Cells retrovirally transfected with fibroblast growth factor-2 isoforms exhibit altered adenylate cyclase activity and G-protein functionality

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Basic fibroblast growth factor (FGF-2) is synthesized as different molecular mass isoforms all lacking the signal-peptide sequence. The high molecular-mass isoforms (21–24 kDa) possess a signal sequence directing their nuclear translocation. The role of each isoform is still poorly understood, however, modifications in intracellular signalling pathways could explain some effects of these peptides. In order to evaluate the role of FGF-2 isoforms on the adenylate cyclase (AC) signalling pathway, we retrovirally infected a rat pancreatic cell line (AR4-2J) with point-mutated FGF-2 cDNAs, allowing the expression of the 18 (A5 cells) or 22.5 kDa isoform (A3 cells) at a low level. In membrane preparations of A3 cells, unscheduled expression of the 22.5 kDa FGF-2 isoform induced a 2-fold decrease in both basal and forskolin-stimulated AC activity. Studies carried out on intact cells also showed decreased accumulation of cAMP in A3 cells in

the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. Both FGF-2 peptides also induced functional modifications of G-proteins without affecting their levels. The 22.5 kDa peptide led to enhanced ADP-ribosylation of both α_s -subunits *in vitro*, whereas the expression of the low molecular-mass 18 kDa peptide resulted in a 2-fold increase in α_{12} and α_0 ADP-ribosylations. Furthermore, control CAT cells (AR4-2J cells transfected with the retrovirus containing the chloramphenicol acetyltransferase gene) and A5 cells were growth-inhibited by 8-Br-cAMP, in contrast to A3 cells. These data provide evidence that the expression of FGF-2 peptides could play a role in cell functions by modifying the AC signalling pathway. FGF-2 peptides are able to modulate both AC activity and the regulatory G-proteins. Finally FGF-2 expression may interfere with cAMP-regulated cell proliferation.

INTRODUCTION

Basic fibroblast growth factor or FGF-2, belongs to a family of growth factors characterized by their affinities for heparin [1]. FGF-2 is distributed in many cell types. It is a key regulator of cell growth and differentiation and also modulates endocrine and neuronal functions in some cells [2–3]. Interestingly, FGF-2 lacks the signal-peptide sequence to direct its secretion. In FGF-2-producing cells, this factor is highly concentrated and the mechanisms involved in its secretion remain poorly understood [4].

At present, four different FGF-2 isoforms have been well characterized, their molecular masses ranging from 18 to 24 kDa. Molecular cloning of FGF-2 cDNAs demonstrated that these isoforms resulted from an alternative initiation of translation [5]. These endogenous peptides have distinct intracellular distributions. Whereas the 18 kDa form is mostly found in the cytosol, the larger forms are translocated to the nucleus since they possess an N-terminal extension corresponding to a nuclearization sequence [6]. The 18 kDa FGF-2 can be secreted and activates a family of plasma-membrane FGF receptors possessing tyrosine kinase activities [7]. Complementary to their intrinsic kinase activity, tyrosine kinase receptors have been reported to be directly and/or indirectly coupled to other second messenger systems, including cAMP, through cross-talk pathways [8]. In addition, the FGF-2 isoforms with their intracellular concen-

trations and subcellular localizations, have been suggested to play a role in 'intracrine' regulations [9].

There is increasing evidence that some cellular responses induced by activation of FGF receptors involve pertussis toxin (PTX)-sensitive G-proteins, for example, FGF-2-stimulated cell movement [10]. Indeed, G-proteins appear to be key components in some cellular signalling pathways [11,12]. The heterotrimeric G-proteins are composed of α -, β - and γ -subunits. Different types of subunits exist, including G_s -subunits, which stimulate adenylate cyclase (AC) activity, and G_i -subunits which are responsible for the inhibition of AC [13].

