Oncogenic potential of TAR RNA binding protein TRBP and its regulatory interaction with RNA-dependent protein kinase PKR

Monsef Benkirane, Christine Neuveut, Rene F.Chun, Stephen M.Smith, Charles E.Samuel¹, Anne Gatignol² and Kuan-Teh Jeang^{1,3}

Molecular Virology Section, Laboratory of Molecular Microbiology, NIAID, NIH, Bethesda, MD 20892-0460, ¹Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA 93106, USA and ²Institut Cochin de Genetique Moleculaire, 22 rue Mechain, 75014 Paris, France

³Corresponding author

TAR RNA binding protein (TRBP) belongs to an RNA binding protein family that includes the double-stranded RNA-activated protein kinase (PKR), *Drosophila* Staufen and *Xenopus* xlrpba. One member of this family, PKR, is a serine/threonine kinase which has anti-viral and anti-proliferative effects. In this study we show that TRBP is a cellular down-regulator of PKR function. Assaying expression from an infectious HIV-1 molecular clone, we found that PKR inhibited viral protein synthesis and that over-expression of TRBP effectively countered this inhibition. In intracellular and in cell-free assays we show that TRBP directly inhibits PKR autophosphorylation through an RNA binding-independent pathway. Biologically, TRBP serves a growth-promoting role; cells that over-express TRBP exhibit transformed phenotypes. Our results demonstrate the oncogenic potential of TRBP and are consistent with the notion that intracellular PKR function contributes physiologically towards regulating cellular proliferation.

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Introduction

Gene expression is regulated at many levels, including transcription (initiation and/or elongation), mRNA stability and translation. Translational control is one important step in controlling the abundance of many essential and highly expressed proteins (reviewed in Kaufman, 1994). Initiation of the translation of mRNAs is believed to be a key regulatory point in protein synthesis (reviewed in Hershey, 1991). While there are many proteins that contribute positively to the translational machinery, one factor that negatively regulates translation is the interferon-inducible double-stranded RNA (dsRNA)-activated protein kinase, PKR.

PKR is found constitutively in low amounts in many eukaryotic cells. Its activity in cells can be potentially induced by treatment with interferon α (IFN- α) (Meurs *et al.*, 1990; Tanaka and Samuel, 1994). PKR is a serine/threonine kinase that has two distinct activities: an

autophosphorylation activity and a *trans*-kinase activity for exogenous substrates (Hovanessian, 1989; Clemens, 1996). It is proposed that upon binding to dsRNA, PKR phosphorylates the α subunit of protein synthesis eukaryotic initiation factor 2 (eIF-2 α). Phosphorylation of eIF-2 α blocks the eIF2B-mediated exchange of GDP with GTP in the inactive eIF-2GDP complex (Rowlands *et al.*, 1988; Ramaiah *et al.*, 1994; for a review see Hershey, 1991), a reaction required for catalytic re-utilization of eIF-2. These events lead to a limitation in eIF-2 function, which facilitates the binding of initiator tRNA^{met} to the initiating ribosomal subunit. Thus, activation of PKR triggers a series of events that culminate in inhibition of protein synthesis (reviewed in Samuel, 1993).

Inhibition of translation is used as one defense by cells against viral infections. Indeed, PKR serves an antiviral function in affecting the efficiency with which viral mRNAs are translated (Samuel, 1991). Not unexpectedly, some viruses have evolved means for escaping PKR-mediated inhibition. Examples of virus escape strategies include the expression of dsRNA binding proteins (Whitaker-Dowling and Youngner, 1984; Imani and Jacobs, 1988), synthesis of viral RNAs that inhibit PKR activation (Kitajewski *et al.*, 1986; O'Malley *et al.* 1986), sequestration of PKR (Dubois and Hovanessian, 1990) and/or induction of its degradation (Black *et al.*, 1989), production of substrates with structural similarity to eIF-2 (Davies *et al.*, 1992) and activation of cellular inhibitors of PKR (Lee,T.G. *et al.*, 1990). Studies on these virus-cell interplays have helped to illustrate and to elucidate important aspects of viral and cellular metabolism (reviewed in Sonenberg, 1990; Katze, 1992).

The antiviral aspect of PKR is, perhaps, only one part of its cellular function(s). Indeed, the action of PKR in regulating protein synthesis suggests an important role in cellular growth and differentiation (Petryshyn *et al.*, 1984, 1988; Chong *et al.*, 1992; Koromilas *et al.*, 1992; Dever *et al.*, 1993; Lee,S.B. *et al.*, 1993; Lee,T.G. *et al.*, 1994). Recent evidence supports the critical contributions of translational control in regulating cellular proliferation (reviewed in Sonenberg, 1993). Perturbing the homeostatic balance of translation can probably tip the cell towards either transformation or programmed cell death. Evidence supportive of this hypothesis comes from over-expression studies using dominant negative mutants of PKR or a mutated eIF-2 which induced malignant transformation of mouse cells (Koromilas *et al.*, 1992; Lengyel, 1993; Meurs *et al.*, 1993; Barber *et al.*, 1995; Donze *et al.*, 1995). The direct mechanistic explanation for this observation has been postulated to be the facilitated translation of mRNAs encoding growth control genes which are normally repressed biosynthetically (reviewed in Sonenberg, 1993).

Considering the critical role of PKR in cellular metabolism, cells have presumably developed mechanisms to

regulate its activity that are at least as intricate as those evolved by viruses. Possibly, multiple factors exist to modulate PKR. In fact, a 58 kDa cellular protein has been described as an inhibitor of PKR autophosphorylation (Lee, T.G. *et al.*, 1990, 1992, 1994; Barber *et al.*, 1994) and a second protein, TAR RNA binding protein (TRBP), has also recently been suggested as a regulator of PKR function (discussed in Gagnol *et al.*, 1993; Park *et al.*, 1994; Blair *et al.*, 1995). TRBP is a cellular RNA binding protein isolated by its ability to bind HIV-1 TAR RNA (Gagnol *et al.*, 1989, 1991). Functional studies show that TRBP augments protein expression from the HIV-1 LTR and a number of other viral promoters (HTLV-1 LTR, Visna LTR and SV40 early promoter; Gagnol *et al.*, 1991). An RNA binding protein motif in PKR (McCormack *et al.*, 1992) is conserved in TRBP (Gagnol *et al.*, 1993; Kozak *et al.*, 1995). Thus, in principle, these two proteins would overlap in RNA binding properties. Indeed, like TRBP, PKR also binds TAR RNA and binding to TAR RNA activates PKR function (Edery *et al.*, 1989; SenGupta and Silverman, 1989; Maitra *et al.*, 1994).

