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The adaptor Grb7 links netrin-1 signaling to regulation of mRNA translation

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We previously reported a novel biological activity of Netrin-1 in translational stimulation of kappa opioid receptor (KOR). We now identify Grb7 as a new RNA-binding protein that serves as the molecular adaptor for transmitting Netrin-1 signals, through focal adhesion kinase (FAK), to the translation machinery. Grb7 binds specifically to the first stem loop of *kor* mRNA 5′-UTR through a new RNA-binding domain located in its amino terminus. Upon binding to its capped, target mRNA, Grb7 blocks the recruitment of eIF4E, rendering mRNA untranslatable. The RNA-binding and translation-repressive activity is reduced by FAK-mediated hyperphosphorylation on two tyrosine residues of its carboxyl terminus. This study reports an adaptor protein Grb7 that transmits the stimulating signals of Netrin-1 to the translational machinery to rapidly regulate mRNA translation.

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Introduction

Netrins constitute a family of extracellular proteins that regulate neuronal migration and axonal growth (Baier and Bonhoeffer, 1994). Netrin-1, an axon guidance cue (Kennedy *et al*, 1994), is known to act on its membrane receptor DCC (deleted in colorectal cancer) that interacts with the focal adhesion kinase (FAK) (Li *et al*, 2004; Liu *et al*, 2004; Ren *et al*, 2004), which results in axon attraction and outgrowth. Netrin-1 has also been shown to be involved in chemotropic response by stimulating protein synthesis through the p42/44 MAPK pathway (Campbell and Holt, 2001, 2003), a process required during re-sensitization of retinal axons (Piper *et al*, 2005). Recently, we identified a novel activity of Netrin-1 in stimulating the translation of a G protein-coupled, transmembrane narcotic drug receptor not known for either cell migration or axon growth, the kappa opioid receptor (KOR) (Tsai *et al*, 2006). This novel activity of Netrin-1 on KOR translational activation required the classical FAK pathway and differential partition of *kor* mRNA in various polysomal fractions. However, it was unclear as to the mechanism for transmitting Netrin-1 signals to the translation machinery. It was also unknown how *kor* mRNA was maintained in a silenced state in the cells before stimulation by Netrin-1.

Three types of opioid receptors bind to opioid drugs and endogenous opioid ligands to affect pain sensation, consciousness and autonomic functions (Minami and Satoh, 1995; Law *et al*, 1999; Pasternak, 2004). Despite apparent active transcription of these genes in fully differentiated neurons and embryonal carcinoma stem cells as well as their differentiated neurons (Bi *et al*, 2001; Park *et al*, 2002; Wei and Loh, 2002; Hu *et al*, 2004), a significant portion of these mRNAs were usually maintained in a silent state. With KOR as a model system, we uncovered Netrin-1 as one important stimulator for active translation (Tsai *et al*, 2006). In this current study, we aimed to determine the molecular effectors that could trans-repress *kor* mRNA by carrying out a three-hybrid screening experiment, aiming to identify specific RNA-binding proteins that could repress KOR translation. In this endeavor, a surprising target was identified as a potent translational repressor of KOR that appeared to be a new RNA-binding protein, Grb7.

Grb7 is the founding member of an emerging family of signaling adaptors called growth factor receptor bound proteins 7 (Grb7) (Daly, 1998). Grb7 consists of an amino-terminal proline-rich region, a central GM region and a carboxyl-terminal src-homology 2 (SH2) domain. The central GM domain plays an essential role in mediating cell migration, whereas the SH2 domain is responsible for its association with the FAK. However, no biological function was assigned to the amino-terminal proline-rich region (Han *et al*, 2001). This study revealed the location of the specific RNA-binding domain of Grb7 within this previously mysterious, proline-rich, amino-terminal domain and reported Grb7 as a new RNA-binding protein that served as the molecular adaptor to transmit Netrin-1 signals through FAK to regulate kor translation.

Results

A functional role for Grb7 in regulation of translation by Netrin-1

Previously we identified a novel activity of Netrin-1 in stimulating KOR protein synthesis in both P19 embryonal carcinoma stem cells and primary neurons through the mediator of Netrin-1, FAK (Tsai *et al*, 2006). In a three-hybrid screening experiment with the 5′-UTR of *kor* mRNA as the bait, we identified Grb7 as a specific RNA-binding protein (see later Figures 3 and 4). FAK was known to act on Grb7 by tyrosine phosphorylation (Han and Guan, 1999; Han *et al*, 2000). To determine if the translation-stimulating effect of Netrin-1 involved Grb7, we first confirmed the expression of Netrin-1 receptor, DCC, in P19 cells (Supplementary Figure S1), and then compared the kinetics of the activation of FAK and of Grb7 in P19 cells stimulated by Netrin-1 to that of the expression of endogenous KOR (Figure 1A). Tyrosine phosphorylation was significantly elevated for both FAK and Grb7

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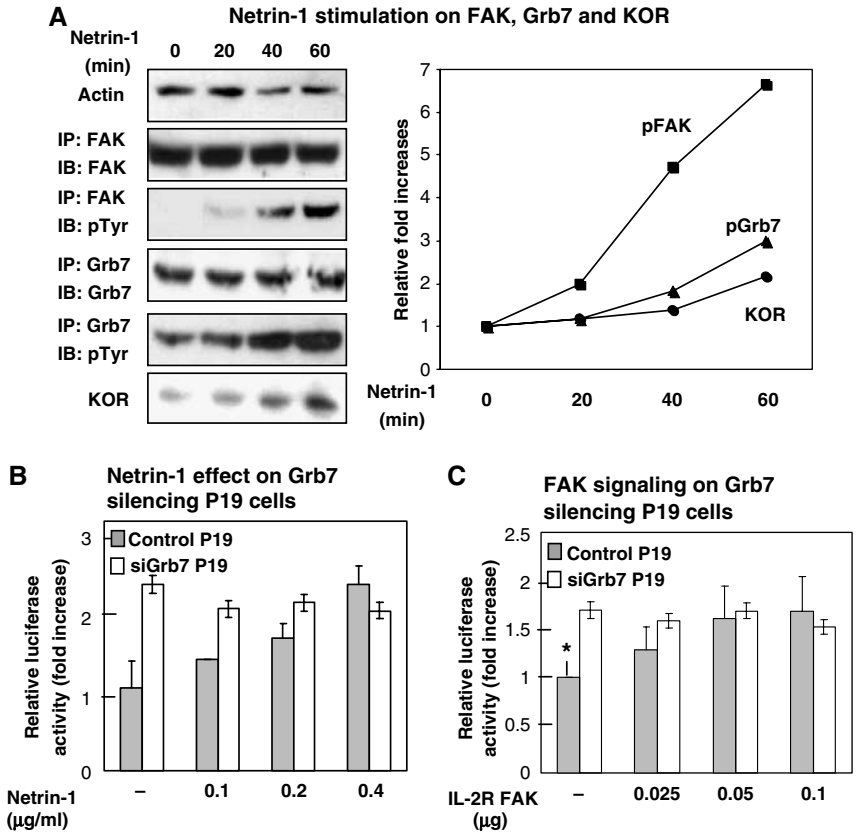

