Journal of Cellular Biochemistry 98:838–850 (2006)

Dual Involvement of Protein Kinase C δ in Apoptosis Induced by Syndecan-2 in Osteoblasts

Armelle Orosco, Olivia Fromigué, Eric Haÿ, Pierre J. Marie, and Dominique Modrowski*

Laboratory of Osteoblast Biology and Pathology, INSERM, Lariboisière Hospital, University Paris 7, Paris, France

Abstract Syndecans are proteoglycans that act as signaling molecules. Previously, we showed that syndecan-2 (SYND2) is involved in the control of osteoblastic (OB) cell apoptosis. Here, we show a novel functional interaction between SYND2 and protein kinase C δ (PKCδ). Overexpression of SYND2 in MG63 OB cells resulted in increased PKCδ protein level without change in PKCδ mRNA production. In SYND2-transfected cells, the increase in PKCδ was restricted to the cytosolic compartment, threonine 505-PKCδ was underphosphorylated and immunoprecipitated PKCδ showed decreased capacity to phosphorylate histone, indicating that SYND2 decreased PKCδ activity. Inhibition of PKCδ by Rottlerin or a dead-kinase dominant negative (DN) construct activated effector caspases and increased the number of apoptotic cells. In addition, rescue of kinase activity with a construct coding, the PKCδ catalytic domain (CAT) reduced SYND2-induced apoptosis. This indicates that PKCδ acts as a pro-survival kinase and that SYND2 inhibits the anti-apoptotic action of PKCδ in OB cells. We also showed that overexpression of PKCδ wild type (WT) induced osteoblast apoptosis. Moreover, inhibition of PKCδ by siRNA resulted in increased apoptosis in control cells but reduced apoptosis in SYND2-overexpressing osteoblasts, indicating that SYND2 requires PKCδ accumulation to induce apoptosis. These results show that SYND2 modulates PKCδ actions by inhibition of the canonical allosterical activation pathway that plays an anti-apoptotic role in OB cells, and promotion of a pro-apoptotic role that may depend on PKCδ protein level and that participates to the induction of cell death by SYND2. This establishes a functional interaction between SYND2 and PKCδ in osteoblasts. J. Cell. Biochem. 98: 838–850, 2006.

Key words: syndecan-2; PKCδ; apoptosis; osteoblast; signaling

Bone formation is dependent on the proliferation, differentiation, and survival of osteoblastic (OB) cells. It is now widely accepted that bone cell apoptosis is a key event that participates to bone turnover regulation [Manolagas, 2000]. On the one hand, increased apoptosis of osteoblasts and osteocytes contributes to bone loss due to glucocorticoid treatment [Weinstein et al., 1998]. On the other hand, dysregulation of cell apoptosis is believed to participate to tumorigenesis and to resistance to chemotherapy in cancer. This is exemplified by Fas system, which was found functional in normal OB cells [Kawakami et al., 1997] whereas altered Fas expression is involved in the ability of osteosarcoma cells to form metastasis [Lafleur et al., 2004]. It is, thereby, crucial to understand the mechanisms that control OB cell life span in normal and dysregulated osteogenesis. Recently, the anabolic effects of estrogens and PTH in osteoporotic patients were found to result from positive actions on OB cell survival [Jilka et al., 1999]. In addition to this hormonal control, local factors such as FGFs, BMP-2, or GM-CSF were shown to regulate OB cell survival [Hay et al., 2001; Postiglione et al., 2003; Debiais et al., 2004].

We recently showed that syndecan-2 (SYND2) is involved in the control of apoptosis in OB cells [Modrowski et al., 2005]. SYND2 is an heparan sulfate transmembrane proteoglycan, which serves as a receptor for various extracellular soluble and matrix components [Bernfield et al., 1999]. Numerous data suggest

Grant sponsor: The Cancer and Solidarity Foundation, Geneva, Switzerland; Grant sponsor: Association Rhumatisme et Travail, Paris, France.

*Correspondence to: Dominique Modrowski, Unit 606 INSERM, Hopital Lariboisière, 2 rue Ambroise Paré, 75475 cedex 10, Paris, France.
E-mail: dominique.modrowski@larib.inserm.fr

Received 12 December 2005; Accepted 15 December 2005
DOI 10.1002/jcb.20826

the involvement of syndecan core protein in signaling events. Indeed, some studies pointed out interactions between the different cytoplasmic domains of syndecans and signaling molecules [Hsueh et al., 1998; Granes et al., 1999; Munesue et al., 2002]. Previously, we showed that SYND2 is involved in modulation of MAP kinases activation [Modrowski et al., 2000, 2005]. This supports that SYND2 may control downstream signaling molecules to exert its pro-apoptotic action.

Protein kinase C (PKCs) control many cellular events in which syndecans are involved, such as matrix assembly, focal adhesion formation, and cell migration. SYND2 is subjected to phosphorylation by PKCαβγ [Oh et al., 1997], and PKCγ regulates SYND2-mediated inside-out signaling [Kramer et al., 2002]. PKCs were shown to be diversely involved in apoptotic signaling. PKCδ, a novel PKC isoform, which contains the DAG binding sites but lacks the calcium-binding domain, displays pro-apoptotic functions since overexpression of this kinase in keratinocytes and in prostate cancer cells led to TPA-dependent induction of apoptosis [Li et al., 1999; Fujii et al., 2000]. Other studies support an opposite role for PKCδ in cell survival. Indeed, in breast cancer cells, this kinase promotes growth and metastasis and displays a pro-survival function [McCracken et al., 2003], suggesting that the pro- and anti-apoptotic functions of PKCδ are tissue specific. The role of PKCδ in the control of OB cell survival is unknown. In this study, we show that SYND2 modulates PKCδ activation and kinase activity. We also demonstrate a dual role of PKCδ in the control of apoptosis in OB cells.

MATERIALS AND METHODS

Materials

Mouse anti-SYND2 antibody (10H4) was a generous gift from Dr. Guido David (University of Leuven, Leuven, Belgium). This antibody recognizes the ectodomain of SYND2 after heparan sulphate chain digestion. Rabbit anti-SYND2 antibody was from Zymed (San Francisco, CA). This rabbit anti-SYND2 antibody that reacts with the cytoplasmic domain of SYND2 is able to recognize the different species (glycosylated or not glycosylated forms). Using this rabbit anti-SYND2 antibody for Western blot analysis results in detection of low molecular species (about 25 and 50 KDa) and high molecular species. We analyzed the 50 KDa band that is representative of the SYND2 expression level. Rabbit anti-PKCδ antibody that recognizes the carboxy terminus of PKCδ was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phospho-PKCδ that reacts with PKCδ when phosphorylated at threonine 505, was from Cell Signaling (Beverly, MA). Rabbit anti-actin antibody was from Sigma-Aldrich (Saint Louis, MI).

Cell Culture and Transfections

Human OB cells MG63 were obtained from the ATCC (USA) and cultured at 37°C in humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal calf serum and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). Human SYND2 cDNA was obtained from Dr. Guido David (University of Leuven). A 1,469 bp sequence was cloned into pcDNA 3.1 (Invitrogen, San Diego, CA) to express full length SYND2. PKCδWT, PKCδDN, and PKCδCAT constructs in pcDNA 3.1 were a generous gift from Dr. Soh (Herbert Irving Comprehensive Cancer Center, Columbia University, NY) [Soh et al., 1999]. In some experiments, cells were transfected with the empty vector (EV) pEGFP-C1 (Clontech, Paolo Alto, CA) to visualize control transfected cells. Transfections were performed as previously described [Modrowski et al., 2005]. Briefly, 3 μg of the expression plasmids were mixed to Transfast reagent (Promega, San Luis Obispo, CA) in serum-free medium and applied to pre-confluent MG63 cells. When SYND2 was co-transfected with a PKCδ construct, 1.5 μg of each plasmid was mixed and pcDNA 3.1 (EV) served to achieve the same total amount of plasmid DNA per transfection. Fetal calf serum was added to the medium 1 h after transfection and cells were cultured for 2 days. In some experiments, transfected cells were treated with 2.5 μg/ml Rottlerin (Merck Biosciences, Darmstart, Germany) or its solvent, DMSO, 1 day after transfection and for 24 h.

