Akt binds prohibitin 2 and relieves its repression of MyoD and muscle differentiation

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Summary

In a yeast two-hybrid screen using the full-length Akt as bait, we found that prohibitin 2 (PHB2) specifically interacts with Akt. The C terminus of Akt (amino acids 413-480) and a central region of PHB2 (amino acids 120-232) are responsible for their mutual interaction. PHB2 acts as a transcriptional repressor in cells. PHB2 interacts with both MyoD and MEF2, and represses both MyoD- and MEF2-dependent gene transcription. Furthermore, binding of PHB2 to both MyoD and MEF2 significantly decreases upon myogenic differentiation. When stably expressed in C2C12 myogenic cells, PHB2 inhibits myogenin induction and phenotypic muscle differentiation. PHB2 was found to specifically recruit histone deacetylase 1, which is probably responsible for its repressive activity. Co-expression of Akt can partially reduce PHB2 binding to MyoD and relieve the repressive effect of PHB2 on myogenic reporters, which could be one of the mechanisms underlying Akt-mediated MyoD activation and accelerated muscle differentiation.

Key words: Prohibitin 2, Akt, Myogenesis, MyoD, MEF2, Histone deacetylase

Introduction

Skeletal-muscle differentiation is an excellent model system for studying developmental problems associated with cell proliferation, signal transduction and cell fate determination. Two families of transcription factors, the myogenic regulatory factors (MRFs) and the MEF2s, play key and decisive roles during muscle differentiation (Molkentin and Olson, 1996; Puri and Sartorelli, 2000; Yun and Wold, 1996). There are four MRFs: Myf5, MyoD, myogenin and MRF4. Whereas both Myf5 and MyoD are important for establishing the myogenic fate of muscle precursor cells, myogenin and MRF4 are crucial for executing the differentiation program (Arnold and Winter, 1998; Molkentin and Olson, 1996; Yun and Wold, 1996). In muscle cell culture, expression of myogenin signals that cells have irreversibly withdrawn from the cell cycle and the differentiation program starts (Andres and Walsh, 1996). For the MEF2 family of proteins, they are also made up of four members: MEF2A, MEF2B, MEF2C and MEF2D (Black and Olson, 1998). MRFs (together with the ubiquitously expressed E proteins) and MEF2 proteins specifically bind to the consensus E box and the MEF2 site, respectively, and increase the transcription of many muscle-specific genes containing these sites in their promoters (Molkentin and Olson, 1996; Puri and Sartorelli, 2000; Yun and Wold, 1996). Moreover, MRFs and MEF2s can physically interact with each other to synergistically activate many muscle-specific genes and are involved in regulating each other's expression (Molkentin et al., 1995; Molkentin and Olson, 1996).

The insulin-like growth factors (IGFs) are potent inducers of muscle differentiation both in cell culture and in vivo (Fernandez et al., 2002; Florini et al., 1996; Musaro et al., 2001). In response to IGF stimulation, several intracellular signaling pathways are activated. Among them, the main pathway that mediates the effect of IGFs involves a lipid kinase phosphatidylinositol-3-kinase (PI3K) and a serine/threonine protein kinase Akt (LeRoith, 2000; Oldham and Hafen, 2003). In response to IGF binding, IGF receptor I is activated and tyrosine phosphorylated, which recruits the phosphotyrosine binding (PTB)-domain-containing insulin-receptor substrates (IRS) and results in IRS phosphorylation on certain tyrosine residues (Whitehead et al., 2000). This in turn recruits the Src homology 2 (SH2)-domain-containing p85 subunit of PI3K, resulting in activation of the p110 catalytic subunit of PI3K. The 3′-phosphorylated phosphoinositides generated by PI3K bind the pleckstrin homology (PH) domain of PDK1 and Akt, resulting in membrane localization of these serine/threonine kinases (Alessi and Cohen, 1998; Chan et al., 1999; Marte and Downward, 1997). PDK1 and an unidentified PDK2 then phosphorylate and activate Akt on or near the membrane. The active Akt can then translocate to either the cytosol or the nucleus to phosphorylate its diverse targets [e.g. Bcl-2 antagonist of cell death (BAD), forkhead transcription factor (FKHR)] (Chan et al., 1999; Vanhaesebroeck and Waterfield, 1999). The IGF-PI3K-Akt pathway has been shown to stimulate myogenic differentiation by transcriptionally upregulating myogenin mRNA (Florini et al., 1991; Xu and Wu, 2000). We further show that the IGF-PI3K-Akt pathway can target MyoD and MEF2, and enhance their transcriptional activity (Tamir and Bengal, 2000; Xu and Wu, 2000). Whereas a constitutively active Akt stimulates myogenic differentiation, a kinase-dead Akt inhibits IGF-

induced myogenic differentiation and myogenin upregulation (Jiang et al., 1999; Xu and Wu, 2000). However, neither MyoD nor MEF2 is a direct substrate of Akt (Xu and Wu, 2000). To look for molecules that interact directly with Akt and might mediate its stimulatory effect on MyoD and MEF2, we initiated a yeast two-hybrid screen to look for Akt-interacting clones from a human skeletal-muscle cDNA library. Prohibitin 2 (PHB2) was found as a specific Akt-interacting protein.

PHB2 is a highly conserved, ubiquitously expressed protein and its homologs are found in bacteria, yeast, plants, *Drosophila* and mammals (Nijtmans et al., 2002). Although it has been a decade since its discovery, the biological function of the PHB2 is still poorly understood. Originally identified as a 37 kDa protein associated with IgM in B cells (BAP37), PHB2 was also shown to interact with the estrogen receptor and repress its transcriptional activity (Delage-Mourroux et al., 2000; Montano et al., 1999; Terashima et al., 1994). Prohibitin 1 (PHB1), a homolog of PHB2, forms oligomeric complexes with PHB2 in mammalian cells and was shown to bind both Rb and E2F1, and repress the transcriptional activity of E2F1 (Wang et al., 1999a; Wang et al., 1999b). In yeast, PHB2 also complexes with PHB1 and the PHB complex acts as a chaperone for newly synthesized mitochondrial proteins and is required for yeast replicative lifespan (Coates et al., 1997; Nijtmans et al., 2000).