One mechanism that TRBP might impose on PKR is to compete for common RNA substrate(s) (Consentino *et al.*, 1995; McCormack and Samuel, 1995). Here, we present evidence for an RNA binding-independent process through which TRBP regulates PKR function in cells. We show that TRBP and PKR form a hetero-complex through direct protein-protein contact and that this contact prevents PKR autophosphorylation and PKR-mediated inhibition of viral protein synthesis. We further show that TRBP subverts IFN- α activation of PKR, and we define the IFN- α /PKR/TRBP axis as one important intracellular check to cellular proliferation. Disturbance of this critical balance in NIH 3T3 cells through over-expression of TRBP results in malignant transformation.

Results

IFN- α treatment inhibits wild-type HIV-1 but not HIV-1 expressing TRBP

Because we are interested in mechanisms regulating virus replication, we investigated the functions of TRBP during HIV-1 infections of T cells. Since IFN- α treatment of cells has been shown to repress HIV-1 replication (Francis *et al.*, 1992; Coccia *et al.*, 1994; Agy *et al.*, 1995; Der and Lau, 1995; for a review see Pitha, 1994) and since this effect is largely attributed to the induction of PKR, we examined the role that TRBP expression might contribute to this regulated pathway. To assess this in a biologically relevant manner, one needs to synthesize TRBP coincidentally with peak periods of viral gene expression. We did this by creating an infectious HIV-1 genome in which a TRBP cDNA was expressed in *nef* (Figure 1A). As controls, chimeric viruses (Figure 1A) were constructed that express PKR [pNLPR, a dominant negative PKR mutant with amino acid substitution of arginine for lysine at position 296 (pNLPR^{K296R}), TRBP (pNLTRBP) or a TRBP mutant deleted in the critical RNA binding domain (pNLTRBP ^{Δ 227-270}; Gagnol *et al.*, 1993). Transfections were performed using C8166-45 cells, which do not express detectable amounts of basal PKR activity (Figure 1B). Wild-type PKR, the K296R mutant of PKR and TRBP expressed from the viral

genome (vTRBP) were readily detected in cells transfected with each of the respective viral genomes (Figure 1B).

Next, the effect of TRBP was compared in parallel treatments of CEM (12D7) cells infected with either HIV-1 wild-type or HIV-1 expressing TRBP. Wild-type HIV-1 replication, measured by the production of reverse transcriptase (RT) from infected cells, was inhibited by IFN- α in a dose-dependent manner, with significant effects observed at 100 and 500 U/ml (Figure 2A); in contrast, replication of HIV-1 TRBP was not affected by IFN- α treatment, even at the highest dose of 500 U/ml (Figure 2B). One interpretation of these findings is that the antiviral effect mediated through the presumptive activation of PKR by IFN- α is down-modulated by TRBP.

TRBP releases PKR-mediated inhibition of HIV-1 protein synthesis

We examined whether the effect of TRBP on IFN- α treatment was at the level of PKR function. Prior studies have established that a major portion of the IFN- α antiviral effect is through PKR activation (reviewed in Samuel, 1991). Activated PKR differentially influences the translatability of viral mRNAs, thereby selectively affecting replication of viruses such as encephalomyocarditis and vaccinia virus (Meurs *et al.*, 1992; Lee, S.B. *et al.*, 1993). Experimentally, we assessed if the anti-IFN- α effect of TRBP could be linked to a direct modulation of PKR.

We determined the effect of TRBP on PKR function as measured by inhibition of HIV-1 protein synthesis. PKR-mediated inhibition of HIV-1 protein synthesis was visualized by immunoblotting using hyperimmune AIDS patient serum (Figure 3A). We compared the amount of viral proteins expressed from HeLa cells transfected with the infectious genomes individually (Figure 3A, lanes 5 and 6) or in combination (Figure 3A, lanes 1-4, 7 and 8), in the presence (Figure 3A, lanes 1, 3, 5 and 7) or absence (Figure 3A, lanes 2, 4, 6 and 8) of IFN- α . We found that normalized transfection with pNL alone (Figure 3A, lane 6) or co-transfection of pNL with pNLTRBP (Figure 3A, lane 8) produced large amounts of HIV-1-specific proteins. In contrast, co-transfection of pNL with a pNLPR genome reduced HIV-1 protein synthesis to undetectable levels (Figure 3A, lane 4). This suggested that the pNLPR genome produced functional PKR that inhibited its own expression *in cis* and the expression of a separate genome (pNL) *in trans*. Interestingly, while pNLPR was effective on wild-type HIV-1 (pNL; Figure 3A, lane 4), the same co-introduction of pNLPR with pNLTRBP had little effect on the synthesis of HIV-1 proteins (Figure 3A, lane 2). These findings are best explained by a direct role of TRBP (Figure 3A, lanes 1, 2, 7 and 8) in mitigating PKR inhibition on HIV-1-specific mRNAs.

While TRBP effectively counters PKR over-produced from an exogenous HIV-1 vector (pNLPR; Figure 3A, lanes 1 and 2), we wondered about the functional ability of TRBP to interact with endogenous PKR induced by IFN- α treatment (Samuel, 1993; Clemens, 1996). Hence, we compared HIV-1 protein synthesis from cells treated (Figure 3A, odd numbered lanes) or mock-treated (Figure 3A, even numbered lanes) with IFN- α (500 U/ml). Indeed, treatment markedly inhibited protein synthesis from wild-type pNL virus (Figure 3A, compare lane 6 with 5), however, the same process produced little inhibitory effect

Fig. 1. Construction of HIV-1 that expresses PKR or TRBP. (A) Schematic representations of seven molecular genomes. All constructs contain insertions into *nef* of pNL4-3 (pNL) wild-type (wt) virus (Adachi *et al.*, 1986) with the wild-type PKR cDNA (pNLPR), the mutant PKR 296 cDNA (pNLPR^{K296R}), the wild-type TRBP cDNA (pNLTRBP) or the mutant TRBP cDNA with deletion of amino acids 227–270 (Gatignol *et al.*, 1993) in the correct (pNLTRBP^{Δ227-270}) or inverted orientation (pNLTRBP^{Δ227-270}antisense). (B) Western blot analysis of expression of PKR and TRBP by pNL constructs. Because we found that C8166-45 cells do not express endogenous PKR (M.Benkirane and K.-T.Jeang, unpublished data), we used this cell line to analyze expression of PKR by the pNLPR genome. C8166-45 cells were mock-transfected (lane 1) or transfected with pNL (lanes 2 and 5), pNLPR^{K296R} (lane 3), pNLPR (lane 4), pNLTRBP^{sense} (lane 6) or pNLTRBP^{antisense} (lane 7). Cell extracts prepared 24 h after transfection were resolved by SDS–PAGE and analyzed by immunoblotting using anti-PKR (lanes 1–4) or anti-TRBP (lanes 5–7). Endogenously expressed TRBP is designated cTRBP (for cellular TRBP) and TRBP expressed by virus is designated vTRBP (for viral TRBP). The difference in size between vTRBP and cTRBP is due to the expression of a shorter cDNA encoding for functional TRBP in the HIV-1 vectors (Blair *et al.*, 1995).

on cells that express or co-express the pNLTRBP genome (Figure 3A, lanes 1, 2, 7 and 8). Thus TRBP can function to counter both exogenous PKR (over-expressed from viral vector, pNLPR; Figure 3A, lanes 1 and 2) and IFN- α -activated endogenous PKR (Figure 3A, compare lane 5 with 7).

