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Cyclic AMP Inhibits the Activity of c-Jun N-Terminal Kinase (JNKp46) but Not JNKp55 and ERK2 in Human Helper T Lymphocytes

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The cyclic AMP (cAMP) elevating agent PGE_2 and dibutyryl cyclic AMP (dBcAMP) affect T cell functions. Using human helper T cell clones, we examined effects of cAMP on c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), which are assumed to play a role in T cell regulation. When we analyzed the effects of dBcAMP on activities of mitogen–activated protein kinase (MAPK) family members ERK2, JNKp55 and JNKp46, dBcAMP did not inhibit the activities of ERK2 and JNKp55 induced by PMA/A23187 stimulation. JNKp46 activity was, however, inhibited by dBcAMP. JNK phosphorylates c-Jun on Ser-63 and Ser-73, the result being induction of its transcriptional activity. We found that dBcAMP inhibited the phosphorylation of c-Jun Ser-63 induced by PMA/A23187 stimulation. We suggest a different mechanism of regulation of JNKp55 and JNKp46 activities and that JNKp46 is a specific c-Jun kinase by which the activity of c-Jun is regulated in T lymphocytes.

Key Words: **T cells; signal transduction.**

T cell activation through the T cell receptor (TCR)/CD3 complex recruits multiple protein complexes consisting of several tyrosine kinases and tyrosine phosphorylated adapter proteins (1). One of the signal transduction pathways leads to the activation of phospholipase C (PLC)-γ1. PLC-γ1 catalyzes the hydrolysis of inositol phospholipids and thereby generates diacylglycerol and inositol polyphosphate, that activates protein kinase C (PKC) and enhances intracellular calcium level respectively. These two signal pathways, PKC activation and Ca^{2+} mobilization, cooperatively regulate cytokine production in T lymphocytes by activating several transcription factors. Signal transduction pathways downstream of PKC leads to activation of parallel mitogen activated protein kinase (MAPK) pathways, ERK2 (2) and c-Jun N-terminal kinase (JNK) (3). Both ERK and JNK are activated by phosphorylation of specific tyrosine and threonine residues common to MAPK family members. ERK2 activates the transcription factor TCF/Elk-1 which induces c-*fos* gene transcription (4). JNK was found to be the kinase that phosphorylates specific serine residues (positions at 63 and 73) of c-Jun thereby leading to the induction of its transcription activity (5). JNK also phosphorylates transcription factors ATF-2 and TCF/Elk-1 (6, 7). Thus, these two kinases are important for expression and activation of the AP-1 complex, consisting of Fos/Jun family members. Three JNK isoforms, JNK1, JNK2 and JNK3 have been isolated and all phosphorylate three substrates, c-Jun, ATF-2 and Elk-1 (6, 7). JNK2 has a higher affinity for c-Jun and ATF-2 than JNK1 and JNK3 (5, 6). It remained to be determined if JNK isoforms have differences in substrate specificity and functions *in vivo.*

Prostaglandin E_2 (PGE_2) elevates intracellular cyclic AMP (cAMP) levels through the activation of adenylate cyclase. dBcAMP can permeate through the plasma membrane and mimic the function of cAMP, an event which can exert a wide range of biological activities in various tissues. In the immune system, PGE_2 can function as an immunosuppressant and increases intracellular cAMP levels resulting in the activation of protein kinase A (PKA) in lymphoid cells. The increase of intracellular cAMP inhibits tyrosine phosphorylation of Grb2-associated proteins (8), ZAP70 (9) and PLC-γ1 leading to the inhibition of their enzymatic activities in T cell lines. Upon phorbol myristate acetate (PMA)/calcium ionophore stimulation, the increase of intracellular cAMP also inhibits the activity of JNK in certain T cell lines (10, 11). Thus, cAMP apparently acts on multiple targets in the TCR-mediated signal pathway, both upstream and downstream of PKC and Ca^{2+}

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mobilization, and thereby regulates the production of several cytokines induced by TCR-mediated signaling.

