INSULIN-LIKE GROWTH FACTOR I STIMULATES TRANSCRIPTION OF THE *C-JUN* PROTO-ONCOGENE IN BALB/C 3T3 CELLS

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Treatment of quiescent Balb/c 3T3 cells with insulin-like growth factor I (IGF I) resulted in the stimulation of proto-oncogene *c-jun* transcription. Cells exposed to cycloheximide and IGF I together showed super-induction of *c-jun* transcripts. Nuclear run-off assay revealed that IGF I up-regulated *c-jun* only while cycloheximide was present. The stability of *c-jun* mRNA was markedly increased in the cells treated with IGF I. These results suggest that IGF I controls the expression of *c-jun* by increasing the transcriptional activity and stabilizing the existing transcripts.   © 1992 Academic Press, Inc.

Insulin-like growth factor I (IGF I) was first identified as a cationic polypeptide from human serum with cell division stimulating capacity for Balb/c 3T3 cells (1,2). The growth factor contains single polypeptide chain with 70 amino acid residues which displays obvious homology to proinsulin (3). *In vivo*, IGF I stimulates growth in hypophysectomized rats, suggesting that IGF I mediate the action of growth hormone, according to somatomedin hypothesis (4, 5). Regulations of cellular growth in different tissues, such as the regulation of embryogenesis, were suggested to be exerted by coordination of several growth factors (6-10).

Many oncogenes, such as *fos, jun, myc, rel, erbA,* and *myb,* encode nuclear proteins which contain DNA binding abilities or act as transcription factors (11). Moreover, several of these oncogenes are rapidly expressed in quiescent cells treated with mitogens. These are immediate early genes or primary response genes (12-16). Recently, many primary response genes were found to play essential roles in cell proliferation and differentiation (15, 16).

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The proto-oncogene *c-jun* is a cell-derived sequence that was first identified as the oncogene of avian sarcoma virus 17 (ASV-17), which neoplastically transforms chick embryo fibroblasts (17, 18). Further characterization indicated that *c-jun* encodes the transcription factor AP-1 or a closely related protein (17, 18). It has been proposed that rapid increase in expression of immediate early gene such as *c-jun* is an important mechanism to establish the long-term transcriptional response of cells to growth factors (14).

It is well known that the receptor for IGF I exhibits high degree of structural and functional homology with that of insulin (19). After growth factor binding, the receptors undergo autophosphorylation and enhancement of their tyrosine kinase activity (20). The possible substrates for the receptor tyrosine kinase include some insoluble cytoskeleton-associated protein pp175 (21) and the cellular protein pp185 which was phosphorylated within 10 seconds after IGF I or insulin binding to their respective receptor (22). It is believed that activation of normal p21 H-*ras*, a member of G protein family, may be involved in the signal transduction pathway of IGF I stimulation (20, 23). In the cell nucleus, it has been reported that IGF I caused an elevation of *c-fos* transcripts in L6 cells (24). However, the linkage between intracellular signal tranmission and cell proliferation in the mechanism of IGF I action remains to be established.

Since fos protein is responsible for transcription regulation by means of heterodimer formation with c-jun/AP-1, genes which contain AP-1 binding site in the promoter region, such as collagenase or metallothionein gene, can be regulated by fos-jun complex (25, 26). To test whether fos-jun heterodimer is involved in the signal transduction pathway of IGF I stimulation, we examined the transcription levels of *c-jun* after quiescent cells were stimulated by IGF I. We propose herein that IGF I have dual functions in *c-jun* stimulation, namely stabilization of the existing transcripts and enhancement of the transcription activity.