We demonstrate in this report that PHB2 can inhibit muscle differentiation by repressing the transcriptional activity of both MyoD and MEF2. We also find that PHB2 specifically recruits HDAC1, which could be responsible for its repressive activity. Co-expression of Akt and PHB2 partially decreases the binding of PHB2 to MyoD, which could be one of the mechanisms by which Akt activates MyoD and stimulates muscle differentiation.

Materials and Methods

Cell lines, DNA constructs, antibodies and other reagents

Cos-7 cells and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin in a 37°C incubator with 5% CO_2. C2C12 cells were grown in DMEM with 20% FBS and antibiotics (also called growth medium, or GM) and were induced to differentiate in DMEM containing 2% horse serum and antibiotics (also called differentiation medium or DM) when cells were near confluent. 3×MEF2-luc, 4RE-luc, 5×Gal4-luc, Gal4-MEF2C, Gal4-MyoD, HA-Aktca, HA-Aktkm, HA-MKK6, Flag-MEF2C, Flag-MyoD, Flag-Six1, xp-JNKK2, Flag-HDAC4 and Flag-HDAC1 have been described previously (Chan et al., 2003; Wu et al., 1997; Xu and Wu, 2000). PHB2/pCMV is a generous gift from B. S. Katzenellenbogen (University of Illinois, Urbana, IL). Xp-PHB2 and xp-PHB2(57-299) were constructed by inserting the cDNA fragments amplified by polymerase chain reaction into pcDNA3.1HisC (Invitrogen) and verified by sequencing. Anti-Flag (M2) and anti-β-actin antibodies were from Sigma; anti-Xpress (Omni-probe, M21), anti-HA, anti-MEF2 (B-4) and anti-Akt1/2 (N-19) antibodies were from Santa Cruz Biotechnology; anti-sarcomere myosin heavy chain (MF20) and anti-myogenin (F5D) antibodies were from Developmental Studies Hybridoma Bank (University of Iowa); anti-MyoD (5.8A) was from BD Biosciences. D(–)-Luciferin was purchased from Roche Applied Science. Puromycin, Polybrene, ATP and tetracycline were purchased from Sigma.

Yeast two-hybrid analysis

MATCHMAKER GAL4 Two-Hybrid System 3 (BD Biosciences) was used for cDNA library screening. The full-length kinase-dead form of mouse Akt [AktK(179)M)] was used as bait. It was cloned into pGBKT7 bait vector and expressed as a fusion protein to the GAL4 DNA-binding domain in the yeast strain AH109. A MATCHMAKER human skeletal muscle cDNA library (BD Biosciences) expressed as fusion proteins to the GAL4 activation domain in pACT2 and pretransformed into the yeast strain Y187 was screened by mating with AH109/pGBKT7-Akt(K179M) following the manufacturer's instructions. Positive clones were selected on synthetic dropout plates lacking leucine, tryptophan, histidine and adenine (SD/–his/–leu/–trp/–ade).

To test the interaction between two known proteins in the yeast two-hybrid assay, the two corresponding cDNAs were separately inserted into pGBKT7 and pGADT7 (BD Biosciences), respectively, and were sequentially transformed into the host strain AH109. Positive clones were selected as described above. Both pGBKT7 and pGADT7 without inserts were used as negative control.

Transfection, cell lysis and luciferase reporter assays

Cells (about 85% confluent) were first transfected with various plasmids using LipofectAMINE™ Plus reagents (Invitrogen) following the manufacturer's instructions and cultured in either DMEM with 10% FBS (for Cos-7 cells) for another 36 hours or GM for 36 hours followed by culturing in DM for another 24 hours (for C2C12) before harvest. Cells were then lysed in the lysis buffer [50 mM HEPES pH 7.6, 1% (v/v) Triton X-100, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 100 mM NaF, 20 mM *p*-nitrophenylphosphate, 20 mM β-glycerophosphate, 50 μM sodium vanadate, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg ml^{-1} aprotinin, 0.5 μg ml^{-1} leupeptin, 0.7 μg ml^{-1} pepstatin], followed by removal of insoluble debris with a benchtop centrifuge at 16,000 ***g*** for 2 minutes to obtain whole cell extracts (WCEs). For luciferase assays, 20 μl of WCEs were added to 150 μl of freshly made luciferase reaction buffer (0.1 M Tris-acetate, pH7.8, 1 μM EDTA, 10 mM magnesium acetate, 66 μM D(–)-luciferin and 2 mM ATP). Luciferase activity was determined with a LB9507 luminometer (EG&G Berthold). Luciferase units were normalized against total protein amount present in each sample determined by protein assay reagent from Bio-Rad (Hercules, CA).

Generation of retrovirus and stable C2C12 cells

To generate retrovirus, *PHB2* cDNA was first cloned into pBPSTR1, a tetracycline-regulated retroviral vector (Paulus et al., 1996). pBPSTR1-PHB2 and pBPSTR1 (a negative control) together with pCLeco (a packaging vector) (Naviaux et al., 1996) were then separately co-transfected into 293T cells. The supernatant containing retroviruses was collected 48 hours after transfection, filtered through 0.45 μm filters and stored in a –80°C freezer. 50% confluent C2C12 cells in a 35-mm plate were infected with 3 ml of viruses in the presence of Polybrene (4 μg ml^{-1}) and tetracycline (2 μg ml^{-1}). 8 hours later, the medium was aspirated and fresh medium was added. After growing for another 24 hours, cells were split 1:5 into selection medium containing puromycin (1.5 μg ml^{-1}) and tetracycline (3 μg ml^{-1}). After 7-10 days, single colonies of cells were picked and expanded. To induce PHB2 expression, stable cells were grown in GM in the presence of puromycin without tetracycline until cells were 95-100% confluent, followed by culturing in DM for either 12 hours or 24 hours before harvest. 30 μg of cell lysates were subjected to sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and western blot.

