# Syndecan-4 regulates localization, activity and stability of protein kinase C-$\alpha$

Eunyoung KEUM*, Yeonhee KIM*, Jungyean KIM*, Soojin KWON*, Yangmi LIM*, Innoc HAN† and Eok-Soo OH*[1]

*Department of Life Sciences, Division of Molecular Life Sciences and Center for Cell Signaling Research, Ewha Womans University, Daehyun-dong, Seodaemoon-Gu, Seoul 120-750, South Korea, and †Research Institute, National Cancer Center, Gyeonggi Goyang 411-764, South Korea

During cell–matrix adhesion, syndecan-4 transmembrane heparan sulphate proteoglycan plays a critical role in the formation of focal adhesions and stress fibres. We have shown previously that the syndecan-4 cytoplasmic domain directly binds to and activates PKC-$\alpha$ (protein kinase C-$\alpha$) *in vitro* [Oh, Woods and Couchman (1997) J. Biol. Chem. **272**, 8133–8136]. However, whether syndecan-4 has the same activity *in vivo* needs to be addressed. Using mammalian two-hybrid assays, we showed that syndecan-4 interacted with PKC-$\alpha$ *in vivo* and that this interaction was mediated through syndecan-4 cytoplasmic domain. Furthermore, the activation of PKC increased the extent of interaction between syndecan-4 and PKC-$\alpha$. Overexpression of syndecan-4, but not a mutant lacking its cytoplasmic domain, specifically increased the level of endogenous PKC-$\alpha$ and enhanced the translocation of PKC-$\alpha$ into both detergent-insoluble and membrane fractions. In addition, rat embryo fibroblasts overexpressing syndecan-4 exhibited a slowed down-regulation of PKC-$\alpha$ in response either to a prolonged treatment with PMA or to maintaining cells in suspension culture. PKC-$\alpha$ immunocomplex kinase assays also showed that syndecan-4 overexpression increased the activity of membrane PKC-$\alpha$. Taken together, these results suggest that syndecan-4 interacts with PKC-$\alpha$ *in vivo* and regulates its localization, activity and stability.

Key words: activation, cytoplasmic domain, fibroblast growth factor (FGF), membrane localization, protein kinase C, syndecan-4.

## INTRODUCTION

Interaction of cells with an extracellular matrix generates a series of signalling events that can regulate adhesion, migration, proliferation and differentiation [1,2]. Adhesive interactions between cells and an extracellular matrix are mediated primarily by the integrins, a family of cell-surface receptors [2], which transduces biochemical signals to various targets such as focal adhesion kinases, tyrosine kinases and PKC (protein kinase C) [3,4].

PKC comprises a family of phospholipid-dependent serine–threonine kinases [5,6]. The 12 known PKC isoforms can be divided into three groups, based on their cofactor requirements for activation. The conventional PKCs (-$\alpha$, -$\beta$1, -$\beta$2 and -$\gamma$) require $Ca^{2+}$, diacylglycerol and phosphatidylserine for full activation [7,8]. The novel PKCs (-$\delta$, -$\varepsilon$, -$\eta$, -$\theta$, -$\nu$ and -$\mu$) are $Ca^{2+}$-independent. The third group, comprising atypical PKCs (-$\zeta$ and -$\lambda/\iota$), is insensitive to both $Ca^{2+}$ and diacylglycerol [7,8]. The distribution of PKC isoforms is tissue- and cell-type-specific, and the role of a given PKC isoform can be different from one cell type to another [7–9]. PKC participates in a wide variety of biological activities, including adhesion-mediated cytoskeletal organization [8,10]. Since spatial and temporal organization of signal transduction complexes is essential in determining the precise signalling events, PKC translocation to the plasma membrane, where the intracellular signalling cascade starts, is a crucial step for specific functions. In parallel with the activation of cytosolic PKC, membrane association of PKC has been generally attributed to the specific interactions with phosphatidylserine [8,10]. Although PKC activity is known to be crucial for adhesion-mediated signal transduction [8,10,11], the mechanism that regulates PKC in this process remains unclear.

Several reports have proposed the role of transmembrane proteins on the translocalization of PKC into the plasma membrane near their select substrates. Three distinct groups of transmembrane proteins have been identified as PKC-binding partners. These include the RACKs (receptors for activated PKC), the transmembrane-4 superfamily (TM4SF; tetraspanins) and syndecan-4. Interestingly, all of them function as integrin co-receptors and are involved in actin organization during cell adhesion processes. Zhang et al. [12] have shown that the translocation of PKCs to the plasma membrane leads to their specific interaction with TM4SF, implicating TM4SF as an adaptor protein that links PKC to specific $\beta$1 integrins. RACK is also known to link PKC to $\beta$1 integrin, providing a mechanistic link between PKC activation and integrin-mediated adhesion [13].

It has been shown that syndecan-4, one of the syndecan family members, plays critical roles in PKC signalling in focal adhesion and stress-fibre formation [14–17]. Specifically, a syndecan-4 cytoplasmic domain interacts with PKC-$\alpha$ [5,17–21] and activates its kinase activity in the presence of $PIP_2$ (phosphatidylinositol 4,5-bisphosphate) *in vitro* [5,22]. Therefore it is highly probable that syndecan-4 regulates translocation of PKC to the cytoskeleton. In the present study, we demonstrate that syndecan-4 directly interacts with PKC-$\alpha$ *in vivo*, and that this interaction regulates localization of PKC-$\alpha$ into the cytoskeleton. In addition, syndecan-4 stabilizes and activates the activity of PKC-$\alpha$, resulting in a sustained PKC activity.

Abbreviations used: EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; FBS, foetal bovine serum; FGF, fibroblast growth factor; bFGF, basic FGF; GFP, green fluorescent protein; HEK-293 cells, human embryonic kidney 293 cells; $\alpha$-MEM, $\alpha$-modified Eagle's medium; PDGF, platelet-derived growth factor; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; RACK, receptor for activated PKC; REF, rat embryo fibroblast; TM4SF, transmembrane-4 superfamily.

[1] To whom correspondence should be addressed (e-mail OhES@ewha.ac.kr).