We analyzed the mechanisms of inhibition of TCR signaling by cAMP at molecular level in human helper T cell clones. We found that the cAMP signal inhibits the phosphorylation of Ser-63 of endogenous c-Jun. While ERK2 and JNKp55 activities are apparently not affected by cAMP, JNKp46 activity is inhibited by the cAMP signal. These observations suggest that activities of JNKp55 and JNKp46 are differentially regulated in T cells and JNKp46 may be the main regulator of the phosphorylation of c-Jun in T cells.

MATERIALS AND METHODS

Reagents. PMA and A23187 were obtained from Calbiochem (La Jolla, CA). dBcAMP was obtained from Sigma (St Louis, MO). Anti-ERK2 (C-14), anti-JNK1 (C-17), anti-JNK2 (FL), anti-phosphorylated-Ser-63-c-Jun (KM-1) and anti-c-Jun (N) antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA). The plasmid encoding glutathione *S*-transferase (GST) fusion protein containing the region between amino acids 1 to 223 of c-Jun (GST-cJun (1–223)) (5) was kindly provided by Dr. M. Karin (University of California, San Diego, CA). GST-cJun (1–223) fusion protein was expressed in *Escherichia coli* and purified on a glutathione-Sepharose affinity column (Pharmacia Biotech, Uppsala, Sweden). Recombinant human IL-2 was a kind gift from Dr. R. Kastelein (DNAX Research Institute, Palo Alto, CA).

Cell lines. The human $CD3^+CD4^+CD8^-$ T cell clone SP-B21 (12), which is specific for tetanus toxin and produces Th0 type of cytokines, was cloned by limiting dilution and screened for the specificity, as described. The human $CD3^+CD4^+CD8^-$ T cell clone TA23, which is specific for purified protein derivative from Mycobacterium tuberculosis and produces Th1 type of cytokines, was isolated from a healthy donor using the same protocol as for SP-B21 cells. These cells were cultured by bimonthly stimulation with a feeder cell mixture consisting of irradiated (4000 rad) allogenic peripheral blood mononuclear cells, an irradiated (5000 rad) Epstein-Barr virus transformed B cell line and PHA (0.1 μg/ml; Wellcome, Beckenham, UK), in Yssel's medium (13) supplemented with 1% human AB^+ serum, as described (14). Three or four days after stimulation, the cultures were split and further expanded in medium containing 20 U/ml recombinant human IL-2. All experiments were done using T cells that had been cultured for 10–14 days after the last re-stimulation with feeder cells.

Whole cell lysate preparation. T cells (5×10^6) stimulated by different protocols were collected and washed with cold PBS and resuspended in 200 μl of lysis buffer (50 mM HEPES, pH 7.5, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and 0.1% Triton X-100). After mixing vigorously by pipetting, samples were centrifuged at 15000 r.p.m. for 20 minutes by a microcentrifuge and the supernatants were stored at −80°C, prior to testing.

Immunoprecipitation and Western blotting. Whole cell lysates prepared by the methods described above were incubated with appropriate antibody and protein A-sepharose at 4°C for 2 h in HEPES binding buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA and 0.05% Triton X-100). For Western blot analysis, whole cell lysate or the immunoprecipitated proteins were separated by 10% SDS polyacrylamide gels (PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane filter (Millipore, Bedford, MA). The membrane was blocked in 5% skim milk in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20), and then probed with an appropriate antibody diluted in TBST for 1 h. After washing in TBST and incubating with second antibody conjugated to horseradish peroxidase for 1 h, the membrane was washed and visualized by the ECL detection system according to the manufacturer's instruction (Amersham, Arlington Heights, IL).

