The EMBO Journal vol.14 no.15 pp.3828–3834, 1995

Abrogation of translation initiation factor eIF-2 phosphorylation causes malignant transformation of NIH 3T3 cells

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The interferon induced double-stranded RNA-activated kinase, PKR, has been suggested to act as a tumor suppressor since expression of a dominant negative mutant of PKR causes malignant transformation. However, the mechanism of transformation has not been elucidated. PKR phosphorylates translation initiation factor eIF-2α on Ser51, resulting in inhibition of protein synthesis and cell growth arrest. Consequently, it is possible that cell transformation by dominant negative PKR mutants is caused by inhibition of eIF-2α phosphorylation. Here, we demonstrate that in NIH 3T3 cells transformed by the dominant negative PKR mutant (PKRΔ6), eIF-2α phosphorylation is dramatically reduced. Furthermore, expression of a mutant form of eIF-2α, which cannot be phosphorylated on Ser51 also caused malignant transformation of NIH 3T3 cells. These results are consistent with a critical role of phosphorylation of eIF-2α in control of cell proliferation, and indicate that dominant negative PKR mutants transform cells by inhibition of eIF-2α phosphorylation.

Keywords: cell transformation/eIF-2/phosphorylation/PKR/translation initiation

Introduction

Regulation at the level of translation plays an important role in the control of expression of many genes. In eukaryotes, translational control occurs mainly at the level of initiation, which is usually the rate-limiting step in translation (reviewed in Hershey, 1991). The translation initiation factor eIF-2, is a major target for translational regulation under disparate conditions. eIF-2 is a multimeric complex composed of three non-identical subunits (α, β and γ), whose molecular masses are 36, 38 and 52 kDa, respectively. eIF-2 forms a ternary complex with the initiator Met-tRNA$_i$ and GTP, and functions to promote the binding of Met-tRNA$_i$ to the 40S ribosomal subunit. Prior to 60S subunit joining, eIF-2–GTP is hydrolyzed to eIF-2–GDP. eIF-2 is recycled for subsequent rounds of initiation by displacement of GDP by GTP, a process that is catalyzed by eIF-2B or GEF (reviewed in Proud, 1986).

Phosphorylation of the α subunit of eIF-2 on Ser51 results in inhibition of translation, probably by sequestering the limiting factor eIF-2B as an inactive complex (Rowlands *et al.*, 1988b; Hershey, 1991; Ramaiah *et al.*, 1994). Small changes in eIF-2α phosphorylation have dramatic effects on protein synthesis. An increase in phosphorylation of eIF-2α of 5–10% (to 25–30%) is sufficient to cause a strong inhibition of translation in reticulocyte lysates (Pain, 1986). Phosphorylation of eIF-2α plays a major role in the regulation of eukaryotic protein synthesis and has been implicated in numerous translational control mechanisms (Hershey, 1993). Increases in eIF-2α phosphorylation and inhibition of protein synthesis occurs in response to serum starvation (Duncan and Hershey, 1985), heat shock (Scorsone *et al.*, 1987; Duncan and Hershey, 1989), virus infection (O'Malley *et al.*, 1989) and plasmid transfection (Kaufman *et al.*, 1989). Decrease in eIF-2α phosphorylation and stimulation of protein synthesis occur during cell proliferation induced by serum or growth factors (Duncan and Hershey, 1985; Montine and Henshaw, 1989)

Two protein kinases that phosphorylate eIF-2α in mammalian cells have been characterized: a heme-sensitive eIF-2α kinase, referred to as HCR or HRI (Chen *et al.*, 1991), and an interferon-induced, cAMP-independent, serine/threonine protein kinase, designated PKR, also known as P1 kinase, p68 kinase, DAI, dsI (Samuel, 1993). PKR is a double-stranded RNA (dsRNA) dependent protein kinase which manifests two distinct kinase activities: one for autophosphorylation (regulated by dsRNA) and the other for phosphorylation of eIF-2α. PKR plays an important role in cell growth control and differentiation (Petryshyn *et al.*, 1984, 1988; Chong *et al.*, 1992; Lee *et al.*, 1993). Recently, a tumor-suppressor activity for PKR has been invoked, inasmuch as expression of dominant negative mutants of PKR in mouse NIH 3T3 fibroblasts causes malignant transformation (Koromilas *et al.*, 1992; Lengyel, 1993; Meurs *et al.*, 1993). Also, expression of wild-type PKR in yeast causes increased phosphorylation of eIF-2α and results in inhibition of cell growth (Chong *et al.*, 1992; Dever *et al.*, 1993). Furthermore, overexpression of a protein inhibitor of PKR, p58, also transforms NIH 3T3 cells (Barber *et al.*, 1994). The mechanism by which mutant PKR induces transformation is not understood. An attractive hypothesis is that dominant negative PKR mutants down-regulate PKR activity by forming inactive heterodimers. This results in reduction of eIF-2α phosphorylation, enhanced translation and deregulation of cell growth. However, other mechanisms for transformation by PKR have not been excluded. Recently, a member of the I-κB family of inhibitors that control NF-κB activity has been shown to be a PKR substrate *in vitro* (Kumar *et al.*, 1994). Moreover, PKR activity is required in the dsRNA-mediated signalling of NF-κB (Maran *et al.*, 1994).