Figure 1 Netrin-1/FAK signaling induces KOR expression. (**A**) Netrin-1 induced FAK and Grb7 phosphorylation. P19 cells were treated with 0.2 μg/ml netrin-1 and subjected to immunoprecipitation/Western blot with specific antibodies. The intensities of phospho-FAK (pFAK), phospho-Grb7 (pGrb7) and KOR were quantified and plotted on the right. (**B**) Relative KOR reporter activities in the control or Grb7-silenced P19 cells. The reporter activity in the control cells without netrin-1 was given an arbitrary value of 1 ($^*P<0.05$). (**C**) The relative reporter activities in the control or Grb7-silenced P19 cells supplemented with constitutively active FAK, IL2R-FAK. Relative reporter activities were determined as described in (B) ($^*P<0.05$). The error bars represent the standard deviations from three independent experiments.

at 20–40 min after the treatment of Netrin-1, in consistence with the increase of KOR protein. The functional role of Grb7 in mediating the effect of Netrin-1 was addressed by employing a KOR UTR-containing reporter in both the control P19 and its stable clones with the endogenous Grb7 silenced. The KOR UTR reporter contains the intact 5′-UTR and 3′-UTR, fused in frame to a luciferase coding sequence. As shown in Figure 1B, this UTR reporter was stimulated by Netrin-1 in a dose-dependent manner in control P19 (filled bars), whereas this UTR reporter failed to respond to Netrin-1 in the Grb7-silenced P19 clones (empty bars). To determine the role of FAK, a constitutively active FAK (IL2R-FAK) (provided by Dr K Yamada) (Akiyama *et al*, 1994) that could bypass the treatment of Netrin-1 was employed (Figure 1C). The result confirmed the activation of this UTR reporter by the constitutively active FAK without the addition of Netrin-1. As predicted, this reporter failed to respond to the constitutively active FAK in the Grb7-silenced cells (empty bars). These results showed that Grb7 was required for Netrin-1 stimulation of KOR expression, which was mediated by the classical FAK signaling pathway.

Grb7 as a new RNA-binding protein and constitutive translational repressor

The higher UTR reporter activity in Grb7-silenced cell (Figure 1B and C) suggested that Grb7, without hyperphosphorylated by FAK, might downregulate KOR expression. To examine whether this repressive effect was at the level of transcription, translation or protein stability, we first supplemented P19 cells with exogenous Grb7 in the absence of Netrin-1, and determined the endogenous levels of *kor* mRNA and protein (Figure 2A). In these Grb7-overexpressing cells, the expression of endogenous KOR protein (the second panel on the left), but not its mRNA (the bottom panel on the left), was severely blunted. On the contrary, in Grb7-silenced (for approximately 50% in protein level) P19 clones, the level of KOR protein was significantly elevated (the third panel on the right). This result was verified with two additional pairs of Grb7 siRNA (data not shown). Thus, Grb7 acted at a post-transcriptional level. The repressive activity of Grb7 was further verified using a KOR UTR reporter (Figure 2B) that contains only the Grb7-targeted 5′-UTR of *kor* mRNA (see following Figures 3 and 4), which was effectively repressed by Grb7 (open bars). As a control, the 3′-UTR reporter was not affected (filled bars). We further determined the rescuing effect in siGrb7-treated cells using the 5′-UTR reporter and an siRNA-resistant Grb7 that is altered in siRNA-targeted sequence without changing its amino-acid sequence. The data confirmed repression of this reporter by the siRNA-resistant Grb7 (Supplementary Figure 2). This finding strongly supports the notion that Grb7 exerts its repressive effect at the level of translation, rather than protein stability. To

