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Regulation of IGF-1/PI3K/Akt signalling by the phosphoinositide phosphatase pharbin

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Pharbin, a 5-phosphatase that induces arborization, is one of the phosphoinositide 5-phosphatases that is highly mutated in patients with Joubert syndrome. Pharbin can hydrolyse PI(4,5)P_2 and PI(3,4,5)P_3 and has the same substrate specificity as SHIP2 and SKIP, which negatively regulate PI3K signalling. Here, we investigated the role of pharbin in IGF-1/PI3K signalling. Ectopic expression of pharbin markedly suppressed the IGF-1-induced activation of Akt without affecting p42/44 MAP kinase phosphorylation. In contrast, pharbin silencing by RNA interference increased the IGF-1-induced phosphorylation of Akt, suggesting that pharbin negatively regulates PI3K/Akt signalling. Pharbin expression also inhibited the phosphorylation of p70 S6 kinase and 4E-BP1 as well as the subsequent protein synthesis in response to IGF-1 treatment. Taken together, these results indicate that pharbin is an important negative regulator of IGF-1/PI3K/Akt signalling and protein synthesis.

Keywords: Akt/IGF-1/Pharbin/PI3 kinase/protein synthesis.

Abbreviations: Grb2, growth factor receptor-bound protein 2; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; p70 S6K, 70-kDa ribosomal protein S6 kinase; PDK1, phosphoinositide-dependent kinase 1; pharbin, a 5-phosphatase that induces arborization; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; PI(3,4,5), P_3, phosphatidylinositol 3,4,5-trisphosphate; PI(4,5), P_2, phosphatidylinositol 4,5-bisphosphate; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SHIP2, SH2 domain containing inositol phosphatase 2; SKIP, skeletal muscle and kidney enriched inositol polyphosphate phosphatase.

Insulin-like growth factor-1 (IGF-1) plays a pivotal role in regulating the growth and development of many normal tissues in many organisms (*1*). The binding of a ligand to the IGF-1 receptor results in the activation of the receptor tyrosine kinase, followed by the tyrosine phosphorylation of insulin receptor substrates (IRS 1–4) and Shc (*2*, *3*). Once phosphorylated, IRS and Shc further propagate downstream signalling via the assembly of signalling complexes, growth factor receptor-bound protein 2 (Grb2)/SOS, and the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (p85 PI3K), which leads to activation of the PI3K pathway (*2*, *3*). The activated PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] to transiently produce phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3] on the membrane. PI(3,4,5)P_3 is a phosphoinositide that regulates a wide range of cell behaviours by recruiting phosphoinositide binding proteins to the membrane (*4*). Akt is recruited to the membrane by binding to PI(3,4,5)P_3 where it is phosphorylated at Thr308 and Ser473 by phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin 2 (mTOR2) complex, respectively (*5*, *6*). Activated Akt regulates a wide range of cellular functions including cellular proliferation, survival and metabolism.

IGF-1 is involved in the regulation of protein synthesis of several cells including neurons, cardiomyocytes, muscle cells, epithelial cells and fibroblasts; the PI3K/Akt signalling pathway contributes greatly to this process (*7–10*). Oligodendrocyte progenitors, in particular, produce the IGF-1 that is necessary for the growth and development of oligodendrocytes (*11*). IGF-1-triggered mRNA translation is regulated by several proteins termed 'translation initiation factors' (*12*). The eukaryotic translation initiation factor 4E (eIF4E) is a key regulator in the initiation of mRNA translation. eIF4E binds to the mRNA cap structure and interacts with eIF4G, which is a large scaffold protein required for the assembly of eIF4E and eIF4A to form the eIF4F complex (*13*, *14*). The coupling of the eIF4F complex to the mRNA 5′-cap structure is essential for anchoring the mRNAs to the ribosome (*12*). Under the resting conditions, the binding of 4E-BP1 (eIF4E binding protein 1) to eIF4E inhibits its association with eIF4G thereby suppressing cap-dependent translation (*13*, *14*). IGF-1 stimulation causes the phosphorylation of 4E-BP1 via the PI3K/Akt/mTOR signalling pathway, promoting its dissociation from eIF4E. Once 4E-BP1 binding is relieved, eIF4E enhances cap-dependent translation (*14*, *15*).

A 70-kDa ribosomal protein S6 kinase (P70 S6K), which phosphorylates ribosomal protein S6, is also a regulator of protein translation initiation. IGF-1-induced S6 phosphorylation initiates the preferential translation of mRNAs containing the 5′-terminal oligopyrimidine tract (5′-TOP) such as ribosomal proteins and translation elongation factors (*16*, *17*), which are also dependent on PI3K. Therefore, the regulation of PI3K signalling plays a crucial role in IGF-1-mediated protein synthesis.

PI(3,4,5)P_3 phosphatase comprised nine molecules that dephosphorylate PI(3,4,5)P_3, thus antagonizing PI3K-induced biological responses (*18*–*20*). While the physiological roles of PI(3,4,5)P_3 phosphatases have been extensively studied, the involvement of these enzymes in IGF-1-induced protein synthesis remains largely unknown.

Pharbin, a 5-phosphatase that induces arborization, is a rat phosphoinositide 5-phosphatase that induces dendritic structures in fibroblasts (*21*). It is also called a 72-kDa 5-phosphatase (72 k-5ptase) (mouse) or type IV 5-phosphatase (human). We previously found that pharbin preferentially hydrolyses 5-phosphate from PI(4,5)P_2. However, it is also reported that pharbin is necessary for PI(3,4)P_2 and PI(3)P generation via the hydrolysis of PI(3,4,5)P_3 and PI(3,5)P_2, which promotes GLUT4 translocation to the plasma membrane (*22*, *23*). Recently, some missense mutations in the pharbin-encoding gene were found in individuals with Joubert and MORM syndrome (*24*, *25*). These diseases are characterized by primary ciliary instability. Primary cilia are antenna-like structures that protrude from cell surfaces and participate in extracellular signalling. Missense mutations are predominantly found within the phosphatase domain.

In this study, we examined the role of pharbin in the IGF-1 signalling pathway. We found pharbin to be a potent negative regulator of IGF-1-induced protein synthesis and membrane ruffling via suppression of the PI3K/Akt pathway.

Experimental Procedures

Materials

Human IGF-1 was purchased from Sigma Chemical Co. (MO, USA). The antibodies against phospho-Akt (Ser473), phospho-Akt (Thr308), p70 S6 kinase, phospho-p70 S6 kinase (Thr389), phospho-p70 S6 kinase (Thr421/Ser424), p42/44 MAP kinase, phospho-p42/44 MAP kinase (Ser202/Tyr204), 4E-BP1 and eIF4E were purchased from Cell Signaling Technology (Beverly, MT). The antibodies against c-Myc, Akt1/2, and IGF-1Rβ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A monoclonal anti-phosphotyrosine antibody (pY20) was purchased from Transduction Laboratories (Lexington, KY, USA). Phosphoinositides [PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2 and PI(3,4,5)P_3] were purchased from Cell Signaling Inc. (Columbus, OH, USA). Glutathione-Sepharose 4B^{TM}, 7-methyl-GTP-Sepharose 4B and Redivue Pro-mix L-[^{35}S] *in vitro* cell labelling mix (>37 TBq/mmol) were purchased from GE Healthcare (Piscataway, NJ, USA). Lipofectamine 2000, pFASTBAC1 baculovirus expression vector and Sf900 serum-free medium were purchased from Invitrogen Inc. (Carlsbad, CA, USA).

