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Regulation of Fibronectin Fibrillogenesis by Protein Kinases in Cultured Rat Osteoblasts

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ABSTRACT

Fibronectin (Fn) plays an important role in the regulation of adhesion, migration, and maturation of osteoblasts. Fn fibrillogenesis is involved in the process of bone mineralization. To elucidate the regulatory role of protein kinases in the formation of fibrillar Fn matrix, Fn synthesis and assembly were examined in cultured osteoblasts. Osteoblasts assembled the endogenously released soluble Fn into immobilized form on the substratum in a time-dependent manner. Both 12-O-tetradecanoylphorbol-13 acetate (TPA) and forskolin increased the synthesis of Fn. However, the extracellular assembly of Fn fibril from both endogenously released and exogenously applied soluble Fn was increased by TPA but decreased by forskolin. Protein kinase C (PKC) inhibitors, such as H7, Ro 318220, and Gö 6976, inhibited Fn fibrillogenesis. These results suggest that the dynamic of Fn fibrillogenesis is differentially regulated by the activation of PKC and protein kinase A (PKA). Both classic and novel isoforms of PKC are involved in the action of TPA in

osteoblasts. It has been reported that $\alpha 5\beta 1$ integrin is related to Fn fibrillogenesis. Immunocytochemistry and flow cytometry showed that TPA and forskolin increased and inhibited, respectively, the clustering and surface expression of $\alpha 5$ integrins. TPA and forskolin did not affect protein levels of $\alpha 5$ integrins. The Western blot and reverse transcriptase-polymerase chain reaction showed that protein and mRNA levels of $\beta 1$ integrins also were not affected by TPA and forskolin. These results suggest that TPA and forskolin may affect the surface expression of $\alpha 5\beta 1$ integrins. cAMP response element-binding protein phosphorylation is involved in the action of forskolin but not that of TPA. Our results suggest that PKC activation enhanced Fn fibrillogenesis, whereas PKA activation inhibited extracellular Fn fibrillogenesis in primary cultured osteoblasts. Cytosolic Fn synthesis and extracellular Fn assembly may be differentially regulated by the activation of PKA.

The interaction of extracellular matrix (ECM) with cells plays a key role in the regulation of cell adhesion, migration, and proliferation, as well as differentiation. The components and structure of ECM provide essential information for the induction of cellular physiological events. Fn is a heterodimeric ECM glycoprotein that has been shown to regulate various kinds of physiological events during embryogenesis, angiogenesis, thrombosis, inflammation, and wound healing (Hay, 1991). Assembly of soluble Fn into matrix is a multistep process under cellular control. Among the membrane components implicated in Fn matrix assembly, integrins have been firmly demonstrated to have a central role (Sakai et al., 1998). Integrins are receptor $\alpha\beta$ heterodimers

with overlapping specificity toward ECM components (Hynes, 1992). Integrin-mediated cell-ECM interactions promote the assembly of cytoskeletal and signaling molecule complexes at sites called focal adhesions. Integrin-focal adhesion kinase signaling complexes have been implicated in the regulation of anchorage-dependent cell survival.

Continuous remodeling of bone through resorption and formation enables the skeleton to maintain strength. Small changes in modeling and remodeling activities by bone-forming osteoblasts and bone-resorbing osteoclasts can have significant functional consequences on skeletal integrity. Osteoblasts, the cells responsible for the formation of new bones, first differentiate from precursors adjacent to bone surfaces. The ECM produced by osteoblasts is complex and consists of several different classes of molecules that may regulate the modeling and remodeling of bone. The ECM contains struc-

ABBREVIATIONS: ECM, extracellular matrix; Fn, fibronectin; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; H-8, *N*-(2-(methylamino)ethyl)-5-isoquinolinesulfonamide; TPA, 12-O-tetradecanoylphorbol-13 acetate; Ro 318220, {3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide, bisindolylmaleimide IX, Methanesulfonate; Gö 6976, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c] carbazole; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; CREB, cAMP response element-binding protein; PKA, protein kinase A; PKC, protein kinase C; RT-PCR, reverse transcriptase-polymerase chain reaction.

tural components such as type I collagen and Fn, as well as proteases that degrade the matrix (Nordahl et al., 1995; Winnard et al., 1995; Robey, 1996). There is strong evidence to suggest a role for Fn in the early stages of osteogenesis. The distribution of Fn in areas of skeletogenesis suggests that it may be involved in early stages of bone formation (Moursi et al., 1996, 1997). The expression of Fn mRNA increases during the early stages of osteoblast differentiation and is reduced during cell maturation (Stein et al., 1990; Winnard et al., 1995; Moursi et al., 1997). Fn is synthesized and deposited in the areas of bone tissue at which recruitment and commitment of osteoblast precursors occur; therefore, Fn is highly localized to sites of early osteogenesis, where the ECM undergoes a great deal of turnover and organization. Fn can interact extensively with itself, and with other matrix components, through its collagen-, fibrin-, and glycosaminoglycan-binding domains. However, the signaling pathways leading to Fn fibrillogenesis underneath the osteoblasts are poorly understood. Cells isolated from fetal rat calvaria and grown in culture provide a useful model of Fn fibrillogenesis. In this study, we investigated the regulation of Fn fibrillogenesis in cultured osteoblasts by protein kinases A and C. It was found that PKC increased and PKA inhibited extracellular Fn assembly. Both protein kinases may play important roles in the dynamic changes of Fn matrix and bone tissue remodeling.

Materials and Methods

Chemicals and Solutions. 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7), N-(2-(methylamino)ethyl)-5-isoquinolinesulfonamide (H-8), forskolin, 12-O-tetradecanoylphorbol-13 acetate (TPA), and leupeptin were obtained from Sigma-Aldrich (St. Louis, MO). Ro 318220 and Gö 6976 were from Calbiochem (San Diego, CA), and soluble human Fn was from Invitrogen (Carlsbad, CA).

Primary Osteoblast Cultures. The primary osteoblastic cells were obtained from the calvaria of 18-day-old fetal rats. In brief, the fetal rats were put under anesthesia using intraperitoneal injection of pentobarbital. The calvaria were then dissected by aseptic technique. The soft tissues were removed under a dissecting microscope. The clavaria were divided into small pieces and were treated with 0.1% collagenase solution for 10 min at 37°C. The next two 20-min sequential collagenase digestions were then pooled and filtered through a 70-μm nylon filter (Falcon; BD Biosciences, San Jose, CA). The cells were then grown on plastic cell culture dishes in 95% air-5% CO_2 with Dulbecco's modified Eagle's medium, which was supplemented with 20 mM HEPES and 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), pH adjusted to 7.6. The cell medium was changed twice a week. The osteoblast characteristics were confirmed by morphology and alkaline phosphatase expression.

Immunocytochemistry. Osteoblasts were grown on glass coverslips. Cultures were rinsed once with phosphate-buffered saline (PBS) and fixed for 15 min at room temperature in phosphate buffer containing 4% paraformaldehyde. Cells were rinsed three times with PBS. After blocking with 4% BSA for 15 min, cells were incubated with rabbit anti-rat Fn (1:100; Invitrogen) for 1 h at room temperature. Cells were then washed again and labeled with FITC-conjugated goat anti-rabbit IgG (1:150; Leinco Technologies, Inc., Ballwin, MO) for 1 h. Finally, cells were washed, mounted, and examined with a fluorescence microscope. The mean fluorescence under 10 to 15 cells was measured using an LSM 410 confocal microscope (Zeiss, Thornwood, NY). In some experiments, the distribution of intracel-

lular Fn was also examined by incubating the cells with 0.5% Triton X-100 for 10 min to permeabilize the cell after fixation.