The immunoblot results (Figure 3A) were checked with immunoprecipitations using hyperimmune serum of biosynthetically radiolabeled HIV-1 proteins (Figure 3B). Viral molecular genomes were introduced into cells in the indicated combinations (Figure 3B); in each case a pCMV- β -gal plasmid was also included in order to monitor for transfection efficiency (Figure 3C). Half of the cells in each transfection were analyzed by RNase protection for expressed β -gal mRNA; the results confirmed that the six different transfections were comparable in efficiency (Figure 3C). Proteins in the other half of the transfected cells were pulse-labeled with [³⁵S]methionine + cysteine and virus-specific synthesis was visualized by immunoprecipitation followed by SDS–PAGE/autoradiography. The results from radiolabeling confirmed the findings by immunoblotting (Figure 3A). We found that over-expression of PKR potently inhibited HIV-1(pNL)-specific protein synthesis (Figure 3B, lane 4) and that expression of TRBP abrogated this inhibitory activity of PKR (Figure 3B, lane 6).

The virological relevance of these molecular findings on viral protein synthesis in HeLa cells (Figure 3A and B) was examined in HIV-1 replication assays in T

lymphocytes (Figure 3D). We desired to ascertain the effect, if any, that direct over-expression of PKR may have on HIV-1 spread. Instead of quantifying viral protein synthesis, we monitored the RT growth profiles produced by viruses after transfection/co-transfection of infectious genome(s) into replication-permissive 12D7 cells. We found that over-expression of PKR from a pNLPR virus effectively inhibited *in trans* wild-type pNL replication for at least 17 days (Figure 3D, pNL + pNLPR). In contrast, no inhibition was seen when pNL4-3 was introduced into 12D7 cells with a pNLPR^{K296R} (K296R is a catalytically inactive mutant of PKR) genome (Figure 3D, pNL + pNLPR^{K296R}), confirming that catalytically active PKR mediated the anti-replicative effect. Over-expression of TRBP produced no inherent effects on HIV-1 replication (Figure 3D, pNL + pNLTRBP). However, consistent with the HeLa cell findings, we found that a HIV-1 recombinant that expresses TRBP resisted the otherwise *trans*-inhibitory effect on replication of a PKR-expressing virus (pNLPR) (Figure 3D, pNLPR + pNLTRBP).

Protein–protein contact between TRBP and PKR

Because TRBP contains two dsRNA binding domains (Gatignol *et al.*, 1993) and because dsRNA binding reflects one important step in PKR activation (Samuel, 1993; Clemens, 1996), one trivial explanation for TRBP-mediated inhibition of PKR is a simple competitive sequestration of RNA substrates. Alternatively, TRBP could affect

Fig. 2. IFN- α has differential effects on infections of 12D7 cells with HIV-1 and HIV-1 expressing TRBP. 12D7 cells were treated or mock-treated with the indicated concentrations of IFN- α 24 h before infection with 100 TCID₅₀ of pNL4-3 (pNL) (A) or pNLTRBP (B). Cells were maintained in medium containing IFN- α . Virus production was monitored by supernatant RT assay every 3 days.

PKR through direct protein–protein contact. To distinguish between these possibilities, we searched for TRBP–PKR complexes in HeLa cells.

Whole HeLa cell extracts were first treated with RNase A to degrade bulk cellular RNA. The extract was then immunoprecipitated with anti-TRBP (Figure 4, top) or anti-PKR (Figure 4, bottom) serum. Recovered immunoprecipitates were resolved by SDS–PAGE, then transferred to membrane. The presence of PKR in TRBP-specific immunoprecipitates and the presence of TRBP in PKR-specific immunoprecipitates were assessed reciprocally by probing membranes with anti-PKR (Figure 4, top) or anti-TRBP (Figure 4, bottom). In the TRBP-specific immunoprecipitations we visualized PKR (Figure 4, top, lane 3) and in the PKR-specific immunoprecipitates we found TRBP (Figure 4, bottom, lane 3). These complementary results suggested that the two proteins formed hetero-complexes that can be co-immunoprecipitated together from cells.

IFN- α treatment of HeLa cells induces PKR. We wondered how this treatment might influence PKR–TRBP complex formation. Aliquots of 500 U/ml of IFN- α were added to cells for varying durations (12, 24 and 48 h; Figure 4, lanes 4–6) and then we performed reciprocal

immunoprecipitation/immunoblotting with anti-TRBP/PKR sera. In contrast to mock-treated cells (Figure 4, lane 3), IFN- α -treated cells showed progressively reduced PKR–TRBP signals (Figure 4, top and bottom, lanes 4–6). One explanation is that IFN- α treatment induced a dissociation of the PKR–TRBP complex and that this release is a physiological mechanism of PKR activation.

RNase A treatment does leave residual dsRNAs. As such, degradation with this ribonuclease does not strictly exclude the possibility that TRBP–PKR complexes can form through an RNA bridge, where both protein moieties are bound to a common RNA molecule. Hence, we repeated the experiment using cobra venom RNase V1 (Figure 5), which cleaves dsRNA. RNase V1 treatment conditions were optimized by assaying for degradation of predominantly dsRNAs bound to maltose binding protein (MBP)–TRBP fusion protein (Figure 5A). Using excess RNase V1, visible RNA signals were essentially eliminated (Figure 5A, lanes 9–13). Reciprocal co-immunoprecipitations (as in Figure 4) were performed on cell extracts thus treated with RNase V1 (Figure 5B) and the findings (Figure 5B, lane 2) were consistent with those in Figure 4. Taken together, these results are compatible with a direct protein–protein contact between PKR and TRBP that occurs independently of dsRNA.

