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Protein Kinase Cδ Induces Src Kinase Activity via Activation of the Protein Tyrosine Phosphatase PTPα*

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Previously we have shown that protein kinase C (PKC)-mediated reorganization of the actin cytoskeleton in smooth muscle cells is transmitted by the nonreceptor tyrosine kinase, Src. Several authors have described how 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulation of cells results in an increase of Src activity, but the mechanism of the PKC-mediated Src activation is unknown. Using PKC isozymes purified from *Spodoptera frugiperda* insect cells, we show here that PKC is not able to activate Src directly. Our data reveal that the PKC-dependent Src activation occurs via the activation of the protein tyrosine phosphatase (PTP) PTPα. PTPα becomes activated *in vivo* after TPA stimulation. Further, we show that PKCδ phosphorylates and activates only PTPα *in vitro* but not any other of the TPA-responsive PKC isozymes that are expressed in A7r5 rat aortic smooth muscle cells. To further substantiate our data, we show that cells lacking PKCδ have a markedly reduced PTPα and Src activity after 12-*O*-tetradecanoylphorbol-13-acetate stimulation. These data support a model in which the main mechanism of 12-*O*-tetradecanoylphorbol-13-acetate-induced Src activation is the direct phosphorylation and activation of PTPα by PKCδ, which in turn dephosphorylates and activates Src.

Protein kinase C (PKC)[1] is a family of phospholipid-dependent serine/threonine kinases comprising 10 isozymes differing in their molecular domain organization of up to 4 variable and 3 constant regions and in their functions. These PKC isozymes are subdivided into three classes: (i)the "conventional" cPKCs, PKC-α, -β (βI and βII), and -γ, which can be activated by phosphatidylserine (PS), diacyl glycerol (DAG), or phorbol esters through binding to the C1 domain and Ca^{2+} through binding to a Ca^{2+}-binding site in their second constant region, C2; (ii) the "novel" nPKCs, PKC-δ, -ϵ, -η, -θ, which lack the C2 region and thus are Ca-independent but still DAG-, PS-, and phorbol ester-responsive; and (iii) the "atypical" aPKCs, PKC-λ/ι and -ζ, which also lack the C2 region and, in addition, are devoid of a functional DAG-binding site. Hence, the atypical PKCs are only responsive to PS but not to DAG or phorbol ester (reviewed in Refs. 1–3).

PKCs reside in the cytosol in an inactive conformation and translocate to the membrane (or other subcellular sites) upon activation, where they modify various cellular functions through phosphorylation of target substrates. Like other kinases, PKCs have been found to be involved in intracellular signal transduction pathways that regulate cell growth, differentiation, and apoptosis, and they have been implicated in the rearrangement of the cytoskeleton and migration (4).

Src-family tyrosine kinases also comprise a major group of cellular signal transducers. These tyrosine kinases can be activated by various extracellular signals and thus can modulate a variety of cellular functions, including proliferation, survival, adhesion, and migration (5). When phosphorylated at Tyr-527 Src is inactive. Activation is accomplished by dephosphorylation of this tyrosine residue, and the resulting conformational change facilitates an autophosphorylation at Tyr-416. After autophosphorylation Src is in an active state.

As described earlier, TPA activates Src *in vivo* (6–9). Previously, we showed in A7r5 rat aortic smooth muscle cells that PKC-induced reorganization of the actin cytoskeleton involves the activation of Src tyrosine kinase activity (10). It is therefore very likely that PKC acts upstream of Src and mediates its activation, because PKC is known to be the main receptor of TPA in cells. However, neither a model for PKC-mediated Src activation nor even an explanation for the TPA stimulation of Src activity has been described so far.

The tyrosine phosphatase PTPα, a 130-kDa transmembrane PTP, is known to be activated upon TPA stimulation and subsequent phosphorylation (11, 12). Moreover, PTPα has been shown to be a physiological regulator of Src (13–15). Hence, we examined whether the TPA-induced activation of Src involves PTPα. Here we present evidence of PKC-mediated Src activation via PTPα.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The monoclonal Src-antibody (Ab-1) was obtained from Oncogene, the HA-antibody was from Roche Applied Science, and the pY(416) Src antibody to measure Src activity (16–18) was obtained from BIOSOURCE. The PTPα-specific antibody was a kind gift from Jeroen den Hertog (Hubrecht Laboratories, Utrecht, Netherlands) (19). All secondary antibodies were from Dianova. TPA, GF109203X, and phosphatidylserine were obtained from Calbiochem. Thrombin, polyvinylidene difluoride and nitrocellulose membranes, the ECL-kit and [γ-^{32}P]ATP were from Amersham Biosciences. All other

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[1] The abbreviations used are: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PTP, protein tyrosine phosphatase; GST, glutathione *S*-transferase; Sf9, *Spodoptera frugiperda*; MBP, myelin basic protein; pNPP, *p*-nitrophenyl phosphate.

chemicals were obtained from Sigma. All cell culture reagents were from Invitrogen.

