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Differential Interaction of CrkII Adaptor Protein with Platelet-Derived Growth Factor α- and β-Receptors Is Determined by Its Internal Tyrosine Phosphorylation

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CrkII is an intracellular adaptor protein involved in signal transduction by various growth factors. Activation of PDGF α-receptor resulted in its association with CrkII *in vivo*. In contrast, binding of CrkII to the PDGF β-receptor was negligible, despite its becoming prominently phosphorylated. Bacterially expressed GST-CrkII SH2 domain specifically bound to Tyr-762 and Tyr-771 in the activated PDGF α- and β- receptors, respectively. GST fusion protein of full-length CrkII also bound to the activated PDGF β-receptor. However, tyrosine phosphorylation of GST-CrkII diminished its binding to the β-receptor. CrkI, a truncated version of CrkII lacking the phosphorylatable tyrosine residue, could bind to both PDGF α- and β-receptors *in vivo*. In conclusion, tyrosine phosphorylation of CrkII negatively affects its binding to the PDGF receptors. The differential binding of CrkII to the PDGF α- and β-receptors may be a rationale for functional diversity between the two receptors.

Platelet-derived growth factor (PDGF), a potent mitogen for mesenchymal cells, elicits its effect through binding to the cell-surface tyrosine kinase receptors designated as PDGF α- and β-receptors (reviewed in ref. 1). PDGF receptors undergo ligand-induced kinase activation and subsequent autophosphorylation on their own tyrosine residues. Many of the autophosphorylation sites in the PDGF receptors serve as the binding modules for various intracellular signaling molecules such as PI3 kinase, PLC-γ and Src family kinases (1). Genetic as well as cell biological analyses revealed that PDGF α- and β-receptors show both similar and distinct functions (reviewed in ref. 2). Functional difference between α- and β-receptors may lie in their ability to recruit different sets of signaling molecules based on their structural diversity.

We previously identified the binding site for CrkI, CrkII and CrkL in the PDGF α-receptor (3). Crk proteins are cellular homologs of an avian oncoprotein v-Crk. They lack catalytic structure and are believed to function as adaptors. CrkI and CrkII are different splice products of the same gene, and CrkL is encoded by a distinct gene (4, 5). Both CrkII and CrkL have a single SH2 and two SH3 domains. What makes CrkII and CrkL unlike other adaptor proteins such as Grb2 and Nck is that they become tyrosine phosphorylated. Various growth factors induce phosphorylation of CrkII (reviewed in ref. 6). Constitutive tyrosine phosphorylation of CrkL is implicated in development of chronic myelogenous leukemia (7–9). On the other hand, CrkI, a truncated version of CrkII, lacks C-terminal SH3 domain and a phosphorylatable Tyr-221. And CrkI is a transforming protein (4). These findings as well as other reports implicate the role of Crk proteins in mitogenic, motogenic and apoptotic signal transduction (reviewed in 7).

CrkII binds to Tyr-762 in the PDGF α-receptor upon activation, and its tyrosine phosphorylation increases by 2-fold. Activation of the PDGF β-receptor leads to phosphorylation of CrkII even to a higher degree (i.e., 5-fold). However, association between CrkII and the β-receptor has not been demonstrated (3). In this report, we show that tyrosine phosphorylation of CrkII negatively affects its binding to the PDGF receptors. An isolated SH2 domain of CrkII is capable of binding to both activated PDGF α- and β-receptors *in vitro*. However, CrkII preferentially binds to the α- receptor than to the β-receptor *in vivo* because of a less prominent phosphorylation of CrkII by the α-receptor.

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EXPERIMENTAL PROCEDURES

Plasmids and cell culture. Porcine aortic endothelial (PAE) cells expressing either the wild-type PDGF α-receptor, wild-type PDGF β-receptor, Y762F mutant PDGF α-receptor or Y771F mutant PDGF β-receptor have been described earlier (3, 11–13). All the receptors are expressed at similar levels and are kinase active (3, 13). The cells were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum (Life Technologies) and 40 μg/ml gentamycin. The cDNAs encoding Myc-CrkI or Myc-CrkII in the pCAGGS eukaryotic expression vector were described previously (14). Transient transfection of cultured cells with the expression vector pCAGGS-Myc-CrkI or pCAGGS-Myc-CrkII was performed using Effectene transfection reagent (Qiagen) according to manufacturer's instruction. The cells were used for experiments 48 h after transfection.

