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The mouse antiphosphotyrosine immunoreactive kinase, TIK, is indistinguishable from the double-stranded RNA-dependent, interferon-induced protein kinase, PKR

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ABSTRACT

The mouse TIK protein, a serine/threonine kinase, was originally isolated from a murine pre-B cell expression library by its ability to bind anti-phosphotyrosine antibodies (Icely *et al.*, *J. Biol. Chem.* 266, 16073–16077, 1991). The 67 kDa protein was found to have an associated autophosphorylation activity when incubated with ATP. Our results show that TIK is actually the mouse interferon-induced, dsRNA-dependent protein kinase, PKR. We demonstrate that the TIK message is interferon-inducible in mouse L-cells and *in vitro* transcription and translation of the TIK cDNA produces a protein that is capable of binding double-stranded RNA. The *in vitro* synthesized TIK protein migrated as a 65 kDa protein on SDS-PAGE when incubated with ATP, but migrated as a 60 kDa protein when incubated with an inhibitor of PKR, 2-aminopurine. We further show that proteolytic digestion of TIK with *Staphylococcus aureus* V8 protease results in a cleavage pattern identical to that obtained by V8 digestion of authentic PKR. Antiserum to TIK specifically recognized PKR. Cloned TIK had inhibitory activity for replication of EMCV but not VSV. From these observations we conclude that TIK kinase is the mouse interferon-induced, double-stranded RNA-dependent kinase, PKR.

INTRODUCTION

The dsRNA-dependent protein kinase, PKR, is an IFN-inducible protein involved in the inhibition of protein synthesis. This kinase, also known as the P_1/eIF-2α protein kinase, p65 (mouse and rabbit) or p68 (human) kinase, DAI and dsI (2–6), plays a key role in the antiviral action of IFN against several animal viruses (5,7). The dsRNA-dependent protein kinase is synthesized in an inactive form and becomes activated by interacting with dsRNA (8). Activation involves autophosphorylation of the enzyme (9). Incorporation of phosphate leads to a shift in electrophoretic mobility of the activated mouse kinase by SDS-PAGE, from a M_r of approximately 60,000 for the inactive mouse kinase to a M_r of approximately 65,000 for activated mouse kinase (9). Kinase autophosphorylation and, therefore, activation can be blocked by 2-aminopurine (10–12). In its active form, PKR can phosphorylate exogenous substrates including the α subunit of the eukaryotic translation initiation factor eIF-2 (13,14). Phosphorylation of eIF-2α inhibits protein synthesis at the initiation step of translation (15–17). The dsRNA-dependent protein kinase may also phosphorylate other IFN-regulating proteins such as the nuclear transcription factor NF$\varkappa$B (18). NF$\varkappa$B can be activated by dsRNA via the phosphorylation and release of its inhibitor I$\varkappa$B (19, 20). It is possible that the dsRNA-dependent protein kinase may be involved in this activation, or possibly the phosphorylation of other IFN-regulated proteins.

A cDNA clone of the human dsRNA-dependent protein kinase has been isolated (2,3). The human PKR mRNA is induced by IFN-α and produces a protein product of 68 kDa, p68. The deduced amino acid sequence from the human PKR cDNA predicts a protein which contains all of the conserved motifs characteristic of serine/threonine protein kinases (21). The human PKR cDNA was recently used as a probe to screen a mouse cDNA library under low stringency conditions (22). A clone was identified with a predicted amino acid sequence 61% identical to the human kinase. Based on this homology, Feng *et al.* speculated that this cDNA encoded the mouse dsRNA-dependent protein kinase (22), and noted that the cDNA sequence was nearly identical to the mouse TIK sequence (1).