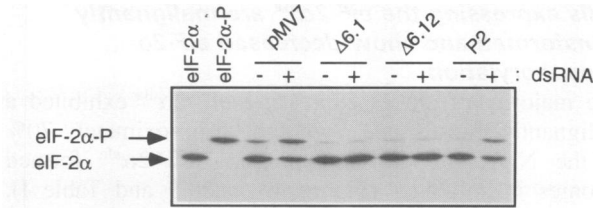

Fig. 1. eIF-2α phosphorylation is inhibited in NIH 3T3 cells expressing the PKRΔ6 mutant. NIH 3T3 cells were grown in the presence or absence of dsRNA (100 μg/ml; polyI:polyC; Pharmacia) for 20 h. Total protein extracts were fractionated by IEF PAGE and subjected to immunoblot analysis using monoclonal antibody to eIF-2α as described in Materials and methods. Lanes marked eIF-2α and eIF-2α-P contain purified eIF-2 that had been incubated in the absence or presence of the heme-regulated eIF-2α kinase to indicate the position of the non-phosphorylated and phosphorylated forms of eIF-2α. Lanes represent the following NIH 3T3 clones: Δ6.1 and Δ6.12 are two different clones of NIH 3T3 cells expressing the PKR-Δ6 mutant; pMV7 represents NIH 3T3 cells transfected with the vector pMV7 alone and P2 is an NIH 3T3 cell line transformed by eIF-4E (Lazaris-Karatzas *et al.*, 1990).

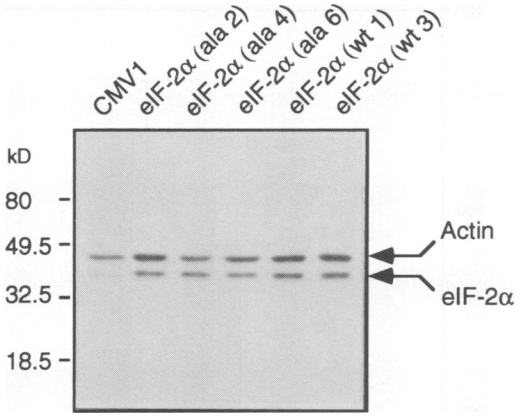

Fig. 2. Expression of human eIF-2α in stably transfected NIH 3T3 cells. NIH 3T3 cells were lysed and eIF-2α was examined in 25 μg of cell extracts by protein immunoblotting with a monoclonal antibody to eIF-2α (Scorsone *et al.*, 1987). The CMV1 lane represents extracts from cells carrying the vector pRc/CMV alone. An antibody against actin (ICN Biomedicals) was used to normalize for loading variability.

There are two important predictions of the model that transformation by PKR is mediated through eIF-2α: (i) eIF-2α phosphorylation should be reduced in cells transformed by dominant negative mutants of PKR and (ii) overexpression of an eIF-2α mutant that cannot be phosphorylated should also cause malignant transformation. To test these predictions, the degree of eIF-2α phosphorylation in NIH 3T3 cells transformed by the trans-dominant negative mutant, PKRΔ6 (Koromilas *et al.*, 1992) was examined. Here, we report that in these cells, eIF-2α phosphorylation is dramatically reduced. Furthermore, we show that expression of the non-phosphorylatable form of human eIF-2α, but not the wild-type eIF-2α, in NIH 3T3 cells inhibits the phosphorylation of endogenous murine eIF-2α and causes malignant transformation.

