The double-stranded (ds) RNA-dependent protein kinase (PKR) regulates protein synthesis by phosphorylating the α subunit of eukaryotic initiation factor-2 (eIF2α). PKR is activated by viral induced dsRNA and thought to be involved in the host antiviral defense mechanism. PKR is also activated by various nonviral stresses such as growth factor deprivation, although the mechanism is unknown. By screening a mouse cDNA expression library, we have identified an ubiquitously expressed PKR-associated protein, RAX. RAX has a high sequence homology to human PACT, which activates PKR in the absence of dsRNA. Although RAX also can directly activate PKR in vitro, overexpression of RAX does not induce PKR activation or inhibit growth of interleukin-3 (IL-3)-dependent cells in the presence of IL-3. However, IL-3 deprivation as well as diverse cell stress treatments including arsenite, thapsigargin, and H$_2$O$_2$, which are known to inhibit protein synthesis, induce the rapid phosphorylation of RAX followed by RAX-PKR association and activation of PKR. Therefore, cellular RAX may be a stress-activated, physiologic activator of PKR that couples transmembrane stress signals and protein synthesis.

Eukaryotic cells rapidly and reversibly halt protein synthesis in response to a variety of stresses including virus infection and cytotoxic chemical injury (1, 2). This fundamental homeostatic mechanism is thought to involve the phosphorylation of the α subunit of eukaryotic initiation factor-2 (eIF2α), which regulates protein synthesis rate at translational initiation. Phosphorylation of eIF2α increases the stability of complexes formed between eIF2 and eIF2B, a guanine-nucleotide exchange factor. eIF2B converts eIF2-GDP to eIF2-GTP binary complex, which further forms a ternary complex with a Met-tRNA and becomes associated with the 40 S ribosomal subunit to initiate translation of mRNA. Because eIF2B exists in cells in relatively low molar quantities with respect to eIF2, phosphorylation of only a limited amount (i.e. 20–25%) of eIF2α is apparently sufficient to sequester virtually all of the eIF2α, resulting in inhibition of protein synthesis (3–5). The interferon-inducible double-stranded (ds) RNA-dependent kinase (PKR) is an ubiquitously expressed eIF2α kinase, which was first identified as a component of the host defense mechanism induced by interferon (5). In vitro, PKR can be activated by synthetic dsRNA, such as poly(I)·poly(C), and natural dsRNA forms, such as reovirus genomic RNA (5). In interferon-treated cells, virus infection leads to activation of PKR by autophosphorylation, followed by eIF2α phosphorylation and inhibition of protein synthesis (5, 6). Thus, protein synthesis inhibition occurring after virus infection is thought to be due to the direct activation of PKR by dsRNA species. However, the mechanism of eIF2α phosphorylation in the absence of infection is not clear.

We previously reported that in interleukin-3 (IL-3)-dependent cells, IL-3 deprivation induced activation of PKR in close association with a decreased rate of total protein synthesis (7). Recently, it has been reported that stresses such as Ca$^{2+}$ depletion from the endoplasmic reticulum, sodium arsenite, or hydrogen peroxide treatments rapidly induce PKR activation (8–10). It was suggested that although PKR is activated by dsRNA in vitro, a dsRNA-independent activation mechanism may exist in cells because it is difficult to envision that such diverse stresses are likely to rapidly change the intracellular levels of dsRNA species. Thus a novel cellular regulator of PKR was sought. By screening a mouse cDNA library using the yeast two-hybrid interacting cloning system, we discovered a PKR-associating protein RAX. RAX appears to be a mouse homologue of PACT, a recently isolated direct activator for PKR (11). We now find that RAX can activate PKR in the absence of dsRNA in a cell-free system as reported for PACT. However, results here indicate that the RAX-PKR association and any resulting cellular activation of PKR may be regulated by an unique, stress-induced signaling mechanism featuring RAX phosphorylation.

**MATERIALS AND METHODS**

**Cloning of RAX**—Using polymerase chain reaction-based mutagenesis, the mouse PKR cDNA (12) was first mutated at lysine 271 to arginine to generate the catalytically inactive PKR(K271R), the mouse equivalent to human PKR(K296R) mutant (13), and then subcloned into pGBT9 (CLONTECH). A random primed NFS/N1.H7 cell cDNA library was generated in pGAD10 (CLONTECH). The yeast two-hybrid screening of the cDNA library was performed according to the Matchmaker Two-Hybrid System protocol (CLONTECH).