PKCδ Knockdown by siRNA

We used target-specific 20–25 nt small interfering RNAs designed to knockdown PKCδ expression (Santa Cruz Biotechnology) and a non-targeting siRNA (scramble sequence) as control. MG63 cells were cultured for 24 h in DMEM containing 10% FCS, then washed twice with serum-free medium that did not contain

antibiotics. Expression plasmids pEGFP or SYND2 (1.5 μg) were mixed with siRNAs (50 pmol) and X-tremeGene transfection reagent (Roche, Indianapolis, IL) in serum- and antibiotic-free medium and applied to MG63 cells. Two hours later FCS was added in the culture medium and culture was continued for 2 days until analysis.

Immunocytochemistry

MG63 cells were seeded on chambered slides (Nunc, Inc., Naperville, IL). Two days after transfection, cells were washed twice with cold phosphate buffered saline (PBS), fixed with 4% paraformaldehyde, permeabilized at −20°C with cold methanol and acetone then treated with heparitinase III (10 mU/ml). After several washes, cells were incubated overnight at 4°C with the primary antibodies. The cells were then incubated for 1 h at room temperature with either donkey Cy3-conjugated anti-mouse or Cy2-conjugated anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA). Controls for specific labelings were performed using non-immune mouse or rabbit IgGs instead of the primary antibodies. Cells were finally washed with PBS and mounted using a fluorescent mounting medium (Dako, Carpinteria, CA). Immunolabeling was analyzed using a Nikon fluorescent microscope and the Histolab software (Microvision, Evry, France).

Immunoprecipitation and Immunoblot

Protein extracts were prepared 2 days after the transfection. Cells were washed with cold PBS and then lysed on ice in 50 mM Tris-HCl pH 8, containing 150 mM NaCl, 5 mM EDTA, a protease inhibitor cocktail (Roche), and 1% Nonidet P-40 (NP-40). Protein content was determined using the DC protein assay (Biorad, Hercules, CA). Immunoprecipitations (IP) were performed using protein A linked to magnetic beads (Dynal Biotech, Oslo, Norway). Rabbit anti-PKCδ or anti-SYND2 IgGs were captured on protein A. The beads were then washed and incubated overnight with 400 μg of cell lysates. After washes, immunocomplexes were suspended in SDS-PAGE loading buffer containing 50 mM dithiothreitol and boiled for 5 min. In each experiment, negative controls for IP were included using normal rabbit immunoglobulin fraction (Dako).

Crude extracts or immunoprecipitated proteins were subjected to SDS-PAGE and electrotransferred onto polyvinylidene difluoride membrane. The membrane was then blocked overnight at 4°C in 20 mM Tris-HCl pH 7.5, 137 mM NaCl, 0.1% Tween 20, 1% Bovine Serum Albumine (TBSTA), incubated with primary antibodies diluted in TBSTA, and then with appropriate horseradish peroxidase-conjugated antibodies. The signals were visualized using a chemiluminescent detection system (Pierce Biotechnology, Rockford, IL).

Membrane Preparation

To detect proteins in subcellular compartments, cells were sonicated in 20 mM Tris-HCl pH 7.2, with 2 mM EDTA, 2 mM EGTA, 0.1% β-mercaptoethanol, and protease inhibitor cocktail. The membrane fraction was then separated from cytosolic fraction by centrifugation at 100,000*g* for 1 h.

PKCδ Kinase Assay

The kinase activity of PKCδ was measured 2 days after transfection as previously described [Chen and Faller, 1999]. Cultures were stopped on liquid nitrogen, cells were lysed in 50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EGTA, 1 mM orthovanadate, protease inhibitor cocktail, and 1% NP-40. PKCδ was immunoprecipitated from 200 μg protein extracts as described above, except that before being boiled with the loading buffer, immunocomplexes were washed twice with the kinase buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) and then incubated for 10 min at 30°C with 25 μl of the kinase buffer containing 10 μM ATP, 0.4 mg/ml histone H1, and 10 μCi of [γ^{32}P]-ATP (6,000 Ci/mmol). Reagents were then separated by SDS-PAGE. Immunoprecipitated PKCδ was detected by Western blot and phosphorylated histone was detected by autoradiography. In control reactions histone H1 was not present.

RT-PCR Analysis

Total RNAs were isolated from the cultured cells using a ready-to-use monophasic solution of phenol and guanidine isothiocyanate. RNA was digested by DNase I to remove genomic or plasmid DNA contamination. Two micrograms were subjected to cDNA synthesis for 1 h at 37°C using 200 U M-MLRV reverse transcriptase (Gibco, Gaithersburg, MD). The cDNA products were submitted to 25 cycles amplification (annealing at 55°C) using the following primers: for PKCδ, sense 5′-AACAATCTATGCGCAGT-

GAGG-3′, anti-sense 5′-GGCATGTCGATGTT-GAAGC-3′; for SYND2, sense 5′-GACGATGA-CTACGCTTCTGC-3′, anti-sense 5′-TTGTAT-CCTCTTCGGCTGG-3′; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense 5′-GGGCTGCTTTTAACT-CTGGT-3′, anti-sense 5′-TGGCAGGTTTTTCTAG-ACGG-3′. Co-amplification of the specific gene and GAPDH as internal control was performed by using two primer sets in a single reaction mixture. Amplified sequences were separated on agarose gel and visualized on UV light.

In Vitro Determination of Caspase Activity

MG63 cells were lysed on ice in 10 mM Tris-HCl, 200 mM NaCl, 5 mM EDTA, 10% glycerol, and 1% NP40. The activity of effector caspases (caspases-3, -6, -7) was determined by the cleavage of synthetic fluorogenic substrates containing the specific amino acid sequence recognized by these caspases (DEVD). Cell extracts were incubated for 2 h at 37°C with 20 μM substrate diluted in 10 mM Hepes/NaOH pH 7.4, 10 mM dithiothreitol, and 0.1 mM PMSF. Fluorescent emission was detected using a spectrofluorimeter (F-2000, Hitachi). Results were expressed as arbitrary units (AU) after correction for protein content.

Detection of Cell Apoptosis-Hoechst Staining

MG63 cells were fixed 2 days after transfection. Cells transfected with SYND2 were submitted to SYND2 immunodetection as described above. Transfected control cells were detected by green fluorescent protein (GFP) expression. To examine nuclear morphology the cells were incubated with 2.5 μg/ml Hoechst 33258 (Sigma-Aldrich) in PBS for 5 min. Cells showing apoptotic chromatin changes (i.e. mainly chromatin condensation) in their nuclei were counted. Only cells that displayed GFP (EV-transfected cells) or high-level of SYND2 expression (SYND2-transfected cells) were considered. At least 100 labeled cells were counted for each condition.

MTT Reduction Assay

Cell-mediated reduction of 3-(4,5-dimethylthiazol-2-yl)-2′5-diphenyl-tetrazo-lium bromide (MTT) in formazan was used as a cell viability assay. MG63 cells were seeded in 96-well plates and transfected as described above. Two days after, 10 μl of 3 mg/ml of MTT were added in each well for 30 min. The insoluble formazan salt that has been producted was then solubilized in DMSO and absorbance was measured at 540 nm.

Statistical Analysis

All experiments were performed at least three times. Photographied bands resulting from Western blot or PCR analysis were quantified using QuantityOne analysis software (Biorad). All values are expressed as mean ± SD. Statistical analysis was performed using the Student's *t*-test to determine the significance between groups ($P < 0.05$).

