Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 298-305

TGFβ INDUCES A SUSTAINED c-fos EXPRESSION ASSOCIATED WITH STIMULATION OR INHIBITION OF CELL GROWTH IN EL2 OR NIH 3T3 FIBROBLASTS

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Received November 3, 1987

We have previously indicated that epidermal growth factor (EGF) plays a fundamental role in the proliferation control of EL2 rat fibroblast line. It is shown here that transforming growth factor β (TGFβ) stimulates both DNA synthesis and proliferation of EL2 cells, while exerting an inhibitory effect on the growth of murine NIH-3T3 fibroblasts. We also report the effect of TGFβ and EGF on c-fos expression in EL2 cells, as compared to that of TGFβ in NIH-3T3 fibroblasts. In EL2 cells EGF induces a transient c-fos expression at both mRNA and protein level, as previously observed in NIH-3T3 fibroblasts treated with platelet-derived or fibroblast growth factor (PDGF, FGF). Conversely, TGFβ induces in EL2 cells a sustained expression of fos mRNA and protein, which are still detectable at least 24 and 7 hr after treatment respectively. In NIH-3T3 fibroblasts TGFβ causes a sustained fos RNA expression, which is not associated, however, with detectable fos protein. We conclude that in fibroblasts stimulated by mitogens c-fos expression may be differentially modulated, depending of the growth factor and the cell line. This is seemingly due to differential regulation of fos gene expression, not only at the transcriptional and/or post-transcriptional level (transient or sustained fos RNA induction by EGF or TGFβ in EL2 cells), but also at the translational level (fos protein(s) induction by TGFβ in EL2 but not NIH-3T3 fibroblasts, possibly related to the stimulatory vs inhibitory effect of this factor on the growth of the former vs the latter line).

Mammalian cell proliferation is controlled by the extracellular environment, largely via interaction of growth factors with specific cell membrane receptors. This interaction induces the activation of proto-oncogenes encoding nuclear proteins (c-myc, c-myb, c-fos) (1, 2, 3), thus suggesting that these genes are directly involved in the regulation of cell growth (4, 5).

One of the earliest events induced by mitogenic stimuli is the activation of c-fos (2, 6, 7), i.e., the cellular homologue of the transforming gene of FBJ-osteosarcoma virus (8), encoding a nuclear protein of unknown function (9). c-fos expression is strongly induced at 10-15 min after growth factor treatment, but then rapidly declines. This pattern has been demonstrated in fibroblast lines (e.g., BALB/c-3T3 (2, 6) and EL2 (10)), pheochromocytoma line

0006-291X/88 $1.50

PC12 (11), thymocytes (12) and epidermal carcinoma line A431 (13). In NIH-3T3 cells the induction of expression is mediated via transcriptional mechanism independent of protein synthesis (14, 15). In fact, protein synthesis inhibitors superinduce fos gene expression, via RNA stabilization and prolongation of transcription (15). An enhancer element in the 5'-flanking region of c-fos has been recently described (16): a transacting factor may in part mediate the induction of c-fos expression by growth factors (17).

We have previously demonstrated (10) that EL2 rat fibroblasts (see 18) require only addition of epidermal growth factor (EGF) for induction to proliferation. Treatment with a "competence" factor, (e.g., fibroblast or platelet-derived growth factor, FGF or PDGF) does not further enhance the mitogenic activity exerted by EGF. We also showed that EGF induces a marked increase of c-fos transcription in EL2 cells.

We report here the effect of TGF β on proliferation and c-fos expression of EL2 fibroblasts, as compared to that exerted in NIH-3T3 cells. Control studies on the effects of EGF on EL2 cell proliferation and fos gene expression are also included.