Co-immunoprecipitation assays

Cos-7 cells were co-transfected with various plasmids. 24-36 hours after transfection, the cells were cross-linked with 200 μg ml^{-1}

dithiobis(succinimidylpropionate) (DSP; Pierce) for 5 minutes followed by lysis in RIPA buffer (25 mM HEPES pH 7.4, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg ml^{-1} aprotinin, 0.5 μg ml^{-1} leupeptin, 0.7 μg ml^{-1} pepstatin) (Baki et al., 2001). Protein-A/Sepharose beads were incubated with 200 μg of extracts and 2 μg of appropriate antibodies for 2 hours at 4°C. After extensive washing with the RIPA buffer, bound proteins were eluted by boiling and subjected to SDS-PAGE and immunoblotting

Alternatively, cell lysates containing xp-PHB2 were mixed separately with C2C12 WCEs harvested before and after differentiation. PHB2 was then immunoprecipitated with the anti-Xpress antibody and the co-precipitated proteins were detected by immunoblot.

Western blot analysis

20-30 μg WCEs or eluted proteins from immunoprecipitation assays were separated by SDS-PAGE, transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore), and probed with various antibodies. Proteins were visualized using an enhanced chemiluminescence kit (ECL; Amersham Biosciences).

Immunostaining and microscopic imaging

Briefly, C2C12 cells were first fixed in 4% paraformaldehyde for 10 minutes. Cells were then permeabilized with 0.2% Triton X-100 in PBS for 15 minutes, blocked with 1% bovine serum albumin for 1 hour and sequentially incubated with MF20 and the Rhodamine-conjugated anti-mouse secondary antibody. Cells were washed with PBS between steps and the coverslips were finally mounted on slides with Mowiol (Calbiochem). The images were acquired using an Olympus IX70 fluorescent microscope linked to a charge-coupled device digital camera (Spot RT; Diagnostic Instruments, MI).

X-Gal staining of cells

After indicated treatment, cells were fixed and washed once with phosphate buffered saline (PBS) followed by staining with freshly prepared 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) solution [4 mM potassium hexacyanoferrate(II) trihydrate, 4 mM potassium hexacyanoferrate(III), 0.4 mg ml^{-1} X-Gal in dimethylformamide, and 8 mM magnesium chloride in PBS] at 37°C for 2 hours to overnight. X-Gal solution was then removed and replaced with PBS.

Results

Akt specifically interacts with PHB2 in yeast and in mammalian cells

We previously showed that Akt stimulates MyoD and MEF2 indirectly during muscle differentiation (Xu and Wu, 2000). In an effort to look for regulators that interact directly with Akt and mediate its stimulatory effect on MyoD and MEF2, we initiated a yeast two-hybrid screen of a human skeletal muscle cDNA library using a full-length kinase-dead Akt [Akt(K179M)] as bait. About a million clones were screened and a cDNA fragment encoding a truncated PHB2 (amino acids 57-299) was found to interact specifically with Akt but not with either an empty bait vector (pGBKT7) or p53 (Fig. 1A). To confirm that PHB2 interacts specifically with Akt in mammalian cells, Cos-7 cells were co-transfected with PHB2 together with either HA-Akt or HA-MKK6 (a negative control). PHB2 was found to be specifically co-immunoprecipitated by Akt but not by MKK6 (Fig. 1B).

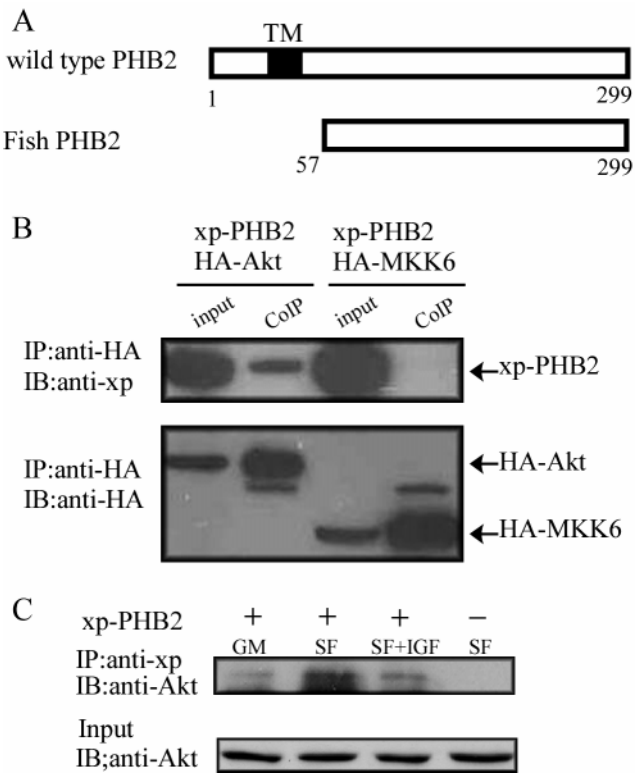

Fig. 1. PHB2 specifically interacts with Akt. (A) Full-length PHB2 and the truncated PHB2 found in the yeast two-hybrid screen. The numbers represent the positions of amino acids. Abbreviation: TM, potential transmembrane domain. (B) Cos-7 cells were co-transfected with xp-PHB2 together with either HA-Akt or HA-MKK6. (C) Cell lysates containing xp-PHB2 were mixed with C2C12 WCEs harvested before and after differentiation. Immunoprecipitation and immunoblot were carried out as indicated. Abbreviations: CoIP, co-immunoprecipitation; GM, growth medium; IP, immunoprecipitation; IB, immunoblot; SF, serum-free medium; Xp, Xpress tag. Input represents 10% of total lysates used for immunoprecipitation.