Antibodies and reagents. Recombinant human PDGF-BB was purchased from R&D Systems. Polyclonal anti-CrkII antibody (C-18), anti-PDGF α-receptor antibody (951) and anti-PDGF β-receptor antibody (958) were obtained from Santa Cruz Biotechnology, Inc. Monoclonal anti-Crk antibody, anti-phosphotyrosine antibody 4G10 and anti-Myc antibody were from Transduction Laboratories, Upstate Biotechnology, and Boehringer Mannheim, respectively.

GST fusion proteins. Fusion proteins of full-length CrkII (GST-CrkII), SH2 (GST-SH2) or SH3 (GST-SH3) domains of CrkII were prepared as described previously (15). Tyrosine phosphorylated GST-CrkII was produced by use of *E. coli* harboring a plasmid-encoded inducible tyrosine kinase gene (Epicurian Coli TKX1 competent cells, Stratagene) according to manufacturer's instruction. Briefly, TKX1 cells transformed with pGEX full-length CrkII were grew in 1x YTG broth for the first step to induce fusion protein expression, and then for the second step in tryptophan-deficient tyrosine kinase-induction medium. Bacteria were pelleted, lysed, and GST fusion protein was purified using Glutathione Sepharose 4B (Amersham-Pharmacia).

Immunoprecipitation and immunoblotting. Cells in a 60-mm plastic dish were serum-starved for 20 h in F-12 medium supplemented with 1 mg/ml bovine serum albumin. The cells were then incubated with or without 50 ng/ml PDGF-BB at 37°C for the indicated time, rinsed once with ice-cold phosphate-buffered saline (PBS), and lysed in a NP-40 lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 2.5 mM EDTA, 500 μM Na_3VO_4, 1% aprotinin and 1 mM PMSF). For immunoprecipitation, 500 μl of the lysates were incubated with the polyclonal anti-CrkII antibody for 2 h at 4°C, followed by incubation with Protein A Sepharose 6MB (Amersham-Pharmacia) for 30 min at 4°C. For anti-Myc immunoprecipitation, Protein G Sepharose (Amersham-Pharmacia) was used instead. The immunoprecipitates were washed three times in the lysis buffer, eluted by boiling in SDS-sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). For immunoblotting, the samples were electrically transferred from the acrylamide gel onto a nitrocellulose filter (Hybond-ECL, Amersham-Pharmacia) followed by blocking in PBS containing 3% bovine serum albumin and 0.1% Tween 20. The blots were incubated with primary antibodies, washed and then incubated with the peroxidase-conjugated sheep anti-mouse (1:2000 dilution) or anti-rabbit (1:2000 dilution) immunoglobulins. After washing, the sites of antibody binding were detected with an enhanced chemiluminescence system (Amersham-Pharmacia).

In vitro association of the autophosphorylated PDGF receptor with GST fusion proteins. PAE cells expressing the wild-type or tyrosine residue-mutated PDGF receptors were serum-starved for 20 h. The cells were then incubated with or without 50 ng/ml PDGF-BB for 10 min at 37°C, and lysed in a Triton-X lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Tx-100, 0.5% deoxycholic acid, 10 mM EDTA, 1% aprotinin and 1 mM PMSF). The lysates were incubated for 2 h at 4°C with 100 ng of GST fusion protein immobilized on Glutathione Sepharose. After washing three times in the same lysis buffer, the samples were subjected to SDS-PAGE and immunoblotted using either anti-PDGF α- or β-receptor antibodies.

