Interaction between Src and a C-terminal Proline-rich Motif of Akt Is Required for Akt Activation*

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Activation of Akt by growth factors is a multistep process. Here, we provide evidence that tyrosine kinase Src is directly associated with Akt through the interaction between its SH3 domain and a conserved proline-rich motif (PXPX) in the C-terminal regulatory region of Akt. Substitution of the proline residues Pro-424 and Pro-427 by alanines results in loss of Akt activity and phosphorylation induced by the epidermal growth factor (EGF), possibly because these mutations disrupt the interaction between Akt and the SH3 domain of Src. This possibility is corroborated by our observation that the Akt mutant lacking these two prolines fails to bind to Src both in vivo and in vitro. We also showed that phosphorylation of Tyr-315 in Akt induced by Src or EGF is dependent on the integrity of this proline-rich motif. Furthermore, the Akt mutant lacking this proline motif fails to block the transcription activity of Forkhead in 293 cells and poorly stimulates the proliferation of Madin-Darby canine kidney cells. Taken together, our data suggest that the interaction between the SH3 domain of Src family kinases and the proline-rich motif in the C-terminal regulatory region of Akt is required for tyrosine phosphorylation of Akt and its subsequent activation. It is noteworthy that this PXXP motif is conserved throughout several members of AGC kinase family, implying that association of this motif with the SH3 domain of an upstream regulator may represent a general mechanism applicable to these kinases as well.

Serine/threonine kinase Akt/protein kinase B (PKB) is a key regulator in a variety of cellular processes including apoptosis, proliferation, intermediary metabolism, and response to inflammatory agents (1–3). Given its critical role in physiological response as well as in malignant transformation, the mechanisms underlying Akt activation are of great interest. Akt belongs to the AGC protein kinase family. Structural analysis showed that Akt contains an N-terminal PH1 domain, a central catalytic domain, and a C-terminal regulatory region. The intact PH domain is essential for activation of Akt. Upon growth factor stimulation, binding of the PH domain to the secondary messenger, PIP3, mediates translocation of Akt to the plasma membrane and the conformational change, which renders the kinase accessible to its upstream kinases such as PDK1 and Src (4–8). As a conserved feature shared by members of AGC protein kinase family, full activation of Akt requires phosphorylation at Thr-308 in the activation loop and Ser-473 in the C-terminal regulatory domain (2, 9, 10). In addition, phosphorylation of Akt at several tyrosine residues is also involved in the regulation of Akt activation (7, 8). Furthermore, protein-protein interaction between signaling molecules and Akt, especially at its C-terminal regulatory region, plays important roles in regulation of its activity. The turn motif of the AGC family kinase is reported to provide a binding site for HSP70, which may stabilize the kinase and allow it to be re-activated (11). The C-terminal modulator protein (CTMP), a recently identified negative regulator of Akt, is also associated with Akt at the C-terminal region encompassing amino acids 411–480 in a phosphorylation-dependent manner (12). These observations suggest an essential role for the C-terminal regulatory region in mediating interaction of Akt with key regulatory molecules during its activation process. It is noteworthy that there are several proline-rich “patches” in the C-terminal region that may potentially serve as protein-protein interaction site(s). However, their functional implications still remain elusive.

In this report, we show that a conserved SH3-domain binding motif (PXXP), located in the C-terminal regulatory region of Akt, is involved in the interaction between Akt and its upstream tyrosine kinase Src and is essential for Akt activation in response to growth factors. Mutation of this motif in Akt dramatically diminished its interaction with Src, which seems to be required for tyrosine phosphorylation of Akt and its subsequent activation by other upstream kinases such as PDK1. Our data further demonstrated the important role of the C-terminal regulatory domain and the direct involvement of Src family kinases in the regulation of Akt activity.

MATERIALS AND METHODS
cDNA Constructs and Antibodies—Mammalian expression constructs of HA-tagged Akt wt and Akt K179M were kindly gifts from Dr. Philip Tsichlis. Full-length Akt cDNA was inserted in pGEX3X for generating the GST-Akt fusion protein. P424A/P427A mutants were generated by a PCR based method using a QuikChange kit (Stratagene). The mouse monoclonal antibody against the HA epitope used for immunoprecipitation and immunoblotting was from Berkeley Antibody. Phospho-specific antibodies against Akt pT308, pS473, and pY315 were from Cell Signaling Technology, Inc. Anti-phosphothreonine PY99 was fromSanta Cruz Biotechnology.

