Regulation of c-jun Gene Expression in Human T Lymphocytes

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The present studies have examined the effects of mitogens on induction of early response gene expression in normal peripheral blood T and Jurkat cells. Pokeweed mitogen (PWM) or anti-CD3 significantly increases c-jun messenger RNA (mRNA) levels in T cells. This transient PWM-related increase in c-jun transcripts is maximal after 15 to 30 minutes of exposure of T cells to PWM. PWM induces c-jun gene expression in a concentration-dependent manner. Moreover, PWM similarly induces expression of other genes coding for leucine zipper transcription factors, i.e., jun-B and c-fos. Nuclear run on assays demonstrate that PWM treatment is associated with an increased rate of c-jun gene transcription. Transient expression assays with c-jun promoter fragments linked to the chloramphenicol acetyltransferase gene suggest that the PWM-induced increase in transcription is mediated by the AP-1 transcription factor complex. Moreover, treatment of T cells with actinomycin D to block further transcription before their culture with PWM suggests that the increase in c-jun gene expression by PWM is also regulated at least in part by a posttranscriptional mechanism. Cycloheximide does not alter c-jun mRNA induction by PWM. Finally, given that PWM induces B-cell differentiation in an interleukin-6 (IL-6)-mediated, T-cell-dependent manner, the relationship of c-jun and IL-6 gene expression in PWM-stimulated T cells was examined. The induction of IL-6 mRNA in T cells stimulated by PWM occurs after maximal induction of c-jun mRNA; at a time when the latter is no longer detectable. These findings suggest that PWM induces c-jun gene expression in T cells by a transcriptional and posttranscriptional mechanism and that AP-1 confers PWM inducibility of this gene. Because the IL-6 promoter has several potential transcriptional control elements, one of which is an AP-1-binding site, future experiments will evaluate the role of c-jun in the regulation of PWM-induced IL-6 synthesis by T cells.

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Materials and Methods

Cells. Human peripheral blood (PB) was obtained from healthy donors and PB mononuclear cells (PBMC) were isolated by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density sedimentation. After three washes with Hanks Balanced Salt Solution (HBSS), the cells were resuspended in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine containing 10% heat-inactivated fetal bovine serum (FBS). Cells were adhered for 1 hour at 37°C on plastic petri dishes to deplete monocytes; nonadherent cells were harvested and T lymphocytes were isolated by double rosetting with sheep red blood cells (SRBC) as previously described. T cells were greater than 99% CD3+ and CD11+ as assessed by indirect immunofluorescence. The Jurkat cell line was maintained in suspension culture in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine.

Cell cultures. The purified T cells were resuspended in RPMI 1640 with 10% FBS and cultured for 3 to 4 hours at 37°C before stimulation with either the RW2-4B6 anti-CD3 monoclonal antibody (MoAb) (1:250), PWM (1:400) (Sigma Chemical, St Louis, MO), or PHA (1 μg/mL) (GIBCO, Gaithersburg, MD) at concentrations known to induce 2×10^6 cpm [3H]thymidine uptake at 72 hours. Cells were also treated with actinomycin D (10 μg/mL, Sigma) or cycloheximide (10 μg/mL, Sigma).

Preparation of RNA and Northern blot hybridization. Total cellular RNA was isolated by a modification of the guanidine-isothiocyanate technique as previously described. Total cellular RNA (20 μg/lane) was subjected to electrophoresis in a 1% agarose/2.2 mol/L formaldehyde gel, transferred to nitrocellulose paper, and hybridized to one of the following 32P-labeled DNA probes: (1) the 1.8-kb BamHI/EcoRI insert of the human c-jun DNA probe containing a 1.0-kb 3’ cDNA and 0.8-kb untranslated sequences purified from a p Bluepc-fos-I plasmid; (2) the 1.8-kb EcoRI fragment of the murine jun-B cDNA purified from the pHS6.20 plasmid; (3) the 0.9-kb Scal/NcoI fragment of the human c-fos DNA consisting of exons 3 and 4 purified from the pc-fos-1 plasmid; (4) the pA1 plasmid containing a 2.0-kb Pst I insert of the chicken β actin gene; and (5) the Ban II-Fag I fragment nucleotides 215 to 657 of a full-length IL-6 cDNA.