To check for TRBP–PKR contact using an independent assay, we tested for reconstitution of complexes *in vitro*. Mutant PKR proteins PKR^{K296R} and PKR^{K64E/K296R} were purified from *Escherichia coli* as GST fusion proteins. PKR^{K296R} is defective in kinase activity, while PKR^{K64E/K296R} is defective in both kinase activity and dsRNA binding activity (Thomis and Samuel, 1992b; McCormack and Samuel, 1995). Both forms of PKR mutant were purified, bound to glutathione–Sepharose beads and packed into 0.5 ml columns (Figure 5C, lanes 6–10 and 11–15). A control column consisting of GST protein (Figure 5C, lanes 1–5) was similarly constructed. Through these columns we passed 1 ml RNase-treated HeLa whole-cell extract. Column flow-throughs (ft, Figure 5C, lanes 1, 6 and 11) were collected, and the columns were washed extensively with buffer (20 mM HEPES, pH 7.9, 20 mM KCl, 1 mM MgCl₂, 2 mM dithiothreitol, 17% glycerol; w, Figure 5C, lanes 2, 7 and 12). Bound proteins were then eluted with stepwise increases of NaCl (0.25, 0.5 and 1.5 M; Figure 5C, lanes 3–5, 8–10 and 13–15). TRBP retained by column matrices was analyzed by immunoblotting of each eluted fraction. We found that TRBP was eluted from the GST–PKR^{K296R} and from the RNA binding-defective GST–PKR^{K296R/K64E} matrices (Figure 5C, lanes 8–10 and 13–15), while no TRBP was found in elutions from the GST-alone matrix (Figure 5C, lanes 3–5). The binding of TRBP to GST–PKR^{K296R/K64E} is further consistent with RNA-independent direct protein–protein contact.

The RNA binding domain of TRBP is not needed for functional interaction with PKR

The RNA-independent biology of PKR–TRBP interaction can be reciprocally assayed using a TRBP mutant (TRBP ^{Δ 227–270}) previously characterized to be deficient in RNA binding activity (Gatignol *et al.*, 1993). The binding phenotype of TRBP ^{Δ 227–270} is biochemically illustrated in Figure 6A. While MBP–TRBP fusion protein (Figure

Fig. 3. Expression of TRBP releases PKR-mediated inhibition of HIV-1 protein synthesis. (A) HeLa cells were mock-treated (lanes 2, 4, 6 and 8) or treated (lanes 1, 3, 5 and 7) with IFN- α (500 U/ml) for 24 h and then transfected with pNL4-3 (pNL) (lanes 5 and 6) and co-transfected with pNL and pNLTRBP (lanes 7 and 8), pNL and pNLPKR (lanes 3 and 4) or pNLPKR and pNLTRBP (lanes 1 and 2). HIV-1-specific viral proteins were assayed 48 h later by immunoblotting using AIDS hyperimmune serum. (B) HeLa cells were transfected with pCMV β -gal (5 μ g) alone (lane 1), pNL (lane 2), pNL and pNLTRBP (lane 3), pNL and pNLPKR (lane 4), pNL and pNLPKR^{K296R} (lane 5) or pNLTRBP and pNLPKR (lane 6). Twenty four hours after transfection, cells were harvested. Half of the cells were pulse-labeled with [³⁵S]methionine + cysteine for 30 min, washed twice in PBS and resuspended in RIPA buffer. Cell extracts were then immunoprecipitated for HIV-1-specific proteins with AIDS hyperimmune serum. The immunoprecipitated products were resolved by 10% SDS-PAGE and analyzed by autoradiography. (C) Verification of transfection efficiency. Half of the cells from the transfection described in (B) were analyzed by RNase protection assay using a probe for β -galactosidase RNA. Lanes 1–6 correspond to the same numbered lanes as in (B). Lane 7 contains intact input probe. Lane 8 contains molecular size markers consisting of ³²P-end-labeled pBR322 *Hpa*II fragments. The lower arrow points to β -galactosidase mRNA signal protected from RNase digestion. (D) 12D7 cells were transfected with pNL (Δ) and co-transfected with pNL and pNLPKR (◇), pNL and pNLPKR^{K296R} (◆), pNL and pNLTRBP (○) or pNLPKR and pNLTRBP (■). Virus production was monitored by supernatant RT assay every 3 days.

6A, lanes 7–10) bound radiolabeled total cellular RNA efficiently, MBP-TRBP ^{Δ 227–270} (Figure 6A, lanes 11–14), like MBP alone (Figure 6A, lanes 3–6), showed no affinity for RNA.

Next, we checked functionally how RNA binding-defective TRBP protein might influence PKR-mediated inhibition of HIV-1 expression. We transfected HeLa cells

individually with pNL (Figure 6B, lane 1), pNLTRBP (Figure 6B, lane 2), pNLTRBP ^{Δ 227–270sense} (Figure 6B, lane 3) or pNLTRBP ^{Δ 227–270antisense} (Figure 6B, lane 4). Each transfection produced similar amounts of HIV-1-specific expression, as measured by release of viral RT into the culture supernatant. Combinations of pNL and pNLTRBP (Figure 6B, lane 5), pNL and

Fig. 4. Co-immunoprecipitation of TRBP and PKR. Extracts from mock-treated (lanes 1–3) or HeLa cells treated with IFN- α (500 U/ml) for 12 h (lane 4), 24 h (lane 5) or 48 h (lane 6) were incubated with RNase A for 1 h at 4°C and were immunoprecipitated using polyclonal antibody against PKR (bottom) or polyclonal antibody against TRBP (top). Immunoprecipitated products were separated by 8% SDS-PAGE and analyzed by immunoblotting using anti-TRBP (bottom) or anti-PKR (top) respectively. Immunoprecipitations using anti-actin (lane 2) or beads alone (lane 1) were performed as controls.

pNLTRBP $\Delta 227-270$ ^{sense} (Figure 6B, lane 6) or pNL and pNLTRBP $\Delta 227-270$ ^{antisense} (Figure 6B, lane 7) produced expected amounts of RT. However, co-transfection into cells of pNL with a PKR-producing vector, pNLPKR, produced dramatically reduced amounts of RT (Figure 6B, lane 8), confirming and consistent with the inhibitory effect of over-expressed wild-type PKR described in the above experiments. In comparison, no such inhibition was seen when pNL and a pNL virus that expresses a catalytically inactive PKR mutant (pNLPKR^{K296R}) were co-transfected into cells (Figure 6B, lane 9). Introduction of pNLPKR together with a pNL virus that expresses wild-type TRBP (pNLTRBP) produced, as expected, normal amounts of RT (Figure 6B, lane 10). Intriguingly, and perhaps somewhat unexpectedly, the simultaneous presence of pNLPKR virus with pNLTRBP $\Delta 227-270$ ^{sense} virus (which expresses an RNA binding-defective TRBP mutant) also yielded (Figure 6B, lane 11) robust amounts of RT. In comparison, control pNLTRBP $\Delta 227-270$ ^{antisense} virus was strongly inhibited by pNLPKR (Figure 6B, lane 12). Taken together, these results agree with an RNA-independent functional interplay between an RNA binding-defective TRBP (TRBP $\Delta 227-270$) and PKR.