Plasmids—To express GST-tagged PKCs in *Spodoptera frugiperda* (Sf9) insect cells, the complete coding regions of the relevant murine PKC cDNAs (20) were cloned in-frame into pAcGHLT-C or a modified pAcGHLT-C vector (BD Biosciences), where the thrombin cleavage site was exchanged for a TEV cleavage site. The accuracy of the inserted PKC sequences was proved by DNA sequencing.

To generate the recombinant baculovirus, PAcGHLT-C-PKC plasmids were cotransfected with Baculo-Gold (BD PharMingen, Dianova) DNA in Sf9 cells according to the manufacturers' recommendations.

Five plaques of each baculovirus were picked and amplified in Sf9 cells. Protein expression in infected cells was examined using immunoblot analysis. The activity of the recombinant protein was examined in an *in vitro* kinase assay, and the respective baculoviruses were used to infect Sf9 cells as described elsewhere (21). The baculovirus for c-Src was a kind gift from Margaret Frame (Beatson Laboratories, Glasgow, UK). The tetracyclin-inducible expression vector for HA-tagged PTPα was a kind gift from David Shalloway (Cornell University, Ithaca, NY) (14).

Cell Culture and Transfections—A7r5 rat vascular smooth muscle cells were grown in Dulbecco's modified Eagle's medium without phenol red containing 10% fetal calf serum and 2 mM glutamine. The medium was changed every 3 days. For transfection, A7r5 cells were seeded freshly and transfections were performed after ~24 h of culture or when cells were grown to 50–70% confluence. The expression vector for HA-PTPα and transactivator-plasmid pTet-tTAk (Invitrogen) were resuspended in the above medium without serum at a ratio of 1:2 (10 μg/10-cm plate). Superfect transfections reagent (Qiagen, Hilden, Germany) was added according to the manufacturer's recommendations. Transfected cells were examined 2 days after transfection.

Primary skin fibroblasts were isolated from newborn PKCδ−/− and PKCδ+/+ mice from the same litter as described earlier (22) and cultured in the same way as A7r5 cells. Experiments were performed between passage 3–5. Sf9 insect cells were cultured at 27 °C without CO_2 in spinner flasks, using Grace's insect medium containing 10% fetal bovine serum, 3.3 g/liter lactalbumin hydrolysate, 3.3 g/liter yeastolate, 50 μg/ml gentamicin, and 2.5 μg/ml fungizone.

PKC Kinase Assays—Recombinant PKC isozymes were prepared from Sf9 cells as described (21), bound to GSSH-Sepharose, and cleaved by thrombin. The activity of each isozyme was standardized in an *in vitro* kinase assay using myelin basic protein (MBP). One μg of MBP was incubated with different amounts (5–20 ng) of the PKC isozymes in the presence of 20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 0.5 μM TPA, 1.5 μM phosphatidylserine, 10 μM ATP, 5 μCi of [γ-^{32}P]ATP (3000Ci/mM, Amersham Biosciences), 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (kinase buffer) in a final volume of 50 μl for 15 min at 30 °C. The reaction was stopped by adding 10 μl of 6× SDS sample buffer and heating to 95 °C for 5 min. The samples were analyzed by SDS-PAGE (12.5%). The gel was stained with Coomassie Brilliant Blue before it was exposed to an x-ray film.

PTPα Activity and Src Activity Assays—A7r5 cells were transfected with HA-tagged PTPα. 48 h after transfection the cells were serum-starved for 6 h, stimulated with 1 μM TPA for 15 min, washed with phosphate-buffered saline, and lysed on ice in RIPA buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1% Triton, 0.1% SDS, 0.5% deoxycholate, 1 mM EDTA, 1 mM NaF, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were cleared by centrifugation for 10 min at 14,000 rpm. Cleared lysates were incubated overnight at 4 °C with 30 μl of protein-G-Sepharose beads and 0.5 μg of HA-antibody or 20 μl of the polyclonal PTPα antibody. Precipitated PTPα immunocomplexes were washed once with RIPA buffer (now containing 500 mM NaCl), twice with PPase assay buffer (0.1 mM succinic acid, pH 6.0, 150 mM NaCl, 1 mM EDTA) and subsequently incubated for 30 min in 200 μl of PPase assay buffer containing 10 μM *p*-nitrophenyl phosphate (pNPP). PTPα immunocomplexes were sedimented by centrifugation, an appropriate volume of 2× sample buffer was added, and the amount of PTPα was estimated by immunoblotting using the PTPα-specific antibody. The supernatant was mixed with 200 μl of 2 M NaOH, and the absorption at 415 nM was measured.

To determine the phosphatase activity toward Src, PTPα was immunoprecipitated from TPA-stimulated or unstimulated cells. Immunocomplexes were washed once with RIPA buffer (500 mM NaCl), twice with PPase assay buffer, and resuspended in 200 μl of PPase-assay buffer. Recombinant Src was isolated from Sf9 cells via glutathione-GST interaction. The GST tag was then cleaved with thrombin and removed by the GSSH-Sepharose chromatography. 1 μg of purified Src was added to each sample, and the sample was incubated at 30 °C for 1 h with agitation.