Expression constructs

Wild-type cDNA encoding rat pharbin (GenBank accession number: AB026288) was isolated as described previously (*21*). A glutathione *S*-transferase (GST) construct for the baculoviral expression system was generated by amplifying full-length GST cDNA from a pGEX-2T vector using specific primers with BglII–BamHI sites and subcloning into the BamHI site of pFASTBAC1. The GST-tagged full-length pharbin construct (GST-Pharbin) was then generated by introducing a full-length rat pharbin cDNA fragment into the BamHI site of the vector. A phosphatase motif-deleted mutant was generated by the deletion of the DNA sequence encoding two motifs that are conserved among the inositol polyphosphate 5-phosphatases. Myc-tagged wild-type (Myc-Pharbin) and phosphatase motif-deleted mutant (Myc-Pharbin-Δphos) constructs of pharbin were obtained by subcloning each type of cDNA fragment into the HindIII–XhoI site of the pCMV-Tag-3B mammalian expression vector (Stratagene, La Jolla, CA, USA).

Cell culture

HEK293 and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum (FBS) in an atmosphere of 5% CO_2 at 37°C. Sf9 cells were grown at 28°C in Sf900 serum-free medium (Invitrogen) containing 5% FBS.

Generation of recombinant adenoviruses

Recombinant adenoviruses expressing wild-type pharbin (Ad-Myc-Pharbin) and a phosphatase-negative pharbin mutant (Ad-Myc-Pharbin-Δphos) as well as a control adenovirus (Ad-Ctl) were generated as described previously (*26*). The recombinant adenoviral plasmids were purified, linearized by digesting with PacI and transfected into HEK293 cells. After incubation for 10 days at 37°C, the suspension in the culture flask was collected and used as the original virus solution. The virus was further amplified several times in order to obtain a virus solution with a sufficient titre for the experiments.

RNA interference

The following stealth siRNAs (Invitrogen) were designed on the basis of the human pharbin cDNA sequence: pharbin siRNA (si-Phar), GCCTGAGCACCTCCTTGCAGGAAAT; control siRNA (si-Ctl), GCCACGACTCCGTTCGGACAGTAAT. HeLa cells were transfected with 120 pmol of siRNA using Lipofectamine 2000 according to the manufacturer's instructions.

Western blotting and immunoprecipitation

Cellular proteins were extracted by washing cells with cold PBS and then scraped into lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA and 1% Triton X-100] supplemented with 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF and 1 mM sodium orthovanadate. After centrifugation at 10,000 *g* for 10 min to remove cell debris, the cell lysates (30 μg each) were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were soaked in blocking buffer (5% skimmed milk and 1% bovine serum albumin in PBS) for 1 h and then immunoblotted with the primary antibody. The membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibody for 30 min and visualized using 5-bromo-4-chloro-indolyl phosphate and nitroblue tetrazolium detecting system. For immunoprecipitation, the cell lysates were incubated with antibodies for 2 h at 4°C, followed by the addition of protein A-agarose and another 1 h of incubation. The agarose beads were washed five times with lysis buffer and used for western blotting analysis. Phosphoinositide phosphatase activities were examined as described previously (*27*).

7-methyl-GTP-Sepharose 4B pull-down assay

HeLa cells were infected with recombinant adenoviruses (Ad-Ctl, Ad-Myc-Pharbin or Ad-Myc-Pharbin-Δphos), and cell lysates were prepared as described previously. The cell lysates (500 μg of total protein) were incubated with 25 μl 7-methyl-GTP-Sepharose 4B beads for 2 h at 4°C. After washing four times with lysis buffer, the pellets were analysed by western blotting.

Measurement of cellular protein synthesis by radioisotope labelling

After serum starved for 16 h, the cells were further incubated with methionine- and cysteine-free DMEM for 3 h, and treated with IGF-1 (50 ng/ml) for 1 h. ^{35}S-methionine and ^{35}S-cysteine (20 μCi/ml) were then added, and the mixture was incubated at 37°C for 1 h. Incorporation was terminated by removing the medium and washing the cells with cold PBS. The cells were lysed, and aliquots of the cell lysate were spotted onto filter paper (Whatman, UK), washed once with ice-cold 5% trichloroacetic acid (TCA), once with boiling 5% TCA, twice with ice-cold TCA and once with 100% ethanol. The filters were dried, and the amino acids incorporated into the cellular proteins were measured by liquid scintillation counting.

Immunofluorescence microscopy

After serum starving for 16 h, HeLa cells transiently transfected with plasmids expressing Myc, Myc-Pharbin or Myc-Pharbin-Δphos were stimulated with IGF-1 (50 ng/ml) for 10 min. The cells were fixed with 3.7% formaldehyde for 15 min, followed by permeabilization with 0.2% Triton X-100 for 10 min. The cells were incubated with anti-Myc antibody for 1 h followed by Alexa Fluor 488 goat anti-rabbit IgG for 30 min. In order to visualize F-actin, the cells were incubated with rhodamine–phalloidin (Molecular Probes, Carlsbad, CA, USA) for 30 min. The images were obtained with a Radiance 2000 laser-scanning confocal microscope (Bio-Rad).

Results

Pharbin hydrolyses PI(4,5)P_2 and PI(3,4,5)P_3 in vitro

Although we previously showed that pharbin hydrolyses the D5-phosphate of PI(4,5)P_2, there are some discrepancies regarding its substrate specificity (*21*). In the present study, we examined its enzymatic activity towards phosphoinositides using recombinant GST and GST-Pharbin proteins (0.5 μg each) *in vitro* (Fig. 1A and B). As shown in Fig. 1C, pharbin exhibited phosphatase activity for PI(4,5)P_2 and PI(3,4,5)P_3 (298.3 ± 16.0 nmol/min/mg and 387.0 ± 16.2 nmol/min/mg, respectively). In contrast, PI(3,5)P_2 was not hydrolysed at all, indicating that pharbin acts as a phosphatase towards PI(4,5)P_2 and PI(3,4,5)P_3. A catalytically inactive mutant of pharbin lacking the phosphatase motifs (GST-Pharbin-Δphos) did not exhibit phosphatase activity for every phosphoinositides.