MATERIALS AND METHODS

Cell cultures

EL2 cells were maintained in Dulbecco's modified Eagle's medium (Gibco) containing 10% calf serum (Gibco) supplemented with penicillin (50 units/ml) and streptomycin (50 ug/ml) in humidified 5% CO_2/95% air at 37°C. In order to grow EL2 cells in serum-free medium we developed the following procedure. Cells were first trypsinized and then grown directly in 4 ml of serum-free medium in Petri's dishes precoated with fibronectin. Several proteins were added: purified human transferrin (Trf, Sigma, USA), 20% saturated with ferric ammonium citrate; purified porcine or bovine insulin (Sigma, USA); EGF purified from male mouse submaxillary glands (Sigma, USA) and PDGF highly purified (a gift of Dr. B. Westermark) from human platelets. Trf was added at 50 ug/ml, insulin at 200 ng/ml, EGF and PDGF at various concentrations (0-20 and 1-40 ng/ml respectively). Highly purified TGF β (kindly provided by Dr. M.B. Sporn) was added in culture at concentrations ranging from 0.01 to 1 ng/ml.

^{3}H-thymidine incorporation.

EL2 cells were grown for 24 hr in the presence of Dulbecco's medium containing 1 ng/ml BSA, 50 ug/ml human Trf, 100 ng/ml insulin. EGF (10 ng/ml) was added and then (^{3}H)-thymidine incorporation was measured at various time intervals, as previously reported (19).

RNA extraction and analysis.

RNA was extracted from density-arrested EL2 cells as described (18). Poly(A)$^+$ RNA was extracted and purified following the guanidinium/cesium chloride method (20), followed by separation on oligo (dT) column. The RNA (5 ug) was run on denaturing (1.2%) agarose gels containing formaldeyde, transferred onto nitrocellulose filter and hybridized to nick-translated specific probes as described (10). The filters were washed in 0.2 x SSC and 0.1% SDS at 45°C and exposed to X-ray film using intensifying screens. The fos oncogene probe was a 1.3 Kb BglII-PvuII fragment containing most of the coding region of v-fos (8).

Immunoprecipitation of c-fos proteins.

Cell labelling and immunoprecipitation were carried out as described by Curran et al. (11). Briefly, quiescent EGF- or TGFβ-

treated EL2 cells were labelled for 15 min in 1.0 ml of DMEM lacking methionine and serum, containing (^{35}S)-methionine (Amersham Laboratories, > 800 Ci/mmole) at a final concentration of 0.3 mCi/ml. At the end of the labelling period, cultures were washed with ice-cold Tris-buffered saline and lysed with 1.0 ml of RIPA buffer (0.15 M NaCl, 1% sodium deoxycolate, 0.1% SDS, 2 mM EDTA, 100 units Trasylol per ml, 10 mM sodium phosphate, pH 7.0). Aliquots of cell lysates were precipitated with 1 ml of 10% TCA and measured in a liquid scintillation counter. For immunoprecipitation, lysates obtained from the same cell number were clarified at 40,000 x g for 30 min at 4°C and incubated with either c-fos peptide antiserum (a gift of Dr. T. Curran) on normal rabbit serum. After 1 hr at 0°C, 20 ul of a 10% suspension of Pansorbin (Calbiochem, San Diego, USA) was added for 20 min. Immunoprecipitates were washed repeatedly by centrifugation in RIPA buffer. Precipitates were analyzed on 8.5% polyacrylamide gels, according to Laemmli (21) and visualized by fluorography.

RESULTS AND DISCUSSION

TGF β action on the growth of EL2 and NIH-3T3 cells.

TGF β exerts either a stimulatory or an inhibitory effect on the growth of cell lines, depending on the cell type, the culture conditions and the spectrum of growth factors in culture (22, 23, 24). In serum-free cultures of quiescent EL2 fibroblasts appropriate concentrations of TGF β induce DNA synthesis, as well as moderate cell proliferation (Table I, II). Combined addition of TGF β and EGF causes an additive enhancement of both DNA synthesis and cell growth (Table I, II). In contrast, TGFβ exerts an inhibitory effect on DNA synthesis and proliferation in NIH-3T3 cells stimulated by PDGF and EGF (Table I, II).

EGF action on c-fos expression in EL2 fibroblasts.

