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REGULAR ARTICLE

Maria Giovanna Sabbieti · Luigi Marchetti · Maria Gabriella Gabrielli · Maura Menghi · Stefano Materazzi · Giovanna Menghi · Lawrence G. Raisz · Marja M. Hurley

Prostaglandins differently regulate FGF-2 and FGF receptor expression and induce nuclear translocation in osteoblasts via MAPK kinase

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Abstract We have previously reported that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its selective agonist fluprostenol increase basic fibroblast growth factor (FGF-2) mRNA and protein production in osteoblastic Py1a cells. The present report extends our previous studies by showing that Py1a cells express FGF receptor-2 (FGFR2) and that treatment with $PGF_{2\alpha}$ or fluprostenol decreases FGFR2 mRNA. We have used confocal and electron microscopy to show that, under $PGF_{2\alpha}$ stimulation, FGF-2 and FGFR2 proteins accumulate near the nuclear envelope and colocalize in the nucleus of Py1a cells. Pre-treatment with cycloheximide blocks nuclear labelling for FGF-2 in response to $PGF_{2\alpha}$. Treatment with SU5402 does not block prostaglandin-mediated nuclear internalization of FGF-2 or FGFR2. Various effectors have been used to investigate the signal transduction pathway. In particular, pre-treatment with phorbol 12-myristate 13-acetate (PMA) prevents the nuclear accumulation of FGF-2 and FGFR2 in response to $PGF_{2\alpha}$. Similar results are obtained by pre-treatment with the protein kinase C (PKC) inhibitor H-7. In addition, cells treated with $PGF_{2\alpha}$ exhibit increased nuclear labelling for the mitogen-activated protein kinase (MAPK), p44/ERK2. Pre-treatment with PMA blocks prostaglandin-induced ERK2 nuclear labelling, as confirmed by Western blot analysis. We conclude that $PGF_{2\alpha}$ stimulates nuclear translocation of FGF-2 and FGFR2 by a PKC-dependent pathway; we also suggest an involvement of MAPK/ERK2 in this process.

Keywords Fibroblast growth factor-2/FGF receptor-2 · Mitogen-activated protein kinases/ERK2 · Prostaglandin $F_{2\alpha}$ · Confocal laser scanning microscopy · Transmission electron microscopy · Rat osteoblasts

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M. G. Sabbieti (✉) · L. Marchetti · M. G. Gabrielli · M. Menghi · G. Menghi
Department of Comparative Morphology and Biochemistry,
Via Gentile III da Varano,
62032 Camerino (MC), Italy
e-mail: giovanna.sabbieti@unicam.it
Tel.: +39-737-402713
Fax: +39-737-402708

S. Materazzi
Department of Chemistry, University "La Sapienza",
Piazzale Aldo Moro,
10 Rome, Italy

L. G. Raisz · M. M. Hurley
Division of Endocrinology and Metabolism, University of Connecticut School of Medicine,
263 Farmington Avenue,
Farmington, CT 06030-1850, USA

Introduction

Prostaglandins (PGs) have both stimulatory and inhibitory effects on bone formation in cell and organ culture (Raisz and Koolemans-Beynen 1974; Raisz and Fall 1990; Raisz et al. 1993). Data concerning structure-activity relations and signal transduction mechanism for the anabolic effects of PGs are conflicting. The stimulation of cyclic adenosine monophosphate (cAMP) production is probably important with respect to the ability of these compounds to increase bone formation (Nagata et al. 1994; Scutt et al. 1995; Woodiel et al. 1996), whereas the activation of protein kinase C (PKC) has been implicated in their mitogenic effect on osteoblasts and their inhibitory effect on collagen synthesis (Quarles et al. 1993; Fall et al. 1994).

The effects of PGs on bone are similar to those of basic fibroblast growth factor-2 (FGF-2). FGF-2 is a member of a protein family of which there are 23 members; these proteins function in an autocrine or paracrine manner to modulate the proliferation and differentiation of cell types of epithelial, neuroectodermal and mesenchymal origin (Hurley et al. 2002). FGF-2 is produced by cells of the osteoblast lineage and can act as a local regulator of bone remodelling; like PGs, FGF-2 stimulates bone cell replication (Hurley et al. 2002; Globus et al. 1988, 1989),

increases bone resorption (Shen et al. 1989), reduces alkaline phosphatase activity (Rodan et al. 1989) and inhibits type I collagen synthesis in osteoblasts (McCarthy et al. 1989; Hurley et al. 1993). Intermittent FGF-2 treatment stimulates bone formation in vitro (Canalis et al. 1988) and in vivo (Norrdin et al. 1990; Aspenberg et al. 1991; Nagata et al. 1994). We have previously reported that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its selective agonist fluprostenol (Flup) regulate the expression of FGF-2 in immortalized rat osteoblastic Py1a cells and that Flup is able to induce nuclear translocation of FGF-2 (Sabbieti et al. 1999). The signalling pathway for FGF-2 is complex and involves the binding and activation of one or more high affinity tyrosine kinase receptors at the cell surface resulting in ligand/receptor activation of downstream genes.

Some authors have demonstrated a role for extracellular signal-regulated kinase (ERK) in the biological responses to both PGs and FGF-2 in osteoblasts. ERK is a member of the family of mitogen-activated protein kinases (MAPKs) involved in the regulation of cell growth, differentiation and apoptosis (Nishida and Gotoh 1993; Matsuda et al. 1998). Activation of the ERK signalling pathway has recently been shown to be involved in the induction of cyclo-oxygenase (Cox-2), the rate-limiting enzyme in the production of PGs (Wadhwa et al. 2002), and in anabolic responses to growth factors in osteoblast-like cells (Chaudhary and Avioli 1998). Since earlier studies have demonstrated that acidic FGF and phorbol esters preferentially activate ERKs in COS cells (Klingenberg et al. 2000), we have examined whether the activation of ERK is important in the PG regulation of the expression of FGF-2 and FGFR2.

In addition to the activation of intracellular signalling proteins, recent studies have shown that certain FGFs lacking nuclear localization sequences can be transported to the nucleus following internalization from the cell surface (Mehta et al. 1998). In this report, we have extended our studies to examine whether PGs also regulate FGFR mRNA expression and the signalling pathway involved. Confocal laser scanning and electron microscopy have been utilized to demonstrate that PG induces the nuclear translocation of FGF-2/FGFR2 via the MAPK, p44/ERK2 signalling pathway in rat osteoblastic Py1a cells.

Materials and methods

Cell cultures

Immortalized rat osteoblastic Py1a cells were plated at 5,000 cells/cm^2 in 6-well culture dishes in F-12 culture medium with 5% non-heat inactivated fetal calf serum (FCS; Invitrogen, Milan, Italy), penicillin and streptomycin (Sigma-Aldrich, Milan, Italy). Cells were grown for 6 days to approximately 80% confluence. These cells were then pre-cultured for another 24 h in serum-free F-12 with antibiotics and 1 mg/ml BSA before treatment with $PGF_{2\alpha}$ (10^{-5} M; Sigma-Aldrich) and Flup (10^{-8} M; Biomol Research Laboratories, Plymouth). Control cultures were treated with appropriate vehicles (Sabbieti et al. 1999).

mRNA levels

Total RNA was extracted from cells by the method of Chomczynski and Sacchi (1987). Cells were scraped into 4 M guanidinium thiocyanate and extracted with phenol/chloroform isoamyl alcohol (24:1) and total RNA was precipitated with isopropanol. For Northern analysis, 20 μg total RNA was denaturated and fractionated on a 1% agarose/2.2 M formaldehyde gel, transferred to nylon membrane by positive pressure, and fixed to the filter by UV irradiation (Sambrook et al. 1989; Towbin et al. 1979). After 4 h pre-hybridization, filters were hybridized overnight with a random primer [^{32}P]dCTP-labelled cDNA probe for the mRNA of interest (Mansukani et al. 1990). Filters were washed in 1× SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0), 1% SDS solution at room temperature and then three times at 65°C in 0.1% SDS and finally exposed to XAR-5 film at −70°C. Signals were quantified by densitometry and normalized to the corresponding values for glyceraldehyde 3-phosphate dehydrogenase (G3PDH).