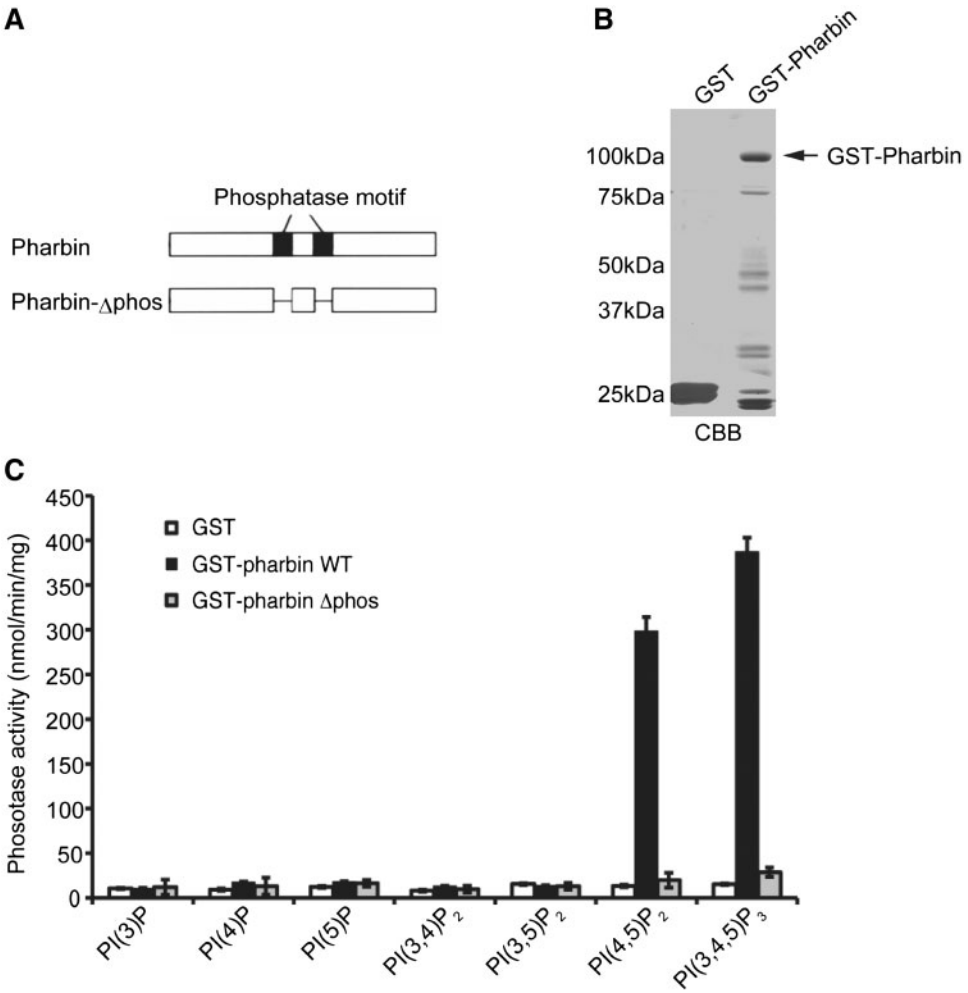


Fig. 1 Phosphatase activity assay of pharbin. (A) Schematic representation of constructs expressing Myc-tagged wild-type pharbin (Myc-Pharbin) and Myc-tagged phosphatase motifs-deleted mutant pharbin (Myc-Pharbin-Δphos). (B) The recombinant GST and GST-Pharbin expressed in Sf9 cells were separated with SDS–PAGE, and detected by staining with Coomassie brilliant blue. (C) 50 μM of different phosphoinositides were incubated with 0.5 μg GST or GST-Pharbin protein at 37°C for 10 min. Amounts of hydrolysed inorganic phosphate were measured by malachite green assay. Pharbin hydrolysed PI(4,5)P_2 and PI(3,4,5)P_3.

Adenovirus-mediated pharbin expression and phosphatase motifs-deleted pharbin mutant in HeLa cells

To obtain a useful model for the functional research of pharbin, we generated Ad-Myc-Pharbin and a phosphatase-inactive mutant (Ad-Myc-Pharbin-Δphos). The expressions of both wild-type and mutant pharbin were increased ~5-fold and above compared with endogenous levels (Fig. 2A). Next, the PI(3,4,5)P_3 phosphatase activity of wild-type and mutant pharbin proteins were determined by immunoprecipitation with anti-Myc antibody after expression in HeLa cells (Fig. 2B), confirming that wild-type pharbin exhibits intact enzymatic activity for PI(3,4,5)P_3 while the mutant does not. To further confirm that pharbin hydrolyses PI(3,4,5)P_3 in IGF-1-stimulated Hela cells, we employed DsRed2-conjugated biosensors containing PI(3,4,5)P_3-specific GRP1 PH and PI(3,4)P_2-specific TAPP1 PH domain. In control GFP-expressing cells, GRP1 PH domain bound uniformly to the plasma membrane, but very weakly binding in pharbin-expressing cells (Fig. 2C). In phosphatase-negative mutant expressing cells, GRP1 PH bound strongly to the plasma membrane. PI(3,4)P_2 localization could be observed punctately at the plasma membrane in IGF-1-treated Hela cells in control or pharbin-Δphos expressing cells. In wild-type pharbin expressing cells, PI(3,4)P_2 was localized uniformly at the plasma membrane (Fig. 2D), confirming that pharbin hydrolysed PI(3,4,5)P_3 to PI(3,4)P_2 in IGF-1-stimulated Hela cells.

Pharbin suppresses the IGF-1-induced phosphorylation of Akt but not p42/44 mitogen-activated protein kinase

IGF-1 regulates a variety of cell behaviours mainly by binding the IGF-1 receptor (IGF-1 R), followed by the activation of PI3K/Akt and mitogen-activated protein kinase (MAPK) signalling pathways (*2*, *3*). To examine the effect of pharbin on the upstream signalling events of IGF-1, we first immunoprecipitated the IGF-1 R β-subunit and tested its tyrosine phosphorylation by immunoblotting with an anti-phosphotyrosine antibody (pY20). As shown in Fig. 3A, IGF-1-treatment phosphorylated the IGF-1 R β-subunit, and the phosphorylation level was not significantly affected by pharbin expression. Next, we examined the effect of pharbin on the phosphorylation of Akt and MAPK. As shown in Fig. 3B, the IGF-1-tiggered phosphorylation of Akt reached a maximum within 5 min; this high level was maintained for up to 20 min. However, the expression of wild-type pharbin remarkably reduced the phosphorylation of Akt both at Thr308 and at Ser473 (Fig. 3B) compared with that of the control cells (Ad-Ctl). In contrast, pharbin expression did not cause any significant changes in the phosphorylation of MAPK (Fig. 3C). These results suggest that pharbin plays an inhibitory role in PI3K/Akt but not MAPK signalling.

