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Identification of Myosin II as a Binding Protein to the PH Domain of Protein Kinase B

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Myosin II was identified as a binding protein to the pleckstrin homology (PH) domain of protein kinase B (PKB) in CHO cell extract by using the glutathione *S*-transferase-fusion protein as a probe. When myosin II purified from rabbit skeletal muscle was employed, myosin II was shown to bind almost exclusively to the PH domain of PKB among the PH domain fusion proteins examined. The purified myosin II bound to the PH domain of PKB with a K_d value of 1.1×10^{-7} M. Studies with a series of truncated molecules indicated that the whole structure of the PH domain is required for the binding of myosin II, and the binding to the PH domain was inhibited by phosphatidylinositol 4,5-bisphosphate. These results suggest that myosin II is a specific binding protein to the PH domain of particular proteins including PKB.

Pleckstrin homology (PH) domain was initially recognized as internal repeat sequences in pleckstrin, the major protein kinase C substrate in platelets, and is found in numerous intracellular signaling and cytoskeletal proteins (1–3). Three dimensional structural studies of the PH domain of different proteins, including pleckstrin (4), β-spectrin (5), dynamin (6–8), phospholipase Cδ (9), SOS1 (10), and β-adrenergic receptor kinase (11), revealed that the domain has a common structure of a seven-stranded β-sandwich formed by two orthogonal antiparallel β-sheets and an amphiphilic α-helix in the amino- and carboxyl-terminal portions, respectively. It has been reported that different substances bind to the PH domain such as inositol phospholipids (12–14), inositol phosphates (9, 15–18), the βγ subunits of the heterotrimeric GTP-binding proteins (19–22), and protein kinase C (22–26), and analysis using mutated molecules has indicated that these substances bind to the distinct sites within the PH domain (19, 21, 25, 26). Therefore, the PH domains seem to be involved in various signaling processes through their interactions with different substances.

Protein kinase B (PKB, also named Akt and RAC-protein kinase) is a serine/threonine protein kinase having a PH domain in its amino-terminal region, and three subtypes, termed α, β and γ, have been isolated (for reviews see Refs. 27, 28). PKB is shown to be activated as a downstream target of phosphatidylinositol (PI) 3-kinase in the cells stimulated with growth factors. Namely, PKB is proposed to be activated by the binding of PI 3-kinase products to its PH domain or by phosphorylation in the protein kinase domain by an upstream kinase that is activated by PI 3-kinase. On the other hand, cellular stresses including heat shock and hyperosmolarity are shown to activate PKB in a manner independent of PI 3-kinase (29), indicating that there are at least two different pathways leading to activation of PKB. As the binding proteins to the PH domain of PKB, the βγ subunits of the heterotrimeric GTP-binding proteins (22) and protein kinase C isoforms (22, 24, 25) have been identified. In addition to these proteins, we have previously found that an unidentified 200-kDa protein in CHO cell extract associates with the PH domain of PKB fused with glutathione *S*-transferase (GST) (25). It seems that the analysis of the binding proteins to the PH domain may provide a clue to clarify the activation mechanism and the physiological roles of PKB. In this study, we revealed that the 200-kDa protein is the heavy chain of myosin II, that binds specifically to the PH domain of PKB.

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Abbreviations used: CBB, Coomassie brilliant blue; GST, glutathione *S*-transferase; PH, pleckstrin homology; PI, phosphatidylinositol; PKB, protein kinase B.