Cell culture for Western blotting

Py1a cells were plated at 5,000 cells/cm^2 in 100-mm culture dishes, in Ham's F-12 culture medium with 5% non-heat inactivated FCS, penicillin and streptomycin. Cells were grown for 5 days to confluence. The cells were subsequently serum-deprived 24 h before addition of $PGF_{2\alpha}$ (10^{-5} M).

Protein levels

Proteins were extracted with Cytobuster Protein extraction reagent (Calbiochem-Inalco, Milan, Italy), resolved by SDS-polyacrylamide gel electrophoresis (12%) and transferred onto polyvinylidene fluoride (Hybond-P) membrane (Amersham Biosciences). The subsequent steps were performed with the ECL Advance Western Blotting Detection Kit (Amersham Biosciences); in brief, membranes were blocked with Advance Blocking agent in PBS-T (phosphate-buffered saline containing 0.1% Tween 20) for 1 h at room temperature. The membranes were then incubated with a polyclonal rabbit anti-ERK2 antibody (Calbiochem-Inalco) diluted 1:500 in blocking solution for 1 h at room temperature. After being washed with PBS-T, the blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Amersham Biosciences) diluted 1:100,000 in blocking solution for 1 h at room temperature. After further washes with PBS-T, immunoreactive bands were visualized by using luminol

reagents and Hyperfilm-ECL film (Amersham Biosciences) according to the manufacturer's instructions.

Confocal laser scanning microscopy

FGF-2 and FGFR2 protein immunodetection

Py1a cells were plated at 3,500 cells/cm^2 in 6-well culture dishes containing coverslips (previously cleaned and sterilized) in F-12 culture medium with 5% FCS (Sabbieti et al. 2000). Cells were grown for 4 days to approximately 80% confluence and then pre-cultured for another 24 h in serum-free F-12 with antibiotics and 1 mg/ml BSA before treatment with $PGF_{2\alpha}$ (10^{-5} M) and Flup (10^{-8} M). Control cultures were treated with appropriate vehicles. After stimulation, cells were briefly rinsed with PBS 0.1 M, pH 7.4 and fixed in 4% paraformaldehyde (PFA) diluted in PBS for 25 min at room temperature. Cells were washed three times with PBS, permeabilized with 0.3% Triton X-100 for 30 min and incubated with 0.5% BSA diluted in PBS for 20 min at room temperature. Cells were incubated with a 1:100 dilution of a polyclonal rabbit anti-FGF-2 antibody (Sigma-Aldrich) in PBS for 2 h at room temperature. After being rinsed, cells were incubated with a 1:75 dilution of goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC; Sigma-Aldrich) in PBS for 90 min at room temperature. To detect FGFR2, fixed and permeabilized cells were incubated with a 1:14 dilution of a polyclonal goat anti-C-term-FGFR2 antibody (Bek C-17; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) in PBS for 2 h at room temperature. After being washed, cells were incubated with a 1:150 dilution of Alexa Fluor 594 donkey anti-goat IgG conjugate (Molecular Probes, USA) diluted in PBS for 90 min at room temperature. Control experiments were performed by using a non-immune goat IgG, by complexing the primary antibody with a relative blocking peptide or by omitting the primary antibody. After a washing step, coverslips were mounted on slides with PBS/glycerol (1:1).

Double-labelling for FGF-2 and FGFR2

Fixed and permeabilized cells were incubated sequentially with anti-FGF-2 antibody, anti-FGFR2 antibody, Alexa Fluor 594 donkey anti-goat conjugate and goat anti-rabbit IgG FITC conjugate to prevent cross-reactivity of the secondary antibodies. Control experiments for double-labelling included additional tests; in order to test the cross-reactivity of the secondary antibodies, incubations with only the Alexa Fluor 594 donkey anti-goat IgG conjugate and the goat anti-rabbit IgG-FITC were performed.

ERK2 immunodetection

After fixation and permeabilization as above, cells were incubated with 1:60 dilution of a polyclonal rabbit anti-ERK2 (Calbiochem-Inalco) in PBS for 2 h at room temperature. After being washed, cells were incubated in a 1:75 dilution of goat anti-rabbit IgG conjugate with FITC in PBS for 90 min at room temperature. Control experiments were performed by using a non-immune rabbit IgG and by omitting the primary antibody. After a wash step, coverslips were mounted on slides with PBS/glycerol (1:1).

Confocal analysis

FGF-2-binding sites were visualized by means of a Nikon Diaphot-TMD-EF inverted microscope with a 60× oil immersion lens with a numerical aperture 1.4 Plan Apo objective. The microscope was attached to a Bio-Rad MRC 600 confocal laser imaging system (Bio-Rad, Hertfordshire, UK) equipped with a krypton/argon laser. Black level, gain and laser intensity, Kalman averaging, excitation intensity, pinhole aperture and Z-series analysis of cells were carried out as previously detailed (Sabbieti et al. 2000). Sections were examined and original images were stored as a PIC format file and then printed with an Epson Stylus Photo 890 on Epson glossy photo paper.

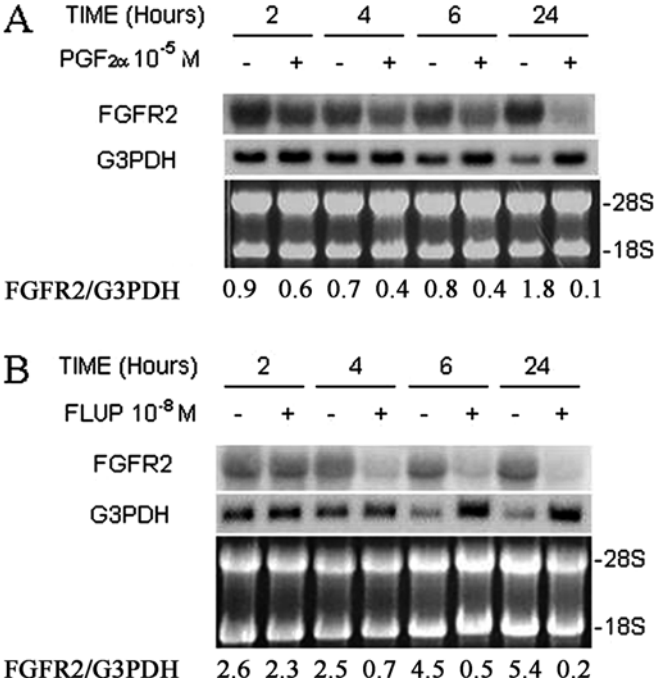

Fig. 1 Time-course effect of $PGF_{2\alpha}$ (**a**) and Flup (**b**) on FGFR2 mRNA expression in Py1a cells. Cells were treated with effectors for the indicated times. Total RNA was extracted from cells and a sample of 20 μg was utilized for Northern analysis. Filters were probed for FGFR2 and bands were quantified by densitometry and normalized to G3PDH