RESULTS AND DISCUSSION

We first examined the time-course of CrkII binding to the PDGF α- and β-receptors following ligand stimulation. PAE cells expressing either the wild-type PDGF α- or β-receptors were treated with PDGF-BB at 37°C for 1–15 min. The cells were lysed and subjected to immunoprecipitation with anti-CrkII antibody, analyzed by SDS-PAGE, and immunoblotted with an anti-phosphotyrosine antibody. As shown in Fig. 1A, upper panel, a 170 kDa phosphorylated protein previously identified as PDGF α-receptor (3) appeared in CrkII immunoprecipitates 1 min after ligand stimulation, and gradually increased up to 15 min. A 190 kDa phosphoprotein most likely representing the activated PDGF β-receptor, although in a small amount, was also detected in CrkII immunoprecipitates 1 min after stimulation (Fig. 1B, upper panel). The coprecipitation decreased thereafter. The 190 kDa phosphoprotein comigrated with activated PDGF β-receptor, but could not be detected by anti-PDGF β-receptor blotting possibly due to low sensitivity of the antibody (data not shown). Interestingly, degree of CrkII tyrosine phosphorylation was inversely related to its receptor association. Phosphorylation of CrkII significantly and time-dependently increased upon ligand stimulation of PDGF β-receptor, whereas activation of PDGF α-receptor led to a subtle change in CrkII phosphorylation (Fig. 1A and B, middle panels). Immunoblotting of the same filter using anti-Crk antibody confirmed equal loading of Crk II in each lane (Fig. 1A and B, lower panels). The results indicate that PDGF β-receptor binds CrkII for a short period following ligand stimulation, and rapidly dissociates from it in parallel to increased tyrosine phosphorylation of CrkII.

We next tested whether bacterially-expressed GST-fusion protein of CrkII is able to bind activated PDGF α- and β-receptors *in vitro.* PAE cells expressing the wild-type or Y762F mutant PDGF α-receptors, wild-type or Y771F mutant β-receptors were treated or untreated with PDGF-BB and lysed. The lysates were incubated with GST fusion protein of CrkII SH2 domain (GST-SH2) immobilized on Glutathione Sepharose. After extensive washing, the precipitated materials were subjected to SDS-PAGE and immunoblotted using anti-PDGF α- or β-receptor antibodies. As shown in Fig. 2A, association of the wild-type but not Y762F mutant PDGF α-receptor with GST-CrkII was observed upon ligand-stimulation. The results indicate that the SH2 domain mediates the binding of CrkII to the phosphorylated Tyr-762 in the PDGF α-receptor. The activated wild-type PDGF β-receptor was also found to associate with GST-SH2 (Fig. 2B). In contrast,