The localization of α5 integrins was also investigated by fixing the cells with acetone for 30 s. After fixation, cells were washed with PBS and then incubated with 4% BSA for 1 h. Cells were then incubated with rabbit anti-rat α5 integrin (1:500; Chemicon, Temecula, CA) for 3 h and FITC-conjugated goat anti-rabbit IgG for 1 h at room temperature.

To observe Fn assembly apart from Fn synthesis by rat osteoblasts, human soluble Fn (30 μg/ml) was bath-applied to the cultures overnight. After fixation and blocking with 4% BSA for 15 min, cells were incubated for 1 h at room temperature with mouse anti-human Fn (1:50; Transduction Laboratories, Lexington, KY), which does not recognize endogenously released rat Fn. Cells were then washed again and labeled with FITC-conjugated goat anti-mouse IgG (1:150; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h. Finally, cells were washed, mounted, and examined with a Zeiss fluorescence microscope.

Western Blotting Analysis. Osteoblasts were plated on six-well (35-mm) dishes. Cells were incubated with various drugs for 15 h [7 min for phospho-cAMP response element-binding protein (CREB) detection] and then washed in PBS lysed for 30 min at 4°C with radioimmunoprecipitation assay buffer. Thirty micrograms (50 μg for phospho-CREB detection) of protein was applied per lane, and electrophoresis was performed under denaturing conditions on a 7.5% (10% for phospho-CREB detection) polyacrylamide-SDS gel and then transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA) at 4°C overnight. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-rat antibodies against Fn (1:1500), α5 integrin (1:100), and phospho-CREB (1:1000; Upstate Biotechnology, Lake Placid, NY) or mouse anti-rat β1 integrin (1:2000; Transduction Laboratories) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit or sheep anti-mouse peroxidase-conjugated secondary antibody (1:2000; Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). For normalization purposes, the same blot was also probed with mouse anti-rat α-tubulin antibody (1:1000; Oncogene Science, Boston, MA).

For the study of PKC translocation, cells were rinsed with PBS and suspended in homogenization buffer (20 mM Tris-HCl, 5 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin, pH 7.5) and then sonicated on ice. The lysates were separated into cytosolic and pellet fractions by centrifugation at 40,000g for 45 min. Membrane-bound PKC was extracted from the pellet with 1% Triton X-100 in the above-mentioned buffer for 20 min at 4°C. The resultant suspension was centrifuged at 100,000g for 30 min, and the supernatant was used as the membrane fraction of cellular PKC. Equal amounts (80 μg) of each protein from cytosolic and membrane fractions were separated by 7.5% polyacrylamide-SDS gel and then electrotransferred to polyvinylidene difluoride membranes. The washed membranes were incubated overnight at room temperature with mouse monoclonal antibodies against various isoforms of PKC (1:1000; Transduction Laboratories). After washing with PBS, the blots were incubated for 1 h at room temperature with sheep anti-mouse peroxidase-conjugated secondary antibody (1:2000).

Flow Cytometry. Osteoblasts were plated in six-well (35-mm) dishes. The cells were then washed with PBS and detached with trypsin at 37°C. Cells were fixed for 10 min in PBS containing 1% paraformaldehyde. After rinsing in PBS, the cells were incubated with rabbit anti-rat α5 or mouse anti-rat β1 integrin antibody for 1 h at 4°C. Cells were then washed again and incubated with FITC-conjugated secondary IgG for 45 min and analyzed by flow cytometry using FACSCalibur (BD Biosciences).

mRNA Analysis by RT-PCR. Total RNA was extracted from osteoblasts using a TRIzol kit (MDBio, Inc., Taipei, Taiwan). Five

hundred nanograms of RNA from osteoblasts was used for reverse transcriptase-polymerase chain reaction (RT-PCR) by using a One-Step RT-PCR kit (CLONTECH, Palo Alto, CA). Amplification was accomplished with 17 cycles, which was within a linear range in a PCR reaction (Biometra, Göttingen, Germany). PCR products were then separated electrophoretically in a 2% agarose DNA gel and stained with ethidium bromide. β1-integrin mRNA levels were normalized to levels of β-actin. The PCR primers used were as follows: β1 integrin: forward primer, GGACAGGAGAAAATGGACGA; reverse primer, TCTGACCATTTGTCGCTACG; β-actin: forward

primer, TGTCACCAACTGGGACGATA; reverse primer, TCTCCG-GAGTCCATCACAAT.

The values given are means $\pm$ S.E.M. The significance of difference between the experimental group and control was assessed by Student's t test. The difference is significant if the p value is <0.05.

Results

Fn Fibrillogenesis by Cultured Rat Osteoblasts. The immobilized form of fibrillogenesis from the endogenously
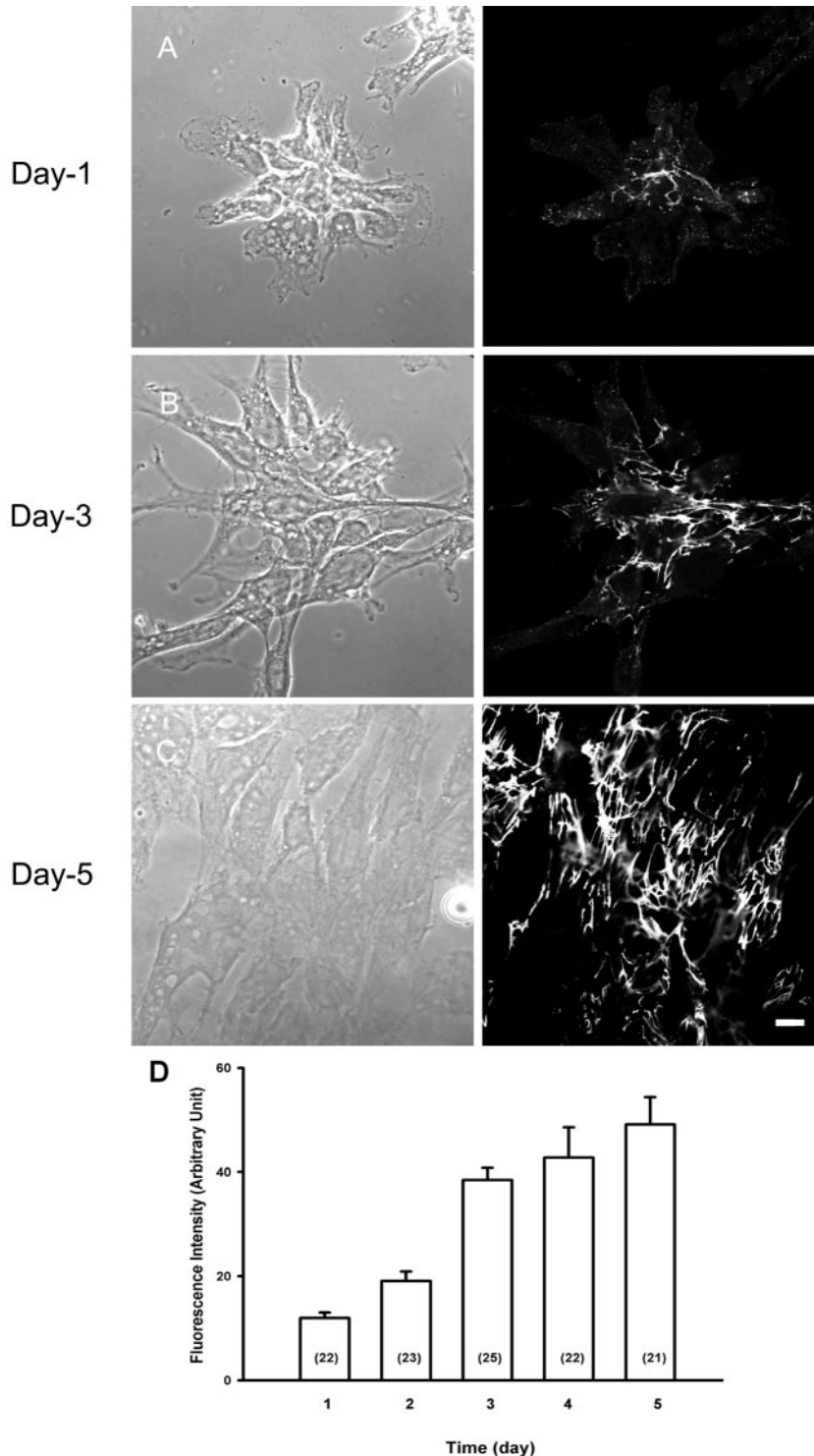