Pharbin silencing significantly increases the IGF-1-induced activation of Akt

To assess the specific role of pharbin on PI3K/Akt signalling, we examined the effect of pharbin silencing on the IGF-1-induced phosphorylation of Akt. The transfection of pharbin siRNA (si-Phar) suppressed the endogenous expression by ~47% (Fig. 4A). Pharbin silencing resulted in significant increases in Akt phosphorylation at Thr308 and Ser473 by 36.0% and 32.3%, respectively, compared with that of the control cells (si-Ctl) (Fig. 4B and C). These results demonstrate that pharbin is a negative regulator of the IGF-1/PI3K/Akt signalling pathway.

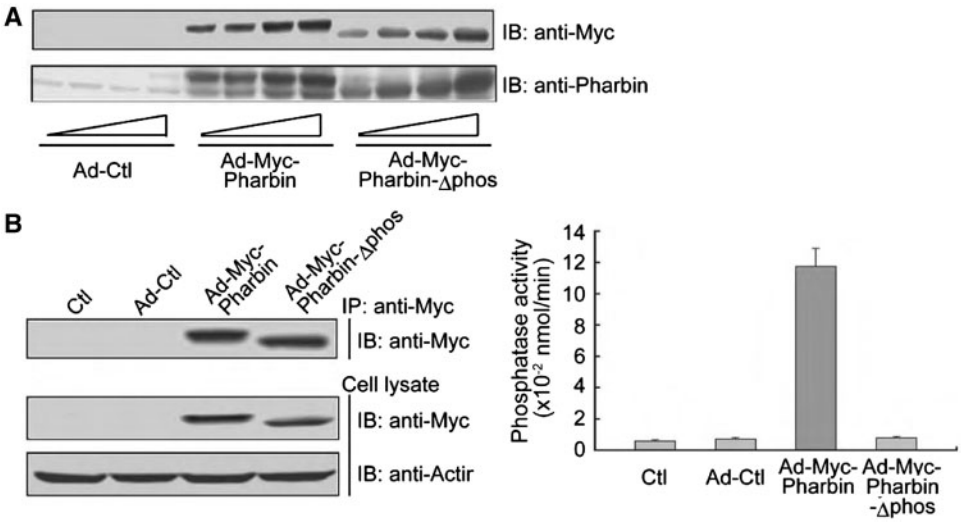
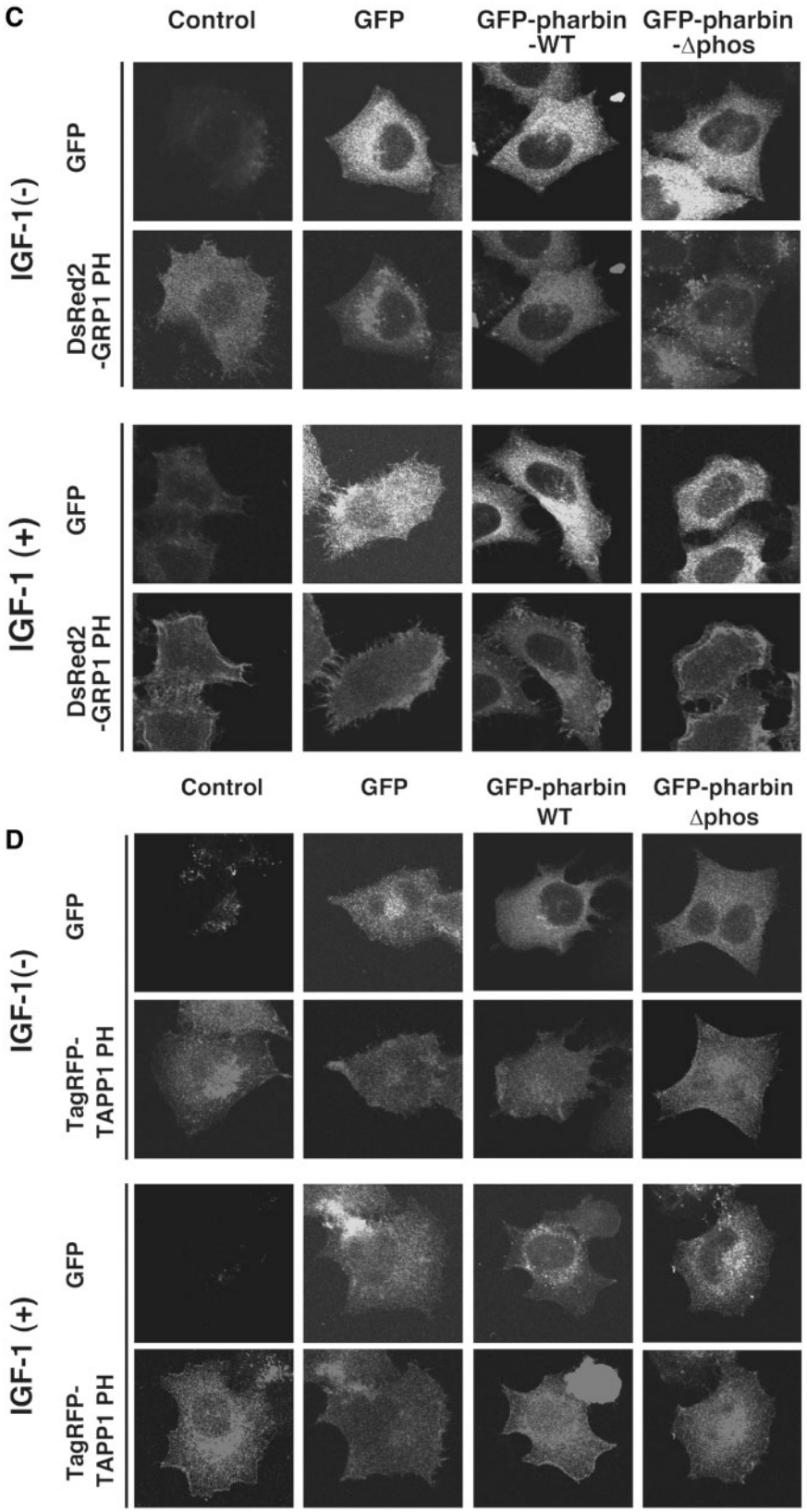


Fig. 2 Adenovirus-mediated expression of pharbin and its phosphatase-negative mutant in HeLa cells. (A) Adenovirus-mediated expression of pharbin constructs. HeLa cells were transfected with control adenovirus (Ad-Ctl) and adenoviruses expressing Ad-Myc-Pharbin or Ad-Myc-Pharbin-Δphos in a dose-dependent manner. The cell lysates were immunoblotted with anti-Myc or anti-Pharbin antibody. (B) Phosphatase activities of Myc-Pharbin and Myc-Pharbin-Δphos. Cell lysates from those transfected with Ad-Ctl, Ad-Myc-Pharbin and Ad-Myc-Pharbin-Δphos were subjected to immunoprecipitation with anti-Myc antibody, and the immunoprecipitates were probed with anti-Pharbin antibody. These immunoprecipitates were incubated with PI(3,4,5)P_3 at 37°C for 10 min, and the released PI was measured by malachite green assay. Myc-Pharbin hydrolysed PI(3,4,5)P_3 but Myc-Pharbin-Δphos did not. (C) Images of GFP-pharbin and DsRed2-GRP1 PH in IGF-1-treated Hela cells. (D) Images of GFP-pharbin and TagRFP-TAPP1 PH in IGF-1-treated Hela cells.