Transmission electron microscopy

Cell cultures for immunoelectron microscopy

Py1a cells were plated at 3,500 cells/cm^2 on 100-mm culture dishes and grown for 6 days in F-12 medium with 5% FCS, penicillin and streptomycin. At confluence, cells were pre-cultured in serum-free F-12 containing antibiotics and 1 mg/ml BSA and then treated with vehicle or $PGF_{2\alpha}$ (10^{-5} M) for 6 h and 24 h. After two rinses in F-12 medium and one rapid wash in 0.1 M cacodylate buffer, pH 7.4, cells were fixed on plates with 4% PFA and 0.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 3 h at 4°C. The cells were rinsed several times for a total of 30 min in 0.1 M PBS, pH 7.4, containing 0.1% BSA and 7% sucrose, at 4°C and subsequently scraped off the plates and collected in Falcon tubes. The centrifuged cells (1,300 rpm for 4 min) were placed on 2.6% agarose and, after centrifugation, cells pre-embedded in agarose were dehydrated in methanol from 50% up to 90% and embedded in Unicryl resin (British Bio Cell International, Cardiff, UK) for 72 h at 4°C under UV lamp. Ultrathin sections (about 60 nm in thickness) of the plastic-embedded cells were cut by means of an LKB Ultrotome V and collected on uncoated 400-mesh nickel grids.

Immunogold labelling for FGF-2 and FGFR2 and transmission electron-microscopy analysis

Floating grids were rehydrated with 0.05 M TRIS-buffered saline (TBS), pH 7.6, and pre-incubated with 1% BSA in 0.05 M TBS, pH 7.6, for 30 min at room temperature. Grids were then incubated with polyclonal rabbit anti-FGF-2 antibody diluted 1:600 in 1% BSA in 0.05 M TBS, pH 7.6, for 3 h at 4°C in a humid chamber or with polyclonal goat anti-C-term-FGFR2 antibody diluted 1:10 in 1% BSA in 0.05 M TBS, pH 7.6, overnight at 4°C in a humid chamber. After being rinsed in 0.05 M TBS, pH 7.6, and pre-incubation with 1% BSA in 0.05 M TBS, pH 7.6, for 10 min, grids were incubated with Auroprobe EM goat anti-rabbit G-10 (10-nm gold labelled IgG; Amersham Life Science, Buckinghamshire, England) diluted 1:15 in 0.05 M TBS, pH 7.6, containing 0.05% Tween 20 or with rabbit anti-goat IgG conjugated to 10-nm gold diluted 1:15 in 0.05 M TBS, pH 7.6, containing 0.05%

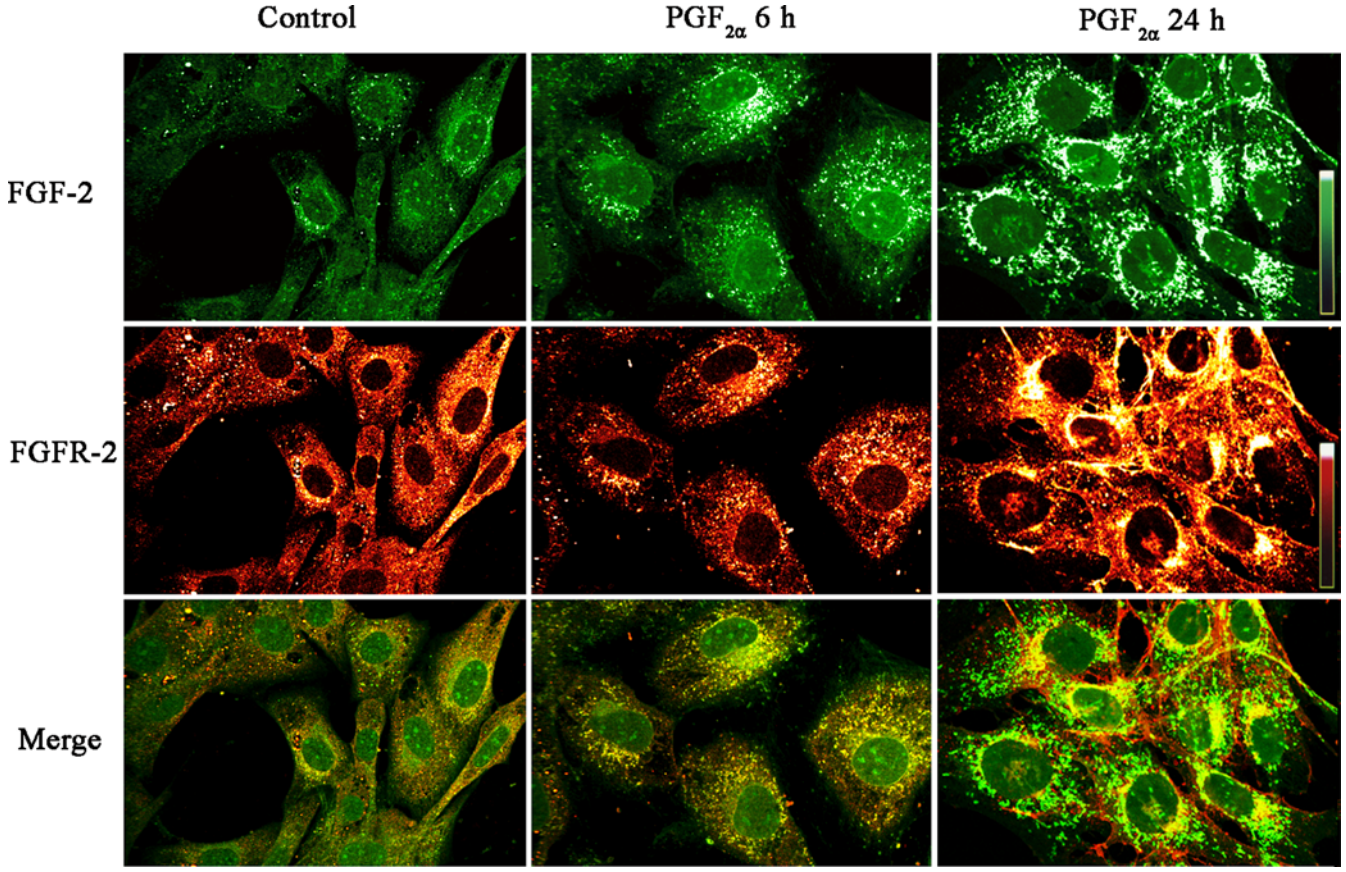

Fig. 2 Untreated Py1a osteoblasts (*left column*) or Py1a osteoblasts treated with $PGF_{2\alpha}$ for 6 h (*middle column*) and 24 h (*right column*). Optical sections obtained on a Bio-Rad MRC-600 confocal laser scanning microscope (CLSM). Micrographs showing cells double-stained with FGF-2 (*green pseudo colour*) and FGFR2 (*red pseudo colour*) in the same area. Colocalization of the two signals corresponding to FGF-2 (*green* FITC staining) and FGFR2 (*red* Alexa Fluor 594 staining), assessed by confocal analysis of single optical sections, is shown as a *yellow pseudo colour* in the composite merged images. The intensity-coded scales, with *white* being the most intense, are shown *right*. Note that the basal labelling for FGF-2 and FGFR2 changes after treatment with $PGF_{2\alpha}$ for 6 h and 24 h when both proteins can also be detected in some cells. ×400

