cfos and cjun antisense oligonucleotides block mitogenesis triggered by fibroblast growth factor-2 and ACTH in mouse Y1 adrenocortical cells

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Abstract

In G_0/G_1 cell cycle-arrested mouse Y1 adrenocortical cells, short pulses (30 min to 2 h) of fibroblast growth factor-2 (FGF2) (5 pM to 1 nM) caused induction of cFos protein by 2 h and onset of DNA synthesis stimulation by 8–9 h. FGF2 dose–response curves for cFos induction (percent labeled nuclei with a specific anti-cFos antibody) and DNA synthesis stimulation (bromodeoxyuridine labeling index) were linearly correlated with a correlation coefficient of 0·969. Inhibition of cFos and cJun protein induction with antisense oligodeoxynucleotides (ODNs) to cfos and cjun mRNAs blocked DNA synthesis stimulation by FGF2. Pulses (up to 2 h) of synthetic $ACTH_{39}$ (1 pM to 1 nM) and natural porcine corticotropin A (10 pg/ml to 1 μg/ml) also induced cFos protein and DNA synthesis in G_0/G_1-arrested Y1 adrenal cells. ACTH dose–response curves for cFos induction and DNA synthesis stimulation were not correlated. But cfos and/or cjun antisense ODNs blocked DNA synthesis stimulation by ACTH. Thus, signals initiated in FGF2 and ACTH receptors appear to converge to the induction of cfos and cjun genes to trigger DNA synthesis stimulation.

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Introduction

The molecular mechanisms by which adrenocorticotropin (ACTH) causes its growth effects on adrenal cortex remained obscure for decades. In the intact organism ACTH seems to be a mitogen, but in cell lines and primary cultures of mouse, rat, cow and human adrenocortical cells ACTH appears to be a growth inhibitory hormone (Hornsby 1985).

We recently reported, for the first time, that short pulses of ACTH trigger a mitogenic response in G_0/G_1-arrested Y1 adrenocortical cells, resembling fibroblast growth factor-2 (FGF2), albeit with a reduced intensity (Armelin *et al.* 1996, Lotfi *et al.* 1997). These observations suggested that the mouse Y1 adrenocortical cell line (Yasumura *et al.* 1966, Schimmer 1981) is a cell system experimentally useful to access cellular and molecular mechanisms of growth control activated by ACTH receptors in adrenocortical cells.

Both ACTH and FGF2, respectively, induce the fos and jun genes in G_0/G_1-arrested Y1 adrenocortical cells (Kimura & Armelin 1990, Kimura *et al.* 1993, Lotfi *et al.* 1997), following the same pattern of induction displayed by G_0-arrested Balb3T3 fibroblasts stimulated by FGF2. Fos (cFos, FosB, Fra1 and Fra2) and Jun (cJun, JunB and JunD) proteins dimerize via a leucine zipper domain to form heterodimers (Fos-Jun) and homodimers (Jun-Jun), making up the series of transcription factors denominated AP1 (for review see Angel & Karin 1991). AP1 factors seem to play a critical role in the control of $G_0 \rightarrow G_1 \rightarrow S$ transition of the cell cycle. Quiescent G_0-arrested fibroblasts exhibit very low levels of AP1 factors and upon stimulation by peptide growth factors, like FGF2 and platelet-derived growth factor, rapidly induce the fos and jun genes in an ordered fashion (Kovary & Bravo 1991*a*,*b*, Cook *et al.* 1999). Blocking of cFos and cJun proteins induction with an antisense technique (Holt *et al.* 1986, Nishikura & Murray 1987) or their activities with specific antibodies (Riabowol *et al.* 1988, Kovary & Bravo 1991*b*) is sufficient to block the $G_0 \rightarrow G_1 \rightarrow S$ transition in G_0-arrested fibroblasts stimulated with growth factors.

In this paper we show that blocking cFos and cJun protein synthesis with antisense phosphorotioate oligodeoxynucleotides (ODNs) is sufficient to inhibit DNA synthesis stimulated by pulses of FGF2 and/or ACTH in G_0/G_1-arrested Y1 adrenocortical cells. These results indicate that cFos and cJun protein induction is an obligatory step in the mechanisms triggered by FGF2 and ACTH to elicit a mitogenic response in Y1 adrenocortical cells.

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Materials and Methods

Cells and culture procedures

Y1 mouse adrenocortical carcinoma cells (Yasumura *et al.* 1966, Schimmer 1981) are a clonal cell line obtained from the American Type Culture Collection (Rockville, MD, USA) in 1973. Frozen stocks were kept in liquid nitrogen and routinely grown in Dulbecco's modified Eagle's medium with heat-inactivated 10% fetal calf serum (FCS), at 37 °C in a humidified atmosphere of 5% CO_2, 95% air.