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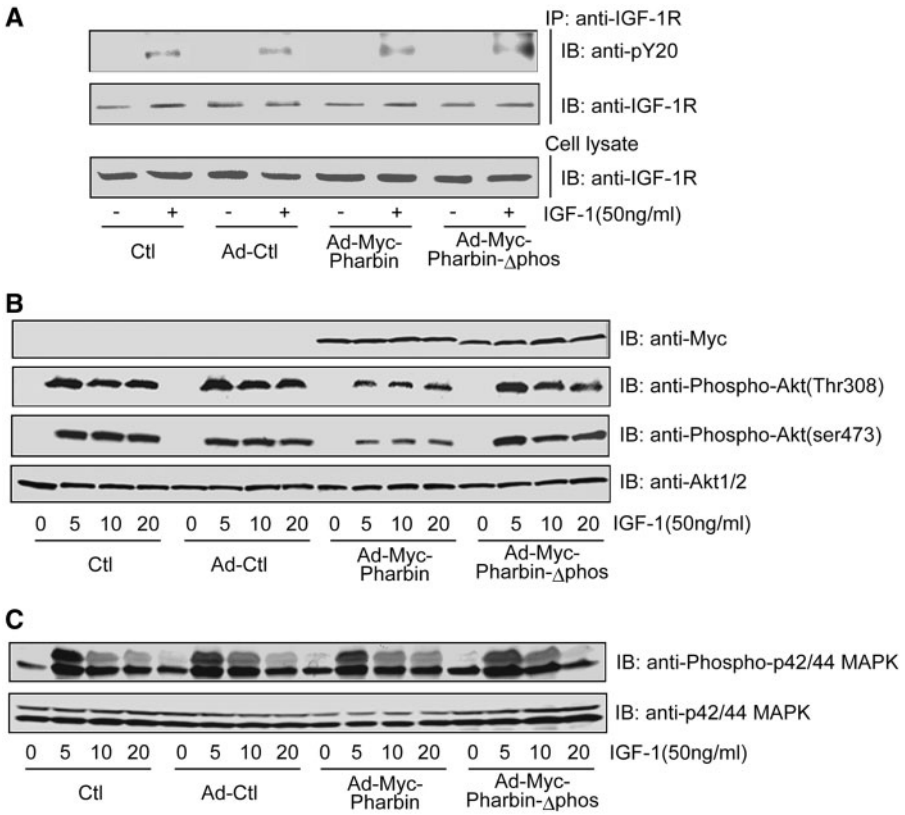

Fig. 3 Pharbin inhibited the IGF-1-induced phosphorylation of Akt but not p42/44 MAP kinase. (A) The phosphorylation of IGF-1 Rβ was not significantly affected by pharbin expression. HeLa cells infected with control adenovirus (Ad-Ctl) and adenoviruses expressing Ad-Myc-Pharbin or Ad-Myc-Pharbin-Δphos were serum starved and stimulated with IGF-1 (50 ng/ml) for 20 min. Cell lysates were immunoprecipitated with anti-IGF-1Rβ antibody and the immunoprecipitates were analysed by immunoblotting with antibodies against phosphotyrosine (upper panel) or IGF-1Rβ (middle panel). (B and C) Following serum-starvation, HeLa cells infected with Ad-Ctl, Ad-Myc-Pharbin or Ad-Myc-Pharbin-Δphos adenoviruses were treated with IGF-1 (50 ng/ml) for the indicated times. The lysates were subjected to immunoblotting with antibodies against Myc, phospho-Akt(The308), phospho-Akt(Ser473), Akt1/2 (B) phospho-p42/44 MAP kinase (Ser202/Tyr204) and p42/44 MAP kinase (C).

Pharbin attenuates IGF-1-induced activation of p70 S6 kinase and 4E-BP1, thus inhibiting protein synthesis

IGF-1-dependent protein synthesis, which is mediated by PI3K signalling, is implicated in a number of physiological and pathological conditions (*1*–*3*). To elucidate whether pharbin is involved in the regulation of PI3K signalling and how, we examined its effect on the signalling molecules downstream of the PI3K/Akt pathway, particularly, p70 S6 kinase and 4E-BP1, which play critical roles in initiating mRNA translation (*15*, *17*). We first tested the effect of pharbin on the IGF-1-induced activation of p70 S6 kinase. As shown in Fig. 5A, the phosphorylation of Thr389, a critical residue for p70 S6 kinase activation (*28*, *29*), was markedly inhibited by the expression of wild-type pharbin, whereas the phosphorylation of Thr421/Ser424, which expose the Thr389 residue, did not change. Next, the phosphorylation of 4E-BP1 was also analysed under the same conditions. There are three forms of 4E-BP1 (α, β and γ) that are defined by their phosphorylation states and exhibit different electrophoretic mobility (*15*). Under resting conditions, majority of 4E-BP1 (>80%) exists in the α form with only small amounts of β and γ forms. Meanwhile, IGF-1 stimulation causes the phosphorylation of 4E-BP1, which is correlated with a mobility shift from α- to β- and γ-bands (Fig. 5B, upper panel). Wild-type pharbin expression (Ad-Myc-Pharbin) reduced the IGF-1-induced phosphorylation of 4E-BP1 to 58.6% of that observed in the control cells (Ad-Ctl) (Fig. 5B, low panel). Since the phosphorylation of 4E-BP1 leads to the dissociation of the 4E-BP1 and eIF4E, which is a crucial step in the initiation of mRNA translation (*13*, *14*), we analysed the effect of pharbin on the formation of the 4E-BP1–eIF4E complex using a pulldown assay with 7-methyl-GTP-Sepharose 4B beads. As shown in Fig. 5C, pharbin expression inhibited the dissociation of 4E-BP1 from eIF4E, indicating that IGF-1 signalling leading to mRNA translation is effectively blocked by pharbin expression. Consequently, we directly investigated the effect of pharbin on protein synthesis by measuring the incorporation of ^{35}S-labelled methionine and cysteine into total cellular protein. As shown in Fig. 6, HeLa cells expressing wild-type pharbin exhibited IGF-1-induced protein synthesis 16.4% lower than that of the control cells (Ad-Ctl), suggesting that pharbin suppresses IGF-1-triggered protein synthesis.

