

# Inhibition of cap-dependent translation via phosphorylation of eIF4G by protein kinase Pak2

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**Translation is downregulated in response to a variety of moderate stresses, including serum deprivation, hyperosmolarity and ionizing radiation. The cytosolic p21-activated protein kinase 2 (Pak2)/ $\gamma$ -PAK is activated under the same stress conditions. Expression of wild-type Pak2 in cells and addition of Pak2 to reticulocyte lysate inhibit translation, while kinase-inactive mutants have no effect. Pak2 binds to and phosphorylates initiation factor (eIF)4G, which inhibits association of eIF4E with m<sup>7</sup>GTP, reducing initiation. The Pak2-binding site maps to the region on eIF4G that contains the eIF4E-binding site; Pak2 and eIF4E compete for binding to this site. Using an eIF4G-depleted reticulocyte lysate, reconstitution with mock-phosphorylated eIF4G fully restores translation, while phosphorylated eIF4G reduces translation to 37%. RNA interference releases Pak2-induced inhibition of translation in contact-inhibited cells by 2.7-fold. eIF4G mutants of the Pak2 site show that S896D inhibits translation, while S896A has no effect. Activation of Pak2 in response to hyperosmotic stress inhibits cap-dependent, but not IRES-driven, initiation. Thus, a novel pathway for mammalian cell stress signaling is identified, wherein activation of Pak2 leads to inhibition of cap-dependent translation through phosphorylation of eIF4G.**

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## Introduction

Translation is a fundamental process of gene expression and plays a critical role in development, cell differentiation and apoptosis (Mathews *et al*, 2000; Dever, 2002). Translation and transcription share a number of common features in regulation (Sachs and Buratowski, 1997), but translation is more flexible and rapid in response to environmental signaling, especially in systems lacking transcription, such as oocytes, early embryos, reticulocytes and platelets (Mathews *et al*, 2000). Since the eukaryotic translation path-

way and mechanisms have been clarified, the last decade has witnessed an explosion of studies on the regulation of translation by signal transduction.

One of the most studied steps in the regulation of initiation is the phosphorylation of initiation factor (eIF)2, where the  $\alpha$  subunit is phosphorylated by the heme-regulated inhibitor (HRI), the RNA-activated protein kinase (PKR) or the PKR-like endoplasmic reticulum protein kinase (PERK) at Ser 51 (Clemens, 2001; Dever, 2002). This phosphorylation enhances the affinity of binding of eIF2 to eIF2B, preventing GTP/GDP exchange and inhibiting translation. eIF4F is another main checkpoint in the regulation of translation. The eIF4E subunit of eIF4F binds the 5'-cap (m<sup>7</sup>GpppN) of mRNA, and is phosphorylated by MAPK-interacting kinase (Mnk) or protein kinase C (PKC), enhancing translation in response to mitogenic stimuli (Morley and Traugh, 1989, 1990; Pyronnet *et al*, 1999). The eIF4G subunit of eIF4F is a scaffold protein that binds eIF4E and eIF4A, interacts with eIF3 and the poly(A)-binding protein (PABP), and recruits mRNA to the 40S ribosomal subunit (Mathews *et al*, 2000). eIF4G is phosphorylated in response to serum stimulation through the PI-3 kinase and FRAP/mTOR pathways (Raught *et al*, 2000). eIF4G is phosphorylated *in vitro*, by multipotential S6 kinase (MS6K), PKC and p21-activated protein kinase 2 (Pak2) (Tuazon *et al*, 1989; Morley and Traugh, 1990). eIF4GII is phosphorylated during mitosis (Pyronnet *et al*, 2001), and is phosphorylated by calmodulin-dependent kinase I at Ser 1156 (Qin *et al*, 2003). These studies suggest that phosphorylation of eIF4G can be involved in a wide spectrum of physiological regulatory events.

Translation is optimal in dividing cells and is significantly reduced in response to serum starvation, ionizing radiation, DNA-damaging drugs and hyperosmolarity (Morley, 2000; Patel *et al*, 2002; Proud, 2002). The protein kinase Pak2/ $\gamma$ -PAK is ubiquitous and is transiently activated by moderate stresses that inhibit cell division by binding of the small G protein Cdc42(GTP) to the regulatory domain, releasing the autoinhibition. Pak2 is also constitutively activated by apoptotic stresses, thus promoting cell death (Roig and Traugh, 1999, 2001; Roig *et al*, 2000a). In contrast, Pak1/ $\alpha$ -PAK is involved in promotion of cell growth and proliferation through cytoskeleton reorganization and cell motility, while Pak3/ $\beta$ -PAK is brain specific (Bokoch, 2003).

Expression of wild-type (WT) Pak2, but not kinase-inactive mutants, specifically inhibits cell division in mammalian cells (Jakobi *et al*, 2001; Huang *et al*, 2003). Injection of active Pak2 into *Xenopus* oocytes inhibits cell cleavage by arresting G<sub>2</sub>/M progression (Rooney *et al*, 1996). Recently, Pak2 has been shown to phosphorylate cMyc, inhibiting growth-related gene transcription, cell proliferation and cell transformation (Huang *et al*, 2004). This evidence, along with the fact that Pak2 phosphorylates several translation eIFs and is highly active in the endoplasmic reticulum (ER) (Tuazon *et al*, 1989; Huang *et al*, 2003; Orton *et al*, 2004), strongly suggests that Pak2 could downregulate translation.

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In this study, we show that Pak2 directly inhibits translation in reticulocyte lysate and cultured cells. eIF4G is a major target for Pak2, wherein phosphorylation of eIF4G by Pak2 reduces binding of eIF4F to the m<sup>7</sup>GTP cap, and the interaction between eIF4G and eIF4E, both *in vitro* and *in vivo*. The inhibitory effect of Pak2 is confirmed with RNA interference (RNAi) specific for Pak2. Using a bicistronic mRNA, cap-dependent translation is significantly and specifically reduced when Pak2 is activated by hyperosmotic stress. Thus, the data define a role for phosphorylation of eIF4G by Pak2 in the downregulation of translation initiation.

## Results

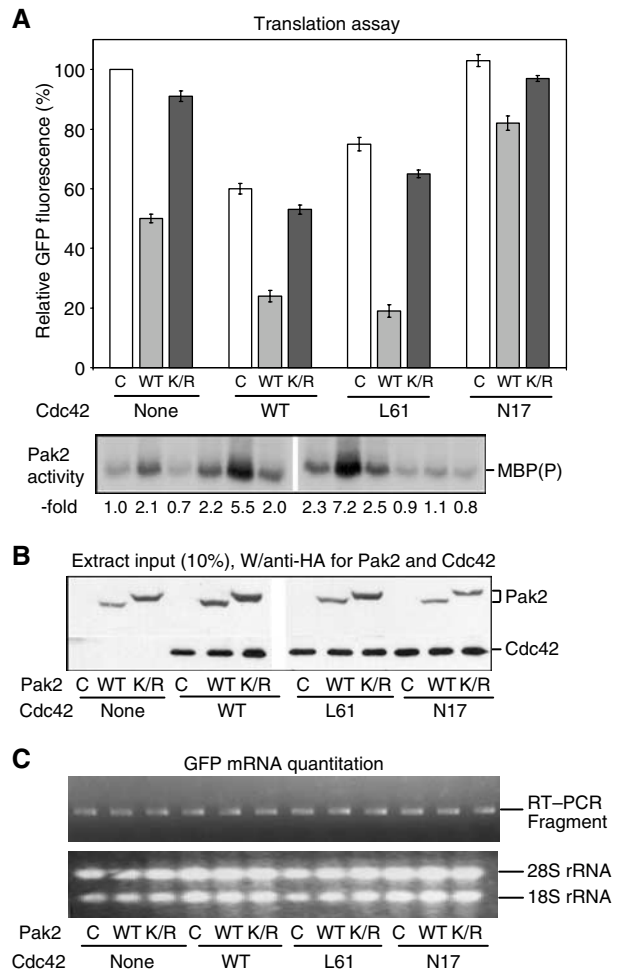
### Pak2 inhibits translation in mammalian cells and reticulocyte lysates

To directly address the role of Pak2 in regulation of translation, 293T cells were cotransfected with Cdc42 and HA-tagged WT Pak2 or the kinase-inactive mutant K278R (K/R). Translation was measured using the GFP reporter gene, located on the same vector as the gene for Pak2, but controlled by a different promoter. WT Cdc42, the constitutively active mutant L61 and dominant-negative N17 were used. As shown in Figure 1A (upper panel), WT Pak2 alone inhibited translation, indicating that the protein kinase had partial activity; K/R had little effect. When WT Pak2 was cotransfected with WT Cdc42, translation was inhibited by 60%; cotransfection with constitutively active Cdc42 L61 inhibited translation by 75%. In contrast, with dominant-negative Cdc42 N17, Pak2 was primarily inactive, and translation was reduced by only 20%.