It is well documented that transfection of different cell lines with cDNAs encoding FGF-2 leads to morphological and biological modifications [14]. Expression of the 22.5 kDa, but not the 18 kDa, FGF-2, was found to be able to induce immortalization and autonomous cell proliferation [15]. Most of these changes were neither reproduced with exogenous FGF-2 nor abolished by neutralizing anti-FGF-2 antibodies, suggesting the involvement of intracellular isoforms [14,15]. The role of intracellular FGF-2 peptides in regulating expression and/or function of the AC signalling pathway is largely unknown. In order to address this question we used a rat pancreatic cell line AR4-2J [16], since expression of FGF-2 and FGF receptors and their implication in the secretory processes have been reported in normal pancreatic cells [17]. AR4-2J cells express FGF-2 receptors but do not synthesize detectable amounts of FGF-2 [16]. In

Abbreviations used: AC, adenylate cyclase; FGF-2, basic fibroblast growth factor; CTX, cholera toxin; DTT, dithiothreitol; FK, forskolin; HMM, high molecular-mass; IBMX, 3-isobutyl-1-methylxanthine; PACAP, pituitary adenylate cyclase activating peptide; PTX, pertussis toxin; CAT, chloramphenicol acetyltransferase; Gpp (NH)p, guanosine 5'-[β , γ -imido]-triphosphate.

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addition, these cells possess cell surface receptors for pituitary adenylate cyclase activating peptide (PACAP), which are positively coupled to AC, and for somatostatin, which mediate AC inhibition [18]. In order to obtain low-level expression of the 18 or 22.5 kDa FGF-2, a retroviral infection system was used [16].

We have shown that the expression of FGF-2 was able to interfere with the AC pathway by acting on AC activity and on G-proteins, and could reduce cAMP-mediated growth inhibition.

MATERIALS AND METHODS

Cell cultures

AR4-2J cells and all subsequent clones issued from transfected AR4-2J cells were routinely grown as previously described [16,19] in Dulbecco's modified Eagle's medium containing 4.5 g/litre glucose and 10% (v/v) fetal calf serum (Gibco, Grand Island, NY, U.S.A.). All cell lines were fed every 2 days and subcultured every 5 days. The absence of mycoplasma contaminations was checked routinely.

Transfection and infection

The transfections of the packaging CRIP cells and the following retroviral infection of AR4-2J cells have been described previously [16]. Briefly, the constructs were point-mutated FGF-2 cDNAs inserted into the mammalian retroviral expression vector PINA downstream of the β -actin promoter. After transfection, the geneticin-resistant cells were cloned. The clone A3F1, called A3, expressed only the 22.5 kDa FGF-2. The clone A5C12, called A5, allowed the expression of only the 18 kDa peptide. As a control, AR4-2J cells were transfected with the retrovirus containing the chloramphenicol acetyltransferase (CAT) gene and the clone CAT1 was used and is called CAT in the text.

Cell proliferation in the presence of 8-Br-cAMP

Cells were seeded at a density of 50 000 cells/cm² in complete medium, and 16 h later the medium was replaced with fresh complete medium containing increasing concentrations of 8-Br-cAMP. Cell were counted 48 h later.

Membrane preparations

Confluent cultures were washed twice in 20 ml of PBS, scraped, pelleted by centrifugation at 200 *g* for 5 min, then lysed with 1 mM NaHCO₃ and rapidly frozen in liquid nitrogen [20]. After thawing, the suspension was centrifuged at 1000 *g* for 10 min and the supernatant was recentrifuged at 15 000 *g* for 10 min. The resulting pellet was suspended in 1 mM NaHCO₃ and kept at -70 °C. Proteins were determined by the Bradford method (protein assay kit; Bio-Rad).

Adenylate cyclase assay

Assay on cell membranes

AC activity was determined as previously described [20]. Membrane proteins (20 μ g) were incubated in a total volume of 60 μ l containing 0.5 mM [α -³²P]ATP (Amersham, Les Ulis, France), 5 mM MgCl₂, 0.5 mM EDTA, 1 mM cAMP, 1 mM theophylline, 10 mM phosphoenolpyruvate, 30 μ g/ml pyruvate kinase, 30 mM Tris/HCl (pH 7.5) and 10 μ M GTP, when hormones were tested. The reaction was initiated by addition of membranes and was terminated after a 15 min incubation at 37 °C by adding 0.5 ml of 0.5% SDS solution containing 0.5 mM ATP, 0.5 mM cAMP and 20 000 c.p.m. of cyclic [8-³H]AMP (Amersham) to evaluate the cAMP recovery. cAMP was separated from ATP by two