RESULTS

Syndecan-2 and PKCδ Interact in Transfected Osteoblastic Cells

To determine whether PKCδ is involved in the pro-apoptotic signaling induced by SYND2, we first examined the distribution of the two proteins in OB cells. To this aim, MG63 cells that display a low-level of SYND2 expression were transfected to overexpress SYND2. The proteoglycan and PKCδ were then detected by double immunofluorescence. In cells with low-level of SYND2 (Fig. 1A, arrow heads) and in SYND2-overexpressing cells (Fig. 1A, arrow), the proteoglycan was present over the whole cell surface. In these cells, PKCδ was also present at the cell membrane. In SYND2-overexpressing cells, PKCδ expression was markedly increased in cytosolic perinuclear compartments (Fig. 1A). At the membrane level there was no coincident distribution of the two proteins. However, as shown by yellow staining in merge views, the kinase and the proteoglycan displayed partial overlapping distribution in cytosolic compartments where SYND2 is present during protein maturation and glycosylation (Fig. 1A). To determine if this partial co-localization may reflect a molecular interaction, SYND2 and PKCδ were immunoprecipitated with specific antibodies and associated proteins were analyzed by Western blot. As shown in Figure 1B, PKCδ co-immunoprecipitated with SYND2. Furthermore, low molecular weight proteins recognized by the anti-SYND2 antibody were found associated with PKCδ. These data suggest that PKCδ can associate with SYND2 in OB cells.

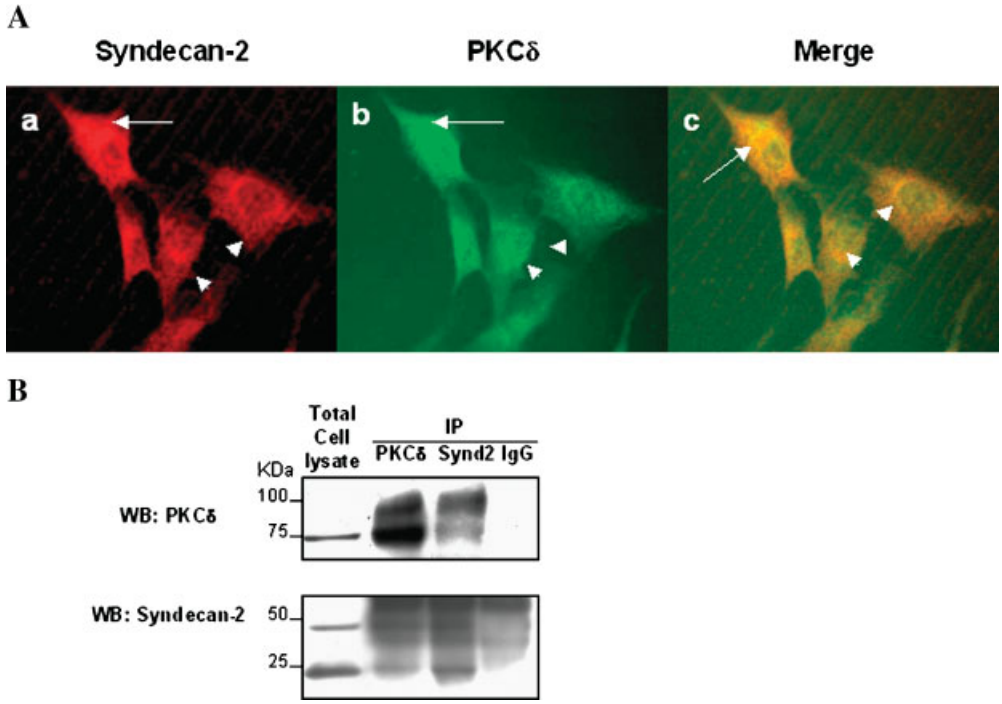

Fig. 1. SYND2 and PKCδ distribution in MG63 OB cells. **A**: Double immunofluorescence was performed on MG63 cells that were transiently transfected with SYND2, using a mouse monoclonal antibody (10H4) that recognizes the ectodomain of SYND2 after heparitinase III digest (**a**) and a rabbit anti-PKCδ antibody (**b**). Arrow heads indicate cells with low-level of SYND2. Arrows indicate cells that overexpress SYND2. Yellow staining indicates overlapping distribution in merge views (**c**). **B**: PKCδ and SYND2 were immunoprecipitated from total lysates of cells transfected with SYND2. Rabbit IgG were used as IP control. Total cell lysate and immunoprecipitated proteins were separated by SDS-PAGE and electrotransferred. The upper part of the resulting membrane was incubated with anti-PKCδ antibody and the lower part with anti-SYND2.

Syndecan-2 Induces Accumulation of PKCδ in Osteoblastic Cells

Since the double immunolabeling of SYND2 and PKCδ (Fig. 1A) suggests that PKCδ level was increased in SYND2-overexpressing cells, we examined the effect of SYND2 overexpression on endogenous PKCδ level by Western blot analysis of proteins extracted from transiently transfected MG63 cells. Using a liposome system, the transfection resulted in 15–25% of overexpressing cells in the culture, as previously observed [Modrowski et al., 2005]. Transfection with SYND2 resulted in a significant increase in SYND2 level as compared with control cells transfected with the EV (Fig. 2A). The increase in SYND2 level was associated with a higher level of PKCδ (Fig. 2A). Analysis of four independent experiments indicated that the increase in PKCδ level was about threefold, similar to the increase in SYND2. Transfection with SYND2 also resulted in a marked increase in SYND2 mRNA level as shown by the RT-PCR analysis (Fig. 2B). However, no change in PKCδ mRNA levels was found in cells transfected with SYND2 as compared to cells transfected with EV (Fig. 2B). These results suggest that SYND2 regulates PKCδ expression at a post-transcriptional level to induce PKCδ accumulation in OB cells.

Syndecan-2 Overexpression Inhibits PKCδ Activation and Activity in Osteoblastic Cells

PKCδ is a kinase that is responsive to DAG, and translocation to the membrane is required for its activation [Gschwendt, 1999]. We, therefore, investigated whether SYND2 could modify the recruitment of PKCδ at the membrane. To this aim, SYND2 and PKCδ were detected by immunoblotting after fractionation of the membrane and cytosol proteins of transiently transfected cells. As expected, SYND2 was mainly increased in the membranes in SYND2-transfected cells. Moreover, the high molecular weight smear was increased in these cells, indicating that recombinant SYND2 was glycosylated (Fig. 3A). A high-level of PKCδ was