The effects of Pak2 on translation were coincident with increased protein kinase activity, as shown by immunoprecipitation of Pak2 with N-19 antibody and assay with myelin basic protein (MBP). Phosphorylation of MBP was increased 2.1-fold with Pak2 alone as compared to the controls (Figure 1A, lower panel). Kinase activity was increased 5.5-fold with WT Cdc42, and 7.2-fold with constitutively active L61, as compared to the vector control; the stimulation was negated with N17 (1.1-fold). Cdc42 was expressed to a similar extent under all conditions (Figure 1B), whereas the expression of WT Pak2 was significantly less than that of the kinase-inactive mutant K/R, due to the tight regulation of Pak2 activity by degradation through the proteasome pathway (Roig *et al*, 2000b). Pak2 activated *in vivo* migrated faster than inactive Pak2 due to structural changes related to phosphorylation.

The effects of Pak2 on translation were not due to changes in transcription, as shown with GFP reporter mRNA. Total RNA was extracted from the transfected cells and RT-PCR was carried out using GFP-specific primers. There was no change in GFP mRNA levels (Figure 1C). A similar pattern of Pak2 inhibition of translation was observed using luc reporter mRNA transcribed *in vitro* and transfected into cells (data not shown).

The inhibitory effect of Pak2 on translation was evaluated further using a modified reticulocyte lysate system. To enhance regulation, excess amounts of eIFs, especially eIF4F, were reduced with m<sup>7</sup>GTP-Sepharose, as developed by Svitkin *et al* (1996). Low levels of mRNA were used to ensure that translation was susceptible to 5'cap-3'poly(A)

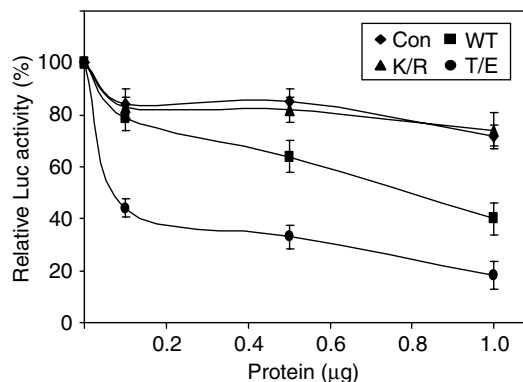


**Figure 1** Pak2 inhibits translation in 293T cells. HEK 293T cells were cotransfected with HA-tagged WT Pak2 or the kinase-inactive mutant K/R in pTracer, and HA-WT Cdc42/pcDNA3.1 +, constitutively active Cdc42 L61 or dominant-negative N17. The control (C) was a mixture of pTracer and pcDNA3.1 + vectors. At 2 days post-transfection, cells were collected and the extracts were prepared. (A) Upper panel, translation was monitored by GFP fluorescence. The data are the mean of three experiments; error bars represent the standard deviation. Lower panel, following immunoprecipitation of Pak2 with N19 antibody from the extract (800 µg), Pak2 activity was assayed with MBP. The autoradiogram is shown. (B) Western blot of cell extract (50 µg) with HA antibody to detect expression of Pak2 and Cdc42. (C) GFP mRNA was measured by RT-PCR with gene-specific primers as described in Materials and methods. Upper panel, analysis of RT-PCR product by agarose gel electrophoresis. Lower panel, input control with rRNA.

synergy. Purified WT Pak2, K/R and the constitutively active mutant T402E were added to reticulocyte lysate. Increasing levels of WT Pak2 inhibited translation by up to 60%, while T/E was a potent regulator of translation with up to 82% inhibition; little effect was observed with K/R (Figure 2). Thus, the level of inhibition was consistent with the activity of Pak2, indicating that the inhibitory effect was exerted through the protein kinase activity.

### Pak2 interacts with eIF4G

The interaction of Pak2 with eIF4F was examined in 293T cells transfected with Pak2, K/R or the dominant-negative mutant T402A (T/A). Using antibody to eIF4G, WT Pak2 and



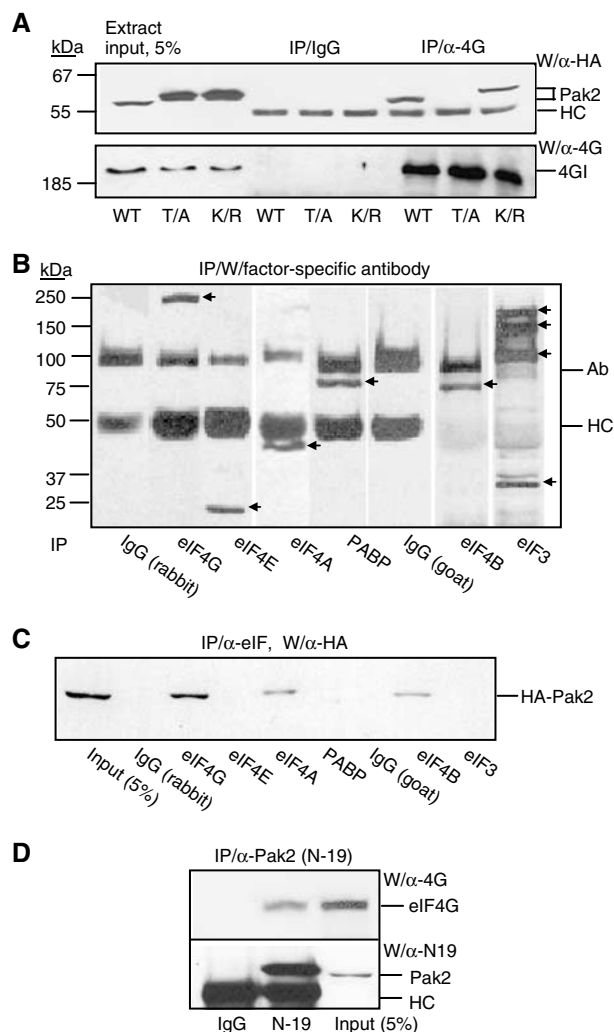
**Figure 2** Pak2 inhibits translation in reticulocyte lysate. Increasing amounts of purified WT GST-Pak2, K/R and T/E were added to the reticulocyte lysate as indicated. Protein synthesis was monitored using luc reporter mRNA (2.5 µg/ml). The data are the average of three experiments; standard deviations are shown by error bars.

K/R coimmunoprecipitated with eIF4G, whereas there was no signal with T/A or the preimmune IgG control, as shown by Western blotting (Figure 3A). When calculated on a molar basis, approximately 80% of the WT Pak2 was bound to eIF4G, as compared to 30% of K/R. Since the dominant-negative T/A was not associated with eIF4G, and the binding of K/R was significantly reduced, the data suggest that WT Pak2 preferentially bound to eIF4G, as compared to the kinase-inactive mutants.

Similar experiments were carried out by immunoprecipitation with antibody specific for eIF4E, eIF4A, PABP, eIF4B and eIF3. Each of these antibodies efficiently immunoprecipitated the cognate eIFs (Figure 3B). eIF4A and eIF4B were found to interact with WT Pak2, but to a lesser extent than eIF4G (Figure 3C). No interaction between WT Pak2 and eIF4E, eIF3 or PABP was observed. Approximately 5% of the total eIF4G was detected in the immunoprecipitate with anti-eIF4A, suggesting that the association could be partially through eIF4G. With antibody to eIF4B, no eIF4G was coimmunoprecipitated, suggesting a direct interaction between eIF4B and Pak2. To confirm the interaction between eIF4G and Pak2, nontransfected 3T3-L1 cells were used to examine the interaction between the endogenous proteins. As shown by Western blotting, eIF4G was associated with Pak2 following immunoprecipitation with N-19 antibody (Figure 3D). Taken together, these data define an interaction between Pak2 and the major eIFs involved in mRNA recruitment to the 40S subunit, which is the rate-limiting step in translation initiation.