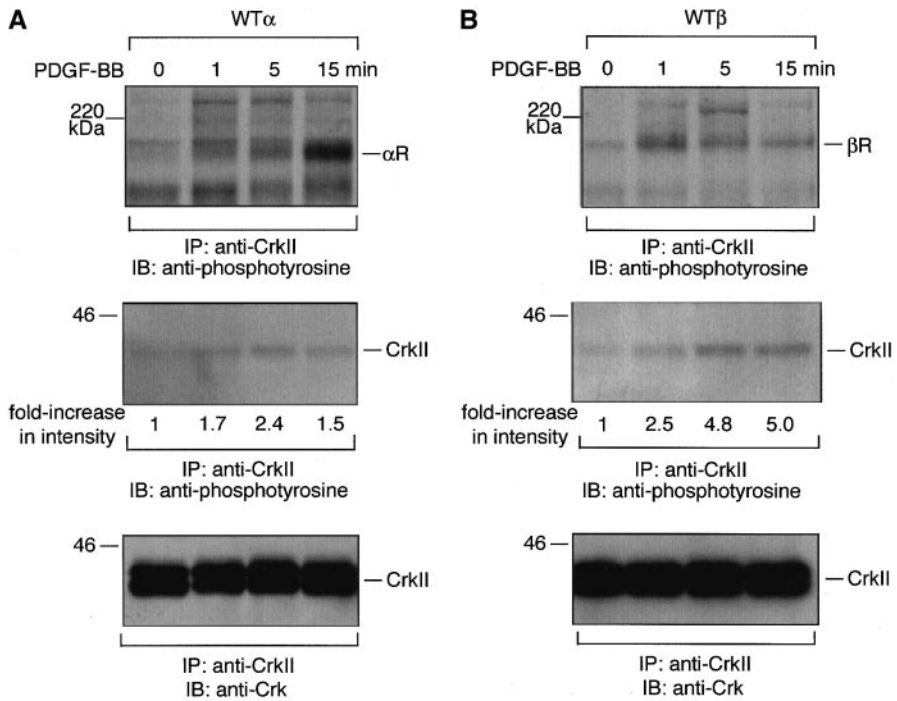

FIG. 1. Time course of PDGF-induced tyrosine phosphorylation and receptor association of CrkII. (A) PAE cells expressing the wild-type PDGF α-receptors (WTα) or (B) PDGF β-receptors (WTβ) were incubated without (−) or with (+) 50 ng/ml PDGF-BB for 1, 5, and 15 min at 37°C. The cells were lysed and subjected to immunoprecipitation using polyclonal anti-CrkII antibody. The immunoprecipitates were analyzed by SDS-PAGE and then transferred onto nitrocellulose filter. The filter was immunoblotted with an anti-phosphotyrosine monoclonal antibody (upper and middle panels). Density of the phosphorylated CrkII bands was measured using NIH image, and indicated in the figure as fold increase. After stripping, the same blot was reprobed with anti-Crk monoclonal antibody (lower panel). Migration positions of the PDGF α-receptor (αR), PDGF β-receptor (βR), and CrkII (CrkII) are indicated.

Y771F mutant PDGF β-receptor did not bind to GST-SH2 (Fig. 2B). Tyr-771 in the PDGF β-receptor is localized in an analogous position as Tyr-762 in the PDGF α-receptor. Amino acid sequences carboxyterminal to the Tyr-762 in the α-receptor and Tyr-771 in the β-receptor are Yp-D-R-P and Yp-M-A-P, respectively (Yp as phosphotyrosine residue). Both sequences satisfy requirement for Crk SH2 binding site, Yp-X-X-P, predicted by degenerated peptide library analysis (16). However, the α-receptor sequence seems to be even more suitable for the binding by having an aspartic acid residue at the second position to phosphotyrosine (16). Neither the wild-type nor mutant PDGF receptors bound GST-SH3 (data not shown). These data indicate that both PDGF α- and β-receptors upon ligand-stimulation are capable to bind CrkII SH2 domain *in vitro*.

CrkII protein itself contains a phosphorylatable tyrosine residue (Tyr-221) within the region between two SH3 domains (4, 14). Phosphorylation of Tyr-221 in CrkII generates a binding site (pY-A-Q-P) for Crk SH2 domain (14). Indeed, an NMR spectroscopic analysis demonstrated an intramolecular interaction between SH2 and the phosphorylated Tyr-221 of CrkII *in vitro* (17). Since a molecule of SH2 domain can only bind a single phosphotyrosine residue at a time, it is speculated that the Crk SH2 domain occupied by Tyr-221 would not bind the PDGF receptor any longer. Therefore, we next examined the effect of tyrosine phosphorylation of recombinant CrkII protein on its association with PDGF β-receptor. Tyrosine phosphorylated GST-full length CrkII fusion protein (GST-CrkII) was produced in bacteria as described in Experimental Procedures. Phosphorylated or unphosphorylated fusion proteins were immobilized on Glutathione Sepharose beads, and incubated with the lysates of ligand-stimulated or unstimulated PAE cells expressing PDGF β-receptors. The materials were subjected to SDS-PAGE and immunoblotted with anti-PDGF β-receptor antibody. As shown in Fig. 2C, upper panel, unphosphorylated GST-CrkII bound ligand-activated PDGF β-receptor *in vitro*. In contrast, phosphorylation of GST-CrkII inhibited its binding to activated PDGF β-receptor. Blotting of the filter with anti-GST antibody (Fig. 2C, middle panel) and with anti-phosphotyrosine antibody (Fig. 2C, lower panel) confirmed that the fusion proteins were equally loaded in each lane and that phosphorylation of GST-CrkII had successfully been done. The results confirmed that tyrosine phosphorylation of CrkII is a negative regulatory factor for the association with PDGF β-receptor.