successive chromatographies on Dowex 50W X8 and neutral alumina. AC activity remained linear for 15 min under these conditions. To test calmodulin-activated AC activity, membranes were washed twice with 2 mM EGTA at pH 7.0 and then the activity was determined in the absence of EGTA in the classical medium containing 50 μ M CaCl₂ and 0.3 μ M calmodulin. Calmodulin-activated AC activity was measured under a number of different conditions: either basal, or in the presence of guanosine 5'-[β , γ -imido]-triphosphate [Gpp(NH)p] or forskolin (FK) or calcium-calmodulin or calcium-calmodulin + FK or calcium-calmodulin + Gpp(NH)p.

Assay on intact cells

cAMP levels were measured in cells incubated in Krebs-Hepes-buffered medium containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). After a 30 min incubation at 25 °C the reaction was stopped by addition of ethanol and cAMP was determined by radioimmunoassay (Amersham).

ADP ribosylation by cholera toxin (CTX)

Membrane proteins (25 μ g) were resuspended in 20 μ l of 200 mM phosphate buffer, pH 6.8, containing 10 mM MgCl₂, 1 mM ATP, 0.1 mM GTP, 10 mM thymidine and 3 μ Ci of [³²P]NAD⁺ (Amersham). Pre-activated CTX (40 μ g) (Sigma, St. Louis, MO, U.S.A.) was added and ADP-ribosylation was carried out for 30 min at 37 °C. The reaction was stopped with 20 μ l of a 20% (v/v) trichloroacetic acid solution. After centrifugation (10 000 *g*, 5 min) the pellets were washed twice with diethyl ether. Pellets were resuspended in 25 μ l of Laemmli loading buffer, incubated for 30 min at 50 °C, boiled for 5 min at 100 °C and analysed by 10% SDS/PAGE. The gels were dried and exposed to X-OMat Kodak films (Rochester, NY, U.S.A.) at -70 °C. Autoradiographs were quantified by the computerized image analyser Biocom (Biocom, Les Ulis, France).

ADP-ribosylation by PTX

Membrane proteins (25 μ g) were resuspended in Tris/HCl buffer containing 1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.05% lubrol (v/v) and were incubated for 1 h at 30 °C in the presence of 3.71 $\times 10^4$ Bq of [³²P]NAD⁺ (2960 $\times 10^{10}$ Bq/mmol; Amersham) with 100 ng of preactivated PTX in a final volume of 60 μ l. The reaction mixture contained 70 mM Tris/HCl (pH 8), 0.5 μ M NAD⁺, 1 mM ATP, 0.1 mM GTP, 10 mM thymidine, 0.1 mM MgCl₂, 1 mM EDTA, 1 mg/ml myristyl phosphatidylcholine, 10 mM nicotinamide and 25 mM DTT. The reaction was stopped by addition of 2 μ g of BSA in 0.04% (w/v) SDS and proteins were precipitated by 70 μ l of 10% (v/v) trichloroacetic acid. Pellets were treated as for CTX-catalysed ribosylations.

Immunoblot analysis

Membranes proteins (50 μ g) were solubilized in 25 μ l of SDS sample buffer [60 mM Tris/HCl, 10% (v/v) glycerol, 3% (w/v) SDS, pH 6.8] and separated on a 12% polyacrylamide gel. After transfer, the nitrocellulose sheets were soaked in PBS containing 0.5% Tween 20 and non-fat dry milk (5% w/v). Antisera were diluted in the same buffer. The blots were incubated overnight at 4 °C. After washing, the nitrocellulose sheets were incubated with ¹²⁵I-Protein A and after washing in PBS/0.5% (w/v) Tween the immunoblots were autoradiographed. Anti-G-protein α -subunits, α_{12} (or α_{11}) and α_{13} (closely related to α_0), were produced against the C-terminal decapeptides [21], and α_s (identical for the 45 and 52 kDa forms of α_s) was a gift from Dr. Y. Audigier

(CNRS 9925, Toulouse, France). Anti- β -subunit antibodies directed against the amino acid sequence 130–145 of β_1 , were from Gramsh Laboratories (Schwabhausen, Germany)

RESULTS

Effect of FGF-2 expression on AC activity

The AC system comprises at least three possible levels of regulation: (1) the catalytic unit; (2) the regulatory G-proteins; and (3) the hormone receptors. We compared the effects of the two isoforms of FGF-2 at these three different levels.