## EXPERIMENTAL

### Materials

[$\gamma$-$^{32}$P]ATP was purchased from Pharmacia Biotech (Arlington Heights, IL, U.S.A.). Antibodies against PKC isoenzymes and ERK1/ERK2 (extracellular-signal-regulated kinase) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The ECL® immunodetection reagent was purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Reagents for cell culture, including FBS (foetal bovine serum), $\alpha$-MEM ($\alpha$-modified Eagle's medium) and trypsin, were purchased from Gibco BRL Life Technologies (Grand Island, NY, U.S.A.). Luciferase activity assay and $\beta$-galactosidase enzyme assay kits were from Promega (Madison, WI, U.S.A.). Anti-$\alpha$-actinin antibody, histone III-S and other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

### Cell culture

Both REFs (rat embryo fibroblasts) and REF-Syn4 (REF over-expressing syndecan-4 core protein) were maintained in $\alpha$-MEM supplemented with 5 % (v/v) FBS, penicillin (1000 units/ml) and streptomycin (1 mg/ml). NIH3T3, COS7 and HEK-293 (human embryonic kidney 293) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % FBS, penicillin and streptomycin. For suspension culture, after detachment using 0.05 % trypsin/0.53 mM EDTA, both REFs and REF-Syn4 cells were resuspended in 5 % (v/v) $\alpha$-MEM, plated on heat-inactivated BSA-coated Petri dishes, and incubated for the indicated periods of time at 37 °C.

### Subcellular fractionation and immunoblotting

After washing twice with PBS (500 $\mu$l/10 cm diameter plate), 150 $\mu$l of hypo-osmotic solution [20 mM Tris/HCl (pH 7.5)/2 mM 2-mercaptoethanol/5 mM EGTA/2 mM EDTA] containing a protease inhibitor cocktail (1 $\mu$g/ml aprotinin, 1 $\mu$g/ml antipain, 5 $\mu$g/ml leupeptin, 1 $\mu$g/ml pepstatin A and 20 $\mu$g/ml PMSF) was applied to the culture plates. Cells were subsequently scraped off the plates, and homogenized on ice. The homogenate was centrifuged at 13 000 ***g*** for 15 min at 4 °C. The resulting supernatant represented the cytosolic fraction. The membrane fraction was collected by solubilizing the remaining pellet in RIPA buffer [50 mM Tris/HCl (pH 8.0)/150 mM NaCl/1 % (v/v) Nonidet P40/10 mM NaF/2 mM $Na_3VO_4$] containing a protease inhibitor cocktail (1 $\mu$g/ml aprotinin, 1 $\mu$g/ml antipain, 5 $\mu$g/ml leupeptin, 1 $\mu$g/ml pepstatin A and 20 $\mu$g/ml PMSF). RIPA lysates were centrifuged at 15 000 ***g*** for 15 min at 4 °C, denatured with SDS sample buffer [50 mM Tris/HCl (pH 7.8), 100 mM dithiothreitol, 2 % SDS, 0.1 % Bromophenol Blue and 10 % glycerol], boiled and analysed by SDS/PAGE. Proteins were transferred on to Immobilon-P membranes and probed with appropriate primary antibodies followed by species-specific horseradish-peroxidase-conjugated secondary antibodies (Amersham Biosciences). Signals were detected by enhanced chemiluminescence (ECL® Plus™).

### Immunoprecipitation and *in vitro* PKC assay

Each cell lysate (containing 200–1000 $\mu$g of total protein) was incubated with PKC-$\alpha$ antibody for 2 h at 4 °C, followed by the addition of Protein G–Sepharose beads (Amersham Biosciences) and incubation for an additional 2 h. Immunocomplexes were collected by centrifugation, washed twice with RIPA buffer and then washed with 50 mM Hepes solution. The standard kinase reaction mixture (total 20 $\mu$l) contained 50 mM Hepes (pH 7.3), 7.5 mM $CaCl_2$ and cell lysates in either the presence or absence of 1 $\mu$g of histone III-S as the substrate. Reactions were started by adding a mixture of 3 mM $MgCl_2$ and 200 $\mu$M ATP (5 $\mu$Ci of [$\gamma$-$^{32}$P]ATP). After 10 min at room temperature (25 °C), the reaction was stopped by adding 5× SDS/PAGE sample buffer and heating at 95 °C for 5 min. Samples were subsequently analysed by SDS/PAGE (12 % gel) and autoradiography.

### Plasmids and transfection

Transient transfection of COS7 and NIH3T3 cells was performed essentially as described previously [23]. A full-length syndecan-4 cDNA (Syn 4W) and a mutant cDNA lacking the cytoplasmic domain (Syn 4R) were subcloned into pcDNA 3.1 vectors. Cells ($8 \times 10^5$) were plated on 6 cm diameter culture dishes, incubated at 37 °C for 24 h and then transfected with 2 $\mu$g of empty vector or with pcDNA3.1 containing syndecan-4 cDNA derivatives. LIPOFECTAMINE™ reagent (Invitrogen, San Diego, CA, U.S.A.) and effectene (Qiagen, Hilden, Germany) were used for NIH3T3 and COS7 cells respectively. The transfected cells were incubated for 24 h in a medium containing G418 (2 $\mu$g/$\mu$l).

### Mammalian two-hybrid assays

HEK-293 cells ($2 \times 10^6$) were seeded in 60 mm culture dishes, and 24 h after cell seeding, 1 $\mu$g of pM-PKC-$\alpha$, 3.5 $\mu$g of pVP16-Syn 4W (or pVP16-Syn 4R), 1 $\mu$g of reporter pG5-Luc and 1 $\mu$g of pCMV-$\beta$-gal were co-transfected using alkaline $CaCl_2$ reagents. Cells were subcultured in G418-containing media 48 h after transfection. Confluent cells were washed twice with PBS and lysed in 1 × RLB [reporter lysis buffer; 25 mM Tris/phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra-acetic acid, 10 % glycerol and 1 % Triton X-100]. After incubation at 4 °C for 15 min, cells were scraped from the plates and cell lysates were centrifuged at 16 000 ***g*** for 5 min at 4 °C. For luciferase assays, 60 $\mu$g of cell lysates was mixed and incubated with 100 $\mu$l of luciferase assay reagent. Mixtures were vortex-mixed and the activity was measured for a period of 20 s in a luminometer. For $\beta$-galactosidase enzyme assays, 40 $\mu$g of protein was incubated with diluted $\beta$-galactosidase and 2× assay buffer for 30 min at room temperature. The reaction was stopped by adding 150 $\mu$l of 1 M sodium carbonate. Absorbance was read by plate reader at 420 nm.