#### Identification of the Pak2-binding region on eIF4G

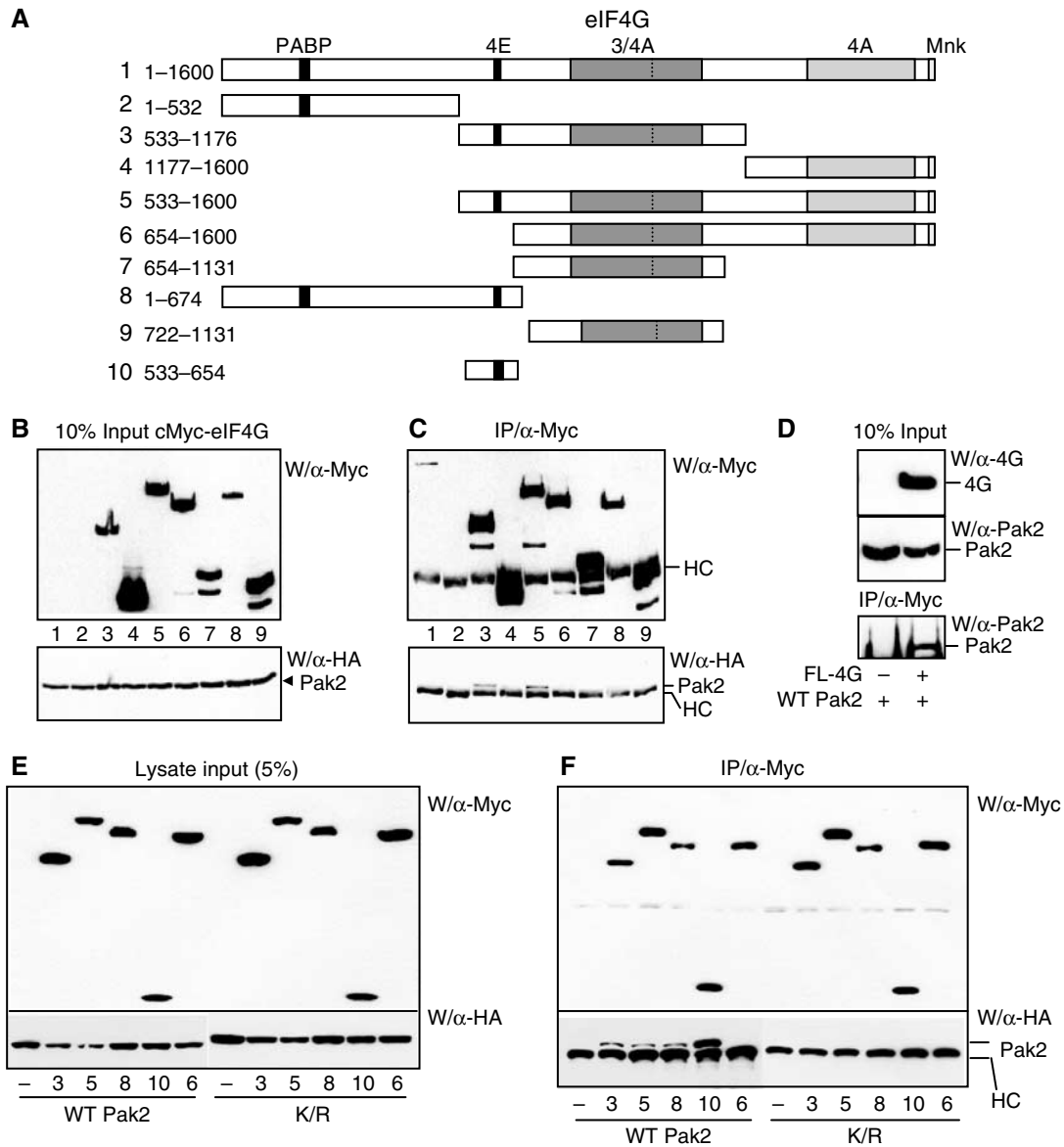
Pak2 binding to eIF4G was mapped using the series of deletion mutants described in the schematic in Figure 4A. 293T cells were cotransfected with HA-WT Pak2 and Myc-tagged full-length eIF4G (FL-4G, 1600 aa) or N-terminal and/or C-terminal deletion mutants of eIF4G. Following immunoprecipitation of eIF4G with anti-Myc and analysis by SDS-PAGE, eIF4G was identified by Western blotting with anti-Myc and Pak2 with anti-HA. Pak2 was expressed to the same extent when transfected with FL-4G or the deletion mutants (Figure 4B). The deletion mutants were expressed at a high level, except for mutant 2 (aa 1–532).



**Figure 3** Interaction between Pak2 and eIFs in 293T cells. (A) The interaction between Pak2 and endogenous eIF4G was analyzed by transfection of HA-Pak2 (WT, K/R, and T/A) into 293T cells; eIF4G was immunoprecipitated from cell extracts with eIF4G antibody and analyzed by SDS-PAGE. Upper panel, Western blot with HA-tag antibody. Lower panel, reprobe of the same membrane with eIF4G antibody. (B) Following transfection with HA-WT Pak2, immunoprecipitation was with antibodies specific for the individual eIFs, as indicated. The lanes were probed with antibody to the corresponding factor. Arrows indicate each eIF. (C) Coimmunoprecipitation of Pak2 was shown by Western blotting of (B) with anti-HA-Pak2. (D) The interaction between endogenous eIF4G and Pak2 in 3T3-L1 cells was analyzed following immunoprecipitation with N-19 antibody. Lower panel, Western blot with N-19; upper panel, the membrane was stripped and reprobed with antibody to eIF4G.

Pak2 was associated with three mutants, 3 (aa 533–1176), 5 (aa 553–1600), and to a lesser extent 8 (aa 1–674) (Figure 4C, lower panel). In contrast, the N-terminal deletion mutants 6 (aa 654–1600) and 7 (aa 654–1131) did not interact with Pak2. The three eIF4G mutants that interacted with Pak2 had a region (aa 533–645) that contained the eIF4E-binding site (aa 610–643), whereas the remainder of the mutants did not.

FL-4G was detected only after immunoprecipitation (Figure 4C, upper panel). The extended N-terminus, consisting of 40 amino acids, contains a proline-rich domain that would lead to rapid degradation of eIF4G, thus explaining



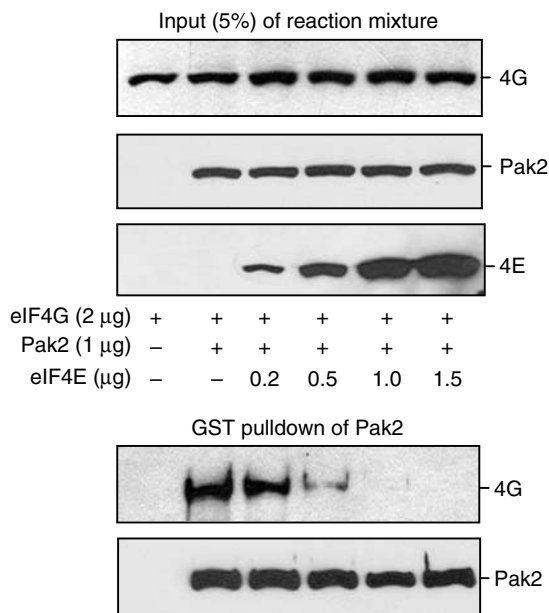
**Figure 4** Mapping of the Pak2-binding site on eIF4G. (A) A schematic diagram for human full-length eIF4G and deletion mutants. (B) Expression of eIF4G and Pak2 was analyzed by SDS-PAGE using equal amounts of protein (50 µg). Western blotting was with Myc antibody for eIF4G and HA antibody for Pak2. (C) Upper panel, eIF4G was immunoprecipitated with anti-Myc, and probed with anti-Myc to define immunoprecipitation efficiency. Lower panel, the blot was reprobed with anti-HA to identify coimmunoprecipitation of Pak2. (D) The interaction between FL-4G and WT Pak2 was examined with a five-fold increase in the number of transfected cells. (E) Expression levels of eIF4G deletion mutants 3, 5, 6, 8 and 10, and WT Pak2 and KR were probed with anti-Myc and anti-HA, respectively. (F) Following immunoprecipitation with anti-Myc, eIF4G and Pak2 were identified by Western blotting.

the low expression of full-length eIF4G. When a larger-scale transfection of FL-4G was carried out, Pak2 interacted to a similar extent with FL-4G as the mutants (Figure 4D). In other experiments, Pak2 associated with a mutant of eIF4G in which the N-terminal PABP-binding site was deleted (aa 160–1560), suggesting that the N-terminal region of eIF4G was not required for interaction with Pak2 (data not shown).

To experimentally prove that the eIF4E-binding region was the target for binding of Pak2, a peptide containing the eIF4E-binding site (aa 533–654) (Lejeune *et al*, 2004) was analyzed along with the three larger constructs containing the eIF4E-binding site and one that did not (Figure 4E). The data clearly showed that this region was responsible for Pak2 binding

(Figure 4F). This strongly suggested that Pak2 inhibited translation through interference of the interaction between eIF4E and eIF4G. It also showed that WT Pak2 associated with the eIF4G mutant, but not K/R, confirming the specificity of the interaction between Pak2 and eIF4G.

To examine further the association between eIF4G and Pak2, a competition experiment was carried out with constant amounts of purified His/Flag-eIF4G and GST-Pak2, to which increasing concentrations of eIF4E were added (Figure 5, upper panel). Using a pulldown assay with Pak2, the increased levels of eIF4E resulted in a concomitant decrease in the interaction between eIF4G and Pak2 (Figure 5, lower panel). Thus, eIF4E competed with Pak2 by binding to the same region on eIF4G.