MATERIALS AND METHODS

Cell culture. Balb/c 3T3 (clone A31) cells were obtained from American Type Culture Collection. Cells were grown in DMEM (Dulbecco's modified Eagle's medium) with 10% calf serum. For growth factor stimulation experiments, cells were grown to confluency and then placed in DMEM / F12 (1:1) with bovine serum albumin (1mg/ml) , transferrin (50 μg/ml), soybean lipid (8 μg/ml) and sodium selenite (3 x 10^{-8} M) for 24 hours. Cells were stimulated by the addition of IGF I (25 ng/ml, human recombinant, Boehringer Mannheim), cycloheximide (10 μg/ml, Boehringer Mannheim), or IGF I plus

cycloheximide, and harvested at 30, 60, 90, 120 minutes after stimulation as indicated. For RNA stability assay, quiescent cells were treated with actinomycin D (1 µg/ml, Sigma) for 15 minutes prior to the administration of growth factor. The level of c-jun mRNA was then analysed by Northern blotting (27). All chemicals for cells culture are purchased from Boehringer Mannheim or Sigma.

Plasmids. The plasmid pSV-jun containing cDNA of c-jun was used to prepare labeled probes as described (28). PstI fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (29) subcloned into pGEM-3Z was used as internal control.

Northern blotting. Total RNA was isolated by the guanidinium/cesium chloride centrifugation method (42). Twenty µg of total RNA were electrophoretically seperated through formaldehyde/ 0.9% agarose gel and transferred to nylon membranes by standard protocol (43). RNA was immobilized to the membrane by UV crosslinking at 1.2 J. The membranes were prehybridized in hybridization buffer (50% deionized formamide , 5X Denhardt's solution, 5X SSPE, 0.2% SDS and 100 µg/ml denatured salmon sperm DNA, 1X SSPE = 0.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM EDTA pH7.4) at 42°C for 2 hours. The heat denatured, random primer labelled 1.8 kb c-jun fragment from clone pSV-jun was added to hybridization buffer and hybridization was performed for 20 hours at 42°C. The membranes were washed for 30 minutes with 5X SSPE plus 0.1% SDS at room temperature and 30 minutes with 1XSSPE plus 0.1% SDS at 37°C , then finally with 0.1XSSPE plus 0.1% SDS at 65°C for 30 minutes. Autoradiograms were obtained by exposing X-ray films at -70°C with intensifying screens.

Nuclear run-off assay. Isolation of Balb/c 3T3 nuclei and run-off transcription are performed as described by Greenberg et al (12) with slight modification. For each run-off transcription assay, 5 X 10 [7] cells were starved for 24 hours in DMEM/F12 (1:1) as described in Cell culture section. After 30 minutes of IGF I stimulation, cells were washed twice with ice-cold PBS (phosphate buffered saline) and harvested by scraping with cell scrapper. Cycloheximide treatments were performed at 1 hour before addition of IGF I. Cells were then lysed by gentle vortexing with NP-40 lysis buffer (10 mM Tris•Cl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40) and nuclei were harvested by centrifugation at 500Xg for 5 minutes at 4°C. The lysis and centrifugation procedures were repeated once to ensure uniform lysis of the cells. Nuclei pellets were then suspended in 200 µl of glycerol storage buffer (50 mM Tris•Cl pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA) and stored in liquid nitrogen. For run-off transcription, we thawed 200 µl of frozen nuclei at room temperature. RNA synthesis reactions were carried out at 30°C for 30 minutes, with 5 mM Tris•Cl pH 8.0, 2.5 mM $MgCl_2$, 150 mM KCl, 2.5 mM DTT, 0.5mM ATP, 0.5mM CTP, 0.5mM GTP, 0.6 µM UTP and 100 µCi [α- ^{32}P]UTP (3000 Ci/mmol, Amersham). The reaction was terminated by digestion with RNase-free DNase I and proteinase K, and subsequently extracted with phenol/chloroform. Radioactive labelled RNA were isolated as described elsewhere (44). Before ethanol precipitarion, [^{32}P]-labelled RNA was treated with a final concentration of 0.2 M NaOH for 10 min on ice. The solution was neutralized by the addition of HEPES (free acid) to a final concentration of 0.24 M and the RNA was precipitated with ethanol at -20°C overnight. After centrifugation, the [^{32}P]-labelled RNA pellet was resuspended in TES solution (10 mM TES [N-tris(hydroxymethyl)methyl-2-aminoethansulfonic acid], pH 7.4, 10 mM EDTA, and 0.2% SDS) at 5 X 10^{6} c.p.m. /ml. This RNA solution was mixed with equal volume of TES/NaCl (10 mM TES, pH 7.4, 10 mM EDTA, 0.2% SDS, and 600 mM NaCl), and 2 ml of the RNA solution was hybridized at 65°C for 36 hour to DNA immobilized on nitrocellulose. In a given experiment, each filter was hybridized with [^{32}P]-labelled RNA of the same c.p.m. After the hybridization reaction, the filters were washed with 2XSSPE for 2 hours at 65°C and incubated at 37°C in 2XSSPE with 10 µg/ml RNase A for 30 minutes. Finally, the filters were washed with 2 X SSPE at 37°C for 1 hour, air dried and exposed to X-ray film.