Synthetic ACTH peptides, natural porcine corticotropin A and recombinant bovine FGF2

Synthetic $ACTH_{39}$, $ACTH_{24}$ and $ACTH_{7–38}$ and natural porcine corticotropin A batch 96H0687 (90 U/mg) were purchased from Sigma (St Louis, MO, USA). These Sigma peptide products were analyzed by HPLC and chemically characterized by mass spectrometry and N-terminal sequencing in the laboratory of Prof. L Juliano, at the Escola Paulista de Medicina (UNIFESP, São Paulo), where synthetic ACTH peptides were also synthesized. Bovine recombinant FGF2 was prepared in the laboratory of Prof. A Gambarini, from this Department of Biochemistry.

Phosphorotioate ODNs, antibodies and other reagents

18-mers of phosphorotioate ODNs were purchased from Gibco BRL (Life Technologies, Inc., São Paulo, Brazil) and Bio-Synthesis, Inc. (Lewisville, TX, USA) with the following sequences: cfos antisense 5′-GGCGTTGAAA CCCGAGAA-3′ and respective sense; cjun antisense 5′-CGTTTCCATCTTGCAGT-3′ and respective sense (Colotta *et al.* 1992). Rabbit polyclonal antibodies to mouse cFos and JunD proteins were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); mouse monoclonal anti-bromodeoxyuridine (BrdU) from Amersham Pharmacia Biotech (São Paulo, Brazil); lipofectin from Gibco BRL; and Vectastain Elite ABC kit for immunoperoxidase staining from Vector Labs Inc. (Burlingame, CA, USA).

Protocols of antisense experiments

Y1 cells were seeded on coverslips and starved in serum-free medium (SFM) for 48 h. Fresh solutions of ODNs (0·2–40 μM) and lipofectin (20 μg/ml), in SFM, were mixed in equal volumes and incubated for 30 min to allow for complex formation. Pairs of coverslips were pre-incubated for 3–4 h in 1 ml fresh SFM containing pre-formed ODN-lipofectin complexes, with ratios of ODN to lipofectin increasing from 0·1 μM:10 μg to 20 μM:10 μg. At time zero, 100 mU/ml porcine corticotropin A or 1 nM FGF2 were added to the medium and incubated for 2 h. To test the effect of ODNs in cFos synthesis the experiment was terminated at 2 h by rinsing the coverslips with PBS and fixing the cells with 3·7% formaldehyde in PBS for 20 min. On the other hand, when the aim was to estimate the effects of ODNs in S-phase entry by BrdU labeling, the experiment continued until 24 h. In this case, at 2 h the medium was sucked off and the coverslips were rinsed twice with PBS and re-incubated with fresh SFM containing ODN-lipofectin complexes. At 12 h, 100 μM BrdU was added to the medium and at 24 h the cells were fixed with methanol and stored in a refrigerator for further processing.

Immunocytochemistry for cFos or JunD protein detection and BrdU labeling indices

Nuclear cFos or JunD immune complex was visualized by immunoperoxidase staining using the Vectastain Elite ABC kit and diaminobenzidine. Nuclei positive for immune complex were stained heavily brown, whereas negative nuclei appeared bluish stained with Harris' hematoxylin and differentiated with a saturated solution of Li_2CO_3. Percent of BrdU-labeled nuclei was estimated with anti-BrdU antibody as previously described (Lotfi *et al.* 1997). Coverslips were randomly coded and 500–600 nuclei per coverslip were blindly counted. Results of independent experiments were pooled and statistically analyzed by Chi-square with 1 degree of freedom.

Results

Pulses of FGF2 and ACTH stimulate G_0/G_1-arrested Y1 adrenal cells to induce the cFos protein and to enter the S-phase of the cell cycle

Forty-eight hours of serum starvation in SFM caused cell cycle arrest at the G_0/G_1 boundary in Y1 adrenal cells. G_0/G_1-arrested Y1 cells resumed growth upon FCS re-feeding or FGF2 sustained treatment, displaying an onset of DNA synthesis initiation by 8–9 h (Fig. 1). Short pulses of FGF2 (30 min to 2 h) were sufficient to trigger this growth response, exhibiting the same kinetics for S-phase entry (Fig. 1 insert).

Two-hour pulses of synthetic $ACTH_{39}$ and natural porcine corticotropin A resembled FGF2, inducing cFos protein and stimulating DNA synthesis, but the pattern of the dose–response curves were different (Fig. 2). The dose–response curves for DNA synthesis stimulation and cFos induction by FGF2 were asymptotic and linearly correlated, displaying a coefficient of linear correlation of 0·969 and an ED_{50} of 6–18 pM (Fig. 2a). On the other hand, $ACTH_{39}$ and corticotropin A showed maximal stimulation of DNA synthesis at physiological concentrations, whereas the curves for cFos induction were shifted towards higher hormonal concentrations (Fig. 2b and c).