The samples were washed twice with 600 μl of buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl, 5% glycerol, 1% Triton-X-100, 2 mM Na_3VO_4, 2 mM β-glycerophosphate, 2 mM NaF, 2 mM pyrophosphate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, and the supernatant was placed in a new tube. Src was immunoprecipitated from the sample using 1 μg of the Src-specific antibody AB-1 coupled to protein-G-Sepharose for 2–4 h at 4 °C. Src-containing beads were washed twice with kinase buffer (20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.1%Nonidet P-40, 2 mM β-glycerophosphate, 2 mM NaF, 2 mM pyrophosphate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and was permitted to undergo autophosphorylation in 50 μl of kinase buffer containing 5 μM ATP for 15 min at 37 °C. The reaction was stopped by adding 10 μl of 6× SDS-sample buffer and heated to 95 °C for 5 min. Equal amounts of the sample were separated in parallel by SDS-PAGE (10%) and subjected to immunoblot analysis, using the anti-Src antibody Ab-1 to quantitate the levels of c-Src and the Src pY(416) antibody to determine the activation state of Src (16–18). To prove that equal amounts of PTPα were analyzed in each sample, an appropriate volume of 2× sample buffer was added to PTPα immunocomplexes, and the amount of PTPα was estimated by immunoblotting using the PTPα -specific antibody.

To analyze the activity of PTPα after direct phosphorylation by PKC isozymes, HA-PTPα was immunoprecipitated from serum-starved unstimulated A7r5 cells. PTPα immunocomplexes were washed once with RIPA buffer (500 mM NaCl) and twice with kinase buffer and were subjected to the kinase assay with ~20 ng of recombinant PKC isozymes in the presence of 0.5 μM TPA, 1.5 μM phosphatidylserine, and 10 μM ATP for 30 min at 30 °C. For direct analysis of the phosphatase activity on pNPP, the phosphorylated phosphatase was washed twice with PPase assay buffer and incubated for 30 min in 200 μl of PPase assay buffer containing 10 μM pNPP. PTPα immunocomplexes were sedimented by centrifugation, an appropriate volume of 2× sample buffer was added, and the amount of PTPα was again estimated by immunoblotting using the PTPα-specific antibody. The supernatant was mixed with 200 μl of 2 M NaOH, and the absorption at 415 nM was measured.

To measure the activity of PTPα on Src after direct PKC phosphorylation, PTPα immunocomplexes were washed twice with PPase assay puffer. The immunocomplexes were resuspended in 200 μl of PPase assay buffer containing 1 μg of purified Src, and the sample was incubated at 30 °C for 1 h with agitation. The analysis of the Src activity was performed as described above. The Src kinase assays shown in Figs. 1*A* and 4*B* were done as described earlier (10)

RESULTS

Src Is Not Directly Activated by PKC—As shown previously (10), TPA stimulation of A7r5 cells leads to an increase of Src kinase activity *in vivo*. Src was immunoprecipitated from serum-starved A7r5 cells before and after TPA stimulation for 15 min, and the activity of Src was determined in immunoblot analysis using the pY(416)-specific antibody for Src. A robust increased immunoreactivity of Src from TPA-stimulated cells with the pY(416)-specific antibody was observed (Fig. 1*A*).

To ensure that the measurement of Src kinase activity with the Src pY(416)-specific antibody is a valid method to determine the activity of Src, we analyzed the Src activity in parallel directly in an *in vitro* kinase assay. Src was immunoprecipitated from A7r5 cells before and after TPA stimulation, and the kinase activity of Src was determined using acid-denatured enolase as an exogenous substrate. As shown in Fig. 1*A*, the kinase activity of Src measured on enolase correlates directly with the immunoreactivity of the Src pY(416)-specific antibody. A 2–3-fold increase of Src kinase activity could be observed after TPA stimulation.

Then we asked whether PKC isozymes could directly activate the kinase activity of Src, because Src has been described as a PKC substrate (23). To this end, Src was phosphorylated *in vitro* with the five TPA-responsive PKC isozymes that are present in wild-type A7r5 cells (α, βI, δ, ε, η), (24). Subsequently, autophosphorylation of Src at Tyr-416 was examined as a marker for Src activity utilizing a pY(416)-specific anti-