Cell Culture and Transfection—COS1 cells (from ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were transfected with FuGENE 6 (Roche Molecular Biochemicals) following the manufacturer’s instruction. 1 μg...
of Akt wt or mutants together with 1 μg of vector or Src were used. At 24 h post-transfection, the medium was removed, and the cells were serum starved for 24 h. The cells were then left untreated or treated with 100 ng/ml of EGF for 10 min. **Transient Transfection Reporter Assay**—Human embryonic kidney 293 cells grown in 12-well plates were transfected with 3XIRS-luc (kindly provide by Dr. K.-L. Guan’s lab) (13), FLAG-tagged FKHR, and HA-tagged Akt or its mutants, pRl-TK, a control reporter, was cotransfected as an internal control. The total amount of DNA was kept consistent using empty vector. Cells were lysed 24 h later, and Dual luciferase assays were performed according to the manufacturer’s instruction (Promega, Madison, WI). **Cell Proliferation Assay**—MDCK cells were transfected with vector, HA-tagged myristoylated (myr) Akt wt, and myr Akt P424A/P427A, respectively. 24 h later, the transfected cells were seeded on 96-well plates and incubated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 6 h to allow the cells to attach. The cells were then maintained in serum-free medium for another 12 h. The proliferation rate was measured using WST-1 reagent (Roche Molecular Biochemicals). An aliquot of cells was lysed and followed by Western blotting with anti-HA to monitor the transfection efficiency. **Immunoprecipitation, Western Blot, and in Vitro Kinase Assay**—The transfected cells were washed twice with ice cold phosphate-buffered saline and then lysed with lysis buffer (20 μM Tris, pH 7.4, 150 mM NaCl, 1 μM EDTA, 1 μM EGTA, 1% TritonX-100, 2.5 μM sodium pyrophosphate, 1 μM Na3VO4, 1 μM leupeptin, and 1 μM phenylmethylsulfonyl fluoride) at 4 °C for 30 min. The cell lysates were centrifuged to remove cell debris before incubation with anti-HA at 4 °C for 1 h. The immunocomplexes were collected with protein G-Sepharose beads. The immunoprecipitates for Western blot were resuspended in SDS sample buffer and resolved by SDS-polyacrylamide gel electrophoresis. Immunoblotting was performed as described previously (7, 14). The immunoprecipitates for in vitro kinase assay were washed twice with kinase buffer (20 μM Tris, pH 7.4, 10 mM MgCl2, and 2 mM dithiothreitol) and then incubated at room temperature with kinase buffer containing 3 μg of H2B, 2 μg ATP, and 10 μCi of [γ-32P]ATP. The reaction was terminated by adding 30 μl of 2X SDS sample buffer and boiling for 10 min. The reaction mixtures were separated by 12% SDS-polyacrylamide gel electrophoresis. The gel was dried, and the phosphorylation of H2B as indicated by γ-32P incorporation was visualized by autoradiography (15). **In Vitro Binding Assay**—The expression and purification of GST-fusion proteins containing wild type Akt or Akt P424A/P427A were described previously (7, 14, 16). The bacteria were harvested and resuspended in PBS containing protease inhibitors and lysed by sonication. After centrifugation (13,000 × g for 10 min), the fusion proteins were collected by incubating the supernatants with glutathione-Sepharose 4B beads (Amersham Biosciences) at 4 °C for 1 h with continuous shaking. The beads were washed three times to remove the unbound proteins. The GST-fusion proteins attached to glutathione-Sepharose 4B beads were mixed with the lysates of COS1 cells transfected with c-Src for 1 h at 4 °C by rotating. The beads were washed three times, and the bound proteins were immunoblotted with anti-Src antibody.