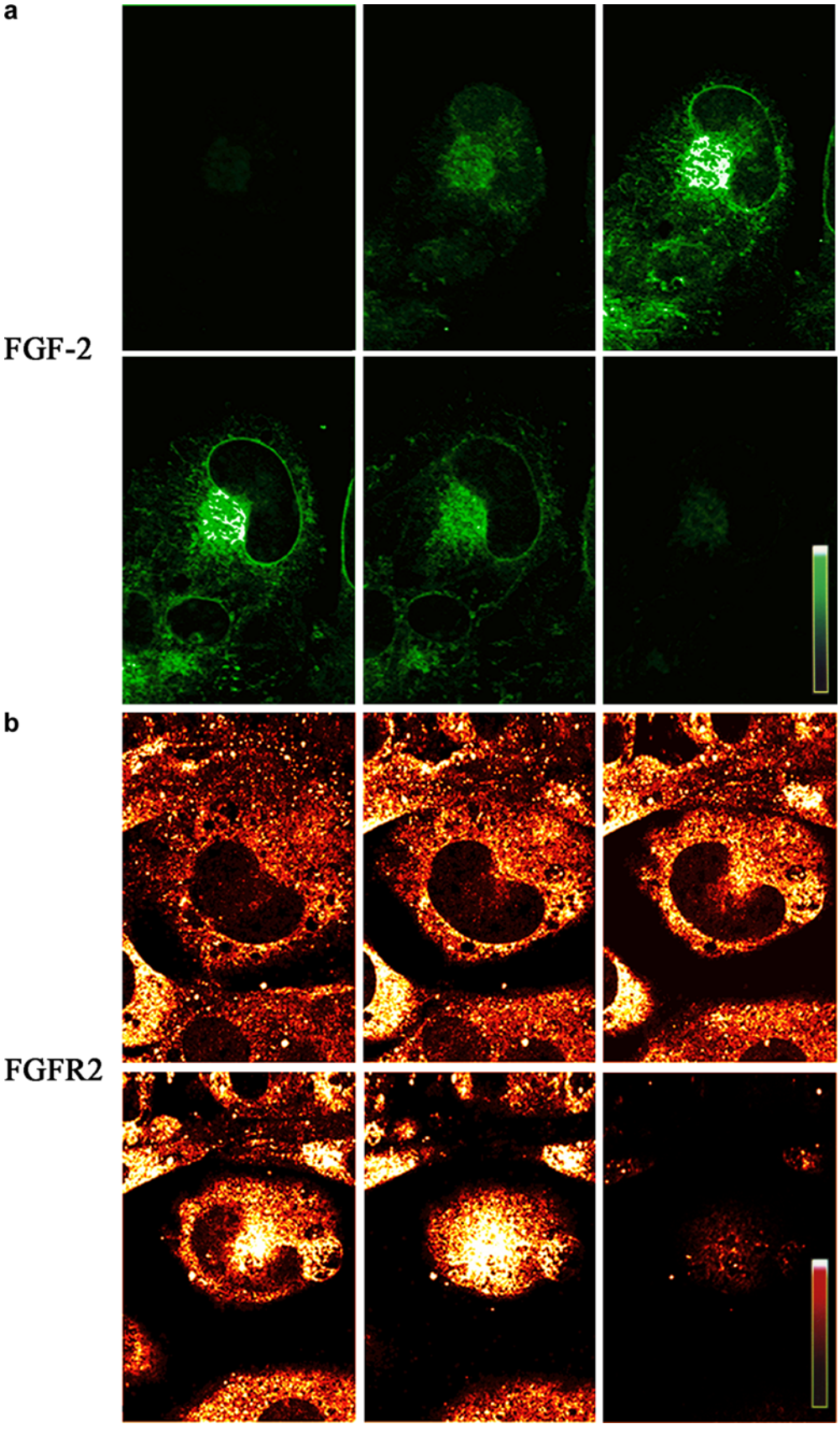

Fig. 3 Py1a cells treated for 24 h with $PGF_{2\alpha}$. CLSM-scanned optical sections obtained from the base (*top left*) to the apex (*bottom right*) of representative cells labelled for FGF-2 (**a**) and FGFR2 (**b**). The nuclear translocation of both proteins seems to involve a restricted region of the nuclear envelope. ×750

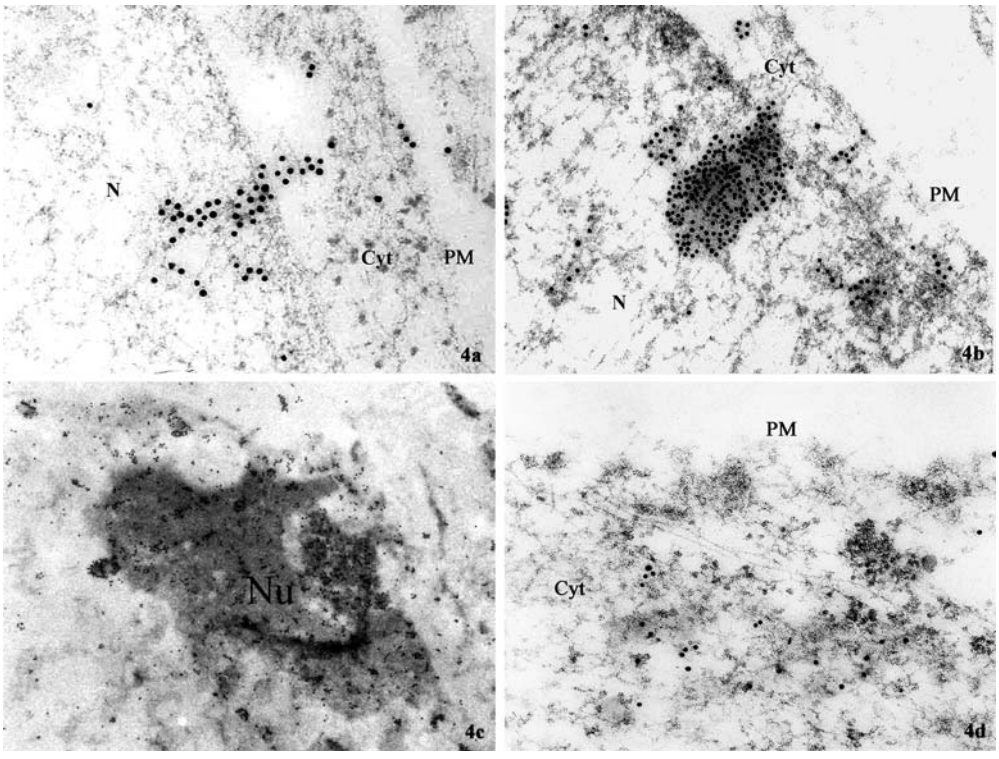

Fig. 4 Electron-microscopic immunolabelling for FGF-2 (**a**, **c**) or FGFR2 (**b**, **d**) in Py1a osteoblasts treated for 24 h with $PGF_{2\alpha}$ (**a–c**). The immunoreactivity (IR) with rabbit anti-FGF-2 or with goat anti-C-term-FGFR2 were detected with 10-nm colloidal gold particles conjugated to goat anti-rabbit IgG or to rabbit anti-goat IgG, respectively. A patchy distribution of gold particles reflecting FGF-2 (**a**) and FGFR2 (**b**) nuclear entry was also located at nucleolus level (**c**). Untreated cells showed a basal cytoplasm labelling for FGFR2 (**d**). FGF-2 IR showed a similar distribution in untreated cells (*PM* plasma membrane, *Cyt* cytoplasm, *N* nucleus, *Nu* nucleolus). ×80,250 (**a**), ×67,800 (**b**), ×15,300 (**c**), ×47,000 (**d**)

Tween 20 and 5% fetal bovine serum for 90 min at room temperature in a humid chamber. Sections were then washed several times in 0.05 M TBS, pH 7.6, and distilled water. Control experiments were carried out by omitting the primary antibody or by using a non-immune rabbit IgG. Sections were finally counterstained with uranyl acetate (5 min) and lead citrate (2 min) at room temperature. All specimens were analysed by means of a Philips EM 201C electron microscope at an accelerating voltage of 60 kV.

