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Regulation by growth factors (IGF-I, b-FGF and TGF-β) of proto-oncogene mRNA, growth and differentiation of bovine adrenocortical fasciculata cells

I. Viard, C. Jaillard and J.M. Saez

INSERM-INRA U 307, Hôpital Debrousse, 29 Rue Soeur Bouvier, 69322 Lyon Cedex 05, France

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Nuclear proto-oncoproteins have been implicated in the regulation of gene expression by peptidic hormones and growth factors during cell proliferation and differentiation. In the present study we have investigated in bovine adrenal cells (BAC) the effects of basic fibroblast growth factor (b-FGF), insulin-like growth factor 1 (IGF-I) and transforming growth factor β (TGF-β) on c-jun, jun-B and c-fos mRNA levels and on cell growth and differentiation. Factors able to enhance the three proto-oncogenes (IGF-I, b-FGF and angiotensin II (A-II)) stimulate cell growth, whereas those inhibiting cell growth (TGF-β and ACTH) decrease c-jun mRNA level. These results suggest that expression of c-jun may be required to induce cell proliferation. The relation between proto-oncogenes and the expression of differentiated functions appears to be more complex. Whereas IGF-I, b-FGF and A-II increase the three nuclear proto-oncogenes, the effects of IGF-I and b-FGF on cytochrome P_{450} 17α-hydroxylase mRNA levels are opposite to those of A-II. On the other hand, while TGF-β and A-II have inhibitory effects on P_{450} 17α mRNA level, they have opposite effects on c-jun mRNA.

Nuclear proto-oncogene; Proliferation; Differentiation

1. INTRODUCTION

Corticotropin (ACTH) and angiotensin-II (A-II) play a central role in the control of adrenal cell growth and function. In bovine adrenal fasciculata cells (BAC), A-II and ACTH not only cause an acute increase in steroidogenesis, but also have opposite, long-term effects on steroidogenic responsiveness and DNA synthesis. Whereas ACTH exerts long-term stimulatory influences on adrenal cell differentiated functions [1,2] and inhibits DNA synthesis [3,4], A-II inhibits differentiated functions [5,6] and increases DNA synthesis [7]. One mechanism which has been proposed to link hormone–receptor interaction with long-term effects is the induction of transcription regulatory proteins, and in particular the Fos and Jun nuclear proto-oncoproteins [8]. In BAC, we have demonstrated that both hormones increase c-fos and jun-B mRNA levels, whereas they have opposite effects on c-jun mRNA levels, A-II being stimulatory and ACTH inhibitory [9].

In addition, BAC growth and function can be modulated by growth factors, specifically transforming growth factor (TGF-β1) [10], basic fibroblast growth factor (b-FGF) [11] and insulin-like growth factor 1 (IGF-I) [12]. In the present report, we have examined the effects of the different growth factors on *c*-fos, *c*-jun and jun-B nuclear proto-oncogene mRNA levels, and have tried to establish a correlation between the early proto-oncogene regulation and the biological processes, namely differentiation and proliferation.

Correspondence address: J.M. Saez, INSERM U 307, Hôpital Debrousse, 29 Rue soeur Bouvier, 69322 Lyon Cedex 05, France. Fax: (33) 78 25 61 68.

2. MATERIALS AND METHODS

Synthetic A-II was obtained from Bachem (Bubendorf, Switzerland); corticotropin (ACTH) (Synacthen) from CIBA (Rueil-Malmaison, France) and porcine TGF-β1 from R&D Systems Inc. (Minneapolis, MN, USA). Human recombinant IGF-I was a gift of Dr. L. Fryklund and R. Gunnarson, Kabi (Stockholm, Sweden), and recombinant bFGF of Dr. M.T. Tauber (Toulouse, France). Insulin and transferrin were obtained from Sigma Chemicals Co. (St Louis, MO); Ham's F-12 medium and Dulbecco's modified Eagle's medium (DMEM) in powder form, nystatin, penicillin/streptomycin, trypsin/EDTA, fetal calf serum from Gibco (Paris, France). Mouse c-jun and jun-B cDNAs were kindly provided by Dr. I. Verma (Salk Institute, San Diego, CA) [13] and Dr. D. Nathans (Johns Hopkins, Baltimore, MD) [14], respectively. Rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA and cytochrome P_{450} 17α hydroxylase (P_{450} 17α) cDNA were gifts from Dr. J.M. Blanchard (Montpellier, France) [15] and Dr. M.R. Waterman (Dallas, USA) [16], respectively. c-fos was purchased from Oncor (Gaithersburg, MD).

