Expression of the High Molecular Weight Fibroblast Growth Factor-2 Isoform of 210 Amino Acids Is Associated with Modulation of Protein Kinases C δ and ε and ERK Activation*

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François Gaubert‡‡, Fabrice Escaffit‡‡, Claudine Bertrand‡, Murray Korc**, Lucien Pradayrol, François Clemente† ‡‡, and Agnès Estival‡

From the ‡INSERM U 531, Institut Louis Bugnard, CHU Rangueil Bat L 3, 31403 Toulouse Cedex 4, France and **Division of Endocrinology, Diabetes and Metabolism, University of California, Irvine, California 92697

The high molecular weight (HMW) fibroblast growth factor (FGF)-2 isoform of 210 amino acids initiated at a CUG start codon possesses a nuclear localization sequence and is not secreted. In contrast, the low molecular weight (LMW) isoform of 155 amino acids initiated at the AUG start codon can be secreted and activates the cell surface FGF receptors. The two isoforms possess different biological properties; however, little is known about the intracellular regulatory mechanisms involved in the biological effects of the HMW FGF-2 isoform. Using pancreatic cells stably transfected with cDNAs leading to the expression of either the HMW FGF-2 (A3 cells) or the LMW form (A5 cells), we provide evidence that the two FGF-2 isoforms differentially modulate PKC levels. The LMW FGF-2 up-regulated the PKC ε levels by 1.6-fold; by contrast the HMW isoform down-regulated the level of this PKC isotype by about 3-fold and increased the amount of PKC δ by 1.7-fold. PKC mRNAs were also modified, suggesting that PKC expression was regulated at a pretranslational level. Additionally, expression of different levels of the HMW FGF-2 with an inducible expression system confirmed the role of this isoform on PKC δ and ε expressions. Increased activation of ERK-1 and -2 was also observed in cells expressing the HMW FGF-2. By using different PKC inhibitors and a dominant negative PKC δ, it was found that ERK activation was PKC δ-dependent. These data indicate that expression of HMW FGF-2 can modify PKC levels by acting at the intracellular level and that the overexpression of PKC δ induces ERK-1/2 activation. The expression of a dominant negative FGFR1 did not reduce ERK-1/2 activation by the HMW FGF-2, suggesting that ERK activation does not require FGFR activity. The signaling cascade downstream of ERK might be involved in the known mitogenic effect exerted by this FGF-2 isoform.

Basic FGF4 (FGF-2) is a protein belonging to the family of heparin-binding growth factors and is produced by many cell types either normal or malignant (for review see Ref. 1). In tumors, it is one of the key factors regulating growth, blood supply, and invasiveness. These pleiotropic effects have been related to the binding of FGF-2 to high affinity FGF receptors or their splice variants and to low affinity binding sites (2). However, FGF-2 possesses some peculiar features supporting the involvement of other regulatory mechanisms in the final biological effects. Indeed, FGF-2 is synthesized from a single mRNA as five different isoforms with molecular masses of 18, 22, 22.5, 24, and 34 kDa through alternative translation at AUG and CUG start codons (3–5) and through internal ribosomal frameshifts (6). The initiation of translation at the AUG codon gives rise to a peptide of 155 amino acids, and the initiation at the four CUG codons is responsible for the synthesis of the other isoforms that possess N-terminal extensions containing nuclear localization sequences (5, 7). Confocal and immunohistochemical analysis show that these nuclear localization sequence-containing isoforms are predominantly localized in the nucleus and more precisely in the nucleoplasmic compartment; no labeling is found at the cell surface and in the extracellular spaces (1, 8–10). Usually the import process across the nuclear pore complex involves the activity of transport factors not yet characterized for FGF-2. All FGF-2 isoforms lack leader consensus secretory sequences. However, the LMW peptide (155 aa) can be secreted through still unclear mechanisms requiring energy and cooperation with heat shock proteins and Na+-K+-ATPase (11, 12). After an endocytotic process small amounts of this LMW peptide can be specifically targeted to the nucleolar compartment (13). The nuclear translocation of FGF-2 seems to be correlated with the nuclear import of the type 1 FGF receptor (FGFR1) (for review see Ref. 14). The selective expression of either the LMW or the HMW form of 210 amino acids in different cell types induces different and even opposite effects. For instance, expression of the LMW form enhances cell migration (10), integrin, and tissue plasminogen activator expressions (15), whereas the HMW FGF-2 does not affect these properties. By contrast, the HMW form specifically promotes, at the difference of the LMW form, growth at low serum con-
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EXPERIMENTAL PROCEDURES

