAG epitope. Duplicate plates of 293 cells were transiently transfected with expression vectors encoding the TRRAP amino acids indicated. Lysates were resolved by 8% SDS-PAGE. Molecular weight markers were run on the same gel and their positions indicated at left in kilodaltons.

(B) Primary REFs were transfected with expression vectors for c-*myc* and H-*ras* and, where indicated, expression vectors encoding fragments of the TRRAP. The TRRAP fragments expressed in this assay encoded amino acids 270–599, 856–1134, 1261–1579, or 3402–3828, as labeled. The number of transformed foci is expressed as a percentage of the number of foci obtained on c-*myc*/H-*ras* plates cotransfected with the parental CMV-FLAG vector (CβF). Bars represent the average of 8 plates for CβF, 2 plates for 270–599, 2 plates for 856–1134, 4 plates for 1261–1579, and 4 plates for 3402–3828. Plate-to-plate variation in the absolute number of foci obtained with a given combination of vectors was minimal (generally 5%–10%), and the average number of transformed foci on the CβF plates was 19.

(C and D) Primary REFs were transfected with expression vectors for c-*myc*, H-*ras*, and a CMV-driven expression vector containing 1.5 kb of the 5′ end of the *TRRAP* cDNA cloned in the antisense orientation (CβS TRRAP antisense). Transfection of the CMV expression vector (CβS) containing no insert served as a control. The number of transformed foci is expressed as a percentage of foci obtained on plates transfected with c-*myc* and H-*ras* and the CβS vector (D). (Each bar in [D] represents the average number of foci on 6 plates, with the average number of transformed foci on CβS being 21.) One representative plate from both control (CβS) and antisense (CβS TRRAP antisense) transfections were stained with crystal violet to visualize foci (C).

(E) Duplicate plates of 293 cells were transiently transfected with either CβS, CβS TRRAP antisense, or no DNA (mock), as indicated. Cells were lysed at 48 hr posttransfection, and the concentration of total protein in the lysates was normalized. Fifty micrograms of protein from each plate was resolved by 8% SDS-PAGE and blotted for TRRAP.

sense strategy serves as an independent confirmation of the essential role of TRRAP in c-Myc-dependent transformation. To confirm that the *TRRAP* antisense vector was functioning by decreasing cellular levels of the TRRAP protein, transient transfection of 293 cells was performed. Duplicate plates of cells received either the empty vector, the *TRRAP* antisense vector, or no DNA. Forty-eight hours after transfection, the cells were lysed, the lysates normalized to equal protein concentration, and the levels of TRRAP determined by Western blotting. The presence of antisense *TRRAP* lowered TRRAP protein levels by approximately 60%–75%, relative to either the empty vector or mock transfection controls (Figure 5E). The 60%–75% reduction in TRRAP levels correlates well with the 50%–80% transfection efficiency routinely obtained with these 293 cells.

An important consideration in both of the experiments above is the potential toxicity of vectors that disrupt the function of an important pathway, such as that associated with c-Myc. To address this question, we tested if any of the expression vectors would inhibit the formation of G418-resistant colonies of HeLa cells. When cotransfected with pRSV-*neo*, approximately equal numbers of colonies were obtained with all of the TRRAP protein fragment expression vectors and with the antisense vector, when compared to the corresponding empty vectors (Table 1). Thus, none of the vectors used to inhibit TRRAP function are generally toxic for cells, and we infer

Table 1. TRRAP Antisense and Transdominant Mutants Fail to Inhibit HeLa Cell Growth

Vector Transfected	G418-Resistant Colonies Per Plate
CβS	29
CβS/anti-sense TRRAP	28
CβF	18
CβF/TRRAP (270–599)	27
CβF/TRRAP (855–1134)	27
CβF/TRRAP (1261–1579)	18
CβF/TRRAP (3402–3828)	26

Duplicate plates of HeLa cells were cotransfected with 1 μg of pRSV-*neo* and 3 μg of CMV-driven TRRAP vector, as indicated. Transfected cells were selected in 400 μg/ml G418 for 14 days, at which time the number of colonies per plate was determined. The table presents data based on the average number of colonies on duplicate plates.