Mouse TIK, a serine/threonine kinase, was originally isolated from a murine pre-B cell expression library by its ability to bind anti-phosphotyrosine antibodies (1). *In vitro* transcription and translation of the cloned TIK cDNA produced a polypeptide of approximately 67 kDa, significantly larger than the predicted molecular mass of the protein (58.5 kDa) (1). The 67 kDa protein was found to have an associated autophosphorylation activity when incubated with ATP (1). Given the sequence similarity to the human dsRNA-dependent protein kinase and the apparent and predicted M_r of TIK, we and others speculated that TIK might be the mouse dsRNA-dependent protein kinase (2,22). Alternatively, these two proteins could represent different members of a kinase gene family. To determine if TIK is the mouse dsRNA-dependent protein kinase, we have compared the biochemical characteristics of TIK to the known properties of

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the dsRNA-dependent protein kinase. We have determined that TIK mRNA is IFN inducible in mouse L-cells. Furthermore, we demonstrate that an *in vitro* translation product of the TIK mRNA bound dsRNA- and poly(rI)·poly(rC)-sepharose. The *in vitro* synthesized TIK protein migrated as a 65 kDa protein on SDS-PAGE after incubation with ATP, but migrated as a 60 kDa protein after incubation with a dsRNA-dependent protein kinase inhibitor, 2-aminopurine. We further show that proteolytic digestion of TIK with *Staphylococcus aureus* V8 protease results in a cleavage pattern identical to that obtained by V8 digestion of authentic mouse dsRNA-dependent protein kinase. Antiserum to recombinant TIK protein specifically recognized an IFN-inducible protein in crude L-cell extracts that co-migrated with authentic PKR and had a peptide map indistinguishable from authentic PKR. Finally, the cloned TIK gene had anti-viral properties similar to human PKR (23). These results demonstrate that TIK cDNA actually encodes the mouse interferon-induced dsRNA-dependent protein kinase, PKR.

MATERIALS AND METHODS

Production of mouse TIK cDNA, cloning, sequencing, and expression of the TIK gene

Mouse L-cells were maintained in suspension in Eagle's suspension minimal essential media MEM (Gibco) supplemented with 5% fetal bovine serum (HyClone Laboratories), 50 μg of gentamicin/ml, 100 μg of glucose/ml, 292 μg of L-glutamine/ml, 110 μg of sodium pyruvate/ml, and 0.1 mM nonessential amino acids. Cells, at a density of 1×10^6 cells/ml, were treated with mouse IFN-β (Lee Biochemical, 1.3×10^8 IU/mg) at 500 units/ml for 12 hours. L-cell mRNA was purified using Fast Track mRNA Isolation Kit (Invitrogen). cDNA was prepared from L-cell mRNA (0.5 μg) by reverse transcription using 1 μg of an oligonucleotide primer (TIK reverse) containing a *Bam*H I restriction site with sequence 5′ GCTTGGATCCCCTCAGA-CTGCA 3′ (complementary to nucleotides 1737−1751 downstream of the mouse TIK coding region), 200 μM dNTPs, 20 U RNasin ribonuclease inhibitor, 1×PCR buffer (Perkin Elmer Cetus) and 25 U AMV reverse transcriptase (Boehringer Mannheim) in a 20 μl volume. Reactions were at 45°C for 1 hour. The TIK gene was cloned from the cDNA reaction mixtures after PCR amplification of the front 2/3 of the gene and the back 1/3 of the gene, respectively. The reverse transcription reaction was diluted 5 fold in PCR reaction buffer for a final volume of 100 μl and divided in half. An oligonucleotide primer (TIK back forward) with sequence 5′ GTCGATACAAACCCGGTGC 3′ (identical to nucleotides 1091−1110 in the coding sequence of the TIK gene), additional TIK reverse primer and 2 units of Taq polymerase (Perkin Elmer) were added to one half of the diluted cDNA reaction mixture. PCR cycles were 94°C for 1 minute, 50° C for 2 minutes, 72° C for 4 minutes, for 25 cycles. An approximately 650 base pair fragment, corresponding to the back 1/3 of the gene, was isolated, cut with *Pst* I and *Eco*R I and cloned into *Pst* I and *Eco*R I cut pBluescript. The front 2/3 of the gene was amplified from the cDNA reaction mixtures as described above using an oligonucleotide (TIK forward) with sequence 5′ CGACCCGGGAAAAATGGCCAGTGAT 3′ (identical to nucleotides 136−153 of the TIK gene and containing a *Sma* I restriction site) and an oligonucleotide (TIK front reverse) with sequence 5′ TTATCACAGAATTCCAT 3′ (identical to nucleotides 1120−1140 of the TIK sequence). The amplified DNA of approximately 1 kbp was cut with *Sma* I and *Eco*R I, and cloned into *Sma* I and *Eco*R I cut pBluescript. To reassemble the full length gene, the front fragment of TIK was isolated after digestion of the plasmid with *Eco*R I and *Sma* I. Gel purified fragment was cloned into *Hinc* II and *Eco*R I cut plasmid containing the back fragment of TIK, downstream from the bacteriophage T3 promoter. The plasmid was designated pBS-TIK. The cDNA insert was sequenced using Sequenase (United States Biochemical) according to the manufacturer's protocol. For *in vitro* transcription of the TIK cDNA, the plasmid was linearized and treated with proteinase K (50 μg/ml) for 30 minutes at 37°C. The DNA was extracted with phenol/chloroform and isolated by centrifugation following ethanol precipitation. Linearized plasmid DNA (2 μg) was transcribed *in vitro* with 10 U of T3 RNA polymerase according to the manufacturers guidelines (Stratagene). The transcription reaction was treated with DNase I (50 U) for 20 minutes at 37°C. The RNA yield was measured by spectrophotometry at 260 nm. RNA was determined to be full-length by visualization of ethidium bromide stained RNA following agarose gel electrophoresis. For *in vitro* translation, reactions (50 μl) contained 70% nuclease-treated rabbit reticulocyte lysate (Promega), 2 μg RNA, 40 U RNasin ribonuclease inhibitor, 20 μM amino acids (minus methionine), and 0.8 μCi of [^{35}S]methionine per μl. Where indicated, 2-aminopurine was also added to the translation reaction at final concentration of 5 mM. Translation was at 30° C for 1 hr. Synthesized proteins were analyzed by SDS-PAGE, through 10% gels, and autoradiography.