Cell Culture—AR4-2J cells and the different transfected cells were maintained in DMEM containing 4.5 g/liter glucose (Life Technologies, Inc.) and supplemented with 10% fetal calf serum (Life Technologies, Inc.). Trypsin (0.05%) and EDTA (0.02%) (Biowhittaker, France) were used to dissociate the cells for successive passages, and medium was replaced every 2 days. Cultures were regularly checked for the absence of contamination by mycoplasma. Cells were routinely plated at the density of 15 000 cells/cm² in DMEM containing 10% fetal calf serum, buffered at pH 7.3 and supplemented with 20 nM Hepes. Media were changed the day before PKC or ERK analysis to standardize the experimental conditions. For FGF-2 stimulation, after an overnight attachment medium was replaced by serum-free DMEM and buffered at pH 7.3 with 20 nM Hepes containing 2 nM FGF-2.

To analyze the effect of the PKC inhibitors, cells were cultured in DMEM and buffered at pH 7.2 with 20 nM Hepes containing 0 or 2.5% fetal calf serum. Gö 6976 (Calbiochem, La Jolla, CA) was used at the concentration of 50 nM to inhibit the conventional PKCs. GF109203X concentration (0–1%) (10, 16, 17), reduces laminin B1 (8), tissue plasminogen activator and plasminogen activator inhibitor-1 expression (18), adenylate cyclase activity (19), and inhibits interleukin-6 promoter activity (20). Furthermore, neutralizing anti-FGF-2 antibodies recognizing the common sequence of all FGF-2 forms (10, 21), suramin (18), a well established inhibitor of FGF-2 binding to FGF receptors (22), and expression of high level of dominant negative FGFR (10) inhibit the biological effects of the extracellular FGF-2, but they do not modify those evoked by the HMW FGF-2. Thus, the LMW isoform regulates cell functions predominantly through auto-paracrine mechanisms, whereas the larger ones exert their biological effects via an “intracrine” pathway, independent of the activation of cell surface FGF receptors (for reviews see Refs. 1, 14, and 23). The signaling pathways involved in intracrine regulations are still poorly known. It has been postulated that the colocalization of HMW FGF-2 and FGFR1 in the nuclear matrix might reflect the existence of FGF receptor activation at the nuclear level (14). We previously reported a reduced adenylate cyclase activity in cells expressing the HMW peptide, which was found to play a positive role on cell growth (19). However, this modification cannot fully explain the increased proliferation in low serum conditions. Because some PKC isotypes are involved either positively or negatively in the proliferation rate of many cell types (24), we sought to examine the effects of the HMW FGF-2 peptide of 210 aa on PKC levels and functions. For that purpose, we analyzed either stably transfected cells or cells transiently transfected with a tetracycline-regulated gene expression system (25), and the cell model used was the well differentiated pancreatic cell line AR4-2J. These cells were transfected with point mutated FGF-2 cDNAs leading to the expression either of the LMW of 155 aa or of the HMW isoform of 210 aa (16). The results reported here demonstrate that the HMW peptide modified the expression of PKC ε and δ and ERK-1 and -2 activities via a receptor-independent pathway.

FIG. 1. Effect of FGF-2 expression on PKC protein levels. PKC levels were measured by Western blots on cell extracts prepared from control CAT cells, A3 cells expressing the HMW FGF-2, and A5 cells expressing the LMW FGF-2. Cell lysates corresponding to 30 μg of protein for PKC α and δ and 60 μg of protein for PKC ε were loaded on SDS-PAGE and immunoblotted with antibodies specific for the different PKC isotypes. Western blots from a single experiment are shown. Quantifications were done with a laser densitometer. Values are the means ± S.D. of five independent experiments in triplicate. PKC levels in control CAT cells were given an arbitrary value of 1. *, p < 0.05; **, p < 0.002, compared with control CAT cells.