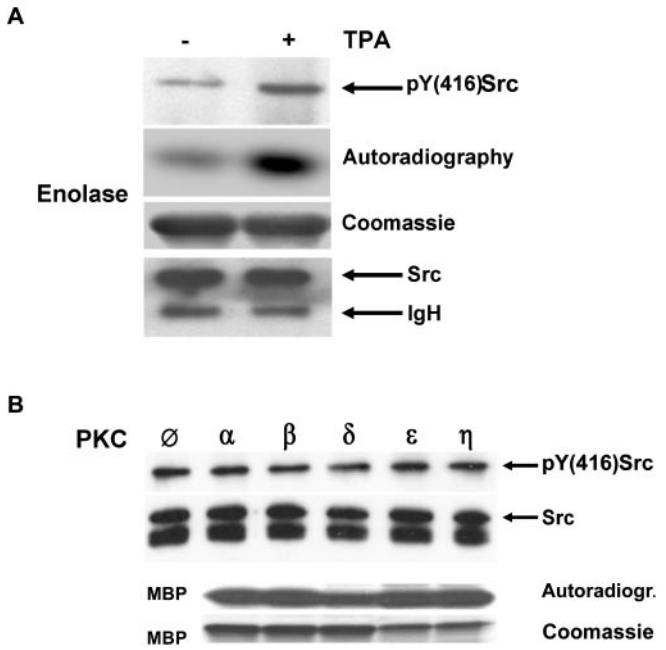

FIG. 1. **Src is not activated directly by PKC phosphorylation.** *A*, Src was immunoprecipitated from serum-starved A7r5 cells before (−) and after (+) TPA stimulation (15 min). The activity of Src was determined using the pY(416)-specific antibody for Src or directly in an *in vitro* kinase assay using acid-denatured enolase to ensure that the immunoreactivity of the pY(416)-specific antibody correlates directly with the kinase activity of Src. In both cases a robust increase of Src kinase activity could be observed after TPA stimulation. As a control, the amount of Src was analyzed using the Src-specific antibody. *B*, Src was immunoprecipitated from serum-starved, unstimulated A7r5 cells and subjected to an *in vitro* kinase assay with the five TPA-responsive PKC isozymes expressed in A7r5 cells (purified recombinant proteins from Sf9 cells) or left untreated as a control. The activity of Src was analyzed using the Src p(Y416)-specific antibody. As a control, the amount of Src was analyzed using the Src-specific antibody. No significant activation of Src could be observed in this assay. The intensity of the signal obtained from the phospho-specific antibody directly correlated with the signal from the Src-specific antibody. As a control for equal activity of the PKC isozymes that were used in this assay, MBP was used as a substrate in an *in vitro* kinase assay. Incubation of MBP with the different PKC isozymes resulted in an equal incorporation of radioactively labeled phosphate, indicating a comparable activity of the five PKC isozymes (*lower panel*). Data shown here are representative of three experiments.

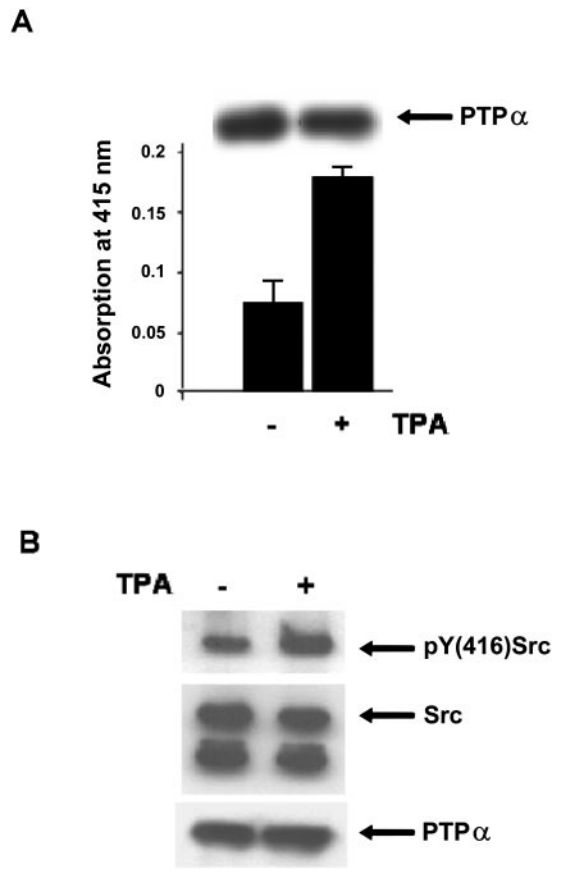

FIG. 2. **PTPα is activated after TPA stimulation.** A7r5 cells were transfected with an HA-PTPα-encoding construct, and PTPα was immunoprecipitated with an HA-specific antibody from either TPA-stimulated (15 min) or unstimulated cells. *A*, the phosphatase activity was assayed using pNPP. A 2–3-fold increase of PTPase activity was detected in immunocomplexes that were derived from TPA-stimulated cells in comparison to unstimulated cells. The *inserted upper panel* shows a Western blot stained for PTPα, indicating that equal amounts of PTPα were assayed. Data shown are mean ± S.E. (n = 3). *B*, the phosphatase activity of PTPα was analyzed with Src as a physiological substrate, and the activation state of Src was determined using the pY(416)-specific antibody. Incubation of recombinant Src with PTPα immunoprecipitated from TPA-stimulated cells resulted in an increase of Src autophosphorylation at Tyr-416 in comparison to Src that was incubated with PTPα from unstimulated cells. As a control that equal amounts of PTPα and Src were analyzed in each sample, the amount of PTPα and Src detected by immunoblot analyses is shown. Data shown here are representative of three experiments.

body (see above). As is evident from Fig. 1*B*, phosphorylation of Src with the different PKC isozymes did not result in any significant differences in the phosphorylation status of Tyr-416. As a control for equal activity of the PKC isozymes that were used in this assay, the activity of PKC isozymes was determined in an *in vitro* kinase assay using MBP as a substrate in the presence of radiolabeled phosphate (Fig. 1*B*, *lower panel*). Incubation of MBP with the different PKC isozymes resulted in an equal incorporation of radioactively labeled phosphate, indicating a comparable activity of the five PKC isozymes (*lower panel*).