Membrane preparations from the different cell lines were tested for basal and stimulated AC activity. As shown in Table 1, the expression of the 22.5 kDa FGF-2 (A3 cells) decreased by 50 % the basal activity of AC. A5 cells expressing the short 18 kDa peptide, exhibited the same activity as control CAT cells. Since FGF-2 is known to mobilize calcium, the activity of a calcium-calmodulin-dependent AC was measured under basal and under different stimulatory conditions (as described in the Materials and methods section) with the aim of detecting the induction by FGF. The enzyme activity was observed in all cell lines but at very low levels (results not shown). To determine whether the altered AC activity induced by FGF-2 expression was related to some modification either of regulatory guanine nucleotide function or of cyclase catalytic unit activity, the effects of FK and a non-hydrolysable GTP analogue, Gpp(NH)p, were examined.

As shown in Table 1, the ability of FK to stimulate AC activity in cells expressing the high molecular-mass (HMM) peptide was significantly attenuated (by about 60 %) when compared with A5 and CAT cells. Since functional modifications could occur during

Figure 2 Effect of Gpp(NH)p on basal and stimulated AC activities

AC activity was determined in membranes isolated from control CAT and FGF-2-expressing cells. cAMP synthesis is reported as pmol/min per mg of protein. (a) Effect of increasing concentrations of Gpp(NH)p on the basal AC activity. (b) Effect of increasing concentrations of Gpp(NH)p on the cyclase activity stimulated by FK (0.01 mM). Results are means of three independent experiments: $\blacktriangle$, CAT; ∇ , A3; $\square$, A5.

membrane preparations, through the disruption of plasma membrane micro-domains [12], experiments were repeated on intact cells in the presence of IBMX. The data reported in Figure 1 show that as observed on cell membranes, the basal value of cAMP was decreased by about 40 % and the FK-stimulated level was also reduced by 50 % in A3 cells. The parallel between the results obtained with membrane fractions and whole cells suggest that AC activity was already impaired in intact cells and was unrelated to any protein or lipid modifications occurring during cell fractionation.

We next examined in cell membranes the effects of Gpp(NH)p on basal and FK-stimulated AC activity. Gpp(NH)p, in the range 10^{-8} – 10^{-6} M, dose-dependently inhibited the basal activity of the enzyme, suggesting the coupling of AC with α_i . The inhibition was weak (around 30 %), reached a maximum at 0.5 – $1 \mu\text{M}$ (Figure 2a) and thereafter the inhibition tended to disappear at higher concentrations of the GTP analogue. By contrast, Gpp(NH)p only inhibited dose-dependently the FK-stimulated cyclase activity (Figure 2b), with IC_{50} values of 4.8×10^{-8} M for A5 cells and 1.7×10^{-7} M for A3 and CAT cells. In A3 cells the amounts of cAMP synthesized always remained lower than in the other cells, reflecting the decreased cyclase activity in these cells.

Identical results were obtained with transfected cell lines before their cloning, providing evidence that the modifications observed were not due to the clones used for these experiments.

Studies at the G-protein level

The reduction of the AC activity indicated that the expression of the 22.5 kDa FGF-2 exerted a control on the cyclase system. This control might also take place at the regulatory G-proteins, therefore we also analysed $\text{G}\alpha_s$ and $\text{G}\alpha_i$ proteins and examined the extent of toxin-catalysed [^{32}P]ADP-ribosylation.

ADP-ribosylation

In membranes isolated from the different cell lines the PTX-catalysed [^{32}P]ADP-ribosylation of $\text{G}\alpha_i$ resulted in the labelling

Table 1 Adenylate cyclase activity in membranes from control and FGF-2 expressing cells

Values are means $\pm$ S.D. for three independent experiments done in triplicate. The values in parentheses indicate the fold increase in activity in the presence of $10 \mu\text{M}$ forskolin.

	cAMP (pmol/min per mg of protein)		
	CAT	A5	A3
Basal	75.8 ± 26	77.2 ± 24	39.4 ± 7.5
Forskolin ($10 \mu\text{M}$)	345 ± 60	362 ± 94	114 ± 14.5
	($\times 4.55$)	($\times 4.7$)	($\times 2.9$)

Figure 1 cAMP levels in control and FGF-2-expressing cells

Intact cells were preincubated for 30 min in the presence of 0.5 mM IBMX and then incubated for a further 30 min either with IBMX (control) or with IBMX and $10 \mu\text{M}$ FK. These experiments were done using three different dishes and repeated three times. Values are means $\pm$ S.D.