FIG. 2. Dose-dependent inhibition of HMW FGF-2 expression by doxycycline and modulation of PKC levels. Control CAT cells were transfected with the tet-off gene expression system. The plasmid containing the TRE encoded the HMW FGF-2. The transfected cells were incubated for 24 h with increasing concentrations of doxycycline and then lysed. Cell lysates corresponding to 40 μg of protein were loaded on SDS-PAGE and immunoblotted with antibodies specific for the different PKC isotypes or with anti-human FGF-2 recognizing all FGF-2 isoforms. Western blots from a single experiment are shown. PKC and HMW FGF-2 relative levels were determined with a laser densitometer. Values are the means ± S.D. of three independent experiments. Protein levels in the absence of doxycycline were given a value of 1. *, p < 0.05; **, p < 0.01, compared with values obtained in the absence of doxycycline.
These drugs were solubilized in a reverse transcriptase buffer contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2 mM each dNTP, 1 unit/10 min at 94 °C, was reverse transcribed at 39 °C in the presence of 1 unit of reverse transcriptase (Life Technologies, Inc.). The reverse transcriptase (Life Technologies, Inc.) was devoid of most of the intracellular domain and lacks kinase activity. The FGFR1dn construct was used to transfect CAT and A3 cells. All transfections were performed by using FuGENE 6 (Roche Molecular Biochemicals). The dominant negative FGFR1 construct (FGFR1dn) corresponds to FGFR1 truncated at residue 405. The resulting protein contains the transmembrane region but is devoid of most of the intracellular domain and lacks kinase activity. The retroviral infection of AR4-2J cells was described previously (16). Genetically-resistant cells were shown to express low levels of FGF-2. Transfected cells synthesizing the HMW FGF-2 isofrom of 210 aa were called A3, and cells expressing the LMW isofrom of 155 aa were called A5. The retroviral infection of AR4-2J cells was performed by using FuGENE 6 (Roche Molecular Biochemicals). The dominant negative FGFR1 construct (FGFR1dn) corresponds to FGFR1 truncated at residue 405. The resulting protein contains the transmembrane region but is devoid of most of the intracellular domain and lacks kinase activity. The retroviral infection of AR4-2J cells was described previously (16). Genetically-resistant cells were shown to express low levels of FGF-2. Transfected cells synthesizing the HMW FGF-2 isofrom of 210 aa were called A3, and cells expressing the LMW isofrom of 155 aa were called A5.

Preparation of Rat PKC Probes—Total RNA was prepared by the guanidinium isothiocyanate method (28). Total RNA, previously heated 10 min at 94 °C, was reverse transcribed at 39 °C in the presence of 1 μM each dNTP, 1 unit/μl RNasin (Promega, Charbonnieres, France), 0.25 μg/μl oligo(dT) and 2 units/μl Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The reverse transcriptase buffer contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2 mM MgCl2 and 1 mg/ml nuclease-free bovine serum albumin. After 10 min at 23 °C and a 1-h incubation at 39 °C, the reaction was stopped at 94 °C, 5 min. Amplification was then carried out in a final volume of 100 μl, with 0.5 μg of cDNA, 2.5 units/μl Taq polymerase (PerkinElmer Life Sciences) in the same buffer, and 500 pmol of each oligonucleotide primer. All reactions were performed for 35 cycles with cycling times of 1 min at 95 °C, 1 min at 65 °C, and 1.30 min at 72 °C. Primers were synthesized by Eurogentec (Liège, Belgium). Primers were chosen on the basis of the cDNA sequences of the rat PKC isoforms (GenBank™ database), and the absence of cross-reactivity among the different PKC was checked.

The following primers were used: for PKC α, 5'-GATGAAATCGGA-CACCTGGG-3' from 397 to 413 bp and 5'-AGGGATGATGATCTTGTGT-3' from 940 to 960 bp; for PKC β, 5'-CCCTGTGAGGTTGCAA-CATTGG-3' from 156 to 174; and 5'-GAAGATCTGACACCCGTGGAT-3' from 638 to 660; for PKC δ, 5'-CATCGTTCTTGTCATCTACTGG-3' from 580 to 601 pb. Sizes of amplified products corresponded to those expected according to the position of the primers in the PKC cDNA sequence. The specificity of the polymerase chain reaction products was further checked by using restriction enzymes selected according to the sequence of each cDNA by using computer analysis (PC/GENE, IntelliGenetics, Mountain View, CA). For each PKC, two sets of restriction endonucleases were used: one able to cut only at one restriction point, and the second one unable to cut the amplified product.
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RESULTS

FGF-2 Expression Modifies PKC Levels—Confocal analysis of AR42J cells stably transfected with the mutated CDNA encoding the FGF-2 of 210 aa (A3 cells) showed its localization in the nucleus but not at the cell surface or in the extracellular compartment (8). We first analyzed total cell extracts by Western blots to find out whether FGF-2 expression modified PKC levels. PKC α, δ, and ε were found expressed in parental AR4-2J and FGF-2 producing cells, and they exhibited apparent mobility different from that in control CAT cells, with a 1.6-fold increase.