These results show, as expected, that the kinase activity of Src is not directly regulated by PKC-mediated phosphorylation. Hence, an indirect mechanism must be responsible for the observed activation of Src *in vivo* after TPA stimulation.

TPA Increases the Activity of PTPα against pNPP and Src—The main regulatory mechanism regulating Src activity is phosphorylation of Tyr-527. Dephosphorylation of this residue is essential for Src kinase activation. Hence, phosphatases represent plausible candidates for signal transmitters from PKC to Src. One such candidate is PTPα, which was reported to be positively regulated by PKC and which also is capable of activating Src (11, 12, 14). To test this scenario, we examined whether the activity of PTPα could be up-regulated in a TPA-dependent manner in A7r5 cells.

To determine whether TPA treatment enhances the phosphatase activity of PTPα, A7r5 cells were transfected with an HA-tagged PTPα expression vector. The transfected cells were stimulated with TPA for 15 min, and HA-PTPα was isolated by immunoprecipitation with an anti-HA antibody and tested for phosphatase activity with pNPP. As is evident in Fig. 2*A*, PKC activation did result in activation of PTPα. The isolated HA-PTPα showed a 2–3-fold increased activity compared with the enzyme that was isolated from cells that were not stimulated with TPA. The *inset* immunoblot shows that equal amounts of PTPα were analyzed in both samples.

We next tested whether PTPα from TPA-stimulated cells is also capable of dephosphorylating and activating Src. To this end, we immunoprecipitated HA-tagged PTPα from A7r5 cells before and after stimulation with TPA for 15 min. Recombinant Src from Sf9 insect cells was incubated with the isolated PTPα *in vitro*. Subsequently, Src was isolated from the reaction mixture by immunoprecipitation. Based on the fact that autophos-

phorylation, and hence activation, of Src depends on the previous dephosphorylation of pY(527), we used the autophosphorylation at Tyr-416 as a measure for the activity of Src. As is evident in Fig. 2*B*, the activity of Src is markedly increased after treatment with PTPα from TPA-stimulated cells, as compared with PTPα from cells that had not been stimulated with TPA.

PKCδ Phosphorylates PTPα in Vitro—Although our data prove that PKC activation *in vivo* results in activation of PTPα, we cannot exclude that another PKC-controlled kinase is responsible for the phosphorylation of PTPα. Hence we examined whether PKC directly phosphorylated the phosphatase *in vitro*. A fusion protein of the cytoplasmic domain of PTPα and GST (GST·PTPαcyt) was used as PKC substrate in an *in vitro* kinase assay. Only the PKC isozymes that are present in wild-type A7r5 cells were tested (α, βI, δ, ϵ, η) (24). The assay was performed with the same conditions used in Fig. 1*B*.

Equal amounts of GST·PTPαcyt were present in the individual assays as judged by Coomassie staining. Although MBP was phosphorylated equally by all five isozymes (see Fig. 1), GST·PTPαcyt was significantly phosphorylated only by PKCδ, as shown in Fig. 3*A*. Hence, the cytoplasmic domain of PTPα serves as a specific substrate for PKCδ only.

To further substantiate these observations, we examined the PKC-phosphorylated PTPα utilizing two-dimensional phosphopeptide maps. Phosphorylation of PTPα with PKCδ *in vitro* resulted in three major spots on the map (not shown). This resembles the data presented by Tracy *et al.* (11), with Ser-180 being responsible for two spots and Ser-204 for one.

In Vitro Phosphorylation of PTPα by PKCδ Increases the Phosphatase Activity Significantly—To examine whether PKCδ was able to activate the PTPα phosphatase activity, HA-tagged PTPα was isolated from serum-starved cells by immunoprecipitation. The isolated protein was phosphorylated *in vitro* with each of the five PKC isozymes expressed in A7r5. After incubation with PKC, the phosphatase activity was determined using pNPP as a substrate for the phosphatase. As clearly evident from Fig. 3*B*, only previous treatment with PKCδ, but not with any other PKC isozyme, increased the phosphatase activity significantly (up to 10-fold).

Next we examined whether the PTPα phosphatase activity was also increased when Src was used as a substrate. To this end, purified Src was subjected to autophosphorylation after PTPα treatment. The activity of Src was then determined in a Western blot utilizing the pY(416)-specific antibody. As is evident from Fig. 3*C*, PTPα that had been treated with PKCδ resulted in a significantly higher activity/autophosphorylation of Src than PTPα that had been treated with other PKC isozymes. These data strongly suggest that the TPA-induced activation of Src is mediated by phosphorylation/activation of PTPα by PKCδ.