**Northern Blot Analysis**—A 405-base pair mouse RAX cDNA fragment (nucleic acid positions 943–1348) was amplified by polymerase chain reaction and radiolabeled using the Prime-A-Probe kit (Ambion, Inc., Austin, TX). A mouse multiple tissue blot was obtained from CLONTECH, and hybridization was performed according to the manufacturer’s instruction.

**Bacterial Expression of RAX and Antibody Production**—The RAX cDNA was cloned into pRSET vector (Invitrogen) to generate a polyhistidine-tagged RAX and used to transform Escherichia coli BL21 (DE3) pLysS (Novagen, Madison, WI). The protein was induced and partially purified with TALON metal affinity resin according to the manufacturer’s instruction (CLONTECH). The fraction containing RAX was incubated with polycl agarose beads (Amersham Pharmacia Biotech),
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FIG. 1. RAX is a ubiquitously expressed protein with dsRNA-binding motifs. A, amino acid sequence of RAX aligned with that of human PACT. Only those amino acids that differ in the two proteins are indicated for PACT. The three dsRNA-binding motifs (bold letters) are aligned with that of human PACT. Only motifs.

and the protein was dissociated by boiling in SDS-PAGE sample buffer and further purified by SDS-PAGE. The gel slice containing the recombinant RAX was used to immunize a rabbit to raise anti-RAX antiserum (COVANCE Research Products Inc., Denver, PA).

Mammalian Expression of RAX—The murine IL-3-dependent NFS/N1.H7 cells (14) were maintained in RPMI 1640 medium supplemented with 20% WEHI-3B cell conditioned medium as described previously (7). The hemagglutinin (HA) epitope-tagged RAX cDNA was subcloned into the expression vector pcDEF3 (15) and transfected into NFS/N1.H7 cells by electroporation. The clones stably expressing HA-RAX were selected as described previously (16).

Immunoprecipitation and Immunoblotting—Cells (1 x 107) were treated with 1 mM sodium arsenite (Sigma), or 10 μM hydrogen peroxide (Poly C), and bound proteins were analyzed by SDS-PAGE as described under “Materials and Methods.” C. Northern blot analysis of poly(A) RNA harvested from various mouse tissues. D, protein samples (100 μg) from various cell lines were subjected to SDS-PAGE and analyzed by immunoblotting using anti-RAX antisemur. RAX/COS7, COS7 transiently transfected with RAX cDNA; Pre, probed with pre-immune serum.

A

| RAX | MSRSRHRAEAPPLQREDSTFSLGKMITAKPQGT |
|-----|-------------------------------------|
| PACT | Q-----E                              |
| PIVLHETG | MKTNFIVYECSERDSQHPHPFTTVGDTCTCGGTSKKLAKHRRAEANIL |
| PACT | ----W-----                  |
| PIGSLGELAHIHGWRLFEYTTLQSGGOPARKKRTTCRCLSEPMTGKGASKQAKRNAAEFPFLAKF |
| PACT | -----E-----                  |
| SNISPENHSITVNGHSLCTWSHLRNSPGKINLKLRSILLSPND |
| PACT | --K-----D----                  |
| YIQLESEAEQGFHITLDIELERSLANGQYQLAEULTSPISTVCHGSISGMAQLDANNLQYL |
| PACT | ----E-----                  |
| KIAABR |                               |
| PACT | ----E-----                  |

B

| pH | g |
|----|---|
| 7.2 | 5 |
| 7.6 | 2 |

C

| protein | brain | heart | lung | spleen | liver | muscle | kidney | testis |
|---------|-------|-------|------|--------|-------|--------|--------|--------|

D

| protein | NFS/N1.H7 | COS7 |
|---------|------------|------|
| Hela | M-2 | DC |

Pre

Anti-RAX

kD

45
33

After treatment, cells (3 x 106) were lysed in 100 μl of buffer A. The acetone-precipitated extract (100 μg) was subjected to the isoelectric focusing and immunoblotting using ECL kit (Amersham Pharmacia Biotech) and a rabbit eIF2α polyclonal antibody raised against a KLH-coupled synthetic peptide corresponding to amino acid residues 298–315 of human eIF2α. For analysis of RAX phosphorylation, Bio-lyte 6/8 (Bio-Rad) and 10 mM ethylenediamine (Sigma) were used for the amphotolys and the cathode buffer, respectively. Before transfer, the gel was treated with 0.1 M Tris-HCl, pH 6.8, 2% SDS, and 10 mM diethothreitol for 30 min at room temperature to enhance the elution efficiency.