Furthermore, PHB2 was also capable of interacting with the endogenous Akt both before and after C2C12 differentiation (Fig. 1C).

Localization of the interaction domains on both Akt and PHB2

To narrow down the regions involved in mutual interaction between Akt and PHB2, deletion analysis was performed and different truncated fragments of Akt and PHB2 were separately cloned into yeast vectors for yeast two-hybrid analysis (Fig. 2). Although the central domain of PHB2 (amino acids 120-232) was required for binding to Akt (Fig. 2A), the C terminus of Akt outside of its kinase domain (amino acids 413-480) was involved in binding PHB2 (Fig. 2B). Of note, the Akt-interacting domain we mapped on PHB2 (amino acids 120-232) overlaps with that involved in interaction with the estrogen receptor (amino acids 175-198) (Delage-Mourroux et al., 2000).

PHB2 is a transcriptional repressor

PHB2 was previously shown to repress the transcriptional

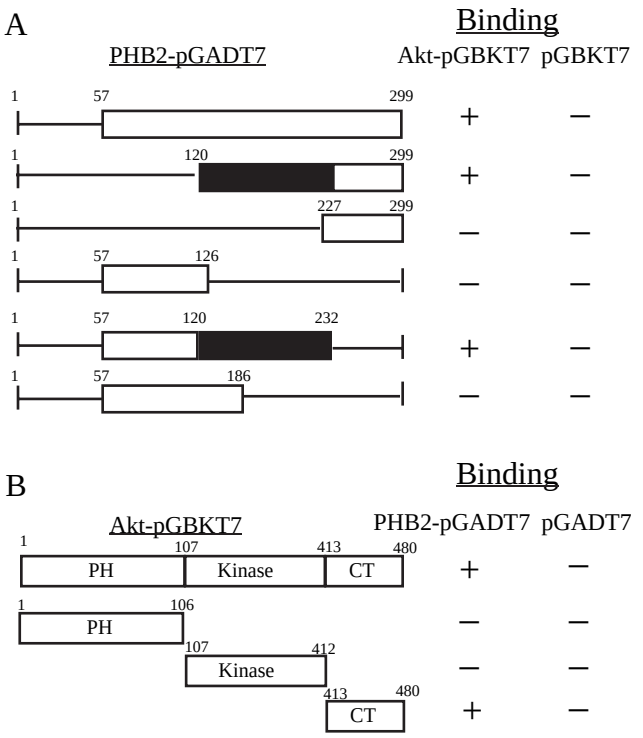

Fig. 2. Further localization of interaction domains on both PHB2 and Akt. Different deletion fragments of the *PHB2* (A) or *Akt* (B) gene were separately cloned into pGADT7 and pGBKT7, respectively. The resulting clones were tested for their mutual interaction using yeast two-hybrid assays. The black bar represents the Akt-interacting region on PHB2. Abbreviations: CT, C terminus; PH, pleckstrin-homology domain. The plus and minus signs denote growth and no growth, respectively, on SD/–his/–ade/–leu/–trp plates. The numbers represent the position of amino acids in either PHB2 or Akt.

activity of the estrogen receptor (Delage-Mourroux et al., 2000; Montano et al., 1999). In addition, PHB1, the binding partner of PHB2, was also shown to repress E2F1-dependent transcription (Wang et al., 1999b). Because PHB2 was found from a skeletal-muscle cDNA library, we tested whether PHB2 represses MyoD- or MEF2-dependent transcription in myogenic cells. C2C12 myoblasts were transfected with either MyoD- or MEF2-dependent reporters (4RE-luc and 3×MEF2-luc, respectively) with or without PHB2. Indeed, both MEF2- and MyoD-dependent transcription were significantly repressed in the presence of PHB2 (Fig. 3A,B). Of note, an N-terminal truncated PHB2 (amino acids 57-299, initially found in our yeast two-hybrid screen) without the potential transmembrane domain was still very repressive (Fig. 3A). In the assays above, either the DNA binding or transactivation functions of MyoD or MEF2 could conceivably be affected by PHB2. To elucidate the repressive mechanism of PHB2, a Gal4-dependent reporter assay was carried out. Both MyoD and MEF2 were fused in-frame to the DNA-binding domain of Gal4 (amino acids 1-147) and the intrinsic DNA-binding domains of MyoD and MEF2 were not required in this assay. C2C12 cells were transfected with gal4-luc and Gal4DBD-MyoD/or Gal4DBD-MEF2 with or without PHB2. Again, PHB2 could significantly repress the transcriptional activity of both MyoD and MEF2 (Fig. 3C), suggesting that the transactivation functions of MyoD and MEF2 were specifically compromised by PHB2.