Fig. 1. Time-dependent Fn fibrillogenesis by cultured rat osteoblasts. The Fn network, which was shown by immunofluorescence, formed underneath the cultured osteoblasts. The assembly of endogenously released Fn by the rat osteoblasts progressively increased day by day. Phase-contrast images are shown on the left. The quantitative data are shown in D. Bar, 10 μm. Data are presented as mean $\pm$ S.E. (n represents the group number of the cells).

released Fn by monolayer osteoblasts was studied using immunocytochemistry. As shown in Fig. 1, rat osteoblasts are able to form an Fn network on the substratum using endog-

enously released Fn. The assembly of Fn by the rat osteoblasts was time-dependent (Fig. 1, B and C). The quantitative data showed a gradual increase of fluorescence intensity
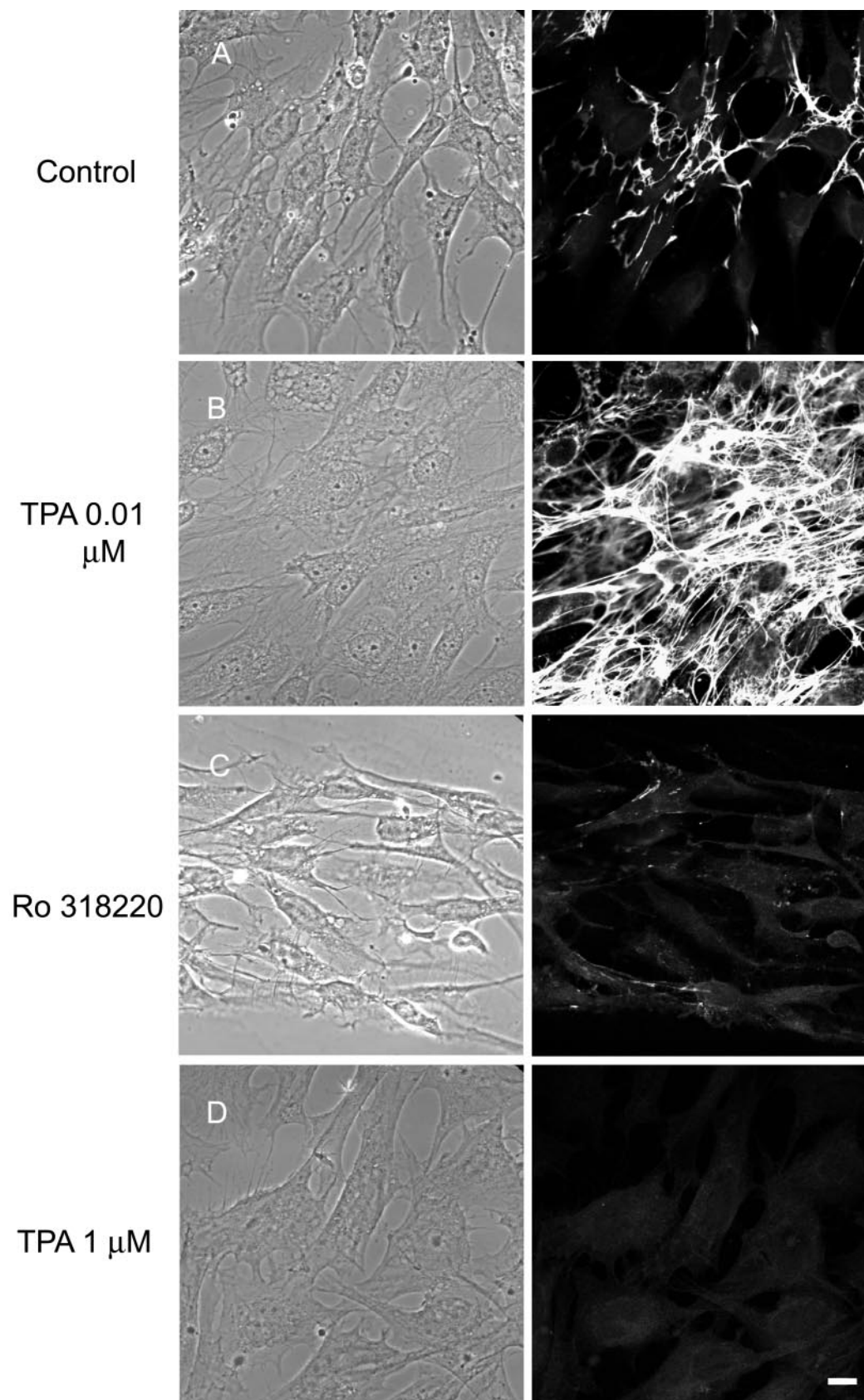
Fig. 2. Effects of PKC activity on the assembly of endogenously released Fn by rat osteoblasts. Compared with control (A), Fn assembly markedly increased after treatment with 0.01 μM TPA for 15 h (B). Fn assembly greatly decreased after 15 h of treatment with PKC inhibitor Ro 318220 (C) or higher concentration of TPA at 1 μM (D). Phase-contrast images are shown on the left. Bar, 10 μm.

day by day (Fig. 1D). The mean fluorescence intensity under 10 to 15 cells was 12.0 ± 1.0, 19.1 ± 1.8, 38.5 ± 2.3, 42.8 ± 5.8, and 49.1 ± 5.2 ($n = 21$–25; n represents the group number of the cells) for cells from days 1, 2, 3, 4, and 5, respectively). The following experiments were performed using cells of days 3~5.