**Figure 5** Competition between eIF4E and Pak2 for binding to eIF4G. Purified His/Flag-eIF4G and GST-Pak2 (expressed in insect cells) were combined, followed by addition of increasing concentrations of eIF4E (expressed in *E. coli*) as indicated. Upper panel, the input proteins were analyzed by SDS-PAGE and Western blotting. Lower panel, Pak2 and eIF4G were detected by Western blotting after GST pull-down.

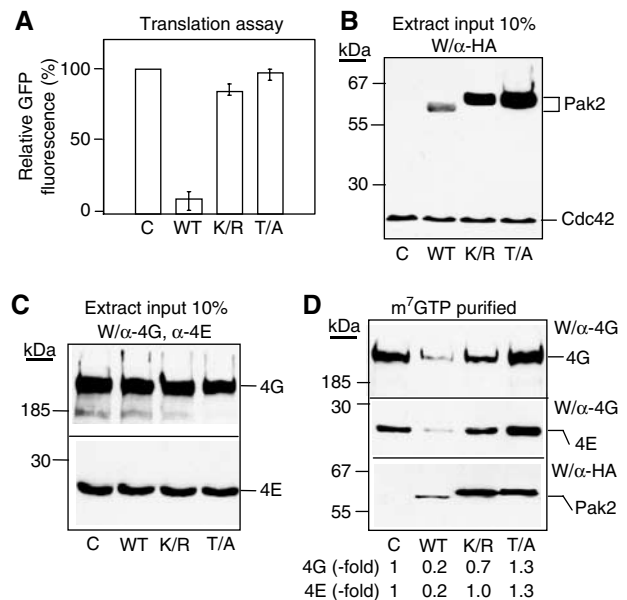
#### Mechanism for inhibition of translation in vivo

The functional mechanisms involved in the inhibition of translation *in vivo* were examined with the GFP reporter by cotransfection of 293T cells with Pak2, K/R or T/A and the active Cdc42 mutant L61. As shown in Figure 6A, activated WT Pak2 inhibited translation by 88% as compared to the control, while K/R and T/A had little effect. Expression of WT Pak2 was significantly lower than that of the inactive mutants, while Cdc42 L61 was expressed at same level in all samples (Figure 6B); the amount of endogenous eIF4G and eIF4E (free and bound forms) was not altered by the expression of WT Pak2 or the mutants (Figure 6C).

eIF4F was affinity purified from the extracts on m<sup>7</sup>GTP-Sepharose and the level of eIF4E and eIF4G was analyzed by Western blotting. A dramatic decrease in the amount of both proteins was observed with WT Pak2 (Figure 6D). As compared to the control, the level of eIF4E and eIF4G bound to m<sup>7</sup>GTP was decreased by approximately 80%. With K/R, there was a 30% reduction in the amount of eIF4G bound to m<sup>7</sup>GTP, but eIF4E binding was not altered. In contrast, the dominant-negative mutant T/A showed a 30% stimulation in binding of eIF4F to the m<sup>7</sup>GTP cap. This was attributed to the inhibition of endogenous Pak2 activity by the dominant-negative T/A mutant. Thus, WT Pak2, K/R and T/A were associated with the eIF4F initiation complex (Figure 6D); however, only active WT Pak2 altered the binding of eIF4G and eIF4E to the m<sup>7</sup>GTP cap. The data indicate that the protein kinase activity was essential for inhibition of translation, and this activity altered the association of eIF4F with the m<sup>7</sup>GTP cap by inhibiting the formation of eIF4F and/or promoting dissociation of the complex.

#### Phosphorylation of eIF4G by Pak2

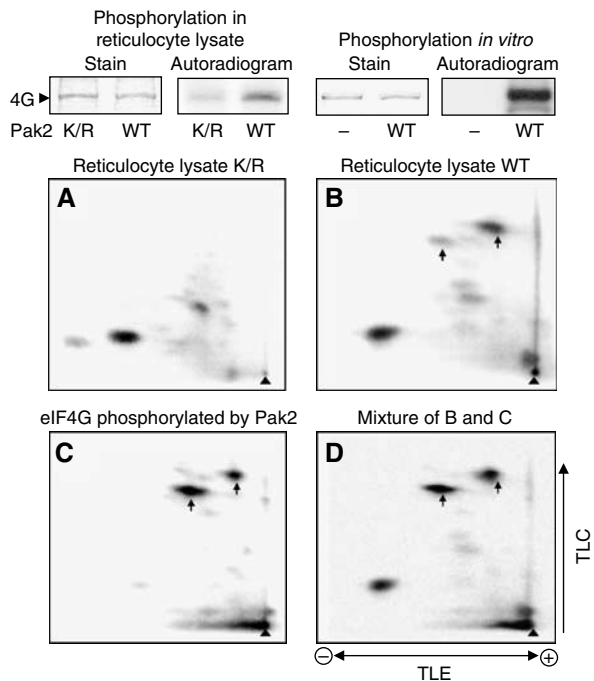
To directly examine the phosphorylation of eIF4G, Pak2 and K/R were added to reticulocyte lysate along with [ $\gamma$ -<sup>32</sup>P]ATP,



**Figure 6** Pak2 decreases the association of eIF4E and eIF4G with m<sup>7</sup>GTP in 293T cells. HA-Pak2/pTracer (WT, K/R and T/A) was cotransfected with Cdc42 L61 into 293T cells. (A) Equal amounts of extract (500 µg) were used to measure the translation rate with GFP. (B) Equal amounts (50 µg) of extract were used for Western blotting with HA. (C) Reprobe of the membrane in panel B with eIF4G and eIF4E antibodies. (D) eIF4F was purified on m<sup>7</sup>GTP-Sepharose; eIF4G, eIF4E and Pak2 were analyzed by Western blotting. eIF4G and eIF4E were quantified using Eagle-eye gel image analysis software (Stratagene). The experiment is representative of six repeats.

and translation was carried out for 20 min. The reaction was terminated by addition of buffer containing phosphatase and protease inhibitors, and endogenous eIF4G was immunoprecipitated with eIF4G antibody; phosphorylation of eIF4G was analyzed by SDS-PAGE and autoradiography. The low level of phosphorylation of eIF4G with K/R was due to background protein kinases in the lysate; phosphorylation was greatly enhanced upon addition of WT Pak2 (Figure 7, top left panel). For the control, purified eIF4G was incubated under phosphorylation conditions in the presence and absence of Pak2 *in vitro*. No phosphorylation was observed in the absence of Pak2, whereas eIF4G was highly phosphorylated by Pak2 (Figure 7, top right panel). To determine whether eIF4G was phosphorylated by Pak2 at the same site(s) in reticulocyte lysate and *in vitro*, the phosphorylated eIF4G bands were excised, subjected to extensive tryptic digestion and analyzed by two-dimensional phosphopeptide mapping. The K/R control showed one major and several minor phosphopeptides (Figure 7A). Following addition of WT Pak2 to the reticulocyte lysate, two new phosphopeptides were detected, as indicated by the arrows (Figure 7B). eIF4G phosphorylated by Pak2 *in vitro* yielded two equivalent spots (Figure 7C), which comigrated with the tryptic phosphopeptides from eIF4G phosphorylated in reticulocyte lysate (Figure 7D). Autophosphorylated Pak2 was present in immunoprecipitates using eIF4G antibody, confirming the interaction between Pak2 and eIF4G (data not shown). Thus, Pak2 specifically phosphorylated eIF4G in reticulocyte lysate, providing evidence that Pak2 inhibited translation through phosphorylation of eIF4G.

Phosphorylation of the eIF4G deletion mutants *in vitro* indicated that a major Pak2 phosphorylation site was

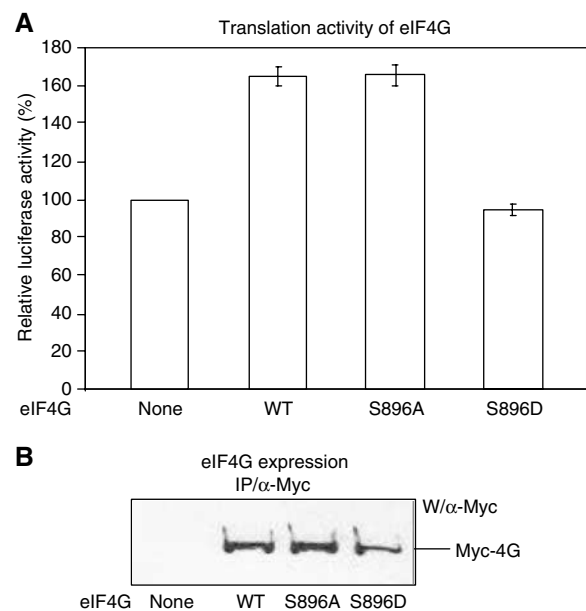


**Figure 7** Analysis of phosphorylation of eIF4G by Pak2 in reticulocyte lysate and *in vitro*. Top left panel, WT Pak2 or K/R and [ $\gamma$ - $^{32}$ P]ATP were added to the reticulocyte lysate just prior to the initiation of translation. Phosphorylation of endogenous eIF4G was analyzed by SDS-PAGE following immunoprecipitation with eIF4G antibody and detected by autoradiography and Coomassie staining after SDS-PAGE. Top right panel, purified His/Flag-eIF4G phosphorylated by Pak2 *in vitro* was the control. eIF4G was excised from the gel, digested with trypsin, and the peptides were analyzed by two-dimensional phosphopeptide mapping; the autoradiograms are shown. (A) eIF4G from reticulocyte lysate containing K/R (B) or WT Pak2. (C) His/Flag-4G phosphorylated *in vitro* by Pak2. (D) Comigration of the peptides shown in panels B and C. Arrows indicate the phosphopeptides specifically phosphorylated by Pak2; the origins are indicated by arrowheads. The experiment was repeated five times.