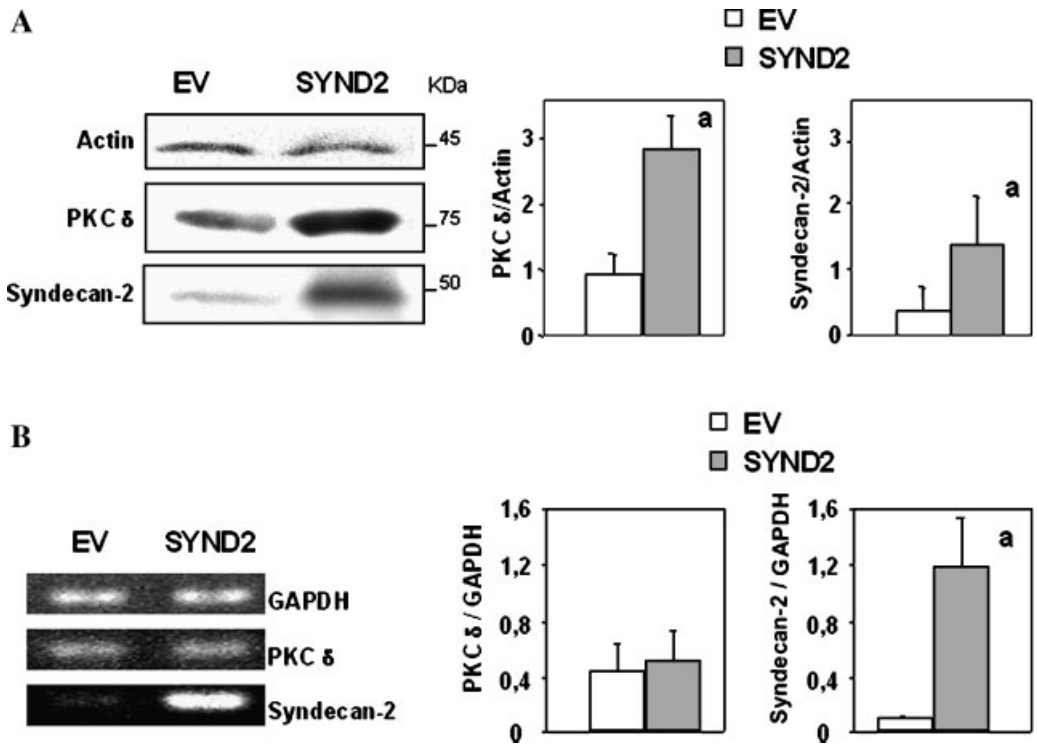

Fig. 2. SYND2 overexpression results in increased PKCδ level. **A**: PKCδ and SYND2 levels were determined by immunoblot in total lysate of cells transfected with an EV or with a vector encoding SYND2. Signals resulting from four independent experiments were analyzed and reported to the actin signal (**B**) level of SYND2 and PKCδ gene expression was determined by RT-PCR using 2 μg of total RNA extracted from MG63 cells transfected with EV or with SYND2. Signals resulting from three independent experiments were analyzed and GAPDH served as control gene to normalize PKCδ and SYND2 mRNA levels. Results are the means ± SD. **a**: indicates a significant difference ($P < 0.05$) with control cells transfected with the EV alone.

found in the membranes of MG63 cells (Fig. 3A). In cells that overexpressed SYND2, membranous PKCδ level was not modified whereas it was markedly increased in the cytosolic fraction (Fig. 3A). Thus, SYND2 transfection induced cytosolic accumulation of the kinase, suggesting that SYND2 may regulate PKCδ activation. To further document the effect of SYND2 on PKCδ activation, the phosphorylation of the kinase was examined using an anti-phospho-PKCδ antibody. Figure 3B shows that at short time after culture medium replacement, phospho-PKCδ level was increased in control cells (EV). In SYND2-transfected cells, phospho-PKCδ level was significantly reduced compared to EV control cells. This confirms that SYND2 inhibits PKCδ activation in OB cells. We then investigated the effect of SYND2 overexpression on PKCδ kinase activity. To this aim, an in vitro kinase assay was performed with PKCδ immunoprecipitated from cells transfected with EV, SYND2, or a plasmid coding the DN mutated form of PKCδ. Phosphorylation of histone was significantly reduced both in DN kinase and SYND2-overexpressing cells as compared to control cells (Fig. 3C). Altogether these data indicate that interaction between SYND2 and PKCδ results in downregulation of the activity of the kinase.

Inhibition of PKCδ Activity Increases Apoptosis in Osteoblastic Cells

The precise role of PKCδ in the control of OB cell life span is not known. To determine whether SYND2-mediated regulation of PKCδ contributes to SYND2-induced apoptosis, PKCδ activity was repressed by specific inhibitors. Rottlerin, a pharmacological agent that was found to inhibit PKCδ activity in OB cells [Li et al., 2002] induced a strong increase in effector caspase activity in MG63 cells (Fig. 4A). Transient expression of a DN mutated form of PKCδ also resulted in a significant enhancement of caspase activity in control cells (Fig. 4B). The 20% increase in caspase activity in PKCδDN-transfected cells corresponds to the 15–25% transfected cells in this system. These results suggest that inhibition of PKCδ activity induces

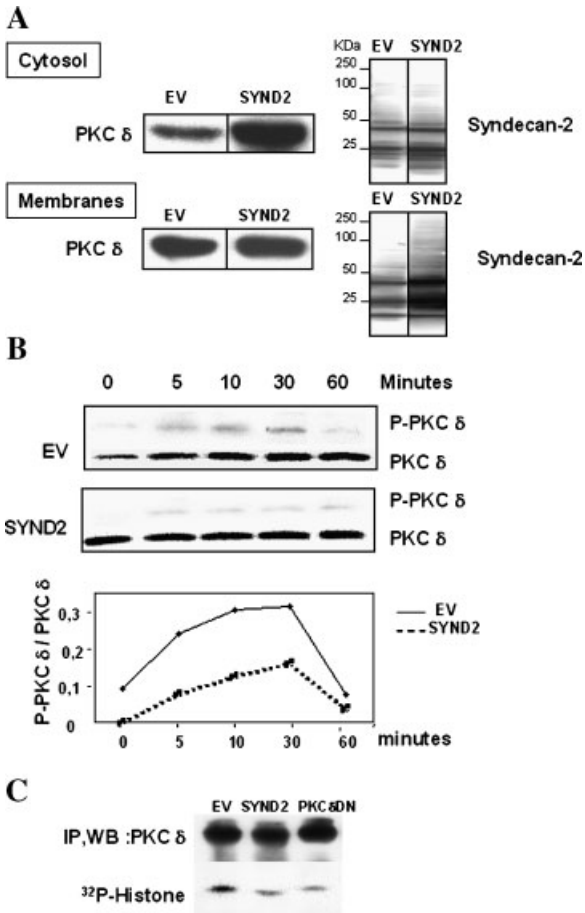

Fig. 3. SYND2 overexpression results in inhibition of PKCδ activation. MG63 cells were transfected with the EV or SYND2. **A**: Immunoblot analysis of PKCδ and SYND2 expression in subcellular compartments. Membranes were separated from cytosol by centrifugation. Unprocessed 75 kDa PKCδ or SYND2 were detected using rabbit polyclonal antibodies. **B**: Levels of (Thr505) phosphorylated PKCδ was detected in MG63-transfected cells. Two days post-transfection medium was replaced and the cells were lysed at different times as indicated. Signals obtained for P-PKCδ were quantified and normalized with the corresponding signal obtained for PKCδ. **C**: A kinase assay was performed using histone H1 as substrate and PKCδ immunoprecipitated from MG63 cells transfected with EV, SYND2, or a DN mutant of PKCδ. Immunoprecipitated PKCδ was detected by immunoblot. Phosphorylation of histone H1 was evaluated by autoradiography.