**RESULTS**

Alignments of the C-terminal regulatory region of Akt kinases from various organisms revealed an evolutionarily conserved SH3 domain-binding motif, PXXP, encompassing the residues 424–427 (Fig. 1A). To test whether this proline-rich motif is of functional importance, we substituted both proline residues (Pro-424 and Pro-427) with alanines. As shown in Fig. 1B, the P424A/P427A mutant failed to respond to EGF stimulation, as evidenced by its lack of phosphorylation at Thr-308 and Ser-473 as well as in vitro kinase activity using H2B as a substrate. These data suggest that the integrity of this proline-rich motif is essential for Akt activity induced by growth factors. We further examined the effects of the mutation of these two proline residues on Akt activation induced by Src, which contains an SH3 domain and can potentially interact with the PXXP motif in Akt. Fig. 1C shows that mutation of Pro-424 and Pro-427 abolished the activation of Akt induced by a constitutively active Src527F. These results prompted us to test whether Src is physically associated with Akt upon growth factor stimulation. As shown in Fig. 2A, we were able to detect the co-precipitation of endogenous Akt with Src in human prostate cancer cell PC3 cells after 5 min of treatment with EGF. This interaction seemed to be transient and peaked at 15 min of treatment and decayed rapidly at 30 min. Similar results were also obtained in COS1 cells. To further confirm this interaction, c-Src and Akt were co-expressed in COS1 cells, and immunoprecipitations were performed using an antibody specific for Src as shown above. The interaction between Akt and the Src SH3 domain appeared to be specific, because no detectable association of Akt with the SH3 domain of p130Cas was observed (data not shown).

Our previous study showed that tyrosine phosphorylation of Tyr-315 and Tyr-326 by Src plays an important role in the regulation of Akt activity (7). We would like to know whether the interaction between Src and Akt could be a prerequisite step for phosphorylating Akt by Src. As shown in Fig. 3A, the mutation of Pro-424 and Pro-427 dramatically diminished the level of tyrosine phosphorylation of Akt induced by Src. These results are further confirmed by using an antibody specific for

**FIG. 1.** The proline-rich motif is required for Akt activation. a, alignments of amino acid sequence of the C-terminal regulatory region of Akt from different species. The evolutionary conserved PXXP motifs are highlighted. The numbers of amino acids are denoted based on mouse Akt. b, the role of proline-rich motif in EGF-induced Akt activation. COS1 cells were transiently transfected with the indicated constructs. At 24 h post-transfection, the cells were serum starved for 24 h and then left untreated or treated with 100 ng/ml of EGF for 10 min. Immunoprecipitations (IP) were performed as described under “Materials and Methods.” The immunoprecipitates were subject to in vitro kinase (IVK) assays or immunoblotting (IB) with phospho-specific antibodies against Akt. The amount of Akt in each immunoprecipitate was monitored by anti-HA antibody. c, a proline-rich motif is necessary for the activation of Akt by Src. COS1 cells were cotransfected with the indicated constructs of Akt and Src527F. At 24 h post-transfection, the cells were serum starved for 24 h. Immunoprecipitation, in vitro kinase assays, and immunoblotting were performed as in panel b.
phosphorylated Tyr-315. Similar results were also obtained when the cells were stimulated with EGF (Fig. 3B). To understand the relationship between tyrosine phosphorylation and Ser/Thr phosphorylation of Akt during its activation, we also examined the tyrosine phosphorylation of Akt derivatives containing two major Ser/Thr phosphorylation site mutations. As shown in Fig 2C, phosphorylation of Tyr-315 was readily detected in wild-type Akt, Akt T308A, and Akt S473A in EGF-treated or Src527F in COS1 cells. The cells were serum starved, and immunoprecipitations were performed with anti-Akt antibody. Tyrosine phosphorylation (pY) and phosphorylation of Tyr-315 (pY315) were determined by immunoblotting (IB) with anti-Akt antibody.  

Taken together, these observations allow us to conclude that the interaction between the PXXP motif of Akt with the SH3 domain of Src family kinases is required for tyrosine phosphorylation of Akt and its subsequent activation by other upstream kinases.  

To investigate whether this proline-rich motif in Akt is required for its biological function, we first examined the effects of the mutation of the proline-rich motif on the transcriptional activity of Forkhead, which is negatively regulated by Akt (13). Fig. 4A shows that co-transfection of wild-type Akt with FKHR into 293 cells dramatically inhibited FKHR activity as evidenced by a marked reduction of the luciferase activity under the control of a reporter containing three copies of the FKHR recognition motif IRS. The mutant Akt P424A/P427A virtually lost its inhibitory effect on FKHR, which is consistent with its lack of kinase activity in the in vitro kinase assays. As another functional assay, we also examined the effects of the mutation of the proline-rich motif on cell proliferation. As shown in Fig. 4B, overexpression of constitutively active myr Akt in MDCK cells resulted in 2-fold increase in cell proliferation, whereas its derivative containing P424A/P427A mutations had very little effect. Our data suggest that the integrity of this proline-rich motif is required for its biological functions.  