Probes were prepared by nick translation and purified by electrophoresis in 0.8% agarose/2.2 mol/L formaldehyde. The purified probes were precipitated two times in 2.5 mol/L ammonium acetate. RNA was purified through a spin column prepared with Sephadex G50 equilibrated in and eluted with column buffer (0.3 mol/L NaCl, 0.1% SDS, 1 mmol/L Na2 EDTA, 10 mmol/L Tris-HCl, pH 7.5).

Plasmid DNAs containing cloned inserts were digested with restriction endonucleases as follows: (1) the 2.0-kb Pst I fragment of the chicken β actin pA1 plasmid; and (2) the 1.8-kb BamHI/EcoRI fragment of the human c-jun DNA. The digested DNA was denatured by heating to 65°C for 15 minutes, separated in 1% agarose gels, and transferred to nitrocellulose filters by the method of Southern. The filters were prehybridized in 5X Denhardt’s solution, 40% formamide, 4X SSC, 5 mmol/L Na2 EDTA, 0.4% SDS, and 100 μg/mL yeast tRNA for 2 to 4 hours. Hybridizations were performed with 106 cpm of 32P-labeled DNA per milliliter of hybridization buffer for 72 hours at 42°C. The filters were then washed in 2X SSC and 0.1% SDS at 37°C for 30 minutes, 10 μg/mL RNase A in 2X SSC at 37°C for 20 minutes, and 0.1% SDS and 0.1X SSC at 42°C for 30 minutes. The autoradiograms were scanned using a laser densitometer.

Reporter assays. AP-1 reporter plasmids were kindly provided by M. Karin. The -132/+170 jun CAT contains the -132/+170 promoter region from the jun gene linked to a promoterless CAT gene. The -132/+170 jun CATAATP-1 is identical, with the exception of four base-pair mutations in the AP-1 region (TRE, TGAAGCTA). The 5X TRE TK CAT contains 5 tandem copies of the TRE linked to the Herpes simplex virus thymidine kinase promoter and the CAT gene (unpublished data). Normal human PB T cells were isolated by using SRBC and E-rosette formation. The cells were cultured for 24 hours in 10% FBS complete media containing 1 μg/mL PHA. Transfections were performed by electroporation (BioRad Gene Pulser, 240 V, 960 μFD; BioRad, Richmond, CA). The cells were harvested 36 hours after transfection, maintained for an additional 6 hours in the presence or absence of PWM, and then harvested and lysed in lysis buffer (0.25 mol/L Tris-HCl, pH 7.8, 1 μmol/L phenylmethylsulfonyl fluoride [PMSF]) by 3 cycles of freeze/thawing. CAT activity was assayed in 200 μL reactions containing 100 μL of cell extract, 0.025 μCi [3H]chloramphenicol (Amersham, Arlington Heights, IL; 57 μCi/mmol), 275 mmol/L Tris-HCl, pH 7.8, and 0.4 mmol/L acetyl-coenzyme A (Sigma) for 2 hours at 37°C. The acetylated products were separated from unacetylated chloramphenicol by thin-layer chromatography (TLC) using chloroform:methanol (95:5, vol/vol) as a solvent system. Both unacetylated and acetylated forms of [3H]chloramphenicol were cut from the TLC plates, and radioactivity was determined by a scintillation counter. Cell extracts contained equal amounts of protein as determined by the BioRad Protein Assay (BioRad).