TPA-induced PTPα and Src Activation Is Decreased in Fibroblasts from PKCδ Knockout Mice—To further substantiate our findings, we set out to examine PTPα and Src activation after TPA treatment of cells that lack PKCδ (22). Fibroblasts from PKCδ knockout mice were prepared and compared with fibroblasts from wild-type mice. These cells do not express PKCδ, as shown by Leitges *et al.* (22) and confirmed by our Western blot analysis (data not shown). Equal numbers of cells were stimulated with TPA for 15 min (or left untreated as a control). PTPα was immunoprecipitated from the cell lysates and analyzed for activity using pNPP as a substrate. As shown in Fig. 4*A*, TPA stimulation of wild-type fibroblasts resulted in activation of PTPα. The isolated PTPα from TPA-stimulated cells showed roughly 2-fold increased activity when compared with PTPα from unstimulated cells. In contrast, we found no

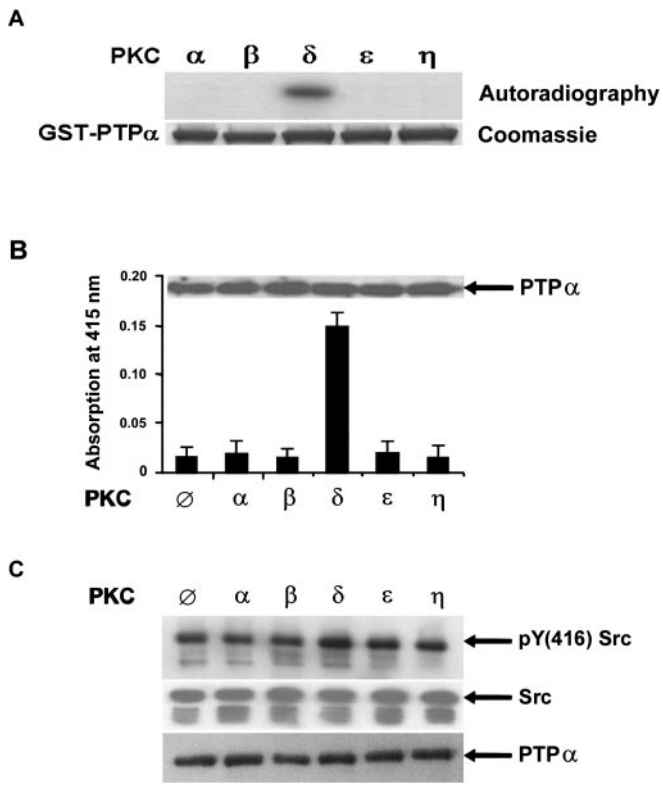

FIG. 3. **PKCδ phosphorylates and activates PTPα *in vitro*.** *A*, 1 μg of a GST·PTPαcyt fusion protein was subjected to an *in vitro* kinase assay with the five TPA-responsive isozymes under the same conditions as in Fig. 1*B*, fractionated by gel electrophoresis, and stained with Coomassie prior to autoradiography. Only the incubation with PKCδ resulted in a strong incorporation of radiolabeled phosphate in PTPα, whereas the other PKC isozymes did not. Data shown here are representative of three experiments. *B*, A7r5 cells were transfected with an HA-PTPα-construct, and PTPα was immunoprecipitated from unstimulated serum-starved cells using the HA-specific antibody. PTPα immunocomplexes were subjected to an *in vitro* kinase assay with the five purified recombinant PKC isozymes tested previously (see Fig. 1*B*) or left untreated as a control, and the phosphatase activity was assayed using pNPP as substrate. Only the incubation with PKCδ resulted in a manifest activation of the phosphatase as indicated by the strong absorbance at 415 nm, whereas the incubation with the other PKC isozymes did not result in any increased activity compared with the basal activity. In the *inserted upper panel* the amount of PTPα protein in each reaction, as determined by immunoblot analysis, is indicated. Data shown are mean ± S.E. ($n = 3$). *C*, Src is a substrate for PTPα. PTPα was subjected to *in vitro* kinase reaction with the different PKC isozymes. Subsequently, 1 μg of purified recombinant Src was incubated with the PKC-phosphorylated PTPα. Src was immunoprecipitated from the sample, and the capacity of Src to undergo autophosphorylation was determined using the pY(416)-specific antibody. As shown, only phosphorylation of PTPα with PKCδ leads to increased Src activity. Results shown are representative of three experiments.

induction of PTPα activity in PKCδ −/− fibroblasts after TPA stimulation. The *inset* immunoblot shows that equal amounts of PTPα were analyzed in both samples.