2.1. *Cell isolation and culture*

BAC were prepared by sequential treatment of adrenal cortical slices with trypsin (0.19%) as previously described [6,9]. The cells were cultured in a chemically defined medium, Ham's-F12/DMEM (1:1), containing transferrin (10 μg/ml), insulin (5 μg/ml), vitamin C (10^{-4} M) and antibiotics. The experiments were carried out on cells cultured for 4 days.

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2.2. *RNA isolation and Northern- and slot-blot analysis*

For each treatment, the culture medium was removed from 25 cm^2 flasks, cells were harvested in guanidine thiocyanate, and total RNA was extracted according to the method of Chomczynski and Sacchi [17]. For Northern blot analysis, total cytoplasmic RNA (10 μg) was subjected to electrophoresis through 1% agarose gels and transferred under vacuum to Hybond-N nylon membrane (Amersham, UK) For each sample analysed by slot-blot, increasing amounts of total cytoplasmic RNA (1–8 μg) were transferred directly to Hybond-N nylon membrane by using a multiwell filtration manifold (BRL). Membranes with bound RNA were baked at 80°C to cross-link the RNA to the filters. Hybridization with DNA ^{32}P-labelled probes and the analysis of blots were carried out as previously described [9].

Analysis of each Northern blot was realized in duplicate. The densitometric values for the proto-oncogene mRNA levels were normalized to those for GAPDH to control for quantity of RNA transferred to the blot.

2.3. *[^{3}H]Thymidine incorporation into DNA*

About 8 h after the addition of growth factors, [^{3}H]thymidine (1 μCi/ml) was added and the incubation continued for 18–20 h. At the end of this period, the medium was removed, the cells were washed three times with 5% trichloracetic acid, and solubilized in 0.5 N NaOH, 0.4% deoxycholate Counting was carried out for 10 min in a liquid scintillation spectrometer with an efficiency of 65%.

2.4. *Statistics*

Data are reported as the mean ± S.E.M. for a minimum of three different cell preparations. Statistical significance was determined with Student's *t*-test. The null hypothesis was rejected when $P < 0.05$ was obtained.

3. RESULTS

In the first series of experiments, we investigated the acute effects of the different growth factors on proto-oncogene mRNA levels (Fig. 1). The maximally active concentration for each growth factor, as well as the time required to induce proto-oncogene mRNA change, were determined in preliminary experiments (data not shown). Both IGF-I and b-FGF significantly increased ($P < 0.05$) the mRNA levels of the three proto-oncogenes, although the effects of b-FGF were consistently higher than those induced by IGF-I. In contrast, TGF-β significantly reduced c-jun mRNA levels, had no effect on c-fos, and stimulated jun-B mRNA.