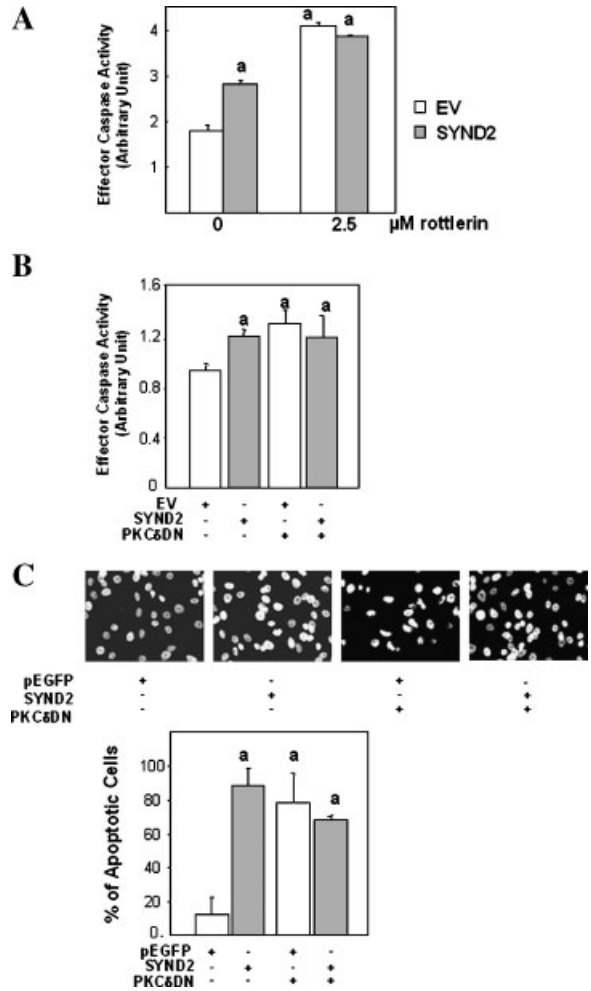

Fig. 4. Inhibition of PKCδ activity results in increased apoptosis. Effector caspase activity was measured using a synthetic fluorogenic substrate. MG63 cells were transfected with EV or SYND2 and treated with 2.5 μM Rottlerin for 24 h (**A**) or MG63 cells were co-transfected with the dominant negative mutant PKCδDN (**B**). **C**: Apoptotic chromatin modifications were evaluated after Hoechst staining in cells co-transfected with pEGFP-C1 or SYND2 and PKCδDN. Cells expressing GFP or high-level of SYND2, as detected by immunofluorescence, and displaying nuclear apoptotic modifications were counted. Results are representative of independent experiments and are the means ± SD of the values obtained from triplicate wells. **a**: indicates a significant difference ($P < 0.05$) with control cells transfected with the EV alone.

apoptosis. To further support this finding, we evaluated the effect of inhibition of PKCδ activity using Hoechst staining to reveal apoptotic changes in chromatin of transfected cells. These cells were detected by GFP expression in control pEGFP-transfected cells or by immunolabeling of SYND2 in SYND2-transfected cells. The rate of apoptotic cells was similar in the total cell population and in GFP expressing cells (12 ± 2 %) indicating that the transfection by itself had no effect on cell apoptosis. The DN kinase increased the number of apoptotic cells among control cells labeled for GFP (Fig. 4C). As previously shown, overexpression of SYND2 resulted in a significant increase in the activity of effector caspases and induced chromatin apoptototic changes (Fig. 4A, B, C). However, inhibition of PKCδ activity by co-transfection

with PKCδDN did not significantly modify SYND2-induced apoptosis (Fig. 4B, C). Overall, these results indicate that inhibition of the kinase activity in MG63 cells induces apoptosis and hence suggest that PKCδ exerts a survival function in these cells.

Rescue of PKCδ Activity in Syndecan-2-Overexpressing Cells Inhibits Apoptosis

To further investigate the role of PKCδ in SYND2-induced apoptosis, we co-transfected MG63 cells with SYND2 and a plasmid coding PKCδCAT, a constitutively active form of the kinase [Soh et al., 1999]. Rescue of PKCδ activity resulted in reduced caspase activation in cells transfected with SYND2 (Fig. 5A). Moreover, as shown in Figure 5B, the nucleus of most cells that overexpressed SYND2 was apoptotic. In contrast, the nucleus of cells that expressed the transgenes after co-transfection with SYND2 and PKCδCAT did not differ from that of control transfected cells that expressed GFP (Fig. 5B). Co-transfection with SYND2 and PKCδCAT allowed to decrease apoptotic cell number by 55 % as compared to cells transfected with SYND2 alone (Fig. 5C). This inhibitory effect of PKCδCAT on apoptosis induced by SYND2 was confirmed using the MTT reduction assay that measures cell viability (Fig. 5D). These data demonstrate that SYND2 induces apoptosis in OB cells in part through inhibition of PKCδ activity.

Overexpression of PKCδ Wild Type Induces Apoptosis

To further document the action of PKCδ during OB cells apoptosis, we transfected MG63 cells with a construct encoding the full length PKCδWT. Overexpression of PKCδ did not modify significantly effector caspase activity in transfected cells (Fig. 6A). However, PKCδWT induced cell death in control cells to the same extent as SYND2 alone, but did not modify SYND2-induced apoptosis (Fig. 6B and C). This pro-apoptotic action of PKCδWT was confirmed with annexin V binding assay (data not shown). These results show that in OB cells, PKCδ displays a pro-apoptotic action beside its pro-survival function.

Effects of PKCδ Suppression by siRNA on Osteoblastic Cell Apoptosis

Since the anti-apoptotic function of PKCδ seems to depend on the catalytic activity whereas the pro-apoptotic action seems to depend on the protein level, we attempted to discriminate the different activities of PKCδ using siRNA to reduce specifically the level of endogenous kinase in these cells. As shown in Figure 7A, co-transfection with a plasmid coding for SYND2 and siRNA targeted against PKCδ induced an increase in SYND2 expression and a marked decrease in PKCδ levels. Inhibition of the kinase expression with siRNA did not significantly modify SYND2-induced effector caspase activity (data not shown). Inhibition of the kinase expression resulted in a strong decrease in cell viability as shown by the MTT reduction test (Fig. 7B). In contrast, co-transfection with SYND2 and PKCδ siRNA increased cell viability as compared to cells transfected with SYND2 and control siRNA (Fig. 7B). Inhibition of the kinase expression also strongly increased the number of control GFP expressing cells showing apoptotic chromatin changes but reduced the number of apoptotic SYND2-expressing cells (Fig. 7C). These results show that suppression of PKCδ resulted in opposite effects in control and SYND2-overexpressing cells. This suggests that a high-level of PKCδ is required for the pro-apoptotic activity of SYND2, and that accumulation of inactive kinase induced by overexpression of SYND2 may be involved in SYND2-induced apoptosis.

DISCUSSION

Recent data indicate that syndecans act as signaling molecules. Notably, SYND2 is connected to key signaling pathways through interactions with kinases, cytoskeleton elements, or scalfolding proteins [Couchman, 2003]. However, SYND2 downstream signals are largely unknown. We recently reported a novel role for this proteoglycan in the control of OB cell apoptosis [Modrowski et al., 2005]. In the current study, we identified downstream signaling events that contribute to the pro-apoptotic signaling effect of SYND2 in OB cells.

The results presented here confirm that recombinant SYND2 expressed in MG63 cells was mainly directed to the membranes. In osteoblasts, SYND2 appeared in the same punctuate pattern at the cell surface and in compartments of the cytoplasm in osteoblasts, as previously shown by Tumova et al. [2000] in fibroblasts. PKCδ was also found to be associated to the plasma membrane and in perinucleus