localized in the region from aa 654 to 1131 in the region of the eIF3/4A-binding domain of eIF4G (data not shown). This region contained a site that coincided with the consensus recognition/phosphorylation site for Pak2, K/RRXS (Tuazon *et al*, 1997); this was the only site in eIF4G that contained the consensus sequence. Ser 896 was mutated to alanine or aspartic acid to mimic the nonphosphorylated and phosphorylated protein. When WT eIF4G and the S896A and S896D mutants were cotransfected into 293T cells with the Luc reporter gene, WT eIF4G and S896A stimulated translation by 2.4-fold. In contrast, S896D inhibited translation to a level below the vector control (Figure 8A). The results indicated that Ser 896 functioned in the downregulation of translation. Western blotting indicated that all three proteins expressed well (Figure 8B).

#### Phosphorylation of eIF4G inhibits the interaction of eIF4E and eIF4G *in vitro*

To link the effects of phosphorylation of eIF4G to regulation of the eIF4F complex, as shown earlier in 293T cells (Figure 6D), the interaction between purified eIF4G and eIF4E was examined *in vitro* following phosphorylation of eIF4G by Pak2 (Figure 9A, upper panel). With eIF4G, 1.0 mol of phosphate was incorporated per mole; eIF4G was not phos-

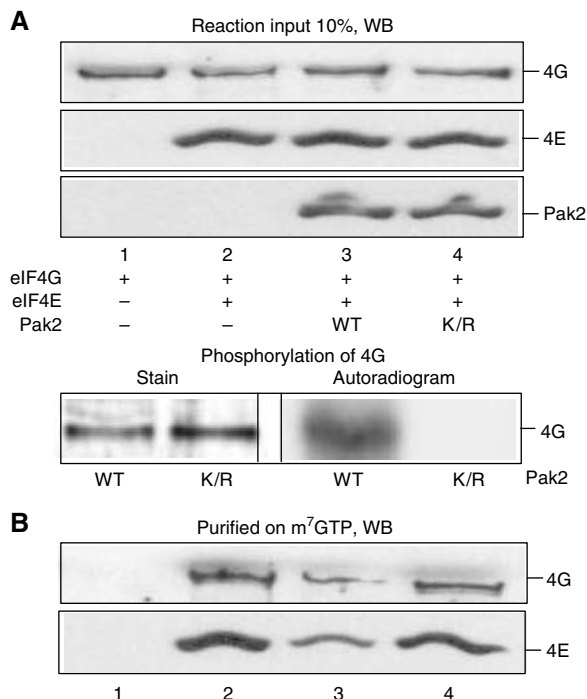


**Figure 8** Analysis of eIF4G mutants on translation. 293T cells were cotransfected with FL-WT eIF4G, S896A or S896D and the bicistronic reporter pRMF. At 30 h post-transfection cells were collected, lysate was prepared and equal amounts of protein (10  $\mu$ g) were used to assay *Renilla* luc activity. (A) Measurement of translation rates by luciferase activity. (B) Expression levels of WT and mutants of eIF4G by Western blotting. Experiments were repeated three times with three replicates. The data represent the mean with error bars for the standard deviation.

phorylated by K/R (Figure 9A, lower panel). eIF4E was added to phosphorylated and nonphosphorylated eIF4G, and the eIF4F complex was analyzed on  $m^7$ GTP-Sepharose 4B. As shown in Figure 9B, phosphorylation of eIF4G by WT Pak2 dramatically reduced the binding of eIF4E to the  $m^7$ GTP cap and the association of eIF4G with eIF4E as shown by Western blotting. Binding of eIF4E to the cap was reduced by 55% and the association with eIF4G by 70%. In contrast, the data with K/R were similar to the nonphosphorylated control; both eIF4G and eIF4E were associated with  $m^7$ GTP to a similar extent. The reduction in the interaction between eIF4E and phosphorylated eIF4G, and the reduced association of eIF4E with the cap structure, indicated that phosphorylation of eIF4G directly interfered with eIF4F complex formation and/or induced dissociation of eIF4E from the cap structure.

#### Phosphorylation of eIF4G inhibits translation in a reconstituted lysate

A reconstituted reticulocyte lysate system was employed to determine whether phosphorylation of eIF4G led directly to translation inhibition. Endogenous eIF4G was completely removed from the lysate by immunoprecipitation using antibody to eIF4G (Figure 10A). The immunoprecipitated eIF4G showed a slight depletion of eIF4E, which is in excess in reticulocyte lysate (Rau *et al*, 1996); no eIF4A coimmunoprecipitated with eIF4G. When equal amounts of immunoprecipitated eIF4G were incubated with ATP alone (control), and with WT Pak2 or K/R, there was a significant increase of phosphorylation only with WT Pak2, and not the control and K/R (Figure 10B). Following removal of Pak2 and ATP, phosphorylated and mock-phosphorylated eIF4G were added

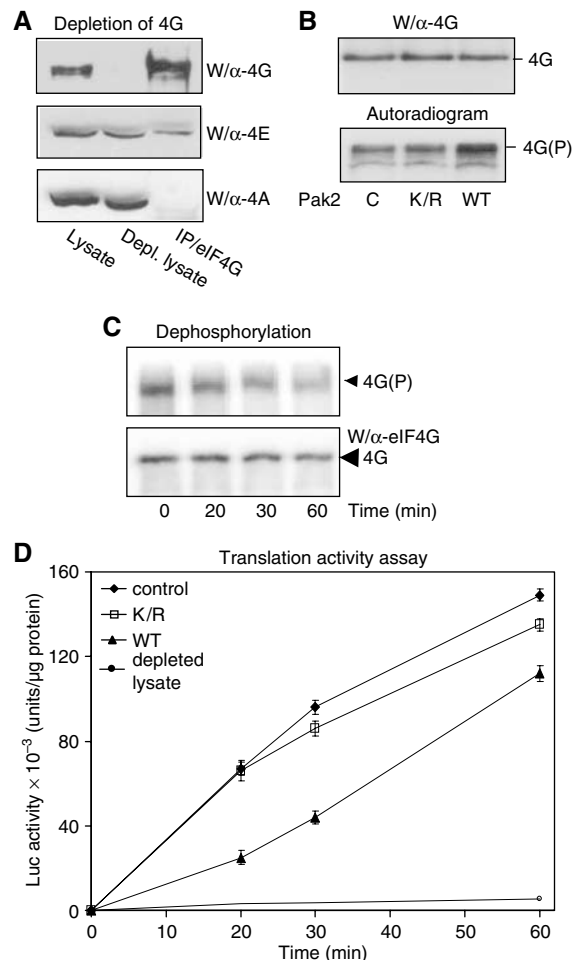


**Figure 9** Phosphorylation of eIF4G by Pak2 alters the interaction between eIF4G and eIF4E *in vitro*. His/Flag-eIF4G (0.2 µg) was phosphorylated by Pak2 (0.03 µg) *in vitro* and purified eIF4E eIF4E (0.2 µg) was added. The complex was purified on m<sup>7</sup>GTP-Sepharose and analyzed by SDS-PAGE. (A) Upper panel, Western blot of the input. Lower panel, stain and autoradiogram of eIF4G phosphorylated with WT Pak2 and K/R. (B) Western blot of the eIF4G·eIF4E complex purified on m<sup>7</sup>GTP-Sepharose.

back to the depleted reticulocyte lysate. The reconstituted lysate was highly active with the mock-phosphorylated eIF4G (control) or when eIF4G was incubated with K/R (Figure 10D). When eIF4G phosphorylated by Pak2 was used to reconstitute the lysate, translation at 20 min was 37% of the mock-phosphorylated control. The eIF4G-depleted lysate had 3% of the translation activity of the reconstituted lysate with mock-phosphorylated eIF4G. At later times, the rate of translation with phosphorylated eIF4G increased, due to dephosphorylation of eIF4G by endogenous phosphatases. As shown in Figure 10C, a slow rate of dephosphorylation was observed over time. The half-life of the phosphate on eIF4G was determined to be about 30 min. From these data it can be concluded that phosphorylation of eIF4G by Pak2 had a direct inhibitory effect on translation.