Binding of plasmids to nitrocellulose.We used plasmid pSV-jun and GAPDH gene in the experiment. The plasmids were first linearized with appropriate restriction enzymes and denatured with 0.2 M NaOH for 30 minutes at room temperature, and by neutralized with 10 volumes of 6 X SSPE at 0°C. Twenty µg of DNA in each well was spotted onto nitrocellulose membrane using dot blot apparatus. The nitocellulose membrane was then baked at 80°C for 2 hours and stored for further use.

RESULTS

Treatment of quiescent, serum-starved Balb/c 3T3 fibroblasts with 25 ng/ml of IGF

I results in an increase in *c-jun* mRNA levels within 30 minutes . The levels of stimulation

are about two folds higher than those in uninduced cells (Fig. 1, lanes 1, 2; Fig. 2c). Both

c-jun species of 2.6-2.7 kb and a 3.4 kb precursor (15) can be induced by IGF I (Fig. 1).

We used GAPDH as an internal control to ensure that equal amounts of RNA were loaded

in each well.

Addition of IGF I in the presence of cycloheximide led to an induction of *c-jun*

mRNA about 5 folds higher than that observed in the absence of the protein synthesis

inhibitor (Fig. 1, lanes 2, 4; Fig. 2c). Our observation is consistent with the induction by

serum as described by Ryseck et al. (14) and the induction by epidermal growth factor

(EGF) as reported by Quantin and Breathnach (15). This supports the notion that several

growth factors may share the common signal transduction pahtways. The super-induction

of *c-jun* by IGF I plus cycloheximide shown in Fig. 1 indicates that the stimulation is a

primary response.

The levels of c-jun RNA during G0/S transition state after stimulation by IGF I

were examined by Northern analyses. Total RNA at different time point after IGF I

stimulation in the absence and presence of cycloheximide were analyzed. The results

showed that in the absence of protein synthesis inhibitor the stimulatory effect of IGF I was

first observed in 30 minutes after stimulation (Fig. 2a). The peak of *c-jun* transcripts

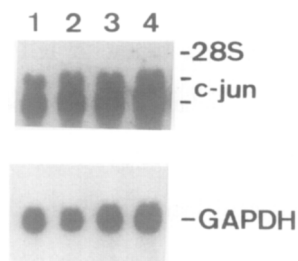persisted for about 120 minutes and reached the maximal amount for about 60 minutes after