### Fluorescence microscopy

COS7 cells transfected with pEGFP-PKC-$\alpha$ cDNA, as described above, were seeded on to glass coverslips in 12-well dishes at a density of $4 \times 10^4$ cells/well and incubated for 48 h. Cells were washed twice with PBS, fixed with 3.5 % (w/v) paraformaldehyde in PBS for 10 min at room temperature and mounted using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, U.S.A.). Images were obtained by digital imaging fluorescence microscopy using a cooled charge-coupled-device camera (Carl Zeiss, Oberkochen, Germany).

## RESULTS

### REF cells express PKC-$\alpha$, -$\beta$1, -$\delta$ and -$\varepsilon$

We have shown that PKC activity is important for syndecan-4 function in REFs and that PKC-$\alpha$ from REF cell lysates interacts with syndecan-4 cytoplasmic domain *in vitro* [21]. To verify the interaction between PKC-$\alpha$ and syndecan-4, we first determined the exact isotypes of PKC that were expressed in REFs. Western-blot analysis of whole-cell lysates using isotype-specific

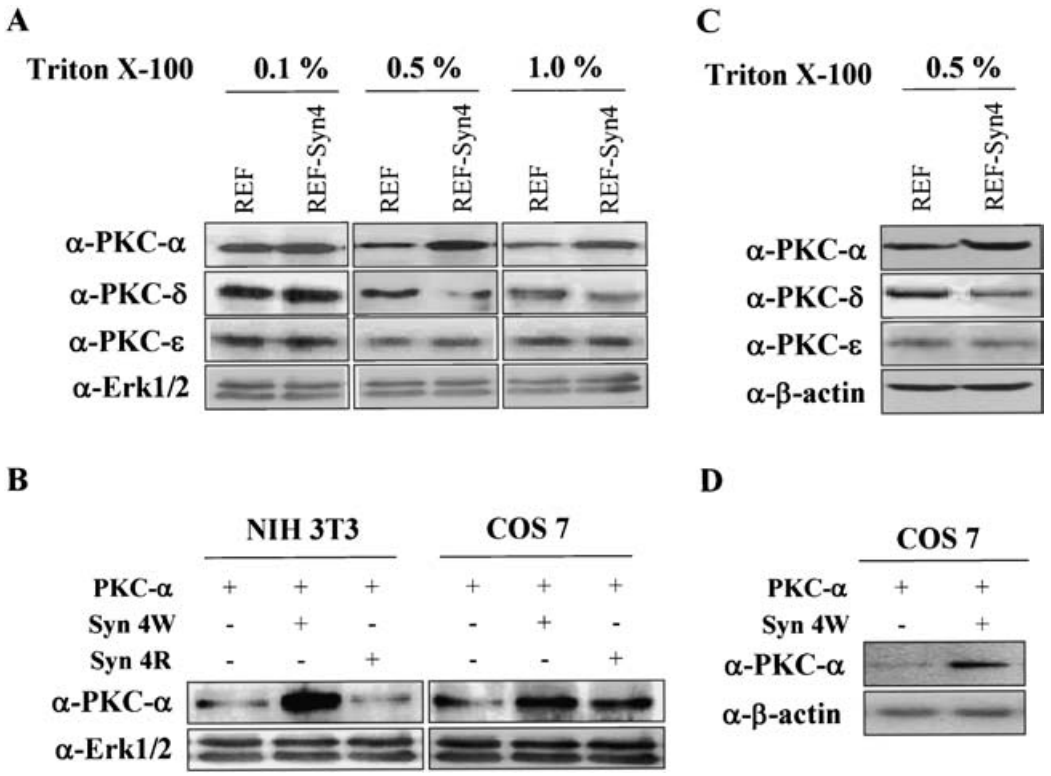


**Figure 1 Syndecan-4 enhances the localization of PKC-α into Triton-insoluble fraction**

(**A**) Both REFs and REF-Syn4 cells were lysed using RIPA lysis buffer containing 0.1, 0.5 or 1.0 % Triton X-100. Each Triton-insoluble protein (20 μg) was resolved by SDS/PAGE and subjected to immunoblotting with isotype-specific antibodies against PKC (anti-PKC-α, anti-PKC-δ and anti-PKC-ε). Erk1/Erk2 was used as a loading control. (**B**) Either Syn 4 or Syn 4R cDNA was transiently transfected into NIH3T3 cells (left panel) or COS7 cells (right panel). Cells were lysed using RIPA lysis buffer containing 0.5 % Triton X-100, and the amount of PKC-α in Triton-insoluble fractions was analysed by immunoblotting with the specific antibody. Erk1/Erk2 was used as a loading control: α-Erk1/Erk2, anti-ERK1/ERK2. (**C**) Both REF and REF-Syn4 cells were fractionated and membrane fractions were collected as described in the Experimental section. The amount of PKC-α in membrane fractions was analysed by immunoblotting with antibody against PKC-α. A representative sample from at least three independent experiments is shown. β-Actin was used as a loading control. (**D**) COS7 cells were co-transfected with PKC-α and either with or without Syn 4W. Confluent cells (80 %) were lysed and fractionated, and 20 μg of lysates from each membrane fraction was resolved by SDS/PAGE and subjected to immunoblotting with antibody against PKC-α. β-Actin was used as a loading control. A representative sample from at least three independent experiments is shown.

antibodies revealed that two of the conventional isotypes, PKC-α and -β1, and two novel isotypes, PKC-δ and -ε, were expressed in REF cells. However, we could not detect the other known isotypes (results not shown).