In Vitro Kinase Assays—PKR was freshly isolated from the exponentially growing NFS/N1.H7 cells by immunopurification using anti-PKR antibody (Ab-2) (7, 19). PKR was recovered by competitive elution with the synthetic peptide (7). The HA-RAX was immunoprecipitated with anti-HA antibody as described above and was recovered from the immunocomplex by competitive elution with 1 mg/ml HA peptide (Roche Molecular Biochemicals). After dialysis against Tris-buffered saline, the purified HA-RAX was quantitated by immunoblotting with bacterially expressed RAX as a reference. Samples were incubated for 10 min at 30 °C in 50 μl of reaction buffer (25 mM Tris-HCl, pH 7.6, 2 mM MgCl2, 2 mM MnCl2, 1 mM dithiothreitol, 20 μM ATP, 5 μCi of [γ-32P]ATP, 0.1 μg of rabbit reticulocyte eIF2α). Proteins were separated by SDS-PAGE (10%), and 32P incorporation was quantitated by autoradiography or directly measured by InstantImagerTM (Packard, Meriden, CT).

DNA Fragmentation Assay—Cells (1.5 x 106) were washed twice with Tris-buffered saline and lysed in 100 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 0.5% Triton X-100). The lysate was centrifuged at 13,000 rpm for 10 min, and the supernatant was treated with 1 μg of RNase A and 40 μg of proteinase K for 2 h at 37 °C, respectively. The DNA was precipitated with 0.5 M NaCl and 50% isopropanol at −70 °C and analyzed by electrophoresis in a 2% agarose gel containing 10 μl/ml ethidium bromide.
RESULTS

To identify novel cellular proteins that could potentially interact with and modulate PKR, we screened a mouse IL-3-dependent NFS/N1.H7 cell cDNA library using the yeast two-hybrid interactive cloning system (20). As bait, the mouse PKR(K271R), which is deficient in kinase activity (13), was used because wild type PKR inhibits yeast growth (21). From $4 \times 10^6$ yeast transformants screened, eight positive clones were obtained. The sequence analysis revealed that six clones are derived from the same novel gene. Clone c35 contained the longest (1080 base pairs) cDNA and encoded a putative 313-amino acid polypeptide terminated by TAG stop codon (Fig. 1A). This gene is referred to as RAX (PKR-associated protein X). Further 5' and 3' rapid amplification of cDNA ends studies using poly(A) RNA from NFS/N1.H7 cells showed that full-length RAX cDNA is 1.6 kilobases. Computer search (BLAST) analysis indicated that both cDNA and amino acid sequences of RAX are highly homologous to those of human PACT (95 and 98% identity, respectively), a recently identified PKR-associated protein (11) (Fig. 1A). The striking similarity suggests that RAX is a murine counterpart of PACT. RAX also has significant homology to two other dsRNA-binding proteins, including a Xenopus dsRNA-binding protein Xlrpba (22) (69% homology) and the mammalian TAR-RNA-binding protein (23) and its murine counterpart, Prbp (24) (60% homology). As featured in these proteins, RAX also contains three dsRNA-binding motifs arranged in tandem whose function is not yet clear (25) (Fig. 1A). As predicted, recombinant RAX was able to specifically bind dsRNA because virtually no binding to DNA or single-stranded RNA was observed (Fig. 1B).

Northern blot analysis of poly(A) RNA prepared from mouse tissues showed that RAX mRNA is expressed in all tissues tested (Fig. 1C). Furthermore, an polyclonal antibody raised against recombinant RAX recognized a 35-kDa protein in the lysates from cell lines derived from different species including mouse NIH3T3, IL-3-dependent NFS/N1.H7, rat pheochromocytoma PC-12, and monkey COS7 cells (Fig. 1D). The migration at the 35-kDa position on SDS-PAGE gel corresponds well with a calculated molecular mass of RAX (34 kDa). Importantly, an increased signal was observed in COS7 cells after transfection of RAX cDNA, indicating that the 35-kDa immunoreactive protein is RAX. The 35-kDa band was detected in human HeLa and MCF-7 cell lysates, indicating that the antibody can react with human PACT (Fig. 1D). These results indicate that RAX/PACT is an ubiquitously expressed and well conserved gene between the species.