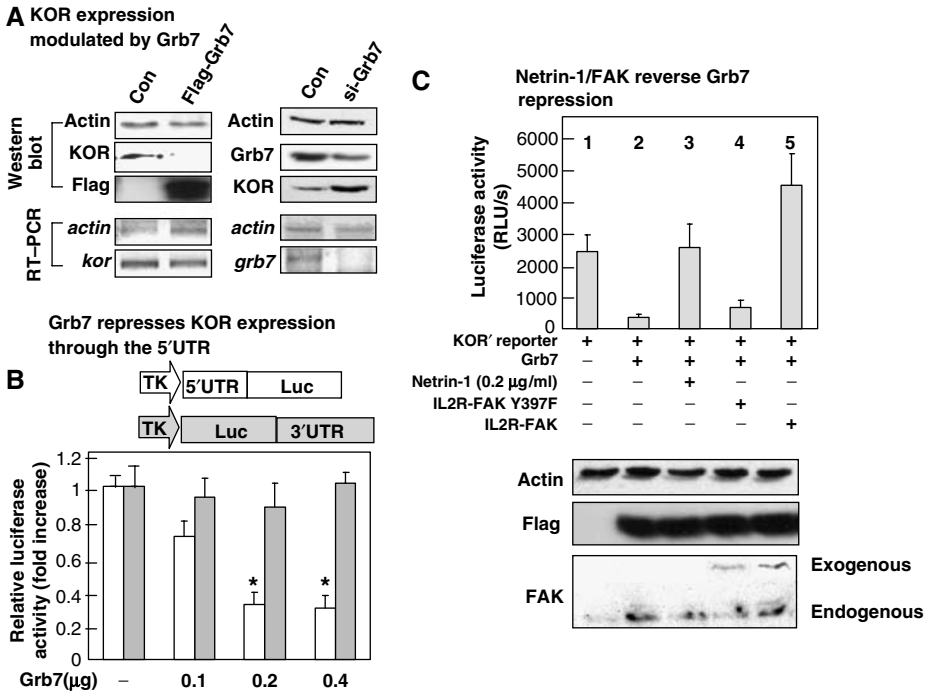

Figure 2 Grb7 represses KOR translation by acting on its 5′-UTR. (**A**) Decreased KOR translation by elevating Grb7 (left) and increased KOR translation by silencing the endogenous Grb7 in P19 cells (right). The steady-state level of *kor* mRNA was not affected significantly (bottom). (**B**) The effects of Grb7 on the reporters of the 5′- or 3′-UTR of *kor* mRNA. (*$P<0.05$). (**C**) The KOR reporter activities in the presence of Grb7 and its upstream signal Netrin-1 constitutively active (IL2R-FAK), or inactive (IL2R-FAK-Y397F) FAK. The error bars represent the standard deviations from three independent experiments.

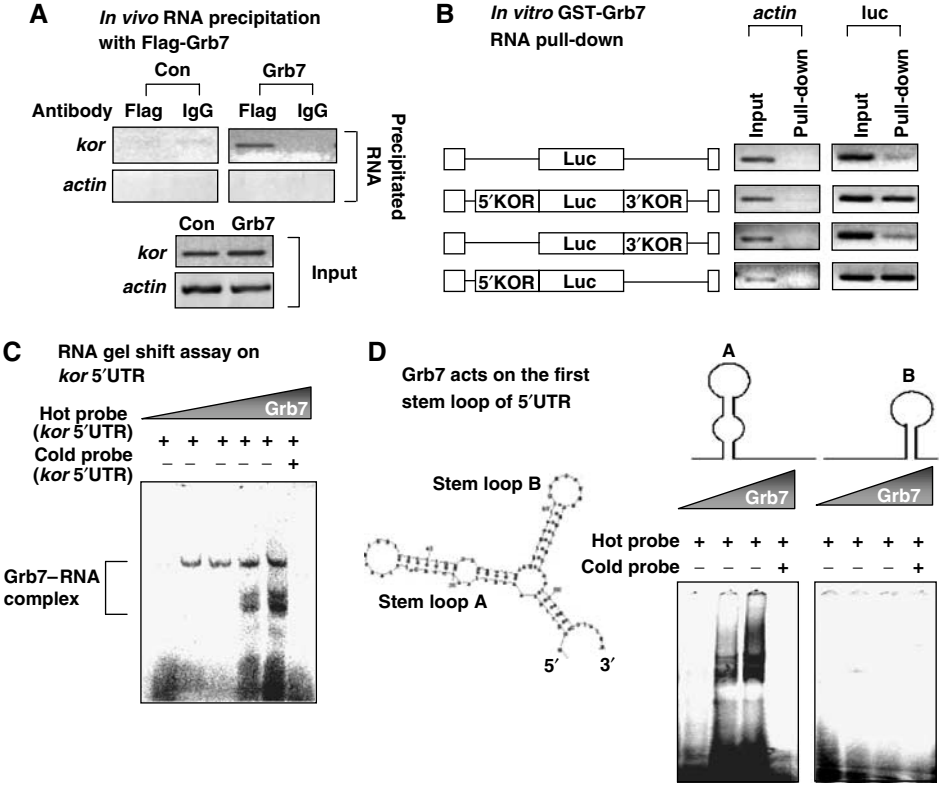

Figure 3 Grb7 binds to the 5′-UTR of *kor* mRNA. (**A**) Grb7-immunoprecipitation of the endogenous *kor* and *actin* (negative control) mRNAs from the control (left) or Flag-Grb7 transfected (right) P19 cells. Input was shown at the bottom. (**B**) GST-Grb7 pull-down of RNAs carrying various *kor* sequences produced in P19 cells. The precipitated RNA was quantified with RT–PCR. (**C**) RNA gel-shift using ^{33}P-labeled *kor* 5′-UTR and increasing amounts of GST-Grb7. (**D**) The predicted *kor* RNA secondary structure (left) and RNA gel-shift assays of two specific stem loops in the 5′-UTR of *kor* mRNA.

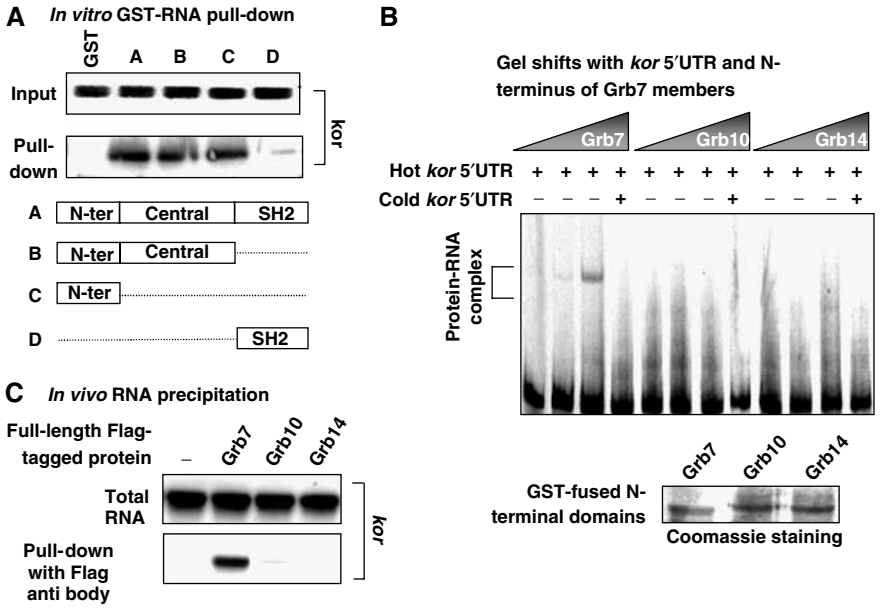

Figure 4 The amino-terminus of Grb7, but not Grb10 or Grb14, exhibits the specific RNA-binding property. (**A**) Southern blot quantification of the GST-RNA pull-down assay. Different deletions of Grb7 were used to pull down *kor* mRNA from P19. (**B**) RNA gel-shift using ^{33}P-labeled *kor* 5′-UTR and the amino-terminus of Grb7, Grb10 and Grb14. Commassie blue staining of purified amino-terminal proteins was shown at the bottom. (**C**) RT–PCR/Southern blot analyses of *kor* mRNA immunoprecipitated from P19 transfected with Grb7, Grb10 or Grb14.

delineate the signaling pathway of Netrin-1, the KOR 5′-UTR reporter was used to examine the key steps, including Netrin-1, FAK and Grb7 (Figure 2C). As predicted, Grb7 functioned as a constitutive repressor for KOR expression (column 2), which could be reversed by Netrin-1 (column 3) or the constitutively active FAK, IL2R-FAK (column 5), but not by the constitutively inactive FAK, IL2R-FAK (Y397F) (column 4). It was confirmed that repression by Grb7 could be relieved by Netrin-1 stimulation, or the activation of FAK.