Regulation of Matrix Assembly from Endogenously Released Fn by PKC and PKA. The effects of PKC and PKA on the assembly of endogenously released Fn were compared in the rat osteoblasts using quantitative immunofluorescence (Figs. 2 and 3). The osteoblasts from days 3~5 were changed to serum-free medium and incubated with various drugs for 15 h. The mean immunofluorescence of 10 to 15 cells of the monolayer osteoblasts was measured using a confocal microscope. For the study of PKC regulation on the assembly of endogenously released Fn, the cultured osteoblasts were treated with PKC activator TPA or PKC inhibitors H7 and Ro 318220, respectively. Compared with control (Fig. 2A), Fn fibrillogenesis increased markedly after treatment with 0.01 μM TPA for 15 h (Fig. 2B). The mean fluorescence intensity increased from 42.6 ± 2.1 to 73.0 ± 4.6 ($n = 16$–56; Fig. 2B). The Fn assembly was greatly inhibited by chronic treatment with PKC inhibitors Ro 318220 (1 μM; Fig. 2C) and H7 (10 μM). The mean fluorescence intensity

was 28.4 ± 2.9 and 27.3 ± 3.7, respectively ($n = 16$–56). Fn assembly also decreased when the cultures were treated with TPA at a higher concentration of 1 μM for 15 h (18.0 ± 3.2, $n = 19$; Fig. 2D), probably resulting from the down-regulation of PKC. There was no obvious cytotoxicity in osteoblasts after prolonged incubation with 1 μM TPA for 15 h. For the investigation of PKA activation on the assembly of endogenously released Fn, osteoblasts were treated with forskolin, an adenylate cyclase activator. Compared with control (Fig. 3A), the assembly of endogenously released Fn greatly decreased after treatment with 10 μM forskolin for 15 h (Fig. 3B). The mean fluorescence intensity was 42.6 ± 2.1 and 15.2 ± 2.7 ($n = 22$–31) for control and forskolin-treated cells, respectively (Fig. 3C).

The Western blotting analysis was then used to examine the effects of protein kinases on the protein level of Fn. Cultured cells from days 3~5 were changed to serum-free medium and incubated with various drugs for 15 h, and the cultured medium was then replaced with lysis buffer to collect protein samples. These protein samples may contain both the cytosolic soluble form and the extracellular immobilized form of Fn. As shown in Fig. 4A, the amount of Fn increased in response to chronic treatment with 0.01 or 0.1 μM TPA. However, treatment with TPA at a higher concen-
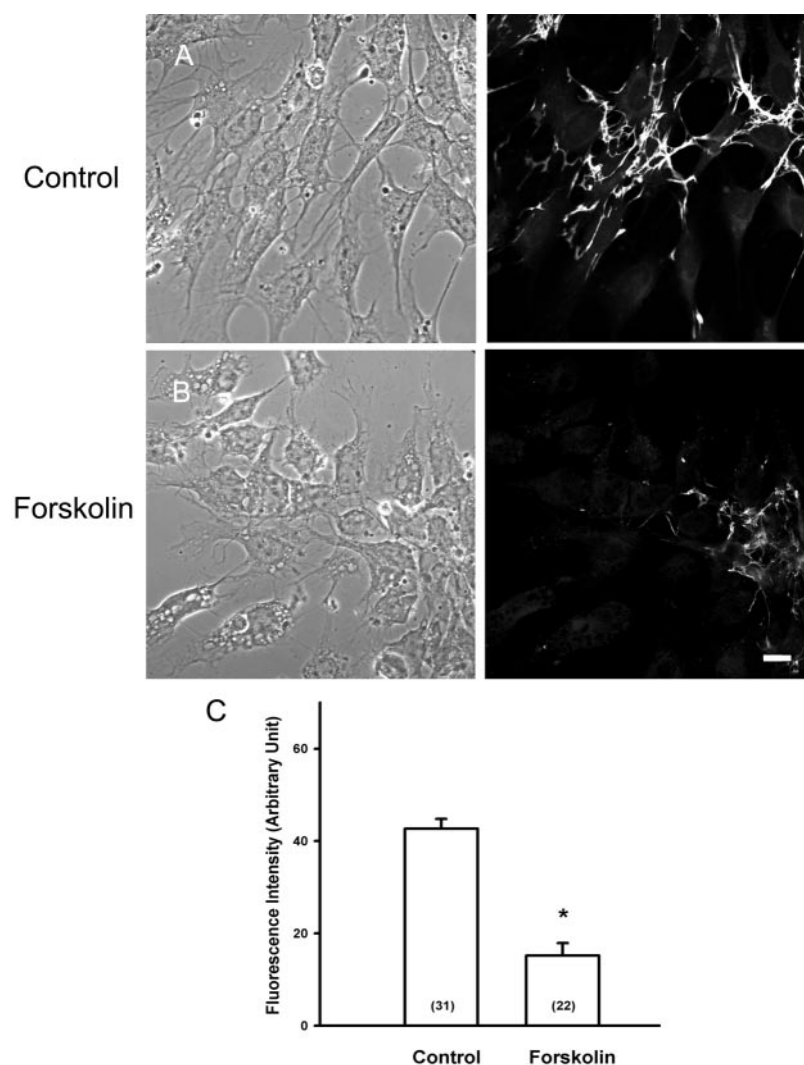

Fig. 3. Inhibition of forskolin on the assembly of endogenously released Fn by rat osteoblasts. Compared with control (A), treatment with forskolin for 15 h of inhibited Fn fibrillogenesis (B). The quantitative data are shown in C. Phase-contrast images are shown on the left. Bar, 10 μm. Data are presented as mean $\pm$ S.E. (n). $\star$, $p < 0.05$ compared with control.

tration of 1 μM, as well as PKC inhibitors H7 and Ro 318220, decreased the level of Fn protein. In addition, Gö 6976 (0.1 μM), which is an inhibitor of classic PKC isoforms (Gschwendt et al., 1996), also decreased the protein level of Fn (Fig. 4B), indicating that Fn fibrillogenesis is regulated by basal PKC activity. Gö 6976 also inhibited the increasing action of TPA on the level of Fn protein (Fig. 4B). We further examined the isoforms of PKC in osteoblasts. Figure 4C demonstrates the membrane translocation of PKCα and PKCβ after incubation with TPA for 30 min in a dose-dependent manner (Fig. 4C). Because all of the fibrillogenesis experiments were carried out for 15 h, we investigated the localization of PKCs at 15 h. As shown in Fig. 4D, the mem-

brane translocation of PKCα and PKCβ slightly increased after treatment with 0.01 μM TPA for 15 h. The novel forms of PKCδ and PKCϵ also exist in osteoblasts and translocated to membrane in response to TPA application (Fig. 4D). Incubation with a high concentration of TPA (1 μM) for 15 h showed an obvious down-regulation of these cytosolic PKC isoforms including PKCα, PKCβ, PKCδ, and PKCϵ (Fig. 4D). PKCγ and PKCθ were undetectable in the cytosolic fraction under a control situation. Therefore, the decrease of Fn assembly underneath the cells after prolonged incubation with higher concentrations of TPA is consistent with this result. On the other hand, forskolin (10 μM) increased the protein level of Fn despite its inhibition on extracellular Fn assembly (Fig. 5A). In contrast, PKA inhibitor H-8 decreased the protein level of Fn (Fig. 5A). CREB phosphorylation was enhanced by forskolin but not by TPA (Fig. 5B). These results suggest that forskolin may increase the synthesis of Fn but inhibit the extracellular assembly of the Fn network. To further confirm the increase of cytosolic Fn by forskolin, Fn immunocytochemistry was performed after permeabilization of cells with 0.5% Triton X-100 for 10 min. As shown in Fig. 6B, treatment with forskolin for 15 h caused an increase of Fn fluorescence intensity in the cytoplasm. When the cytosolic fraction was collected and used to examine the protein level of Fn, it was found that Fn increased in response to forskolin or TPA (Fig. 6C). These results suggest that the synthesis and assembly of Fn may be differentially regulated by the activation of PKA.