Figure 3 PTX-catalysed ADP-ribosylation of α -subunits

Membranes were prepared from control CAT (Cat) and FGF-2-expressing cells. Membrane proteins (25 μ g) were ADP-ribosylated in the presence of PTX as described in the Materials and methods section. Proteins were then resolved by SDS/PAGE and autoradiography. Three labelled bands were found. Molecular masses were determined by the position of pre-stained molecular-mass markers. Results are representative of two similar experiments each run in duplicate.

Figure 4 CTX-catalysed ADP-ribosylation of α_s -subunits

Membrane proteins (25 μ g) were ADP-ribosylated in the presence (+) and in the absence (–) of the toxin. After SDS/PAGE and autoradiography, two bands at 52 and 45 kDa were labelled, corresponding to the α_s -subunits. Results are representative of two independent experiments each carried out in duplicate. Cat, CAT.

of three bands with apparent molecular masses of 39, 40 and 41 kDa corresponding to α_0 -, α_{12} - and α_{13} -subunits. As shown in Figure 3, ADP-ribosylation was increased in A5 cells expressing the short FGF-2 form. Quantitative analysis performed on autoradiographs showed that in A5 cells the amounts of [32 P]ADP-ribose incorporated after PTX treatment was doubled in both the 39 and 40 kDa bands compared with control and A3 cells. No significant differences between control and A3 cells were observed.

The CTX-catalysed ADP-ribosylation of α_s -subunits resulted in the labelling of two bands of apparent molecular mass 52 and 45 kDa, with a stronger labelling of the 52 kDa form (Figure 4). Cells expressing the HMM FGF-2 peptide incorporated 1.65-fold more [32 P]ADP-ribose in the two α_s -subunits than the other cells.

Thus compared with control cells, ADP-ribosylation appeared to be modified in FGF-2-expressing cells. Indeed α_s ribosylation was increased in A3 cells whereas α_i and α_0 ribosylation was

Figure 5 Effect of PACAP-27 and SMS on AC activity

Membranes prepared from the different cell lines were incubated with increasing concentrations of SMS or PACAP-27, in the presence of 0.01 mM GTP. cAMP synthesis is reported as pmol/min per mg of protein. (a) Dose-dependent inhibition of AC activity by SMS. (b) Dose-dependent stimulation of AC activity by PACAP-27. Data presented correspond to experiments performed on two different cell preparations: $\blacktriangle$, CAT; ∇ , A3; $\square$, A5.

increased in A5 cells. These data suggested some modifications in either the subunit levels or functions.

Immunoblotting of G-proteins

In order to determine whether increased ribosylation was related to an increase in G-protein subunit content, we examined the steady-state amounts of the different α_s - and α_i -subunits by Western blots. For the α_s -subunits the levels of both 52 and 45 kDa species appeared to be unmodified (results not shown). Similarly, immunoblot analysis of G α_i -subunits with antibodies recognizing either α_{11-2} - or α_{13-0} -subunits revealed no differences in the expression of these subunits (results not shown). Additionally, antibodies directed against the common sequence of β -subunits did not reveal any differences in the steady-state levels among the cell lines (results not shown).

Thus, enhanced toxin-catalysed ADP ribosylation and Western blot analysis revealed functional G-protein alterations rather than abnormal expression of the α - or β -subunits.

Effect of FGF-2 expression on AC responses to hormonal stimulations

AC activity can be modulated in AR4-2J cells in both a positive and negative manner by G $_s$ - and G $_i$ -coupled receptors respectively. In order to analyse the modifications of the AC response to hormone stimulations, we measured on cell membranes the AC responses to PACAP, which is positively coupled to the AC system, and to the somatostatin-stable analogue SMS, which is negatively coupled to the cyclase [18].