PCR quantitation of the TIK message from interferon-induced and uninduced L-cells

Polyadenylated RNA was isolated (Fast Track, Invitrogen) from 1×10^8 IFN-induced (500 units/ml for 12 hours) or 1×10^8 uninduced mouse L-cells. The yield of mRNA was quantitated by OD_{260}. For reverse transcription, 4 μg, 2 μg, 0.4 μg, and 0.2 μg of mRNA from IFN-induced or uninduced cells was used. The 100 μl reactions contained 1 μg of TIK reverse primer, 1×PCR buffer (Perkin Elmer Cetus), 250 μM dNTP mixture, 20 U RNasin (Promega), and 25 U AMV reverse transcriptase (Boehinger Manheim). Reverse transcription was performed at 45°C for 1 hour. For PCR, 1 μg of TIK back forward primer and 2U of Taq polymerase (Perkin Elmer Cetus) were added directly to the 100 μl reverse transcription reaction and 25 cycles of amplification were performed. Amplification of the 660 base pair 3′ TIK fragment in the IFN-induced and uninduced samples was analyzed by agarose gel electrophoresis and ethidium bromide staining.

Preparation of antiserum to recombinant TIK

The bacterial strain XL-1 Blue was transformed with plasmid pBS-TIK. Cells were grown in Luria Broth containing 50 μg/ml ampicillin. When cells reached a density of $OD_{600}=0.4$, IPTG was added to a final concentration of 5 mM. Cells were induced for one hour, pelleted, and resuspended in sonication buffer (50 mM Tris [pH 8.0], 0.2 mM EDTA, 10% glycerol, 2.5 mM $MgCl_2$, 150 mM NaCl, 4 mM PMSF). The cells were briefly sonicated at 4°C. SDS solubilization buffer was added to the extracts, and the proteins were separated by SDS-PAGE and visualized by Coomassie stain. The induced protein band was cut from the gel and emulsified in Freunds Complete Adjuvant. Approximately 20 μg of protein was injected into two rabbits. Five weeks later, rabbits were boosted with 20 μg of protein

emulsified in Freunds Incomplete Adjuvant. Serum was collected seven days later and tested for antigen recognition by Western blot analysis.