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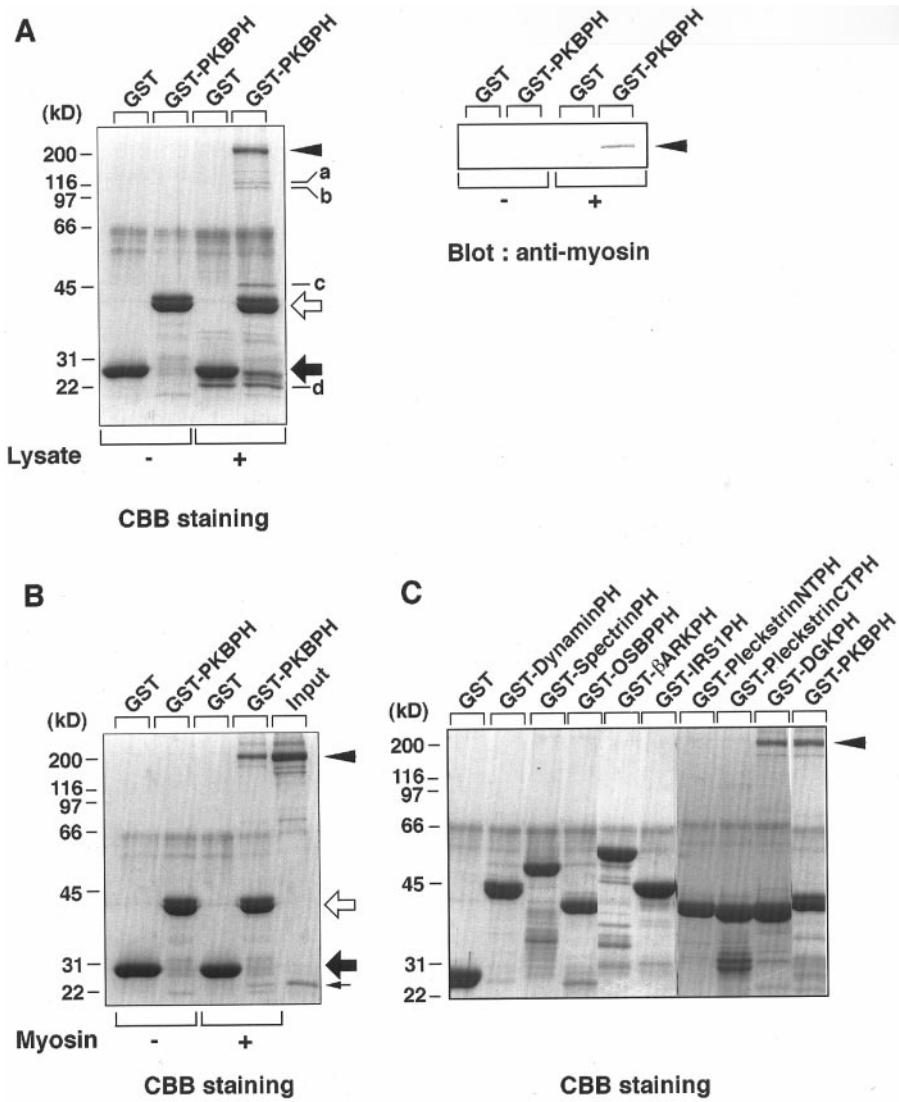

FIG. 1. Binding of myosin II to the PH domain of PKB. The CHO cell extract and purified rabbit skeletal muscle myosin II were incubated with either GST or GST-fusion proteins immobilized onto glutathione-conjugated resin. Proteins bound to the resin were separated by SDS–PAGE, and either stained with CBB or subjected to immunoblot analysis. Positions of the molecular weight markers are indicated in kDa. (A) Binding proteins to the PH domain of PKB in CHO cell extract. Left, CBB staining after SDS–PAGE. GST and GST-PKBPH are indicated by filled and open arrows, respectively. The specific binding protein of an approximate molecular mass of 200 kDa (200-kDa protein) is indicated by an arrowhead. Minor binding proteins are indicated by alphabets (a–d). Right, immunoblot analysis using the anti-myosin antibody. The position of the 200-kDa protein is indicated by an arrowhead. (B) Binding of rabbit skeletal muscle myosin II to the PH domain of PKB. Myosin II purified from rabbit skeletal muscle (4 μg) was employed for the binding assay. Input lane contains protein of rabbit skeletal muscle myosin II (4 μg). GST and GST-PKBPH are indicated by filled and open arrows, respectively. Myosin heavy and light chains are indicated by an arrowhead and a small arrow, respectively. (C) Specificity of the PH domains for the binding of myosin II. Myosin II purified from rabbit skeletal muscle (4 μg) was employed for the binding to the GST-fusion proteins of the PH domain of dynamin, spectrin, oxysterol-binding protein (OSBP), β-adrenergic receptor kinase (βARK), insulin receptor substrate-1 (IRS-1), pleckstrin in its amino-terminal half (pleckstrin NT), pleckstrin in its carboxyl-terminal half (pleckstrin CT), diacylglycerol kinase (DGK), and PKB. Myosin heavy chain is indicated by an arrowhead.