**DISCUSSION**

Protein-protein interactions, which control the organization of the complex network of molecules involved in signal transduction, are mediated via interactions of various structure domains and their recognition motifs. In this study, we demonstrated, for the first time, that a PXXP motif, which is evolutionarily conserved in Akt kinases among organisms from *Caenorhabditis elegans* to human, plays a crucial role in regulating Akt activity. This PXXP motif is located in the C-terminal regulatory region of Akt. When it is mutated, Akt is no longer activated by EGF. Because phosphorylation of Thr-308 of this proline-rich motif on cell proliferation. As shown in Fig. 4B, overexpression of constitutively active myr Akt in MDCK cells resulted in 2-fold increase in cell proliferation, whereas its derivative containing P424A/P427A mutations had very little effect. Our data suggest that the integrity of this proline-rich motif is required for its biological functions.
and Ser-473 is necessary for Akt activation, we then examined the effects of the mutation of the PXXP element on the phosphorylation state of these two critical sites. As expected, EGF failed to induce phosphorylation of either site. According to the current model of Akt activation, translocation of Akt from cytosol to the plasma membrane is required for its phosphorylation by upstream kinases. The loss of response of the mutant to growth factors may result from incapability of translocation to the plasma membrane, because the interaction between proline-rich motif and SH3 domain has been implicated in plasma membrane localization of some proteins (17–19). However, we found that the mutant Akt P424A/P427A can still be efficiently recruited to the plasma membrane as the wild-type (data not shown). These data suggested that this PXXP element regulates Akt activation through mechanisms other than affecting its recruitment to plasma membrane. Recently, we and others have reported that, in addition to phosphorylation of Thr-308 and Ser-473, tyrosine phosphorylation is also implicated in the regulation of Akt activation (7, 8). In our previous study, we provided evidence showing that Src directly regulates Akt activity by phosphorylating Tyr-315 and Tyr-326 in the activation loop of Akt. Because Src contains an SH3 domain, it is likely that Src could potentially interact with the PXXP motif in Akt. In this report, we showed that endogenous Akt is associated with Src in response to EGF. Such interaction was confirmed by both in vivo and in vitro binding assays. As expected, the mutation of the PXXP motif markedly reduced the amount of Src associated with Akt. This indicated that binding of the PXXP motif on Akt and the SH3 domain of Src plays a major role in mediating their interaction. We then demonstrated that this interaction could be a prerequisite for the Src family kinases to phosphorylate the two critical tyrosine residues in the activation loop of Akt, because replacement of the two proline residues with alanines dramatically blocked the tyrosine phosphorylation of Akt. Moreover, the phosphorylation of Tyr-315 was also significantly reduced. Furthermore, we showed that that EGF- or Src527F-induced phosphorylation of Tyr-315 is not affected in Akt T308A or Akt S473A, suggesting that tyrosine phosphorylation of Akt may occur prior to serine/threonine phosphorylation, at least in COS1 cells. The interaction between Akt and Src is functionally important because the mutant Akt P424A/P427A failed to inhibit FKHR transcriptional activity and can hardly stimulate cell proliferation. Our data are consistent with the model in which Akt translocates to the plasma membrane through the binding of its PH domain to the second messenger PIP3 generated by phosphatidylinositol 3-kinase, where Akt meets with membrane-bound Src via the interaction between its PXXP motif in the C-terminal regulatory region and the SH3 domain of Src. This allows Src family kinase(s) to phosphorylate Akt at Tyr-315 and Tyr-326, which is followed by phosphorylation of Akt at Thr-308 and Ser-473 by other upstream kinases such as PDK1. Our results further support the notion that Src family tyrosine kinases can directly regulate Akt activity in concert with phosphatidylinositol 3-kinase and PDK1. Most intriguingly, this PXXP motif is conserved throughout several members of the AGC kinase family including protein kinase A, protein kinase C, and S6K, suggesting that the association of this motif with its recognition domain in the corresponding upstream regulator may be a general mechanism applicable to these kinases.

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