We tested the effect of the synthetic inactive ACTH fragment 7–38, a well-known competitive inhibitor of $ACTH_{39}$ (Li *et al.* 1978), on DNA synthesis stimulated by both $ACTH_{39}$ and corticotropin A, under the same

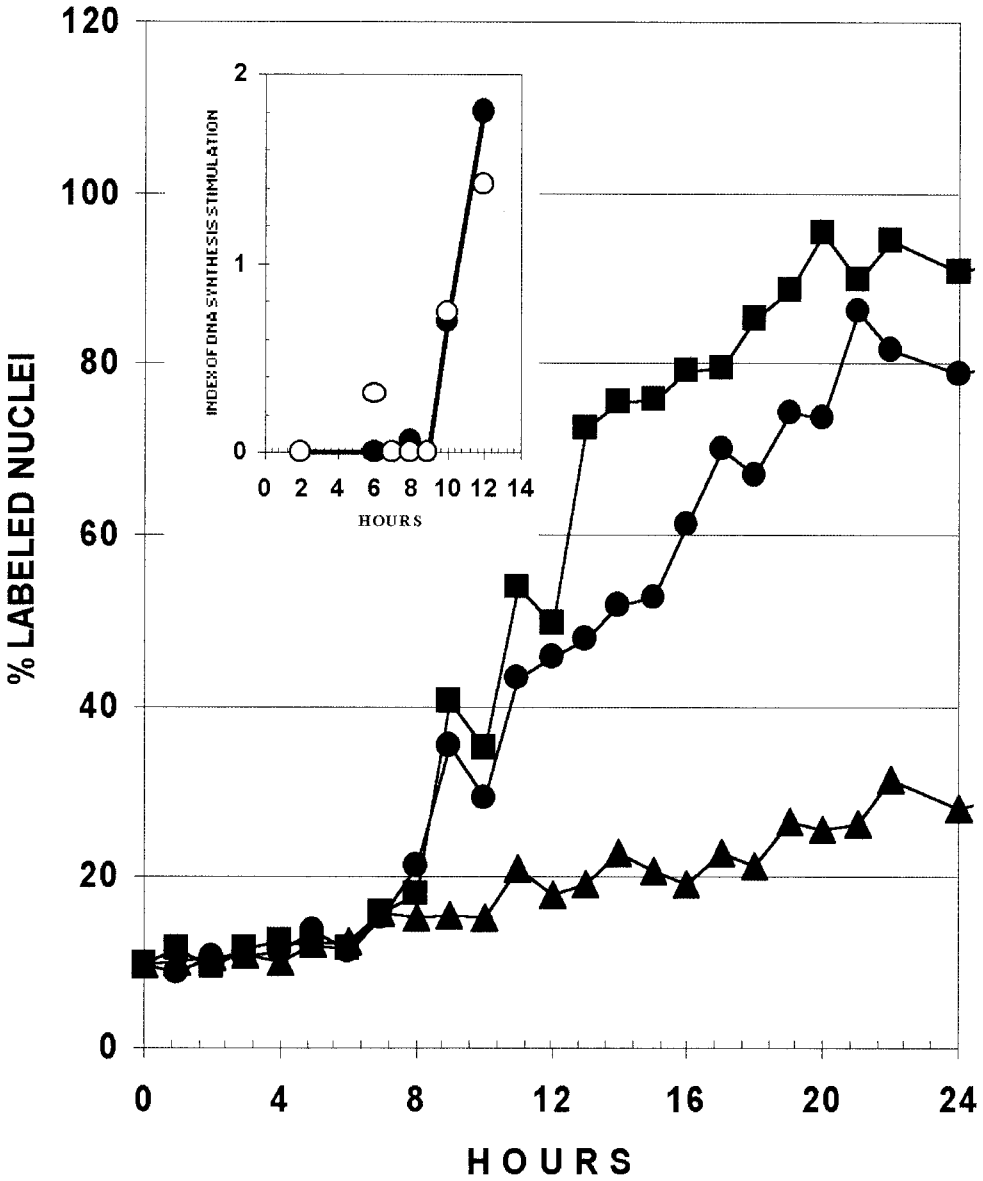

Figure 1 Kinetics of S-phase entry in G_0/G_1-arrested Y1 adrenocortical cell growth stimulated by treatment with FCS or FGF2. FCS (10%), 1 nM FGF2 and 100 μM BrdU were added to the SFM at zero time; every hour a pair of coverslips was collected for each condition and processed to estimate percent BrdU-labeled nuclei. Results from two independent experiments were pooled and plotted as percent labeled nuclei as a function of time. (▲) Control; (●) FGF2; (■) FCS. Insert: kinetics of S-phase entry after a sustained treatment or an initial 2 h pulse of FGF2. For sustained treatment (●), 1 nM FGF2 and 100 μM BrdU were added to SFM at zero time. For pulse treatment (○), 1 nM FGF2 was added for the first 2 h, coverslips washed twice with PBS, incubated with fresh SFM and 100 μM BrdU was incorporated in the last hour before collecting coverslips. The index of DNA synthesis stimulation for each time plotted in the graph is given by the expression: (percent labeled nuclei of treated cells minus percent labeled nuclei of untreated control cells) divided by percent labeled nuclei of untreated control cells.