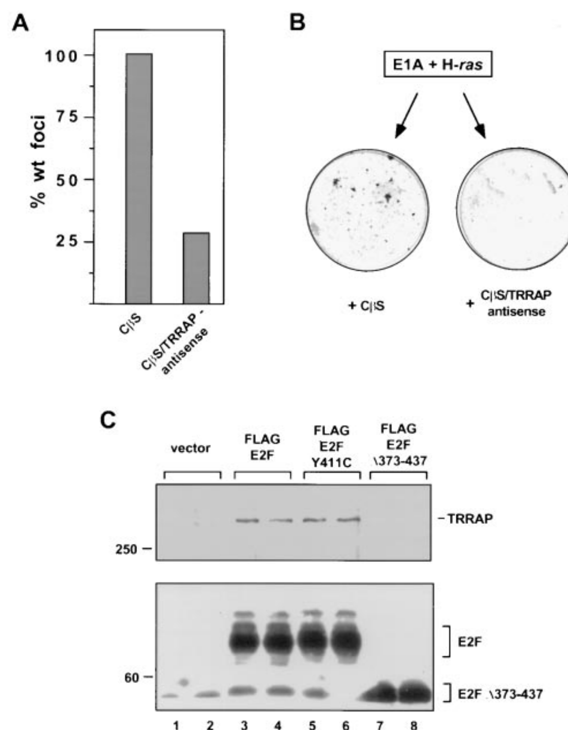

Figure 6. TRRAP Is Essential for Transformation by E1A and Interacts with the Transactivation Domain of E2F-1 In Vivo

(A) Primary REFs were transfected with expression vectors for E1A, H-*ras*, and the TRRAP antisense expression vector described in Figure 5 (CβS TRRAP antisense). Transfection of CβS containing no insert served as a control. The number of transformed foci was determined and expressed as a percentage of foci obtained on plates transfected with E1A, H-*ras*, and the CβS vector. (Each bar represents the average number of foci obtained on 4 plates, with the average number of transformed foci in the presence of CβS being 18.)

(B) Representative plates from an E1A/H-*ras* transformation assay done in the presence of either CβS or TRRAP antisense vectors were stained with crystal violet to visualize foci.

(C) CMV-driven expression vectors were created encoding either a FLAG epitope–tagged human E2F-1 protein, a similarly FLAG-tagged E2F-1 protein with a point mutation (Y→C) at amino acid 411, or an E2F-1 mutant truncated at amino acid 373 (Δ373–437). These vectors were transiently transfected into 293 cells. Lysates from duplicate transfections were prepared and immunoprecipitated with anti-FLAG antibody. Precipitated proteins were resolved by SDS-PAGE and Western blotted for either TRRAP (top) or the FLAG epitope of the transfected E2F-1 proteins (bottom). Lanes 1 and 2, lysates of cells transfected with an expression vector producing only the FLAG epitope. Lanes 3 and 4, lysate from cells expressing full-length E2F-1. Lanes 5 and 6, lysates from cells expressing FLAG-E2F-1-Y411C. Lanes 7 and 8, lysates from cells expressing FLAG-E2F-1 truncated at amino acid 373. Size (in kilodaltons) at the left was determined by coelectrophoeresis of protein MW markers (Novex).

that TRRAP becomes rate-limiting in Myc-dependent transformation but not in normal cell growth where TRRAP may be in excess.

TRRAP Is Essential for E1A-Mediated Oncogenic Transformation

Previous studies demonstrated that overexpression of the c-Myc N terminus could inhibit transformation by the E1A oncogene and that inhibition was dependent on MbII (Brough et al., 1995). To determine if TRRAP was the rate-limiting cellular factor in this experiment, a transformation assay of primary REFs transfected with the E1A and H-*ras* oncogenes was supplemented with the antisense *TRRAP* vector or with the empty vector as a control. Focus formation was monitored after 16–18 days posttransfection. The antisense *TRRAP* vector inhibited E1A-mediated focus formation by greater than 70% (Figures 6A and 6B), comparable to the activity of the same vector in Myc-mediated transformation. Thus, TRRAP is an essential, rate-limiting factor for two distinct oncogenic pathways (c-Myc and E1A).