Results

eIF-2α phosphorylation is dramatically reduced in cells expressing a transdominant negative mutant of PKR

The degree of eIF-2α phosphorylation in NIH 3T3 cells overexpressing a transdominant negative mutant of PKR, PKRΔ6, was examined. This PKR mutant has been inactivated by the deletion of six conserved amino acids in catalytic domain V and has been shown to cause malignant transformation of NIH 3T3 cells (Koromilas *et al.*, 1992). eIF-2α phosphorylation was studied by isoelectric focusing (IEF) followed by immunoblotting (Maurides *et al.*, 1989; Carroll *et al.*, 1993). The two independent clones expressing PKR-Δ6 (1 and 12) examined here showed a strong diminution in the phosphorylation of eIF-2α as compared with control cells expressing the vector pMV7 alone (Figure 1). The decrease was much more pronounced when eIF-2α phosphorylation was compared in dsRNA treated cells: no increase was observed for the cells expressing PKRΔ6 (Figure 1). The reduction of eIF-2α phosphorylation is not a consequence of cell transformation *per se*, since for another transformed cell line (P2: an NIH 3T3 cell line overexpressing eIF-4E: Lazaris-Karatzas *et al.*, 1990), the eIF-2α phosphorylation level upon dsRNA treatment was similar to control cells (compare pMV7 and P2 in Figure 1). Moreover, the interferon treated human adenovirus transformed 293 cell line showed a normal level of eIF-2α phosphorylation (Barber *et al.*, 1995). Inhibition of eIF-2α phosphorylation has also been reported for NIH 3T3 cells transformed by PKR mutants lacking the first RNA binding domain (PKR-M6, PKR-M7; Barber *et al.*, 1995) or by p58 (Barber *et al.*, 1994). These data are consistent with the hypothesis that PKR mutants induce malignant transformation through reduction of eIF-2α phosphorylation. However, they do not rule out the possibility that failure to phosphorylate some other unidentified protein(s) is responsible for the transformation.

Cell lines expressing the human wild-type or mutant eIF-2α

To study directly the role of phosphorylation on Ser51 of eIF-2α in cell growth regulation, a mutated form of human eIF-2α (eIF-2α^{ala}: Ser51 changed to alanine) was stably expressed in NIH 3T3 cells (Ernst *et al.*, 1987; Pathak *et al.*, 1988). When expressed transiently in COS cells, eIF-2α^{ala} is incorporated into the eIF-2 complex, and it mitigates the inhibitory effect of PKR on translation by reducing the extent of phosphorylation of eIF-2α (Kaufman *et al.*, 1989; Choi *et al.*, 1992). We stably transfected NIH 3T3 cells with plasmids containing cDNAs encoding the human wild-type and mutant eIF-2α under the control of the cytomegalovirus (CMV) promoter. Transfection with the vector alone (pRc/CMV) was used as a control. G418-resistant cells expressing eIF-2α^{ala} (12 clones) and wild-type eIF-2α (eight clones) were isolated and characterized. The level of expression of the human eIF-2α protein in different G418-resistant NIH 3T3 clones was determined by Western blotting. The amount of eIF-2α in cells transfected with pRC/CMV-eIF-2α^{ala} or pRC/CMV-eIF-2α^{wt} was increased ~2-fold as compared with a cell line carrying the vector pRc/CMV alone (normalized against actin; Figure 2).

Transient transfection of COS cells results in the activation of PKR and specific translational inhibition of the transfected plasmid (Kaufman *et al.*, 1989). Expression of