Results

Effect of CD3 and PWM treatment on c-jun mRNA levels in T cells. The effects of T-cell mitogens on c-jun expression were studied in normal human PB T cells. PB T cells isolated by SRBC rosetting and maintained in culture for 3 to 4 hours expressed low to undetectable levels of the 2.7-kb c-jun transcript (Fig 1). Triggering of these cells through the T-cell receptor with anti-CD3 (Fig 1A) or with PWM (Fig 1B) demonstrated a 20- to 30-fold increase in c-jun transcripts by 15 to 30 minutes. These effects were associated with little change in the levels of actin transcripts. Similar findings were obtained when Jurkat cells were treated with 2.5 μg/mL PWM. The c-jun mRNA transcripts were transiently induced by 30 minutes and peaked after 3 hours of exposure to PWM.
whereas longer exposures were associated with downregulation of c-jun expression (data not shown).

Similar studies were performed with varying concentrations (0.625 to 2.5 µg/mL) of PWM (Fig 2). While exposure of normal human PB T cells to 1.25 µg/mL PWM for 30 minutes also resulted in increases in c-jun transcripts, 0.625 µg/mL PWM had minimal effects. Taken together with the absence of detectable changes in actin mRNA levels, these results indicate that PWM regulates c-jun expression in a concentration-dependent manner.

Effect of PWM on jun-B and c-fos mRNA levels in T cells. Because the jun-B gene shares significant homology with c-jun and encodes for a protein that activates multimeric TREs, the effect of PWM treatment on jun-B transcripts was similarly assayed. As can be seen in Fig 3, jun-B mRNA was present at low to undetectable levels in T cells. Moreover, treatment of these cells with 2.5 µg/mL PWM was associated with transient eightfold increases in the 2.0-kb jun-B transcripts at 15 to 30 minutes. Because binding of both c-Jun and Jun-B to TREs is enhanced by the formation of heter-
Induction of the jun-B and c-fos genes in PWM-treated T cells. T cells were treated with 1.25 pg/mL PWM for the indicated times. Total cellular RNA (20 µg/lane) was hybridized to the 32P-labeled jun-B and c-fos cDNA probes. Actin signal demonstrated equal loading of the lanes (data not shown).

Nuclear run-on assays. Run-on assays were performed to determine whether the effects of PWM on c-fos expression in T cells are related to increases in the rate of transcription of this gene. The actin gene was constitutively transcribed in T cells and PWM treatment had no effect on the transcription rate of this gene (Fig 4). A low level of c-jun gene transcription was detectable in T cells cultured in media alone. However, exposure to PWM (2.5 µg/mL) for 5 minutes was associated with a fivefold increase in the rate of c-jun gene transcription (Fig 4). These results suggest that the induction of c-jun expression by PWM in T cells is regulated at least in part by a transcriptional mechanism.

Reporter assays. It has already been reported that c-jun gene is autoinduced by its product Jun/AP-1. Consequently, studies were performed to determine whether the effects of PWM involve activation of the AP-1 consensus sequence in the c-jun promoter. This issue was addressed by performing CAT reporter assays using the plasmids -132/+170 jun-CAT and -132/+170 jun-CAT Δ AP-1. In -132/+170 jun-CAT transfected T cells, the basal level of CAT activity was increased 1.5-fold (n = 5, P < .025) after PWM treatment (data not shown). In contrast, PWM treatment of T cells transfected with the -132/+170 jun-CAT Δ AP-1 plasmid demonstrated no increase in CAT activity (data not shown). To confirm the apparent requirement for an AP-1-binding site, the 5X TRE CAT reporter construct was used in similar experiments. CAT activity increased threefold to fourfold (n = 5, P ≤ .01) after PWM treatment of cells transfected with this construct (Fig 5). These data suggest that induction of jun transcription by PWM requires, at least in part, the AP-1-binding region in the jun promoter.