### Overexpression of syndecan-4 enhances localization of PKC-α into the membrane/cytoskeleton

Since translocation of PKC-α from the cytosol to the membrane is a hallmark of its activation [7,8] and syndecan-4 is found in the plasma membrane/cytoskeleton fraction of the cell lysates [1,16], we investigated whether syndecan-4 is involved in the localization of PKC-α into the membrane/cytoskeleton fraction. To analyse the translocation pattern, REFs were compared with REF-Syn4 cells. Both REFs and REF-Syn4 cells were lysed using lysis buffer containing 0.1, 0.5 or 1.0 % Triton X-100, and PKC isoforms in equal amounts of Triton-insoluble proteins were analysed (Figure 1A). Compared with the amount of PKC-α in REF cell lysates, it was increased in REF-Syn4 in all three Triton-insoluble fractions, whereas the amount of PKC-δ or -ε was less or similar in two cell lines. These results were similar to what was seen in NIH3T3 and COS7 cells transiently transfected with wild-type syndecan-4 cDNAs (Syn 4W), implying that syndecan-4 might regulate localization of PKC-α into the cytoskeleton *in vivo*. In contrast, a syndecan-4 mutant lacking the cytoplasmic domain (Syn 4R) did not affect the amount of PKC-α in Triton X-100-insoluble fraction (Figure 1B). In addition, an increased amount of PKC-α was detected in the membrane fraction from cells both stably (Figure 1C) and transiently (Figure 1D) transfected with syndecan-4 cDNAs. Therefore it seemed that overexpression of syndecan-4 caused increased localization of PKC-α, but neither PKC-δ nor PKC-ε, into membrane/cytoskeleton in cell lines.

To investigate further the effect of syndecan-4 on membrane localization of PKC-α *in vivo*, GFP–PKC-α (where GFP stands for green fluorescent protein) was constructed and transiently transfected into COS7 cells either with or without Syn 4W. Cells showing fluorescence in the plasma membrane were counted (Figure 2). Overexpression of PKC-α slightly increased its membrane localization. However, syndecan-4 overexpression enhanced further the membrane localization of PKC-α by approx. 2-fold compared with the mock-transfected cells (Figure 2A). Immunoblotting with anti-GFP antibody confirmed that an increased amount of GFP–PKC-α was present in the membrane fraction (Figure 2B). Taken together, these results strongly suggest that syndecan-4 regulates membrane/cytoskeleton localization of PKC-α, and the interaction with syndecan-4 cytoplasmic domain is critical for its localization.

### Syndecan-4 interacts with PKC-α *in vivo*

To investigate the interaction of syndecan-4 cytoplasmic domain with PKC-α *in vivo*, a mammalian two-hybrid assay system was

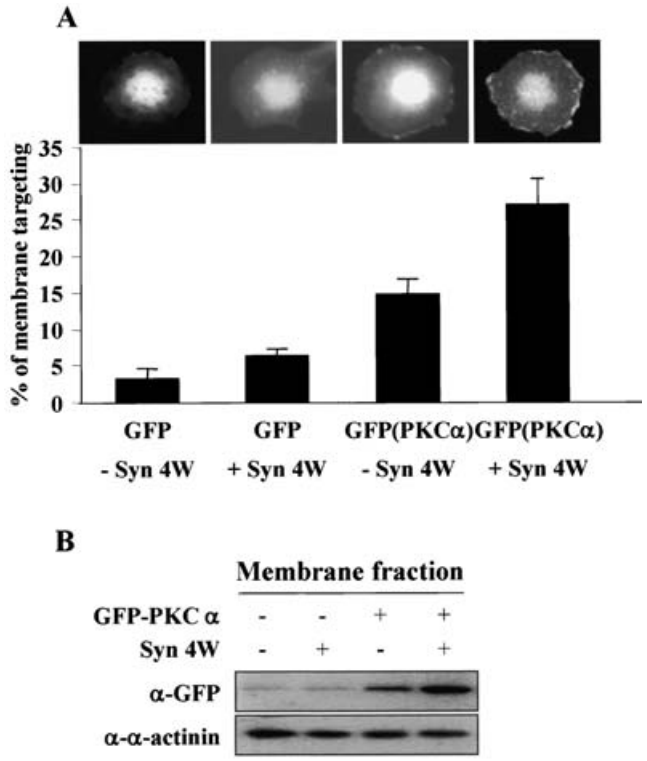


**Figure 2 Overexpression of syndecan-4 increases membrane localization of PKC-$\alpha$ *in vivo***

(**A**) COS7 cells were co-transfected with GFP–PKC-$\alpha$ cDNA and either Syn 4W or vector cDNAs. The localization of GFP–PKC-$\alpha$ was monitored, and photographs were taken under a fluorescence microscope attached to a digital camera. (**B**) Confluent cells (80 %) were lysed and fractionated, and 20 $\mu$g of lysates from each membrane fraction was resolved by SDS/PAGE and subjected to immunoblotting with antibody against GFP. $\alpha$-Actinin was used as a loading control. A representative sample from at least three independent experiments is shown.