TRRAP Binds to the Transactivation Domain of the E2F-1 Transcription Factor

Both the dominant inhibitory activity of the c-Myc N terminus (Brough et al., 1995) and the similar inhibition by the *TRRAP* antisense vector are expected to create a TRRAP loss-of-function, indicating that TRRAP is an essential component of the E1A pathway. It is well established that a major downstream effector of E1A is the E2F family of transcription factors. Because of the functional similarity between c-Myc and E2F, it was of interest to determine whether the TRRAP requirement in E1A transformation was due to a direct interaction between TRRAP and E2F-1. Since the interaction of TRRAP with c-Myc involves the transactivation domain, it was of particular interest to test if the transactivation domain of E2F-1 might also mediate binding. To this end, E2F-1 expression vectors with FLAG epitope tags were constructed for effective immunoprecipitation. One vector expressed full-length E2F-1, while a second vector expressed E2F-1 with a C-terminal truncation (deleting amino acids 373–437). The latter construct deletes the transactivation domain of E2F-1 but preserves the DNA-binding and DP dimerization domains, and similar C-terminal deletions of E2F-1 are inactive for most biological activities. Transient expression of full-length E2F-1 in 293 cells led to the coprecipitation of endogenous TRRAP protein (Figure 6C, lanes 3 and 4). Notably, the binding of TRRAP to E2F-1 was dependent on the transactivation domain because the C-terminal truncation

mutant failed to coprecipitate endogenous TRRAP protein (Figure 6C, lanes 7 and 8) even though the expression level was comparable to that of the full-length protein. Thus, TRRAP binds to essential functional domains of two different oncogenic transcription factors.

The transactivation domain of E2F is also the site of Rb binding, which masks transactivation. To determine whether TRRAP binding to E2F-1 was distinguishable from that of Rb, a third FLAG-tagged E2F-1 expression vector was constructed that contained the Y411C mutation. This mutation (and other missense mutations at the same position) eliminates Rb binding and enhances the ability of E2F to promote S phase progression (Helin et al., 1993; Shan et al., 1996). Transient expression of the E2F(Y411C) mutant in 293 cells led to coprecipitation of endogenous TRRAP, and the binding was undiminished compared to the wild-type E2F-1 protein (Figure 6C, lanes 3–6). Since E2F-1 carrying the Y411C mutation is still biologically active, it must retain the capacity to recruit positive cofactors. Thus TRRAP binding to the Y411C mutant is consistent with a role for TRRAP as an essential E2F-1 cofactor.

Discussion

Through studies of an evolutionarily conserved and functionally essential region of the c-Myc N terminus, we have identified a novel cofactor, designated TRRAP, whose interaction correlates with cell transformation by this oncoprotein. Since both the TRRAP-binding and the DNA-binding domains of both c-Myc and E2F are essential for virtually all functional activities, the simplest model is one in which these oncogenic transcription factors (as c-Myc/Max or E2F/DP heterodimers) recruit TRRAP to specific chromosomal sites to modulate gene expression. The sequence of TRRAP has no homology with proteins that have been implicated in gene regulation and hence offers little insight into its precise function. TRRAP has no apparent DNA-binding motif, so it is unlikely that it binds to DNA in the absence of recruitment by c-Myc or other factors. Extensive searches for motifs that might predict a function for the unique section of TRRAP (92% of the protein), such as acetylation, deacetylation, methylation, etc., have proven negative to date. The lack of obvious motifs conferring biochemical activity and its unusually large size suggest that TRRAP may serve as a scaffold for the assembly of a multiprotein complex at Myc/Max– and E2F/DP–binding sites in the genome.

Genetic evidence indicates that the MbII domain of c-Myc performs an essential function in cell transformation, apoptosis, blocking differentiation, and other biological activities associated with the c-*myc* oncogene (see Introduction). There have been contradictory reports as to whether or not the MbII domain is required for transcriptional regulation by c-Myc (Kato et al., 1990; Bello-Fernandez et al., 1993; Brough et al., 1995; Desbarats et al., 1996). The most straightforward interpretation of these inconsistent findings is that some, but not all, Myc-regulated promoters may require the recruitment of the TRRAP protein, although this model requires further experimentation.