MATERIALS AND METHODS

Expression plasmids and purification of GST-fusion proteins. The PH domain of PKB β and its truncated regions were cloned into pGEX-4T-1 expression vector (Pharmacia) as described, and the fusion proteins of GST-PKB PH, GST-β1, GST-β1-6, GST-$\Delta\beta$1, and GST-$\Delta\beta$1-6 include the amino acid residues 1–113, 1–18, 1–81, 19–113, and 81–113 of PKB β, respectively (25). The GST-fusion protein expression plasmids encoding the PH domain of diacylglycerol kinase (δ-isozyme), spectrin, oxysterol-binding protein, β-adrenergic receptor kinase, insulin receptor substrate-1, and pleckstrin in its amino-terminal half, were obtained as described (19, 30). The PH domain of dynamin (Met^{596}–Glu^{656}) (31) was isolated from a rat brain cDNA library and cloned into pGEX-2T vector. The GST-fusion protein expression plasmid encoding the PH domain of pleckstrin in its carboxyl-terminal half (Ile^{246}–Lys^{350}) was donated by Dr. Tetsuya

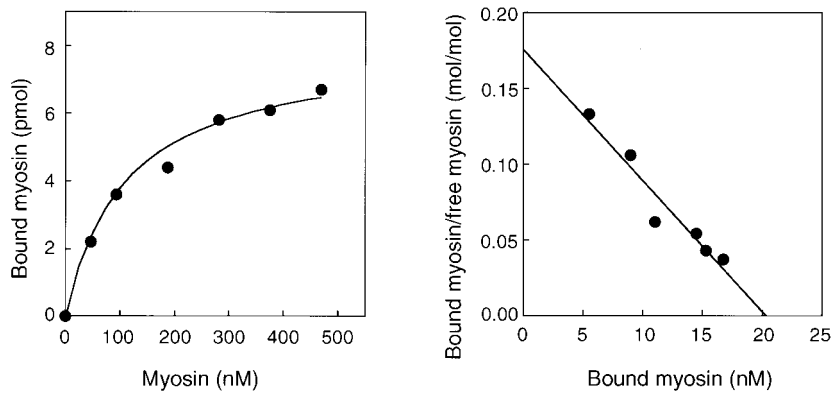

FIG. 2. Kinetics of the binding of rabbit skeletal muscle myosin II to the PH domain of PKB. Myosin II purified from rabbit skeletal muscle (4–38 μg) was incubated with GST-PKBPH immobilized onto glutathione resin, and myosin heavy chain bound to the resin was quantitated by densitometry. The concentrations and amounts of myosin heavy chain were calculated by using the molecular weight of 200 kDa of myosin heavy chain. A representative of three independent experiments is shown. Left, bound myosin heavy chain as a function of total myosin heavy chain applied. Right, Scatchard plot analysis.

Inazu (Fukui Medical School). The GST-fusion constructs were introduced in *Escherichia coli* strain DH5α, and the GST-fusion proteins were purified by successive chromatography on glutathione-Sepharose and Mono Q HR 5/5 columns (Pharmacia).