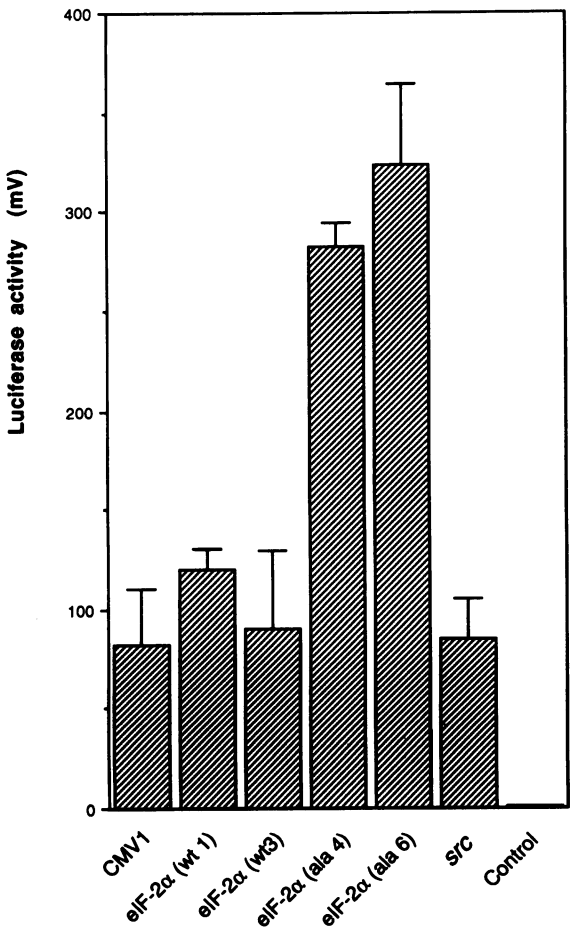

Fig. 3. Translational enhancement in NIH 3T3 cells expressing eIF-2α^{ala}. Cells were transfected with plasmid pcDNA3-luc. Luciferase activity was measured 48–72 h after transfection in mV in a luminometer (Bioorbit) using the luciferase assay system (Promega). The experiments were carried out three times and the error bars indicate the standard deviation from the mean. The luciferase activities shown are normalized to the amount of mRNA using an RNase protection assay. *src* and control indicate NIH 3T3 cells expressing v-*src* transfected with pcDNA3-luciferase plasmid and NIH 3T3 cells expressing eIF-2α^{ala} which were transfected with no DNA, respectively.

the eIF-2α^{ala} mutant in COS (Kaufman *et al.*, 1989; Choi *et al.*, 1992), human 293 (Davies *et al.*, 1989) or CHO cells (Murtha-Riel *et al.*, 1993) and in yeast (Chong *et al.*, 1992), reversed the translational inhibitory effect of PKR. It was anticipated therefore, that translation from a transiently transfected plasmid would be enhanced in eIF-2α^{ala} expressing NIH 3T3 cells. Two cell lines (ala4 and 6) expressing eIF-2α^{ala} were transfected with the luciferase gene under the control of the CMV promoter. In these cells, luciferase mRNA was translated 3- to 4-fold better as compared with its translation in cells expressing wild-type eIF-2α (wt 1 and 3) or the vector alone (CMV1) (Figure 3). The observed increase was post-transcriptional since luciferase activities were normalized to the amount of mRNA using an RNase protection assay (data not shown). The observed effect is not likely to be a consequence of transformation since no increase in luciferase expression was observed when the plasmid was transfected in NIH 3T3 cells transformed by v-*src* (Figure 3) (Frederickson *et al.*, 1991).

Cells expressing the eIF-2α^{ala} are malignantly transformed and show decreased eIF-2α phosphorylation

The majority of clones expressing eIF-2α^{ala} exhibited a malignantly transformed phenotype. Approximately 70% of the NIH 3T3 clones expressing eIF-2α^{ala} formed colonies in soft agar (Figure 4, panel F and Table I), whereas no colonies were observed in cells transfected with eIF-2α^{wt} (Figure 4, panel E) or the vector alone (Table I). Cells expressing eIF-2α^{ala} exhibited other hallmarks of a transformation phenotype, including increased refractility (panel B), high saturation density (Table I) and the formation of foci on a monolayer of cells (panel D). In contrast, cells transfected with eIF-2α^{wt} (panel A) or with the vector alone (data not shown) exhibited a flat morphology, indistinguishable from parental NIH 3T3 cells. In addition, cells overexpressing the mutant eIF-2α grew faster (~20%) than wild-type eIF-2α overexpressing cells or control cells. Tumorigenicity was tested by injection into athymic nude mice. All nude mice (n = 24) injected with cells from four independent eIF-2α^{ala}-expressing cell lines developed tumors (>1 cm) within 30 days. Tumors were not observed in mice (n = 24) injected with cells carrying the wild-type eIF-2α or the vector alone (except for two tumors out of 12 mice arising 45 and 50 days after injection with cells from two wild-type clones).

The expression of a non-phosphorylatable mutant of eIF-2α is expected to cause a decrease in the extent of phosphorylation of endogenous eIF-2α as was observed for clones overexpressing the inactive form of PKR (Figure 1), and in COS cells overexpressing the eIF-2α mutant (Kaufman *et al.*, 1989; Choi *et al.*, 1992). The extent of eIF-2α phosphorylation was monitored by IEF (Carroll *et al.*, 1993). In NIH 3T3 cells expressing the vector alone or eIF-2α^{wt}, phosphorylation of eIF-2α was increased after treatment with dsRNA (Figure 5: lanes CMV1, WT1 and 5). In contrast, cells expressing the eIF-2α^{ala} mutant showed only a slight increase in phosphorylation upon dsRNA treatment (lanes ala 1, 2 and 6): a 3- to 7-fold reduction in phosphorylation of eIF-2α was observed. Taken together, our results indicate that transformation of NIH 3T3 and stimulation of reporter protein synthesis by eIF-2α^{ala} are readily explained by the failure of eIF-2α to become phosphorylated.