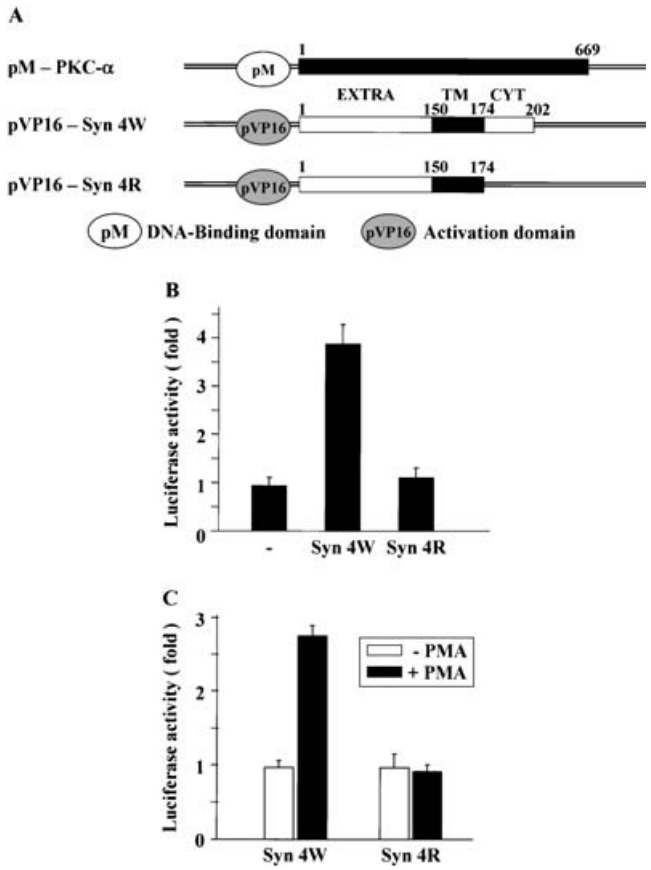


**Figure 3 Syndecan-4 cytoplasmic domain interacts with PKC-$\alpha$ *in vivo***

(**A**) A strategy to detect the interaction using mammalian two-hybrid system. The Gal4 DNA-binding domain was fused to PKC-$\alpha$ cDNA (pM-PKC-$\alpha$) and the VP16 transcription activation domain was fused with either whole syndecan-4 cDNA (pVP16-Syn 4W) or mutant cDNA lacking the cytoplasmic domain (pVP16-Syn 4R). (**B**) HEK-293 cells were grown in a 60 mm cell culture plate and transfected with 1 $\mu$g of pM-PKC-$\alpha$, 1 $\mu$g of pCMV-$\beta$-gal, 1 $\mu$g of pG5-Luc and 3.5 $\mu$g of either pVP16-Syn 4W or pVP16-Syn 4R. After culturing for an additional 48 h period, cells were lysed with 1 $\times$ RLB and luciferase activity was determined. Transfection efficiency was normalized using $\beta$-galactosidase activity as described in the Experimental section. (**C**) Each transfected cell culture was starved for 24 h in the culture medium containing 0.2 % FBS, and then incubated either in the absence (control, open bars) or in the presence (PMA, black bars) of 100 nM PMA for 5 h. Luciferase activities were measured using Luminoskan TL Plus (Labsystems). After normalizing the transfection efficiency using $\beta$-galactosidase activity, the relative luciferase activities are shown.

used (Figure 3A). The pM-PKC-$\alpha$ is a fusion protein expression vector, which consists of the DNA-binding domain of yeast Gal4 and the full-length human PKC-$\alpha$ cDNA. The transcription activation domain of VP16, which directs RNA polymerase II to transcribe the gene downstream of the DNA-binding domain, was fused either with the whole syndecan-4 cDNA (pVP16-Syn 4W) or with a deletion mutant cDNA lacking the cytoplasmic domain (pVP16-Syn 4R). The pG5-Luc is a reporter vector, which contains the luciferase gene downstream of five Gal4-binding sites. These plasmids were transiently co-transfected into HEK-293 cells together with pCMV-$\beta$-gal to normalize the transfection efficiency. The co-transfection of pVP16-Syn 4W with pM-PKC-$\alpha$ in HEK-293 cells resulted in a 3.5-fold increase in the luciferase activity over that seen after transfection with the pVP16 alone. In contrast, no significant increase in the luciferase activity was seen in pVP16-Syn 4R- and pM-PKC-$\alpha$-transfected cells (Figure 3B). Therefore it is probable that syndecan-4 interacts with PKC-$\alpha$ and that the cytoplasmic domain of syndecan-4 mediates specific interaction with PKC-$\alpha$ *in vivo*.

We have shown that activation of PKC with PMA increases the level of interaction of syndecan-4 with PKC-$\alpha$ *in vitro* [21]. We investigated whether PMA increased the interaction of syndecan-4 with PKC-$\alpha$ *in vivo* (Figure 3C). In the same experimental conditions as above, cells were treated with 100 nM PMA for 5 h and then luciferase activity was measured. For cells co-transfected with pVP16-Syn 4W and pM-PKC-$\alpha$, luciferase activity was increased by approx. 2.8-fold in response to PMA, compared with PMA-untreated cells. However, the luciferase activity in pVP16-Syn 4R and pM-PKC-$\alpha$ trasfected cells remained unchanged. Therefore, consistent with the previous results [5,19–22], PKC activation seemed to increase its interaction with syndecan-4. Taken together, these results indicate that syndecan-4 directly interacts with PKC-$\alpha$ and enhances the localization of PKC-$\alpha$ into membrane/cytoskeleton *in vivo*.

### Syndecan-4 regulates stability of PKC-$\alpha$

We next investigated whether syndecan-4 was involved in the regulation of PKC-$\alpha$ stability. Three different cell lines were transfected with syndecan-4 core protein, and PKC protein levels were analysed using immunoblotting with isotype-specific antibodies. As shown in Figure 4, increased protein levels of PKC-$\alpha$ were detected in cell lines both stably and transiently transfected with syndecan-4. It appeared to be specific to PKC-$\alpha$, since there was no change in protein levels of PKC-$\varepsilon$ and -$\gamma$ (Figures 4A and

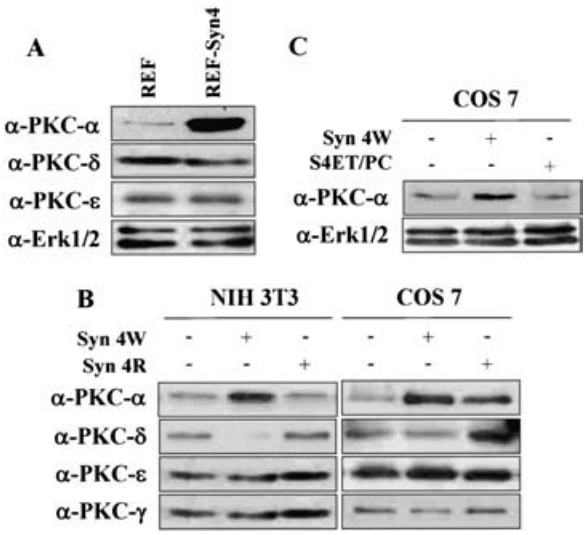


**Figure 4 Syndecan-4 overexpression increases PKC-α protein level**

(**A**) REF and REF-Syn4 cells were lysed using RIPA buffer, and PKC isotypes in TCL (total cell lysate) were determined by immunoblotting with isotype-specific PKC antibodies. Erk1/Erk2 was used as a loading control. (**B**) Either Syn 4W or Syn 4R cDNA (2 μg) was transiently transfected into either NIH3T3 cells (left panel) or COS7 cells (right panel) as described in the Experimental section. After 3 days of culture, cells were lysed using RIPA buffer, and the amount of each PKC isotype in TCLs was analysed by immunoblotting with isotype-specific PKC antibodies. A representative sample from at least three independent experiments is shown. (**C**) COS7 cells were transfected with 2 μg of either Syn 4W or Syn4-PDGF chimaera cDNA, and the amount of each PKC isotype in TCLs was analysed as described in (**B**). Erk1/Erk2 was used as a loading control.