To investigate the biological function of RAX, we first transfected HA epitope-tagged RAX into IL-3-dependent NFS/N1.H7 cells (14) and obtained more than 10 clones that stably express RAX, an Activator for PKR

mouse NIH3T3, IL-3-dependent NFS/N1.H7, rat pheochromocytoma PC-12, and monkey COS7 cells (Fig. 1D). The migration at the 35-kDa position on SDS-PAGE gel corresponds well with a calculated molecular mass of RAX (34 kDa). Importantly, an increased signal was observed in COS7 cells after transfection of RAX cDNA, indicating that the 35-kDa immunoreactive protein is RAX. The 35-kDa band was detected in human HeLa and MCF-7 cell lysates, indicating that the antibody can react with human PACT (Fig. 1D). These results indicate that RAX/PACT is an ubiquitously expressed and well conserved gene between the species.

Results from the yeast two-hybrid assay suggest that RAX can bind to PKR. To examine the effects of RAX on PKR activity, an in vitro reconstitution kinase assay was developed using proteins isolated from mammalian cells. HA-RAX was immunopurified from exponentially growing cells (clone 13; Fig. 2). The purified HA-RAX contained no associated kinase activity, indicating that any potentially associated PKR was removed during purification (Fig. 3A, first lane). PKR was purified separately from exponentially growing parental NFS/N1.H7 cells. We found that HA-RAX could potently activate PKR(K271R), which is deficient in kinase activity (13), was used because wild type PKR inhibits yeast growth (21). From $4 \times 10^6$ yeast transformants screened, eight positive clones were obtained. The sequence analysis revealed that six clones are derived from the same novel gene. Clone c35 contained the longest (1080 base pairs) cDNA and encoded a putative 313-amino acid polypeptide terminated by TAG stop codon (Fig. 1A). This gene is referred to as RAX (PKR-associated protein X). Further 5' and 3' rapid amplification of cDNA ends studies using poly(A) RNA from NFS/N1.H7 cells showed that full-length RAX cDNA is 1.6 kilobases. Computer search (BLAST) analysis indicated that both cDNA and amino acid sequences of RAX are highly homologous to those of human PACT (95 and 98% identity, respectively), a recently identified PKR-associated protein (11) (Fig. 1A). The striking similarity suggests that RAX is a murine counterpart of PACT. RAX also has significant homology to two other dsRNA-binding proteins, including a Xenopus dsRNA-binding protein Xlrpba (22) (69% homology) and the mammalian TAR-RNA-binding protein (23) and its murine counterpart, Prbp (24) (60% homology). As featured in these proteins, RAX also contains three dsRNA-binding motifs arranged in tandem whose function is not yet clear (25) (Fig. 1A). As predicted, recombinant RAX was able to specifically bind dsRNA because virtually no binding to DNA or single-stranded RNA was observed (Fig. 1B).

