Increased intracellular Ca^{2+} is necessary for maximal expression of the proto-oncogene *c-jun* in the Jurkat T-cell line

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The time course and signal-transduction requirements for proto-oncogene *c-jun* expression in T-cells were investigated. Expression of *c-jun* mRNA was evident at 30 min after stimulation. Both the activation of Ca^{2+} /phospholipid-dependent kinase as well as an increased intracellular free Ca^{2+} concentration were necessary for the maximal induction of *c-jun* mRNA and synthesis of Jun protein 1 h after stimulation.

INTRODUCTION

Two major intracellular signal-transduction pathways, the activation of Ca^{2+} /phospholipid-dependent kinase (C-kinase) and a sustained increase in intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), have been implicated in the induction of certain genes upon T-cell activation. Cellular stimulation leads to elevated phosphoinositide turnover, the formation of the Ca^{2+} -mobilizing agent inositol trisphosphate, and diacylglycerol, which activates C-kinase. Simultaneously, a sustained increase in $[\text{Ca}^{2+}]_i$ is achieved by a Ca^{2+} flux into the cell from extracellular sources. These events finally culminate in autocrine T-cell proliferation through the secretion of interleukin-2 and the expression of its cell-surface receptor (reviewed in [1] and [2]).

Two nuclear *trans*-acting factors, NFAT-1 and NFIL-2A, mediating the Ca^{2+} -dependent activation of the interleukin-2 gene, have been described [3]. A *trans*-acting factor responsible for mediating the C-kinase signal in T-cells has not yet been identified. However, the interleukin-2 gene contains a putative binding sequence for the *trans*-acting factor AP-1, responsible for C-kinase-dependent gene activation in certain cell types [4]. The major 44 kDa product of AP-1 has been shown to be encoded by the *c-jun* proto-oncogene [5]. Jun protein associates with the oncogene *c-fos* gene product [6,7], which is expressed early in the T-cell mitogenic response [8].

T-cell mitogenesis is of prime importance in the immune response. It is, however, not known whether *c-jun* expression occurs in T-cells. In fibroblasts *c-jun* can be induced with epidermal growth factor, transforming growth factor β or serum factors [9–11]. We investigated the expression and nature of induction of *c-jun* in the human T-cell line Jurkat. Our results showed that, in T-cells, C-kinase activation stimulated *c-jun* expression, but also that a Ca^{2+} signal co-mediated expression of this proto-oncogene.

MATERIALS AND METHODS

Materials

Phytohaemagglutinin (PHA), phorbol 12-myristate 13-acetate ('TPA'), Ca^{2+} ionophore A23187 and cycloheximide (CHX) were all purchased from Sigma (St. Louis, MO, U.S.A.). [α - ^{32}P]dCTP

(3000 Ci/mmol) and L-[^{35}S]methionine (1300 Ci/mmol) were obtained from Amersham International (Little Chalfont, Bucks., U.K.), and random-primed labelling kits were bought from Boehringer Mannheim (Mannheim, Germany). Guanidinium isothiocyanate was from Merck (Darmstadt, Germany). Culture-medium components were from Gibco (Grand Island, NY, U.S.A.). GeneScreen Plus membranes were obtained from New England Nuclear Corp. [via du Pont (U.K.) Ltd., Stevenage, Herts., U.K.]. All other reagents were of the highest analytical grade available.

Cell culture and stimulation

Jurkat cells were cultured in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated fetal-calf serum (FCS) and antibiotics at a cell density of 10^6 /ml in 250 ml tissue-culture flasks in an air/ CO_2 (19:1) environment at 37 °C. Before stimulation, cells were transferred to RPMI containing 50 μM - β -mercaptoethanol, 1% FCS and antibiotics and cultured for 24 h at 37 °C [12] to decrease the high basal levels of *c-jun* expression of cells cultured in medium with 10% FCS (results not shown). Cell viability was more than 95% as assessed by Trypan Blue exclusion. Control cells received no further additions, whereas stimulated cultures were incubated with various combinations of PHA (10 $\mu\text{g}/\text{ml}$), PMA (10 ng/ml), cycloheximide (10 $\mu\text{g}/\text{ml}$), A23187 (1 μM) and EGTA (2 mM) for the indicated time periods (Figs. 1 and 2 below).

Northern-blot analysis

After stimulation, cells were collected by centrifugation, washed with phosphate-buffered saline (0.01 M-sodium phosphate/0.15 M-NaCl, pH 7.4), and total RNA was isolated by guanidinium isothiocyanate extraction and centrifugation over a 5.7 M-CsCl cushion [12]. RNA concentration was determined from A_{260} . Total RNA (30 $\mu\text{g}/\text{lane}$) was electrophoresed in formaldehyde/agarose gels and transferred to GeneScreen Plus membranes as indicated by the manufacturer. Filters were hybridized to cDNA probes, radiolabelled [sp. radioactivity $(1-3) \times 10^9$ c.p.m./ μg] using the random-primed method. Stringency washes were carried out in $0.1 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M-NaCl/0.015 M-sodium citrate) at room temperature after post-

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; C-kinase, Ca^{2+} /phospholipid-dependent kinase (protein kinase C); CHX, cycloheximide; FCS, fetal-calf serum; PHA, phytohaemagglutinin; PMA, phorbol 12-myristate 13-acetate ('TPA').

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washing in $2 \times$ SSC containing 1 % SDS at 60 °C. After revealing the signals by means of autoradiography, the membranes were 'scrubbed' (95 °C, 10 min) and rehybridized with τ -actin cDNA in order to control for the amount of RNA present in each lane. The *c-jun*-cDNA-containing plasmid (ph-cJ-1) was kindly provided by Dr. Peter Angel [13].