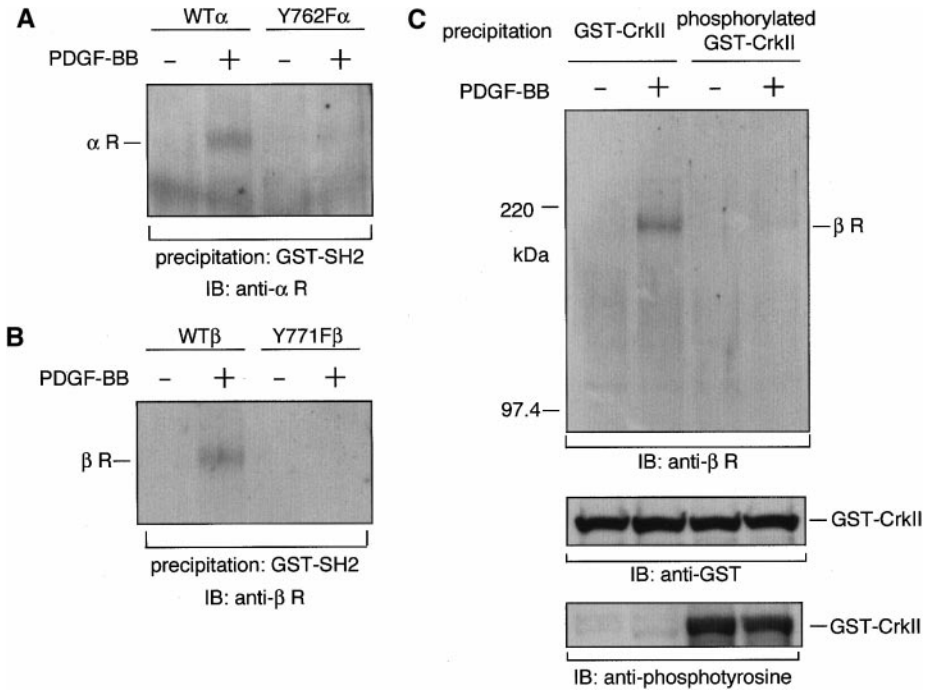

FIG. 2. *In vitro* association of GST-CrkII SH2 fusion protein with the wild-type or tyrosine residue-mutated PDGF α- or β- receptors. (A) PAE cells expressing the wild-type PDGF α-receptor (WTα) or Y762F mutant PDGF α-receptor (Y762Fα) were incubated without (−) or with (+) 50 ng/ml PDGF-BB for 10 min at 37°C and lysed. The lysates were incubated for 2 h with 100 ng of GST-CrkII SH2 domain fusion protein immobilized on Glutathione Sepharose. The precipitated materials were subjected to SDS-PAGE and then immunoblotted using anti-PDGF α-receptor antibody. The position of the PDGF α-receptor (αR) is indicated. (B) PAE cells expressing the wild-type PDGF β-receptor (WTβ) or Y771F mutant PDGF β-receptor (Y771Fβ) were incubated without (−) or with (+) 50 ng/ml PDGF-BB for 10 min at 37°C and lysed. The lysates were incubated for 2 h with 100 ng of GST-CrkII SH2 domain fusion protein immobilized on Glutathione Sepharose. The precipitated materials were subjected to SDS-PAGE and then immunoblotted using anti-PDGF β-receptor antibody. The position of the PDGF β-receptor (βR) is indicated. (C) PAE cells expressing the wild-type PDGF β-receptor were incubated without (−) or with (+) 50 ng/ml of PDGF-BB for 10 min at 37°C and lysed. The lysates were incubated with nonphosphorylated or tyrosine-phosphorylated GST full-length CrkII fusion protein immobilized on Glutathione Sepharose beads. Materials bound on beads were subjected to SDS-PAGE followed by electrical transfer onto nitrocellulose membrane. The membrane was then immunoblotted with anti-PDGF β-receptor antibody (upper panel). The low molecular weight part of the blot was probed with anti-GST antibody (middle panel). After stripping of the anti-GST antibody, the blot was reprobed using anti-phosphotyrosine antibody (lower panel). Migration positions of the PDGF β-receptor (βR) and GST-CrkII (GST-CrkII) are indicated.