The HeLa cell results (Figure 6B) were replicated with virus growth assays in T lymphocytes (Figures 6C and D). In the latter setting, viral growth was monitored over a period of 17 days after transfection of the indicated genomes, alone or in combination, into 12D7 cells. We followed the spread of virus by plotting the amount of viral RT released from infected cells. From the RT profiles (Figure 6C and D) it was evident that with respect to PKR activity, in the setting of replicating viruses, RNA binding-deficient TRBP $\Delta 227-270$ functioned like wild-type RNA binding-competent TRBP.

IFN- α -regulated expression of TRBP and PKR

The suggestion that IFN- α might modulate TRBP–PKR complex formation (Figures 4 and 5) prompted us to

examine how such treatment might affect the basal expression of PKR and TRBP. We treated HeLa cells with 500 U/ml IFN- α . Extracts from mock-treated HeLa cells or cells treated for 12 or 24 h were prepared and resolved by SDS-PAGE. The amount of PKR/TRBP in each extract was analyzed by immunoblotting using anti-PKR (Figure 7A) or anti-TRBP (Figure 7B) serum. We observed an inverse correlation. Treatment of HeLa cells with IFN- α up-regulated steady-state levels of PKR (Figure 7A) and down-regulated the steady-state amount of TRBP (Figure 7B). Mechanistically, this suggests that IFN- α alters the intracellular stoichiometry of PKR and TRBP and that such change adversely affects the formation of PKR–TRBP hetero-complexes.

PKR activity can be regulated at many levels, including repression of gene transcription and modulation of phosphorylation state. Since PKR can form homodimers (Cosentino *et al.*, 1995; Patel *et al.*, 1995; Ortega *et al.*, 1996) and can also hetero-complex with TRBP (Figures 4 and 5; Blair *et al.*, 1995; Cosentino *et al.*, 1995), it was possible that the nature of complex formation influences the state of PKR phosphorylation. To explore this possibility, we isolated, using neomycin co-selection, HeLa cell clones that express a sense-oriented CMVTRBPs or a reverse sense-oriented CMVTRBPas (Figure 8). Cells selected for *neo* alone (none, Figure 8) and cells selected for sense (CMVTRBPs, Figure 8) or reverse sense (CMVTRBPas, Figure 8) TRBP were mock-treated (Figure 8, lanes 1, 3 and 5) or treated (Figure 8, lanes 2, 4 and 6) with IFN- α (500 U/ml) for 12 h. This was followed by radiolabeling using either [³²P]orthophosphate (Figure 8A) or [³⁵S]methionine + cysteine (Figure 8B) for an additional 8 h. Equivalent count-containing samples were prepared and immunoprecipitated using anti-PKR antibody. The immunoprecipitates were resolved by SDS-PAGE and PKR-specific 68 kDa protein was visualized by phosphorimaging.

We found that compared with mock-treated cells, cells treated with IFN- α modestly increased (4-fold) the amount of ³⁵S-labeled PKR (Figure 8B, compare lane 6 with 5), however, the same treatment resulted in a larger (9-fold) increase in ³²P-labeled PKR (Figure 8A, compare lane 6 with 5). In comparison, the HeLa cell line expressing reverse-oriented TRBPas (Figure 8, lanes 3 and 4) when treated with IFN- α produced a 4.5-fold increase in ³⁵S-labeling and a 4-fold increase in ³²P-labeling. Furthermore, while INF- α induced a 5-fold increase in ³⁵S-labeled PKR from cells that express CMVTRBPs (Figure 8B, lanes 1 and 2), such treatment failed to elicit much increase (1.5-fold) in ³²P-labeled PKR (Figure 8A, lanes 1 and 2). These results suggest that one primary level of TRBP action is to influence PKR phosphorylation.

To determine whether inhibition of PKR autophosphorylation was a direct effect of TRBP, we reconstituted this finding *in vitro* (Figure 8C). We immunoprecipitated PKR from HeLa cells and incubated this preparation with MBP (Figure 8C, lane 1) or a MBP–TRBP fusion (Gatignol *et al.*, 1993; Figure 8C, lanes 2 and 3) or buffer alone (none; Figure 8C, lane 4). *In vitro* autophosphorylation of immunoprecipitated PKR in each of these incubations was commenced by adding 1 μ g/ml poly(I)·poly(C) with 100 μ Ci/ml [γ -³²P]ATP for 20 min at 30°C. The resulting phosphorylated moieties were resolved by SDS-PAGE

Fig. 5. TRBP-PKR interaction occurs in a dsRNA-independent manner. (A) Total cellular RNA was isolated from H9 cells after labeling with [32 P]orthophosphate. The radiolabeled RNA was equilibrated with bead-bound MBP-TRBP, as described in Materials and methods. The beads were divided into two aliquots which were mock-treated (lanes 4–8) or treated with RNase V1 (lanes 9–13). After RNase treatment, bound RNAs were eluted in a stepwise fashion using binding buffer containing 0.5 M (lanes 4 and 9), 1.0 M (lanes 5 and 10), 1.5 M (lanes 6 and 11), 2.0 M (lanes 7 and 12) or 2.5 M (lanes 8 and 13) NaCl. Eluted RNAs were concentrated by ethanol precipitation and visualized in a denaturing 6% polyacrylamide gel. Lane 1 contains molecular size markers. Total [32 P]-labeled RNA isolated from H9 cells that were mock-treated (lane 2) or treated with RNase V1 (lane 3) is shown. (B) Co-immunoprecipitation of PKR and TRBP after treatment of cell extract with RNase V1. Immunoprecipitations followed by Western blotting were performed as described in Figure 4, except that the extracts were treated with RNase V1 prior to immunoprecipitation. (C) Protein column chromatography demonstrates an interaction between TRBP and a PKR mutant (K64E/K296R) defective for dsRNA binding. PKR mutants K296R and K64E/K296R were produced in *E. coli* as GST fusion proteins (GST-PKR^{K296R} and GST-PKR^{K64E/K296R}). GST-PKR^{K296R}, GST-PKR^{K64E/K296R} or GST alone were saturated on glutathione-Sepharose beads. Extracts from HeLa cells were separately equilibrated overnight at 4°C with each of the three protein-bound beads. After this incubation, the beads were washed several times with buffer (20 mM HEPES, pH 7.9, 20 mM KCl, 1 mM MgCl₂, 17% glycerol, 2 mM dithiothreitol; lanes 2, 7 and 12). Washed beads were eluted with stepwise increases of NaCl (0.25 M, lanes 3, 8 and 13; 0.5 M, lanes 4, 9 and 14; 1.5 M, lanes 5, 10 and 15). The presence of TRBP in the eluates were assessed by Western blotting using anti-TRBP polyclonal antibody. Lane ft, 'flow through' after equilibration with beads.

and incorporation of 32 P was visualized by autoradiography (Figure 8C). We found MBP-TRBP (Figure 8C, lanes 2 and 3) but not MBP (Figure 8C, lane 1) nor buffer alone (Figure 8C, lane 4) in the reactions efficiently prevented PKR autophosphorylation. The experiment shown in Figure 8C was repeated substituting MBP-TRBP $^{\Delta 227-270}$ in place of MBP-TRBP (Figure 8D). In this phosphorylation assay, the RNA binding-defective TRBP $^{\Delta 227-270}$ again exhibited the same phenotype as RNA binding-competent wild-type TRBP.