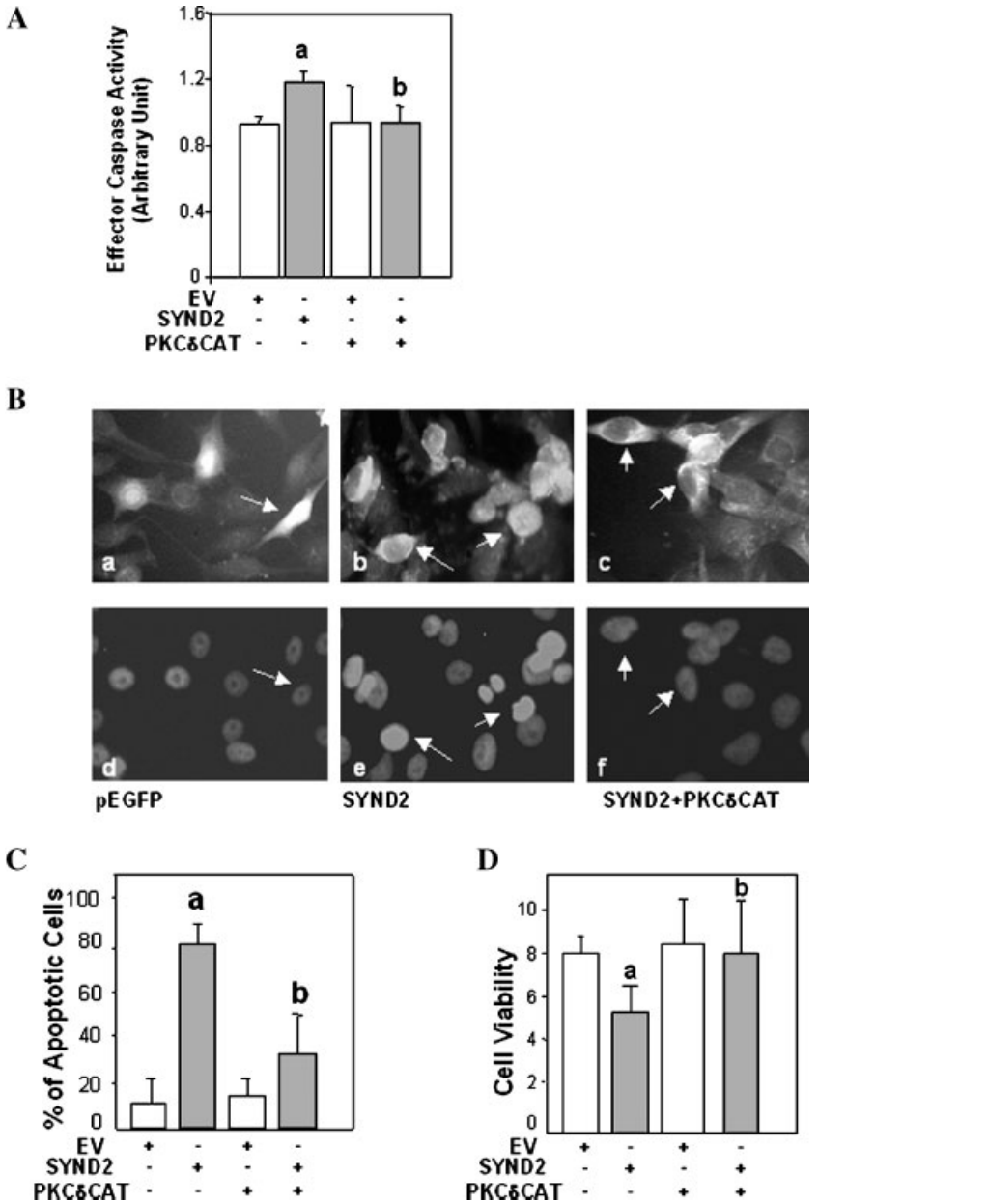

Fig. 5. Rescue of PKCδ activity results in inhibition of SYND2-induced apoptosis. **A**: Effector caspase activity was measured using a synthetic fluorogenic substrate in MG63 cells co-transfected with EV or SYND2 and the sequence coding the catalytic domain of the kinase (PKCδCAT). **B**: Two days after transfection SYND2 was detected by immunofluorescence using the rabbit antibody in cells co-transfected with SYND2 plus EV or PKCδCAT. Apoptotic chromatin changes were examined in control transfected cells that expressed GFP or in SYND2-overexpressing cells (arrows) after Hoechst staining. **C**: MG63 cells were transfected with the indicated plasmids and the cells expressing GFP or high-level of SYND2 and displaying nuclear apoptotic modifications were counted. **D**: Cell viability was assessed in transfected cells by a MTT reducing assay. Results are repesentative of independent experiments and are the means ± SD of the values obtained from triplicate wells. **a**: indicates a significant difference ($P < 0.05$) with control cells transfected with the EV alone. **b**: indicates a significant difference ($P < 0.05$) with cells transfected with SYND2 alone.

compartments. Although PKCδ and SYND2 did not display any overlapping distribution at the plasma membrane level, they were both present in cytoplasmic compartments. Moreover, co-IP analysis suggested that SYND2 can associate with PKCδ in OB cells. Several types of association have been described between syndecans and PKCs or other kinases. In particular, PKCδ and PKCγ are able to phosphorylate serines of the C1 domain of syndecan-4 and the V domain

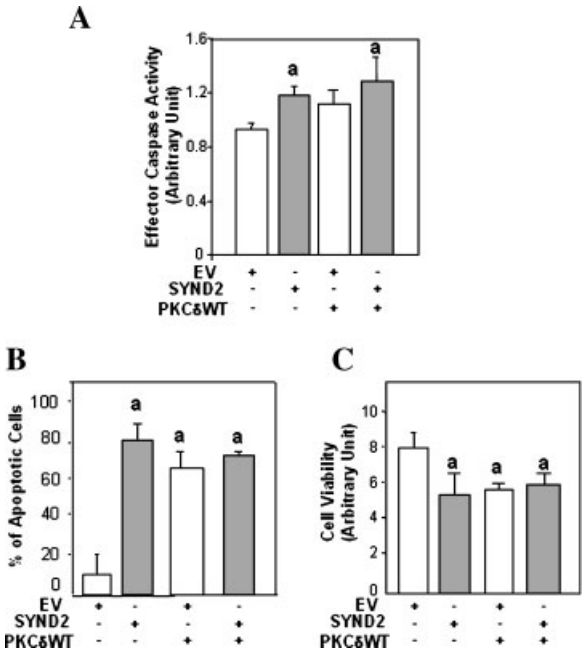

Fig. 6. PKCδWT induces apoptosis in MG63 cells. **A**: Effector caspase activity was measured using a synthetic fluorogenic substrate in MG63 cells co-transfected with EV or SYND2 and a construct coding the full length PKCδWT. **B**: Two days after transfection, SYND2 was detected by immunofluorescence in MG63 cells transfected with the indicated plasmids and the cells expressing GFP or high-level of SYND2 and displaying nuclear apoptotic modifications were counted. **C**: Cell viabilty was assessed in transfected cells by a MTT reducing assay. Results are repesentative of independent experiments and are the means ± SD of the values obtained from triplicate wells. **a**: indicates a significant difference ($P < 0.05$) with control cells transfected with the EV alone.

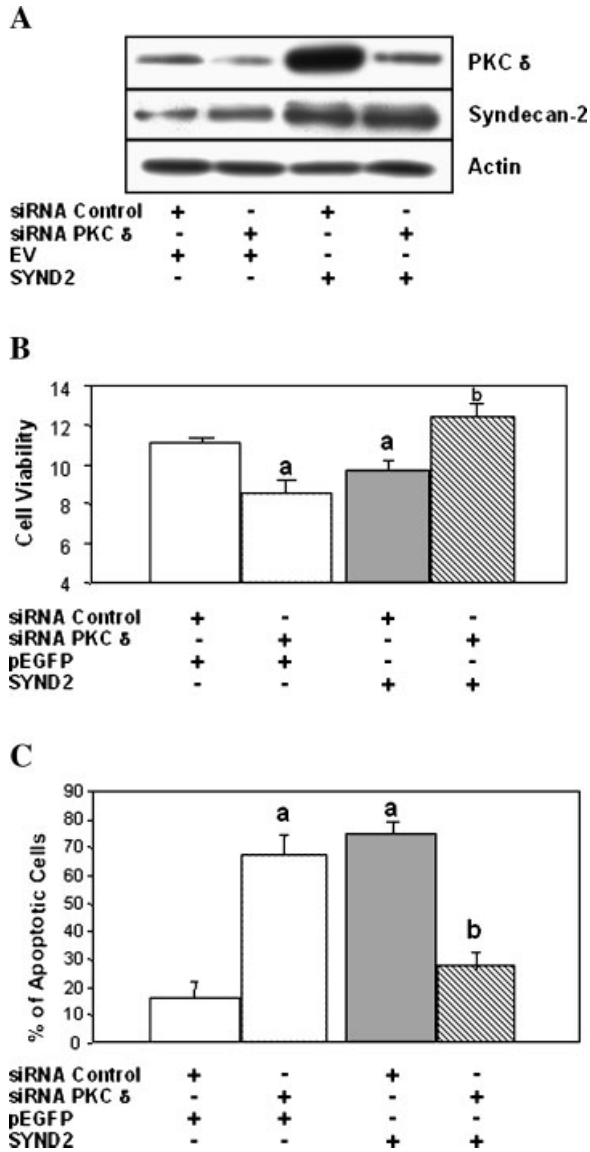

Fig. 7. SYND2 requires PKCδ expression to induce apoptosis. siRNA were used to inhibit PKCδ expression in SYND2-overexpressing cells. MG63 cells were transfected with EV or SYND2 and control scramble siRNA or PKCδ targeted siRNA. Inhibition of PKCδ expression was checked by immunoblot (**A**). Apoptosis was assessed by MTT reducing assay (**B**). The number of apoptotic cells was evaluated by counting GFP or SYND2-overexpressing cells that presented a condensed chromatin (**C**). Results are repesentative of independent experiments and are the means ± SD of the values obtained from at least triplicate wells. **a**: indicates a significant difference ($P < 0.05$) with control cells transfected with the EV and control siRNA. **b**: indicates a significant difference ($P < 0.05$) with cells transfected with SYND2 and control siRNA.