Grb7 was cloned from a three-hybrid screening experiment for proteins binding to the 5′-UTR of *kor* mRNA. This was verified in several *in vivo* and *in vitro* RNA–protein interaction assays. Figure 3A shows co-immunoprecipitation of the endogenous *kor* mRNA with ectopically expressed Flag-Grb7 in P19 cells. Figure 3B shows a GST protein pull-down assay using partially purified GST-Grb7 protein and RNA templates containing various regions of *kor* mRNA sequence, which confirmed that Grb7 bound, primarily, to the 5′-UTR of *kor* mRNA. Figure 3C shows an RNA gel-shift assay using *in vitro* prepared Grb7 protein and the labeled 5′-UTR of *kor* mRNA (Figure 3C), confirming a direct RNA-binding activity of Grb7. With the help of Mfold software (Zuker, 1999), the secondary structure of this 5′-UTR was predicted, which could form two major stem loops, A and B (left of Figure 3D). The 5′-UTR was truncated into loop A and loop B, and each was tested in RNA gel-shift experiments (Figure 3D). According to this result, the specific Grb7-binding region was mapped to loop A of this 5′-UTR. A series of loop A mutants defected in the pairing of the stem were made and found to lose their abilities to interact with Grb7, whereas the loop B mutants still effectively interacted with Grb7 (unpublished data). Therefore, it is likely that Grb7 binds to the upstream region of *kor* 5′-UTR that can potentially adopt a stem loop structure.

While Grb7 appeared to bind a specific KOR sequence, it encoded none of the previously reported RNA-recognition motifs. Grb7 was then dissected into three segments, that is, amino-, central and carboxyl domains according to earlier study (Han *et al*, 2001), and tested in both GST protein-RNA pull-down (Figure 4A) and RNA gel shift (Figure 4B) assays. Figure 4A revealed the amino-terminal domain as the smallest RNA-interacting domain of Grb7 (construct C). This was further confirmed in the RNA gel shift assay where the GST-tagged amino-terminal domain of Grb7 was used (Figure 4B). Since three Grb7 members have been reported, Grb7, 10 and 14, the amino-terminal domain of Grb7 was then compared to that of Grb10 and Grb14 in the same gel shift assay. It appeared that only Grb7 could bind specifically to *kor* mRNA. This was confirmed in the co-immunoprecipitation assay using the full-length proteins (Figure 4C) where neither the full-length Grb10 nor the full-length Grb14 was able to be associated with *kor* mRNA. These experiments confirmed Grb7 as a specific RNA-binding protein that bound, minimally, to the sequence that can potentially adopt a stem loop in the 5′-UTR of *kor* mRNA.

The target of Grb7 in translation

Grb7 repressed KOR expression primarily at the level of translation (Figures 1 and 2). *In vitro* translation was carried out to directly evaluate the effect of Grb7 on KOR translation (Figure 5A). As shown, translation of templates containing the 5′-UTR, but not those deleted in the 5′-UTR, was effectively blocked by Grb7. We further determined if Grb7 affected uncapped RNA templates. As shown in Figure 5B, translation of the capped templates was effectively blocked by Grb7 (lanes b) whereas translation of the uncapped templates, despite a much higher amount (five-fold) of RNA template was provided, was not affected (lanes a). This series of experiments revealed a translationally repressive role for Grb7 that targeted at the 5′-UTR of the capped *kor* mRNA. To identify the step in translation that might be targeted by Grb7, we first examined if Grb7 affected ribosome loading on the target RNA in a series of sedimentation analyses of ribosome-