Regulation of Protein Kinases on the Matrix Assembly from Exogenously Applied Soluble Fn. To exclude the factor of endogenous Fn synthesis, we further examined the effects of TPA and forskolin on the assembly of exogenously applied soluble Fn by rat osteoblasts. Exogenous soluble human Fn (30 μg/ml) was bath-applied concomitantly with TPA or forskolin for 15 h. The immunocytochemistry of
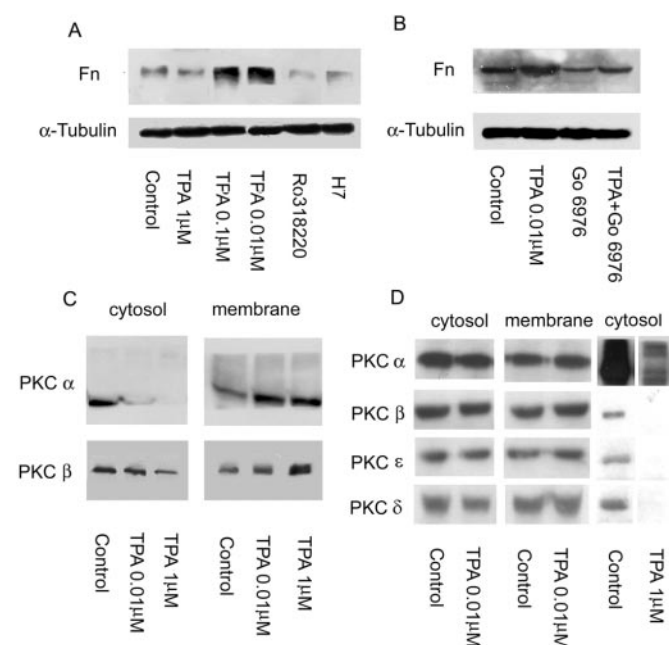

Fig. 4. Increase of Fn protein levels by PKC activation. A, Western blot showed that activation (TPA at 0.01 and 0.1 μM) and inhibition of PKC (Ro 318220 and H7) for 15 h increased and decreased Fn protein levels, respectively. B, classic type PKC inhibitor Gö 6976 also inhibited Fn protein levels in both resting and TPA-stimulated cells. C, treatment with TPA for 30 min showed an obvious translocation of cytosolic PKCα and PKCβ to cell membrane in a concentration-dependent manner. D, treatment with 0.01 μM TPA for 15 h caused a slight increase of membrane translocation of PKC isoforms, including PKCα, PKCβ, PKCϵ, and PKCδ. However, treatment with 1 μM TPA for 15 h exerted an obvious down-regulation of cytosolic PKCα, PKCβ, PKCϵ, and PKCδ.
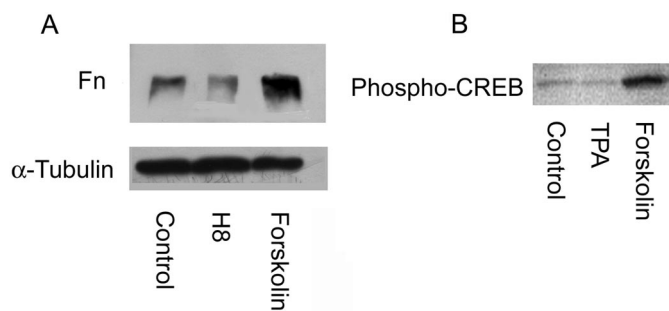

Fig. 5. Effects of forskolin on Fn protein levels and CREB activation. A, Western blot showed that treatment with forskolin or H-8 for 15 h, respectively increased and decreased Fn protein levels in cultured rat osteoblasts. Fn proteins include both cytosolic and extracellularly immobilized forms. B, treatment with forskolin but not TPA for 7 min increased the phosphorylation of CREB.
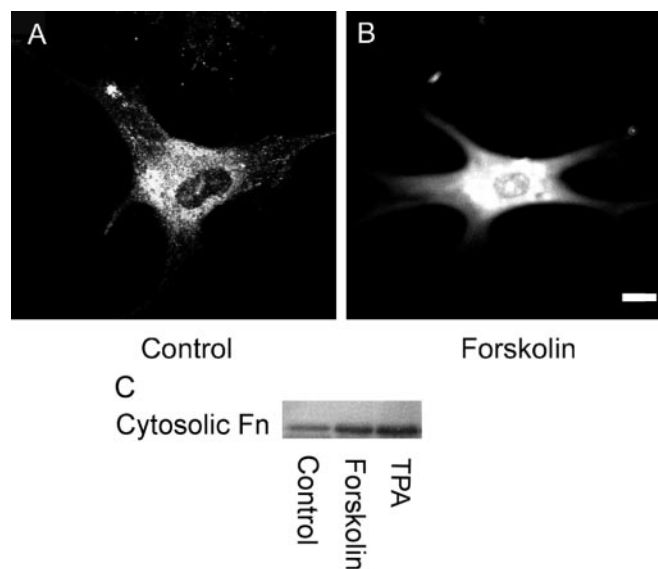

Fig. 6. Effect of forskolin on intracellular Fn. To observe intracellular Fn, the osteoblasts were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Compared with control (A), treatment with forskolin for 15 h significantly increased cytosolic Fn (B). The cells were treated with forskolin (10 μM) or TPA (0.01 μM) for 15 h and the cytosolic fraction was obtained by centrifugation. The Western blot from cytosolic fraction showed that treatment with forskolin increased Fn protein levels. Treatment of osteoblasts with TPA was used for comparison (C).

Fn was performed by using mouse antihuman Fn monoclonal antibody, which does not recognize endogenously released rat Fn. We thus are able to simply compare the effects of protein kinases on the extracellular assembly of Fn excluding Fn synthesis. As shown in Fig. 7A, TPA still exerted a stimulatory effect on the assembly of exogenous Fn underneath the cells (Fig. 7B), whereas forskolin inhibited Fn-like immunoreactivity (Fig. 7C). The mean fluorescence intensity was 32.8 ± 2.2, 56.5 ± 6.9, and 16.5 ± 3.1 ($n = 22$–31) for control, TPA-treated, and forskolin-treated cells, respectively (Fig. 7D). These results further confirm that extracellular Fn as-

sembly is potentiated by PKC and inhibited by PKA activation, respectively.