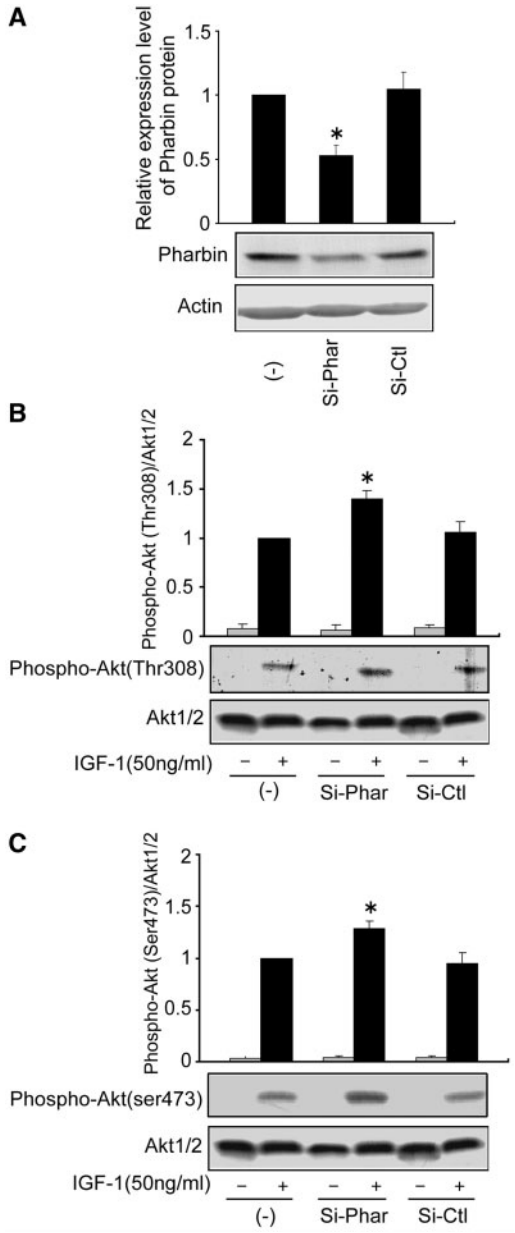

Fig. 4 Pharbin silencing increased IGF-1-induced phosphorylation of Akt. (A) Silencing of pharbin by RNA interference. HeLa cells were transiently transfected with pharbin siRNA (si-Phar) or control siRNA (si-Ctr). Cell lysates were immunoblotted with anti-Pharbin antibody, and the expression level of endogenous pharbin was analysed by densitometry. (B and C) HeLa cells transfected with pharbin siRNA (si-Phar) or control siRNA (si-Ctr) were serum starved and exposed to IGF-1 (50 ng/ml) for 10 min. The lysates were subjected to immunoblotting with anti-phospho-Akt (Thr308), anti-phospho-Akt (Ser473) or anti-Akt1/2 antibodies. Pharbin silencing significantly increased Akt phosphorylation at both Thr308 and Ser473. The amounts of Akt phosphorylated at Thr308 and Ser473 were analysed by densitometry. Results represent the mean ± SEM of three independent experiments. $*P < 0.05$ compared with control siRNA (Si-Ctl).

Pharbin expression attenuates IGF-1-induced membrane ruffling formation

The induction of actin rearrangement and membrane ruffle formation is a well-characterized role of IGF-1 that requires PI3K signalling (*30*). To examine the effect of pharbin on IGF-1-triggered membrane ruffling, we transiently expressed Myc-Pharbin and Myc-Pharbin-ΔPhos in HeLa cells (Fig. 7A). In the resting condition, ~10% of the cells exhibited membrane ruffling. However, after treatment with IGF-1 for 10 min, this population increased to ~80% of the total in both the control and mutant-expressing cells. Only 35.8% of the wild-type-expressing cells (Myc-Pharbin) exhibited membrane ruffling compared with 75.3% of Myc-expressing cells (vector) and 82% of the mutant-expressing cells (Myc-Pharbin-Δphos) (Fig. 7C). Thus, these results indicate that pharbin also negatively regulates IGF-1-induced membrane ruffle formation.

Discussion

To gain insight into the regulation of IGF-1-mediated protein synthesis, we investigated the role of pharbin in IGF-1 signalling. Pharbin is a 5-phosphatse which contains two catalytic motifs, WXGDXNXR and PXWCDRXL, evolutionally conserved among phosphoinostide 5-phosphatases. While several researchers report that pharbin can hydrolyse $PI(3,5)P_2$, $PI(4,5)P_2$ and $PI(3,4,5)P_3$, there are some discrepancies. Pharbin contains an N-terminal proline-rich region, a 5-phosphatase catalytic domain and a C-terminal CAAX motif. We have first reported that rat pharbin could hydrolyse 5-phosphate of $PI(4,5)P_2$ and $Ins(1,4,5)P_3$, while others have reported that a mouse 72 kDa 5-phosphtase, which share 74% amino acid identity, hydrolysed $PI(3,4,5)P_3$ and $PI(3,5)P_2$ (*22*, *23*). They have described that 72 kDa 5-phosphtase-regulated $PI(3,4,5)P_3$ and $PI(3,5)P_2$ levels in 3T3-L1 adipocytes, We first determined the *in vitro* substrate specificity of pharbin and found that $PI(4,5)P_2$ and $PI(3,4,5)P_3$ were its critical substrates—the same as skeletal muscle and kidney enriched inositol polyphosphate phosphatase (SKIP) and SH2 domain containing inositol phosphatase 2 (SHIP2). However, $PI(3,5)P_2$ was not likely to be a substrate for pharbin because it was not hydrolysed *in vivo* at all. The separate observation that membrane translocation of DsRed2-GRP1 PH was markedly repressed in wild-type-expressing cells indicated that pharbin hydrolysed $PI(3,4,5)P_3$ *in vivo*. We have previously reported that pharbin was a 5-phosphatase, not a 4-phosphatase; therefore, we could conclude that pharbin was a $PI(3,4,5)P_3$ 5-phosphatase to generate $PI(3,4)P_2$ (*21*). It is important to note that pharbin expression markedly reduced IGF-triggered Akt phosphorylation; conversely, pharbin silencing significantly increased Akt activation. This finding provides strong evidence that pharbin negatively regulates Akt activation by reducing the generation of $PI(3,4,5)P_3$. In addition, the partial membrane location of pharbin expressed in HeLa cells provides spatial convenience for the rapid hydrolysis of $PI(3,4,5)P_3$, which may account for the observation that pharbin expression leads to a rapid decrease in Akt phosphorylation (within 5 min). Pharbin also contains a proline-rich motif at its N-terminus, which raises the possibility that pharbin interacts with Grb2 and interferes with the formation of the IRS1/Shc/Grb2 complex, which