Preparation of mouse dsRNA-activated kinase

Protein extracts containing mouse dsRNA-dependent protein kinase were prepared from L-cells treated with 100 units/ml of recombinant human IFN-α A/D for 18 hours. The dsRNA-dependent protein kinase was partially purified from the RSW fraction of these cells as previously described (23). The RSW fraction was incubated with poly(rI)·poly(rC)-sepharose for 1 hour at 4° C. Proteins bound to resin were washed 3× with buffer A (20 mM HEPES, pH 7.5; 50 mM KCl; 5 mM MgOAc; 1 mM DTT; 1 mM Benzamidine; 10% glycerol). The poly(rI)·poly(rC) bound proteins were further incubated with buffer A containing [γ^{32}P]ATP (3×10^{-2} μM) for 15 minutes at 30°C. Proteins were denatured in SDS solubilization buffer and separated by SDS-PAGE. Activated mouse dsRNA-dependent protein kinase was visualized by autoradiography.

Binding of mouse TIK protein to poly(rI)·poly(rC)-sepharose

Mouse TIK cDNA was transcribed *in vitro*, and the resulting products were translated in a rabbit reticulocyte system. The protein products were analyzed by affinity chromatography to determine their ability to bind poly(rI)·poly(rC)-agarose. For each binding, 35 μl of translation reaction was incubated with an equal volume of poly(rI)·poly(rC)-sepharose for 1 hour at 4° C. The bound proteins were washed 3× with buffer A. Where indicated, samples were then incubated in 20 μl Buffer A containing 100 μM ATP for 15 minutes at 30°C.

Staphylococcus aureus V8 protease peptide mapping

Peptide mapping was performed as previously described (24,25). Briefly, [^{35}S]methionine-labeled *in vitro* translated TIK protein or [γ^{32}P]ATP-phosphorylated RSW kinase were separated by SDS-PAGE, stained briefly in Coomassie Brilliant Blue R-250, and destained in methanol/acetic acid. The 65 kDa bands were cut from the gel and equilibrated in buffer (125 mM Tris, pH 6.8; 0.1% SDS; 1 mM EDTA; 1 mM dithiothreitol) for 20 minutes at 25°C. The gel fragments containing the 65 KDa proteins were then placed into the wells on a second 12% SDS polyacrylamide gel and overlaid with sample buffer containing 10% v/v glycerol and 25 ng of V8 protease (Boehringer Mannheim). The protein samples were electrophoresed through the stacking gel at 30 mA. Electrophoresis was stopped, and V8 protease digestion was allowed to occur for 30 minutes. Electrophoresis was then continued through the resolving gel. Protein fragments were visualized by Coomassie staining and autoradiography.

Expression of TIK in HeLa cells

The TIK gene was subcloned from pBS-TIK into pMTVa−. pBS-TIK was cleaved with *Kpn* I, and ends were blunted with T4 DNA polymerase. Linearized blunted pBS-TIK was cleaved with *Sma* I and the 1.7 kbp fragment was gel purified and cloned into *Eco*R I cut, blunted (T4 DNA polymerase), phosphatase-treated pMTVa−. Orientation was determined by cleavage with *Pst* I. A clone with the TIK gene oriented downstream from the adenovirus major late promoter was designated pMT-TIK. pMT-TIK, purified with a Qiagen Plasmid Kit according to the manufacturers instructions, was transfected into HeLa cells by a modification of the calcium phosphate precipitation technique (26, 27). Briefly, 20 μg of pMT-TIK in 500 μl of 250 mM $CaCl_2$ was added dropwise to an equal volume of 2×HBS, pH 7.05, while continually applying a gentle airstream to the surface of the 2×HBS. After allowing the mixture to stand undisturbed for 10−20 min, the solution was layered under the surface of the growth medium (DMEM (Gibco), containing 10% fetal bovine serum (HyClone Laboratories), and 50 μg of gentamicin/ml) covering a just confluent monolayer of HeLa cells. After 8 hours the medium was removed from the monolayer and the cells were washed three-times with complete medium. Twelve hours later cells were infected with either EMCV or VSV at an MOI of 5 pfu/cell. After 18 hours progeny virus was harvested and assayed by plaque formation onto monolayers of HeLa cells.