Effect of TPA and Forskolin on the Distribution and Synthesis of Integrins. The assembly of extracellular Fn matrix underneath the cells may be related to integrins (Wu et al., 1993; Dzamba et al., 1994). Integrins are a family of dimeric transmembrane receptors that contain α and β subunits. The different combinations of α and β chains form different receptors for various kinds of ECM molecules. $\alpha 5 \beta 1$ integrin is a specific receptor for Fn. We thus used immunocytochemistry to visualize the localization of $\alpha 5$ integrins in
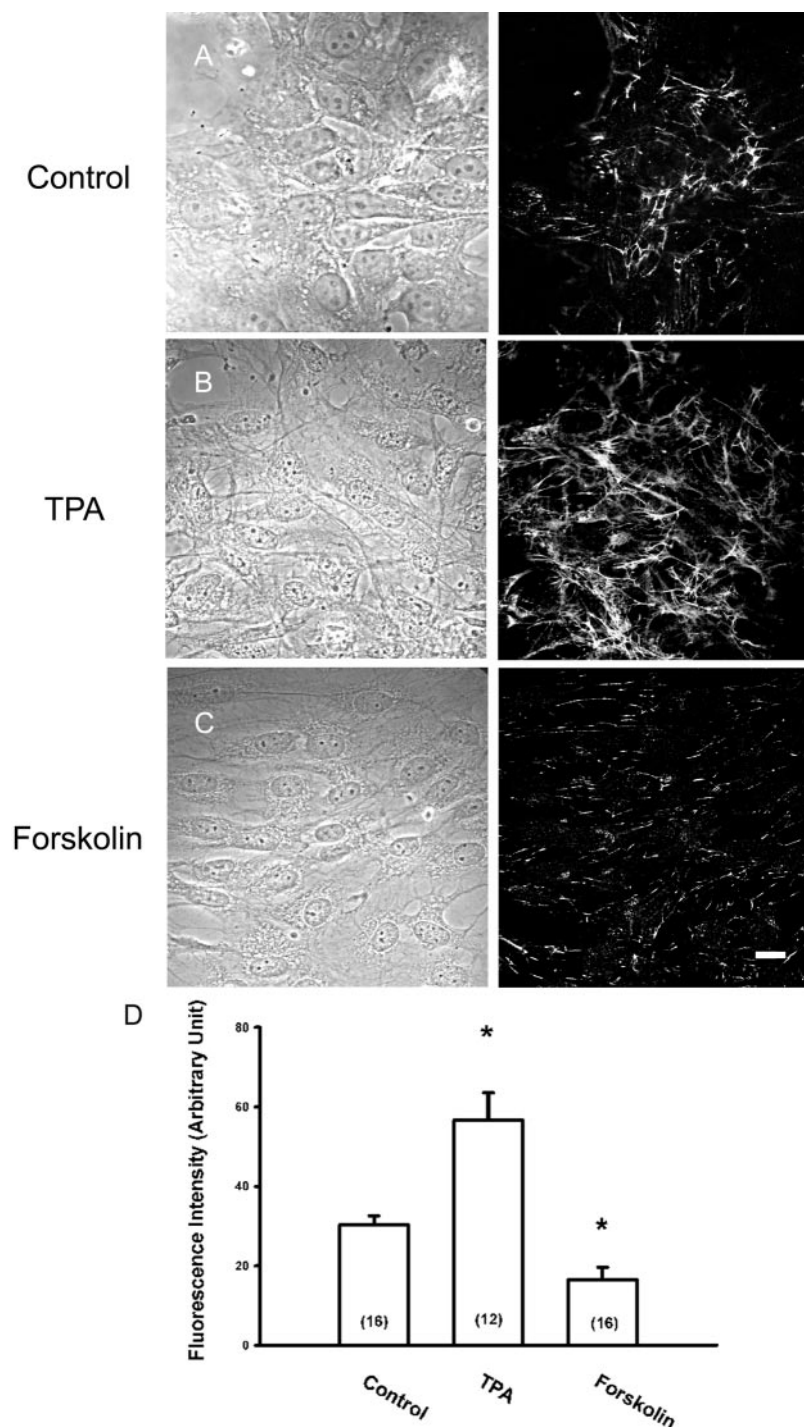

Fig. 7. Effects of TPA and forskolin on the assembly of exogenously applied soluble human Fn. Exogenous soluble human Fn (30 μg/ml) was bath-applied to osteoblast cultures for 15 h and the immunocytochemistry was performed using mouse anti-human Fn, which does not recognize rat Fn. Compared with control (A), TPA increased (B) and forskolin inhibited (C) the assembly of exogenous soluble Fn underneath the cells. Phase-contrast images are shown on the left. The quantitative data are shown in D. Bar, 10 μm. Data are presented as mean $\pm$ S.E. (n). $\star$, $p < 0.05$ compared with control.
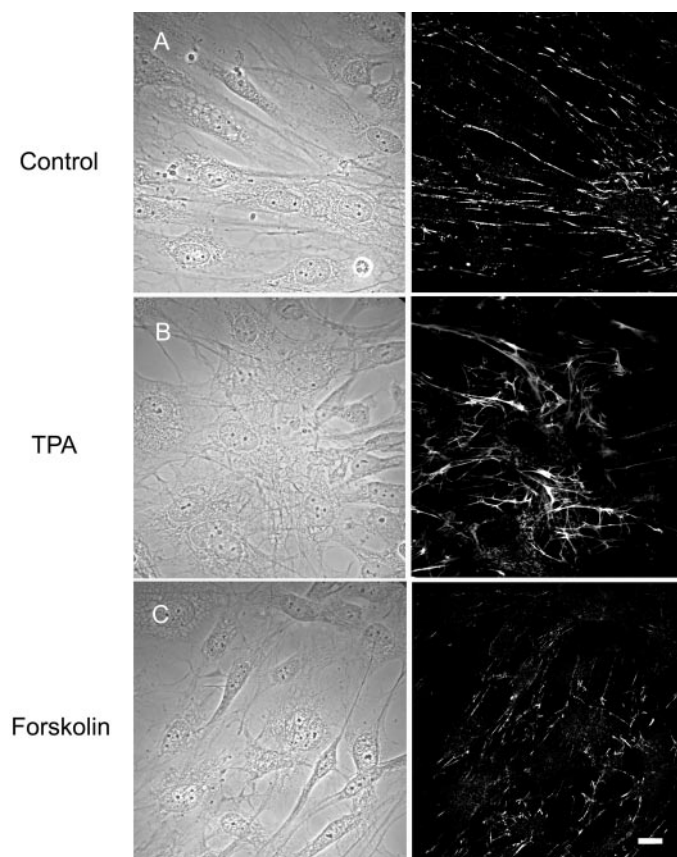
Fig. 8. Effects of TPA and forskolin on the clustering of $\alpha 5$ integrins. $\alpha 5$ integrin, which was shown by immunofluorescence, clustered underneath the cultured osteoblasts. Compared with control (A), treatment with TPA (B) or forskolin (C) for 15 h enhanced and inhibited the clustering of $\alpha 5$ integrins, respectively. Phase-contrast images are shown on the left. Bar, 10 μm.

cultured rat osteoblasts. The fluorescence was difficult to detect unless the integrin molecules aggregated to form clustering. The control osteoblasts showed the clustering of $\alpha 5$ integrins underneath the cell (Fig. 8A). Treatment with 0.01 μM TPA for 15 h greatly enhanced the fluorescence intensity of $\alpha 5$ integrins (Fig. 8B). By contrast, forskolin showed an inhibitory effect on the fluorescence intensity of $\alpha 5$ integrins (Fig. 8C). These results are consistent with the actions of TPA and forskolin on the extracellular assembly of Fn.