Cell Treatment with Suramin and Neutralizing Anti-FGF-2 Antibodies—Cells plated at 100,000 cells/35-mm dish were grown in Hepes-buffered (20 mM) serum-free medium at pH 7.3. Cells were incubated with suramin (400 μg/ml) (Upstate Biotechnology, Lake Placid, NY) in the absence or presence of 0.1 nM rFGF-2 for 24 h. Cells were harvested, and ERK1/2 phosphorylation was analyzed as described above.

FIG. 5. ERK-1 and -2 levels and phosphorylations in CAT cells expressing the HMW FGF-2 under the control of doxycycline. Control CAT cells were transfected with the tet-off system leading to the expression of the HMW FGF-2 under the control of tTA. Cells were then incubated for 24 h with increasing concentrations of doxycycline before cell lysis. Equal amounts of protein (20 μg) were analyzed by SDS-PAGE, and the membranes probed with an antibody recognizing ERK-1 and -2 (top panel). Blots were reprobed with an anti-phosphorylated ERK-1 and -2 antibody (middle panel). ERK activity was determined on the myelin basic protein (MBP) (Sigma) as substrate (30). After cell lysis, equal amounts of protein were incubated overnight at 4 °C with the anti-ERK-1/2 antibody and protein A-agarose beads (Sigma). The immunoprecipitated complexes were incubated with MBP in the presence of [γ-32P]ATP (1 mCi/assay). The labeled MBP was quantified with a PhosphorImager. Cell Treatment with Suramin and Neutralizing Anti-FGF-2 Antibodies—Cells plated at 100,000 cells/35-mm dish were grown in Hepes-buffered (20 mM) serum-deprived medium at pH 7.3. Cells were incubated with suramin (400 μg/ml) or neutralizing anti-FGF-2 antibodies (5 μg/ml) (Upstate Biotechnology, Lake Placid, NY) in the absence or presence of 0.1 nM FGF-2 for 24 h. Cells were harvested, and ERK1/2 phosphorylation was analyzed as described above.

PKC Localization—Cells seeded at the concentration of 100,000 cells/35-mm dish were transfected with the tat-off expression system. After transfection, cells were grown in Hepes-buffered (20 mM) serum-deprived medium at pH 7.3, with or without doxycycline. 24 h after the beginning of doxycycline treatment, cells were washed twice with cold phosphate-buffered saline and then harvested at 4 °C in 0.5 ml of the 25 mM hypotonic Tris-HCl buffer, pH 7.5, containing inhibitors of proteases (Pefabloc) and phosphatases (25 mM NaF and 10 mM sodium orthovanadate). Then cells were sonicated three times for 5 s at 4 °C and spun at 100,000 × g for 5 min to discard the nonlyzed cells. The supernatant corresponding to the total PKC was then centrifuged at 100,000 × g for 30 min. The 100,000 × g pellet was resuspended in the same buffer containing 1% Triton X-100. 100,000 × g pellet pellet corresponds to the particulate fraction (31). The two fractions were subjected to SDS-PAGE electrophoresis, transferred to a polyvinylidene difluoride membrane, and revealed with the anti-PCDP-δ antibody as described above. To analyze the serine-phosphorylated PKC δ, equal amounts of protein were immunoprecipitated with the anti-δ PKC antibody, separated on SDS-PAGE, and revealed with an anti-serine antibody (Zymed Laboratories Inc. Laboratories, San Francisco, CA).

Statistics—Statistical significance was determined according to the unpaired Student’s t test.
the tet-off transcription regulation system to regulate the expression of the HMW FGF-2 (25). In this system the transactivator tTA regulates a synthetic promoter composed of the tetracycline operator. The plasmid expressing the tTA transactivator was cotransfected with the plasmid containing the HMW FGF-2 cDNA inserted downstream the tetracycline operator. The presence of tetracycline or its analogue doxycycline prevents binding of the transactivator to the promoter and reduces the expression of the HMW FGF-2. Transfected cells displayed a doxycycline-dependent down-regulation of the HMW FGF-2 expression (Fig. 2). Only this FGF-2 isoform was found produced whatever the doxycycline concentration (Fig. 2), indicating that the HMW FGF-2 did not modulate in the transfected cells the expression of endogenous FGF-2.