conditions of the assays of Fig. 2b and c respectively. In the range 10^{-12}-10^{-9}M $ACTH_{39}$ and 10–1000 pg/ml corticotropin A, stimulation of DNA synthesis was inhibited 60–80% by $ACTH_{7-38}$ at 10^{-7} M; by itself, $ACTH_{7-38}$ at 10^{-7} M had no effect on DNA synthesis. These results suggest that stimulation of DNA synthesis by $ACTH_{39}$ and corticotropin A, in G_0/G_1-arrested Y1 cells, is mediated by specific ACTH receptors.

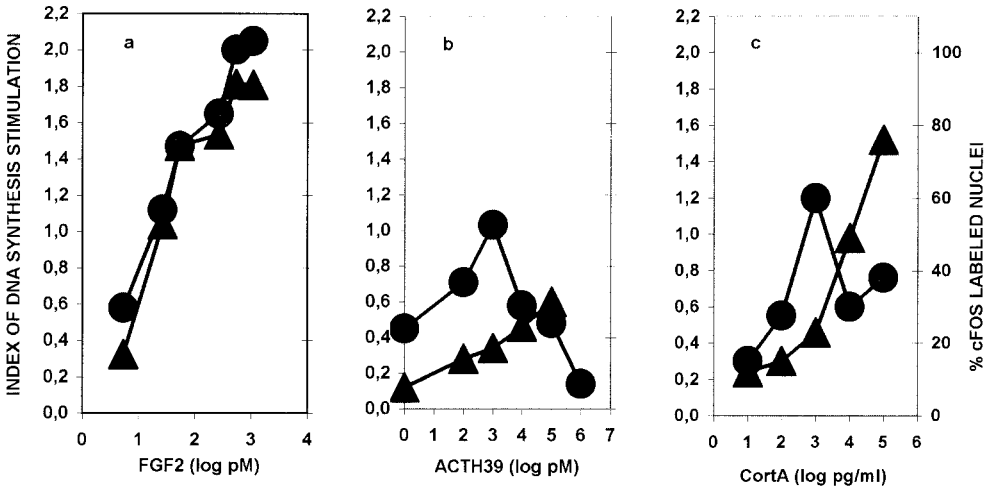

Figure 2 Dose–response curves for DNA synthesis stimulation and cFos protein induction in G_0/G_1-arrested Y1 adrenocortical cells by 2 h pulses of FGF2, synthetic $ACTH_{39}$ or natural porcine corticotropin A. Y1 cells G_0/G_1-arrested in SFM were pulsed for 2 h with FGF2, $ACTH_{39}$ or corticotropin A at the indicated concentrations. To estimate cFos protein induction (▲), a pair of coverslips for each condition was collected at 2 h and processed to count percent of nuclei stained with the anti-cFos antibody (see Materials and Methods). To estimate DNA synthesis stimulation (●), 2 h pulse-treated Y1 cells were washed twice with PBS and incubated in fresh SFM; 100 μM BrdU was incorporated between 12 and 24 h and at 24 h pairs of coverslips for each one of all conditions were collected and processed to count percent BrdU-labeled nuclei. (a) Data from three independent experiments were aggregated to estimate percent labeled nuclei to generate the figures plotted for each point in the curves; 3000–4000 were counted per point. All points in the curves are statistically significant with respect to control ($P<0{\cdot}01$, at least). (b) Five independent experiments; 5000–6500 nuclei counted per point ($P<0{\cdot}01$, at least). (c) Three independent experiments; 3000–3500 nuclei counted per point ($P<0{\cdot}01$, at least). Index of DNA synthesis stimulation as defined in legend of Fig. 1.

Antisense ODNs to cfos and cjun mRNAs block Y1 adrenal cell entry into S-phase stimulated by ACTH and FGF2

The complex 20 μM cfos antisense/10 μg/ml lipofectin reduced cFos labeling by 30%, whereas 0·1 μM cfos antisense/10 μg/ml lipofectin caused 80% reduction in cFos labeling, (compare data in Table 1). Thus, with a low ODN to lipofectin ratio (0·1 μM:10 μg) we achieved a high degree of inhibition of cFos induction. Lipofectin alone and antisense ODN alone had respectively minimal or no effect on cFos labeling (see Fig. 3 and Table 1). The cFos labeling reduction effect of the cfos antisense-lipofectin complex was sequence specific: cjun antisense and sense ODNs had no effect (Table 1). In addition, cfos antisense and sense and cjun antisense and sense had no effect on JunD labeling (not shown).