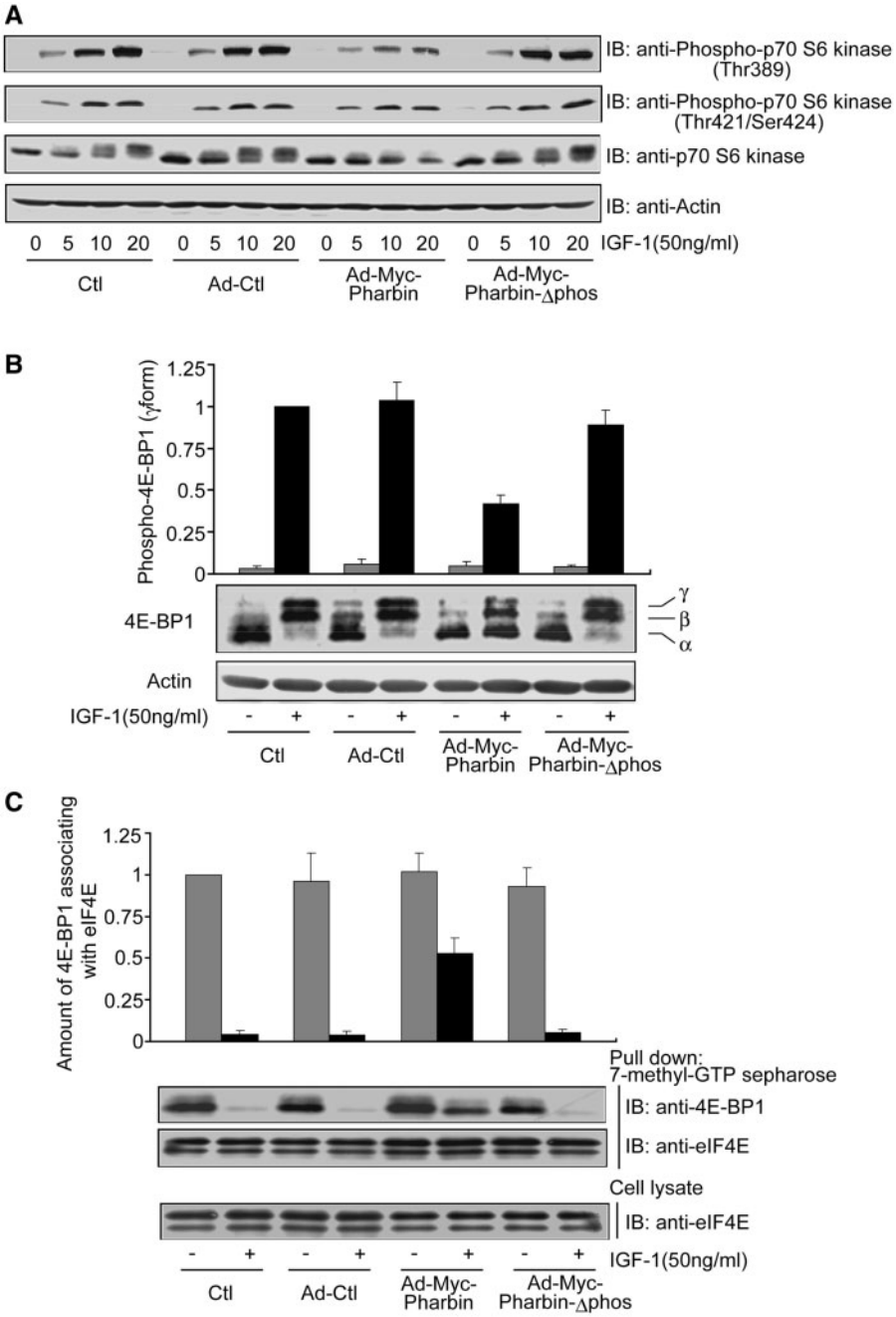

Fig. 5 Pharbin inhibited IGF-1-induced phosphorylation of p70 S6 kinase and 4E-BP1 and blocked the dissociation of 4E-BP1–eIF4E complexes. (A) HeLa cells infected with control adenovirus (Ad-Ctl) and adenoviruses expressing Ad-Myc-Pharbin or Ad-Myc-Pharbin-Δphos were serum starved and exposed to IGF-1 (50 ng/ml) for up to 20 min. Cell lysates were immunoblotted with antibodies against phospho-p70 S6 kinase (Thr389), phospho-p70 S6 kinase (Thr421/Ser424) and p70 S6 kinase. Pharbin suppressed the IGF-1-dependent activation of p70 S6 kinase. (B) HeLa cells infected with Ad-Ctl, Ad-Myc-Pharbin and Ad-Myc-Pharbin-Δphos adenoviruses were serum starved and treated with IGF-1 (50 ng/ml) for 20 min. Cell lysates were immunoblotted with anti-4 E-BP1 antibody. The α- and β-bands represent the least phosphorylated and faster migrating 4 E-BP1, respectively; the γ-band represents the slower migrating and hyperphosphorylated 4 E-BP1. Pharbin inhibited IGF-1-induced 4 E-BP1 phosphorylation. (C) HeLa cells infected with Ad-Ctl, Ad-Myc-Pharbin and Ad-Myc-Pharbin-Δphos adenoviruses were serum starved and treated with IGF-1 (50 ng/ml) for 20 min. Cell lysates were subjected to a pull-down assay with 7-methyl-GTP Sepharose beads, followed by immunoblotting with antibodies against eIF4E or 4E-BP1. Pharbin expression blocked the IGF-1-induced dissociation of 4E-BP1–eIF4E complexes. The amounts of the phosphorylated γ form (C, upper panel) and 4E-BP1 associating with eIF4E (C, lower panel) were quantified by densitometry. Results represent the mean ± SEM of three independent experiments.

is required for MAPK activation (*31*). However, pharbin expression did not impair MAPK phosphorylation (Fig. 3C). Thus, unlike SHIP and phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which attenuate MAPK phosphorylation by impeding the formation of the IRS1/Shc/Grb2 complex (*32*, *33*), pharbin specifically downregulates the PI3K signalling pathway.

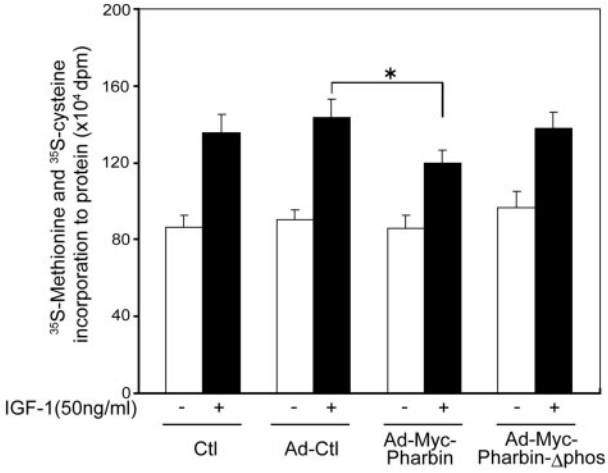

Fig. 6 Pharbin suppressed IGF-1-triggered protein synthesis. HeLa cells infected with adenoviruses (Ad-GFP, Ad-Myc-Pharbin and Ad-Myc-Pharbin-Δphos) were cultured in serum-derived medium for 12 h, followed by methionine- and cysteine-free medium for 4 h, and then treated with IGF-1 (50 ng/ml) for 1 h. Cell lysates were prepared, and the amounts of ^{35}S-methionine and ^{35}S-cysteine incorporated into protein were measured as described in the experimental procedures. Results represent the mean ± SEM of four independent experiments. $*P < 0.05$.