Cell culture and preparation of cell extract. CHO cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and proline (35 μg/ml) at 37°C in a 5% CO_2 incubator. The cell extract was prepared at 0–4°C as described (25, 29). Briefly, the cells were washed with phosphate-buffered saline, and lysed in 20 mM Tris–HCl at pH 7.5 containing 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, and 50 μg/ml phenylmethylsulfonyl fluoride (lysis buffer). After centrifugation for 10 min at 18,000*g*, the resulting supernatant was employed as a cell extract.

Binding assay. GST and GST-fusion proteins (4 μg) immobilized onto glutathione-Sepharose were employed. The CHO cell extract (400–600 μg protein) and myosin II purified from rabbit skeletal muscle (Calzyme Laboratories) were incubated with either GST or GST-fusion protein on the resin in 400 μl of lysis buffer with shaking at 4°C for 4 h. The mounts of purified myosin II were indicated in each figure legend. Where indicated, GST-PKBPH was preincubated for 30 min with either PI (Sigma), PI 4-phosphate (Boehringer-Mannheim), PI 4,5-bisphosphate (Boehringer-Mannheim), phosphatidylserine (Doosan Serdary Research Laboratories) or phosphatidylcholine (Sigma). Phospholipids were sonicated in an ultrasonic processor and then employed for the binding assay. After washing 10 times with 20 mM Tris–HCl at pH 7.5 containing 1% Triton X-100 and 150 mM NaCl at 4°C, the proteins bound to the resin were released by boiling in SDS-sample buffer, separated by SDS–PAGE, and either stained with Coomassie brilliant blue (CBB) or subjected to immunoblot analysis. Protein concentrations were determined by BCA protein assay reagent (Pierce) using bovine serum albumin as a standard. Where indicated, the amounts of myosin heavy chain bound to the resin were quantitated by densitometry using Image Gauge Program Version 3.0 (Fuji film) calibrating with known amounts of myosin heavy chain stained with CBB after SDS–PAGE.

Immunoblot analysis. After SDS–PAGE, proteins were transferred to an Immobilon P membrane (Millipore). Immunoblot analysis was carried out using an anti-myosin polyclonal antibody (Biomedical Technologies) which recognizes non-muscle cell myosin II and an anti-actin monoclonal antibody (Boehringer-Mannheim) as primary antibodies. The alkaline phosphatase-conjugated anti-rabbit and anti-mouse antibodies (Promega) were employed as secondary antibodies, and the color reaction was carried out using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium as substrates (25, 29).

RESULTS AND DISCUSSION

Several proteins binding to the GST-fusion protein of the PH domain of PKB β were found in CHO cell extract (Fig. 1A, left), including a protein of an approximate molecular weight of 200 kDa (25). These binding proteins apparently bound specifically to the GST-fusion protein except for the protein indicated as (d), which also associates with GST. During the characterization of these binding proteins, the 200-kDa protein was revealed to be recognized by the antibody against myosin II (Fig. 1A, right). Myosin II purified from rabbit skeletal muscle was then employed for the binding assay to examine whether myosin II binds directly to the PH domain of PKB. The heavy chain of rabbit skeletal muscle myosin II bound to the fusion-protein but not to GST (Fig. 1B). Although the R_f value of the protein band (d) in Fig. 1A roughly corresponds to that of myosin light chain in Fig. 1B, the protein band (d) may contain some other protein as the protein band binds to GST. This protein indicated as (c) in Fig. 1A was recognized by the antibody against actin (data not shown), that is consistent with the fact that actin associates with myosin. The properties of the proteins indicated as (a) and (b) in Fig. 1A are not clear. Similar results were obtained when the fusion proteins of the PH domain of PKB α and γ were employed.

The specificity of the PH domain for the association with myosin II was studied. In Fig. 1C, GST-fusion proteins containing the PH domain of various proteins were employed for the binding assay of rabbit skeletal muscle myosin II. The binding of myosin II was detected only for the PH domain of PKB and the δ-isozyme of diacylglycerol kinase, but not for the do-

main of other proteins such as pleckstrin in its amino- and carboxyl-terminal halves, insulin receptor substrate-1, β-adrenergic receptor kinase, oxysterol-binding protein, spectrin, and dynamin. The heavy chain of nonmuscle myosin II in CHO cell extract bound less efficiently to the PH domain of the diacylglycerol kinase isozyme (data not shown). These results suggest that myosin II may not be a general binding protein to the PH domains but bind to the PH domain of particular proteins such as the PKB subtypes and the δ-isozyme of diacylglycerol kinase.