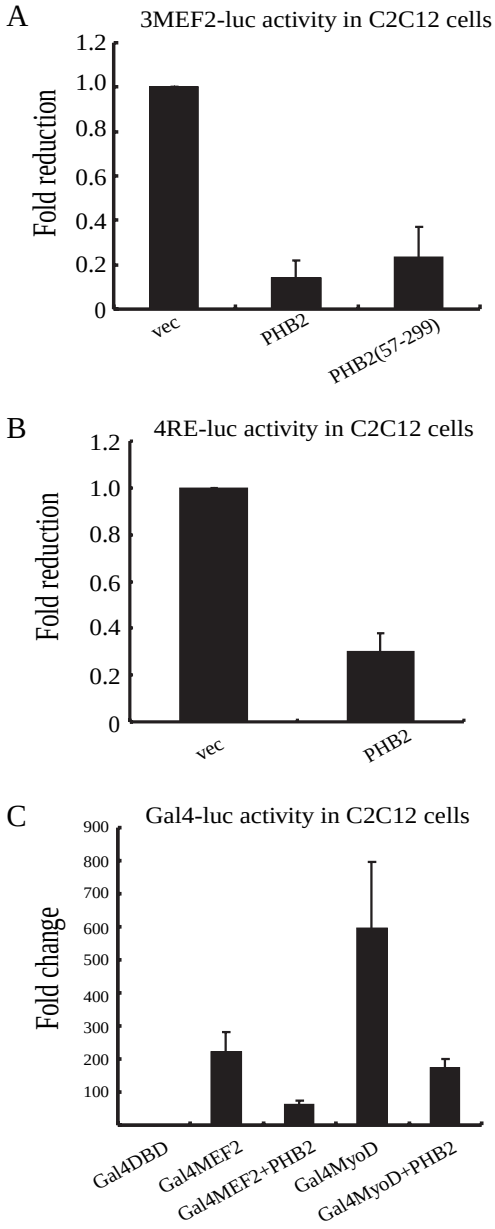

Fig. 3. PHB2 represses the transcriptional activity of MyoD and MEF2. C2C12 cells were separately co-transfected with 3×MEF2-luc (A), 4RE-luc (B) or gal4-luc (C), together with various expression vectors as indicated. After 36 hours of growth in GM and 24 hours in DM, cells were harvested and the luciferase activity in each sample was determined. Fold change was calculated as the ratio of the luciferase activity in samples transfected with either PHB2 (A,B) or Gal4MEF2/Gal4-MyoD with or without PHB2 (C) to that of samples transfected with either an empty vector (A,B) or Gal4DBD (C). Abbreviations: DBD, DNA-binding domain (amino acids 1-147 of Gal4); vec, an empty vector. All experiments were done independently three times and the results were presented as mean±s.d.

PHB2 binds MyoD and MEF2 in myogenic cells

Because PHB2 represses both MyoD- and MEF2-dependent transcription, we then tested whether PHB2 could interact with MyoD and MEF2 in cells. Cos-7 cells were separately co-transfected with various expression vectors. As shown in

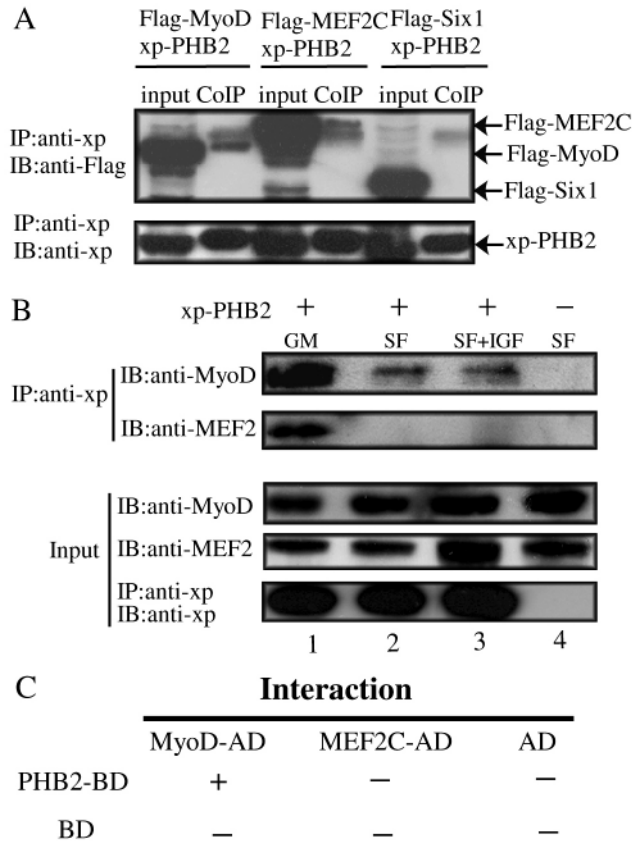

Fig. 4. PHB2 interacts with both MyoD and MEF2 in cells. (A) Cos-7 cells were co-transfected with various expression vectors as indicated. (B) Equal amounts of Cos-7 cell lysates expressing xp-PHB2 were separately incubated with C2C12 cell lysates harvested before and after differentiation. Xp-PHB2 was immunoprecipitated and the co-precipitated MyoD and MEF2 were detected by immunoblot. Abbreviations: GM, growth medium; SF, serum-free medium. Input represents 10% of total lysates used for immunoprecipitation. (C) Yeast AH109 cells were transformed with expression vectors as indicated. The plus and minus signs denote growth and no growth, respectively, on SD/–his/–ade/–trp/–leu plates. AD, pGADT7 vector encoding the Gal4 transcription-activation domain; BD, pGBKT7 vector encoding the Gal4 DNA binding domain.