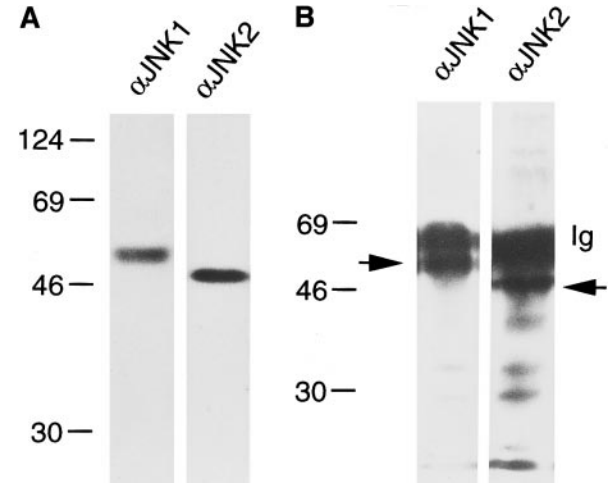

FIG. 1. Reactivity of JNK1 and JNK2 in cloned human T cells. SP-B21 whole cell lysate (A) or immunoprecipitates obtained by using either anti JNK1 or JNK2 antibodies from SP-B21 whole cell lysate (B) were separated by 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was blotted with anti JNK1 or JNK2 antibodies and bands were visualized by the ECL detection system. The strong upper bands of B correspond to the immunogloblin heavy chain and are indicated as Ig. The arrows indicate bands corresponding to molecular weights of 55 kDa (left lane) and 46 kDa (right lane).

In vitro immune complex kinase assay. The immune complex ERK kinase and JNK kinase assays were done according to the previous reports (5, 15). Briefly either ERK2 or JNK was resuspended in kinase reaction buffer (20 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM dithiothreitol) containing 20 μM ATP, 1 μCi [γ-^{32}P]ATP and 5 μg myelin basic protein (MBP) or GST-cJun (1–223) as the substrate of ERK2 or JNK respectively, in a final volume of 30 μl. The reaction of ERK2 was run at 30°C for 30 minutes, and that of JNK was for 30°C for 20 minutes. These reactions were terminated by adding Laemmli sample buffer, and the phosphorylated proteins were separated by SDS-PAGE and analyzed by FUJI image analyzer (BAS2000, FUJIFILM, Tokyo, Japan).

RESULTS

cAMP Inhibits the Kinase Activity of JNKp46, but Not That of JNKp55

We have reported that cAMP differentially modulates DNA binding activity of NF-κB, NF-AT and AP-1 in cloned human T cells (16, 17). For further analysis of effects of cAMP, we examined JNKp55 and JNKp46 activities in the presence or absence of cAMP using Th0 type human T cell clone SP-B21. We first analyzed the reactivity of antibodies used in this work, anti JNK1 antibody (C-17, Santa Cruz) and JNK2 antibody (FL, Santa Cruz). Whole cell lysate of SP-B21 was prepared and western blotting was done using these antibodies. As shown in Fig. 1A, the anti JNK1 anti-