Total RNA was extracted from EL2 cells in the quiescent state and at various time intervals after mitogen, and its polyadenilated fraction analyzed by Northern blot. Treatment with EGF (10 ng/ml) rapidly induced a transient increase of the 2.2 Kb fos mRNA, which was already abundant at 15 min after the growth stimulus, but

Table I - Effect of TGF β on DNA synthesis of density-arrested EL2 and NIH-3T3 cells

Treatment	% value	
	EL2	NIH-3T3
Control	100	100
EGF (10 ng/ml)	938	102
EGF (10 ng/ml) + PDGF (10 ng/ml)	1080	980
TGFβ (0.1 ng/ml)	355	105
TGFβ (0.1 ng/ml) + EGF (10 ng/ml)	1450	110
TGFβ (0.1 ng/ml) + PDGF (10 ng/ml) + EGF (10 ng/ml)	1380	312

Cells were recovered and processed for ^{3}H-thymidine incorporation as previously reported (19).

Results are expressed in terms of (^{3}H)-thymidine incorporation values, as percentage of control level. Mean values from three separate experiments are presented.

Table II - Effect of TGFβ on the growth of EL2 and NIH-3T3 cells in serum-free cultures

Cell line	Medium	Cell number (x 10^{-4}) day 0	day 4
EL2	BSA, Trf, Insulin	0.5	0.42
"	" " " EGF	0.5	6.5
"	" " " TGFβ	0.5	1.25
"	" " " EGF, TGFβ	0.5	8.9
NIH 3T3	BSA, Trf, Insulin	0.5	0.33
" "	" " " EGF, PDGF	0.5	7.2
" "	" " " TGFβ	0.5	0.29
" "	" " " EGF, PDGF, TGFβ	0.5	2.9

EL2 or NIH-3T3 cells were grown as described in Materials and Methods. Cells were counted daily after trypsinization.

decreased at 1 hr (Fig. 1A) (see also 10). Simultaneous addition of EGF and a protein synthesis inhibitor (cycloheximide, CHX, 10 ug/ml) did not apparently enhance the peak level of fos RNA (re-