RESULTS

The TIK message is induced by interferon

In a quantitative PCR assay, we compared the amount of TIK cDNA, obtained from reverse transcribed mRNA, in IFN-treated and untreated L-cells (Figure 1). Total polyA-enriched RNA, isolated from IFN-treated L-cells and untreated L-cells, was reverse transcribed. The reverse transcription reactions contained 0.2 μg, 0.4 μg, 2 μg or 4 μg of polyA-enriched RNA. The amount of TIK cDNA obtained from the reverse transcription reaction was then amplified by 25 cycles of PCR. Since reverse transcription reactions may not consistently yield full-length cDNA, PCR primers were constructed such that only the 3′ end of the TIK cDNA was amplified. For each of the various mRNA concentrations, more TIK cDNA was amplified from IFN-treated L-cells than from untreated L-cells. For example, reverse transcription of 4 μg of total mRNA, followed by PCR amplification of the 3′ end of the TIK cDNA, yielded substantially more of the 660 base pair TIK fragment in cells treated with IFN than in untreated cells (Figure 1, lanes 7−8). The logarithmic amplification of cDNA by PCR appeared to result in approximately comparable amounts of DNA from 0.2 μg of IFN-treated L-cell total mRNA and 4 μg of untreated L-cells total mRNA (Figure 1, lanes 1 and 8). Therefore, we estimate that IFN-treated L-cells contain approximately 20 fold higher levels of TIK mRNA than untreated cells.

Cloning, expression, and sequencing of the mouse TIK gene

Oligonucleotide primers specific for non-translated regions 5′ and 3′ to the coding region of the published TIK sequence and overlapping internal primers (1) were synthesized. 5′ and 3′ fragments of mouse TIK cDNA were amplified from total L cell cDNA by PCR. The TIK cDNA was reconstructed in pBluescript

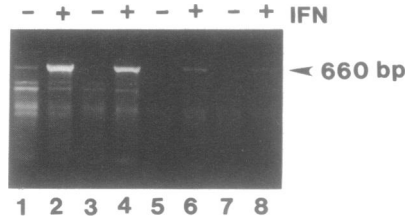

Figure 1. PCR amplification of 660 b.p. of the TIK cDNA from IFN-β-treated and untreated L-cells. Reverse transcription reactions containing 4 μg lanes 1−2), 2 μg (lanes 3−4), 0.4 μg (lanes 5−6) and 0.2 μg (lanes 7−8) polyA-enriched RNA were used to PCR amplify the 3′ end of the TIK cDNA. IFN treatment (500 U/ml for 12 h) of L-cells prior to mRNA isolation is as shown.

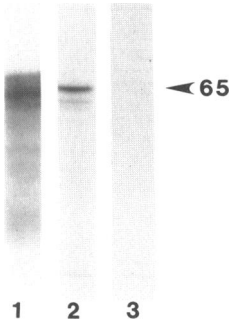

Figure 2. *In vitro* transcribed and translated TIK cDNA. ^{35}S-Methionine- labeled proteins were separated by SDS-PAGE and visualized by autoradiography. Lane 1: Total translation product. Lane 2: Translation products which bound poly(rI)·poly(rC)-sepharose. Lane 3: Translation products which bound sepharose alone. The approximate molecular weight (in kDa) of the major dsRNA-binding protein is indicated.

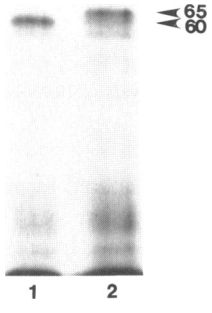

Figure 3. The effect of 2-aminopurine on TIK mobility through SDS-PAGE. TIK cDNA was translated in the presence (lane 1) or absence (lane 2) of 5 mM 2-aminopurine. [^{35}S]Methionine-labeled proteins which bound to poly(rI)·poly(rC)-sepharose were separated by SDS-PAGE and visualized by autoradiography.

downstream from the bacteriophage T3 promoter. *In vitro* transcription and translation of the TIK clone in a cell free rabbit reticulocyte system produced several protein products in the 55−65 kDa range (Figure 2, lane 1).

Comparison of our PCR amplified TIK sequence to the published TIK sequence identified several nucleotide discrepancies which would affect, most notably, the translation stop site as well as two amino acid in the putative dsRNA binding domain of the protein. The sequence we amplified using PCR primers to the TIK non-translated region was identical to the cDNA obtained by Feng *et al.* (22).