#### RNAi for Pak2 releases the suppression of translation during contact inhibition

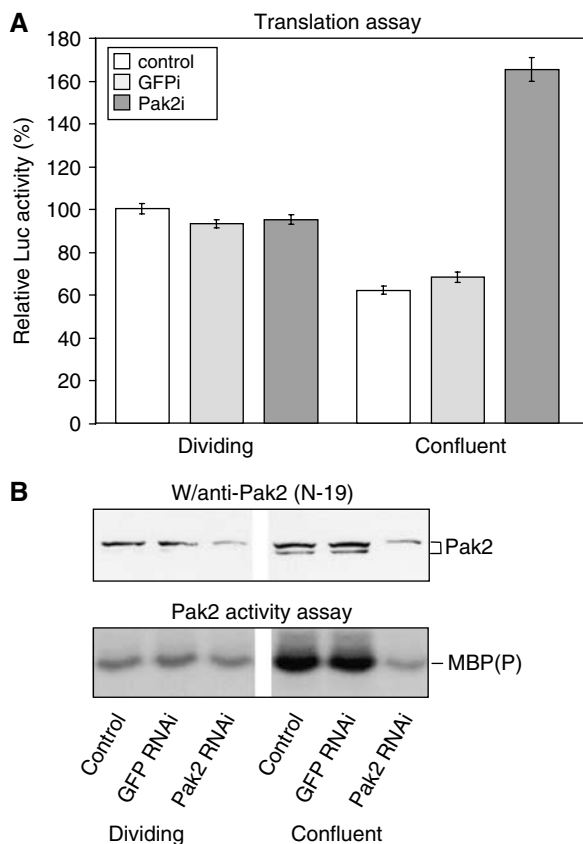
To confirm that Pak2 inhibits translation, RNAi was employed to reduce the level of endogenous Pak2. Exponentially growing 293T cells were cotransfected with the luc reporter gene and the Pak2 RNAi construct, the GFP RNAi construct (non-specific control), or the RNAi vector (control). The cells were collected 24 h post-transfection (dividing state) or at 55 h (confluent state). When dividing cells containing the vector control were set at 100%, the rate of translation (as measured with Luc) was essentially the same as that with RNAi for GFP or for Pak2. This indicated that endogenous Pak2 had little effect on translation in dividing cells (Figure 11A). In con-



**Figure 10** Pak2 inhibits translation in a reconstituted reticulocyte lysate by phosphorylation of eIF4G. (A) Western blot showing the depletion of eIF4G and associated eIFs from reticulocyte lysate. (B) eIF4G was incubated alone (control), with K/R or with WT Pak2 under phosphorylation conditions, as shown by Western blotting and autoradiography. (C) Dephosphorylation of eIF4G (1.0 µg), phosphorylated *in vitro* by Pak2, was monitored over time following addition to reticulocyte lysate and analyzed by SDS-PAGE and autoradiography. (D) Phosphorylated and mock-phosphorylated eIF4G were added to the depleted lysate and translation activity was measured using Luc reporter mRNA. The data are the average of three experiments; the standard deviations are shown by error bars.

fluent cells, the level of translation in the vector control and with GFP RNAi was reduced to 62 and 68% of the vector control in dividing cells. Thus, the rate of translation in confluent cells was significantly reduced, as compared to that in dividing cells. RNAi for Pak2 enhanced the rate of translation over the vector control by 2.7-fold in confluent cells and 1.7-fold in dividing cells. These changes were directly correlated with changes in Pak2 protein levels and activity.

As shown by Western blotting, the level of expression of Pak2 in confluent cells was significantly higher than in dividing cells (Figure 11B, upper panel). RNAi for Pak2 reduced the expression of Pak2 by 70% in dividing cells and 85% in confluent cells as compared to the vector controls. Endogenous Pak2 was activated only in confluent cells, as shown by the appearance of a lower band, which is the active form of Pak2 *in vivo* (Corneillie S, Rooney RD,



**Figure 11** Release of suppression of translation by Pak2 by RNAi. (A) The luciferase reporter gene was transfected into 293T cells (35% confluent) with Pak2 siRNA/pSilencer1.0, with the GFP RNAi construct, or with vector alone as a control. The translation rate was analyzed at 24 h (dividing) and 55 h (confluent) following transfection. Luc activity was measured with equal amounts of cell extract. The experiment was repeated three times. (B) Upper panel, the effects of RNAi on the expression of endogenous Pak2, as measured by Western blotting with 50  $\mu$ g of cell extract. Lower panel, Pak2 activity was quantified following immunoprecipitation with N-19 antibody using MBP as substrate.

Ling J and Traugh JA, unpublished data.). Measurement of protein kinase activity with immunoprecipitated endogenous Pak2 showed that Pak2 was not activated in dividing cells; therefore, removal of inactive Pak2 by RNAi had no effect on translation. In contrast, Pak2 was highly active in confluent cells, with a five-fold stimulation of activity, as compared to the activity in dividing control cells (Figure 11B, lower panel). Thus, the reduction of Pak2 protein and activity by RNAi released the suppression of translation, further indicating that the protein kinase activity of Pak2 was required for translation inhibition.

#### Effects of Pak2 on cap-dependent and IRES-driven translation in dividing and stressed cells

Endogenous Pak2 is primarily inactive in dividing cells and is activated in response to stress (Roig and Traugh, 1999; Roig *et al*, 2000a; Huang *et al*, 2003; Miah *et al*, 2004). Thus, it was of interest to determine whether Pak2 had a role in regulating the initiation of cap-dependent and/or cap-independent translation. A bicistronic reporter with a cap-dependent *Renilla* luc gene and a c-myc IRES-driven firefly luc gene (Chappell *et al*, 2000) was used for this analysis (Figure 12A).

To examine the effects of Pak2 on translation, 293T cells were transfected with WT Pak2, active p34 or the dominant-negative mutant T/A. The effects of Pak2 on translation were measured with or without hyperosmotic stress (0.4 M sorbitol for 30 min) (Roig *et al*, 2000b). The rates of cap-dependent and IRES-driven translation in dividing cells (control) were each set at 100% (Figure 12B, left panel). In dividing cells, there was a 45% reduction in cap-dependent translation with WT Pak2, and a similar inhibition with p34. In contrast, expression of T/A had a 10% stimulatory effect on cap-dependent translation in dividing cells. When cells were treated with sorbitol for 30 min, the control was reduced to 60% of the nonstressed control. Expression of WT Pak2 and p34 reduced the cap-dependent translation to 30 and 20% of the control for dividing cells, respectively. As shown in Figure 12C, T/A was expressed at a level >10-fold higher than that of WT Pak2 and p34. This was due to the tight regulation of Pak2 inside the cell (Roig *et al*, 2000b). The activity of Pak2, as measured with MBP, was consistent with the inhibition of translation (Figure 12D). The data shown in Figure 12B were supported by analysis of the luc mRNA by RT-PCR (Figure 12E), indicating that Pak2 functioned to regulate translation rather than transcription of the reporter gene.