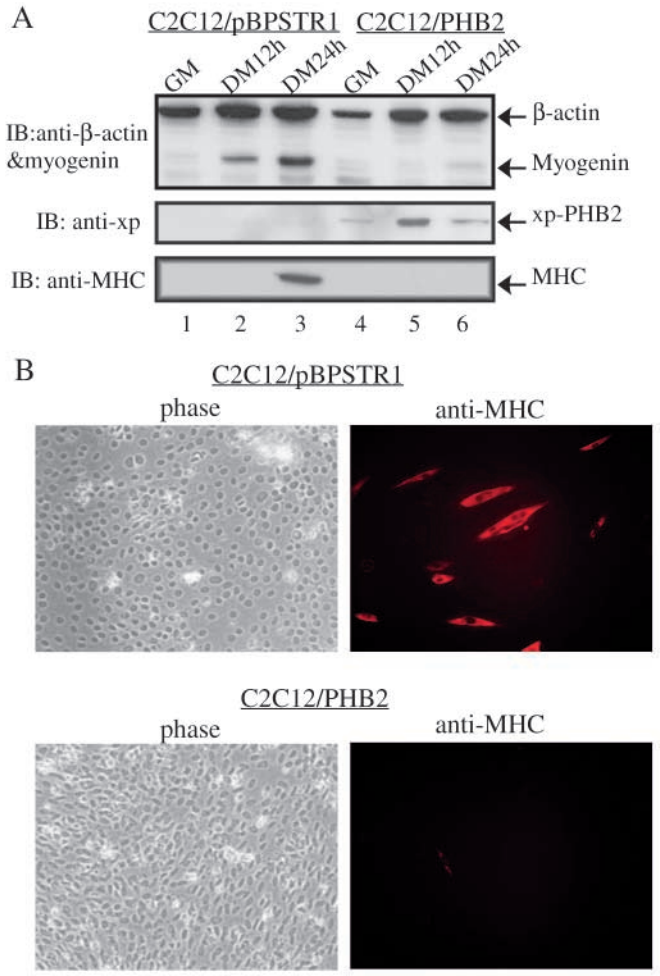

Fig. 5. Stable expression of PHB2 in C2C12 inhibits muscle differentiation. Near-confluent stable C2C12 cells expressing either an empty vector or PHB2 were allowed to differentiate in DM for the indicated times. (A) Cells were harvested and 30 μg WCEs were resolved by SDS-PAGE and subjected to immunoblotting. (B) After 24 hours in DM, cells were fixed, subjected to immunostaining and microscopic imaging. Abbreviations: DM, differentiation medium; GM, growth medium; MHC, myosin heavy chain; Phase, phase contrast.

Fig. 4A, transfected MyoD and MEF2 but not Six1 (control) were found to be specifically co-immunoprecipitated by PHB2. We then asked whether PHB2 can interact with the endogenous MyoD and MEF2 in myogenic cells and assessed their interaction before and after myogenic differentiation. We found that the endogenous MyoD and MEF2 in C2C12 cells were indeed specifically retained in the co-immunoprecipitation assays in a PHB2-dependent manner (Fig. 4B, lanes 1 and 4). Interestingly, PHB2 binding to either MyoD or MEF2 significantly decreased after differentiation (Fig. 4B, lanes 2 and 3). To further examine whether the interactions between PHB2 and MyoD or MEF2 are direct or not, yeast two-hybrid assays were performed. We found that PHB2 directly interacts with MyoD but not MEF2 in yeast (Fig. 4C)

Stable expression of PHB2 in C2C12 cells represses myogenin induction and muscle differentiation

Because PHB2 interacts with both MyoD and MEF2, and represses their transcriptional activities, we further tested whether PHB2 represses phenotypic muscle differentiation when stably expressed in myoblasts. Tetracycline-inducible vectors with or without the PHB2 insert were stably introduced into C2C12 myoblasts by infection with recombinant retroviruses. Several single clones were chosen for further analysis and they all showed similar effect on muscle differentiation. The results of two representative clones were presented here (Fig. 5). Indeed, when PHB2 was induced to express in C2C12 cells, myogenic differentiation was significantly inhibited as indicated by a lack of myogenin and myosin heavy chain (MHC) expression and the absence of elongated myotubes (Fig. 5A,B). Our data indicated that

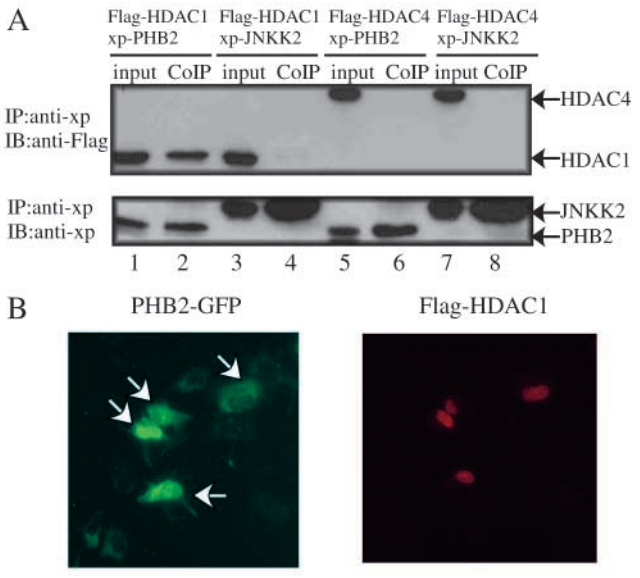

Fig. 6. PHB2 specifically interacts with HDAC1 in cells. (A) Cos-7 cells were co-transfected with various expression vectors as indicated. Either PHB2 or JNKK2 (control) were first immunoprecipitated with the anti-Xpress antibody and the co-precipitated HDAC1 was detected by immunoblot with the anti-Flag antibody. Input represents 10% of total lysates used in the immunoprecipitation. (B) C2C12 cells were co-transfected with PHB2-GFP and Flag-HDAC1. PHB2 was visualized by autofluorescence (green) of GFP and HDAC1 was visualized by indirect immunofluorescence (red) with the anti-Flag antibody. Arrows indicated cells containing both PHB2 and HDAC1. Abbreviations: GFP, green fluorescent protein. Notice the two PHB2-positive cells at the bottom left corner without HDAC1 showing predominant mitochondrial staining.

overexpression of PHB2 is capable of repressing phenotypic muscle differentiation.

PHB2 exerts its repressive activity by specifically recruiting HDAC1

Because PHB2 contains no domains known to be involved in transcription repression, we hypothesized that PHB2 represses transcription by recruiting other repressors, such as histone deacetylases (HDACs). We chose a representative member from both class I and class II of the HDACs for further analysis. In Cos-7 cells, we found that HDAC1 (a class I HDAC) was specifically co-precipitated by PHB2 but not by JNKK2 (control) (Fig. 6A, lanes 2 and 4). By contrast, HDAC4 (a class II HDAC) was not co-precipitated by either PHB2 or JNKK2 (Fig. 6A, lanes 6 and 8). Moreover, when HDAC1 and PHB2-GFP were co-expressed in cells, HDAC1 induced more PHB2 to enter the nucleus (Fig. 6B). This suggests that some PHB2 might shuttle between the cytoplasm and the nucleus.