**Fig. 6.** Effect of suramin on ERK-1 and -2 phosphorylation by the HMW FGF-2. Control CAT cells were transfected with the tet-off system leading to the expression of the HMW FGF-2 under the control of doxycycline. Cells were then incubated for 24 h with 0 or 2 μg/ml doxycycline, with or without suramin (400 μM), and with or without rFGF-2 (0.1 nM). Equal amounts of protein (20 μg) were analyzed by SDS-PAGE, and the membranes were probed with an anti-phosphorylated ERK-1 and -2 antibody. Membranes were stripped and reprobed with an antibody recognizing ERK-1 and -2. The relative intensities of bands were determined by laser densitometry. A, suramin inhibits phosphorylation of ERK-1 and -2 induced by exogenous rFGF-2 in cells grown with 2 μg of doxycycline to inhibit the expression of the HMW FGF-2. B, effect of suramin on ERK-1/2 phosphorylation in cells expressing (0 ng of doxycycline) or not (2000 ng/ml doxycycline) the HMW FGF-2. Values are the means ± S.D. of two independent experiments performed in duplicate.

**Fig. 7.** Effect of neutralizing anti-FGF-2 antibodies on ERK-1 and -2 phosphorylation by the HMW FGF-2. Control CAT cells were transfected with the tet-off system leading to the expression of the HMW FGF-2 in the absence of doxycycline. Cells were then incubated for 24 h with 0 or 2000 ng/ml doxycycline, with or without neutralizing anti-FGF-2 antibodies (5 μg/ml). To check the efficiency of the anti-FGF-2 antibody, cells were stimulated with rFGF-2 (0.1 nM). Equal amounts of protein (30 μg) were analyzed by SDS-PAGE, and the membranes were probed with an anti-active ERK-1 and -2 antibody. Membranes were stripped and reprobed with an antibody recognizing ERK-1 and -2. The relative intensities of bands were determined by laser densitometry. Values are the means ± S.D. of two independent experiments performed in duplicate.
maximal level of HMW FGF-2 expressed in transiently transfected cells was comparable with that of A3 cells, as observed by SDS-PAGE (data not shown). PKC \( \delta \) and \( \epsilon \) levels were modulated by the concentration of the HMW FGF-2 (Fig. 2), and as observed in A3 cells, these PKCs were differentially regulated. Taken together, these data show that the HMW isoform regulates PKC \( \delta \) and \( \epsilon \) levels and also indicate that the secretory and nonsecretory FGF-2 behave differently with respect to PKC levels.

FGF-2 Expression Modifies PKC mRNA Levels—To analyze whether these modifications were related to mRNA changes, the levels of the PKC transcripts were determined by using rat-specific PKC probes synthesized by reverse transcriptase-polymerase chain reaction. Northern blot analysis revealed that PKC \( \alpha \) and \( \delta \) were expressed as transcripts of about 3.4 kilobases, and PKC \( \epsilon \) was expressed as a transcript of about 7 kilobases (32). A PKC \( \alpha \) mRNA species of about 8 kilobases (32) was also detected as a faint band. As shown in Fig. 3, the level of the \( \epsilon \) isotype mRNA was decreased by a factor of 2.5 in A3 cells; by contrast, that of PKC \( \delta \) was increased by a factor of 2. In contrast, in A5 cells the PKC \( \epsilon \) mRNA was found increased by a factor of 1.6 (Fig. 3). Thus, the expression of FGF-2 modifies both PKC protein and mRNA levels.