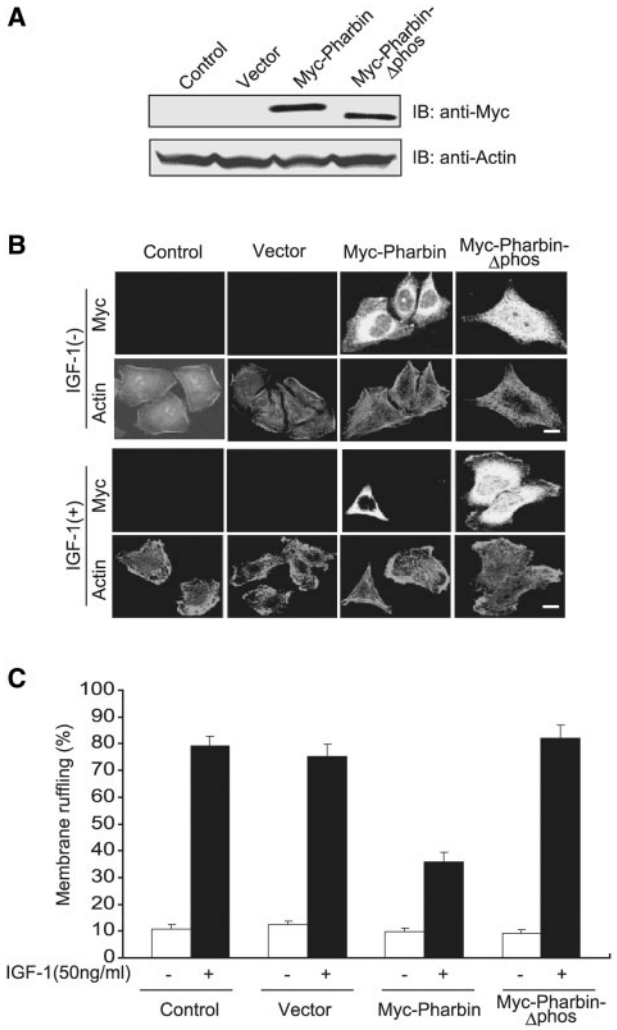

Fig. 7 Pharbin inhibited IGF-1-induced membrane ruffling. (A) HeLa cells were transiently transfected with Myc, Myc-Pharbin, and Myc-Pharbin-Δphos plasmids. Protein expression was detected by immunoblotting with anti-Myc antibody. (B) HeLa cells expressing Myc, Myc-Pharbin or Myc-Pharbin-Δphos were serum-starved and treated with IGF-1 (50 ng/ml) for 10 min. The cells were fixed, permeabilized and immunostained with anti-Myc antibody (green) and rhodamine-phalloidin (red). Images were obtained from a confocal microscope. Scale bar, 10 μm. (C) The number of cells exhibiting membrane ruffling was counted. The IGF-1-induced membrane ruffling formation was inhibited by pharbin expression.

Recently, the PI3K/Akt/mTOR pathway has emerged as a central axis that responds to IGF-1 to regulate protein synthesis in skeletal muscle cells (*34*). As expected, pharbin expression significantly inhibited global protein synthesis (Fig. 6); the suppression of PI3K/Akt signalling is the most reasonable explanation for this. Moreover, the IGF-1-induced phosphorylation of p70 S6 kinase and 4E-BP1, two crucial downstream substrates of Akt that are essential for mRNA translation—were vigorously attenuated by pharbin expression (*15*, *17*). However, it should be noted that pharbin suppressed the phosphorylation of p70 S6 kinase at Thr389 by PDK1, while PI3K-independent phosphorylation at Thr421 and Ser424 residues was not altered at all (*28*, *29*). Our results indicate that pharbin specifically regulates the PI3K signalling pathway.

Pharbin also remarkably suppressed the phosphorylation of 4E-BP1, which restrained the dissociation of 4E-BP1 from the 4E-BP1–eIF4E protein complex. The eIF4F complex comprises the cap-binding protein eIF4E, scaffold protein eIF4G and RNA helicase eIF4A (*13*). The coupling of this complex to the 7-methy-guanosine-cap structure of mRNA, mediated by eIF4E, is essential for the loading of mRNAs onto the 40S ribosomal subunit and the subsequent initiation of translation (*14*, *15*). Thus, by suppressing the dissociation of the 4E-BP1–eIF4E complex, pharbin interferes with the binding of eIF4E to eIF4G and the subsequent formation of the eIF4F complex, inhibiting mRNA translation.

Actin cytoskeletal rearrangement is another important hallmark of IGF-1 signalling: it leads to stress fibre breakdown and membrane ruffling (*30*). In our model, pharbin expression efficiently blocked IGF-1-dependent membrane ruffling. Importantly, the expression of phosphatase-negative mutant did not affect this phenotype, implying that the negative regulation of IGF-1-induced membrane ruffling depends on the 5-phosphatase activity. Similarly, PTEN and SKIP inhibit insulin-induced membrane ruffling in L6 myoblast cells (*35*, *36*). We think that the inhibition of the PI3K pathway by these phosphoinositide phosphatases may at least in part be responsible for suppressing the formation of membrane ruffling, because $PI(3,4,5)P_3$ formation facilitates the activation of Cdc42 and Rac via the binding of the PH domains of their guanine nucleotide exchange factors such as Tiam 1 and Vav1. (*37*, *38*).

Taken together, our results suggest that pharbin inhibits IGF-1-induced protein synthesis and membrane ruffling by suppressing PI3K signalling. Abnormalities in IGF-1/PI3K/Akt signalling are implicated in a variety of pathological conditions, such as those related to neuronal and muscle cell development (*3*, *39*). It was recently found that hypomorphic mutations in human pharbin genes cause Joubert syndrome, providing the first evidence of a link between phosphoinositides and ciliopathies (*24*, *25*). Although it remains unknown whether the developmental defects in Joubert syndrome are the results of cilia withdrawal, it is possible that pharbin is involved in this process because it is localized in the cilia. It is also known that growth factor receptors, including PDGF receptors, are localized in the cilia, indicating a link between growth factor receptors and pharbin (*40*). Our results show that pharbin negatively regulates IGF-1/PI3K/Akt signalling. This signalling pathway is known to be required for the development of neuronal or muscle cells. Further analysis will identify the molecular mechanisms by which pharbin acts in IGF-1 signalling.

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Conflict of interest

None declared.

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