Akt antagonizes the repressive effect of PHB2 by disrupting the binding of PHB2 to MyoD

Whereas PHB2 represses MyoD, MEF2 and muscle differentiation, Akt does the opposite (Jiang et al., 1999; Xu and Wu, 2000). This suggests that Akt might antagonize the repressive effect of PHB2. To test this hypothesis, we first carried out reporter assays using either 3×MEF2-luc or G133-luc, a 133-bp native myogenin promoter that is both MyoD and MEF2 dependent owing to the presence of an indispensable E box and MEF2 binding site (Xu and Wu, 2000). Although PHB2 repressed both myogenic reporters, this repression could be reversed by the co-transfected constitutively active Akt (Fig. 7A). To examine directly the interplay between IGF/Akt and PHB2 on myogenic differentiation, we used a *LacZ* reporter gene under the control of the myosin light chain promoter (*MLC-lacZ*) to mark the differentiated cells. We showed that PHB2 repressed differentiation as indicated by the reduced number of X-Gal-positive cells. Importantly, addition of IGF1, which activates the endogenous Akt, effectively relieved PHB2-mediated repression on myogenic differentiation (Fig. 7B). Because both Akt and MyoD interact directly with PHB2, we tested whether Akt competes with MyoD for binding to PHB2. Cos-7 cells were co-transfected with various constructs as indicated. When Akt was absent, Flag-MyoD but not Flag-Six1 (control) was specifically co-precipitated by PHB2 (Fig. 7C, top, lanes 2 and 5) as we showed above (Fig. 4). When Akt but not MKK6 (control) was co-transfected with MyoD and PHB2, significantly less MyoD was co-precipitated by PHB2 (Fig. 7C, top, lanes 3 and 4). The decrease in the level of co-precipitated MyoD was concomitant with the increase in the level of co-precipitated Akt (Fig. 7C, bottom, lanes 1 and 3). Of note, the kinase activity of Akt was not required for this effect, because both a kinase-dead Akt (Aktkm) and the constitutively active Akt (Aktca) were equally effective in disrupting interaction between PHB2 and MyoD (Fig. 7C, top, lanes 1 and 3). This result was in line with the fact that the C terminus of Akt outside the kinase domain is involved in binding PHB2 (Fig. 2).

Discussion

PHB2 is a transcription repressor

Although a major pool of PHB2 has been shown to localize to mitochondria (Coates et al., 1997; Ikonen et al., 1995; Nijtmans et al., 2000), a small proportion of PHB2 can also be found in the nucleus (Wang et al., 2002) (our unpublished results). This correlates well with diverse roles found for the PHB complex. Although the PHB complex in mitochondria is involved in chaperoning newly synthesized mitochondrial proteins (Nijtmans et al., 2000), the PHB complex in the nucleus seems to serve as a transcriptional repressor (Montano et al., 1999; Wang et al., 2002; Wang et al., 1999b). Although it is possible that the mitochondria-localized PHB2 could indirectly repress nuclear transcription by interfering with normal mitochondria function, our data do not support this hypothesis, because an N-terminally truncated PHB2 (amino acids 57-299) that lacks the putative transmembrane domain (amino acids 18-36) required for mitochondrial localization still effectively represses MEF2-dependent transcription (Fig. 3A). Whereas PHB1 has been shown to bind both Rb and E2F1, and to repress E2F1 transcriptional activity, PHB2 has been shown to bind the estrogen receptor directly and to repress its transcriptional activity (Delage-Mourroux et al., 2000; Montano et al., 1999; Wang et al., 2002; Wang et al., 1999b). Interestingly, homologous domains in PHB1 and PHB2 are implicated in binding various target proteins. For example, we