Over-expression of TRBP morphologically transforms NIH 3T3 cells

An increasing number of biological examples illustrates the critical role of translational control in homeostatic cell

growth (Sonenberg, 1993). In principle, a perturbation of this homeostasis might contribute adversely to cellular proliferation. Indeed, it has been shown that NIH 3T3 cells engineered to over-express an exogenous PKR mutant (a substitution of Lys296 with arginine or proline, PKR296) produced tumors rapidly when injected into nude mice. Thus, mechanistically, a disturbance of the PKR translational control (Koromilas *et al.*, 1992; Lengyel, 1993; Meurs *et al.*, 1993; Donze *et al.*, 1995) is sufficient to account for cellular transformation. By analogy, we queried whether TRBP, when over-expressed, would transform NIH 3T3 cells by inhibiting PKR activity.

NIH 3T3 cells were selected for expression of pCMVTRBP or expression of *neo* alone (Figure 9). These cells were subjected to morphological and functional com-

One of our primary interests is to understand the process(es) through which IFN- α affects HIV-1 replication. Although the exact mechanism of action remains under

parisons. We examined three growth criteria: (i) contact inhibition; (ii) anchorage-independent growth; (iii) tumor formation in animals. NIH 3T3 cells expressing TRBP (Figure 9B, D and F) were found to exhibit characteristics consistent with transformation, including loss of contact inhibition (Figure 9D) and colony formation in soft agar (Figure 9F). In comparison, NIH 3T3 cells (data not shown) and NIH 3T3-*neo* cells were contact inhibited and failed to grow into sizable colonies in soft agar (Figure 9C and E).

Tumorigenicity in animals was assessed by injecting 1×10^6 cells into the posterior neck region of nude CD1/CD1 mice. Five out of five mice injected with NIH 3T3-TRBP developed tumors (>1.5 cm) within 15 days (Figure

Fig. 7. IFN- α affects steady-state levels of TRBP. HeLa cells were mock-treated (lane 1) or treated with IFN- α (500 U/ml) for 12 h (lane 2) or 24 h (lane 3). Cell extracts were prepared as described in Materials and methods and fractionated by 10% SDS-PAGE and analyzed by immunoblotting using anti-PKR (A) or anti-TRBP (B) polyclonal antibody.

discussion, studies by others (Edlin *et al.*, 1992; Francis *et al.*, 1992; Coccia *et al.*, 1994; for a review see Pitha, 1994) make it clear that IFN- α , in various settings, suppresses productive HIV-1 infection of permissive cells. Unquestionably, there are PKR-independent pathways in cells through which IFN- α signals are transduced (for a review see Williams, 1991). Our findings here establish a tight correlation between IFN- α and PKR in biosynthetically repressing HIV-1 protein synthesis and replication (Figures 2–6).

The linkage between PKR and IFN- α was largely established using TRBP. TRBP is an HIV-1 TAR RNA binding protein that shares a dsRNA binding motif (Gatignol *et al.*, 1993; Kozak *et al.*, 1995) with PKR (McCormack *et al.*, 1992). Given this commonality in RNA binding and given that TRBP is highly conserved across species and expressed in all tissues (Kozak *et al.*, 1995), it was reasonable to suppose that TRBP and PKR could compete in cells for binding to the same substrate RNA. Evidence supportive of this reasoning is illustrated by the example of TAR RNA, which can be bound by both TRBP (Gatignol *et al.*, 1991) and PKR (McCormack *et al.*, 1992) and which activates the function of the latter (Edery *et al.*, 1989; SenGupta and Silverman, 1989; Roy *et al.*, 1991; Maitra *et al.*, 1994). Indeed, this similarity in RNA binding has prompted others to propose that RNA substrate competition accounts for the functional interaction between PKR and TRBP (Cosentino *et al.*, 1995).

Our data using replicating HIV-1s expressing either PKR or TRBP indicate that in physiologically productive infections of T lymphocytes, TRBP has a potent ability to counter the inhibition of viral protein synthesis mediated by PKR (Figures 3 and 6). This finding agrees with and extends the *in vitro* reporter assays previously reported by Park *et al.* (1994). It also suggests that TRBP belongs functionally to a class of cellular inhibitors of PKR (reviewed in Lee, T.G. and Katze, 1994) that includes p58

Fig. 8. Inhibition of PKR phosphorylation *ex vivo* and *in vitro* by TRBP. (A and B) HeLa cells (lanes 5 and 6), HeLa cells stably expressing pCMVTRBP (lanes 1 and 2) or HeLa cells expressing pCMVTRBPΔ (lanes 3 and 4) were treated (lanes 2, 4 and 6) or mock-treated (lanes 1, 3 and 5) with IFN- α (500 U/ml). Twelve hours later, cells were labeled with [32 P]orthophosphate (A) or [35 S]L-cysteine + methionine (B) for 8 h. Cells extracts were prepared and immunoprecipitated using anti-PKR serum. The immunoprecipitated products were analyzed by 10% SDS-PAGE. (C and D) PKR was immunoprecipitated from HeLa cells using anti-PKR polyclonal antibody. The immunoprecipitated product was used for *in vitro* phosphorylation with poly(I)-poly(C) (1 μ g/ml) as dsRNA activator in the presence of [γ - 32 P]ATP. Autophosphorylation was performed in the absence (C and D, lane 4) or in the presence of MBP-TRBP (C, lanes 2 and 3) fusion protein, dsRNA binding-defective MBP-TRBP Δ 227–270 (D, lanes 2 and 3) fusion protein or MBP protein (C and D, lane 1).

(Lee, T.G. *et al.*, 1990, 1992, 1994; Barber *et al.*, 1994), a 15 kDa 3T3-F442A cell protein (Judware and Petryshyn, 1991) and a Ras-inducible inhibitory factor from KBALB cells (Mundschau and Faller, 1992).

Details on how cellular inhibitors of PKR function are

Fig. 9. Morphological transformation of cells by TRBP. NIH 3T3-*neo* (A, C and E) and NIH 3T3 TRBP-*neo* (B, D and F) were plated at 1×10^5 cells/100 mm dish in DMEM supplemented with 10% FBS and G418 (500 μ g/ml). (A and B) Cells in exponential growth; (C and D) cells maintained in culture for 5 days after reaching confluency. (E and F) Anchorage-independent growth of NIH 3T3-*neo* (E) and NIH 3T3 TRBP-*neo* (F) in soft agar.