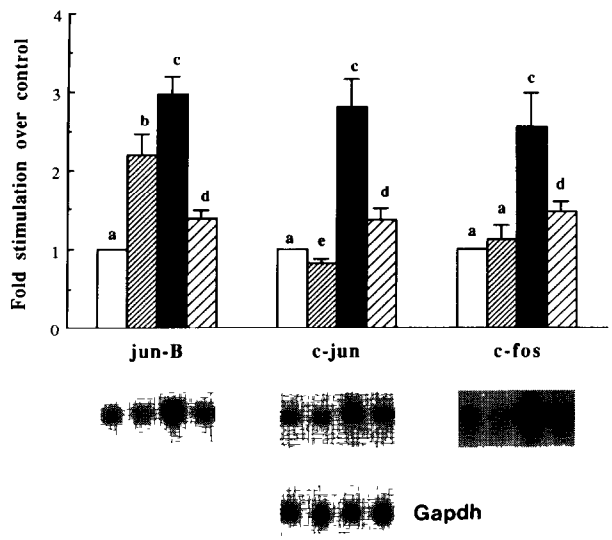

Fig. 1. Effects of growth factors on proto-oncogene mRNAs. On the fourth day of culture cells were incubated for one hour with fresh medium without (open bar) or with TGF-β (1 ng/ml) (thin hatched bar), b-FGF (10 ng/ml) (filled bar), IGF-I (50 ng/ml) (wide hatched bar). The cells were harvested, the RNA extracted, and 10 μg RNA/lane were electrophoresed, transferred to nylon membrane, and hybridized as outlined in section 2. (Top panel) Mean ± S.E M. of 3–11 experiments. Values with different letters are significantly different. (Lower panel) Representative Northern blots.

Table I

Effect of growth factors on [^{3}H]thymidine incorporation into BAC DNA

	cpm/well	
	− Insulin	+ Insulin
Control	810 ± 120	4,500 ± 340[b]
b-FGF 10 ng/ml	18,500 ± 800[a]	32,400 ± 1,200[a,b]
IGF-I 50 ng/ml	5,200 ± 460[a]	5,400 ± 350
TGF-β 1 ng/ml	790 ± 140	3,100 ± 210[a,b]

[a] $P < 0.05$ compared to Control of the same column.
[b] $P < 0.001$ compared to values of the same line.

Cells were seeded at 2×10^6 cells/well in complete medium supplemented with 0.5% fetal calf serum (FCS). Then the medium was removed and replaced by fresh medium (Deprivation medium: without FCS and without insulin). After 24 h the medium was replaced by fresh medium without or with insulin (10 μg/ml) in the absence or presence of the indicated growth factor. After 8 h [^{3}H]thymidine 1 μCi/ml was added and the incubation continued for 18–20 h. The incorporation of [^{3}H]thymidine into DNA was measured as indicated in section 2. The results are the mean ± S.E.M. of three experiments

Since the proteins encoded by the proto-oncogenes might play a role in the long-term response of cells to several stimuli, we investigated the effects of growth factors on BAC growth and differentiation. In the absence of insulin, b-FGF, and to a lesser extent IGF-I, enhanced [^{3}H]thymidine incorporation into DNA, whereas TGF-β had no effect (Table I). Insulin alone at micromolar concentrations, had a significant stimulatory effect, similar to that induced by IGF-I at nanomolar concentrations, suggesting that the mitogenic effect of insulin at this concentration was mediated through IGF type I receptors. This was confirmed by the fact that when both peptides were added together the effects were similar to those induced by each peptide alone. The mitogenic effect of b-FGF was, however, potentiated by insulin. In contrast, TGF-β slightly but significantly inhibited the mitogenic action of insulin (Table I) and that of b-FGF (data not shown).

The effects of growth factors on BAC differentiation were evaluated by measuring the mRNA levels of cytochrome P_{450} 17α-hydroxylase, a specific marker of BAC differentiation (Fig. 2). Insulin at micromolar concentrations enhanced P_{450} 17α mRNA levels about 2-fold. IGF-I (50 ng/ml) produced similar effects (data not shown). In the presence of insulin, b-FGF further en-