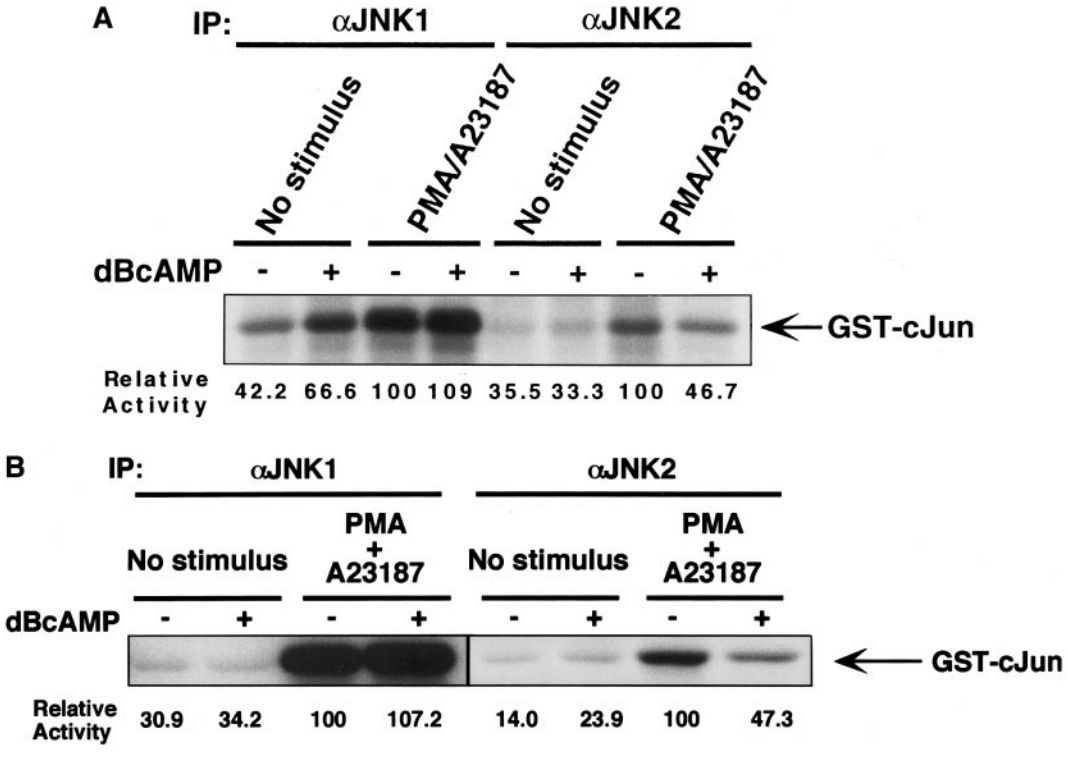

FIG. 2. Effects of dBcAMP on JNKP55 and JNKP46 activities. SP-B21 cells (A) or TA23 cells (B) were pretreated with dBcAMP (1 mM) or medium for 1 h. Then cells were stimulated with a combination of PMA (1 ng/ml) and A23187 (500 ng/ml). Fifteen minutes after stimulation, cells were lysed and immunoprecipitation was done using either anti JNK1 or JNK2 antibodies. Kinase assays of JNKp55 or JNKp46 immunoprecipitants were done with GST-cJun (1–223) fusion proteins (5 μg) and [γ-^{32}P]ATP at 30°C for 20 min. The reactions were separated by SDS-PAGE and the phosphorylated GST-cJun was visualized using a FUJI image analyzer (BAS2000). Relative kinase activities of JNKp55 and JNKp46 are expressed under the lanes. Experiments were done three times and essentially the same results were obtained.

body reacted with a single band corresponding to a molecular weight of 55 kDa, and the anti JNK2 antibody recognized a single band with a molecular weight of about 46 kDa. We next did immunoprecipitation using these antibodies. Figure 1B shows the western blotting pattern of immunoprecipitates and it was also shown that the antibodies recognized either p46 or p55 molecules. In the following experiments, we refer to JNK recognized by either antiJNK1 or JNK2 antibodies as JNKp55 and JNKp46 respectively. We next analyzed kinase activities by an *in vitro* immune complex kinase assay with GST-cJun containing region between amino acid positions between 1 and 223 as the substrate. After stimulation of SP-B21 cells for 15 minutes, JNKp55 and JNKp46 proteins in whole cell lysates were immunoprecipitated in the presence of isoform specific antibodies and kinase assay was performed using GST-cJun (1–223). The activities of both JNKP55 and JNKP46 were induced after PMA/A23187 stimulation (Fig. 2A). Kinetics of the kinase activity peaked at 15 minutes and was reduced 30 minutes after the stimulation (data not shown). Kinase activities of JNKp55 in SP-B21 cells were not inhibited by the addition of 1 mM dBcAMP 1 h prior to the stimulation. In contrast, JNKp46 activation in SP-B21 cells induced by PMA/A23187 stimulation was inhibited by the same pretreatment with 1 mM dBcAMP. We next performed the same experiments using Th1-like human T cell clone TA23 cells. Similar to the SP-B21, PMA/A23187 induced JNKp46 but not JNKp55 activity was inhibited by the addition of cAMP (Fig. 2B).