Furthermore, we used flow cytometry to investigate the effects of TPA and forskolin on the surface expression of $\alpha 5$ integrins in nonpermeabilized rat osteoblasts. As shown in Fig. 9, A and C, incubation with 0.01 μM TPA for 15 h enhanced the fluorescence of $\alpha 5$ integrins. In contrast, treatment with 10 μM forskolin inhibited significantly the fluorescence of $\alpha 5$ integrins (Fig. 9, B and C). The Western blot showed that the protein levels of $\alpha 5$ integrins were not affected by TPA and forskolin (Fig. 9D), indicating that TPA and forskolin may affect the cell surface expression of $\alpha 5$ integrins.

We also examined the effect of TPA and forskolin on $\beta 1$ integrins using flow cytometry. Similar to the effect on $\alpha 5$ integrins, treatment with 0.01 μM TPA and 10 μM forskolin for 15 h enhanced and inhibited, respectively, the fluorescence intensity of $\beta 1$ integrins (Fig. 10, A and B). The Western blot and RT-PCR showed that protein and mRNA levels of $\beta 1$ integrins were not affected by treatment with TPA or forskolin for 15 h (Fig. 10, C and D). Treatment of cells with TPA or forskolin for 10 or 30 min also did not affect the mRNA levels of $\beta 1$ integrins (data not shown). These results suggest that TPA and forskolin may also affect the cell surface expression of $\beta 1$ integrins.
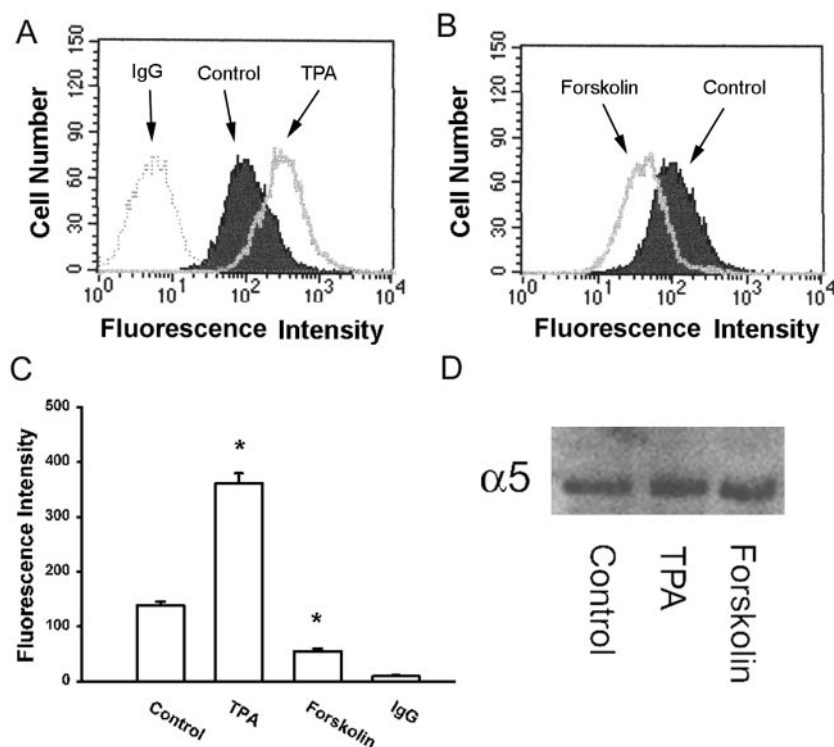

Fig. 9. Effect of TPA and forskolin on the cell surface expression of $\alpha 5$ integrins. Compared with control, treatment with TPA (A) or forskolin (B) for 15 h enhanced and inhibited, respectively, the fluorescence intensity of $\alpha 5$ integrins, using flow cytometric analysis. The quantitative data are shown in C. Protein levels of $\alpha 5$ integrins were not affected by either TPA or forskolin (D). Data are presented as mean $\pm$ S.E. ($n = 5$). $\star$, $p < 0.05$ compared with control.

Discussion

Interfering with interactions between Fn and integrin Fn receptors in immature fetal rat calvarial osteoblasts suppressed formation of mineralized nodules in vitro and delayed the expression of tissue-specific genes, including osteocalcin (Moursi et al., 1996, 1997). It also has been reported that osteoblasts become increasingly dependent on Fn for survival when they differentiate and form nodules (Globus et al., 1998). The present study has demonstrated the regulation of protein kinases on the synthesis and assembly of Fn in primary rat osteoblasts. PKC activation enhanced the synthesis of Fn assembly from both endogenously released and exogenously applied soluble Fn. In contrast, PKA activation inhibited the Fn assembly from both endogenously released and exogenously applied soluble Fn. The synthesis of Fn, however, is increased by forskolin. Our results have provided important information about the regulation of Fn dynamic changes by protein kinases in osteoblasts.

The physiological process of Fn matrix formation is complex. The soluble Fn dimmer is compact and maintained by intramolecular binding. The dimmer then binds to integrin heterodimers at the cell surface to immobilize Fn dimmers and induce conformational changes. The clustering of integrin and binding of additional Fn leads to formation and elongation of Fn fibrils, and then connects to the neighboring cells to form a dense network (Schwarzbauer and Sechler, 1999). The turnover of Fn concentration and affinity of Fn receptors, as well as the components of ECM molecules, may affect cell-Fn interactions. With regard to Fn fibrillogenesis in osteoblasts, immunocytochemistry study showed the assembly of endogenously released Fn. In this study, we found that PKC activation enhanced Fn fibrillogenesis. TPA is a direct stimulator of classic and novel types of PKCs. Acute treatment with TPA causes translocation of classic and novel