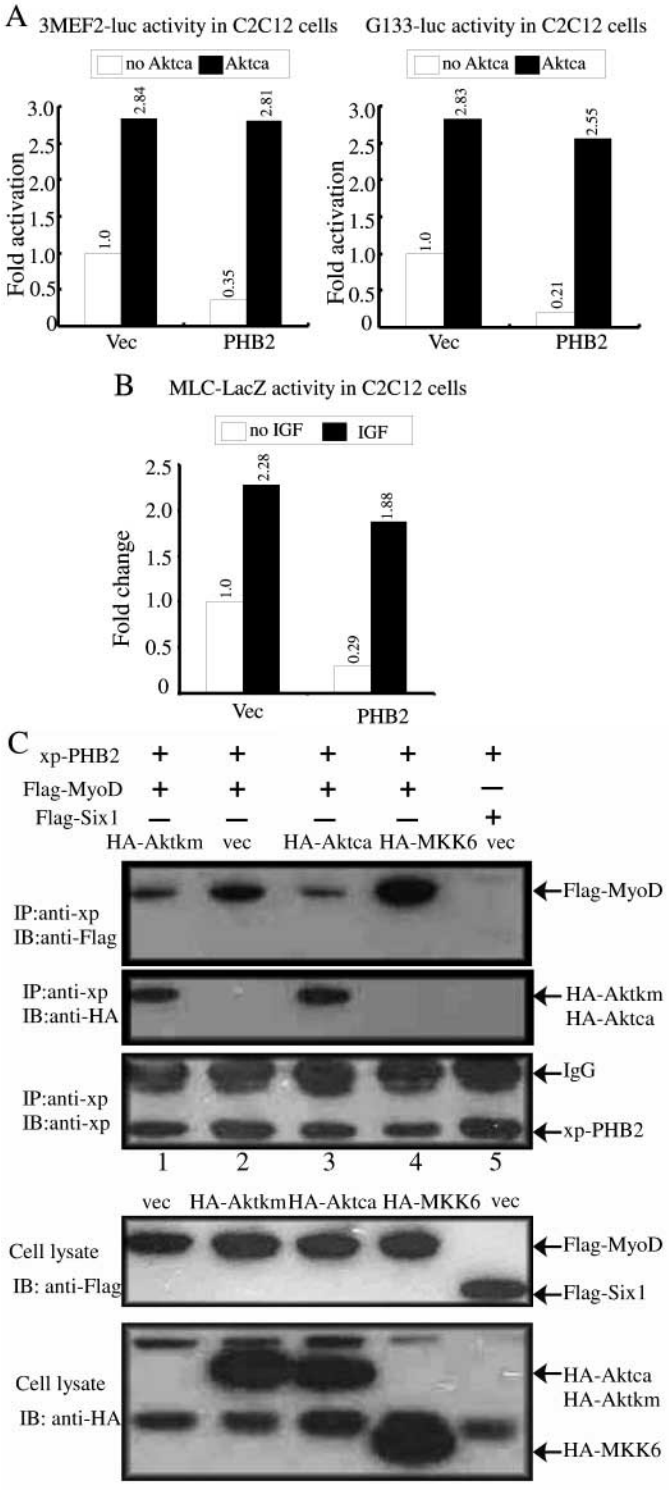

Fig. 7. Akt relieves repressive effect of PHB2 and reduces PHB2 binding to MyoD. (A) C2C12 cells were co-transfected with myogenic reporters together with an empty vector, PHB2 and Aktca in various combinations as indicated. After 36 hours of growth in GM and 24 hours in DM, cells were harvested and the luciferase activity determined. The experiments were performed independently three times with similar results. The results from a representative experiment are presented. (B) C2C12 cells were co-transfected with *MLC-lacZ* together with either an empty vector or PHB2. At the start of differentiation, either DMSO (vehicle) or IGF1 (100 ng ml^{-1}) were added to DM. After 24 hours, cells were fixed and stained with X-Gal. The numbers on top of each bar denote fold changes in either the luciferase activity (A) or the number of X-gal-positive cells (B) of a sample versus that of the controls. (C) Cos-7 cells were co-transfected with various expression vectors as indicated. PHB2 was immunoprecipitated by the anti-Xpress antibody and the co-precipitated Flag-MyoD and HA-Akt were detected by immunoblot. The expression levels of Flag-MyoD, Flag-Six1, HA-Akt, HA-Aktkm and HA-MKK6 are shown in the bottom two panels. The identities of the bands on each panel are indicated next to arrows. Abbreviations: ca, constitutively active; km, mutation of lysine 179 to methionine; MLC, myosin light chain; vec, vector.

showed that amino acids 120-232 of PHB2 bind Akt. Others have shown that amino acids 175-198 of PHB2 and amino acids 185-214 of PHB1 interact with the estrogen receptor and E2F1, respectively (Delage-Mourroux et al., 2000; Wang et al., 1999b). Our finding that PHB2 specifically recruits HDAC1 is also supported by a recent finding in which PHB1 was found to interact with HDAC1 (Wang et al., 2002). Because PHB1 and PHB2 normally associate with each other in vivo, the functions previously ascribed to either PHB1 or PHB2 alone are presumably the normal functions of the PHB complex.

Many myogenic repressors have been identified so far. These myogenic repressors function through distinct mechanisms. For example, Id (a member of the helix-loop-helix family of proteins) represses myogenesis by competing for either MRFs or E proteins and disrupting the MRF/E-protein complex (Puri and Sartorelli, 2000; Yun and Wold, 1996). Class II HDACs inhibit myogenesis by direct binding to MEF2 and repressing MEF2-dependent gene transcription (McKinsey et al., 2001). We demonstrate in this report that the PHB2-PHB complex inhibits muscle differentiation by recruiting HDAC1 to repress both MyoD- and MEF2-dependent gene transcription.

Phosphorylation-independent function of Akt in muscle differentiation

Three classes of Akt-interacting proteins have been characterized so far: the first class serves as direct kinase substrates of Akt, the second functions to regulate Akt activity and the third is regulated simply by binding to Akt (Brazil et al., 2002). Most Akt-interacting proteins fall into the first class. For example, Akt can directly phosphorylate BAD, caspase-9 and Forkhead transcription factors to protect cells from apoptosis (Chan et al., 1999; Vanhaesebroeck and Waterfield, 1999). In addition, Akt can also directly phosphorylate GSK-3, TSC-2 and $p21^{cip1}$ to modulate cellular processes ranging from glucose metabolism and cell growth to cell cycle control (Brazil et al., 2002; McManus and Alessi, 2002; Zhou et al., 2001). Several Akt-interacting proteins including C-terminal modulator protein (CTMP) and TCL1, fall into the second class, because their binding to Akt either inhibits or enhances Akt activity (Laine et al., 2000; Maira et al., 2001; Pekarsky et al., 2000). JNK-interacting-protein 1 (JIP1) is a founding member of the third class of Akt-interacting proteins. Binding of Akt to JIP1 prevents JIP1 from efficiently scaffolding the JNK complex in neurons (Kim et al., 2002).

As for PHB2, although there is a consensus Akt phosphorylation site in PHB2 (86-RPRKIS-91), we cannot detect PHB2 phosphorylation either in vitro by immune-complex kinase assays or in vivo using an antibody raised against the phosphorylated peptides containing Akt recognition sites (L.S., L.L., X.-J.Y. and Z.W., unpublished data). Moreover, overexpression of PHB2 has no significant effect on Akt kinase activity (L.S., L.L., X.-J.Y. and Z.W., unpublished data). Thus, our results identify PHB2 as a new member of the third class of Akt-interacting proteins in which Akt functions by competitive binding to prevent its interacting proteins from binding other proteins. As discussed above, the nuclear pool of PHB2-PHB complex is thought to mediate transcriptional repression. In order to bind the nuclear PHB2-PHB complex and to relieve its repressive effect on gene transcription, Akt has to enter the nucleus. Although the major pool of inactive Akt resides in the cytosol, once activated, a proportion of the activated Akt can translocate to the nucleus (Andjelkovic et al., 1997) (L.S., L.L., X.-J.Y. and Z.W., unpublished data). We suggest that Akt-mediated competitive binding to the PHB2-PHB repressive complex could be one of the mechanisms by which Akt stimulates MyoD- and MEF2-dependent gene transcription and promotes muscle differentiation.

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