SMS dose-dependently inhibited cAMP synthesis (Figure 5a) in all cell lines by similar levels (40–50%). This inhibition was maximal at 1 μ M.

PACAP-27 dose-dependently elevated cAMP synthesis in control cells as well as in FGF-2-expressing cells. EC $_{50}$ values, which were of the order of 1–5 nM, were similar in all cell lines and identical with the values already described [18]. Maximal cAMP responses were increased over basal values 2.9-fold for CAT, 3.3-fold for A3, and 2.2-fold for A5 cells (Figure 5b). Binding experiments performed on cell membranes with 125 I-PACAP-

Figure 6 Effect of 8-Br-cAMP on cell proliferation

Cells were treated for 48 h with increasing concentrations of the cAMP analogue 8-Br-cAMP. Then cells were harvested by trypsin treatment and counted. Data are the means of two independent experiments each with triplicate determinations.

27 did not show any differences in the density of the cell surface receptors (results not shown). Thus, the stimulatory action of PACAP induced a high cAMP response in HMM FGF-2-expressing cells in the absence of any modification of the receptor level.

Effect of exogenous FGF-2

FGF-2 has been observed at the cell surface of A5 cells but not of A3 cells. The FGF-2 secreted by A5 cells activates the FGF receptors. In order to evaluate the effect on the AC system of the 18 kDa FGF-2 produced by A5 cells, control CAT cells were treated for 3 days with 1 nM recombinant FGF-2 before membrane preparation. AC activity, either under basal conditions or after FK and Gpp(NH)p stimulation, was similar to that found in untreated CAT cells (results not shown). Therefore, activation of the tyrosine kinase-coupled FGF receptors by the ligand does not explain the results obtained with A5 cells.

Role of cAMP in cell proliferation

In order to understand better the biological consequences of the modifications of the cyclase system induced by FGF-2 expression, we analysed the effect of cAMP on cell proliferation. Cells were grown in the presence of increasing concentrations of 8-Br-cAMP and were counted 48 h later. As shown in Figure 6, nucleotide treatment had no effect on A3 cells growth while it decreased by 40 % the proliferation of the two other cell lines. All these data suggest that unregulated expression of the 22.5 kDa FGF-2 counteracts the inhibitory action of cAMP. Similar modifications in cell growth were observed with uncloned transfected cells, further confirming that the mitogenic responses were unrelated to the clones analysed, as already found for cyclase activity.

DISCUSSION

In view of the fact that many cell types synthesize FGF-2 as different isoforms and that this growth factor exerts multiple effects, it is of interest to know the specific role of each FGF-2 isoform. It has already been established that the HMM FGF-2 isoforms which possess a nuclearization signal are able to

immortalize cells, unlike the 18 kDa form. The 18 kDa isoform has also been shown to be nuclearized through receptor-mediated pathways [22,23]. When co-expressed in cells, high and low molecular-mass peptides are able to induce cell transformation, suggesting cooperation between these isoforms [15]. Nuclear translocation occurring through receptor-coupled endocytotic pathways has already been reported for several growth factors, such as epidermal growth factor, platelet-derived growth factor and transforming growth factor- α [24], however, the biological functions exerted by these factors at the nuclear level remain to be established. The present study was undertaken in order to analyse the role of two FGF-2 isoforms. We developed pancreatic acinar cell lines expressing only one single FGF-2 isoform, either the 18 kDa or the 22.5 kDa form [16]. These cell lines infected by a replication-defective retrovirus containing point-mutated FGF-2 cDNAs were found to produce low amounts of FGF-2, unable to modify cell morphology, cell differentiation, biosynthesis and storage of digestive secretory enzymes and cell regulation by glucocorticoids [16].