Results

Expression of FGFR2 mRNA in osteoblastic Py1a cells

Time-course studies of the effects of PGs on FGFR2 mRNA expression in osteoblastic Py1a cells were determined by Northern analysis. Representative experiments are shown in Fig. 1a, b). In the absence of serum, Py1a cells expressed a 4-kb FGFR2 mRNA transcript. $PGF_{2\alpha}$ (10^{-5} M) and Flup (10^{-8} M) caused a reduction in FGFR2 mRNA levels at 4 h and both effectors caused a maximal down-regulation at 24 h.

Immunodetection of FGF-2 and FGFR2 proteins

Double-labelling of Py1a cells (Fig. 2, left column) that had been serum-deprived for 24 h showed a prominent basal cytoplasmic staining for both FGF-2 and FGFR2. Treatment with $PGF_{2\alpha}$ (10^{-5} M) or Flup (10^{-8} M) for 6 h

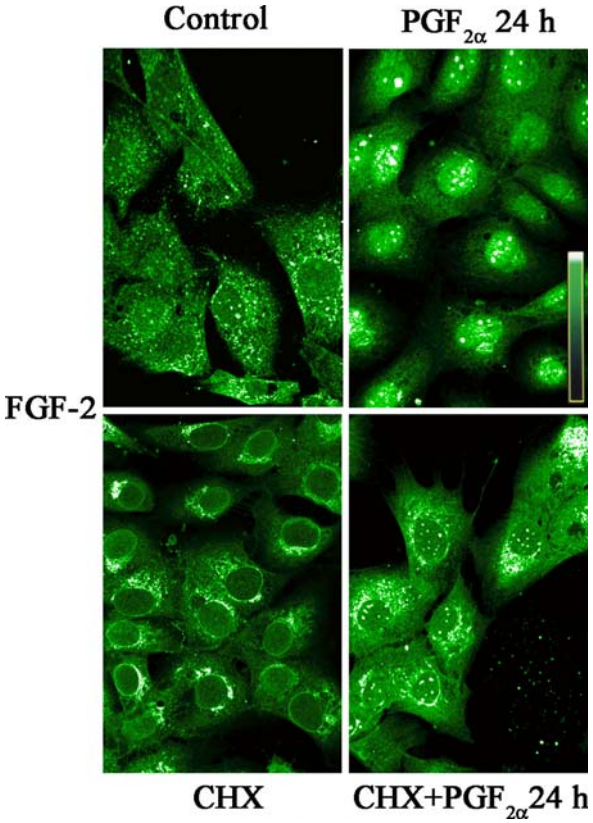

Fig. 5 Control Py1a cells or Py1a cells treated with $PGF_{2\alpha}$ for 24 h and labelled for FGF-2. Optical sections illustrating the effects of $PGF_{2\alpha}$ on FGF-2 nuclear accumulation in the absence or in the presence of CHX. Cells that had been serum-deprived for 24 h were pre-treated with CHX for 1 h; $PGF_{2\alpha}$ was then added for an additional 24 h. Note that CHX was able to block FGF-2 nuclear translocation and, in particular, nucleolar indicative binding in cells treated for 24 h with $PGF_{2\alpha}$. ×450

(Fig. 2, middle column) resulted in the perinuclear accumulation of FGF-2 and FGFR2. After 24 h of treatment, cells showed nuclear internalization of both proteins inside the nucleus. The initial nuclear translocation of FGF-2 (green) and FGFR2 (red) is shown in Fig. 2 (right column) and in detail in the higher magnification of a single cell (Fig. 3a, b) in which optical sectioning indicates that both proteins are translocated in a similar manner through a limited region of the nuclear envelope.

These data were also supported by immunogold electron microscopy. After 24 h of treatment with $PGF_{2\alpha}$, cells showed clusters of gold particles indicating labelling for FGF-2 (Fig. 4a) and FGFR2 (Fig. 4b). FGF-2 labelling was also documented at the nucleolar level (Fig. 4c). In