We finally tested the binding of CrkI, a naturally occurring C-terminal truncated form of CrkII (Fig. 3A) and lacks Tyr-221, to the PDGF receptors. Since an antibody able to distinguish CrkI from CrkII was unavailable, we took advantage of using epitope-tagged Crk proteins. PAE cells expressing either the wild-type PDGF α- or β-receptors were transiently transfected with pCAGGS plasmids encoding Myc-tagged CrkII or Myc-tagged CrkI. The cells were treated with PDGF-BB, lysed and immunoprecipitated with anti-Myc antibody. The samples were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 3B, upper panel, the activated PDGF α-receptor but not β-receptor coprecipitated with CrkII upon ligand stimulation (lane 1, 2, 5, 6). In contrast, CrkI was found to coprecipitate both α-receptor and β-receptor upon ligand-stimulation (lanes 3, 4, 7, 8). Figure 3B, lower panel, shows equal loading of CrkI and CrkII. It is interesting to note that CrkI coprecipitated even a larger amount of PDGF α-receptor compared to what CrkII did. These results suggest that the region encompassing Tyr-221 in CrkII, which is absent in CrkI, negatively regulates association between CrkII and PDGF receptors *in vivo*. It seems that PDGF β-receptor compared to the α-receptor is more sensitive to the regulatory mechanism.

Recently, Hashimoto *et al.* reported that ligand-treated EGF receptor phosphorylates CrkII on Tyr-221 and that the phosphoryation results in an immediate dissociation of CrkII from the EGF receptor (14). This is likely due to formation of intramolecular binding between the phosphotyrosine and SH2 domain in CrkII (14, 17, 18). The finding is analogous to what we observed upon activation of the PDGF β-receptor (Fig. 4). Unlike EGF or PDGF β-receptors, PDGF α-receptor maintained its association with CrkII up to 15 min after ligand-stimulation. We assume that the relatively inefficient phosphorylation of CrkII by the PDGF α-receptor leaves CrkII SH2 domain free to bind the

receptor, and thus enables formation of stable complex between the two molecules (Fig. 4).

Tyr-771 in the PDGF β-receptor has well been characterized as the binding site for RasGAP (19). On the other hand, RasGAP does not bind PDGF α-receptor (3, 20, 21). Therefore, competitive binding of RasGAP to Tyr-771 may also contribute to a trivial binding of CrkII to the β-receptor.

It remains to be elucidated why CrkII becomes more efficiently phosphorylated by PDGF β-receptor than by the α-receptor. One possible explanation is that CrkII serves as a better substrate for the PDGF β-receptor kinase than for the α-receptor kinase. Alternatively, CrkII phosphorylation may be mediated by a downstream effector specific for the PDGF β-receptor. For example, Blakesley *et al.* reported involvement of sphingosine kinase in PDGF-BB-induced tyrosine phosphorylation of CrkII (22).