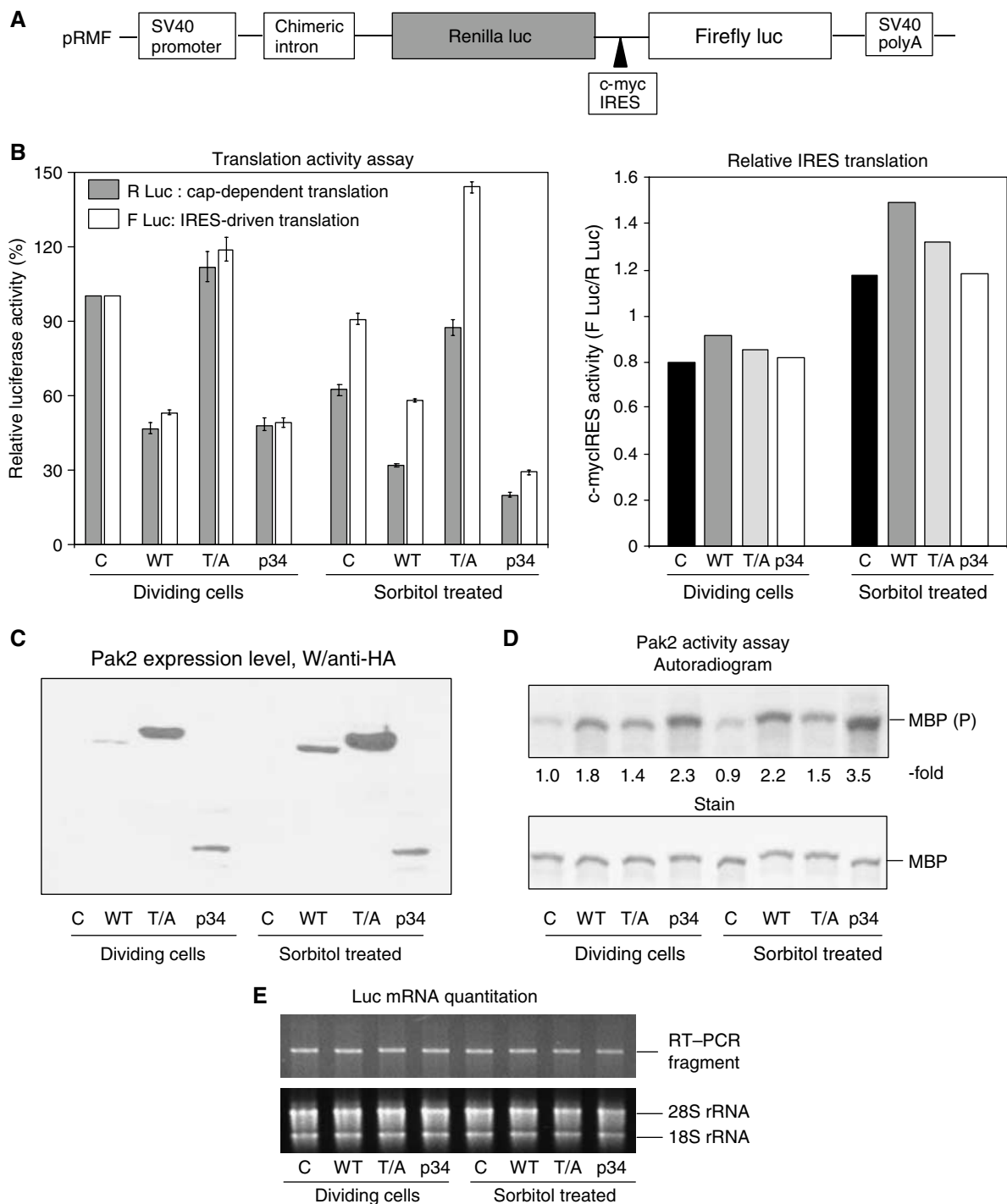
To analyze the IRES-driven translation, firefly luc activity was normalized to *Renilla* luc, as shown in Figure 12B (right panel). There was little or no effect of WT Pak2 or the Pak2 mutants on cap-independent translation in dividing cells. Cells treated with sorbitol had a slightly higher level of c-myc-IRES-driven translation, as compared to dividing cells, suggesting that Pak2 may slightly enhance cap-independent translation during stress. Similar results were obtained with a different bicistronic reporter containing the EMCV-IRES sequence in response to serum starvation in 3T3-L1 cells (data not shown).

## Discussion

Pak2 activity is low in dividing cells, and becomes activated under moderate stress conditions (Roig and Traugh, 2001). Expression of WT Pak2 inhibits cell division and induces a cytostatic response (Huang *et al*, 2003). Pak2 mediates a number of pathways in the stress response by association with and phosphorylation of key regulatory proteins, including the inhibition of transcription of growth-related genes via phosphorylation of c-Myc (Huang *et al*, 2004).

This study shows that Pak2 directly inhibits translation during the cytostatic response to moderate stress, by association with and phosphorylation of eIF4G by Pak2, resulting in decreased binding of eIF4F to m<sup>7</sup>GTP. In contrast, the inactive mutant T/A enhances binding of eIF4F to m<sup>7</sup>GTP, with little or no inhibition of translation. Reconstitution experiments using an eIF4G-depleted reticulocyte lysate confirm that phosphorylation of eIF4G by Pak2 limits translation, while mock-phosphorylated eIF4G fully restores translation. Ser 896 is identified as a major site for regulation of eIF4G by Pak2, as shown by site-directed mutagenesis. S896A stimulates translation 2.5-fold, while S986D has a slight inhibitory effect.

Pak2 binds to the region on eIF4G (aa 533–654) that contains the eIF4E-binding site (aa 610–643), as shown in a competition experiment for eIF4G between eIF4E and Pak2. This suggests that phosphorylation of eIF4G could have



**Figure 12** Activation of Pak2 inhibits cap-dependent translation. 293T cells were cotransfected with WT Pak2, mutants of Pak2 and the bicistronic reporter pRME. At 30 h post-transfection, the cells were collected and luc activity was assayed. (A) Schematic diagram for the structure of the reporter construct. (B) Left panel, relative luc activities were shown by setting *Renilla* and firefly luciferase at 100% (control); right panel, normalization of c-myc IRES activity as described (Chappell *et al*, 2000). (C) Western blot to show the expression levels of HA-Pak2. (D) HA-Pak2 was immunoprecipitated with cell lysate and the kinase activity was measured with MBP. (E) Luc mRNA was quantitated by RT-PCR.

several effects on translation, including inhibition of the formation of eIF4F and the binding of eIF4F to m<sup>7</sup>GTP. In addition, phosphorylation of Ser 896 in the eIF3/4A-binding site of eIF4G could prevent the association of eIF3 and eIF4A with eIF4G and further block translation initiation.

RNAi provides further support for translational control by Pak2. In dividing cells endogenous Pak2 is inactive and RNAi

has no effect on translation, whereas in contact-inhibited cells Pak2 is highly active, and the reduction of Pak2 protein stimulates translation. In response to moderate stress, Pak2 is translocated and activated in the ER (Roig *et al*, 2000a; Huang *et al*, 2003), and thus is poised to phosphorylate eIF4G and newly translated proteins. This effect is specific for Pak2, as overexpression of Pak1 is not inhibitory, but results in a slight

stimulation of translation (data not shown). This is consistent with the growth-promoting properties of Pak1.

Pak2 specifically inhibits cap-dependent translation. These data are consistent with previous studies that show that phosphorylation of c-Myc by Pak2 inhibits the transcription of growth-related mRNAs (Huang *et al*, 2004). Thus, concomitant downregulation of growth-related transcription and translation would contribute to the cytostatic response and cell survival.

Pak2 binds directly to eIF4G in the absence of eIF4E, and is present in the eIF4F initiation complex. The two kinase-inactive mutants do not associate tightly with eIF4G, but are present in the complex. This suggests an association of Pak2 with other eIFs or proteins in the complex. In this regard, we note that Pak2 phosphorylates the N-terminal region of Mnk, blocking binding of Mnk to eIF4G, thus limiting the growth-related phosphorylation of eIF4E (Orton *et al*, 2004).

Phosphorylation of eIF4G by Pak2 could help explain the molecular mechanisms involved in regulating the binding of eIF4F to the 5'-cap of mRNA. There are currently two models; the prevailing model proposes that eIF4F is assembled prior to interacting with the cap structure (Gingras *et al*, 1999). eIF4E alone exhibits a relatively lower affinity for cap analogs, while eIF4G dramatically increases the binding of eIF4E to the cap (Haghighat and Sonenberg, 1997). The other model proposes that eIF4E binds to the cap, then is recruited to the 43S complex, which already contains eIF4G; this is based on the observation that eIF4G associates with the 43S initiation complex in the absence of mRNA (Joshi *et al*, 1994). Our data support the first model of eIF4F assembly by explaining how Pak2 could influence the binding of eIF4E to the mRNA cap via phosphorylation of eIF4G. Gross *et al* (2003) solved the solution structure of the complex between eIF4E, m<sup>7</sup>GTP and eIF4G (aa 393–490) containing the eIF4E-binding site and an adjacent N-terminal region. This eIF4G fragment forms a right-handed helical ring that wraps around the N-terminus of eIF4E, which allosterically enhances and stabilizes the association of eIF4E with the cap. Pak2 phosphorylation could induce a conformation change on eIF4G, regulating binding of eIF4E to the mRNA cap.

Although eIF4G is multiply phosphorylated, the effects of phosphorylation on translation are not well understood. Raught *et al* (2000) identified three sites phosphorylated in response to serum stimulation, serines 1108, 1148 and 1192; other unidentified sites were phosphorylated in response to serum deprivation. Phosphorylation of eIF4G at Ser 896 mediates the downregulation of translation by altering the interaction between eIF4E and eIF4G. Pyronnet *et al* (2001) show that eIF4GII is hyperphosphorylated during mitosis, decreasing the interaction with eIF4E, and inhibiting cap-dependent translation.

This is the first report showing that Pak2 directly inhibits translation through phosphorylation of eIF4G, providing experimental evidence that Pak2 acts downstream of stress signaling to inhibit translation. This is consistent with the Pak2-induced inhibition of cell division and induction of cytoarrest (Rooney *et al*, 1996; Huang *et al*, 2003, 2004). In addition to eIF4G, Pak2 phosphorylates eIF4B and subunits of eIF3 (Tuazon *et al*, 1989). The potential universality of translational inhibition by Pak2 is consistent with the fact that Pak2 is ubiquitous in all mammalian cells and tissues examined (Roig and Traugh, 2001), providing a com-

mon mechanism to downregulate cap-dependent translation in response to stress.