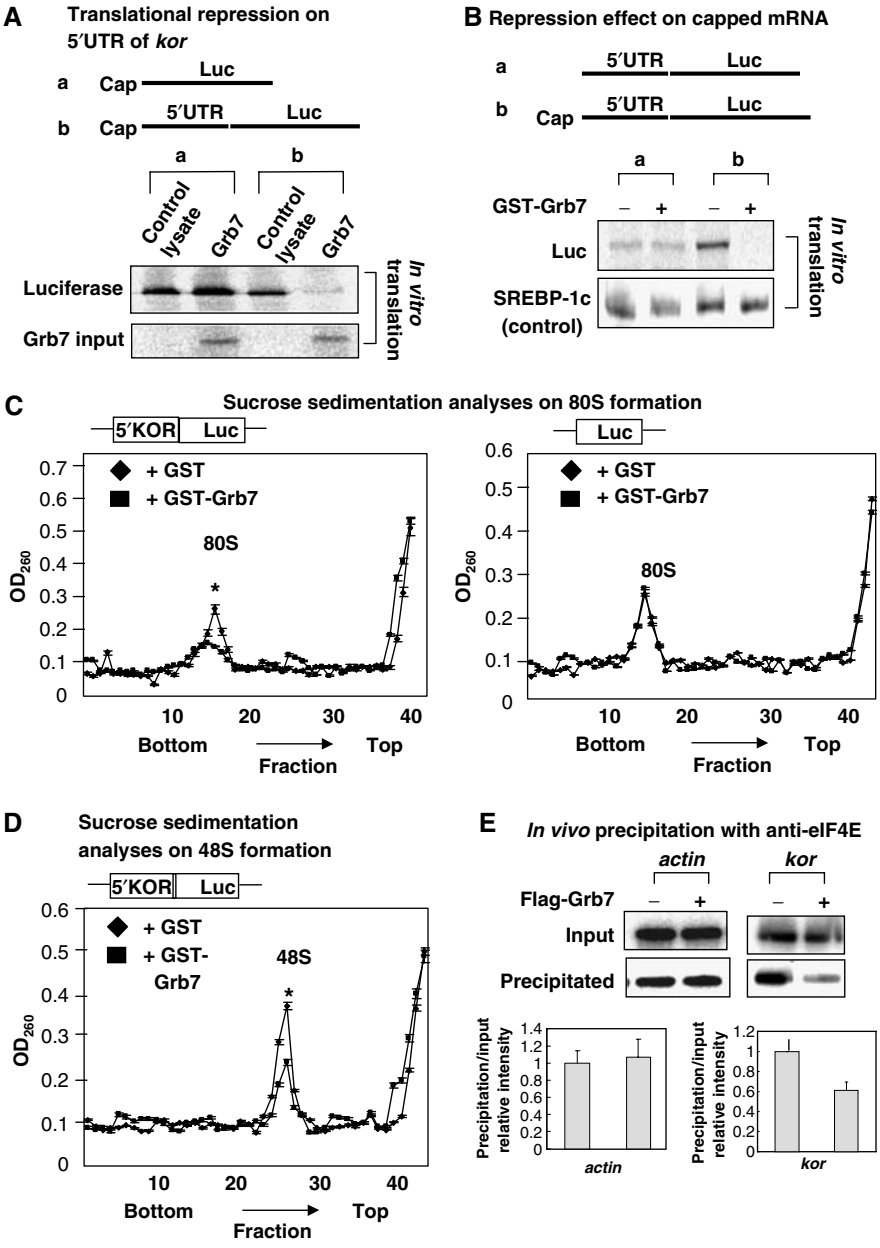

Figure 5 Grb7 represses KOR translation by blocking its 5′-end recognition of the translation machinery. (**A**) *In vitro* translation of reporter mRNA in the absence or presence of *kor* 5′UTR and Grb7. Bottom: Grb7 input. (**B**) *In vitro* translation of non-capped (a) and capped (b) KOR reporters with or without Grb7. Capped and uncapped mRNAs of sterol regulatory element binding protein-1c (SREBP-1c) (bottom) were included as the controls. (**C**) Sucrose sedimentation analyses of transcripts carrying the 5′-UTR of *kor* (left) or a negative control (right). *In vitro* synthesized transcripts were incubated with GST (diamond) or GST-Grb7 (square) in the presence of cycloheximide, and subjected to sucrose gradient analyses. (**D**) Sucrose sedimentation analyses of transcripts carrying the 5′-UTR of *kor* in the presence of GMP-PNP. (**E**) Precipitation of *kor* and *actin* mRNAs by anti-eIF4E from the control (−) or Flag-Grb7 expressing (+) P19 cells. Quantified results are shown at the bottom ($*P<0.05$). The error bars represent the standard deviations from three independent experiments.

associated *kor* mRNAs. With cycloheximide to stall the elongation step in translation, it was found that Grb7 reduced the association of the 80S ribosome subunit with *kor* transcripts (Figure 5C, left). For the control template without the 5-UTR, the association of 80S ribosomes was not affected (right). It was noticed that a small peak appeared after the 80S position in the experiment of Grb7 association with the 5′-UTR, which was probably from non-ribosomal aggregates of kor transcripts with either GST-Grb7 or some contaminating proteins in the GST-Grb7 preparation. The result suggested that Grb7 represses the step before elongation proceeds but after capping of the RNA. With GMP-PNP to prevent GTP hydrolysis and 60S subunit joining, it was found that Grb7 significantly reduced the formation of 48S subunit on the template (Figure 5D), suggesting a target for Grb7 prior to start codon (ATG) recognition. The earlier steps in translation initiation were speculated, such as the recognition of the capped mRNA by eIF4F members and the subsequent 40S subunit recruitment. Figure 5E showed that Grb7 caused a significant reduction in the amount of eIF4E-associated, endogenous *kor* mRNA (right panel), but it exerted no effect on eIF4E loading to the *actin* mRNA (left panel). This result

demonstrated that Grb7 acted on *kor* mRNA by blocking eIF4E recognition of the capped 5′-end of *kor* mRNA, thereby reducing or hindering the formation of preinitiation complex.

Activation of Grb7 by FAK and concurrent stimulation of translation

To both the endogenous *kor* mRNA and the KOR UTR reporter, Grb7 acted as a constitutive translational repressor in P19 cells (Figure 2). However, Netrin-1 exerted a rapid stimulating effect on KOR translation and the concurrent activation (phosphorylation) of its mediator FAK, as well as the phosphorylation of the substrate of FAK, Grb7 (Figure 1A). While Grb7 appeared to be phosphorylated following Netrin-1 stimulation, or FAK activation, it was unclear how Grb7 was activated by phosphorylation and where were/was its site(s) of phosphorylation.

To determine the effects of Grb7 phosphorylation (by FAK) on its RNA-binding activity, phosphorylated Grb7 was prepared (two top panels of Figure 6A) and tested in a direct RNA interaction test (lower panels of Figure 6A). According to the quantitative analysis shown on the right, it appeared that phosphorylation of Grb7 severely blunted its direct interaction with *kor* mRNA (pGrb7 panel). Consistently, the repressive activity of Grb7, as determined by *in vitro* translation assay (Figure 6B), was also abolished by its phosphorylation (pGrb7 panel). These data showed that phosphorylation of Grb7 by FAK severely hindered its direct binding to *kor* mRNA, thereby releasing *kor* mRNA for the loading of the translation initiation complex.

To map the sites of phosphorylation by FAK, the three domains (amino-terminal, central and carboxyl terminal) of Grb7 were each tagged with GST and subjected to *in vitro* phosphorylation by FAK in the presence of γ-^{32}P-ATP (Figure 7A). Interestingly, the site of FAK-triggered phosphorylation on Grb7 was located outside its RNA-binding domain, but in its carboxyl terminal, SH2 domain (arrow, construct D). Within this domain, two potential residues for tyrosine phosphorylation were located at Y483 and Y495. Three mutated carboxyl terminal domains were then produced, including two single mutants (Y483F and Y495F) and a double mutant (Y483F/Y495F), and each was tested *in vivo* in the presence of the constitutively active FAK, IL2R-FAK (Figure 7B). It appeared that all three mutants were drastically defected in phosphorylation by FAK, despite they all exhibited a similar FAK-binding ability (Figure 7B, the bottom panel).