of SYND2, respectively [Kramer et al., 2002; Murakami et al., 2002]. Another type of association is exemplified by the interaction between syndecan-4, phosphatidylinositol 4,5-biphosphate (PIP2), and PKCα that results in activation of the kinase [Horowitz and Simons, 1998]. Although SYND2 does not have the appropriate V domain to complex directly PIP_2, this proteoglycan is able to interact with PDZ containing proteins, such as syntenin, that were shown to bind PIP_2 [Grootjans et al., 1997]. Indeed, the best described mode of activation for the novel PKCs involves conformational changes induced by interaction with the membrane lipids, that allows release of the inhibitory pseudosubstrate domain and facilitates the PKC-mediated phosphorylation of membrane substrates [Gschwendt, 1999]. Conversely, we show here that SYND2 induced cytosolic accumulation of PKCδ. Moreover, we found a lower level of PKCδ-Thr505 phosphorylation in SYND2-overexpressing cells. In contrast, PMA and other activators of PKCδ induce PKCδ-Thr505 phosphorylation in different cell types [Rybin et al.,

2004] which increases the catalytic activity of the membrane-associated activated kinase [Stempka et al., 1999]. We also showed that PKCδ immunoprecipitated from cells transfected with SYND2 had a reduced ability to phosphorylate histone in vitro. Overall, these results indicate that overexpression of SYND2 decreases activation of PKCδ through the membrane pathway and decreases PKCδ catalytic activity in OB cells, which provides a novel functional interaction between a syndecan and a kinase. We cannot exclude however the possibility that inhibition of PKCδ by SYND2 may not result from the direct physical interaction but may depend on intermediate signals. Since activation of PKC by phorbol ester treatment enhances its degradation via the ubiquitin-proteasome pathway [Lu et al., 1998], the accumulation of full length PKCδ protein may be related to its reduced activation via the membrane pathway that results from SYND2 overexpression.

To determine whether the altered PKCδ activity can account for the pro-apoptotic action of SYND2 overexpression, we examined the effect of inhibition of the PKCδ kinase activity on OB cell apoptosis. We found that either a low dose of Rottlerin, a kinase-dead PKCδ, or inhibition of PKCδ expression by specific siRNA induced cell death in control cells, indicating that PKCδ has a pro-survival function in OB cells. Anti-apoptotic functions for this kinase were described in other cell types including malignant breast cells in which PKCδ activation triggers the MEK/ERK survival pathway [McCracken et al., 2003]. PKCδ was also found to be involved in the anti-apoptotic effects of TNFα and FGF-2 through modulation of the JNK and p38 pathways [Brodie and Blumberg, 2003]. Interestingly, we show here that, as compared to transfection with SYND2 alone, co-transfection of SYND2 with DN PKCδ mutant had the same effect on cell death. The absence of any additive effect of PKCδDN and SYND2 overexpression suggests that the induction of cell death might be mediated through a common pathway that involves inhibition of PKCδ activity. Consistently, the rescue of PKCδ activity in SYND2-overexpressing cells resulted in reduced apoptosis. Altogether, these results demonstrate that SYND2 induces apoptosis in OB cells, at least partly, by inhibiting the pro-survival catalytic activity of PKCδ.

Our data also reveal that overexpression of PKCδWT results in increased number of apoptotic MG63 cells. This is not specific to MG63 cells because overexpression of PKCδWT in human primary pre-OB cells also decreased cell viability (data not shown). We speculated that, in the absence of allosteric activators, overexpression of PKCδ might result in accumulation of full length PKCδ in the soluble fraction, comparable to that occuring in SYND2-overexpressing cells. Since treatment with siRNA that inhibited SYND2-induced accumulation of PKCδ resulted in reduced apoptosis, we suggest that PKCδ may have a pro-apoptotic function that is dependent on the non-processed protein and is independent of the membrane activating pathway, and that SYND2 may also induce apoptosis through PKCδ accumulation. To our knowledge this is the first demonstration that PKCδ could display both a pro- and an anti-apoptotic role in the same cell type. This dual effect is consistent with the finding that PKCδ mediates distinct functions of the pro-apoptotic cAbl and the anti-apoptotic Atm in OB cells subjected to oxidative stress. Indeed, these kinases were found to destabilize and stabilize PKCδ, respectively [Li et al., 2004]. Our data are also consistent with the finding that the catalytic form of PKCδ free from the regulatory domain and the full length PKCδ display different lipid requirements and substrate specificity [Hamaguchi et al., 2003; Steinberg, 2004], indicating distinct functions for the different forms of the kinase. Hence, SYND2 may drive a switch from a catalytic activity of PKCδ to another activity. Another possibility is that SYND2 may promote a kinase independent activity of PKCδ. Indeed, PKCδ is able to induce apoptosis in vascular smooth muscle cells independently of its kinase activity [Goerke et al., 2002]. Other functions that do not seem to be linked to kinase action were described for PKCδ, such as Dishevelled recruitment [Kinoshita et al., 2003] or docking function for Src kinase and associated proteins [Zang et al., 1997]. It is worth noting that inhibition of the catalytic activity by Rottlerin or the kinase-dead construct induced cell death and activation of effector caspases whereas the apoptotic activity of PKCδ was not associated with marked changes in caspase activity in OB cells. This is consistent with the finding that the apoptotic activity of PKCδ in vascular smooth muscle cells is independent of caspase activation [Goerke

et al., 2002], and supports the idea that PKCδ may have opposite actions involving independent mechanisms.

In conclusion, our data indicate that overexpression of SYND2 modulates PKCδ action, by inhibition of the canonical allosterical activation pathway that plays an anti-apoptotic role in OB cells, and promotion of a pro-apoptotic role that participates to the induction of cell death by SYND2, which establishes a novel functional interaction between SYND2 and PKCδ.

ACKNOWLEDGMENTS

We thank Dr. Soh (Herbert Irving Comprehensive Cancer Center, Columbia University, NY) and Dr. Jürgen Dittmer (Halle-Wittenberg University, Saale, Germany) for providing PKCδ constructs.

REFERENCES

Bernfield M, Götte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M. 1999. Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem 68: 729–777.

Brodie C, Blumberg PM. 2003. Regulation of cell apoptosis by protein kinase c delta. Apoptosis 8:19–27.

Chen CY, Faller DV. 1999. Selective inhibition of protein kinase C isozymes by Fas ligation. J Biol Chem 274: 15320–15328.

Couchman JR. 2003. Syndecans: Proteoglycan regulators of cell-surface microdomains? Nature Review 4:926–936.

Debiais F, Lefèvre G, Lemmonier J, Le Mee S, Lasmoles F, Mascarelli F, Marie PJ. 2004. Fibroblast growth factor-2 induces osteoblast survival through a phosphatidylinositol 3-kinase-dependent, -beta-catenin-independent signaling pathway. Exp Cell Res 297:235–246.