Discussion

This study provides evidence that regulation of cell growth by PKR is mediated through eIF-2α and thus by translation initiation. The role of eIF-2α as reported in this study is consistent with numerous earlier studies demonstrating a correlation between increased cell growth and decreased eIF-2α phosphorylation (Duncan and Hershey, 1985; Rowlands *et al.*, 1988a; Ito *et al.*, 1994; Redpath and Proud, 1994). The importance of eIF-2α in the control of cell growth is also reinforced by its regulation at the transcriptional and post-transcriptional levels during changes in cell growth: the level of eIF-2α mRNA is increased following c-*myc* activation (Rosenwald *et al.*, 1993) and in proliferating human T lymphocytes (Cohen *et al.*, 1990).

Inhibition of eIF-2α phosphorylation also occurs in

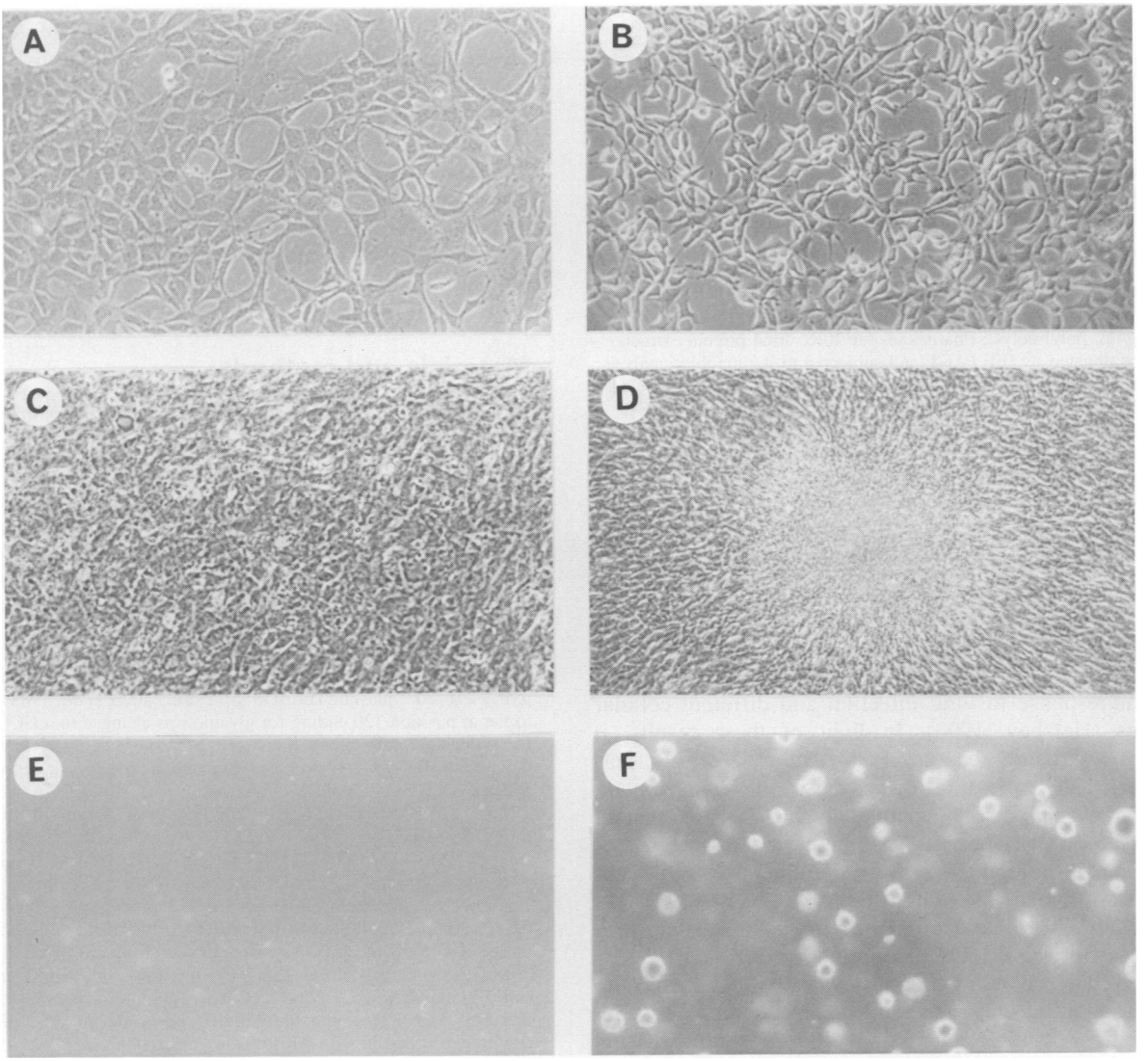

Fig. 4. Morphological characteristics of eIF-2α-expressing cells. NIH 3T3-eIF-2α^{wt} (A, C and E) and NIH 3T3-eIF-2α^{ala} cells (B, D and F) were plated at 1×10^5 cells per 100 mm dish in DMEM supplemented with 10% fetal bovine serum and G418 (250 μg/ml). (**A** and **B**) Cells in exponential growth. (**C** and **D**) Cells maintained in culture for 5 days after reaching confluency. (**E** and **F**) Anchorage-independent growth of cells in soft agar.