To confirm that the decreased phosphorylation of mutants was not caused by altered property or protein quality of the SH2 domain in living cells, *in vitro* reaction of GST-Grb7 mutants (Y483F, Y495F and Y483F/Y495F) was conducted where the quality and quantity of proteins was also monitored (Figure 7C). The result was consistent with that of the *in vivo* study. This was further confirmed with *in vitro* Grb7-RNA interaction tests where, following *in vitro* FAK phosphorylation, the mutated GST-Grb7s remained binding to *kor* mRNA whereas the wild-type GST-Grb7 bound *kor* mRNA very poorly (Supplementary Figure S3). In the *in vitro* translation assay (Figure 7D), only the wild-type GST-Grb7, following FAK-phosphorylation, failed to repress kor translation (Figure 7D). All these results supported that both Y483 and Y495 residues of Grb7 were critical for its phosphorylation by FAK that regulated its RNA-binding and translation-repressive activity.

To further verify the effects of FAK-mediated phosphorylation of Grb7 on its RNA-binding and its repressive activity *in vivo*, two experiments were carried out using the wild type and mutant Flag-Grb7s constructs. Figure 7E showed the RNA–protein interaction test conducted in P19 activated by the constitutive active FAK. As predicted, the wild-type Grb7 (Grb7 WT) failed to efficiently interact with *kor* mRNA in these FAK-activated cells, but all the three mutants remained effectively associated with *kor* mRNA even in the presence of the active FAK (lower three panels on the right), supporting that mutation of Grb7 on either one of the two FAK sites disrupted its release from the RNA target. Figure 7F showed the effect of phosphorylation on the biological activity of Grb7 as a translational repressor for the KOR UTR reporter. Consistently, this UTR reporter was dramatically repressed by the wild-type Grb7 in P19, which was reversed by the addition of activating FAK (the WT group). All three Grb7 mutants remained as effective repressors even in the presence of the activating FAK (groups of Y483F, Y495F and Y483F/Y495F), further supporting that phosphorylation of Grb7, by FAK, abolished its activity as a translational repressor, coinciding with the effect on its RNA-binding activity.

This series of experiments demonstrated that Grb7 acted as a translational repressor by binding to its RNA target through its amino-terminal domain and blocking eIF4E loading. Its activity could be reversed by FAK-triggered hyperphosphorylation on its SH2 domain, which could be activated by Netrin-1. Thus, Grb7, an adaptor of FAK and a new RNA-binding protein, served as an important translational repressor of its target mRNA in cells when extracellular stimulus was absent,

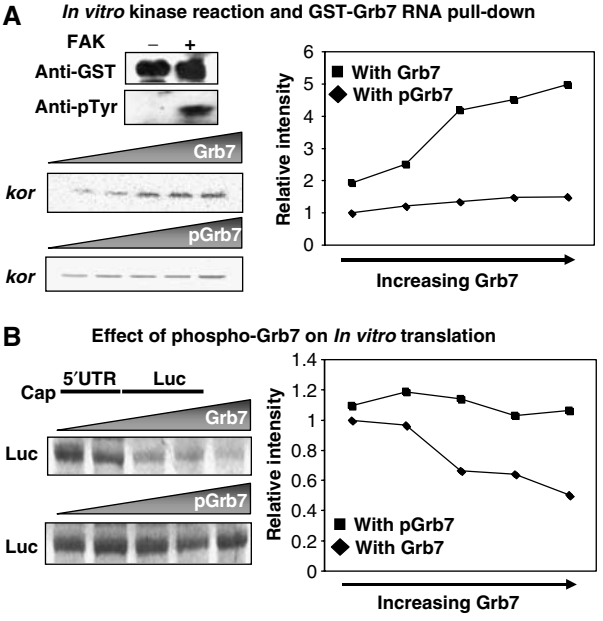

Figure 6 Grb7 responds to FAK activation by tyrosine phosphorylation, relieving translational repression. (**A**) The effects of Grb7 phosphorylation on its interaction with *kor* mRNA. Top: *in vitro* phosphorylated. Lower: GST-Grb7 pull-down of *kor* mRNA using various amounts of *in vitro* prepared Grb7 and its phosphorylated counterpart (pGrb7). Quantitative measurement is shown on the right. (**B**) *In vitro* translation assays of the reporter in the presence of Grb7, or its phosphorylated counterpart (pGrb7). Quantitative measurement is shown on the right.