Table I. TRBP induces tumors in nude mice

Cells	Animals with tumors	Tumor size	Latency (days)
NIH 3T3	1/5	2–3 mm	15–18
NIH 3T3CMV- <i>neo</i>	0/5		
NIH 3T3CMV-TRBP	5/5	>1.5 cm	15–18

Nude CD1/CD1 mice (4–6 weeks old) were injected s.c. in the posterior neck region with 1×10^6 cells suspended in 100 μ l PBS. The times required to produce visible tumors of the indicated size are listed as the latency period.

not fully understood. We find that TRBP exerts its effects, at least in part, through formation of a hetero-complex with PKR in an RNA binding-independent manner. Three lines of experimentation are consistent with this proposition. First, direct immunoprecipitation from cellular extracts that were first treated with RNase A and/or RNase V1 showed that TRBP is recovered using anti-PKR serum and PKR is recovered using anti-TRBP serum (Figures 4 and 5). This supports the idea that the two proteins exist as a complex in cells. Second, TRBP binds directly to column matrices containing GST-PKR^{K296R} or GST-PKR^{K296R/K64E}, but not to one containing GST alone (Figure 5C). We note that GST-PKR^{K296R/K64E} is a form of PKR deficient for dsRNA binding activity (McCormack *et al.*, 1994; McCormack and Samuel, 1995). The finding that TRBP binds GST-PKR^{K296R/K64E} suggests that the two proteins contact each other directly and are not indirectly tethered via a bridging dsRNA molecule. Third, an RNA binding-defective TRBP mutant (TRBP ^{Δ 227–270}) behaved like wild-type TRBP in effective biochemical (Figure 8D) functional (Figure 6B) interactions with PKR. Taken together, these results strongly suggest that TRBP forms a protein-mediated complex with PKR in a manner

similar to protein-mediated formation of PKR homodimer (Patel *et al.*, 1995; Ortega *et al.*, 1996).

One study (Cosentino *et al.*, 1995) has suggested that the binding of dsRNA by PKR is necessary for homodimerization with PKR itself or heterodimerization with TRBP. This conclusion was largely based on the results of far-Western and yeast two-hybrid analyses using a PKR fusion protein possessing a large deletion (residues 1–97 and 104–157 deleted). In contrast, other investigators have concluded, from studies utilizing various mutant PKR constructs which lack RNA binding activity, that the dimerization of PKR is RNA independent, both when measured *in vivo* (in yeast or COS cells by two-hybrid analyses) or *in vitro* by affinity chromatography and electrophoretic mobility shift analyses (Patel *et al.*, 1995; Ortega *et al.*, 1996). The differences between Cosentino *et al.* and the other groups (Patel *et al.*, 1995; Ortega *et al.*, 1996) most likely derive from the nature of the assays employed and the conformational form of the PKR protein examined. Perhaps an RNA bridge may indeed mediate dimerization in the absence of the protein domain with required conformation that is sufficient to mediate protein–protein complex formation in the absence of RNA.

An evolved primary function of PKR is the regulation of a subset of cellular genes (Petryshyn *et al.*, 1984; Chong *et al.*, 1992; Dever *et al.*, 1993; Lee, S.B. *et al.*, 1993; Lee and Esteban, 1994; for a review see Williams, 1991), thus, the commonly described anti-viral effects of PKR are probably secondary manifestations. In this regard, the ability of PKR to influence NF- κ B-mediated signaling (Kumar *et al.*, 1994) is an important concordant finding. Accordingly, one expects that dysregulated PKR function would not only be reflected in viral measurements in host cells, but also by changes in endogenous cellular metabolic parameters. Indeed, ample evidence supports this presumption. Early findings based on over-expression of trans-dominant mutant forms of PKR showed compellingly that NIH 3T3 cells in this setting progressed to transformed states (Koromilas *et al.*, 1992; Lengyel, 1993; Meurs *et al.*, 1993; Donze *et al.*, 1995). More recently, independent confirmation of transformation was achieved by over-expressing p58, a cellular inhibitor of PKR (Lee, T.G. *et al.*, 1990, 1992, 1994; Barber *et al.*, 1994). Mechanistically, this effect on cellular proliferation can be explained by a disturbance in the balanced regulation of translation of certain growth-related mRNAs (reviewed in Sonenberg, 1993). Indeed, findings from mutations in other elements of the translational machinery support this notion (Donze *et al.*, 1995). Although no evidence of tumor suppressor activity of PKR was observed in studies of ‘knock-out’ mice devoid of functional PKR (Yang *et al.*, 1995), it is conceivable that another form of functional eIF-2 α protein kinase (Chen *et al.*, 1991; Samuel, 1993) remained in the PKR^{–/–} animals which complemented in part the PKR deficiency.

Our results on TRBP provide further support for the critical role of PKR in cellular proliferation. We found that over-expression of TRBP results in loss of contact inhibition, production of anchorage-independent growth and progression to tumor formation when injected into animals (Figures 9 and 10 and Table I). Because, at the protein level, TRBP is completely different from p58 and is insignificantly similar to *trans*-dominant mutant PKRs

Fig. 10. TRBP induces tumors in nude mice. Athymic CD1/CD1 nude mice were injected s.c. in the posterior neck region. Nude mice were injected with 1×10^6 NIH 3T3 TRBP cells (right) or NIH 3T3-neo (left).

(tmPKRs) and because the one property common to the three proteins (TRBP, p58 and tmPKRs) is functional inhibition of wild-type PKR, one can reasonably deduce that PKR is the linchpin in the three discrete approaches to transformation. Growth controls like that engendered by PKR represent important checks to general cellular metabolism. In other biological systems critical checkpoints are commonly redundant (for a review see Hunter, 1995). A recent mouse 'knock-out' study (Yang *et al.*, 1995) provides some indication that developmentally this might also be the case for PKR.

From the perspective of HIV-1, we find it intriguing that TRBP is a potent counter to PKR. Viruses clearly have a vested interest in evolving escape strategies from the antiviral effects of PKR (reviewed in Katze, 1992) and some viruses are more accomplished at this than others. For HIV-1, because the viral leader RNA, TAR, activates PKR function (Edery *et al.*, 1989; Maitra *et al.*, 1994), it seems logical that the virus could use a cellular (TRBP) and a viral (Tat; McMillan *et al.*, 1995a,b) TAR binding protein to counter this activation. Future investigations of this intricate interplay should lead to important insights into viral and cellular metabolic processes.