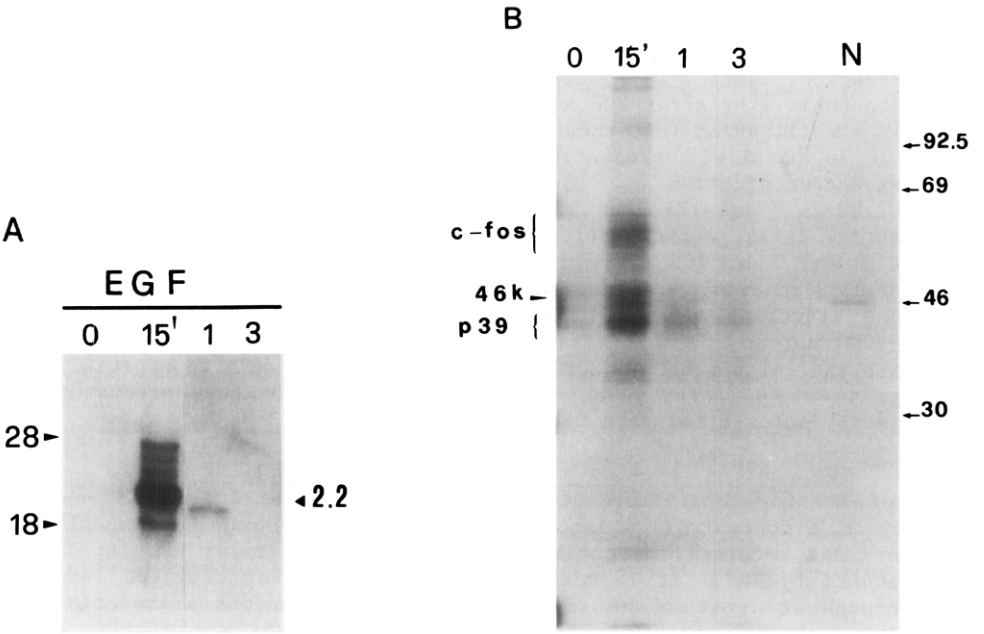

Fig. 1 Kinetics of fos mRNA (panel A) and fos protein(s) synthesis (panel B) in EGF-treated EL2 cells. Cells were unstimulated (0) or stimulated with EGF (10 ng/ml) for 15 min, 1 or 3 hr. Panel A: poly $(A)^+$ RNA was extracted and analyzed as described in Materials and Methods. The 2.2 Kb prominent band of fos mRNA is indicated, together with the position of 28S and 18S rRNA. Panel B: cells were labelled and immunoprecipitated as described in Materials and Methods. Positions of the c-fos protein(s), p39 and 46 Kd proteins are indicated, together with molecular sizes of marker proteins (Amersham Laboratories). N, normal rabbit serum.

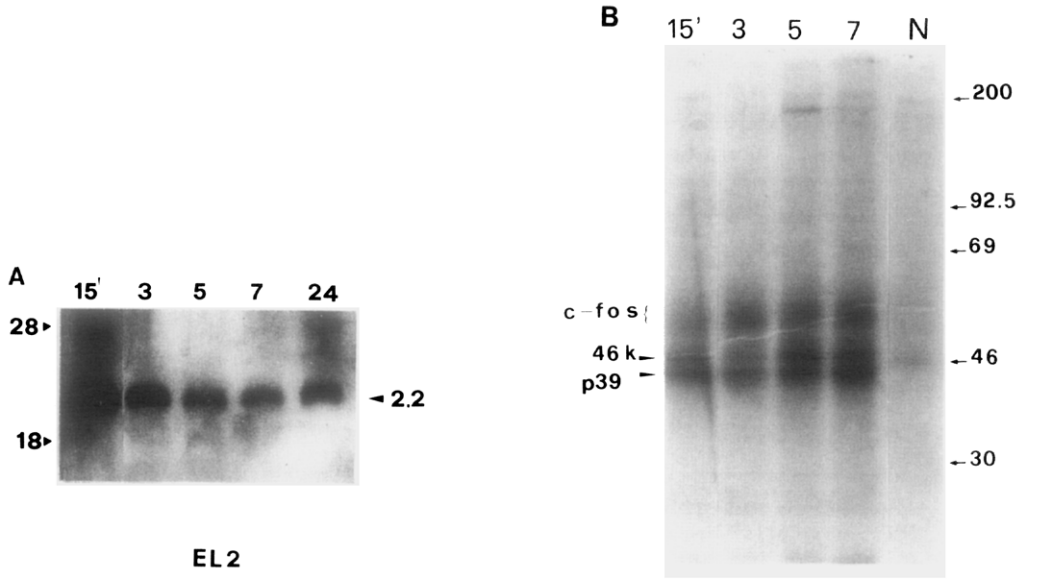

Fig. 2 Kinetics of fos mRNA expression (panel A) and c-fos protein(s) synthesis (panel B) in TGFβ treated EL2 cells. Quiescent EL2 cells were incubated for 15 min, 3, 5, 7 or 24 hr with TGFβ (0,1 ng/ml). For further details see legend of Fig. 1.

sults not presented). However, CHX altered the kinetics of fos RNA induction, since a significant level of this RNA species was still detectable 1 hr after EGF addition, presumably at least in part via RNA stabilization (not shown here).

In parallel, production of fos protein(s) was measured by immunoprecipitation of ^{35}S-labelled proteins from EGF-stimulated EL2 cells (Fig. 1B). The fos protein(s), virtually undetectable in resting cells, was dramatically induced 15 min after EGF addition. At 1 and 3 hr fos protein(s) synthesis was markedly reduced and barely detectable respectively, thus reflecting the kinetics of mRNA induction. In addition, the antiserum also detected both a 39.000 daltons protein (i.e., a cellular protein co-precipitating with the c-fos gene product(s) (25, 9) and a fos-related protein in the 44-46 Kd range, which may represent the r-fos gene product in growth factor-stimulated fibroblasts (7).

TGFβ action on c-fos expression in EL2 and NIH-3T3 cells.