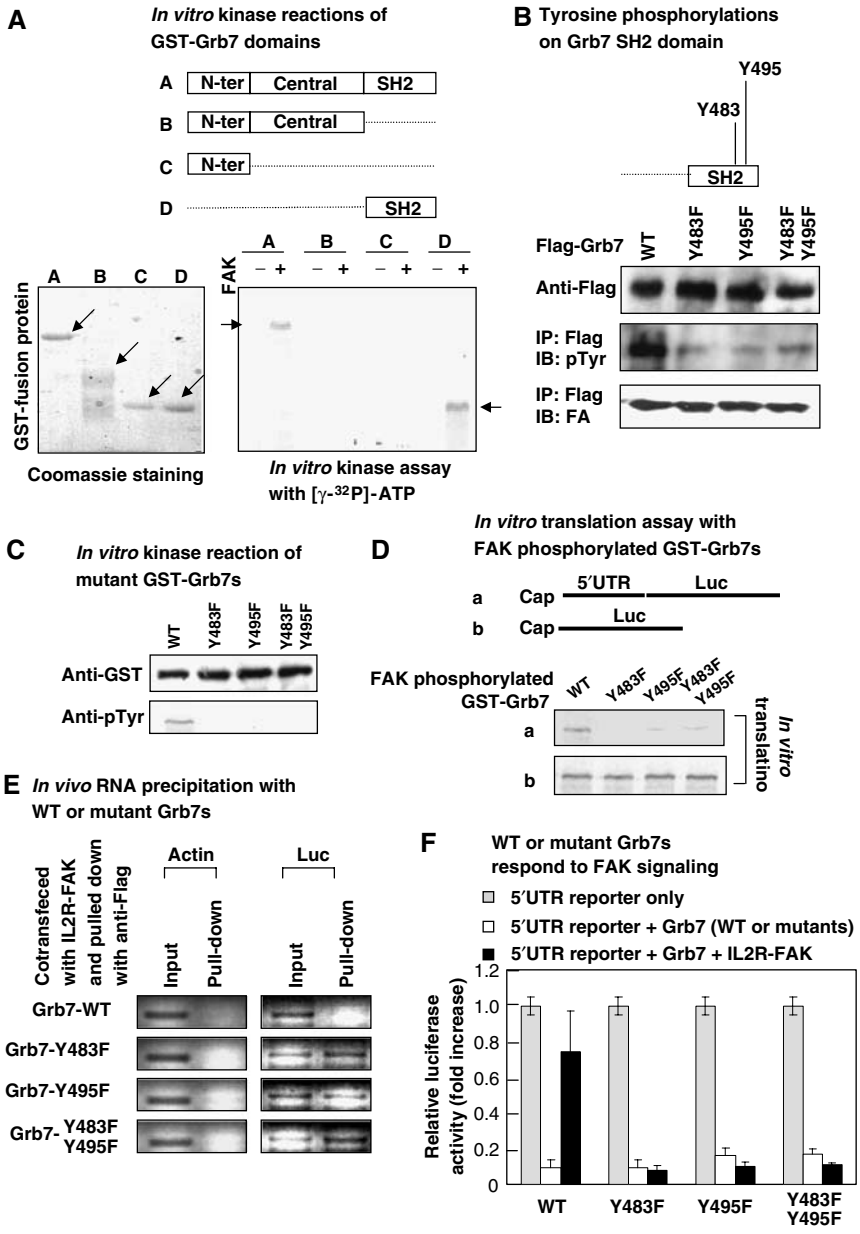

Figure 7 Tyr483 and Tyr495 of Grb7 are critical for FAK-mediated phosphorylation. (**A**) *In vitro* kinase reactions of different GST-Grb7 proteins. (**B**) Immunoprecipitation and Western blot analyses of samples from P19 cells transfected with IL2R-FAK with Flag-Grb7, Flag-Grb7 (Y483F), Flag-Grb7 (Y495F) or Flag-Grb7 (Y483F/Y495F) using anti-Flag, anti-phospho-tyrosine and anti-FAK antibodies. (**C**) Western blot analyses of *in vitro* FAK-phosphorylated wild type, Y483F, Y495F or Y483F/Y495F GST-Grb7 using anti-GST and anti-phospho-tyrosine antibodies. (**D**) *In vitro* translation assays of the control reporter (a) and kor 5′UTR reporter (b) with FAK-phosphorylated wild type, Y483F, Y495F or Y483F/Y495F GST-Grb7. (**E**) Anti-Flag immunoprecipitation of the endogenous *kor* and negative control (actin) mRNAs from P19 cells transfected with Flag-Grb7, Flag-Grb7 (Y483F), Flag-Grb7 (Y495F) or Flag-Grb7 (Y483F/Y495F) and IL2R-FAK. (**F**) Relative reporter activities of the *kor* 5′UTR reporter in the absence or presence of IL2R-FAK, together with various Grb7 constructs. The error bars represent the standard deviations from three independent experiments.

and provided a critical switch to activate translation of its target mRNA when cells were exposed to extracellular signals that activated the FAK pathway.

Discussion

A new functional role for Grb7 in translational regulation

The extracellular signals are known for their effects on various downstream biological processes including translation (Rajasekhar *et al*, 2003). Grb7 is known to be involved in cell migration. This study uncovers a new functional role for Grb7 in mediating extracellular signal transduction to the translational machinery. Further, this functional role depends directly upon its newly discovered molecular feature, the specific RNA-binding property.

It is known that important mRNAs are usually protected by RNA-associated proteins if they are not immediately used for protein synthesis (Huttelmaier *et al*, 2005; Evdokimova *et al*, 2006; Goldstrohm *et al*, 2006). Our previous discovery of the predominantly silent status of *kor* mRNA predicted a translationally repressive apparatus for *kor* mRNA. The three-hybrid

screening experiments allowed us to identify Grb7 as one specific *kor* mRNA-binding protein that is also a translational regulator. Interestingly, while Grb7, without activation by phosphorylation, serves as a constitutive translational repressor by binding directly to its RNA target and preventing eIF4E recruitment, it can be directly activated by FAK-mediated phosphorylation on two tyrosine residues that are outside its specific RNA-binding domain. As a result of this FAK-mediated activation, the phosphorylated Grb7 immediately releases its target message for the loading of the translational initiation complex, allowing active translation to occur within minutes. This provides a most direct and efficient mechanism of translational activation that can very rapidly respond to extracellular stimuli without the need to synthesize new components. The unique property of Grb7, as an RNA-binding repressor of translation and a specific kinase target, provides the cells with a rapidly adjustable capacity for translational regulation by endowing its molecule a dual function which, on the one hand, efficiently represses its target mRNA and, on the other hand, rapidly responds to specific signals by switching off its RNA-binding property. This represents a novel double-edged device that cells employ to respond to extracellular signals and engage in productive actions.

Grb7 as a new regulated RNA-binding protein

The specific RNA-binding domain of Grb7 is located in its previously mysterious amino terminus that exhibits no particular, recognizable structural features. Alignment using the service provided by EMBL (European Molecular Biology Laboratory) and our results confirm this domain as a new RNA-binding domain, which has to be validated by structural studies in the future. Interestingly, its RNA-binding activity is regulated by its status of phosphorylation. Intriguingly, the phosphorylated residues that regulate its RNA-binding property reside outside the RNA-binding domain. It remains to be determined how the phosphorylation of these residues outside the direct RNA-binding domain affects its RNA-binding ability. Presumably, structural changes occur to the RNA-binding domain as a result of its interaction with the carboxyl terminal SH2 domain, which is regulated by its status of phosphorylation by FAK. Alternatively, the phosphorylation of the carboxyl terminus alters its own structure that is then propagated to alter the structure of the amino-terminus directly binding to the RNA. Further studies are needed to explore these scenarios.