Fig.1 Induction of *c-jun* mRNA in quiescent Balb/c 3T3 cells stimulated by IGF I.
Northern blot of RNA from Balb/c 3T3 cells with different treatments. The blot was hybridized with *c-jun* and GAPDH probes. Lane 1 shows total RNA isolated from cells synchronized at quiescent stage. In lane 2, RNA was isolated from quiescent cells after 30 minutes of IGF I (25 ng/ml) treatment. Quiescent cells in lane 3 had been treated with cycloheximide (10 µg/ml) for 1 hour. The cells in lane 4 were treated with cycloheximide as in lane 3 and further subjected to IGF I stimulation for a period of 30 minutes.
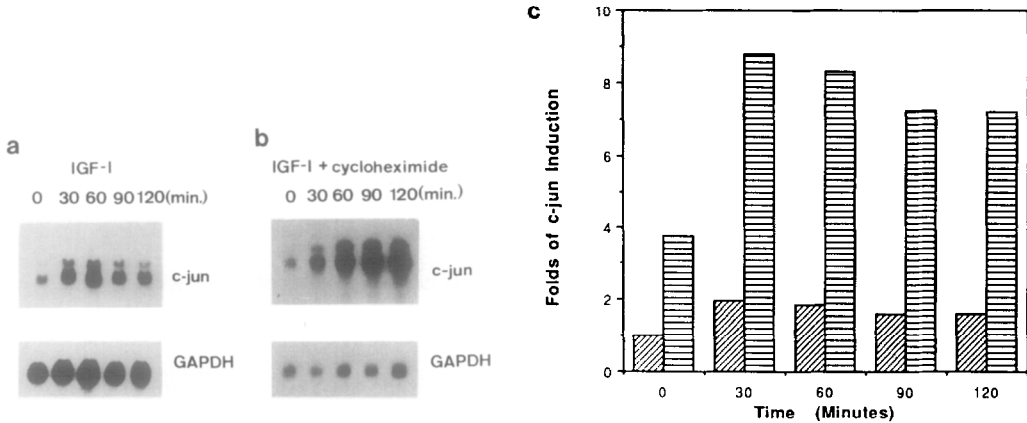
Fig.2 Kinetics of *c-jun* mRNA induction in IGF I stimulated Balb/c 3T3 cells.

Northern blot of RNA from Balb/c 3T3 cells treated with IGF I or IGF I plus cycloheximide harvested at different time point.

a.   Total RNA (10 µg) isolated from quiescent cells incubated with IGF I for different times.

b.   Total RNA(10 µg) isolated from quiescent cells pretreated with cycloheximide for 1 hour and incubated with IGF I for different times.

c.   Northern blots in Fig. 2a and 2b quantified by densitometry scanning. The *c-jun* mRNA levels from IGF I-treated cells (▨) and from IGF I/cycloheximide-treated cells (▤) are compared.

stimulation (Fig. 2a). Addition of IGF I to serum starved quiescent cells caused a 2-fold elevation of *c-jun* mRNA in comparison with basal level expression (Fig. 2c). In the presence of both cycloheximide and IGF I , the transcripts of *c-jun* not only increased within 30 minutes but maintained at high levels for a period of at least 2 hours (Fig. 2b).

To determine whether the effects of IGF I on *c-jun* transcript were due to transcriptional activation of the gene, nuclear run-off analyses were performed as described. To our surprise, we found that IGF I did not activate the transcription of *c-jun* in quiescent cells because only low amounts of newly synthesized *c-jun* mRNA were found in both quiescent cells and cells treated with IGF I alone (Fig. 3 lanes 1, 2). Cycloheximide, by itself, showed stimulatory effect on the synthesis of *c-jun* mRNA (Fig. 3, lane 3). Furthermore, while the cells were treated with both cycloheximide and IGF I, the transcription of *c-jun* dramatically increased (Fig. 3, lane 4). In comparison with cells treated with IGF I only, the presence of both cycloheximide and IGF I showed synergistic stimulation on *c-jun* transcripiton (Fig. 3, lanes 2, 4).

Since IGF I activated *c-jun* transcription efficiently only in the presence of cycloheximide, the increase of *c-jun* mRNA observed in Northern blot analysis of IGF I stimulated cells (Fig. 1, Fig. 2) could be caused by stabilization of pre-existing transcripts.
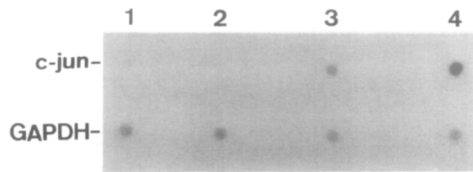
Fig.3 Nuclear Run-off analysis of c-jun mRNA.
Nuclei were isolated from quiescent cells (lane 1) or cells stimulated with IGF I for 30 minutes in the absence (lane 2) or presence (lane 4) of cycloheximide. Lane 3 contained transcripts of nuclei isolated from quiescent cells treated with cycloheximide only.