Table 2 shows antisense inhibition of DNA synthesis stimulated by FGF2 and corticotropin A. To analyze the antisense effect we compared pairs of results obtained with an antisense and its respective sense ODN. The complexes of 0·1 μM ODN:10 μg/ml lipofectin yielded highly significant inhibition for both cfos and cjun antisense ODNs (Table 2). But the pattern of antisense inhibition was different for FGF2 and corticotropin A (Fig. 4). Combination of both cfos and cjun antisense ODNs was required to completely inhibit DNA synthesis stimulation by FGF2; cfos or cjun antisense ODN alone caused only about 50% inhibition (Fig. 4a). Table 2 also shows that, in FGF2-treated cells, percent BrdU labeling for cfos plus cjun sense ODNs was significantly lower than for lipofectin alone, cfos sense ODN and cjun sense ODNs. However, this unspecific inhibitory effect of the sense ODNs is relatively small when compared with the complete inhibition of DNA synthesis stimulation by FGF2 caused by the combination of cfos and cjun antisense ODNs (Table 2 and Fig. 4a).

On the other hand, DNA synthesis stimulated by corticotropin A was essentially abolished by either cfos or cjun antisense ODNs (Fig. 4b). Lipofectin at 10 μg/ml, by itself, caused a significant increase in BrdU labeling of control cultures (Table 2). But this concentration of lipofectin is required to optimize entry of ODNs into the cell, as shown in Fig. 3.

Discussion

FGF2 and FGF1 are the prototypes of the large family of FGFs (Burgess & Maciag 1989), presently consisting of 20 members (Nishimura *et al.* 2000) that are widely expressed in embryo, fetus and adult tissues of vertebrates and invertebrates. It has been recognized for years that forms of

Table 1 Inhibition of cFos protein induction by cfos-antisense phosphorotioate ODN in G_0/G_1-arrested Y1 adrenocortical cells. Y1 cells seeded on coverslips, G_0/G_1-arrested in SFM, were incubated for 4 h with ODN-lipofectin complex, then treated for 2 h with either 1 μg/ml corticotropin A or 1 nM FGF2, rinsed with PBS and fixed with 3·7% formaldehyde. cFos-stained nuclei were estimated by an immunocytochemical reaction with antibody for the mouse cFos protein. In controls, untreated cells, cFos staining is negligible; 3800–4800 nuclei and 1060–1550 nuclei were counted for each condition of respectively corticotropin A- and FGF2-treated cells. These results were pooled from two sets of four and two independent experiments for, respectively, corticotropin A and FGF2

		FGF2		Corticotropin A	
	Ratio ODN : lipofectin	Fraction of cFos-stained nuclei	Inhibition of cFos synthesis (%)	Fraction of cFos-stained nuclei	Inhibition of cFos synthesis (%)
ODN+lipofectin					
cfos sense	20 μM : 10 μg/ml	0·80	0	0·79	4·8
	0·1 μM : 10 μg/ml	ND	ND	0·91	0
cfos antisense	20 μM : 10 μg/ml	0·55	31	0·41	51
	0·1 μM : 10 μg/ml	ND	ND	0·17	80
cfos sense+cjun sense	20 μM : 10 μg/ml	0·79	1·3	0·86	0
cfos antisense+cjun antisense	20 μM : 10 μg/ml	0·54	33	0·62	31
Lipofectin	0 : 10 μg/ml	0·88	0	0·76	8·4
—	0 : 0	0·80	0	0·83	0

ND: not done.

FGF exist as local paracrine and autocrine regulators of basic biological processes like mitogenesis, cellular differentiation, angiogenesis and tissue repair in the adult organism (Burgess & Maciag 1989). In addition, reports over the last few years have involved FGF proteins and their respective set of receptors in mechanisms of classical epithelial–mesenchymal interactions, underlying differentiation and organogenesis in the embryo (Jung *et al.* 1999, Metzger & Krasnow 1999, Meyers & Martin 1999, Miralles *et al.* 1999). For instance, FGF2 has long been considered a candidate to participate in the control of fetal development of adrenal glands and, also, of growth and function of the adult adrenal cortex (Mesiano *et al.* 1991).

Here we show that FGF2 elicits a strong mitogenic response in G_0/G_1-arrested Y1 adrenocortical cells, measured by BrdU labeling (Fig. 1), that linearly correlates with an early cFos induction (Fig. 2), implying a cause–effect relationship. We previously reported (Lotfi *et al.* 1997) that short pulses of ACTH, but not sustained treatment, resemble FGF2, inducing cFos and stimulating DNA synthesis in G_0/G_1-arrested Y1 cells. In sharp contrast with FGF2, ACTH dose–response curves for cFos induction and DNA synthesis stimulation are not correlated (Fig. 2b and c), suggesting that the cFos protein might not be a mediator in the growth response promoted by this hormone. To test whether induction of fos and jun genes are necessary for cell entry into S-phase stimulated by ACTH and FGF2, we blocked cFos and cJun protein synthesis with antisense phosphorotioate ODNs.