4B). Interestingly, the protein level of PKC-δ was decreased in syndecan-4-overexpressing cells. In contrast, overexpression of Syn 4R failed to increase the protein level of PKC-α (Figure 4B), confirming involvement of the syndecan-4 cytoplasmic domain in the regulatory process. Consistently, when a syndecan-4/PDGF-R (platelet-derived growth factor receptor) chimaera (S4ET/PC), in which the syndecan-4 cytoplasmic domain was substituted by that of PDGF receptor, was transfected into cells, the protein level of PKC-α was not affected (Figure 4C). Therefore it is probable that the syndecan-4 cytoplasmic domain is specifically important for the regulation of PKC-α protein level *in vivo*.

The increased amount of PKC-α protein in syndecan-4-overexpressing cells was not due to a change in mRNA stability or to transcription level, as PKC-α mRNA levels were not significantly different in REF and REF-Syn4 (results not shown). We next investigated the effect of syndecan-4 on PKC-α stability, since activated PKC was known to be down-regulated by proteolysis [24]. Cells were either incubated with 100 nM PMA or maintained in suspension for the indicated period of time to induce the PKC down-regulation, and the amount of PKC-α protein was subsequently determined by Western-blot analysis. PKC-α protein level in REF cells in suspension culture was clearly decreased by 120 min. However, PKC-α protein level in REF-Syn4 was maintained at 120 min, and showed decrease only after 180 min (Figure 5A). Similarly, PKC-α was more persistent to PMA-induced down-regulation in syndecan-4-overexpressing cells (Figure 5B). Specifically for REF, PKC-α protein level showed a significant decrease after 3 h, but in REF-syn4, it remained unchanged for 12 h and started to decrease only after 18 h. These results indicate that increased PKC-α level in syndecan-4-overexpressing cells is due to increased stability of PKC-α protein.

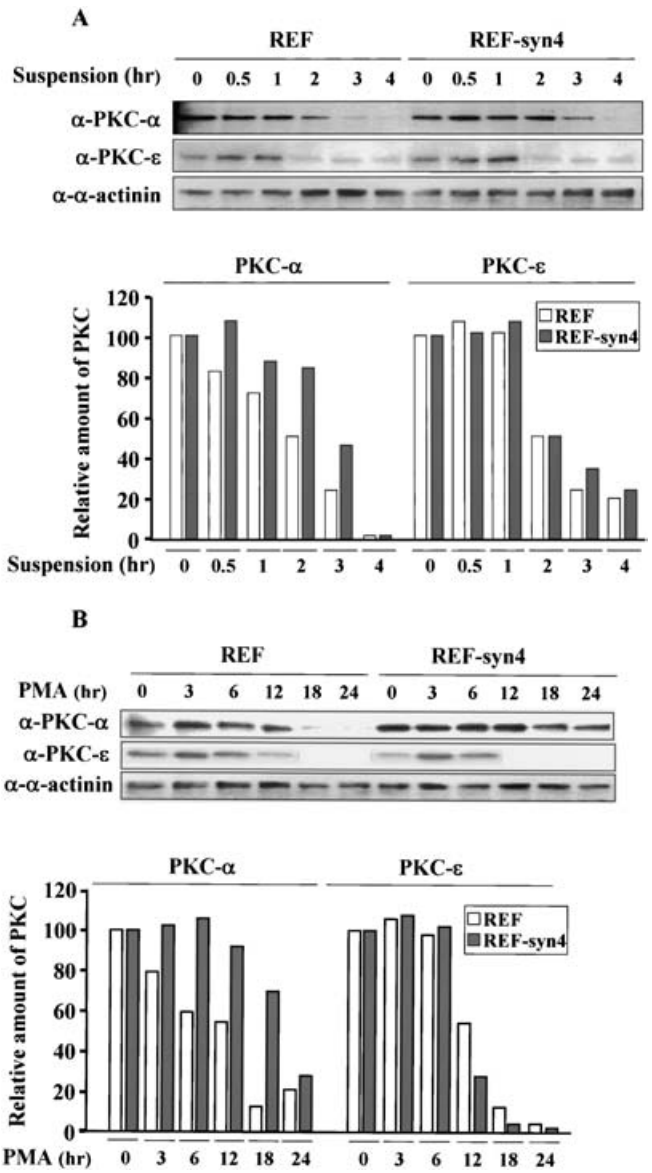


**Figure 5 Syndecan-4 overexpression causes a delay of PKC-α down-regulation**

Both REF and REF-Syn4 cells were either detached and maintained in suspension (**A**) or incubated with 100 nM PMA (**B**). After incubation for the indicated times, cells were lysed using RIPA buffer, and the amount of each PKC isotype in TCLs was analysed by immunoblotting with each PKC antibody (top panels) and quantification of PKC levels at indicated times are shown graphically (bottom panels). α-Actinin was used as a loading control. Representative results from two independent experiments are shown.

### Syndecan-4 regulates the activity of PKC-α

We have shown that the syndecan-4 cytoplasmic domain activates PKC activity *in vitro* [5,21]. To see if syndecan-4 activated PKC similarly *in vivo*, we examined PKC-mediated phosphorylation in REF and REF-Syn4 cells. After fractionation, lysates of both REF and REF-Syn4 membrane fraction were incubated with [$\gamma$-$^{32}$P]ATP to allow phosphorylation, and phosphorylated proteins were analysed by autoradiography. Several potential PKC substrates showed increased levels of phosphorylation in REF-Syn4 cells, indicating that the overall kinase activity in membrane was increased by overexpressing syndecan-4 (Figure 6A). Addition of 100 nM GF109203X, a PKC inhibitor [10], decreased