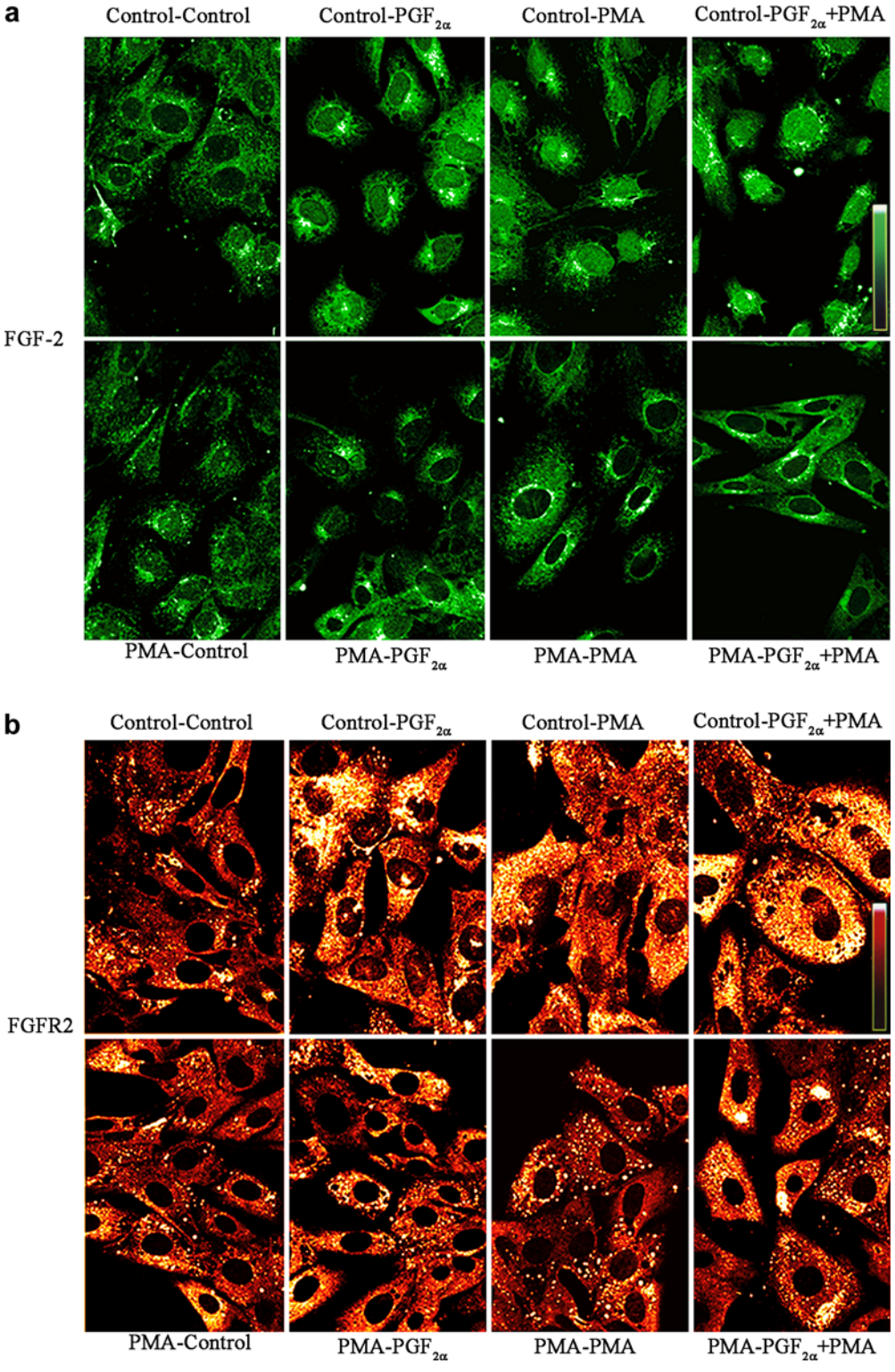

Fig. 6 Optical sections. Labelling for FGF-2 (**a**) and FGFR2 (**b**). Effect of down-regulation of the PKC pathway with 24 h PMA pre-treatment on $PGF_{2\alpha}$ and PMA-mediated FGF-2 and FGFR2 nuclear accumulation. Cells were serum-deprived in the absence (a, b *top*) or in the presence (a, b *bottom*) of PMA pre-treatment. After PMA pre-treatment, the medium was changed and the effectors were added for an additional 24 h. ×400

untreated cells, basal cytoplasmic labelling was observed for FGFR2 (Fig. 4d) and FGF-2 (data not shown).

In order to determine whether new protein synthesis was necessary for the nuclear accumulation of FGF-2, cells were pre-treated with cycloheximide (CHX, 3 μg/ml; Sigma-Aldrich), a protein synthesis inhibitor, for 1 h. $PGF_{2\alpha}$ and Flup were added for an additional 6 h or 24 h. Cells treated with CHX showed a decrease of the FGF-2 labelling present with maximal intensity, indicated by white spots, in nucleolar regions. Pre-treatment with CHX blocked both the perinuclear and nuclear accumulation of FGF-2 in stimulated cells (Fig. 5). These results suggest that new protein synthesis may be required.

Signalling pathway of PG regulation of FGF-2 and FGFR2

To explore the signalling pathway by which $PGF_{2\alpha}$ and Flup regulate FGF-2 and FGFR2 nuclear binding, we examined their nuclear accumulation in the absence or presence of SU5402 (Calbiochem-Inalco), a specific FGFR1 tyrosine kinase inhibitor. Cells were pre-treated for 1 h with SU5402 (2.5×10^{-5} M) and then pulsed with $PGF_{2\alpha}$ or Flup for an additional 24 h. Pre-treatment with SU5402 did not block perinuclear or nuclear accumulation of FGF-2 and FGFR2.

To examine the role of PKC in the nuclear translocation of FGF-2/FGFR2, cells were treated with PMA (10^{-6} M; Sigma-Aldrich), an activator of PKC, for 24 h. PMA alone or combined with $PGF_{2\alpha}$ induced nuclear accumulation of FGF-2 (Fig. 6a, top) and FGFR2 (Fig. 6b, top); conversely, PMA pre-treatment before administration of $PGF_{2\alpha}$ or PMA prevented the nuclear accumulation of FGF-2 (Fig. 6a, bottom) and FGFR2 24 h later (Fig. 6b, bottom). These data were also confirmed at the electron-microscope level. Indeed, cells treated with PMA alone or combined with $PGF_{2\alpha}$ showed a FGF-2 (Fig. 7a) and FGFR2 (Fig. 7b) nuclear labelling that was prevented by PMA pre-treatment before administration of the effectors (Fig. 7c). Moreover, pre-treatment for 1 h with the PKC inhibitor H-7 (5×10^{-5} M; Calbiochem-Inalco) reduced the FGF-2 nuclear accumulation induced by PMA and $PGF_{2\alpha}$ (Fig. 8). Similar results were found also for FGFR2.

Since our previous molecular data had indicated that FGF-2 increased ERK in Py1a cells (Hurley et al. 1996b), we examined whether the PG regulation of ERK accumulation and localization mimicked that of FGF-2. We localized ERK2 in untreated and PG-treated cells. Untreated cells showed cytoplasmic but no nuclear labelling for ERK2. Treatment with $PGF_{2\alpha}$ for 24 h increased ERK2 labelling prominently at the cytoplasm level. In contrast, pre-treatment with PMA for 24 h blocked $PGF_{2\alpha}$-induced labelling for ERK2 (Fig. 9).

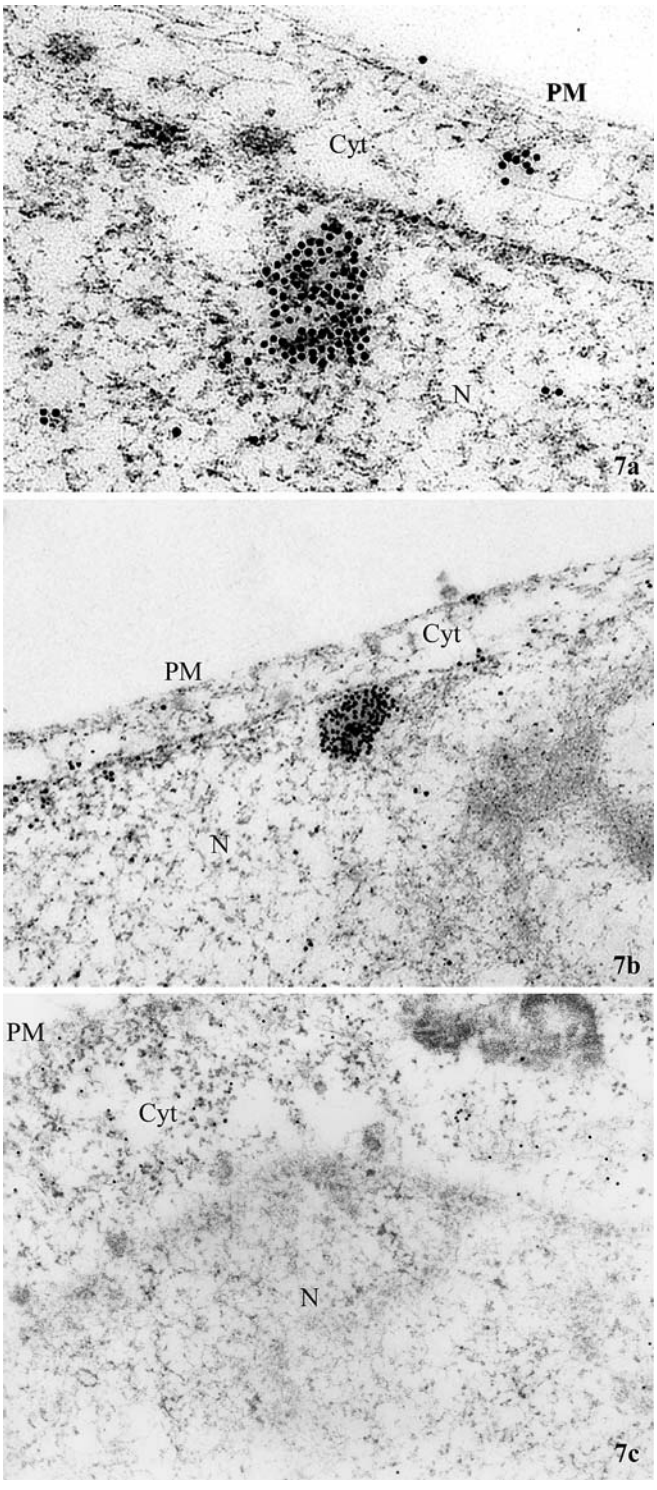

Fig. 7 Electron-microscopic immunolabelling of FGF-2 (**a**, **c**) or FGFR2 (**b**). In cells treated with PMA for 24 h, a patchy distribution of gold particles reflecting FGF-2 IR (**a**) and FGFR2 IR (**b**) was located as in Fig. 4 (*PM* plasma membrane, *Cyt* cytoplasm, *N* nucleus). Cells pre-treated with PMA for 24 h before administration of $PGF_{2\alpha}$ or PMA showed sparse cytoplasmic gold particles reflecting FGF-2 IR (**c**). ×60,500 (**a**), ×27,700 (**b**), ×22,600 (**c**)