TIK protein binds dsRNA and 'shifts' in apparent molecular weight when incubated with ATP

The products obtained by *in vitro* translation of TIK mRNA were incubated with poly(rI)·poly(rC)-sepharose to determine whether any of the newly synthesized proteins could bind to dsRNA. Two proteins bound specifically to poly(rI)·poly(rC)-sepharose, but did not bind to the sepharose resin alone (Figure 2, lanes 2−3). The higher molecular weight protein (approximately 65 kDa) was more abundant than the lower molecular weight protein (approximately 60 kDa). These proteins most likely correspond to the non-phosphorylated and phosphorylated forms of mouse dsRNA-dependent protein kinase. Since the rabbit reticulocyte translation mix contains ATP, it is likely that the mouse kinase

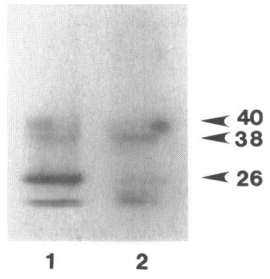

Figure 4. V8 protease mapping of TIK and dsRNA-dependent mouse kinase proteins. [^{35}S]Methionine-labeled*in vitro* transcribed and translated TIK protein (lane 1) and [^{32}P$_i$]-labeled authentic mouse kinase (lane 2) were proteolyzed with V8, separated by SDS-PAGE, and visualized by autoradiography. The approximate molecular weights (in kDa) for each of the fragments is indicated.

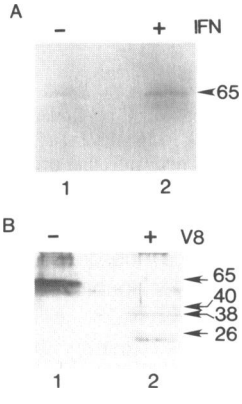

Figure 5. A. Western blot analysis of protein extracts from untreated and IFN-treated L-cells. Antibodies raised against recombinant TIK protein faintly recognize a 65 kDa protein in extracts from untreated L-cells (Lane 1) but strongly recognize the 65 kDa protein in extracts from IFN-treated (100 U/ml for 18 h) L-cells (Lane B). **B.** Western blot analysis of uncleaved dsRNA-dependent protein kinase (Lane 1) and V8 proteolyzed dsRNA-dependent protein kinase (Lane 2) using antibodies raised against recombinant TIK.

would become phosphorylated when bound to poly(rI)·poly(rC)-sepharose, and therefore migrated as a 65 kDa protein through SDS-PAGE.

To determine if the higher molecular weight protein (p65) actually represents a phosphorylated form of the lower molecular weight protein, 5 mM of the PKR inhibitor 2-aminopurine was added at the time of translation. Translation and binding in the presence of 2-aminopurine resulted in visualization of only the lower molecular weight protein (p60) (Figure 3, lane 1). None of the higher molecular weight species was detected. Washing of the bound protein to remove the 2-aminopurine, and addition of exogenous ATP resulted in the protein now migrating as the higher molecular weight form on SDS-PAGE (Figure 3, lane 2).

In vitro synthesized TIK protein has a V8 proteolysis pattern similar to authentic dsRNA-dependent protein kinase

Mouse dsRNA-dependent protein kinase, when digested with *Staphylococcus aureus* V8 protease, has previously been shown to migrate as three peptides by SDS-PAGE (24). The two larger fragments migrate as a doublet of 38−40 kDa, while the third peptide migrates as a smaller fragment of 26 kDa. The *in vitro* synthesized mouse TIK protein (Figure 4, lane 1) gave an