## Materials and methods

### Constructs and antibodies

Pak2 constructs used in these studies include the following: WT Pak2; two kinase-inactive mutants, a mutant of the catalytic site K278R (K/R) and a dominant-negative mutant of the conserved threonine in the activation loop T402A (T/A); a constitutively active mutant of the conserved threonine T402E (T/E), which lacks cytostatic activity and the active caspase cleavage product (p34) of the catalytic domain (Huang *et al*, 2003). HA-tagged Pak2 was subcloned into the pTracer expression vector (Invitrogen) and expressed in 293T cells (Huang *et al*, 2003). GST-Pak2 was subcloned into the pAcG2T baculovirus expression vector (BD Bioscience) and expressed in TN5 insect cells (Walter *et al*, 1998). His/Flag FL-4GI (1600 aa, AY082886) was also expressed in insect cells. Myc-tagged FL-4GI and deletion mutants thereof in pcDNA3.1+ were expressed in mammalian cells. eIF4E in pET11a was expressed in *Escherichia coli* and purified on m<sup>7</sup>GTP-Sepharose. HA-tagged Cdc42 constructs, including WT Cdc42, constitutively active L61 and dominant-negative N17 were subcloned into the pcDNA3.1+ vector. The bicistronic reporter pRMF with a c-myc IRES was used to measure cap-(in)dependent translation (Chappell *et al*, 2000). Antibody to eIF4GI was raised in rabbit against the peptide 920–1396 and to eIF4E against peptide 199–217. Antibodies to eIF4A and PABP were produced in rabbit and antibodies to eIF4B and eIF3 were raised in goat. Preimmune antibodies to rabbit or goat were used as controls. Antibody to the HA-tag (HA.11, 101R) was from Covance. Protein A/G beads and antibodies to GST and Myc-tag were from Santa Cruz Biotechnology.

### Pak2 activity assay

Pak2 activity was assayed as described elsewhere (Roig *et al*, 2000b). Briefly, immunoprecipitated or purified Pak2 (0.1 µg) was preincubated alone or with 0.5 µg GST-Cdc42(GTPγS) and was assayed with the substrate MBP or H4 (1–2 µg) and 0.2 mM (γ-<sup>32</sup>P)ATP, 1000–2000 c.p.m./pmol) for 20 min at 30°C. The reaction was terminated with SDS sample buffer and analyzed by SDS-PAGE on 12% gels, followed by autoradiography.

### Rabbit reticulocyte lysate—translation, phosphorylation and reconstitution

**Translation.** Rabbit reticulocyte lysate (Promega) was chromatographed on m<sup>7</sup>GTP-Sepharose 4B to recover 5'cap-3'poly(A) synergy as described (Svitkin *et al*, 1996). Low levels of the luc reporter mRNA (2.5 µg/ml) were used to mimic regulation of translation *in vivo*. The translation reactions (12 µl) contained 50% lysate, 100 µM amino-acid mixture, 1 U of RNasin (Promega, 40 U/µl) and Pak2; incubation was at 30°C for 1 h. The translation rate was measured by Luc activity, as described (Ling *et al*, 2002).

**Phosphorylation.** Phosphorylation of eIF4G was examined by addition of WT and mutants of GST-Pak2 (2 µg) to a reticulocyte lysate system (50 µl) containing luc reporter mRNA and 20 µCi of [γ-<sup>32</sup>P]ATP (30 Ci/mmol). After 20 min of incubation at 30°C, endogenous eIF4G was immunoprecipitated with eIF4G antibody in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol and 1% NP-40) in the presence of protease inhibitors (2 µg/ml leupeptin, 4 µg/ml pepstatin, 4 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml E64 and 2 mM benzamide) and phosphatase inhibitors (1 mM Na<sub>3</sub>VO<sub>4</sub>, 2 µM okadaic acid, 50 mM NaF and 1 pM cypermethrin), and analyzed by SDS-PAGE on 8% gels, followed by autoradiography.

**Reconstitution.** Reconstituted lysate (50 µl) was prepared by depletion of endogenous eIF4G with eIF4G antibody. For phosphorylation of the depleted eIF4G, equal amounts of eIF4G (1 µg bound to the resin) were incubated alone or with WT Pak2 or K/R (0.1 µg) for 20 min at 30°C. Pak2 and Cdc42 were released by washing the beads with translation compatible buffer B (10 mM HEPES, pH 7.2, 100 mM potassium acetate, 1 mM magnesium acetate, 0.1 mM EDTA, 1 mM dithiothreitol) containing 1% NP-40. eIF4G was added

to the depleted lysate to reconstitute the translation system, and translation was assayed with Luc mRNA.

#### Phosphopeptide mapping

Phosphorylated eIF4G was excised from the gel and subjected to extensive tryptic digestion (Tuazon *et al*, 1989). The digests were analyzed by two-dimensional phosphopeptide mapping, with thin-layer electrophoresis (pH 3.5) in the first dimension and chromatography in the second dimension, followed by autoradiography.

#### Translation assay in 293T cells

293T cells cultured to 35% confluence were transfected with DNA constructs, as described in the figure legends, using the FuGene6 transfection agent (Roche). Translation was assayed by fluorescence. To induce stress, 30 h post-transfection, dividing cells were subjected to sorbitol (0.4 M) for 30 min and collected (Roig *et al*, 2000b).

With the pRMF reporter, luciferase activities were measured using dual-luciferase reporter assay system (Promega). Equal amounts of the extract were used to assay the rate of cap-dependent translation with Renilla luc (R-Luc) or IRES-driven translation with firefly luc (F-Luc). IRES activity was calculated by normalizing the F-Luc activity to R-Luc as described (Chappell *et al*, 2000).

To quantify luc mRNA the 5' primer used was CTGCATAAGGCT ATGAAG, and the 3' primer was GTTGAGCAATTCACGTTTC, with a 283 bp product. For EGFP, the 5' primer was CCCTGAAGTTCAT CTGC and the 3' primer was CTTGTAGTTGAAGTCG; the product was 194 bp. Total RNA was extracted from cells using the RNeasy kit (Qiagen).

#### Site-directed mutagenesis of eIF4G

Serine 896, the Pak2 phosphorylation site in eIF4G1, was mutated using a Quick-change kit (Stratagene). To mutate serine to alanine, the primer was 5'-CCCGCGGGCTCTTTAGGGAATATC. To mutate serine to aspartic acid, the primer was 5'-CCCGCGGGCGATTAGG GAATATCAAG.

#### Assays for protein:protein interactions

*In vivo*. 293T cells were cotransfected with HA-Pak2 and Myc-eIF4G or deletion mutants of eIF4G using 4 µg of total DNA in 10 cm plates. Cells were lysed in buffer A containing protease and phosphatase inhibitors (Roig *et al*, 2000a). Equal amounts of cell extract (500–800 µg) were incubated for 2 h at 4°C with antibodies prepared to eIFs or the Myc tag, then incubated with protein A/G beads for an additional 2 h. The immunoprecipitates were washed three

times with cell lysis buffer and analyzed by SDS-PAGE and Western blotting.

*In vitro*. The interaction between His/Flag-4G (expressed in insect cells) and eIF4E (expressed in *E. coli*) was examined before and after incubation of eIF4G (0.2 µg) with Pak2, the K/R mutant (30 ng) or buffer alone. Following phosphorylation, eIF4E (0.2 µg) was added in buffer C (20 mM HEPES, pH 7.5, 100 mM KCl, 2.5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 3% glycerol and 0.1% dried milk) with protease and phosphatase inhibitors in a final volume of 100 µl and incubated for 1.5 h at 4°C. Association between the two proteins was analyzed by binding to m<sup>7</sup>GTP-Sepharose or by GST pull-down, followed by SDS-PAGE and Western blotting.

#### Knockdown of endogenous Pak2 by RNAi

To make the RNAi construct, the DNA sequence from aa 197–216 (human Pak2, U24153) was selected for the synthesis of a small-hairpin RNA (Sui *et al*, 2002). The sense DNA oligonucleotide used was 5'-GTCATCTCCATATTCAGTTCAAGAGACCTGAGAATATGG AGATGATTTTIG-3'. The antisense strand was 5'-transcription AATTCAAAATCATCTCCATATTCAGTTCTTGAACCTGAGAATAT GGAGATGAC-3'. The restriction site sequences, the loop sequence and the transcription termination sequence are in bold. The sense and antisense DNA oligonucleotides were annealed and subcloned into pSilencer1.0-U6 (Ambion) by *Apal* (blunt end, 5') and *EcoRI* (3') ligation. The RNAi for the GFP control was prepared according to the method reported (Sui *et al*, 2002).

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