The kinetic analysis for the binding of myosin was carried out using rabbit skeletal muscle myosin II (Fig. 2). Myosin II bound to the PH domain of PKB in a saturable manner, and Scatchard plot analysis revealed that the dissociation constant (K_d) for this interaction is 1.1×10^{-7} M. The maximum binding of myosin II was 8 pmol and the amount of the fusion protein in the binding assay mixture was calculated to be 100 pmol from its molecular weight of 40 kDa. A small fraction of the fusion protein of approximately 8% seems to have an appropriate structure to associate with myosin II under the conditions employed. As the K_d values between 10^{-7} to 10^{-6} M for the interaction of polyphosphoinositides to the PH domain of PKB have been reported (32, 33), and polyphosphoinositides and myosin II appear to bind to the PH domain of PKB with similar affinities.

Previous studies have demonstrated that the βγ subunits of the G-proteins and protein kinase C bind to the PH domain (16, 25, 26). The binding sites of these proteins are in the carboxyl- and amino-terminal portions of the PH domain, respectively, indicating that the PH domain interacts with these proteins at distinct sites. To define the binding site for myosin II, the binding assay was performed using a series of truncated mutants of the PH domain (Fig. 3). No obvious binding was detected to the deletion mutant GST-Δβ1 lacking the amino-terminal end β sheet (β1) or the mutant GST-β1-6 deleting the α-helix (α1) at the carboxyl-terminal end. The binding was not detected to the amino- or carboxyl-terminal end sequence such as GST-β1 and GST-Δβ1-6. These results suggest that the whole structure of the PH domain, rather than the short region of the domain, is required for the binding of myosin II.

PI 4,5-bisphosphate has been shown to interact with the PH domain of several proteins including PKB (12–14, 32, 33). It has also been reported that the binding of the βγ subunits of the G-proteins and PI 4,5-bisphosphate to the PH domain of β-adrenergic receptor kinase is cooperative (34, 35). In contrast, PI 4,5-bisphosphate inhibited the binding of skeletal muscle myosin II to the PH domain of PKB in a dose dependent manner (Fig. 4). The 50% inhibitory concentration (IC_{50}) was 40 μM, and the binding was significantly inhibited at 60 μM of the phospholipid. PI 4-phosphate