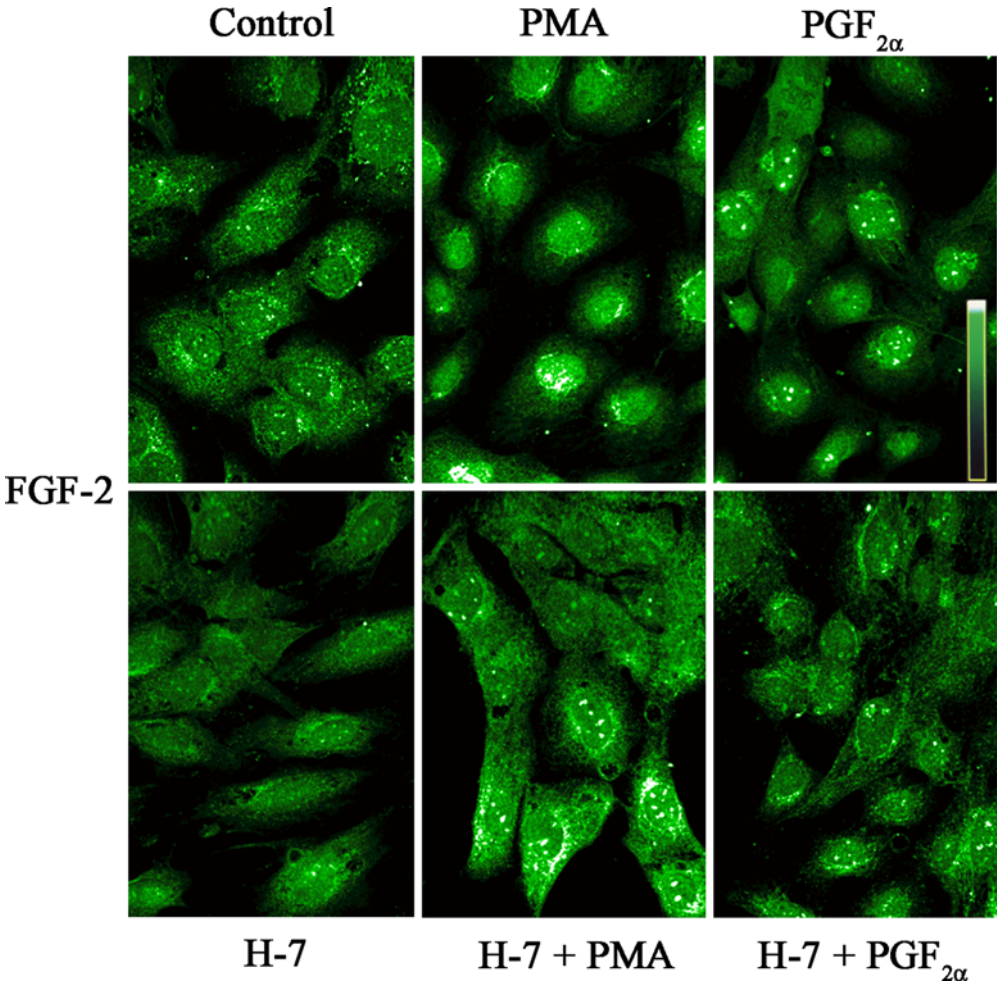

Fig. 8 Control osteoblasts and osteoblasts treated for 24 h with PMA or $PGF_{2\alpha}$. Optical sections demonstrating the effects of H-7 on FGF-2 labelling. Note that H-7 was able to reduce the FGF-2 nuclear accumulation induced by PMA and $PGF_{2\alpha}$. ×450

Expression of ERK2 protein

To support our results obtained at the confocal laser scanning microscopy (CLSM) level, blots probed with anti-ERK2 showed an increase of ERK2 protein after 24 h of treatment with $PGF_{2\alpha}$ and confirmed PKC pathway involvement, as was also indicated by PMA pre-treatment (Fig. 10).

Discussion

We have previously reported that $PGF_{2\alpha}$ and the $PGF_{2\alpha}$ agonist Flup increase FGF-2 mRNA and protein production in bone cells (Sabbieti et al. 1999). The present report extends these studies by examining the effect of $PGF_{2\alpha}$ on FGFR2 expression and the subcellular localization of FGF-2 and FGFR2 proteins in osteoblastic Py1a cells and the signalling pathway involved. We show, for the first time, that $PGF_{2\alpha}$ and Flup are able to regulate FGFR2 mRNA expression in osteoblasts; indeed, a reduction in FGFR2 mRNA level, with a maximal down-regulation after 24 h of treatment, occurs. Taken together, our results suggest that the effect of PGs on FGF-2 expression involves an interaction with FGFR2. Interestingly, we have not detected mRNA for other FGFRs in Py1a cells. This differs from our observation in MC3T3-E1 osteoblasts that expressed both FGFR1 and FGFR2 but not FGFR3 (Hurley et al. 1996a). Conversely, a spatiotemporal expression of FGFR1, FGFR2 and FGFR3 has been found in neonatal rat mandibular condile and calvaria during osteogenic differentiation in vitro (Molteni et al. 1999). The differences in FGFR expression may depend on the species, the state of differentiation of the osteoblasts or whether transformed or non-transformed cell lines are examined.

CLSM experiments performed to examine whether PGs also regulate the occurrence and distribution of FGF-2 and FGFR2 protein further support our molecular data. Treatment of Py1a cells for 24 h with PGs not only increases FGF-2 and FGFR2 levels in the cytoplasm and perinuclear region but also causes nuclear translocation. In addition, double-labelling methods have demonstrated the localization of FGF-2 and FGFR2 in the perinuclear region and internalization into the nucleus in cells stimulated for 6 h and 24 h, respectively. The strategic perinuclear localization of FGF-2 and FGFR2 could represent an "ergonomic system" for traversing the nuclear envelope; studies of the involvement of carrier proteins are now under investigation. A peculiar perinuclear labelling