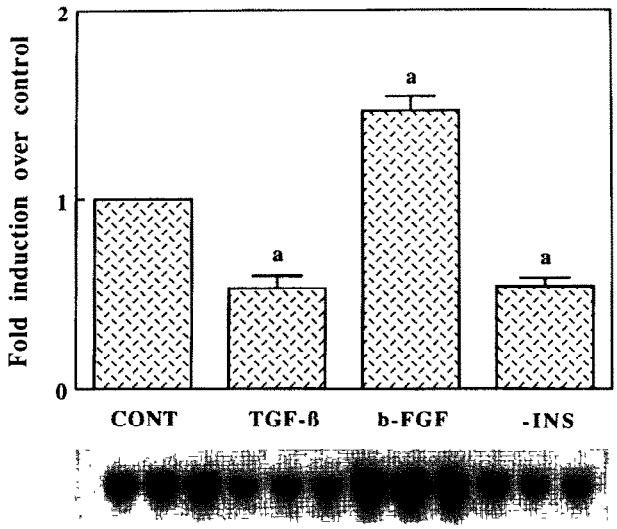

Fig. 2. Effects of growth factors on P_{450} 17α mRNA level. Cells were incubated for 36 h with fresh medium without (−INS) or with insulin (10 μg/ml) alone (CONT), or supplemented with TGF-β (1 ng/ml) or b-FGF (10 ng/ml). Three aliquots of 10 μg RNA were electrophoresed, transferred to nylon membrane, and hybridized as outlined in section 2. (Top panel) Densitometric scanning of 4 different experiments (mean ± S.E.M.). $^{a}P < 0.05$ compared to control. (Lower panel) Representative Northern blot

hanced P_{450} 17α mRNA levels, whereas TGF-β caused a 50% decrease.

In order to investigate the long-term effect of growth factors on BAC responsiveness to specific peptidic hormones, cells were pretreated for 36 h with either the standard culture medium containing insulin or with medium supplemented with b-FGF or TGF-β, and then stimulated with ACTH or A-II. The results of Figs. 3 and 4 show that pretreatment with b-FGF decreases the basal level of the three proto-oncogene mRNAs, but significantly enhanced the stimulatory effects of both ACTH and A-II on jun-B and c-fos mRNAs. Pretreatment with TGF-β reduced proto-oncogene mRNA levels both under basal conditions and following ACTH or A-II stimulation, but none of these values were statistically different from the corresponding values of cells cultured in the standard culture medium.

The steroidogenic responsiveness of BAC to both ACTH and A-II was also modified by pretreatment with growth factors (Table II). Insulin and IGF-I similarly enhanced the response to both hormones, b-FGF further enhanced the effects of insulin, and TGF-β completely blocked the effects of insulin. Indeed, the steroidogenic response of cells pretreated with insulin plus TGF-β was even lower than that of cells cultured in the absence of insulin.

4. DISCUSSION

Evidence has accumulated over the last 5 years that supports the concept that nuclear proto-oncoproteins are part of a complex network of signal transduction systems, and that they might be involved in the regulation of cell growth and differentiation by peptide hormones and growth factors (reviewed in [18,19]). Therefore, in the present study, we have investigated the effects of three growth factors on c-jun, c-fos and jun-B mRNA levels, cell growth, and cell differentiation using a well-differentiated cell type. Both IGF-I and b-FGF acutely increased the proto-oncogene mRNA levels, and enhanced DNA synthesis and cytochrome P_{450} 17α mRNA levels, a very specific marker of adrenal cell differentiation [16,20], in a long-term manner. Moreover, pretreatment with both IGF-I and b-FGF enhanced the biological response (cortisol production) to ACTH and A-II, the two specific hormones that regulate adrenocortical secretion. It is likely that the increased responsiveness of cells pretreated with growth factors is not only due to an increase in P_{450} 17α, but also to an increased expression of genes encoding the receptors for both hormones, since at least IGF-I increases the receptor number for both hormones [12,21]. Although the effects of both growth factors are thought to be mediated by specific membrane tyrosine kinase receptors [22], the effects of b-FGF on cell parameters studied were higher than those induced by IGF-I.