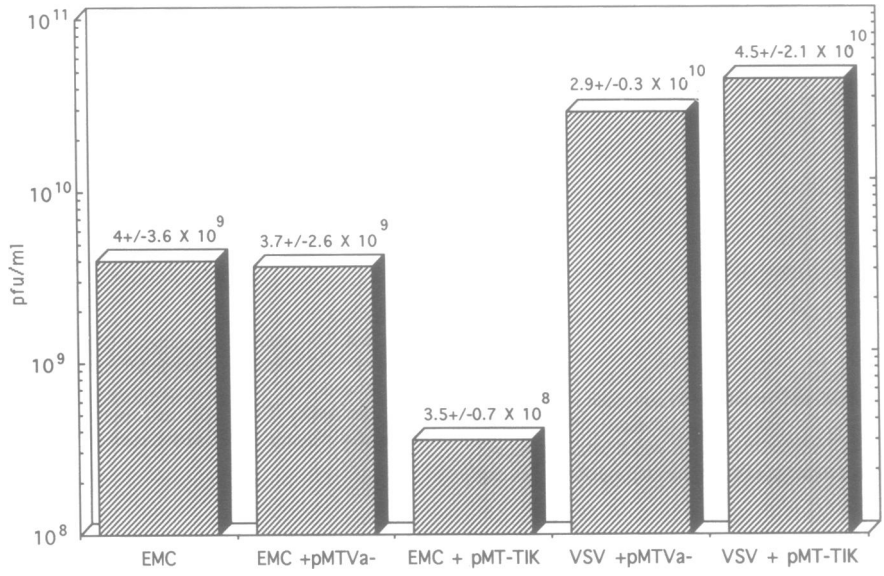

Figure 6. Inhibition of virus replication by cloned TIK. The mouse TIK gene, in a eukaryotic expression vector (pMT-TIK), or the parental plasmid (pMTVa−) were transfected into HeLa cells. Monolayers of cells were mock transfected as controls. 20 hours post-transfection cells were infected with EMCV or VSV at an MOI of 5 pfu/cell. 18 hours after infection progeny virus was harvested and quantitated by plaque assay onto monolayers of HeLa cells. All assays were done in duplicate and titered in duplicate.

identical V8 map to activated ribosomal salt wash purified kinase (Figure 4, lane 2). The upper doublet of both the TIK and RSW kinase migrated at approximately 38−40 kDa; whereas, the lower fragments migrated at approximately 26 kDa.

Antiserum to recombinant TIK protein recognizes the dsRNA-dependent mouse kinase

Antiserum produced against a β-galactosidase-TIK fusion protein produced in *E.coli* specifically recognized the dsRNA-dependent mouse kinase in crude L-cell extracts. Western blot analysis demonstrated that the antiserum, at a dilution of 1:1000, recognized a protein of 65 kDa in crude extracts from IFN-treated L-cells that had been incubated with poly(rI).poly(rC) sepharose and [γ^{32}P]ATP, but only weakly detected the protein in identically prepared extracts from untreated L-cells (Figure 5A). Autoradiography of the Western blot confirmed that the antiserum specifically recognized a protein that migrates identical to the radiolabeled mouse dsRNA-dependent protein kinase (data not shown). In extracts from IFN-treated cells, the antibody recognized both the non-phosphorylated and phosphorylated forms of this protein. In addition, the three proteolytic fragments obtained by V8 digestion of L-cell purified dsRNA-dependent protein kinase were also recognized by antiserum to TIK (Figure 5B).

Inhibition of virus replication by cloned TIK

Cloned human PKR has been reported to inhibit replication of EMCV but not VSV (23) To determine if mouse TIK had similar properties we have subcloned the mouse TIK gene into a eukaryotic expression vector pMTVa− (pMT-TIK). Either parental plasmid (pMTVa−) or plasmid expressing TIK (pMT-TIK) was transfected into HeLa cells using a technique that allows expression of transfected DNA in a high percentage of cells (26,27). Transfected monolayers were subsequently infected with either VSV or EMCV, and progeny virus was quantitated after a single cycle of virus infection. As can be seen in Figure 6, expression of pMTVa− had little effect on replication of EMCV or VSV (data not shown) compared to mock transfected cells. However, constitutive expression of TIK inhibited EMCV replication approximately 10-fold. Expression of TIK had little effect on VSV replication. These results suggest that mouse TIK has an anti-viral activity similar to human PKR.