By examining the stability of *c-jun* mRNA in IGF I-treated cells, we observed that *c-jun* mRNA was more stable than that of quiescent cells (Fig. 4). The half life of *c-jun* mRNA was found to be increased by 30 minutes by IGF I treatment. (Fig. 4c)

DISCUSSION

In this study we demonstrated that IGF I was able to increase the *c-jun* mRNA level in Balb/c 3T3 cells up to 2 folds within 30 minutes. Nuclear run-off analyses revealed that IGF I alnone did not stimulate *c-jun* transcription but would cause a dramatic increase in *c-jun* mRNA level only if the protein synthesis was blocked by cycloheximide. Cycloheximide was also shown to stimulate *c-jun* mRNA synthesis, as has been well-
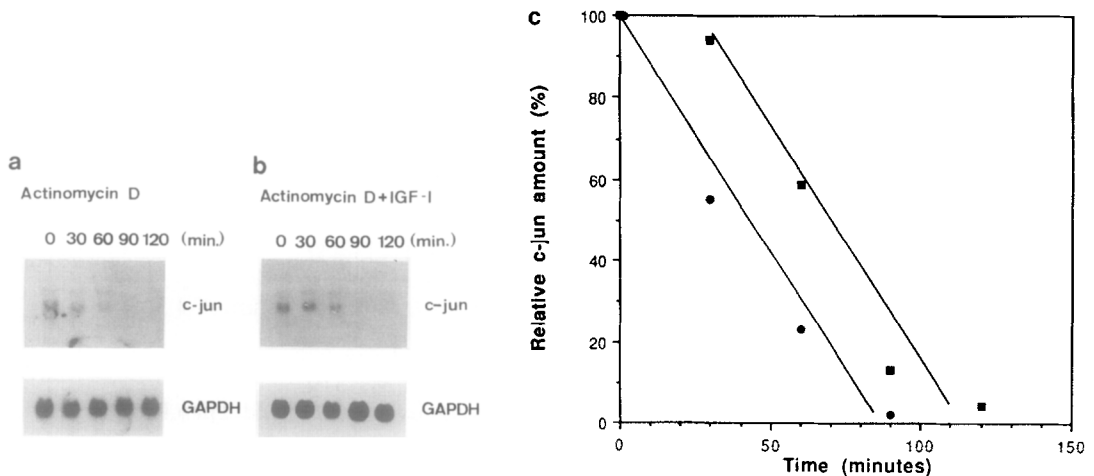

Fig.4 Stability of *c-jun* mRNA after IGF I stimulation.
Quiescent Balb/c 3T3 cells were treated with actinomycin D (1 µg/ml) for 15 minutes before IGF I stimulation. RNA was isolated at indicated time points after administration of growth factor. The level of *c-jun* mRNA was determined by Northern blotting analysis. Ten micrograms of cellular RNA was used in each lane in a and b.
a.   Levels of *c-jun* mRNA at different time points after actinomycin D treatment.
b.   Levels of *c-jun* mRNA at different time points after actinomycin D and IGF I treatment.
c.   Quantification of Northern blottings in Fig. 4a.(●) and Fig. 4b.(■) The levels of *c-jun* mRNA were normalized with respect to GAPDH.

documented. These observations prompted us to hypothesize that the *c-jun* mRNA levels may be regulated by some transciptional repressors and nucleases whose functions are modulated by IGF I. In the quiescent state, the repressors may be switched to active form to repress the *c-jun* transcription. IGF I treatment would inactivate the repressors and at the same time reduce the nuclease activity via post-translational modification (33). The synergistic effects by IGF I and cycloheximide on *c-jun* mRNA level and the stabilization of *c-jun* mRNA by IGF I reported herein indicate that IGF I affects *c-jun* expression by transciptional activation and stabilization of the existing transcripts.

ACKNOWLEDGMENTS

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