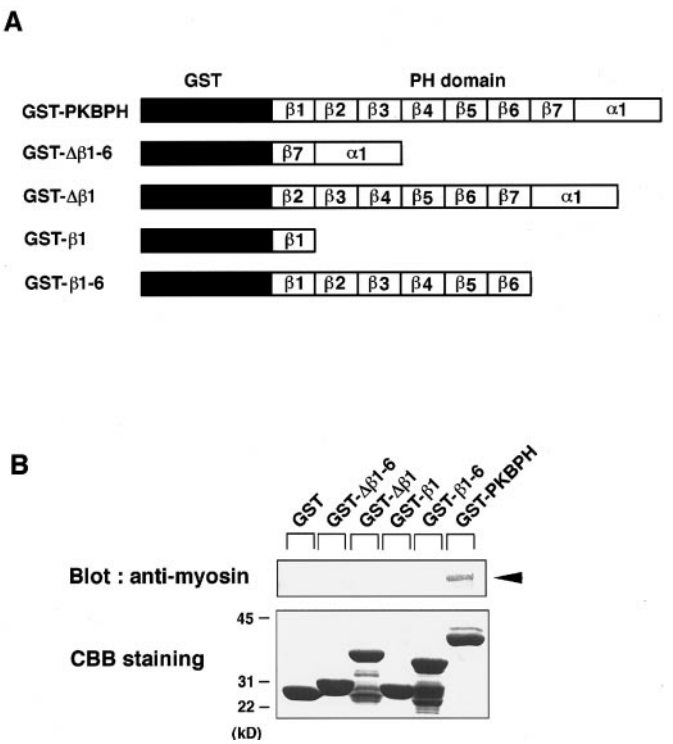

FIG. 3. Deletion analysis of the PH domain of PKB. (A) Deletion mutants of the PH domain of PKB. The regions of the α-helix and β-sheets of the PH domain are indicated as α1 and β1 to β7, respectively. (B) Binding of myosin II in CHO cell extract to the deletion mutants. The CHO cell extract was incubated with either GST or each GST-fusion protein immobilized onto glutathione resin. Upper, myosin II bound was detected by immunoblot analysis using the anti-myosin antibody. Myosin heavy chain is indicated by an arrowhead. Lower, GST and GST-fusion proteins separated by SDS–PAGE were stained with CBB. Positions of molecular weight markers are indicated in kDa.

lowered the binding of myosin to 45% at 60 μM, while other phospholipids such as PI, phosphatidylcholine, and phosphatidylserine at the same concentration did not affect the binding. These results confirm that myosin II binds to the PH domain in a manner distinct from that of the βγ subunits of the G-proteins which recognize the restricted region in the domain.

We here demonstrated that myosin II interacts with the PH domain of PKB *in vitro*. The binding of myosin II to the intact PKB molecule, however, was not detected when the recombinant full-length PKB protein expressed in insect cells was employed (data not shown). The conformational change of the PKB structure induced by extracellular signals might be necessary for the binding of myosin II to the PH domain of PKB. In fact, myosin II was co-immunoprecipitated with the truncated protein having the PH domain of PKB, but not with the full-length PKB transiently expressed in HEK293 cells (data not shown). Further studies are required to examine the association between myosin II and the PH domain of PKB *in vivo*. A

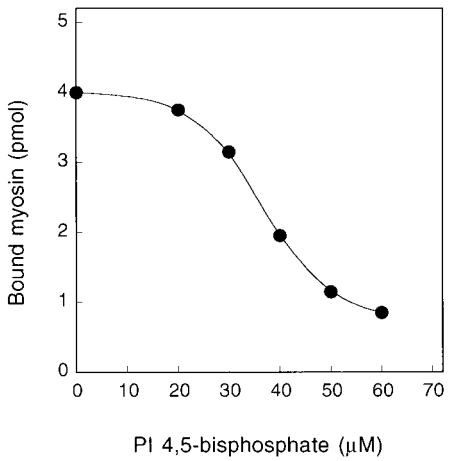

FIG. 4. Inhibition of the binding of rabbit skeletal muscle myosin II to the PH domain of PKB by PI 4,5-bisphosphate. Myosin II purified from rabbit skeletal muscle (12 μg) was incubated with GST-PKBPH in the presence of different concentrations of PI 4,5-bisphosphate, and myosin heavy chain bound to the glutathione resin was quantitated by densitometry. Amounts of myosin heavy chain were calculated by using the molecular weight of 200 kDa of myosin heavy chain.

preliminary experiment indicates that not only myosin II but other myosin isoforms could bind to the PH domain of PKB through their head region, which is conserved among the myosin family (36). PI 3-kinase, the upstream regulator of PKB, is indicated to have an essential role in myogenic differentiation (37), and the δ-isozyme of diacylglycerol kinase, another target protein for the binding of myosin II, is expressed predominantly in skeletal muscle (38). Taken together, it seems possible that the association of myosin to the PH domain proteins may have a role in the muscle cells. Most recently, it was reported that tubulin, a cytoskeletal protein, binds to the PH domain of G-protein-coupled receptor kinase 2 (39,40). The functions of some cytoskeletal proteins might be regulated by the protein-protein interaction through the PH domain. Analysis of the binding proteins to the PH domain is required to clarify the role of the PH domain in the signal transduction.

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