Resting EL2 fibroblasts were exposed to TGFβ for 15 min - 24 hr, at a concentration inducing maximal DNA synthesis (0.1 ng/ml). Fos mRNA, scarcely detectable in quiescent EL2 cells, was rapidly induced by TGFβ at 15 min, and then expressed at elevated level through at least 24 hr (Fig. 2A). Upon simultaneous stimulations with both EGF and TGFβ, the kinetics of fos RNA was similar to that observed in cells treated with TGFβ alone (data not shown).

The pattern of fos protein(s) synthesis following TGFβ addition in EL2 cells is also of interest. A progressive accumulation of c-fos protein(s) was observed starting from 15 min up to at least 7 hr (Fig. 2B), thus in line with results on fos mRNA kinetics. The different molecular weight of the fos protein(s) observed upon stimulation with EGF (M.W., 60,000 D, Fig. 1B) or TGFβ (M.W., 54,000 D, Fig. 2B) probably derives from extensive post-translational modifications of the fos gene product (25, 11, 6).

A similar analysis was performed on the kinetics of fos mRNA in a clone of NIH-3T3 fibroblasts (see 26) treated with TGFβ (Fig.

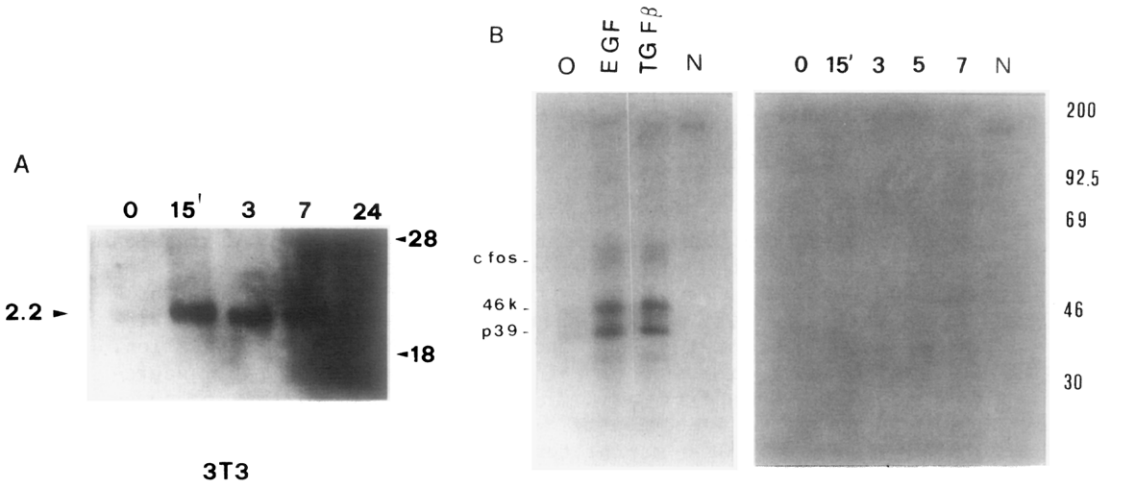

Fig. 3 Analysis of fos mRNA induction (panel A) and c-fos protein(s) synthesis (panel B) in TGFβ treated NIH-3T3 cells. Quiescent NIH-3T3 cells were exposed to TGFβ (0,1 ng/ml) for 15 min, 3, 7 or 24 hr. Panel A: the 2.2 Kb c-fos mRNA and the position 28S and 18S rRNA are indicated. Panel B: right panel: resting NIH-3T3 cells were incubated with TGFβ (0.1 ng/ml) for the indicated time. The c-fos proteins were immunoprecipitated as in Fig. 1. Left panel: in this control experiment, quiescent EL2 cells were untreated or treated with EGF (10 ng/ml) or TGFβ (0.1 ng/ml) for 15 min and immunoprecipitated as in Fig. 1.

3A). Fos RNA, almost undetectable in GO/G1 arrested NIH-3T3 cells, was markedly induced by TGFβ addition, starting from 15 min through at least 24 hr. It is noteworthy, however, than fos protein(s) was never detected in NIH-3T3 cells after TGFβ addition (Fig. 3B).