Effect of PWM on stability of c-jun mRNA in T cells. To determine whether PWM regulates c-jun expression by altering mRNA stability, T cells were treated with 10 µg/mL actinomycin D to inhibit further transcription. Because the expression of c-jun gene in T cells was low, calculation of the half-life of the c-jun mRNA necessitated overexposure of the blots. Using this method, the half-life of c-jun transcripts was approximately 20 minutes (Fig 6A). In contrast, this half-life was increased to 45 minutes when T cells were pretreated with PWM (Fig 6B).

To determine whether protein synthesis alters c-jun mRNA induced by PWM, T cells in the presence of PWM were cultured with and without cycloheximide. As can be seen in Fig 7, cycloheximide did not affect the induction of c-jun mRNA expression in T cells by PWM.
Functional significance of c-jun on IL-6 synthesis in T cells. Given that PWM is a T-cell-dependent B-cell mitogen, it was of interest to examine the relationship of c-jun mRNA to IL-6 mRNA expression in T cells. As can be seen in Fig 8, IL-6 mRNA expression was detectable at as early as 1 hour and increased 11-fold at 6 hours. The peak (20- to 30-fold) increase in c-jun mRNA occurred at 15 to 30 minutes, and this transcript was no longer detectable when IL-6 mRNA became evident. These effects of PWM were not associated with alterations in the levels of actin transcripts.
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Fig 7. Effects of cycloheximide on c-jun mRNA levels. T cells were treated with 1.25 μg/mL PWM for 15 minutes with or without cycloheximide (10 ng/mL). Total cellular RNA (20 μg/lane) was hybridized to 32P-labeled c-jun cDNA probe.

DISCUSSION

In the present studies, both anti-CD3 and PWM induced an increase in c-jun transcripts that occurred early and was transient. This induction of c-jun mRNA was PWM concentration dependent. Nuclear run-on assays further demonstrated that the induction of c-jun gene expression by PWM in T cells is controlled at the transcriptional level. Moreover, this induction of c-jun is conferred, at least in part, through an AP-I-binding site present in the c-jun promoter, as suggested by the CAT reporter assays. Previous work has demonstrated that c-jun expression is regulated at both the transcriptional and posttranscriptional levels by serum, platelet-derived growth factor, and fibroblast growth factor. Our finding that the half-life of c-jun transcript in T cells was prolonged by PWM suggested that the c-jun expression is also controlled by a posttranscriptional mechanism. Treatment with cycloheximide and PWM together did not alter the induction of c-jun transcripts, suggesting that de novo protein synthesis is not required for the induction of this gene by PWM. PWM induces IL-6 mRNA in T cells after maximal c-jun mRNA expression and at a time when c-jun mRNA is no longer detectable. Given that (1) PWM mediates T-cell-dependent, IL-6-mediated B-cell differentiation, and (2) the IL-6 promoter also has an AP-1–binding site, the current studies are a first step to further understanding the role of c-Jun in the triggering of IL-6 in T cells by PWM.

Previous studies have shown that T-cell activation involves transient induction of c-fos and c-myc proto-oncogenes, whose expression may also affect the transcriptional control of other genes. Expression of c-fos has also been shown to be induced by agents capable of initiating T-cell growth and differentiation. Phorbol esters and serum factors are also known to cause the accumulation of c-jun–specific mRNA. In recent studies, it has been shown that human T-cell activation through CD69 enhances the binding of AP-1 to TRE binding complexes with a resultant marked increase in transcription of c-fos, but not c-jun. In contrast, inhibition of protein phosphatases by okadaic acid was found to dramatically increase mRNA transcripts of the jun family, with lesser increases in mRNA transcripts for the fos family. Thus, the pattern of early response gene expression in antigen- or mitogen-triggered T cells is complex, and their functional significance at present remains undefined.