To test whether the diminished activation of PTPα in PKCδ−/− fibroblasts also resulted in a diminished activation of Src after TPA stimulation, we analyzed the activity of Src before and after TPA stimulation. Src was immunoprecipitated from PKCδ +/+ and −/− before and after TPA stimulation for 15 min, and the activity of Src was analyzed by Western blot using the Src pY(416)-specific antibody (Fig. 4*B*, *upper panel*) and directly in an *in vitro* kinase assay using acid-denatured enolase as a substrate (*lower panel*). As shown in Fig. 4*B*, TPA stimulation of wild-type fibroblasts resulted in a roughly 3-fold Src activation as compared with the non-stimulated cells. As expected from our previous results, cells from PKCδ−/− mice

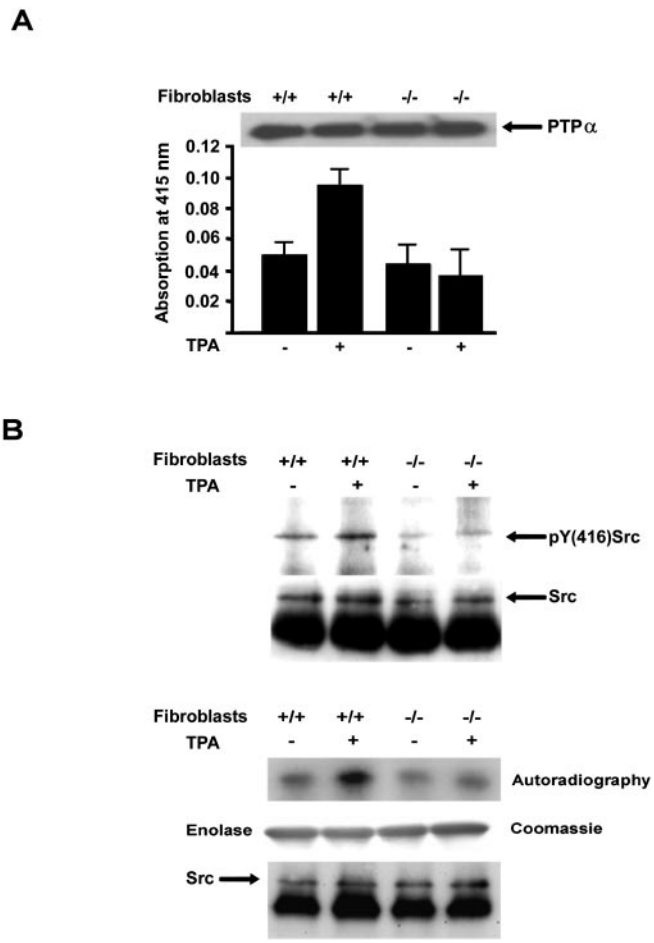

FIG. 4. **Cells from PKCδ knockout mice show decreased TPA-stimulated PTPα and Src activity.** Dermal fibroblasts from PKCδ knockout (−/−) or matching wild-type (+/+) mice were serum-starved overnight and then stimulated with TPA for 15 min or left untreated, and the activity of PTPα and Src was determined. *A*, PTPα was immunoprecipitated from these cell lysates and analyzed for activity using pNPP as a substrate. PKCδ +/+ cells showed an increase of PTPα activity after TPA stimulation, whereas the activity of PTPα from −/− cells was unaffected by the TPA treatment. The *inserted upper panel* shows a Western blot stained for PTPα, indicating that equal amounts of PTPα were assayed. Data shown are mean ± S.E. ($n = 3$). *B*, Src was immunoprecipitated from PKC +/+ and PKCδ −/− cells before and after TPA stimulation for 15 min, and the activity was determined using the Src pY(416)-specific antibody (*upper panel*) or in an *in vitro* kinase assay using acid-denatured enolase (*lower panel*). To demonstrate that equal amounts of Src were present, the level of Src protein was estimated by immunoblotting using the Src-specific antibody. Results shown are representative of three experiments.

showed only a marginal increase of Src activity after TPA stimulation. These data reveal that the lack of PKCδ results in an impaired ability of the cells to activate PTPα, and thereby Src, upon TPA stimulation *in vivo*.

DISCUSSION

Several laboratories, including ours, have shown previously that TPA treatment of cells leads to the activation of Src. The molecular mechanism, however, has long been elusive. Because our initial observations showed that TPA increases Src activity, we first evaluated the effect of TPA on Src. Using specific PKC inhibitors we could easily demonstrate that this effect was transmitted via PKC, while other phorbol ester receptors like β-chimaerin and Ras-GRP have been excluded from being responsible for the observed effects (Ref. 10 and data not shown).

The activation of Src has been described before in detail (14). Src activation is dependent on dephosphorylation at Tyr-527.

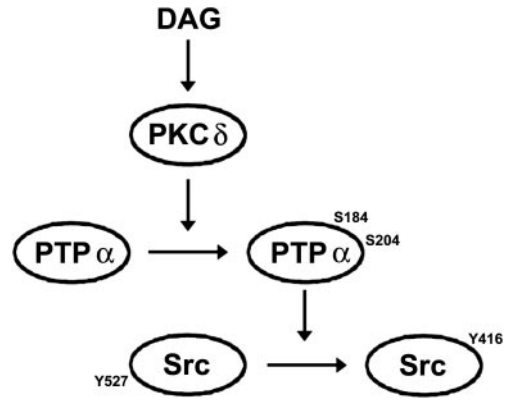

FIG. 5. **Schematic representation of the model for the PKCδ-mediated activation of Src.** Upon activation, PKCδ phosphorylates PTPα on two serine residues, thereby shifting the phosphatase in a active conformation. PTPα dephosphorylates Src at Tyr-527; the resulting conformational changes facilitate an autophosphorylation at Tyr-416. After autophosphorylation Src is in an active state.