The TRRAP protein is remarkably well conserved in evolution, both in size and primary sequence (25% identical/55% similar in *S. cerevisiae*, *S. pombe*, *A. thaliana*, and *C. elegans*) over the entire 3800 amino acids in any pairwise comparison. This extensive identity indicates that these genes are almost certainly true analogs and not simply proteins that share a common structural motif. Interestingly, the TRRAP protein predates the existence of c-Myc in evolution. Searches of the complete *S. cerevisiae* genome database fail to identify any protein with substantial homology to c-Myc, although several DNA-binding proteins with B/HLH or B/Zip motifs are present. Based on these observations, we suggest that the c-Myc protein evolved to recruit the TRRAP protein to specific chromosomal sites in more complex eukaryotes. This scenario has ample precedent in other pathways, with the most relevant being the Myc-related repressor protein, Mad. The Mad protein represses gene expression by recruiting the mammalian homolog of the yeast corepressor, SIN3 (Ayer et al., 1995; Schreiber-Agus et al., 1995). However, like Myc, there is no yeast homolog of Mad, whereas the corepressor SIN3 is conserved. The most primitive homologs of c-Myc and E2F identified to date are in *Drosophila melanogaster*, where the domains linked to TRRAP binding are conserved (Dynlacht et al., 1994; Ohtani and Nevins, 1994; Gallant et al., 1996; Schreiber-Agus et al., 1997). This supports our contention that an interaction with TRRAP was functionally critical even at the earliest point in evolution.

The N-terminal 92% of the TRRAP protein has no significant homology to any other protein in the database. In contrast, the C-terminal 300 amino acids of TRRAP exhibits a striking homology to the "PI-3 kinase" family, whose name derives from the catalytic subunit, p110, of phosphoinositol-3 kinase, which is an enzyme involved in the generation of lipid second messengers and intracellular signaling (Toker and Cantley, 1997). Although p110 is a lipid kinase, other members of the family, including ATM and DNA-PKcs, are S/T protein kinases with no lipid kinase activity (Hartley et al., 1995; Baskaran et al., 1997). The sequence of the TRRAP PI-3 kinase domain adds a perplexing new facet to this enigmatic family. Although the homology between TRRAP and the PI-3 kinase family is readily apparent, close inspection of the TRRAP sequence reveals that this domain may not be catalytically active in any of the five species discussed here. All other members of the ATM family contain the DXXXXN and DFG motifs that constitute critical residues of the catalytic site, and these residues can be oriented with the same invariant motifs in other kinases (Hunter, 1995; Taylor et al., 1995). Furthermore, missense or frameshift mutations that are predicted to abolish kinase activity yield biologically nonfunctional proteins in those systems where they have been tested (Zheng et al., 1995; Danska et al., 1996). The absence of the DXXXXN and DFG motifs in TRRAP (Figure 3B) may imply that this domain serves a function beyond its role as a kinase in other ATM family members. It is of interest to note that at least one other member of the ATM family, DNA-PKcs, is recruited to DNA by a heterodimeric complex of DNA-binding proteins. ATM itself also functions in the nucleus, and a loss of ATM function leads to hypersensitivity to irradiation and a

substantially increased risk of cancer (Lavin and Shiloh, 1996).

The homology between TRRAP and the ATM family may predict a novel function for the c-Myc and E2F oncoproteins. Given the sequence-specific DNA binding activity of these transcription factors and the binding of TRRAP to the transactivation domain, the simplest model for TRRAP function is that of a transcriptional adaptor protein. Yet no member of the ATM family has been previously linked to sequence-specific DNA-binding proteins. One model is that the ATM/PI-3 kinase domain (noncatalytic in TRRAP) has been incorporated into proteins of many disparate families to mediate protein–protein interactions and that its presence does not predict any specific functional overlap. However, the involvement of several ATM-related proteins in the integrity and proper segregation of chromosomes suggests the possibility that TRRAP may alter the chromatin around specific target genes or that it may function in a more global context in regulating chromosome structure or dynamics rather than at selected target genes. Elucidation of the biochemical function of TRRAP may offer novel insight into c-Myc and E2F oncogenesis.