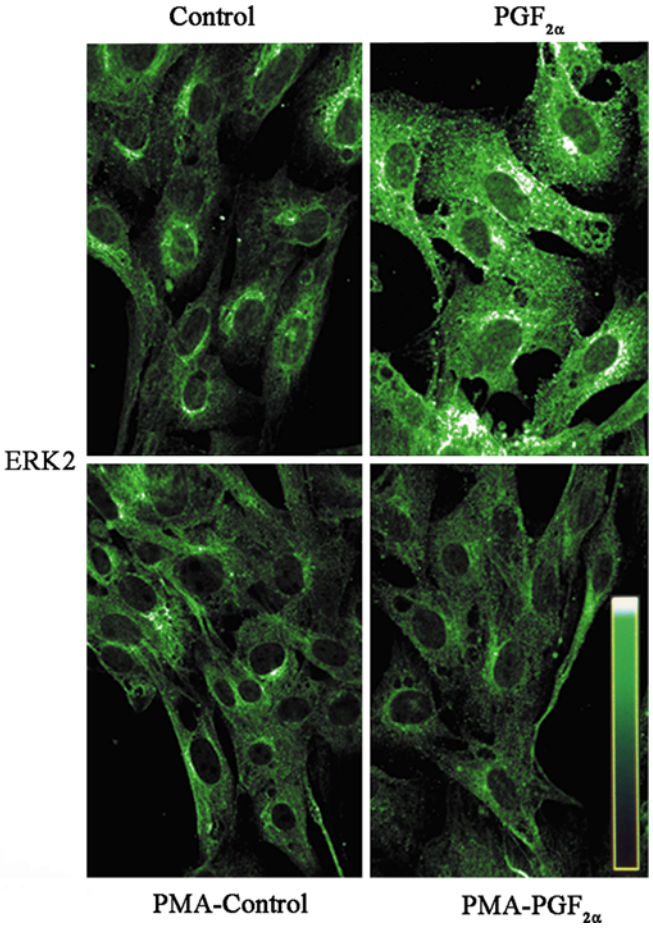

Fig. 9 Control osteoblasts or osteoblasts treated for 24 h with $PGF_{2\alpha}$ and labelled for ERK2. Optical sections showing the effects of $PGF_{2\alpha}$ on ERK2 without or with 24 h PMA pre-treatment. Note that pre-treatment with PMA blocks the increased labelling for ERK2 induced by $PGF_{2\alpha}$. ×450

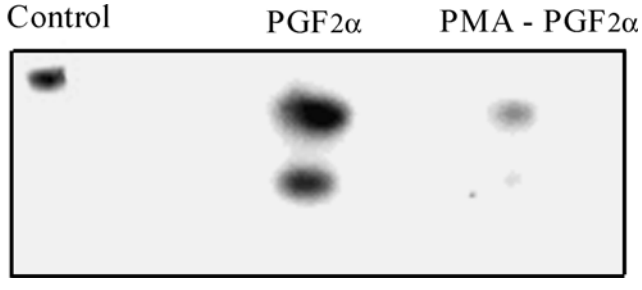

Fig. 10 Western analysis. Effect of the down-regulation of the PKC pathway with a 24-h PMA pre-treatment on $PGF_{2\alpha}$-mediated ERK2 protein expression

has also been observed by Marchese et al. (1998) who have shown, by CLSM, that anti-Bek antibody is localized in restricted perinuclear regions.

One hypothesis for the action of PGs is that they first increase FGF-2 mRNA that, after translation into FGF-2 protein, binds FGFR2 and is translocated into the nucleus. In support of this hypothesis, cells pre-treated with CHX, a protein synthesis inhibitor, do not show nuclear accumulation for FGF-2.

Transmission electron-microscopy (TEM) immunogold labelling has indicated that FGF-2 and FGFR2 are translocated in a similar way through restricted areas of the nuclear envelope and have confirmed the confocal images of FGF-2 and FGFR2 binding patterns. Other authors have reported that, in bovine adrenal medullary cells, FGFR1 occurs in cytoplasmic areas close to the nucleus, traversing the nuclear membrane and inside the nucleus; indeed, they have observed that labelling is concentrated in a few regions on the nuclear envelope (Stachowiak et al. 1994).

In addition, Bouche et al. (1997) have demonstrated the translocation and nucleolar accumulation of FGF-2 in the G_1 phase of the cell cycle in bovine aortic endothelial cells (Baldin et al. 1990) and the nucleolar accumulation of FGF-2 in adrenal cells associated with the transcription of ribosomal genes. These data are in accordance with our immunoelectron-microscopy and CLSM data.

The apparent heavy labelling for FGF-2 and FGFR2 is attributable to the clusters of gold particles in restricted areas and confirms data from CLSM. However, a massive occurrence of receptors has also been observed in an internalization study of a splicing transcript variant of FGFR2, named KGFR, by Marchese et al. (1998). Other ultrastructural studies have demonstrated a patchy distribution of gold particles, reflecting fibroblast growth factor receptor, within the nuclear matrix (Stachowiak et al. 1996).

The signalling cascade by which PGs induce the nuclear accumulation of FGF-2 and its effect at the nuclear level is unknown. We have therefore explored possible signalling pathways by which PGs could regulate FGF-2 and FGFR2 expression in Py1a cells. Recent studies have demonstrated that a selective inhibitor of FGFR tyrosine kinase, SU5402, blocks the stimulation of inorganic phosphate transport induced by FGF-2 in mouse osteoblastic MC3T3-E1 cells (Suzuki et al. 2000). We have therefore examined whether SU5402 can inhibit PG-induced FGF-2 and FGFR2 nuclear accumulation. We have found that FGF-2 and FGFR2 perinuclear accumulation and nuclear translocation is not blocked by this inhibitor. This further supports the lack of FGFR1 in Py1a; indeed, SU5402 has been shown to be a selective inhibitor of this receptor type (Mohammadi et al. 1997). However, we cannot exclude that the PG-mediated response follows other pathways of signal transduction in addition to cell surface tyrosine kinase receptors.

Previous studies have demonstrated that the mitogenic effect of $PGF_{2\alpha}$ in osteoblastic MC3T3-E1 cells involves the activation of the PKC pathway (Quarles et al. 1993). We have also reported that the mitogenic effect of FGF-2 and PMA on Py1a cells is mediated, in part, by the activation of the PKC pathway (Hurley et al. 1996b). In the present study, we have found that the PKC activator PMA mimics $PGF_{2\alpha}$-induced perinuclear accumulation and nuclear internalization of FGF-2 and FGFR2. The involvement of PKC in this process is further supported by experiments with the PKC inhibitor H-7, which decreases the entry of FGF-2 into the nucleus.

Tyrosine kinase receptors activate several intracellular signalling pathways, including MAPKs (Marshall 1995) that modulate cell proliferation or differentiation (Pages et al. 1993). MAPK also phosphorylates nuclear proteins such as c-Myc, c-Jun and c-Fos (Davis 1993). Previously, we have showed that FGF-2 increased ERK1 and ERK2 phosphorylation in rat Py1a cells (Hurley et al. 1996b). Other studies have established that FGF-2 and platelet-derived growth factor-BB (PDGF-BB) also activate ERK1 and ERK2 in normal human osteoblastic cells (Chaudhary and Avioli 1997). In view of these data, we have studied whether PGs regulate the accumulation and localization of ERK2 protein, in a manner similar to that of FGF-2 and FGFR2. We have found increased perinuclear labelling for ERK2 protein in cells treated with PGs. However, labelling for ERK2, induced by PGs, is blocked by PMA pre-treatment. Our findings suggest that ERK2 plays a role in the nuclear accumulation of FGF-2 and FGFR2 in Py1a cells; data originating from Western analysis also support the results from the in situ experiments.

Taken together, our results indicate that, in PG-treated osteoblasts, FGF-2 can bind to FGFR2 and both proteins are translocated into the nucleus by a PKC-mediated mechanism. Our data also suggest a role for ERK activity in this nuclear accumulation. The nuclear FGF-2 and FGFR2 accumulation caused by PG stimulation could be responsible for the down-regulation of FGFR2 mRNA at 24 h. Moreover, the presence of FGF-2 and FGFR2 proteins inside the nucleus could activate the FGF-2 promoter and directly participate in the regulation of transcription, replication, and/or other nuclear events. We conclude that PGs stimulate the nuclear accumulation of FGF-2 and its receptor by a PKC-dependent pathway and we suggest a role for MAP kinase/ERK2 in this process.

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