Table I. Growth properties of cells expressing eIF-2α

Clone	Doubling time (h)[a]	Saturation density (cells $\times10^6$)[a]	Cloning efficiency (%)[b]	Animals with tumors/ animals injected[c]	Latency (days)
NIH 3T3 pRc/CMV-1	24.5 ± 0.7	3.5 ± 0.4	0	0/6	–
NIH 3T3 pRc/CMV eIF-2α^{wt}-1	23.6 ± 1.3	3.4 ± 0.5	0	1/6	45
NIH 3T3 pRc/CMV eIF-2α^{wt}-3	23.8 ± 1.2	2.9 ± 0.4	0	0/6	–
NIH 3T3 pRc/CMV eIF-2α^{wt}-5	23.3 ± 0.9	3.1 ± 0.4	0	1/6	50
NIH 3T3 pRc/CMV eIF-2α^{ala}-1	19.7 ± 0.9	6.2 ± 0.5	26	6/6	27–30
NIH 3T3 pRc/CMV eIF-2α^{ala}-2	18.6 ± 2.2	6.1 ± 0.7	29	6/6	26–28
NIH 3T3 pRc/CMV eIF-2α^{ala}-4	18.8 ± 1.8	5.5 ± 0.2	22	6/6	24–28
NIH 3T3 pRc/CMV eIF-2α^{ala}-6	18.8 ± 1.2	5.0 ± 0.6	27	6/6	23–25

[a]Cells were seeded at 10^5 cells per 60 mm dish and grew in DMEM supplemented with 10% fetal bovine serum (FBS) and G418 (250 μg/ml). Cells were counted every 24 h. Saturation density measures the number of cells in culture 3–4 days after reaching confluency. Numbers are the average of three experiments ± the standard deviation.

[b]Cells (1×10^4) were suspended in 0.35% agar solution in DMEM containing 20% FBS, and overlaid onto 0.5% agar solution in DMEM containing 20% FBS in 35 mm plates. Colonies were scored 2 weeks after plating. Cloning efficiency is calculated as the number of colonies ×100 divided by the number of cells plated. Numbers are the average of three experiments.

[c]Nude mice [4–6 weeks old; CD1/CD1 (Charles River)] were injected subcutaneously on the left side of the lower limb with 1×10^6 cells resuspended in 100 μl of PBS. The time required to produce tumors of 3 mm diameter was considered as the latency period. Tumor formation was monitored for up to 8 weeks.

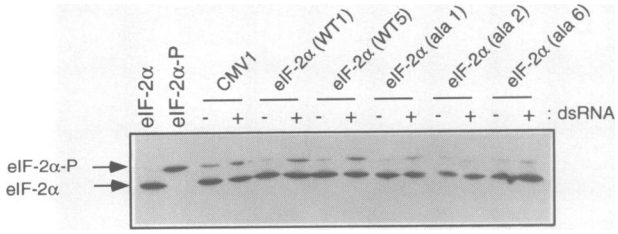

Fig. 5. Overexpression of mutant eIF-2α in NIH 3T3 cells results in diminished eIF-2α phosphorylation. Cells expressing eIF-2α^{wt} and eIF-2α^{ala} were grown in the presence or absence of dsRNA (100 μg/ml; polyI:polyC; Pharmacia) for 20 h. Total protein extracts were fractionated by IEF PAGE and subjected to immunoblot analysis using a monoclonal antibody to eIF-2α as described in Materials and methods. Lanes marked eIF-2α and eIF-2α-P contain purified eIF-2 that had been incubated in the absence or presence of the heme-regulated eIF-2α kinase to indicate the position of the non-phosphorylated and phosphorylated forms of eIF-2α. CMV1 lane indicates NIH 3T3 cells containing the vector alone.