The TGF-β receptor has been recently cloned [23,24], but its mechanism of action still remains unknown [25]. In BAC, TGF-β increased jun-B mRNA, but either had no effect or decreased the mRNA levels of c-fos and c-jun, respectively, and these early effects were associated with long-term inhibition of cell growth and differentiation. The inhibitory effects of TGF-β on P_{450} 17α, similar to that previously reported [26,27], is one of the

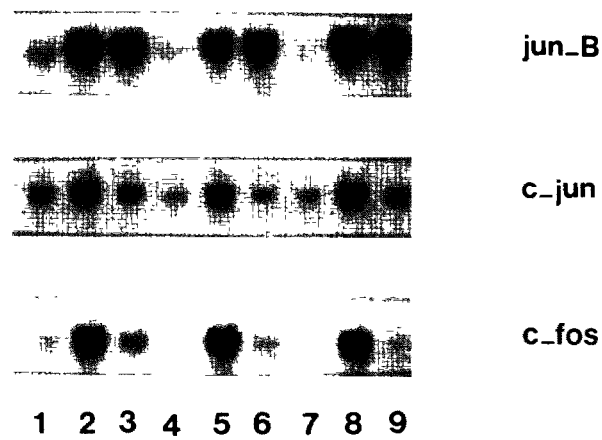

Fig. 3. Effects of long-term treatment with growth factors on basal and ACTH- or A-II-stimulated proto-oncogene mRNA levels; representative Northern blots. On the fourth day of culture, cells were incubated for 36 h with fresh medium without (lanes 1–3) or with TGF-β 1 ng/ml (lanes 4–6), b-FGF 10 ng/ml (lanes 7–9). After this pretreatment the medium was changed and replaced by fresh medium without (lanes 1,4,7) or with 10^{-7} M A-II (lanes 2,5,8), or 10^{-9} M ACTH (lanes 3,6,9) for 1 h. The cells were then harvested and the RNA extracted, and 10 μg RNA/lane electrophoresed, transferred to nylon membrane, and hybridized as outlined in section 2.