In principle, the antisense ODN technique affords the highest possible selectivity for blocking mRNA translation, thanks to the specificity of Watson–Crick base pairing between the antisense ODN and its respective mRNA. Phosphorotioate ODNs are resistant to cellular nucleases and association of ODN with cationic liposomes promotes cell entry, rendering the antisense ODN technique an efficacious approach to suppress synthesis of specific proteins (Wagner 1994). We tested cfos and cjun antisense and sense 18-mers sequences that have worked well for others to suppress cFos and cJun expression (Colotta *et al.* 1992). In Y1 adrenocortical cells, this cfos antisense ODN efficaciously inhibited cFos protein synthesis (Table 1 and Fig. 3). We have not directly monitored cJun protein synthesis, but presumably cJun synthesis was also inhibited by an antisense effect with the cjun antisense ODN used. In addition, cfos and cjun antisense ODNs blocked DNA synthesis stimulation by FGF2 and ACTH (Table 2 and Fig. 4).

Others (Guvakova *et al.* 1995) have reported that phosphorotioate ODNs can bind FGF2 preventing its association to receptors and, consequently, inhibiting its activity. This non-specific interaction between ODNs and FGF2 is unlikely to be important in our experiments. First, except for the cfos antisense, all other ODNs do not inhibit cFos induction by FGF2 (Table 1). Secondly, cfos or cjun sense had no effect on DNA synthesis stimulation by FGF2 (Table 2). Thirdly, in our laboratory, several sense phosphorotioate ODNs for cyclin D1 and c-myc mRNAs were tested and none of them have shown inhibition of FGF2 activity (A P Lepique, T T Schwindt & H A Armelin, unpublished observations). Fourthly, in our procedures ODNs are first complexed with lipofectin before addition to the culture medium (see Materials and Methods); under these conditions ODNs are trapped in ODN-lipofectin complexes and are unlikely to be available for interaction with FGF2. Altogether, these results suggest that we are efficaciously blocking both cFos