The HMW FGF-2 Modifies ERK-1 and -2 Phosphorylations and Activities—ERK-1/2 are involved in cell proliferation. We were then interested on the possible modifications of ERK-1 and 2 levels and activities in cells expressing the HMW FGF-2. ERK-1 and -2 protein levels were found to be comparable in A3 and CAT cells (Fig. 4). By contrast, the anti-active ERK antibody revealed an hyperphosphorylation state of both proteins in A3 cells (Fig. 4). Similar results were obtained in control CAT cells transiently transfected with the cDNA encoding the HMW FGF-2 under the control of the tetracycline-dependent transactivator. Indeed, ERK-1/2 levels were not modified (Fig. 5), whatever the concentration of the HMW FGF-2. In addition, a close relationship was observed between HMW FGF-2 levels and ERK-1/2 hyperphosphorylation state (Fig. 5). For control purposes CAT cells were transfected with the basal tetracycline-regulated expression vectors and treated with doxycycline. The phosphorylation state of ERK was not modified by doxycycline, as expected according to the results obtained in stably transfected cells that were not treated with doxycycline (Fig. 4). All of the data indicate that ERK phosphorylation was indeed the result of the expression of the HMW peptide. The hyperphosphorylation reflected an increased kinase activity as observed on the MBP substrate (Fig. 5).

Activation of FGFRs Is Not Responsible for the Modifications Observed in Cells Producing the HMW FGF-2—The question arises of whether the HMW FGF-2 exerts its effects after release, for instance by cell lysis. The results obtained by confocal analysis (8) that are against a significant release of the HMW FGF-2 as well as the opposite effects of HMW and LMW FGF-2 on PKC \( \delta \) and \( \epsilon \) expressions do not support the involvement of FGFR activation in the effects of the HMW form as also reported for other cell lines (1). Anyway, activation of FGFRs by the HMW form present outside the cells can be determined by
Fig. 9. Effect of PKC inhibitors on ERK-1 and -2 phosphorylation. After transfection CAT cells were cultured for 24 h either in the absence or in the presence of doxycycline and different PKC inhibitors. GF109203X was used at the concentration of 1 μM to inhibit PKC α, δ, and ε. Go6976 was used at the concentration of 50 nM to inhibit PKC α, and rottlerin, which specifically inhibits PKC δ, was used at the concentration of 6 nM. Cell lysates were loaded on SDS-PAGE and probed with the anti-phosphorylated ERK-1/2 and then reprobed with the anti-ERK-1/2 antibody to assess equivalent protein loading. Data from a single experiment are shown for phosphorylated ERK-1/2. Similar results were obtained in three independent experiments. The relative intensities of bands were determined with a laser densitometer. *, p < 0.05; **, p < 0.01, compared with values obtained in untreated cells (7).

analyzing ERK-1/2 phosphorylation because the secreted FGF-2 strongly activates the MAPK pathway. We should notice that expression of LMW and HMW FGF-2 does not modify the subtypes of the FGFR or the variant of FGFR1 (33). CAT cells were transfected with the tet-off expression system and then grown in serum-free medium and treated with 400 μM suramin, which is a well established inhibitor of FGF-2 binding and activation of the cell surface FGFR receptors (34). This treatment was found to strongly inhibit ERK-1/2 phosphorylation induced by the rFGF-2 added to the culture medium (Fig. 6A). By contrast, suramin did not modify the kinase phosphorylation in cells expressing the HMW FGF-2 (Fig. 6B). Similar results were obtained by adding to the culture medium neutralizing antibodies recognizing all FGF-2 forms (Fig. 7). The antibody treatment strongly decreased ERK phosphorylation induced by rFGF-2 but did not affect the basal ERK phosphorylation observed either in control cells (Fig. 7; 2000 ng/ml doxycycline) or in cells expressing the HMW FGF-2 (Fig. 7; 0 ng/ml doxycycline).

Taking into account both the opposite effects of high and low molecular weights FGF-2 on PKC δ and ε expressions and the above results on ERK phosphorylation, all of the data indicate that the activation of cell surface FGFRs is not involved in the effects of the large form of FGF-2, as already reported in other cell types (1).

To analyze the involvement of intracellular FGFRs in the effects of the HMW FGF-2, A3 cells were transfected with increasing concentrations of a cDNA encoding the dominant negative FGFR1 truncated in the cytoplasmic domain (FGFR1dn) (35). The dominant negative FGFR1dn can dimerize with the FGFR1 and conceivably with the other FGFRs and inhibit their signaling pathways (10, 35). FGFR1dn concentration-dependently inhibited ERK-1/2 phosphorylation induced by rFGF-2, in CAT (Fig. 8A) and A3 cells (Fig. 8B). Additionally, the expression of increasing amounts of the truncated receptor did not modify the basal phosphorylation state of ERK-1/2 in the presence of anti-FGF-2 antibodies, either in control CAT cells that do not produce FGF-2 (Fig. 8A) or in A3 cells (Fig. 8B) producing the HMW FGF-2. These results did not support the implication of intracellular FGFRs in the intracellular regulation. They also confirm the previous results excluding the activation of the cell surface FGFRs in the action of the HMW FGF-2.