The present results suggest that PWM or anti-CD3 can trigger c-jun and IL-6 mRNA expression in T cells. Although unfractionated T cells were used in these studies, it is possible that the observed effects were on a subset of T cells. Specifically, the CD4+/45RA– (CDW29+) T-cell subset contains those T cells that proliferate in anamnestic responses to soluble antigens and provide polyclonal and Ag-specific help. It has been demonstrated by multiple observers that PWM-induced B-cell differentiation is IL-6 mediated. Moreover, we have recently demonstrated that PWM triggers IL-6 mRNA in CD4+/45RA– T cells, but not in CD4+/45RA+ T cells. Recent studies also support the view, as was true in our studies, that IL-6 mRNA is not induced in T cells triggered by other mitogens. Specifically, PHA/IL-2 does not induce IL-6 mRNA. Although E-rosette–positive T cells cocultured with PHA and TPA express high levels of IL-6 mRNA, stimulation with either alone induces a negligible increase in IL-6 mRNA expression. Finally, infection of T cells with HTLV-I and HTLV-I–positive cell lines leads to the expression of IL-6 mRNA. Although mitogen-induced genes are subject to multiple pathways of regulation in the initial stages of T-cell activation, their functional significance is not well characterized. To date, several potential transcriptional control elements, such as glucocorticoid-responsive elements (GRE), an AP-1–binding site, a c-fos serum-responsive element homology (c-fos SRE), a cyclic AMP-responsive element (CRE), and an NF-κB binding site have been described within the IL-6 promoter. Future experiments will determine whether Jun bind-

Fig 8. Effects of PWM on IL-6 mRNA levels in T cells. T cells were treated with 1.25 μg/mL PWM for the indicated times. Total cellular RNA (20 μg/lane) was hybridized to the 32P-labeled IL-6 cDNA probe.
ing to the AP-1 site of the IL-6 promoter or binding of other early response gene products to the c-fos SRE, CRE, or NF-κB binding sites within the IL-6 promoter confer PWM inducibility of this gene.

Other hematopoietic (ie, monocyteic) and nonhematopoietic cell types (fibroblasts and endothelial cells) also produce IL-6. IL-6, originally known as B-cell-stimulatory factor-1 (BSF-1) or IL-6, was first isolated and characterized in PWM cultures. Indeed, we have recently demonstrated that IL-6 is produced by PWM-stimulated monocytes, which are not associated with T cells, but also monocytes to be present in culture. Monocytes may therefore be a source of IL-6 in the PWM cultures. Indeed, we have recently demonstrated that PWM-stimulated monocytes produce IL-6. Previous studies in our laboratory have also shown that low levels of c-jun transcripts were detectable in resting human PB monocytes and that human recombinant macrophage colony-stimulating factor (M-CSF) was associated with rapid and transient increases in c-jun mRNA levels. As was true in the current study for T cells, nuclear run-on assays and mRNA stability studies demonstrated that M-CSF regulates c-jun expression by both an increase in transcription rate and a prolongation in the half-life of c-jun transcripts. Although not yet elucidated, it is therefore possible that c-jun regulates IL-6 gene expression in monocytes in a manner similar to that demonstrated in T cells. Similar regulatory mechanisms may also be occurring in other cell types, ie, fibroblasts. B cells can be triggered by T-cell–independent and T-cell–dependent mitogens to differentiate and secrete Ig in vitro. IL-6, originally known as B-cell–stimulatory factor-2 (BSF-2), has been characterized and molecularly cloned as an interleukin that induces the final maturation of B cells to antibody-producing cells. Moreover, the receptor for IL-6 has been shown to be present on activated B cells. Both Staphylococcus Cowan I (SAC) and Epstein-Barr virus (EBV), T-cell–independent B-cell mitogens, can also induce IL-6 mRNA in B cells. Our preliminary studies of highly purified B cells, in the absence of any accessory T cells or monocytes, suggest that PWM triggers an early and transient increase in c-jun mRNA similar to that observed in T cells. Again, only a subset of B cells appear to be PWM responsive, and thus upregulation of c-jun mRNA may be occurring in only a subset of cells. Future studies will determine the role of c-jun in the regulation of IL-6 gene expression and its role in accessory cell-dependent B-cell differentiation.

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