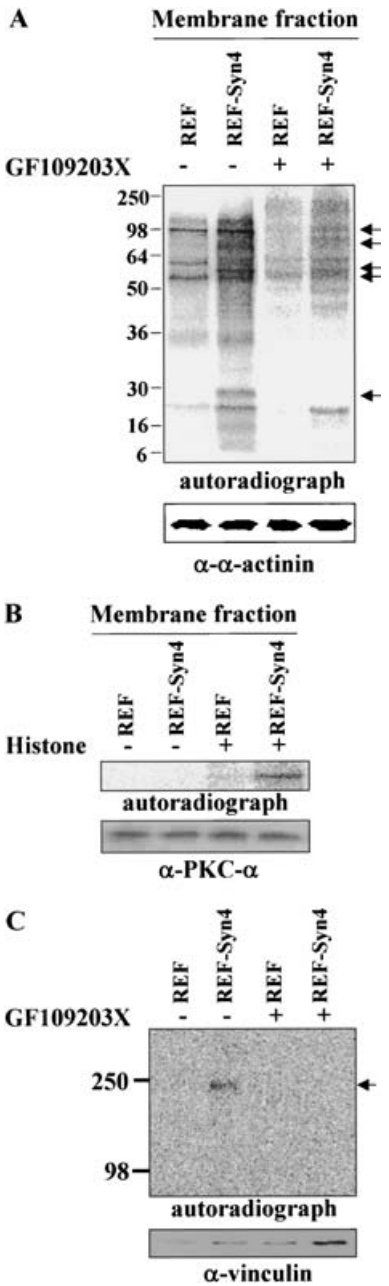


**Figure 6 Syndecan-4 up-regulates endogenous PKC activity**

(**A**) Membrane fractions were prepared from REF and REF-Syn4 cells. Membrane lysate (20 $\mu$g) was incubated with 1 mM ATP, 5 $\mu$Ci of [$\gamma$-$^{32}$P]ATP in PKC reaction mixture in either the presence or absence of 100 nM GF109203X. Representative autoradiography of phosphorylated proteins is shown. Arrows indicate proteins showing increased phosphorylation in REF-Syn4. $\alpha$-Actinin was used as a loading control. (**B**) Each membrane lysate was immunoprecipitated with anti-PKC-$\alpha$ antibody, and *in vitro* immunocomplex kinase assays were performed in either the absence or presence of histone III-S as a substrate. Autoradiography of phosphorylated histone is shown (upper panel). The level of PKC-$\alpha$ present in each immunoprecipitate was determined by immunoblotting with anti-PKC-$\alpha$ antibody (lower panel). (**C**) *In vitro* phosphorylated proteins were immunoprecipitated with anti-vinculin antibody, and representative autoradiography is shown (upper panel). The level of vinculin present in each immunoprecipitate was determined by immunoblotting with anti-vinculin antibody (lower panel). Arrow indicates protein showing increased phosphorylation in REF-Syn4. Molecular mass (in kDa) is indicated to the left of the gels in **A** and **C**.

the level of protein phosphorylation, indicating that the increased phosphorylation in REF-Syn4 cells was due to an increase in the PKC activity. Consistently, PKC-$\alpha$ activity in immunoprecipitates was increased in REF-Syn4 cell lysates, based on histone phosphorylation (Figure 6B). In addition, increased phosphorylation of vinculin-interacting protein was seen in the immunocomplex from REF-Syn4 cell lysates (Figure 6C). Taken together, these results show conclusively that PKC-$\alpha$ activity is up-regulated in syndecan-4-overexpressing cells.

### bFGF (basic fibroblast growth factor), enhances both membrane localization and interaction of PKC-$\alpha$ with syndecan-4

Syndecan-4 is known to function as a co-receptor for an FGF (fibroblast growth factor) receptor. FGF is in turn known to regulate the function of syndecan-4 through PKC activation [16,22,27]. Thus we investigated whether FGF could regulate membrane localization of PKC-$\alpha$. REF cells were starved for 24 h, and then treated with 50 nM bFGF, EGF (epidermal growth factor) or PDGF for the indicated period of time. The level of PKC-$\alpha$ in the membrane fraction was subsequently determined. As expected, bFGF, but neither EGF nor PDGF, induced increased localization of PKC-$\alpha$ into the membrane fraction, based on Western blotting with antibody against PKC-$\alpha$ (Figure 7A) and enhanced membrane localization of GFP–PKC-$\alpha$ *in vivo* (Figure 7B). Consistently, mammalian two-hybrid assays showed that bFGF increased the interaction of syndecan-4 with PKC-$\alpha$ by 3.5-fold, whereas EGF showed a 2-fold increase (Figure 7C). Taken together, syndecan-4 probably regulates PKC-$\alpha$ localization into the membrane in response to bFGF stimulation.

## DISCUSSION

Syndecan-4 is known to regulate the organization of cytoskeleton, including focal adhesion and stress-fibre formation [1,14–16], which are dependent on the PKC activity [16,17,21,22]. It is also known that the syndecan-4 cytoplasmic domain interacts with and potentiates PKC-$\alpha$ *in vitro* [5,17–21], further substantiating the functional interaction between syndecan-4 and PKC. In contrast, not much is known about the regulatory function of syndecan-4 on PKC-$\alpha$ *in vivo*. In the present study, we have shown that syndecan-4 interacts with PKC-$\alpha$ *in vivo*, and regulates its localization to the membrane where the intracellular signalling cascades start and PKC-$\alpha$ is fully activated. Several reports have shown that the syndecan-4 cytoplasmic domain interacts with PKC-$\alpha$. Both recombinant syndecan-4 core protein and its synthetic peptide directly interact with PKC-$\alpha$ *in vitro* [20,21]. Immunohistochemical staining also shows co-localization of PKC with syndecan-4 [17,25]. To demonstrate the direct interaction between syndecan-4 and PKC-$\alpha$ in cells, we utilized a mammalian two-hybrid system which can reveal a direct protein–protein interaction in live cells [26]. Our results clearly show that syndecan-4 directly interacts with PKC-$\alpha$ through the cytoplasmic domain *in vivo* (Figure 3).