Target of Grb7 in translational regulation

As an RNA-binding protein, Grb7 directly represses target translation by blocking the loading of the critical translation initiation factor, eIF4E. This is consistent with the finding that it only represses capped mRNAs that bear its recognition site. Currently, a well-established model for the effect of the iron-regulatory RNA-binding proteins (IRPs) on the iron-responsive elements of *ferritin* mRNA proposes that this interaction blocks the early translational initiation step (Thomson *et al*, 1999). The IRE/IRP complex allows eIF4E binding onto the 5′ cap of *ferritin* mRNA but prevents the 43S preinitiation complex from being loaded to the mRNA. However, in the case of Grb7-RNA complex, the distance between the 5′cap structure and the Grb7 binding region is less than 40 nucleotides, much shorter than 60 nucleotides found in the case of IRE. Thus, the Grb7-mediated repression appeared to employ a mechanism different from that of the IRE-mediated repression.

Through specific binding, Grb7 blocks the recognition of the selected mRNAs by the translational initiator complex, thereby efficiently masking these specific mRNA targets without affecting the general translational machinery. Once it is phosphorylated, Grb7 immediately loses its RNA-binding activity, allowing the translational machinery to access its otherwise masked target messages without synthesizing new components. Thus, Grb7-binding appears to be the rate-limiting step in translational regulation of Grb7-targeted mRNA species. Theoretically, this mechanism could provide one very specific, reversible, and transient mechanisms to modulate only those desired targets without jeopardizing the overall cellular environment. In light of the predominant expression of KOR in neurons, this type of regulatory mechanism can be particularly significant because specific local protein synthesis is critical for neuronal activity. The plasticity of a neuron depends critically on its ability to selectively modulate the expression of certain proteins in specific compartments without drastically altering its cellular machinery. A general translational repressor in certain neuronal compartments would probably be detrimental to its survival and function. As such, a small adaptor molecule like Grb7 can be the most effective and energy-conserving device to transmit extra cellular signals to regulate specific, maybe transient, translation for the synthesis of certain proteins that are needed only when cells are stimulated. Intriguingly, among the known Grb7 family members, only Grb7 can bind to *kor* mRNA. Therefore, it remains to be determined whether the rest of Grb7 members can bind to other RNAs.

Netrin-FAK-Grb7 in opioid biology

Grb7 is the downstream target of FAK and other environmental signals, such as epidermal growth factor (Hauck *et al*, 2001) and integrin (Sawhney *et al*, 2006). Presumably, the activating FAK–Grb7 pathway can integrate multiple signal crosstalks by regulating the translation of specific mRNAs. Our previous study showed that Netrin-1 stimulated KOR translation in both P19 cell and DRG neurons. Interestingly, we also observed Grb7 expression in DRG neuron (unpublished), although Grb7 was not significantly detected in the samples of whole brain (Margolis *et al*, 1992). It will be an important task in the future to determine if Grb7-mediated translational regulation also occurs in neurons such as DRG. Equally important, it should be explored whether Grb7 can regulate other target mRNAs.

KOR is known as a classical narcotic drug receptor, but not a growth factor or axon guidance-cue responder. Our findings that KOR synthesis can be stimulated by Netrin-1 and is mediated by a growth factor adaptor Grb7 invite an important question that whether the classical narcotic drug receptor can potentially be involved in functions other than analgesia. Given the established pharmacological role for KOR in mediating the analgesic activity of certain opioid compounds and its endogenous ligand, it is also important to investigate how the neuronal circuit of analgesia may be regulated by, or integrated with, growth factors or axon guidance cues of neurons.

Materials and methods

Antibodies and reagents

Antibodies were from Santa Cruz Biotechnology (anti-KOR, anti-Grb7 and anti-GST), Cell signaling (anti-eIF4E), Sigma-Aldrich (anti-Flag) and Upstate Biotechnology (anti-FAK, anti-Actin and anti-phospho-Tyr). γ-^{32}P-ATP, ^{33}P-UTP and ^{35}S-Met/Cys were from MP Biomedical Inc.

Yeast three-hybrid screening and plasmid constructs are provided in Supplementary data. Transfection, reporter assay, immunoprecipitation and Western blot were performed as described (Huq *et al*, 2006; Tsai *et al*, 2006).

Sucrose sedimentation, in vitro transcription, translation and RNA gel-shift assays

Sucrose gradient sedimentations and translational analyses were performed as described (Uchida *et al*, 2002). Protein products were analyzed by SDS–PAGE, exposed to Phosphoimager (Molecular Dynamics). Transcripts were synthesized *in vitro* with MAXIscript or mMESSAGE mMACHINE T7 Ultra (Ambion). *In vitro* translation was conducted using TNT Cell-Free Protein Expression (Promega). RNA gel-shift was performed as described (Bouvet *et al*, 1997) using purified GST-fusion proteins. All *in vitro* assays were conducted at least three times.

In vitro kinase reaction

Specific FAK reaction was performed as described (Beggs *et al*, 1997) using immunoprecipitated FAK and washed with a buffer (50 mM Tris–HCl, pH 7.5, 3 mM $MnCl_2$, 3 mM $MgCl_2$, 100 mM NaCl). GST-Grb7 was incubated with precipitated FAK in the presence of γ-^{32}P-ATP for 30 min at 37°C, analyzed by SDS–PAGE, and followed by Phosphoimager (Molecular Dynamics) detection or Western blot with anti-phospho-tyr antibody.

GST-RNA pull-down, RNA-immunoprecipitation and semiquantitative RT–PCR

GST-Grb7 proteins were bound to Glutathione beads, and incubated with total RNA from P19 for 1 h. After washing, RNAs were recovered with TRIzol. For RNA-immunoprecipitation, P19 cells were sonicated, incubated with antibodies for 1 h at 4°C, and with protein-G beads for another 1 h. After three times washing, RNAs were recovered using TRIzol. For RT reactions, 2 μg RNA was used to synthesize cDNA (Invitrogen). Specific primers are 5′-CAT CAT CAG GAA ACT GCA-3′/5′-TGG TCA TGT TTG TCA TC-3′ (*kor*) and 5′-CTT CGC CAA GTA TGA ACT ATT C-3′/5′-GTT GGG CTT GAC ACA GAA GCC-3′ (*grb7*). RT–PCR result was subjected to Southern blot as described (Tsai *et al*, 2006).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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