Pulse-labelling and immunoprecipitation

Cells (5×10^5) were stimulated as described above for 1 h, transferred to methionine-free RPMI and pulse-labelled with 200 μ Ci of [35 S]methionine/ml for 45 min. Cells were lysed, then immunoprecipitated with PEP2, a rabbit anti-Jun antiserum as described in [11]. The PEP2 antiserum was kindly given by Dr. Timothy Bos [5].

RESULTS AND DISCUSSION

Time course of *c-jun* induction and the effect of CHX

RNA extracted from cells stimulated for up to 8 h with PMA and PHA showed maximal *c-jun* expression at 1 h (Fig. 1*a*). mRNA for *c-jun* was evident 30 min after stimulation and had returned to basal levels by 8 h (Fig. 1*a*). τ -Actin expression remained relatively constant over this time period. Addition of the protein-synthesis inhibitor CHX at the time of stimulation resulted in a large increase in *c-jun* expression 1 h after stimulation. This increased expression reached a peak at 4 h, after which mRNA levels declined. Although τ -actin mRNA levels increased in CHX-treated cells stimulated with PMA and PHA, this increase was considerably less than that observed in *c-jun* mRNA levels.

The maximal induction of *c-jun* 1 h after stimulation, and the superinduction by CHX, are similar to that reported in fibroblasts [9, 10]. Furthermore, the expression of *c-jun* in lymphocytes occurs in the same time frame as *c-fos* expression [8]. As the products of these two genes interact to form a potent *trans*-activator [6], their early induction in T-cells could stimulate expression of T-cell-specific genes such as those for interleukin-2 and its receptor.

Signal-transduction requirements for *c-jun* expression

As the activation of C-kinase and an increased $[Ca^{2+}]_i$ have been shown to lead to the expression of lymphocyte-specific genes [1], we examined the relative contribution of these pathways to *c-jun* expression.

Fig. 2. Effect of C-kinase activation and $[Ca^{2+}]_i$ on the expression of *c-jun*.

Jurkat cells were treated for 1 h as indicated. (a) The effect of PMA, PHA and A23187 on *c-jun* expression: (i) extracted RNA was analysed by Northern blot and hybridized with *c-jun* and τ -actin; (ii) immunoprecipitation of Jun protein. Cells were labelled with [35 S]methionine for 45 min after stimulation for 1 h and lysed. Jun protein was immunoprecipitated with PEP2 and analysed by SDS/PAGE and autoradiography (X = immunoprecipitation of this sample was not done). (b) Northern-blot analysis of the effect of EGTA on *c-jun* mRNA levels. Cells were stimulated with PMA and PHA in the presence or absence of EGTA.

Treatment of cells with a C-kinase agonist, PMA, leads to a moderate, yet significant, increase in *c-jun* expression [Fig. 2*a*(i)]. Although PMA is a potent agonist for C-kinase, it is a weak T-

Fig. 1. Time course of *c-jun* induction by TPA and PMA in the presence or absence of CHX.

Jurkat cells, stimulated for the indicated time with PMA and PHA were lysed and the total RNA extracted. RNA was electrophoresed and transferred to GeneScreen Plus membranes. Membranes were hybridized with labelled *c-jun* and τ -actin cDNA probes and autoradiographed. (a) *c-jun* induction without CHX; (b) induction time course of *c-jun* in the presence of CHX alone or CHX plus PMA and PHA.

cell mitogen [14]. PHA, which activates C-kinase and increases $[Ca^{2+}]_i$ through stimulation of cell-surface receptors [14,15], was a more powerful stimulus for *c-jun* expression than PMA alone. A combination of PMA and PHA, however, maximally stimulated *c-jun* mRNA synthesis, possibly by activating both pathways [Fig. 2a(i)]. The importance of the role of a Ca^{2+} signal in the induction of *c-jun* was further investigated by examining the effect of the Ca^{2+} ionophore A23187. A combination of PMA and A23187 reproduced the maximal expression of *c-jun* induced by PMA and PHA. Ionophore could not increase the signal provided by stimulation with PHA. In agreement with the finding that A23187 treatment of T-cells is not mitogenic [16], we showed that, in the absence of PMA, the elevation of $[Ca^{2+}]_i$ did not induce *c-jun* expression [Fig. 2a(i)]. Further evidence for the partial Ca^{2+} -dependence of *c-jun* induction was provided by the addition of EGTA to PHA-treated cells. This decreased *c-jun* mRNA to amounts comparable with those of cells treated with PMA alone (Fig. 2b). EGTA also depressed the maximal PMA/PHA activatory signal. Addition of excess Ca^{2+} (4 mM) to the EGTA-treated cultures abrogated the chelator's effect on *c-jun* expression, indicating that Ca^{2+} is the bivalent cation involved in the stimulation of *c-jun* expression (results not shown). Levels of τ -actin mRNA expression remained relatively constant in all the experiments.

The mRNA response to various combinations of stimuli was correlated with the rate of Jun-protein synthesis [Fig. 2a(ii)]. This confirmed that maximal Jun-protein synthesis was dependent upon the combined action of activated C-kinase and elevated intracellular Ca^{2+} .

In conclusion, *c-jun* is expressed in T-cells and could therefore play an important role in the control of gene expression during cellular activation in these cells. The activation of C-kinase induces moderate *c-jun* mRNA synthesis, but an additional signal, mediated by increased $[Ca^{2+}]_i$, is required for the maximal expression of *c-jun* in T-cells. A better understanding of events affecting these aspects of T-cell activation could be useful for manipulating the immune response.

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