ERK-1 and -2 Phosphorylation Is PKC δ-dependent—It is known that the secretory FGF-2 activates different PKC isotypes through the occupancy of FGFRs (36, 37), and PKCs have been involved in the activation of the MEK pathway (38, 39). Then the question arises of whether ERK phosphorylation by the HMW FGF-2 is PKC-mediated. To answer that question, three PKC inhibitors were used: GF109203X, which inhibits most of the PKC isoforms, among them PKC α, δ, and ε (26–27); Go6976, which only inhibits the conventional PKCs α, β, and γ; and rottlerin, a specific inhibitor of PKC δ when used at the concentration of about 6 μM (40). Because the conventional PKC isotypes β1 and γ were not found by reverse transcriptase-polymerase chain reaction in any of the cell lines used in the present study, Gô 6976 must only decrease PKC α activity. At the concentrations used in the present study, any of these compounds modified cell shapes or attachment, indicating the absence of toxic effects as reported for other cell types (26, 41). Gô 6976 added for 24 h to the cell medium slightly decreased ERK-1 and -2 phosphorylation whatever the doxycycline concentration (Fig. 9). By contrast, GF109203X strongly reduced ERK-1 and -2 phosphorylation in cells producing the HMW FGF-2 (Fig. 9), suggesting that ERK activity was under the control of an unconventional PKC. Rottlerin decreased ERK-1
and -2 phosphorylation in cells producing the FGF-2 of HMW (Fig. 9). To confirm the specific role of the δ isotype, cells were transfected with cDNA encoding either normal or mutated PKC δ. The normal δ isotype induced an increased phosphorylation of ERKs whatever the level of the HMW FGF-2 (results not shown), whereas cell transfection with increasing concentrations of cDNA encoding the nonfunctional PKC δ resulted in a concentration-dependent decrease of ERK-1 and -2 phosphorylation only in cells expressing the HMW FGF-2 (Fig. 10; 0 ng doxycycline). Furthermore, at the maximal concentration of the mutated PKC δ (9 μg), the phosphorylation level was comparable with that of control cells (Fig. 10; 2000 ng of doxycycline). These data clearly indicate that ERK activation was PKC δ-dependent and that this PKC isotype must be in the active state in HMW FGF-2 expressing cells.

According to the fact that phosphorylation and translocation to the particulate cell fraction is recognized as an index of the PKC δ activation (42), we analyzed the effect of the HMW FGF-2 on both parameters. In CAT cells transfected with the void tet-off vectors, doxycycline did not affect PKC δ levels and phosphorylation (Fig. 11A). Furthermore, at the maximal concentration of the mutated PKC δ (9 μg), the phosphorylation level was comparable with that of control cells (Fig. 10; 2000 ng of doxycycline). These data clearly indicate that ERK activation was PKC δ-dependent and that this PKC isotype must be in the active state in HMW FGF-2 expressing cells.

DISCUSSION

The results presented here demonstrate that the expression of the HMW FGF-2 can induce modifications of some intracellular signaling pathways through the regulation of calcium-independent PKC δ and ε levels and ERK-1 and -2 activation. By comparing cells stably producing the HMW FGF-2 to control CAT and LMW FGF-2 producing cells, differences in PKC levels were observed. Indeed, the HMW FGF-2 of 210 aa significantly modified the PKC amounts by increasing the δ isoform by a factor of 1.7 and by decreasing the ε one by a factor of 3. Transient transfection of control CAT cells with the cDNA encoding the HMW FGF-2 under the control of the tetracycline-dependent promoter confirmed these opposite regulations of PKC δ and ε and also showed the correlation between HMW FGF-2 concentration and PKC levels. Otherwise, as mRNA levels paralleled those of proteins, the modifications observed should take place at the pretranslational level. Our data also indicate that the two FGF-2 forms exert opposite effects on PKC expression, strongly suggesting the involvement of different regulatory mechanisms.