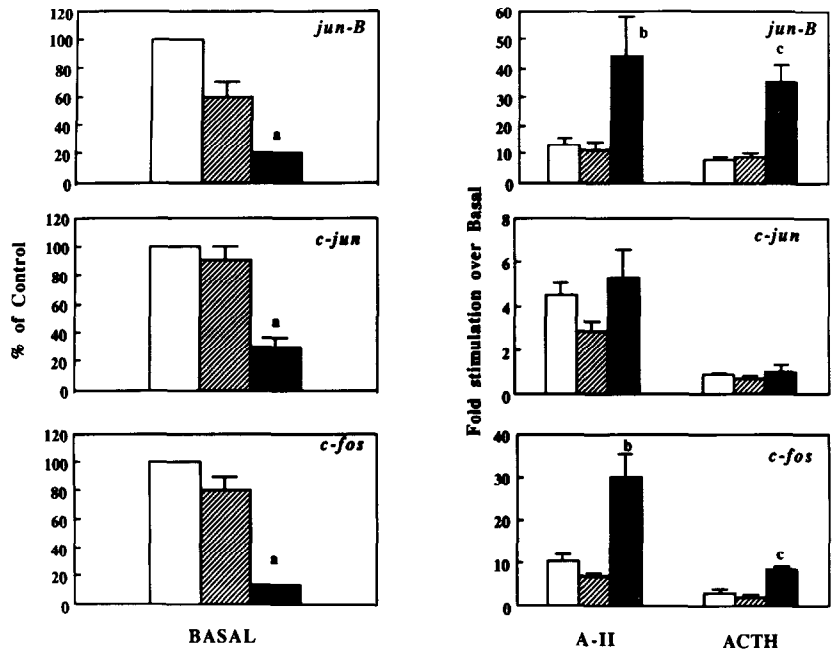

Fig. 4 Effects of long-term treatment with growth factors on basal and A-II- or ACTH-stimulated proto-oncogene mRNA levels; results of densitometric scanning of Northern blots. On the fourth day of culture, cells were incubated for 36 h with fresh medium without (control, open bar) or with TGF-β 1 ng/ml (hatched bar), b-FGF 10 ng/ml (filled bar) After this pretreatment the medium was changed and replaced by fresh medium without (BASAL) or with 10^{-7} M A-II, or 10^{-9} M ACTH for 1 h. The cells were then harvested, the RNA extracted, and 10 μg RNA/lane electrophoresed, transferred to nylon membrane, and hybridized as outlined in section 2. The results are the mean ± S.E.M. of 3–9 different experiments. [a] $P < 0.05$ compared to control/basal. [b] $P < 0.05$ compared to control/A-II. [c] $P < 0.05$ compared to control/ACTH.

pleiotropic inhibitory effects of this peptide on adrenal cells [28,29], which explains the very low responsiveness of TGF-β-pretreated cells to both ACTH and A-II.

Taken together, the above results indicate that factors able to enhance the three proto-oncogenes, stimulate cell growth. This conclusion is confirmed by the fact that A-II, which enhanced the expression of the three proto-oncogenes ([9] and present data), also has a mitogenic action on BAC [7]. On the other hand, ACTH, which stimulates the expression of jun-B and c-fos ([9] and present data) but decreases c-jun, and TGF-β, which also decreases c-jun, inhibit cell multiplication [3,4]. These results therefore suggest that expression of c-jun may be required to induce cell multiplication. In favour of this hypothesis are recent data showing that over-expression of c-jun induces cell multiplication [30].

The relationship between proto-oncogenes and the expression of differentiated functions appears to be more complex. In BAC, ACTH enhances transcription of the genes encoding the enzymes of the steroidogenic pathway (cytochrome P_{450} 17α, P_{450} 21-hydroxylase and P_{450} cholesterol side chain cleavage) [20] through cAMP. The upstream regions of these genes have been found to contain unique cAMP-responsive sequences not observed in other genes for which transcription is regulated by cAMP [31]. Thus in BAC, both inhibition of cell growth [4] and differentiation (P_{450} 17α) can be directly regulated by cAMP. No classical AP-1 binding site has been found in the 5′-flanking region of P_{450} 17α [32], but it is known that the homodimer (Jun) and heterodimer (Fos/Jun) of proto-oncoprotcins may bind to sequences other than the classical (TGACTCA) AP-1 binding site [18]. Moreover, if the proto-oncoproteins (homo and/or heterodimer) are involved in the regulation of P_{450} 17α, it remains to be explained why the effects of IGF-I and b-FGF are opposite to those of A-II, whereas the effects of the three growth factors on

Table II

Long-term effects of growth factors on the steroidogenic responsiveness of BAC to ACTH and A-II

	Cortisol production (ng/10^6 cells/2 h)	
	ACTH	A-II
Control	48 ± 4[a]	51 ± 5[a]
IGF-I (50 ng/ml)	165 ± 15[b]	154 ± 11[b]
Insulin (10 μg/ml)	158 ± 18[b]	148 ± 15[b]
Insulin + b-FGF (10 ng/ml)	225 ± 12[c]	214 ± 13[c]
Insulin + TGF-β (1 ng/ml)	22 ± 4[d]	19 ± 3[d]

Cells were cultured for 36 h with the indicated factors. Then the medium was removed and cells were stimulated with 10^{-9} M ACTH or 10^{-7} M A-II for 2 h, after which cortisol was measured in the medium. Means bearing different superscripts are significantly different ($P < 0.05$). The results are the mean ± S.E.M. of three experiments.

proto-oncogene mRNAs are all stimulatory. Since in some cell models the effects of jun-B and c-jun may be antagonistic [33], a difference in action between these proto-oncoproteins could explain the above discrepancy. It also remains to be demonstrated whether the inhibitory effect of TGF-β on P_{450} 17α expression is solely related to its effect on proto-oncogenes. To clarify the potential role of proto-oncogenes on the expression of genes encoding for BAC differentiated functions, we have started transfection experiments with these proto-oncogenes, either alone or in combination.

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