NIH 3T3 cells transformed by p58, or by PKR mutants lacking the first dsRNA binding domain (Barber *et al.*, 1994, 1995). Taken together with our studies, these data indicate that PKR functions through eIF-2α phosphorylation to effect cell growth, in a similar manner to the cellular response to viral infection and different cellular stresses (Hershey, 1993). In light of these results, it is then surprising that phosphorylation of eIF-2α was unaffected in cells transformed by a different mutant allele, the catalytically inactive domain II PKR mutant (Meurs *et al.*, 1992). It was therefore suggested that PKR can also regulate cell proliferation through other mechanisms, e.g. involving NF-κB (Clemens, 1992). In this regard, it is interesting to note the different growth properties of the PKRΔ6 (Koromilas *et al.*, 1992), PKR-M6 and PKR-M7 (Barber *et al.*, 1995), p58 (Barber *et al.*, 1994) and eIF-2α^{ala} expressing cells as compared with the domain II PKR expressing NIH 3T3 cells (Meurs *et al.*, 1993): the latter cells do not exhibit a transformed phenotype in culture (morphological change, increase in cell density and growth in soft agar), although they are tumorigenic in nude mice.

eIF-2α is the second eukaryotic initiation factor whose deregulation of function causes transformation. The cap binding protein, eIF-4E, which also plays a central role in regulation of cell growth, transforms cells when over-expressed (Lazaris-Karatzas *et al.*, 1990). It has been proposed that transformation by eIF-4E is the consequence of translational derepression of mRNAs that play a role in regulating cell proliferation. A large proportion of these mRNAs, such as those encoding proto-oncogenes, contains long structured 5′UTRs and are therefore inefficiently translated (Sonenberg, 1993). According to the Lodish model for translation initiation, a general down-regulation of translation mediated by eIF-2α phosphorylation would preferentially affect the mRNAs that are poorly translated. Conversely, non-specific stimulation of translation is expected to preferentially increase the translation of weak mRNAs (Lodish, 1974). An alternative mechanism to explain the transformed phenotype in NIH 3T3 cells expressing eIF-2α^{ala} is that eIF-2α phosphorylation up-regulates the translation of mRNAs encoding growth suppressor proteins by a mechanism similar to that operating for yeast GCN4 mRNA. GCN4 mRNA contains four open reading frames in its 5′UTR and phosphorylation of eIF-2α engenders a specific increase in translation of GCN4 mRNA through a mecanism involving ribosome re-initiation (Abastado *et al.*, 1991; Dever *et al.*, 1992). If such a mechanism operates in mammalian cells, then a decrease in phosphorylation of eIF-2α would result in lower amounts of growth suppressor proteins. Thus, translational control of mRNAs that encode growth regulating proteins could be an attractive mechanism by which eIF-2 regulates cell growth.

Materials and methods

Cell culture and production of eIF-2α overexpressing cell lines

NIH 3T3 cells (ATCC) were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum (FCS; GIBCO). Cells were transfected by calcium phosphate precipitation using 15 μg of pRc/CMV plasmid. G418-resistant colonies were isolated after 10 days of selection in medium containing 500 μg/ml of G418. Cells expressing the neomycin resistance gene were maintained in medium containing 10% FCS supplemented with 250 μg/ml of G418 (Geneticin: GIBCO).

Plasmid construction and site directed mutagenesis

We found one nucleotide change from the original sequence in the pSP72-eIF-2α^{ala} plasmid (Ernst *et al.*, 1987; Pathak *et al.*, 1988): a GGG triplet at position 120 coding for glycine was changed to a GCG coding for alanine. We generated a new eIF-2α^{ala} by PCR using four oligos; the 5′ and 3′ oligos contain a *Bsu*36I site and a *Bbv*II site, respectively: 5′-CAAATTTCCTGAGGTGGAAGA-3′ and 5′-TTTGTGAATTTGTC-TTCACATTTGATTG-CTTCC-3′ and the oligos carrying the ala mutation: 5′-ATTCTTCTTAGT-GAATTGGCCAGAAGGCGTATCCGT-3′ and 5′-ACGGATACG-CCTTCTGGCCAATTCACTAAGAAGAAT-3′. The mutation as well as the sequence of the PCR fragment were confirmed by sequencing. The amplified fragment was subcloned into the *Bsu*36I and *Bbv*II sites of pSP65-eIF-2α^{wt} (Pathak *et al.*, 1988).

The eIF-2αwt and ala cDNAs were excised from pSP65-eIF-2α (Ernst *et al.*, 1987; Pathak *et al.*, 1988) by digestion with *Eco*RI and blunt ended with Klenow polymerase. After gel purification, the 1.6 kb fragment was ligated into the *Hin*dIII site of the plasmid pRc/CMV (Invitrogen) filled in with Klenow polymerase.