cAMP Inhibits Ser-63 Phosphorylation of c-Jun

c-Jun is one of the components of AP-1 (18). Both Ser-63 and -73 on the N-terminal portion of c-Jun is phosphorylated by stimuli such as PMA, TNF-α, p21ras and UV irradiation, and this phosphorylation correlates with the transcriptional activity of c-Jun (19, 20, 21). To determine if cAMP modulates the phosphorylation at the N-terminal of c-Jun, we examined the effect of dBcAMP on Ser-63 phosphorylation of endogenous c-Jun in the Th0 clone SP-B21 by using an anti-phosphorylated-Ser-63-specific c-Jun antibody. PMA/A23187 stimulation induced phosphorylation of the Ser-63 residue (Fig. 3) and the peak occurred after 60 minutes of stimulation (data not shown). Pretreatment with dBcAMP for 1 h prior to this stimulation strongly inhibited Ser-63-phosphorylation of c-Jun in-

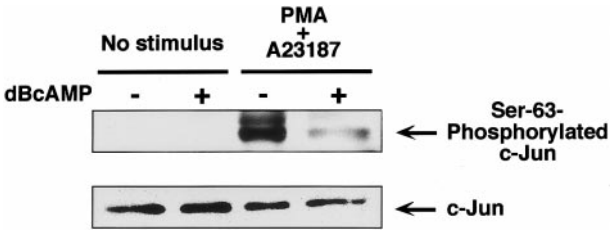

FIG. 3. Effects of dBcAMP on phosphorylation of c-Jun Ser-63. SP-B21 cells were treated with dBcAMP (1 mM) for 1 h and then stimulated with a combination of PMA (1 ng/ml) and A23187 (500 ng/ml). After 60 min of culture, whole cell lysates were prepared and separated in a 12% SDS-PAGE and immunoblotting was done using anti-phospho-specific c-Jun antibody (top) or anti-c-Jun polyclonal antibody (bottom). Bands were visualized by the ECL detection system.

duced by PMA/A23187 stimulation. Addition of dBcAMP did not alter the amount of endogenous c-Jun protein, indicating that post translational modification is affected by cAMP.

cAMP Has no Effect on ERK2 Activity

We next examined the effects of dBcAMP on PMA/Ca^{2+} induced ERK2 activation, which is assumed to play an important role to regulate various transcription factors. ERK2 activation can be detected by its mobility shift, due to the phosphorylation of specific residues (22). We examined ERK2 activities using whole cell lysates from SP-B21 cells, by western blots. Upon stimulation with either PMA alone or with the combination of PMA and A23187 for 5 minutes, the mobility of ERK2 proteins shifted (Fig. 4A), but there was no shifted band with stimulation by A23187 alone (data not shown). This suggests that the PMA-mediated signal can induce ERK2 activation without the Ca^{2+} signal in this cell line. When cells were pretreated with 1 mM dBcAMP for 1 h prior to stimulation, no apparent effect on the mobility shift of ERK2 induced with either PMA alone or the combination of PMA and A23187 was observed (Fig. 4A).

Next, we further confirmed this observation by using ERK2 immune complex kinase assay with MBP as a substrate. After PMA/A23187 stimulation for 5 minutes, ERK2 proteins were immunoprecipitated and kinase assay was done. As shown in Fig. 4B, the phosphorylation of MBP was induced by PMA/A23187 stimulation for 5 minutes. Consistent with the results of the mobility shift assay, the treatment with dBcAMP had no effect on the kinase activities of ERK2. These results indicate that cAMP-mediated signaling does not affect the ERK2 activity in these cells.

DISCUSSION

In the present work, we obtained evidence that cAMP inhibited the phosphorylation of c-Jun Ser-63 as well as JNKp46 activity induced by PMA/A23187 stimulation in cloned human T cells. In contrast, neither JNKp55 nor ERK activities were affected by cAMP. Previous reports indicated that cAMP elevating agents inhibit the kinase activity of JNK in mouse thymoma line EL4 and human T cell leukemia line Jurkat (10, 11). In these reports however, JNKs were precipitated as proteins bound to GST-cJun conjugated with glutathione-agarose, and the activity of the mixture of JNK isoforms was detected. Thus, it remained to be determined if cAMP effects various JNK isoforms differently. The antibodies which detected different isotypes of JNKs allowed us to find that dBcAMP inhibited the activity of JNKp46 but not that of JNKp55 and