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PKR as indicated by eIF2α phosphorylation and PKR auto-phosphorylation in the absence of dsRNA (Fig. 3A). Furthermore, if suboptimum amounts of dsRNA are added to the reaction, RAX synergistically activates PKR (Fig. 3B). Thus, we find HA-RAX purified from growing cells can activate PKR in vitro. However, overexpression of HA-RAX apparently does not affect the growth of NFS/n1.7 cells in the presence of IL-3, suggesting that the overexpressed RAX in vivo may not be activating cellular PKR efficiently under the growing condition. In support of this notion, co-immunoprecipitation studies using anti-PKR and anti-HA reveal that the RAX–PKR association is barely detected in exponentially growing cells even when highly expressed as in the case with clone 13 (i.e. expresses approximately 5–10-fold more than the endogenous level) (Fig. 4A). However, an increase in the association occurs when the cells are deprived of IL-3 for 2.5 h, a time by which protein synthesis is significantly inhibited by PKR activation (7). Moreover, more dramatic and rapid RAX–PKR association is observed when the cells are exposed to various chemical stresses including sodium arsenite (As), H2O2, or thapsigargin (TG), an inhibitor of endoplasmic reticular Ca2+-ATPase (Fig. 4A), agents known to potently inhibit protein synthesis (1, 10). Importantly, the total amount of HA-RAX in the cell lysate is not affected by any of these stress treatments, confirming that any increased association is not due to increased RAX expression (Fig. 4A, lower panel). Furthermore, as previously reported (2, 7, 26), the stress treatments were also found to induce phosphorylation of eIF2α, clearly indicating that PKR, its physiologic kinase, has been activated (Fig. 4B). Significantly, any eIF2α phosphorylation cannot be detected in growing transfected cells, indicating that the simple overexpression of exogenous RAX is not sufficient to activate PKR and inhibit growth. These results suggest that RAX can directly bind to and activate PKR following application of stress conditions. The rapid RAX–PKR association observed after the stress treatments raised the possibility that RAX may be post-translationally modified as a result of any stress signal evoked by the treatments. Isoelectric focusing analysis revealed that exponen-tially growing NFS/n1.7 cells contain only a single species of RAX with an approximate pI of 8.6 (Fig. 5A). Interestingly, IL-3 deprivation or treatment of cells with As or TG induced the prominent acidification of RAX to pI 8.3 (Fig. 5A). A similar RAX mobility shift was also observed when HA-RAX expressing cells were either treated with As, TG, or H2O2 or deprived of IL-3 (Fig. 5B). HA-RAX was more acidic (i.e. pI 7.8) than RAX due to the acidic nature of the HA peptide sequence and was shifted to pI 7.5 following stress treatments. When HA-RAX was immunoprecipitated from the cell lysate and treated with alkaline phosphatase before loading onto the gel, the TG-induced, shifted band was no longer observed whereas migration of RAX purified from growing cells did not change after alkaline phosphatase treatment (Fig. 5C). These findings indicate that growing, unstimulated cells contain only the nonphosphorylated RAX, whereas the stress treatments induced phosphorylation of RAX. Phosphoamino acid analysis revealed that RAX is exclusively phosphorylated on serine residue(s) following As treatment or IL-3 deprivation (Fig. 5D).

Both RAX and eIF2α phosphorylation were detected within 3 min after treatment with TG and As. Significantly, however, phosphorylation of RAX begins and is maximal before that of eIF2α, indicating that RAX phosphorylation precedes PKR activation (Fig. 6A). Because PKR was unable to directly phosphorylate HA-RAX in vitro (Fig. 3), RAX does not appear to be a substrate for PKR. To test whether cellular RAX could be phosphorylated by PKR after its association, we examined whether 2-aminopurine, a selective inhibitor for PKR (27, 28), could affect RAX phosphorylation. Results revealed that the eIF2α phosphorylation caused by IL-3 deprivation was potent inhibited by 2-aminopurine but had little or no effect on RAX phosphorylation (Fig. 6B). These results therefore suggest that RAX is phosphorylated by stress-activated, 2-aminopurine-resistant serine protein kinase(s) before it activates PKR.

DISCUSSION

The paradigm for PKR activation following viral infection indicates that dsRNA activates this important regulator of protein synthesis, but the mechanism of activation in the absence of viral infection is not clear. Several studies have shown
that various stress stimuli can induce eIF2α phosphorylation (2, 26), indicating that activation of PKR may occur as a result of stress signaling, but whether dsRNA is involved is not known. Our results indicate that RAX may be a direct, stress-sensitive activator of PKR. The high sequence similarity between mouse RAX and human PACT suggests that RAX is a mouse homologue of PACT, which was recently identified using a similar interactive cloning strategy (11). Furthermore, the in vitro studies with purified RAX indicate that RAX, like PACT, can directly activate PKR, confirming that PKR can be activated by cellular proteins in the absence of dsRNA.

The current model of PKR activation holds that two molecules of PKR bind to a single dsRNA molecule, thereby leading to homodimerization, which results in autophosphorylation via transphosphorylation (5). RAX-mediated autophosphorylation and activation of PKR indicates that RAX may also bind two PKR molecules to cause homodimerization of PKR. We also found that RAX can synergistically activate PKR in the presence of dsRNA (Fig. 3), indicating an unique mechanism. Although further studies are required, the potential for a functionally synergistic interaction between RAX and dsRNA with respect to PKR activation in vivo is considered high because PKR is associated with ribosomes where dsRNA species may be abundant (5, 29).