PKCs from the cytosol to the membrane, an event that is necessary for activation of at least some PKC members. However, prolonged treatment with high concentrations of TPA results in degradation and loss of expression of some TPA-responsive PKCs. PKCα and PKCδ are reported to be associated with focal adhesion (Jaken et al., 1989; Barry and Critchley, 1994). A role for PKC in regulating integrin-mediated cell spreading has been observed in other cell systems. For example, down-regulation of PKCα and PKCϵ in vascular smooth muscle cells blocked cell spreading on Fn (Haller et al., 1998). Overexpression of a dominant inhibitory mutant of the well-characterized myristoylated alanine-rich C kinase substrate, completely blocks cell spreading of fibroblasts on Fn (Myat et al., 1997). However, the fact that PKC activation increases Fn production has been previously noted in other cell lines, such as human pulmonary fibroblasts, cultured human retinal pigment epithelial cells, and human vascular smooth muscle cells (Osusky et al., 1994; Lee et al., 1996; Kaiura et al., 1999). Activation of PKC results in increased amounts of ^{125}I-labeled Fn binding to the cell surface of fibroblast (Somers and Mosher, 1993). In rat cultured mesangial cells, hyperglycemia activated PKCβ, which then stimulated production of Fn (Koya et al., 1997). In the present study, chronic treatment with low concentrations of the PKC activator TPA enhances the assembly of the endogenously released Fn. Furthermore, TPA increased the cytosolic protein levels of Fn from Western blotting analysis. TPA can induce the membrane translocation of PKCα, PKCβ, PKCδ, and PKCϵ in osteoblasts, indicating that both classic and novel types of PKCs are involved in the effect of TPA. TPA at higher concentrations caused a cytosolic down-regulation of these PKC isoforms and inhibited Fn fibrillogenesis. Furthermore, PKC inhibitors such as H7, Ro 318220, and Gö 6976 also exerted a marked inhibition on Fn fibrillogenesis.
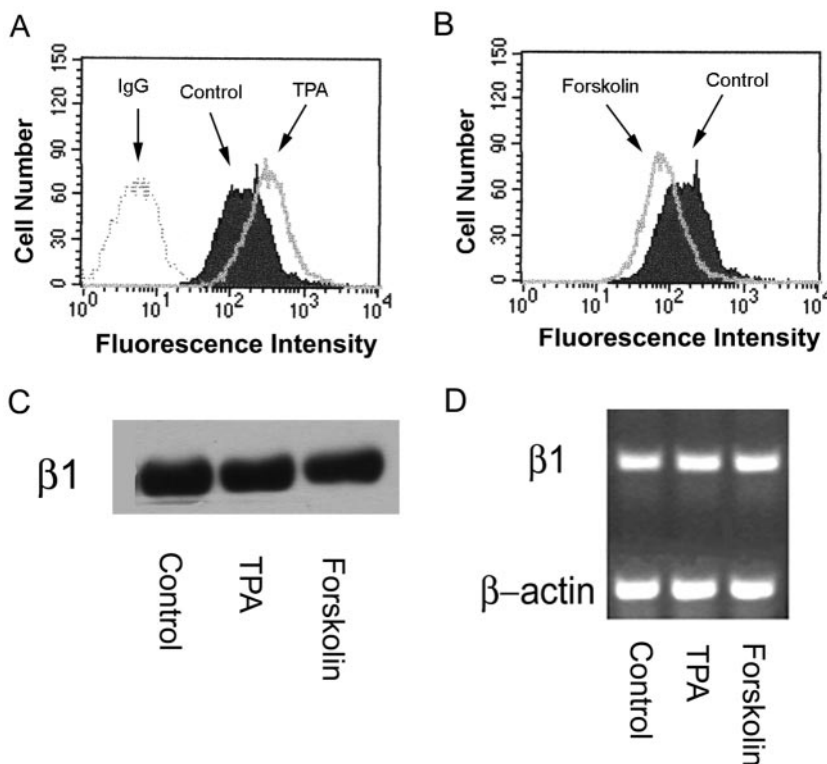

Fig. 10. Effect of TPA and forskolin on the cell surface expression of β1 integrins. Compared with control, treatment with TPA (A) or forskolin (B) for 15 h enhanced and inhibited, respectively, the fluorescence intensity of β1 integrins, using flow cytometric analysis. Protein (C) and mRNA levels (D) of β1 integrins were not affected by TPA and forskolin.

Therefore, the current study suggests that PKC regulates Fn fibrillogenesis in multiple ways.

Forskolin, an adenylate cyclase activator, showed a marked inhibition on Fn fibrillogenesis. This may result from the decrease of Fn synthesis or extracellular Fn assembly and/or the increase of disassembly of Fn matrix. Intracellular staining of Fn and Western blot showed that forskolin increased cytosolic protein levels of Fn. Therefore, inhibitory action on Fn matrix formation by forskolin may be related to the decrease of assembly. Our findings in flow cytometry and immunocytochemistry of $\alpha5$ integrins are correlated to this decrease of assembly. In addition, experiments with exogenously applied soluble Fn also confirmed that forskolin inhibited Fn assembly. However, forskolin caused a disassembly of previously formed Fn fibrils. Therefore, Fn fibrillogenesis is enhanced and inhibited by PKC and PKA activation, respectively. Although forskolin inhibited extracellular Fn fibrillogenesis, it increased Fn synthesis, as demonstrated by immunocytochemistry and Western blotting analysis of intracellular Fn. It has been shown that cAMP inhibits Fn gene expression in granulosa cells of human cytotrophoblasts (Ulloa-Aguirre et al., 1987; Bernath et al., 1990). However, transcription of the Fn gene is stimulated by cAMP in other cells such as HT1080 and JEG-3 (Dean et al., 1988, 1989). We found here that intracellular Fn levels are enhanced by PKA activation in primary osteoblast cultures. CREB phosphorylation is probably involved in the action of PKA. Intracellular Fn synthesis and extracellular Fn assembly may thus be differentially regulated by PKA activation. In contrast, PKC regulates Fn synthesis and assembly in parallel. Our data point to the existence of multiple regulatory circuits involved in the control of Fn expression in osteoblasts. A number of studies have shown that tissue repair in both normal and pathological conditions is linked to an increased activity of neutral proteases. These enzymes are involved in the regulation of proteolysis of ECM and have an important role in tissue remodeling and cell-matrix and cell-cell interactions (Bond and Butler, 1987; Saksela and Rifkin, 1988). Leupeptin and aprotinin partially antagonize the action of forskolin on the disassembly of previously formed Fn fibril, suggesting that matrix protease activity increased in response to PKA activation (data not shown).

Direct osteoblast interactions with the extracellular matrix are mediated by a select group of integrin receptors that includes $\alpha5\beta1$, $\alpha3\beta1$, $\alpha v\beta3$, and $\alpha4\beta1$ (Clover et al., 1992; Grzesik and Robey, 1994). $\alpha5\beta1$ integrin, a specific Fn receptor, mediates critical interactions between osteoblasts and Fn required for both bone morphogenesis and osteoblast differentiation (Moursi et al., 1997). Perturbing cell-Fn interactions suppress osteogenic differentiation of MG-63 osteosarcoma cells, whereas amplification of $\alpha5\beta1$ promotes differentiation of these cells (Dedhar et al., 1987; Dedhar, 1989). Using flow cytometric analysis, we found here that the fluorescence intensity of $\alpha5$ and $\beta1$ integrins was increased and decreased by TPA and forskolin, respectively. However, Western blotting analysis showed that TPA and forskolin do not affect the protein levels of either integrin. In addition, $\beta1$ mRNA level is also not altered by either drug. Therefore, TPA and forskolin may influence the surface expression of $\alpha5$ and $\beta1$ integrins. They may also affect the clustering of integrins, which is consistent with the result derived from immunocytochemistry. TPA increased and forskolin inhib-

ited the clustering of $\alpha5$ integrins, which is correlated to the drug effect on Fn fibrillogenesis.

In conclusion, our results demonstrated that both PKC and PKA are involved in the regulation of Fn fibrillogenesis. PKC activators enhance the Fn fibrillogenesis in multiple ways; i.e., TPA is able to stimulate the synthesis of Fn, assembly of endogenously released and exogenously applied soluble Fn, and clustering of $\alpha5$ and $\beta1$ integrins. Although Fn synthesis is increased by forskolin, activation of PKA inhibited Fn matrix formation via the inhibitory effect on Fn assembly, clustering of $\alpha5$ and $\beta1$ integrins, and enhancement of Fn disassembly. Our results show that PKC and PKA may be involved in Fn dynamic changes and bone tissue remodeling and may provide some information for the development of drugs to treat osteoporosis.

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