Analysis of eIF-2α phosphorylation by IEF

Protein samples from NIH 3T3 cells expressing PKRΔ6, eIF-2α or from control cells were subjected to IEF essentially as described (Maurides *et al.*, 1989). Cells were lysed in 20 mM HEPES, pH 7.2, 5 mM EDTA, 100 mM KCl, 0.05% SDS, 0.5% Elugent (Calbiochem), 10% glycerol, 20 μg/ml chymostatin, 0.5 mM microcystin and 1 mM DTT. The 10 000 *g* supernatant was clarified with BPA-1000 (Toso-Haas, Philadelphia) and eIF-2α was immunopurified using monoclonal antibodies (Scorsone *et al.*, 1987) bound to Unisyn's Avidchrom Mab orientation beads, solubilized in IEF sample buffer and subjected to IEF using a narrow pH range of 4.5–6.1, in the presence of 9 M urea, 50 mM dithiothreitol and 2% CHAPS (Fluka). Proteins were electrophoresed at 2 mA/gel for 16 h, using reverse polarity, with 0.01 M glutamic acid at the anode and 0.05 M histidine at the cathode. Proteins were transferred to Immobilon-P at 60 V for 1 h in 250 mM glycine, 20 mM Tris, 20% (v/v) methanol 0.01% SDS. After transfer, the blots were blocked with BLOTTO: 5% non-fat dried milk, 0.01% antifoam A (Sigma), 0.01% sodium azide in 50 mM Tris–HCl, pH 7.4, 200 mM NaCl and processed as described (Carroll *et al.*, 1993), using affinity-purified polyclonal antibodies against eIF-2α (Maurides *et al.*, 1989).

Luciferase assay

Cells were transfected with 10 μg of pcDNA3-luciferase plasmid obtained by inserting a *Bam*HI–*Xho*I fragment from pGEMluciferase (Promega) into the pcDNA3 plasmid (Invitrogen). Fresh medium was added to cells 18 h after transfection. Cell extracts were prepared 48 h later and luciferase assays were done according to the manufacturer's instructions (Promega). To assess the level of luciferase mRNA, total RNA from transfected cells was isolated by the guanidinium/CsCl method as described (Sambrook *et al.*, 1989). RNA was digested with RNase-free-

DNase I (Boehringer Mannheim) and analysed using 'RNase One' protection assay according to the manufacturer's instructions (Promega).

Protein analysis

Cells (5×10^6) were washed twice with cold phosphate-buffered saline (140 mM NaCl, 15 mM KH_2PO_4, pH 7.2, and 2.7 mM KCl), and incubated on ice with 500 μl of lysis buffer [10 mM Tris–HCl, pH 7.5; 1% Triton X-100, 50 mM KCl, 1 mM dithiothreitol (DTT), 2 mM $MgCl_2$, 0.2 mM phenylmethylsulfonylfluoride and 0.2 mg/ml aprotinin]. The lysate was centrifuged at 10 000 *g* for 5 min and aliquots containing equal amounts of protein (determined by Bradford) were electrophoresed on an SDS–polyacrylamide gel (12.5%). Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) in 25 mM Tris–HCl, pH 7.5, 190 mM glycine, and 20% (v/v) methanol for 1 h at 100 V. Filters were incubated with 5% (w/v) non-fat dried skimmed milk powder in PBS and 0.2% Tween-20 for 30 min at room temperature and then with PBS plus 0.2% Tween-20, 2% BSA containing a mouse monoclonal antibody to eIF-2α (Scorsone *et al.*, 1987) and anti-actin sera (ICN Biomedicals) for 1 h. After washings with PBS and 0.2% Tween-20, the filter was incubated for 1 h with 0.1 mCi of ^{125}I-labeled goat antibody against mouse immunoglobulin G (New England Nuclear). After washings the membrane was dried and exposed to an X-ray film for 4–6 h.

Cell growth, soft agar and tumorigenicity assays

Assays were done as described in Table I.

Acknowledgements

We are grateful to Olga Savinova for technical assistance on the analysis of eIF-2α phosphorylation. We thank L.M.O'Brien for the antibodies to eIF-2α, G.Cosentino, A.Craig and S.Mader for advice and comments on the manuscript and V.Adkar for technical assistance. Supported by a grant from the National Cancer Institute of Canada with funds from the Canadian Cancer Society to N.S., by NIH grant GM22135 to J.W.B.H., by NSF grant MCB9317264 to R.J. and by a scholarship from the American Foundation for AIDS Research (AmFAR) for A.E.K. O.D. was a recipient of a post-doctoral fellowship from the Swiss National Science Foundation.

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Received on March 16, 1995; revised on May 15, 1995