It seems that their interaction has regulatory implications, since activation of PKC with PMA (Figure 3C) and bFGF (Figure 7) treatment enhanced their interaction. PKC is supposed to be present in an inactive conformation in the cytosol and translocates to the plasma membrane on cell activation. To interact with syndecan-4, PKC has to be activated and localized near the plasma membrane. Thus it is highly probable that syndecan-4 interacts only with the active form of PKC-$\alpha$ *in vivo*. To activate PKC, PMA may induce conformational changes in PKC to expose the putative interaction sites for syndecan-4. This could explain why an enhanced interaction of syndecan-4 with PKC-$\alpha$ is seen in PMA-treated cells. Similarly, we have demonstrated that bFGF regulates PKC-$\alpha$ activity and the interaction between syndecan-4 and

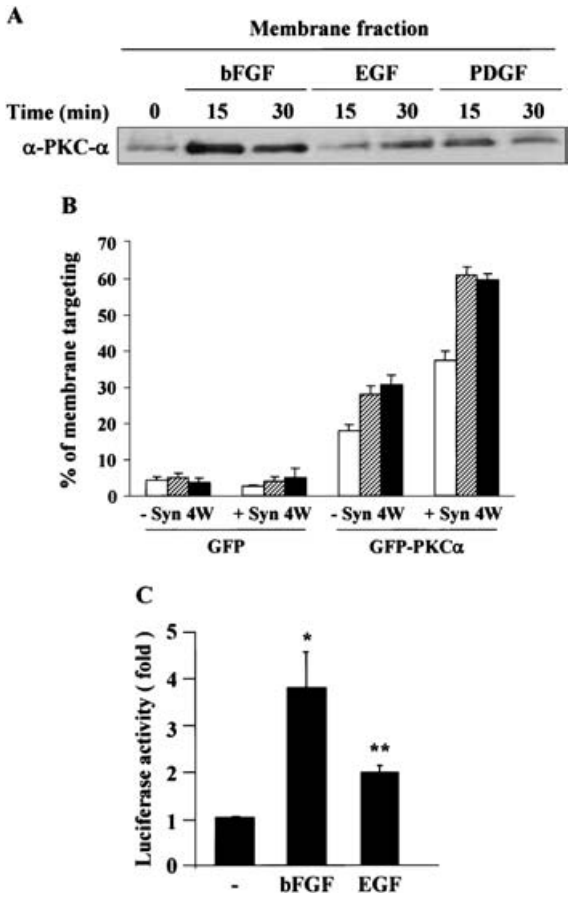


**Figure 7 bFGF enhances membrane localization and interaction of PKC-α with syndecan-4**

(**A**) After serum starvation for 24 h, REF-Syn4 cells were treated with 50 nM PDGF, bFGF or EGF for the indicated periods of time. After subcellular fractionation, the level of PKC in each fraction was determined as described in Figure 3. (**B**) COS7 cells co-transfected with GFP–PKC-α and Syn 4W were treated with bFGF (0 min, open bars; 15 min, hatched bars; 30 min, black bars) as described in (**A**). Localization of GFP–PKC-α was monitored, and photographs were taken under a fluorescence microscope attached to a digital camera. (**C**) Cells transfected with 1 μg of pM-PKC-α, 1 μg of pCMV-gal and 3.5 μg of pVP16-Syn 4W were starved for 24 h in serum-free medium, and then treated with 50 nM of either bFGF or EGF. After 12 h, cells were lysed and luciferase activity was determined. Transfection efficiency was normalized using β-galactosidase activity. $*P < 0.001$, $**P < 0.01$ compared with control (−) after Student's *t* test.

PKC-α (Figure 7C). Recently, a novel mechanism for the pivotal role of syndecan-4 in FGF-mediated signalling has been proposed [27]. In this signalling pathway, FGF2 stimulation induces activation of PKC-α by a complex consisting of syndecan-4 and $PIP_2$. The key regulatory event is the phosphorylation of $Ser^{183}$ in the cytoplasmic domain of syndecan-4, which in turn down-regulates the response to FGF2 by preventing $PIP_2$-dependent oligomerization of syndecan-4 and subsequent activation of PKC-α [5,22,27]. Thus bFGF is required for the oligomerization of syndecan-4 cytoplasmic domain, and this oligomerization may be critical for syndecan-4 functions, including its interaction with PKC-α.

The role of $PIP_2$ in syndecan-4 mediated PKC activation has also been studied [5,18,20]. *In vitro* kinase assay has shown that a syndecan-4 peptide activates PKC-α activity in the presence of $PIP_2$ [5,18,20]. Since oligomerization of syndecan-4 is important for its function, $PIP_2$ may regulate PKC activity through the regulation of syndecan-4 oligomerization. However, we cannot exclude the possibility that $PIP_2$ regulates PKC activity more directly by inducing conformational changes. Several $PIP_2$-binding proteins, including vinculin, talin, filamin and α-actinin, are regulated through conformational changes that affect their ligand-binding activity [6]. In particular, it is well known that $PIP_2$ binding alters vinculin conformation and consequently exposes its ligand-binding sites.

It is clear that syndecan-4 increased the activity of PKC *in vivo*, since the level of phosphorylation of an exogenous substrate histone III-S by PKC-α immunoprecipitates was higher in the membrane fraction of REF-Syn4 when compared with that of REF (Figure 6). In other words, PKC-α is specifically up-regulated by the combination of syndecan-4 and $PIP_2$ [28].

Although PKCs typically translocate to the plasma membrane on activation, their location on membranes is supposed to be transient if not stabilized by other mechanisms. Thus the up-regulation of PKC-α activity requires additional binding sites for PKC in the membrane. Since syndecan-4 is in the plasma membrane, its interactions with PKC-α would lead to a higher level of its activity in the plasma membrane. Such an activity of syndecan-4 is highly reminiscent of anchoring proteins, the RACKs, which function as scaffold proteins in the plasma membrane and regulate subcellular distribution of PKC-α.

Once activated, PKC undergoes down-regulation. A good example is the PMA-induced proteolysis. Prolonged exposure to PMA causes an almost complete depletion of certain isoenzymes of PKC from the cells, presumably as a result of proteolysis [24]. Interestingly, the down-regulation of PKC-α by PMA was delayed in syndecan-4-overexpressing cells (Figure 5). Although we are yet to determine the detailed mechanisms, it appears that complex formation with syndecan-4 renders PKC-α more resistant to degradation. It probably results in the accumulation of more PKC-α in syndecan-4 overexpressing cells. The enhanced expression of PKC-α is apparently not regulated at the transcriptional level, since the mRNA level of PKC-α was not affected by syndecan-4 expression.

In summary, when PKC-α is activated, it translocates into the plasma membrane where, in association with syndecan-4, it is stabilized in an active form. Therefore syndecan-4 might function not only as a PKC-binding protein that regulates the membrane localization and activity, but also as a potentiator of the PKC-α activity that stabilizes the active PKC-α.

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