DISCUSSION

Previous work (22) has identified a mouse cDNA, nearly identical in sequence to TIK (1), that shares 61% homology at the amino acid level with the human dsRNA-dependent protein kinase, PKR (3). The results presented in this paper demonstrate that this mouse cDNA (Genebank accession no. M93567) represents the correct sequence for TIK (Genebank accession no. M6529) and that TIK encodes a protein that exhibits properties identical to murine PKR. Both mouse and human PKR are induced approximately 7−10 fold in cells that have been treated with IFN for 16−24 hours (2,3). Human kinase message is maximally induced by IFN-α in Daudi cells by 16 hours (3). Northern blot analysis depicts a dramatic increase (greater than 10 fold) in human dsRNA-dependent protein kinase message following IFN treatment of Daudi cells (3). Our results indicate that mouse TIK mRNA levels are similarly induced by IFN (approximately 20 fold induction in mouse L-cells of TIK message by IFN-β). The predicted and actual molecular weights of the TIK protein also correspond to the molecular weights of the phosphorylated and nonphosphorylated forms of the dsRNA-dependent protein kinase. The TIK cDNA sequence predicts a protein of 60 kDa. When incubated with ATP and dsRNA, however, products of TIK primed translation migrated on SDS-PAGE as a larger species (65 kDa). This shift in molecular weight was inhibited by 2-aminopurine, an inhibitor of PKR. Similarly, the cDNA sequence of human PKR predicts a protein of 62 kDa, yet authentic human PKR migrates as a 67−68 kDa protein (2). Cleavage of *in vitro* transcribed TIK by V8 protease produced three fragments which were identical in size to the three fragments obtained by digestion of purified mouse PKR with V8 protease.

Antiserum produced against recombinant TIK protein specifically recognized both the nonphosphorylated and phosphorylated forms of the dsRNA-dependent mouse kinase in crude extracts, as well as the three proteolytic fragments of PKR obtained by V8 digestion. Finally, the mouse TIK gene had an anti-viral activity similar to the human PKR, in that expression of TIK inhibited EMCV but not VSV replication.

In addition to homology with the human dsRNA-dependent protein kinase (3, 22) TIK further shares N-terminal homology with several other dsRNA-binding proteins, such as vaccinia virus E3L (28), rotavirus NS34 (29), the tar/RRE binding protein (30,31), and *E.coli* RNase III (32). This region has been shown to be necessary for dsRNA binding of the human dsRNA-dependent protein kinase (22, 3) and for the vaccinia virus E3L-encoded proteins (34). Mouse PKR also contains eleven catalytic domains (21) and a striking nine amino acid region of homology which has been suggested to be involved in eIF-2 recognition (35). The mouse dsRNA-dependent protein kinase sequence further contains the kinase recognition sequence for *cdc2* phosphorylation (Ser/Thr-Pro-X-Arg/Lys) at residues 162–166, specifically Lys-Ser-Pro-Pro-Lys. (36). This kinase recognition sequence is not conserved at the comparable region of the human dsRNA-dependent protein kinase, however, a similar sequence (Lys-Ser-Pro-Glu-Lys) is found C-terminal of the XI catalytic domain (3) of human dsRNA-dependent protein kinase.

It is interesting to note that the TIK cDNA was initially isolated by screening a λgt11 expression library with anti-phosphotyrosine antibodies, and to note that *in vitro* transcribed and translated TIK protein can be detected and immunoprecipitated with three different monoclonal anti-phosphotyrosine antibodies and a polyclonal anti-phosphotyrosine antibody (1). Yet, TIK lacks phosphotyrosyl residues, and under no conditions has this kinase been found to posses a tyrosine phosphorylating ability. It has been suggested that the primary sequence of TIK encodes an epitope resembling phosphotyrosine, and this epitope may represent a binding site for molecules which specifically recognize phosphotyrosyl residues (1). If this is true, the dsRNA-dependent protein kinase may have a yet undefined role in signal transduction and/or cell cycle control.

Transphosphorylation of growth factor receptor tyrosine kinases requires dimerization of the receptor for subsequent signal transduction (37), and mutations in the catalytic sites of receptor tyrosine kinases have dominant negative effects (38). The dsRNA-dependent protein kinase also undergoes dimerization that is phosphorylation-dependent (24) and mutation of the putative eIF-2 recognition region, conserved between the mouse, human and yeast proteins, results in a dominant negative effect on autophosphorylation of this kinase (39). Furthermore, expression of the mutant human dsRNA-protein kinase in NIH 3T3 cells results in transformation of these cells (39), whereas wild type human dsRNA-dependent protein kinase has an inhibitory effect on cell growth (40). Evidence that the human dsRNA-dependent protein kinase may function as a suppressor of cell proliferation and tumorigenesis supports the idea that this protein may share a related function to tyrosine kinases in controlling signal transduction and cell growth.

Note added in proof

While this work was in progress Patel and Sen (*J. Interferon Research* **12**, 389–393, 1992) published work demonstrating that carboxy-terminal TIK domains were functionally equivalent to human PKR domains.

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