Experimental Procedures

Biochemical Purification of c-Myc-Associated Nuclear Proteins

Baculovirus constructs for expression of FLAG epitope–tagged GAL4 and FLAG-GAL4/Myc (generous gift of Chi Dang) were prepared by tailed PCR to the N terminus of the GAL4 domain. Production of appropriate proteins was monitored by Western blotting using anti-FLAG monoclonal antibodies (generous gift of Deborah Morrison, NCI-Frederick). Nuclear extracts from infected Sf9 cells were produced (Dignam et al., 1983). Heat treatment of HeLa cell nuclear extracts was performed by incubation at 55°C for 10 min followed by brief centrifugation. Large-scale binding reactions were prepared with FLAG-GAL4/Myc or FLAG-GAL4, HeLa nuclear extract, and anti-FLAG antibody. The antibody-bound complex was then captured on Protein A/G agarose beads (Santa Cruz Biotechnology). Beads were extensively washed in binding buffer, and the FLAG-GAL4/Myc-TRRAP complex was eluted from the antibody by incubation with a large molar excess of FLAG peptide. Purified protein (80 μg) was concentrated and resolved by 4% SDS-PAGE. Following transfer to PVDF membrane, TRRAP was visualized by staining with Ponceau S and excised from the surrounding membrane. Portions of the PVDF membrane were submitted to the Harvard University Microchemistry Facility and to NCI-Frederick for microsequencing.

The amino acid sequences of ten TRRAP peptides were obtained, and two of these (NYSIAQ and SIPNVIISLIY) were used to design degenerate oligonucleotides for use as probes on a HeLa cell cDNA library (a generous gift of P. Chambon, CNRS INSERM-Strasbourg). Following screening of duplicate lifts of 500,000 phage with these oligonucleotides, a single phage that hybridized to oligonucleotide probes from both TRRAP peptides was obtained. This phage contained a 3.5 kb insert whose 5′ and 3′ ends were used as probes on the same library. Seven rounds of screening yielded 150 independent, overlapping cDNA clones, which were sequenced and aligned.

Cell Culture, Transfection, and Immunoprecipitation

293 cells were cultured on 10 cm plates in Dulbecco's modified Eagle's media (DMEM), supplemented with 10% fetal calf serum (GIBCO BRL). Cells were transfected using the calcium phosphate method and lysed using E1A lysis buffer (Harlow et al., 1986). Lysates were analyzed by Western blotting with anti-FLAG monoclonal antibody to determine the level of protein production or subjected to immunoprecipitation by incubation with anti-FLAG in conjunction with Protein A/G beads. Precipitates were then analyzed by Western blotting with anti-FLAG or a rabbit anti-TRRAP antisera. Rabbit anti-TRRAP was generated by immunization with a peptide corresponding to the 26 C-terminal amino acids of human TRRAP. Antibody detection was performed using ECL (Amersham).

COLO 320 cells (ATCC) were grown in DMEM with 10% fetal calf serum. For the preparation of lysates, confluent 15 cm plates were washed with PBS and then lysed in buffer F (Sommer et al., 1998). Immunoprecipitations contained lysate from one-half of a single 15 cm plate and 5 μg of polyclonal rabbit anti-Max antisera (Santa Cruz) or control rabbit immunoglobulin (Santa Cruz). Precipitations were carried out as described above for 293 cells, except that Western blotting for c-Myc was performed using the monoclonal antibody 9E10.

Transformation assays involving REFs were performed as described previously (Brough et al., 1995). Transfections included c-Myc expression vectors in the form of either CMV promoter–driven FLAG/c-Myc (or various mutants thereof), or EMSV-c-*myc* (1 μg) and H-*ras* (G12V) (1 μg), or the E1A expression vector p1A (1 μg) and H-*ras* (G12V) (1 μg). Where indicated, transfections were supplemented with either the CMV promoter–driven expression vectors containing *TRRAP* cDNA fragments or with the empty vector (3 μg). Following transfection, cells were maintained in DMEM containing 4% fetal bovine serum, and foci were counted at day 12–14 post-transfection. Transfections were performed in duplicate, and the data from multiple experiments were averaged.

For HeLa cell transfection, pRSV-*neo* (1 μg) and CMV-driven *TRRAP* cDNA expression vectors (3 μg) were introduced by the calcium phosphate method. Transfected cells were selected in 400 μg/ml G418 (GIBCO/BRL) for 14 days, at which time the number of colonies per plate was determined.

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The protein TRRAP that is described in this study was previously called TR-AP.

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GenBank Accession Number

The sequence of the human TRRAP protein described here has been entered into GenBank under the accession number AF076974.