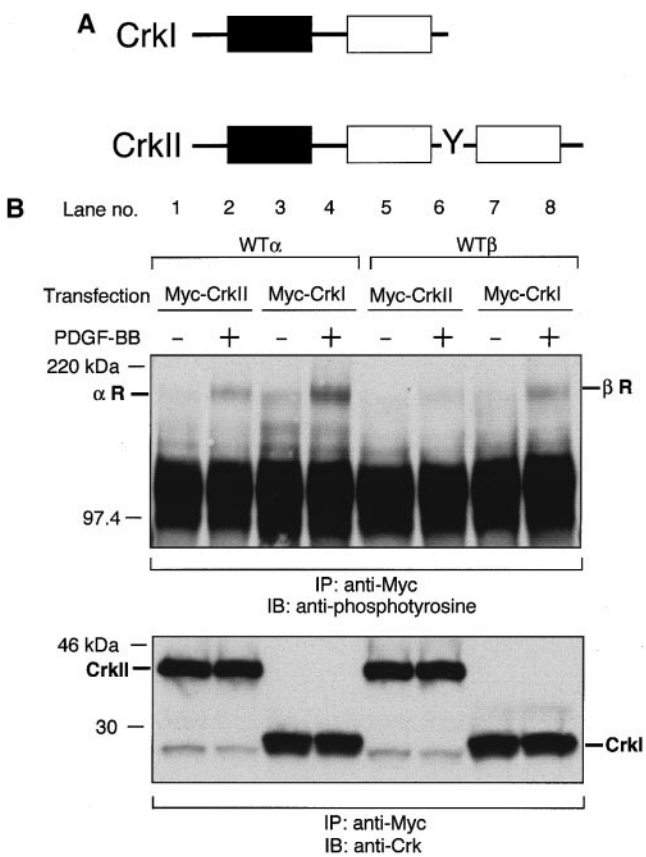

FIG. 3. *In vivo* association of PDGF receptors with CrkI and CrkII. (A) Schematic illustration of CrkI and CrkII. Closed and open boxes represent SH2 and SH3 domains, respectively. Y indicates the position of Tyr-221. (B) PAE cells expressing the wild-type PDGF α-receptors (WTα) or β-receptors (WTβ) were transiently transfected with pCAGGS plasmid containing the Myc-tagged CrkII or CrkI cDNAs. The transfected cells were incubated without (−) or with (+) 50 ng/ml PDGF-BB for 10 min at 37°C and then lysed. The lysates were immunoprecipitated using anti-Myc monoclonal antibody. Eighty percent of the material was separated by 5% SDS-PAGE and subjected to immunoblotting using anti-phosphotyrosine antibody (upper panel). The remaining 20% of the sample was separated by 12.5% SDS-PAGE and analyzed by immunoblotting with the monoclonal anti-Crk antibody (lower panel). The positions of the PDGF receptors (αR, βR) and Myc-tagged Crk proteins (CrkI, CrkII) are indicated.

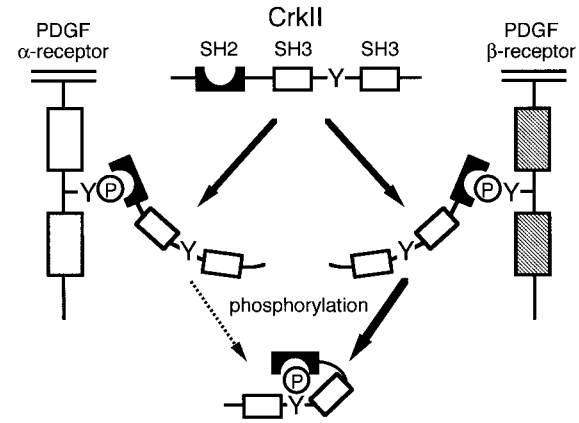

FIG. 4. Schematic model of hypothesized interaction between PDGF receptors and CrkII. In the absence of phosphorylation on Tyr-221, CrkII binds via its SH2 domain to phosphotyrosine residue in the PDGF receptors. Upon phosphorylation, Tyr-221 and the SH2 domain in CrkII binds to each other intramolecularly. As a result, CrkII SH2 domain is no longer able to associate with the PDGF receptor. Since the PDGF β-receptor phosphorylates CrkII efficiently, CrkII tends to dissociate from the receptor. In contrast, CrkII remains in complex with PDGF α-receptor because majority of CrkII molecules are left unphosphorylated.

Because the lack of a catalytic structure, it is likely that Crk proteins function via binding to their effector molecules. It has been shown that C3G, a guanine nucleotide exchange protein for Rap1, DOCK180, an activator of Rac, Sos, a guanine nucleotide exchange protein for Ras and Abl family tyrosine kinases bind the SH3 domain of CrkII (18, 23–25). Ichiba *et al.* reported that membrane targeting of C3G resulted in activation of its guanine nucleotide exchange activity for Rap1 (26). It is well accepted that membrane-translocation is required for Sos to activate Ras (27, 28). These evidences suggest that dissociation of CrkII from the PDGF β-receptor may result in inactivation of the effector functions. In contrast, the stable binding of CrkII to the α-receptor possibly leads to a prolonged activation. In other words, PDGF α-receptor may be less sensitive to the negative feedback regulation of CrkII function compared to the β-receptor. We are currently investigating these possibilities. The differential binding of CrkII to the PDGF α- and β- receptors may be a rationale for functional diversity between the two receptors.

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