Fujii T, Garcia-Bermejo ML, Bernabo JL, Caamano J, Ohba M, Kuroki T, Li L, Yuspa SH, Kazanietz MG. 2000. Involvement of protein kinase C delta (PKCdelta) in phorbol ester-induced apoptosis in LNCaP prostate cancer cells. Lack of proteolytic cleavage of PKCdelta. J Biol Chem 275:7574–7582.

Goerke A, Sakai N, Gutjahr E, Schlapkohl WA, Mishinski JF, Haller H, Kolch W, Saito N, Mischak H. 2002. Induction of apoptosis by PKC d is independent of its kinase activity. J Biol Chem 277:2054–2062.

Granes F, Garcia R, Casaroli-Marano RP, Castel S, Rocamora N, Reina M, Urena JM, Vilaro S. 1999. Syndecan-2 induces filopodia by active cdc42Hs. Exp Cell Res 248:439–456.

Grootjans JJ, Zimmermann P, Reekmans G, Smets A, Degeest G, Durr J, David G. 1997. Syntenin, a PDZ protein that binds syndecan cytoplasmic domains. Proc Natl Acad Sci USA 94:13683–13688.

Gschwendt M. 1999. Protein kinase C delta. Eur J Biochem 259:555–564.

Hamaguchi A, Suzuki E, Murayama K, Fujimura T, Hikita T, Iwabuchi K, Handa K, Withers DA, Masters SC, Fu H, Hakomori S. 2003. Sphingosine-dependent protein kinase-1, directed to 14-3-3, is identified as the kinase domain of protein kinase C delta. J Biol Chem 278: 41557–41565.

Hay E, Lemonnier J, Fromigue O, Marie PJ. 2001. Bone morphogenetic protein-2 promotes osteoblast apoptosis through a Smad-independent, protein kinase C-dependent signaling pathway. J Biol Chem 276:29028–29036.

Horowitz A, Simons M. 1998. Phosphorylation of the cytoplasmic tail of syndecan-4 regulates activation of protein kinase C alpha. J Biol Chem 273:25548–25551.

Hsueh YP, Yang FC, Kharazia V, Naisbitt S, Cohen AR, Weinberg RJ, Sheng M. 1998. Direct interaction of CASK/LIN-2 and syndecan heparan sulfate proteoglycan and their overlapping distribution in neuronal synapses. J Cell Biol 142:139–151.

Jilka RL, Weinstein RS, Bellido T, Roberson P, Parfitt AM, Manolagas SC. 1999. Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. J Clin Invest 104:439–446.

Kawakami A, Eguchi K, Matsuoka N, Tsuboi M, Koji T, Urayama S, Fujiyama K, Kiriyama T, Nakashima T, Nakane PK, Nagataki S. 1997. Fas and Fas ligand interaction is necessary for human osteoblast apoptosis. J Bone Miner Res 12:1637–1646.

Kinoshita N, Iioka H, Miyakoshi A, Ueno N. 2003. PKC delta is essential for dishevelled function in a noncanonical Wnt pathway that regulates Xenopus convergent extension movements. Genes Dev 17:1663–1676.

Kramer KL, Barnette JE, Yost HJ. 2002. PKCgamma regulates syndecan-2 inside-out signaling during xenopus left-right development. Cell 111:981–990.

Lafleur EA, Koshkina NV, Stewart J, Jia SF, Worth LL, Duan X, Kleinerman ES. 2004. Increased Fas expression reduces the metastatic potential of human osteosarcoma cells. Clin Cancer Res 10:8114–8119.

Li L, Lorenzo PS, Bogi K, Blumberg PM, Yuspa SH. 1999. Protein kinase Cdelta targets mitochondria, alters mitochondrial membrane potential, and induces apoptosis in normal and neoplastic keratinocytes when overexpressed by an adenoviral vector. Mol Cell Biol 19: 8547–8558.

Li B, Ishii T, Tan CP, Soh JW, Goff SP. 2002. Pathways of induction of peroxiredoxin I expression in osteoblasts: Roles of p38 mitogen-activated protein kinase and protein kinase C. J Biol Chem 277:12418–12422.

Li B, Wang X, Rasheed N, Hu Y, Boast S, Ishii T, Nakayama K, Nakayama KI, Goff SP. 2004. Distinct roles of c-Abl and Atm in oxidative stress response are mediated by protein kinase C delta. Genes Dev 18:1824–1837.

Lu Z, Liu D, Hornia A, Devonish W, Pagano M, Foster DA. 1998. Activation of protein kinase C triggers its ubiquitination and degradation. Mol Cell Biol 18:839–845.

Manolagas SC. 2000. Birth and death of bone cells: Basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocrine Reviews 212:115–137.

McCracken MA, Miraglia LJ, McKay RA, Strobl JS. 2003. Protein kinase C delta is a prosurvival factor in human breast tumor cell lines. Mol Cancer Ther 2:273–281.

Modrowski D, Baslé M, Lomri A, Marie PJ. 2000. Syndecan-2 is involved in the mitogenic activity and signaling of granulocyte-macrophage colony-stimulating factor in osteoblasts. J Biol Chem 275:9178–9185.

Modrowski D, Orosco A, Thevenard J, Fromigue O, Marie PJ. 2005. Syndecan-2 overexpression induces osteosarcoma cell apoptosis: Implication of syndecan-2 cytoplasmic domain and JNK signaling. Bone 37:180–189.

Munesue S, Kusano Y, Oguri K, Itano N, Yoshitomi Y, Nakanishi H, Yamashina I, Okayama M. 2002. The role of syndecan-2 in regulation of actin-cytoskeletal organization of Lewis lung carcinoma-derived metastatic clones. Biochem J 363:201–209.

Murakami M, Horowitz A, Tang S, Ware JA, Simons M. 2002. Protein kinase C (PKC) delta regulates PKCalpha activity in a syndecan-4-dependent manner. J Biol Chem 277:20367–20371.

Oh ES, Couchman JR, Woods A. 1997. Serine phosphorylation of syndecan-2 proteoglycan cytoplasmic domain. Arch Biochem Biophys 344:67–74.

Postiglione L, Di Domenico G, Giordano-Lanza G, Ladogana P, Turano M, Castaldo C, Di Meglio F, Cocozza S, Montagnani S. 2003. Effect of human granulocyte macrophage-colony stimulating factor on differentiation and apoptosis of the human osteosarcoma cell line SaOS-2. Eur J Histochem 47:309–316.

Rybin VO, Guo J, Sabri A, Elouardighi H, Schaefer E, Steinberg SF. 2004. Stimulus-specific differences in protein kinase C delta localization and activation mechanisms in cardiomyocytes. J Biol Chem 279:19350–19361.

Soh JW, Lee EH, Prywes R, Weinstein IB. 1999. Novel roles of specific isoforms of protein kinase C in activation of the c-fos serum response element. Mol Cell Biol 19:1313–1324.

Steinberg SF. 2004. Distinctive activation mechanisms and functions for protein kinase Cdelta. Biochem J 384: 449–449.

Stempka L, Schnolzer M, Radke S, Rincke G, Marks F, Gschwendt M. 1999. Requirements of protein kinase cdelta for catalytic function. Role of glutamic acid 500 and autophosphorylation on serine 643. J Biol Chem 274: 8886–8892.

Tumova S, Woods A, Couchman JR. 2000. Heparan sulfate chains from glypican and syndecans bind the Hep II domain of fibronectin similarly despite minor structural differences. Int J Biochem Cell Biol 32:269–288.

Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC. 1998. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest 102(2):274–282.

Zang Q, Lu Z, Curto M, Barile N, Shalloway D, Foster DA. 1997. Association between v-Src and protein kinase C delta in v-Src-transformed fibroblasts. J Biol Chem 272: 13275–13280.