PKR activation leads to protein synthesis inhibition and decreased cell growth (5, 21, 30). Because RAX can efficiently activate PKR, RAX-PKR association during cell growth must be tightly regulated to prevent inadvertent activation of PKR. Co-immunoprecipitation studies suggest that the RAX-PKR interaction can be enhanced by stress signals. The mechanism, however, remains unknown. We found a significant correlation between PKR activity and the phosphorylation state of RAX after various stress treatments. Because RAX phosphorylation clearly precedes PKR activation, these data support the idea that the post-translational modification of RAX may be involved in the RAX-PKR association and activation of PKR in vivo. Phosphoamino acid analysis shows that only serine is phosphorylated, indicating that RAX is the substrate for a stress-activated serine/threonine kinase. Arsenite, thapsigargin, hydrogen peroxide, and IL-3 withdrawal all have been reported to activate the mitogen-activated protein kinase sub-family that includes c-Jun N-terminal kinase/stress-activated protein kinase and p38 kinase (31, 32). Whether RAX is a substrate for these kinases will require further studies.

Interestingly, RAX in growing cells is not heavily phosphorylated (Fig. 5), but even unphosphorylated RAX can apparently activate PKR in vitro (Fig. 3). Therefore, these results suggest that RAX phosphorylation per se is not required for PKR activation but may potentially regulate the RAX-PKR association and affect PKR activation, possibly by altering the affinity or accessibility of RAX to PKR. However, the precise regulatory role for RAX phosphorylation and the mechanism by which RAX can activate PKR remain to be elucidated. Because PKR is a ribosomal protein (29, 33), one possibility is that phosphorylation may facilitate ribosomal localization of RAX and thereby association with PKR. Another is that phosphorylated RAX may have a higher affinity for PKR binding than certain PKR inhibitory molecules such as p58 (34) and TAR-RNA-binding protein (35). Identification of the phosphorylation site(s) and subsequent site-directed mutagenesis analysis of RAX are required to test these possibilities.

Patel and Sen (11) have reported that transient transfection of the PACT cDNA increased the level of eIF2α phosphorylation (about 2-fold) in HT-1080 fibrosarcoma cells, and the establishment of cell lines stably overexpressing PACT was unsuccessful. On this basis it was concluded that simple overexpression of PACT activates PKR and suppresses cell growth. By contrast, RAX can be overexpressed (more than 10-fold) in IL-3-dependent NFS/N1.H7 cells with no effect on PKR activity or cell growth, at least in the presence of IL-3. Although the reason for the discrepancy between PACT and RAX with respect to cell growth is not yet clear, it could be due to the different cell types tested. RAX, in the absence of IL-3, accelerates apoptosis in direct proportion to the expression levels (Fig. 2B). Therefore, it seems likely that growth factors such as IL-3 may, at least in part, potentially suppress stress signals (e.g. stress kinase activity) by inhibiting the association between RAX and PKR. It will therefore be important to determine whether PACT can be overexpressed in NFS/N1.H7 cells and not result in cell death in the presence of IL-3.

In addition to PKR, another stress-sensitive eIF2α kinase, termed PERK (36) or PEK (37), has been recently cloned. PERK is associated with endoplasmic reticulum and can be activated by endoplasmic reticulum stresses such as thapsigargin. However, PERK is not activated by cytoplasmic stresses such as arabinosyladenine, ultraviolet irradiation, and heat shock (36). In fact, our recent data indicate that neither arabinosyladenine nor IL-3 deprivation activates PERK in NFS/N1.H7 cells.3 Thus, some stresses may activate only PKR, whereas some may activate both eIF2α kinases through different mechanisms.

In summary, we propose that RAX is part of a novel stress-activated signaling pathway that features inhibition of protein synthesis that occurs as a result of the direct interaction between RAX and PKR. Because PKR activation has been implicated in apoptosis and the stresses applied here can all induce cell apoptosis, activated RAX appears to be a potent negative regulator of cell growth and survival.

Acknowledgments—We thank R. Jages for providing purified eIF2α, J. A. Langer for pcDEV vector, and D. A. Vasquez and S. P. Warnken for technical assistance.

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