These studies indicate that in EL2 and NIH-3T3 fibroblasts different growth factors elicit a sharply different expression of fos mRNA. Thus, transient expression is induced by EGF in EL2 cells, as well as by PDGF or FGF in NIH-3T3 fibroblasts (6), while sustained induction is provoked by TGFβ in both lines. Interestingly, the control of fos protein synthesis is apparently different in these fibroblast lines. Both show a transient fos protein(s) synthesis following EGF (EL2) or PDGF (NIH-3T3) stimulus. However, in EL2 cells stimulated by TGFβ the sustained synthesis of fos RNA is paralleled by a prolonged production of fos protein(s), while in this clone of NIH-3T3 cells the sustained fos RNA level induced by TGFβ is not associated with detectable fos protein(s). The blockade of fos RNA translation in NIH-3T3 fibroblasts may be caused by a different structure of fos RNA and/or a suppressor at its translation.

Recently, Leof et al. (27) showed that treatment of quiescent mouse AKR-2B cells with TGFβ results in early induction of sis mRNA, followed by secretion of a PDGF-like protein in the culture medium. The mitogenic effect of TGFβ was hence attributed to autocrine PDGF synthesis, thus leading to the subsequent stimulation of c-fos, c-myc and other PDGF-inducible genes. Therefore, it seemed of interest to evaluate the level of sis mRNA in EL2 cells after treatment with TGFβ . Induction of sis RNA was not observed (data not shown): it is apparent therefore, that in EL2 fibroblasts the effect of TGFβ on fos RNA induction, as well as

its mitogenic action, is not mediated by autocrine PDGF production. This conclusion is further strengthened by other observations: (i) addition of neutralizing anti-PDGF antibodies does not inhibit the mitogenic effect of TGFβ; (ii) TGFβ does not induce EL2 cells to form colonies in agar, while PDGF induces the growth of EL2 cells in semi-solid medium (10).

In conclusion, these studies indicate that a variety of molecular mechanisms may mediate the induction of c-fos expression by different growth factors in various types of fibroblasts. The spike of fos RNA and protein expression in EL2 or NIH-3T3 fibroblasts following EGF or PDGF treatment is mediated by transcriptional activation of fos gene and efficient fos RNA translation, followed by early blockade of c-fos transcription and rapid degradation of fos RNA and protein. In sharp contrast to this phenomenon, the sustained induction of fos RNA and protein in EL2 fibroblasts exposed to TGF β may be tentatively attributed to prolonged fos gene transcription and RNA translation and/or stabilization of fos RNA and protein (28). Conversely, the sustained fos RNA induction in NIH-3T3 fibroblasts treated with TGFβ, uncoupled with detectable fos protein(s) level, indicates prolonged activation of fos gene transcription and/or stabilization of fos RNA with concomitant blockade of efficient translation. Since the employed dosage of TGFβ induces proliferation of EL2 cells, but inhibits the growth of NIH-3T3 fibroblasts, it is suggested that the efficient translation of fos RNA in the former line is linked to and possibly mediates at least in part the proliferative response of EL2 fibroblasts to TGFβ.

It is hence apparent that a variety of molecular mechanisms, acting at transcriptional, post-transcriptional and translational level, may underlie fos gene expression induced by different growth factors in fibroblast lines. Particularly, efficient or blocked translation of fos RNA after TGFβ treatment might directly correlate with the growth stimulus or inhibition exerted by this factor in different lines.

ACKNOWLEDGEMENTS

We are grateful to T. Curran for c-fos polypeptide antiserum and to M. Sporn for the generous gift of highly purified TGF β . We thank P. Alessandri for typing the manuscript. E.L. is fellow of the "Associazione Italiana per la Ricerca sul Cancro". This work was supported in part by grants from CNR, Rome, Progetti Finalizzati Oncologia", (No. 86.02683.44 to G.B.R. and 86.00699.44 to C.P.), and "Ingegneria Genetica" (No. 86.00101.51 to G.B.R. and 86.00099.51 to C.P.).

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