Several reports have led to the hypothesis that some biological effects of FGF-2 can be modulated through G-proteins and AC [8,10]. In the present study we observed that under basal conditions, CAT and A5 cells exhibited higher levels of AC activity in the membrane fraction (about 2-fold higher) as compared with A3 cells expressing the HMM FGF-2. Similar results were also obtained with intact cells in the presence of a phosphodiesterase inhibitor (IBMX), suggesting that the observed decrease in A3 cells was unrelated to membrane modifications occurring during cell fractionation. By using FK to stimulate intact cells, similar results were also obtained, i.e. the increase of the cAMP level was still significantly lower in A3 cells. It is interesting to note the lack of any evident modification of the cyclase activity in cells expressing the 18 kDa FGF-2. This finding does not agree with the involvement of FGF receptor activation and nucleolar translocation of the secreted 18 kDa form in the modulation of the cyclase activity. In support of this hypothesis, we found that control CAT cells grown in the presence of exogenous FGF-2 did not show modifications of AC activity. These data are not in marked contrast with those previously reported on 3T3 cells since only a small decrease of AC activity (about 19 %) was observed under FGF-2 treatment [8]. In order to check that the slight effect of the 18 kDa form on AC activity was unrelated to the low level of this isoform in A5 cells, we analysed another cell line, bovine endothelial ABAE cells, transfected with the same retroviral vectors [15]. ABAE-A5 cells express about seven times more 18 kDa FGF-2 than AR4-2J-A5 cells, whereas ABAE-A3 cells produce similar amounts of the HMM form as AR4-2J-A3 cells. FK was found to increase the AC activity of the cell membranes 19-fold in ABAE-A5 cells and 11-fold in ABAE-A3 cells. Thus, in endothelial cells producing the 22.5 kDa form, AC activity was about 50 % lower than that of cells expressing the 18 kDa form, as was also observed in pancreatic cells (A. Estival and F. Clemente, unpublished work). These data also support the involvement of the HMM FGF-2 in the decrease in AC activity. Taking into account the fact that the HMM FGF-2 is not secreted, the whole data suggest that the modifications induced by this isoform involve some intracrine mechanisms. The possibility that FGF-2, a known calcium-mobilizer [3], could induce the expression of a calcium-calmodulin-dependent AC was also analysed in AR4-2J-transfected cells, but this enzyme was hardly detectable in all cell lines.

As G-proteins play an important role in the regulation of the cyclase activity, we analysed the levels and functions of G-proteins in the different cell lines. Gpp(NH)p is a well-known

activator of both stimulatory and inhibitory G-proteins, and the AC response results from the activation of both $G\alpha_i$ and $G\alpha_s$. In the present study we observed that this GTP analogue dose-dependently decreased FK-stimulated AC activity, suggesting that in these pancreatic cells the inhibitory component predominates. Some changes were observed in the toxin-catalysed ADP-ribosylation of both α_s - and α_i -subunits. Indeed, CTX-catalysed ADP-ribosylation increased the labelling of α_s -subunits in cells expressing the HMM FGF-2, whereas PTX increased ribosylation of both α_{12} and α_0 in cells expressing the 18 kDa FGF-2 peptide. Some functional changes of different subunits [25–27] are therefore induced by each isoform of FGF-2. Western blot analysis, carried out in order to see whether these data could results from altered G-protein levels, did not reveal any significant difference in the steady-state levels of α - and β -subunits for both G_i and G_s proteins.

Stimulation of AC through receptor occupancy might detect the functional state of these subunits. Somatostatin induced comparable inhibitory effects in all cell lines. In contrast, PACAP evoked some differences in cAMP responses, although the levels of the PACAP receptors were found to be similar in all cell lines. Notwithstanding the decreased AC found in A3 cells, the maximal response was not very different from that observed in the two other cell lines. Whatever the mechanism involved [25,27], these data reflect an increased cyclase response induced by HMM FGF-2 expression.

To evaluate the effect on cell functions of the present observations, experiments were carried out by treating intact cells with cAMP, a growth inhibitor of these pancreatic cells. In 22.5 kDa-expressing cells, cAMP was less efficient at inhibiting cell proliferation.

All the data indicate that expression of the FGF-2 peptides can modify AC activity and induce functional changes in regulatory G-proteins. In addition, the 22.5 kDa isoform modifies cell growth by altering the cellular responses induced by a cAMP analogue. According to the different effects of the two FGF-2 isoforms, these findings also suggest that the co-expression of these isoforms, generally found in normal and malignant cells, exerts some cooperative effects on the regulation of cyclase-related pathways, as already described for cell transformation by FGF-2 [15,28]. Until now, only the cellular effects evoked by exogenous FGF-2 have been extensively analysed, the present data bring new insights into the intracrine regulations involving the intracellular isoforms.

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