Subsequently, Src autophosphorylates at Tyr-416 to obtain full tyrosine kinase activity (Fig. 5). These data led to the assumption that an immediate activator of Src is a phosphatase, rather than a kinase like PKC. As we show here, phosphorylation of Src with purified PKC isozymes does not result in the activation of Src. Hence, PKC must be upstream of a phosphatase and essential for the TPA-induced Src activation.

We have shown evidence that PTPα directly activates Src kinase after previous activation of PTPα only by PKCδ but not by any other PKC isozyme tested. These experiments were performed *in vitro* and *in vivo* to substantiate our conclusions.

PTPα becomes activated after TPA treatment as judged by pNPP dephosphorylation. This is consistent with data published by den Hertog *et al.* (12), who also showed an increase of phosphatase activity of PTPα after TPA treatment of epidermal growth factor receptor-phosphorylated MBP. However, from these data it remained unclear whether the PKC-dependent phosphorylation activates PTPα to recognize and dephosphorylate a physiological substrate like Src. Here we show that PTPα, immunoprecipitated from TPA-stimulated cells, is able to dephosphorylate and activate Src.

The suggestion that PTPα might be a target for PKC has also been made by Tracy *et al.* (11). In that report the authors showed that PTPα becomes phosphorylated on two serine residues (Ser-184/204) within the cytoplasmic domain of the molecule after TPA treatment of NIH3T3 cells. The same results were obtained by the use of purified PKC from rat brain and a GST fusion protein of PTPα in an *in vitro* kinase assay. In addition, it has been shown that changing these serine residues to alanine diminished the ability of PTPα to activate Src after mitotic stimulation *in vivo* (27). Therefore, these phosphorylation sites must be, at least in part, necessary for the regulation and activation of PTPα.

Because the *in vitro* kinase assays by Tracy *et al.* (11) were carried out with only partially purified PKC, it was still unclear whether PKC is directly involved in the phosphorylation of PTPα or whether another kinase that is regulated by PKC is responsible for this phosphorylation. In addition, it was unclear which of the PKC isozymes might be responsible for PTPα phosphorylation.

We used five PKC isozymes, purified from Sf9 cells, to analyze whether PKC isozymes are directly involved in the phosphorylation and activation of PTPα and, if so, which PKC isozyme is responsible for the phosphorylation of PTPα. Because we could show earlier that TPA stimulation of A7r5 cells resulted in a strong activation of Src (10), only PKC isozymes

that are expressed in A7r5 rat smooth muscle cells (24) were used. In these assays, only PKCδ was able to phosphorylate a GST fusion protein containing the entire cytoplasmic domain of PTPα (amino acids 176–802). Similar results were obtained using PTPα immunoprecipitated from A7r5 cells (data not shown).

Analysis of PTPα protein phosphorylated by PKCδ using a two-dimensional phosphotryptic map showed a similar pattern of phosphopeptides that had been shown before by Tracy *et al.* (11). From these data we conclude that the *in vivo* phosphorylation sites within PTPα that have been described before are phosphorylated by PKCδ.

We next set out to determine whether the observed phosphorylation of PTPα by PKCδ resulted in increased activity of the phosphatase. As shown in Fig. 4, PKCδ induced an increase in PTPα activity as measured using pNPP dephosphorylation. It also led to an increase in Src activity, as reflected in pY(416) levels of baculovirus-synthesized Src. Thus, while other kinases may contribute to the activation of PTPα (28), our data show that phosphorylation by PKCδ was sufficient to increase its phosphatase activity.

Utilizing cells derived from PKCδ knockout mice, we were able to further substantiate our findings. As we show here, PKCδ −/− cells showed no induction of PTPα activity after TPA stimulation. In accordance with these data, we found only a marginal induction of Src activity after TPA stimulation in these cells, indicating that the PKCδ-mediated phosphorylation of PTPα is a critical moment for the activation of Src after TPA stimulation.

However, a low but yet significant induction of Src activity can consistently be observed in PKCδ −/− cells after TPA stimulation. This finding indicates that additional, yet less prominent, mechanisms of TPA-induced Src activation exist, the nature of which remains to be identified. However, the reduced basal and induced levels of Src activity might explain why these cells have a severe defect in migration and cell cycle progression).[2]

Because both the colocalization of PKCδ and Src (9, 29) and the colocalization of PTPα and PKCδ (30) have been described, our data suggest a model according to which these molecules (most likely together with additional associated proteins like RACK) reside in a signaling complex, as has been described for the mitogen-activated protein kinases (reviewed in Ref. 26). According to our data, we propose a model in which the main mechanism of TPA-induced Src activation is the direct phosphorylation and activation of PTPα by PKCδ, which in turn dephosphorylates and activates Src.

[2] U. Braun, T. Tennenbaum, and M. Leitges, ununpublished observation.

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Protein Kinase Cδ Induces Src Kinase Activity via Activation of the Protein Tyrosine Phosphatase PTP α

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