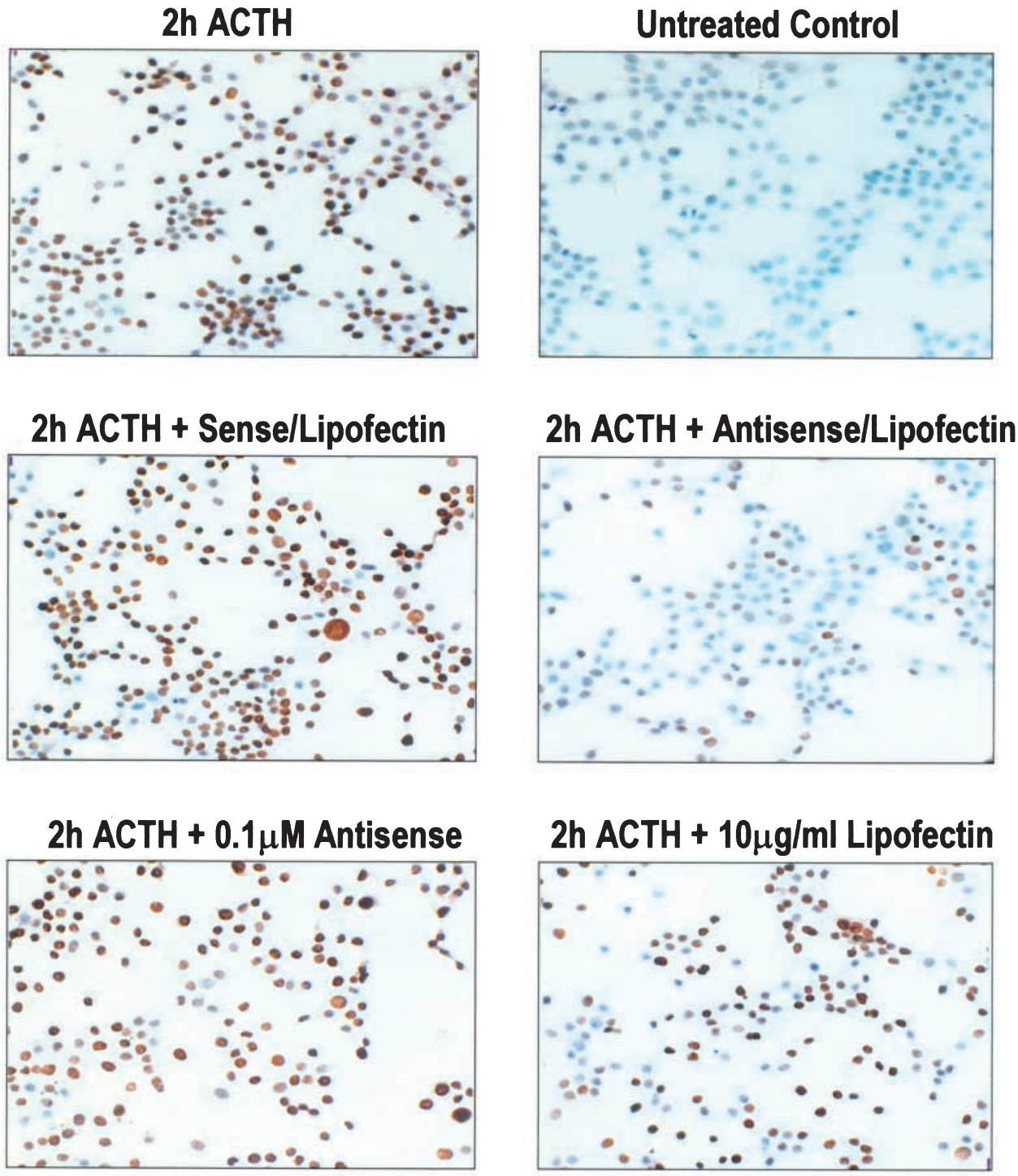

Figure 3 Inhibition of cFos protein induction by cfos antisense phosphorotioate ODN in G_0/G_1-arrested Y1 adrenocortical cells: specificity of the antisense ODN and requirement for lipofectin. Protocols are described in the legend to Table 1 and staining procedures in Materials and Methods. Ratio of ODN to lipofectin for complex formation: 0·1 μM:10 μg/ml respectively. Magnification: × 250.

Table 2 Inhibitory effects of c-fos and c-jun antisense phosphorotioate ODNs on DNA synthesis stimulated by 2 h pulses of respectively FGF2 and corticotropin A in G_0/G_1-arrested Y1 adrenocortical cells. Y1 cells seeded on coverslips and G_0/G_1-arrested in SFM for 48 h, were incubated for 4 h with ODN-lipofectin complex (0·1 μM ODN : 10 μM lipofectin), before treatment with 1 μg/ml corticotropin A and 1 nM FGF2 between 0 and 2 h. After treatment, coverslips were rinsed and incubated in fresh SFM plus the respective ODN-lipofectin complex. BrdU (100 μM) was incorporated between 12 and 24 h. At 24 h cells were fixed and processed to estimate percent BrdU-labeled nuclei. Index of DNA synthesis stimulation as defined in legend of Fig. 1

	FGF2[a]			**Corticotropin A**[b]		
	BrdU-labeled nuclei (%)		Index of DNA synthesis stimulation	BrdU-labeled nuclei (%)		Index of DNA synthesis stimulation
	Treated	Untreated		Treated	Untreated	
ODN+lipofectin						
cfos sense	57·6	35·4	0·63	55·1	39·7	0·40
cfos antisense	45·0	33·3	0·35	38·8	44·9	0
cjun sense	61·7	36·2	0·70	58·1	44·2	0·31
cjun antisense	48·7	39·2	0·24	45·9	45·3	0·01
cfos sense+cjun sense	50·7	34·2	0·48	64·0	43·7	0·46
cfos antisense+cjun antisense	31·5	31·0	0·02	36·2	44·6	0
Lipofectin	58·4	33·4	0·75	58·5	42·3	0·38
—	63·1	25·7	1·46	60·6	36·0	0·68

[a], [b] are pools of two different sets of two independent experiments; 2200–2700 nuclei were counted for each condition.