It is well established in different cell types that the HMW FGF-2 is not exported and exerts some specific biological effects.
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PKCs δ and ε as other PKC isotypes activate various signal transduction pathways, and some of these signaling pathways converge on ERK-1/2 (38). The functional relevance of the modified levels of PKCs was investigated by analyzing ERK-1/2 activation in cells producing the HMW FGF-2. The results obtained either with stably transfected A3 cells or transiently transfected CAT cells expressing variable amounts of HMW FGF-2 show an increased ERK-1 and -2 phosphorylation, correlated to increased ERK-1 and -2 activities. Therefore, our findings indicate that constitutive expression of HMW FGF-2 induces ERK activation.

Three lines of evidence point to the involvement of PKC δ in ERK activation. First, rottlerin, a specific inhibitor of the δ subtype, reduced ERK-1 and -2 activation. Second, overexpression of functional PKC δ in control cells increased ERK-1 and -2 phosphorylation, indicating that this PKC isotype acts upstream from ERK-1 and -2 in these cells. Finally, in cells expressing the HMW FGF-2, the inactivated PKC δ dose-dependently decreased ERK-1 and -2 phosphorylation. Thus, when overexpressed PKC δ activates the signaling cascade leading to ERK phosphorylation in cells producing the large form of FGF-2. Although the mechanism by which PKC δ regulates MEK-1 activation remains to be elucidated, recent studies show a PKC δ-mediated activation of MEK-1/ERK but not of c-Raf, suggesting that PKC δ acts downstream of c-Raf (44, 45). The HMW peptide enhanced ERK activity 2–3-fold, which is low compared with the increase of ERK activity observed early after cell stimulation by rFGF-2. However, the activation evoked by the HMW peptide was not short-lived because it was observed for at least 24 h in the doxycycline-regulated system and at any time in stably transfected A3 cells. Moreover, we found that MEK inhibition by PD98059 also decreased the growth promoting effect of the HMW FGF-2, indicating that the hyperphosphorylated ERK was functional and involved in the mitogenic effect of the HMW FGF-2.²

PKC δ and ε protein expressions were modified by the HMW FGF-2 at a pretranslational step, indicating that regulation occurred either at the transcriptional or at the mRNA half-life levels. We previously showed that the HMW FGF-2 regulated the expression of FGFR1 and laminin B1 by modifying the half-life of their transcripts (33, 8). A similar mechanism could be evoked for the PKCs. By contrast, ERK-1/2 proteins remained constant either after short term transfection with inducible HMW FGF-2 or in stably transfected cells, suggesting that the PKC δ controlled ERK activity but not ERK biosynthesis. However, an increased turnover rate of ERK-1/2 proteins cannot be excluded.

The observation that the HMW FGF-2 exerts opposite effects on PKC δ and ε levels is an important finding in the understanding of the intracrine regulation because these PKCs have been shown to exert opposite effects according to the cell type (46, 47). Recent data demonstrate the mitogenic action of PKC δ and the growth inhibitory effect of PKC ε (48). Opposite regulations have also been reported on the serum-responsive element, which plays an important role in the control of gene expression (49) and on the heat shock protein-27 phosphorylation which is involved in multiple cell functions (50). These

² A. Estival and F. Escalier, manuscript in preparation.
observations suggest that the different expression patterns of PKC δ and ε in cells producing the HMW FGF-2 might facilitate the mitogenic response induced by this growth factor.

The present data and our previous ones on the same cells provide a better understanding of the signaling pathways modulated by the HMW FGF-2. First, the HMW FGF-2 induces the overexpression of FGFRs in vivo (33) as also observed in vitro in different tumor types (51), thereby leading to an increased response to the extracellular FGF-2. Second, the HMW FGF-2 decreases the adenylate cyclase activity and thus reverses the growth inhibition induced by cAMP (19). Third, as shown in the present study, the HMW FGF-2 modulates the levels of PKC δ and ε. Altogether these results give new insights on the broad effects of the HMW FGF-2 on the intracellular signaling pathways, which can be involved in growth promotion, one of the well established biological effects of this FGF-2 isoform (1).

Analysis of the nuclear targets and gene promoters regulated by the HMW FGF-2 may lead to the characterization of the genes that are thereby activated and to further progress in the understanding of the mechanisms involved in the intracellular regulation.

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Expression of the High Molecular Weight Fibroblast Growth Factor-2 Isoform of 210 Amino Acids Is Associated with Modulation of Protein Kinases C \( \delta \) and \( \epsilon \) and ERK Activation

François Gaubert, Fabrice Escaffit, Claudine Bertrand, Murray Korc, Lucien Pradayrol, François Clemente and Agnès Estival

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