Materials and methods

Plasmids

All proviral constructs were derived from the parental pNL4-3 infectious molecular clone (Adachi *et al.*, 1986). Insertions of cDNA into *nef* have been described elsewhere (Joshi and Jeang, 1993; Huang *et al.*, 1994). The PKR cDNA, PKR^{K296R} and PKR^{K296R/K64E} have been described previously (Thomis and Samuel, 1992a,b; McCormack *et al.*, 1994; McCormack and Samuel, 1995). TRBP and TRBP^{A227-270} have been described (Gatignol *et al.*, 1993).

Cell culture, transfection and infection

HeLa cells and NIH 3T3 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Continuous suspensions of human CEM (12D7) cells and continuous suspensions of C8166-45 T-cells were grown in RPMI 1640 with 10% FBS. Transfections of HeLa and NIH 3T3 cells were performed using calcium phosphate. Aliquots of 10 μ g total HIV-1 genome were used for each transfection. Electroporation (Bio-Rad) of 5×10^6 suspension cells in 300 μ l $1 \times$ phosphate-buffered saline (PBS) using 20 μ g total HIV-1 genome was performed at a setting of 250 V and 960 μ F.

Virus stocks were prepared from supernatants of transfected HeLa cells and were quantified by RT activity. For infection of CEM (12D7) cells, 1×10^6 cells were incubated with virus for 1 h at 37°C and then

washed twice with medium before resuspending in medium at a density of 5×10^5 cells/ml.

Reverse transcriptase assay

Reverse transcriptase assays were performed as previously described (Huang *et al.*, 1994). Each reaction contained 5 μ l viral supernatant in 50 μ l RT cocktail [60 mM Tris, pH 8, 75 mM KCl, 5 mM MgCl₂, 0.1% Nonidet P-40, 1 mM EDTA, 5 μ g/ml poly(rA), 0.16 μ g/ml oligo(dT), [α -³²P]dTTP (1 μ Ci/ml)] and was incubated at 37°C for 1 h. For each reaction 5 μ l was spotted onto DEAE paper which was washed four times in $2 \times$ SSC, dried and analyzed using a Fuji phosphorimager.

Antibodies

Anti-PKR and anti-TRBP polyclonal antibodies were as previously described (Thomis and Samuel, 1992a,b; Gatignol *et al.*, 1993). HIV-1 hyperimmune serum was from an AIDS patient.

Protein analysis

For Western analysis, cells were washed with PBS and resuspended in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% desoxycholate, 0.1% SDS, 50 mM Tris, pH 8). The cell extracts were quantified using a Bio-Rad colorimetric protein assay. Identical quantities of protein were resolved in SDS-polyacrylamide gels and were transferred to Immobilon-P (Millipore) membranes, which were blocked with blocking buffer (0.2% I-block 'Tropix' in $1 \times$ PBS, 0.1% Tween-20) and incubated overnight with primary antiserum. Reactive proteins were developed with secondary antibody conjugated to alkaline phosphatase and visualized using chemiluminescence according to the manufacturer's suggestions (Tropix; Jeang *et al.*, 1993).

For immunoprecipitation, identical amounts of protein were suspended in 1 ml RIPA buffer and incubated for 2 h at 4°C with antiserum. Protein A-Sepharose (Pharmacia) was added to each sample followed by 1 h incubation at 4°C, with several washings in RIPA buffer. The immunoprecipitated products were then solubilized in loading buffer (125 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 2% β -mercaptoethanol, 0.01% bromophenol blue) and resolved by SDS-PAGE.

PKR phosphorylation

Cells (5×10^6) were mock-treated or treated with IFN- α (500 U/ml) for 12 h, washed in PBS and then resuspended in 2 ml phosphate-free DMEM or methionine- and cysteine-free DMEM medium. [³²P]Orthophosphate (500 μ Ci/sample) or a mixture of [³⁵S]methionine + cysteine (Translabel; ICN Biomedicals) (200 μ Ci/sample) was added to each 2 ml suspension of cells. After 8 h incubation at 37°C, the cells were washed in PBS and resuspended in RIPA buffer.

For *in vitro* phosphorylation, PKR was purified from 1×10^6 HeLa cells using specific polyclonal antibody with protein A-Sepharose and assayed for protein kinase activity as previously described (Hovanessian *et al.*, 1987). Briefly, immune complex preparations were incubated in 20 μ l buffer (20 mM HEPES, pH 7.5, 50 mM potassium chloride, 5 mM 2-mercaptoethanol, 1.5 mM manganese chloride, 1.5 mM magnesium chloride) containing poly(I):poly(C) (1 μ g/ml) and 2 mM [γ -³²P]ATP (50 Ci/mmol) at 30°C for 20 min. Plasmid pBS-TRBP, encoding TRBP fused to MBP, has been described (Gatignol *et al.*, 1991). MBP-TRBP,

MBP-TRBP^{Δ227-270} and MBP were produced in *E.coli* XL1 Blue as previously described (Gatignol *et al.*, 1991).

RNA binding assay

Samples of 10×10^6 T cells (H9) were cultivated in 5 ml RPMI without phosphate 4 h before addition of 5 mCi [³²P]orthophosphate. After 15 h, total RNA was extracted using a RNeasy extraction kit (Tel-Test). RNAs were incubated with beads containing MBP alone, MBP-TRBP or MBP-TRBP^{Δ227-270}. To prepare the column, MBP alone, MBP-TRBP and MBP-TRBP^{Δ227-270} were expressed in bacteria. Bacterial lysates were prepared and incubated overnight with amylose resin (Biolabs). Equivalently normalized amounts of fusion and non-fused proteins were used. The resins were then packed into columns and the columns washed twice with binding buffer (20 mM HEPES, pH 7.3, 40 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol). The same quantity of [³²P]orthophosphate-labeled RNA resuspended in 1.5 ml RNA binding buffer was passed three times through each column. The columns were washed three times with 5 vol. binding buffer. RNAs were then eluted in stepwise fashion using binding buffer containing 0.5, 1.0, 1.5 or 2.0 M NaCl. RNAs were precipitated with ethanol and visualized on a denaturing 6% polyacrylamide gel. Where indicated, columns were washed and treated for 30 min at room temperature with 10 U/ml RNase V1 (Pharmacia). Additional washes were performed and the bound RNAs were eluted as described above.

RNase protection assay

Total RNA from transfected HeLa cells was extracted using a RNeasy extraction kit. The RNase protection assay was performed according to protocol suggested by the manufacturer (RPA II kit; Ambion).

Cell growth, soft agar and tumorigenicity assays

Cells (1×10^4) were suspended in 0.8% agarose solution in DMEM containing 20% FBS and overlaid onto 1.4% agarose solution in DMEM containing 20% FBS on 35 mm plates. Colonies were scored 10 days after plating. Tumorigenicity assays were as described in Table I. Nude CD1/CD1 mice were purchased from Charles River Laboratory (CRL, Boston, MA).

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