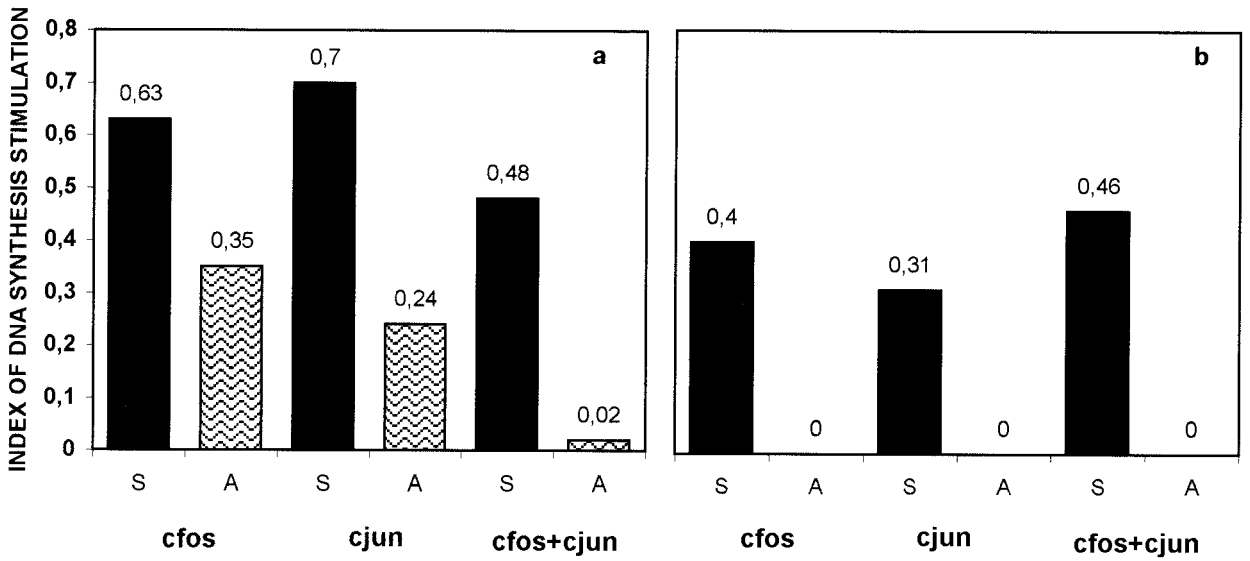

Figure 4 Inhibition of DNA synthesis stimulation by cfos and/or cjun antisense phosphorotioate ODN in G_0/G_1-arrested Y1 adrenocortical cells stimulated by 2 h pulses of respectively FGF2 and natural porcine corticotropin A. The indices of DNA synthesis stimulation shown in the histograms are from Table 2. (a) Y1 cells stimulated with 1 nM FGF2 and (b) Y1 cells stimulated with 1 μg/ml porcine corticotropin A. S and A stand for respectively sense and antisense ODN. All differences between S and A are statistically significant ($P<0·01$, at least). Protocols are summarized in the legend of Table 2.

protein induction and DNA synthesis stimulation by an antisense mechanism.

cfos or cjun antisense ODNs are sufficient to completely abolish stimulation of DNA synthesis by ACTH, but not by FGF2 (Fig. 4). Only a combination of both cfos and cjun antisense ODNs can extinguish DNA synthesis stimulated by FGF2 in G_0/G_1-arrested Y1 cells (Fig. 4). These results are not surprising, since Kovary & Bravo (1991*b*) have shown that only micro-injections of combinations of antibodies against Fos and Jun proteins can block G_1-phase traversing in G_0-arrested mouse 3T3 fibroblasts growth stimulated with serum.

Thus, the results of our antisense experiments support the notion that the induction of cFos and cJun proteins is a necessary step in the triggering mechanisms by which ACTH or FGF2 drives G_0/G_1-arrested Y1 cells to enter

the S-phase. Consequently, FGF2 and ACTH receptors must activate pathways that converge to cFos and cJun induction. The Ras-Raf-MEK-ERK cascade of kinases is a highly conserved pathway that integrates mitogenic signals initiated in tyrosine kinase and G protein-coupled receptors (Gutkind 1998). Actually, in G_0/G_1-arrested Y1 cells, FGF2 rapidly and strongly triggers transient phosphorylation of ERK1/2, whereas $ACTH_{39}$ is a poor ERK1/2 activator. But, the MEK1 inhibitor, PD98059 (50 μM), inhibits cFos induction and DNA synthesis stimulation by both $ACTH_{39}$ and FGF2, suggesting that ERK1/2 activation mediates the strong and the weak mitogenic effect of, respectively, FGF2 and $ACTH_{39}$ (Lotfi *et al.* 1997, 2000). In addition, we have previously reported that the weak mitogenic effect of $ACTH_{39}$ does not depend on the cAMP/PKA pathway (Lotfi *et al.* 1997).

On the other hand, one must keep in mind that ACTH behaves as a strong anti-mitogen in cultures of adrenocortical cells from a variety of species (Hornsby 1985). How can we mechanistically reconcile this strong anti-mitogenic action of ACTH with its weak mitogenic effect that we are focusing on in this report? In Y1 adrenal cells, this ACTH anti-mitogenic effect is dependent on the cAMP/PKA pathway (Masui & Garren 1971, Rae *et al.* 1979, Lotfi *et al.* 1997). But the mechanisms underlying ACTH anti-mitogenic effects are still essentially unknown. We recently observed three cAMP/PKA-dependent effects of ACTH concurring to block Y1 cell cycle at G_1-phase, namely: (i) degradation of the cMyc protein (Lepique *et al.* 2000); (ii) dephosphorylation/deactivation of the Akt/PKB enzyme (Forti & Armelin 2000); and (iii) induction of the p27 Kip1 protein, an inhibitor of cyclin-dependent kinases (T. Schwindt, F. Forti & H. Armelin, unpublished observations). In conclusion, ACTH receptors initiate signals leading to dual effects promoting and inhibiting cell cycle progress. It remains to be explained how these antagonistic effects of ACTH are balanced to regulate growth and function of the adrenal cortex. Studies on the interactions between ACTH and FGF2 in G_0/G_1-arrested Y1 adrenocortical cells are useful to elucidate circuits that integrate signals initiated in respectively ACTH receptors and FGF2 receptors that should be relevant to the mechanisms underlying regulation of the $G_0 \rightarrow G_1 \rightarrow S$ transition in adrenal cell cycle.

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