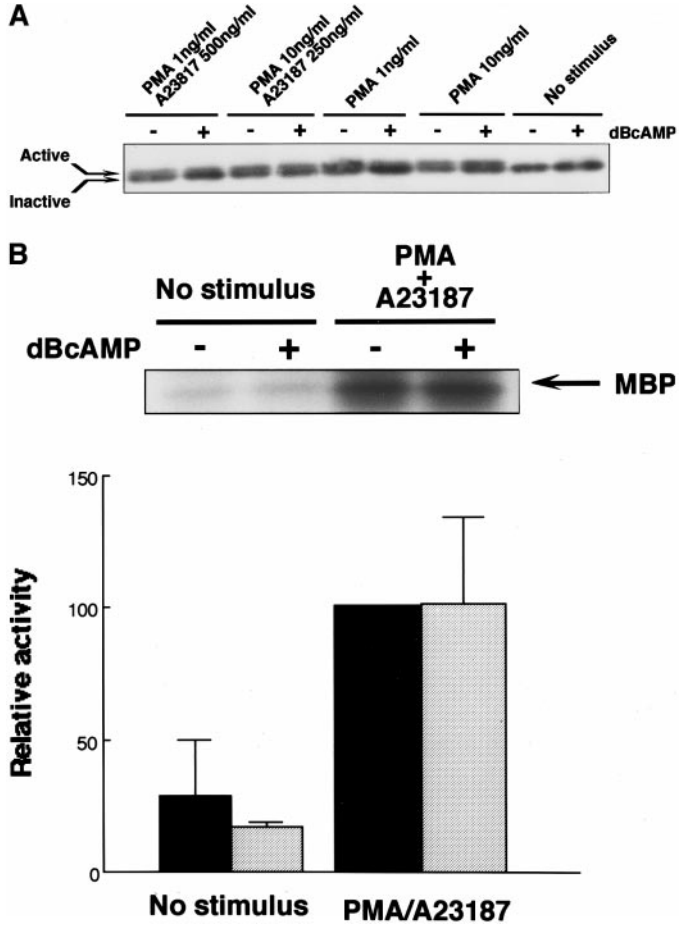

FIG. 4. Effect of dBcAMP on ERK2 activity in SP-B21 cells. SP-B21 cells were stimulated with PMA (1 ng/ml) and/or A23187 (500 ng/ml) after pretreatment in the absence or presence of dBcAMP (1 mM) for 1 h. Five minutes after stimulation, whole cell lysates were prepared. (A) The lysates were separated through 10% SDS-PAGE and transferred to a PVDF membrane. Western blotting was done using anti ERK2 antibody as described under Materials and Methods. (B) ERK2 proteins immunoprecipitated from the same whole cell lysates were subjected to kinase assay using MBP (5 mg) and [γ-^{32}P]ATP at 30°C for 30 min. Phosphorylated proteins were separated in 15% SDS-PAGE and analyzed by a FUJI image analyzer (BAS2000). Experiments were done three times and the averages of relative values with standard deviations are indicated.

the inhibition of c-Jun phosphorylation paralleled the inhibition of JNKp46 activity. These observations indicate that cAMP inhibits only the JNKP46-mediated signal transduction pathway in T lymphocytes, the result being inhibition of c-Jun phosphorylation. Since phosphorylation of c-Jun Ser-63 and the kinase activity of JNKp46 were inhibited by cAMP, it is speculated that JNKp46 rather than JNKp55 may be the main regulator for c-Jun phosphorylation *in vivo*. The molecular weight of JNKp55 and p46 which we observed in this work corresponds to that of authentic JNK1 and JNK2, respectively, suggesting the possibility that JNKp55 which was detected by the anti JNK1 antibody (C-17) and JNKp46 which is detected by the anti JNK2 antibody (FL) are authentic JNK1 and JNK2, respectively. This notion is supported by the observation that JNK2 has a higher affinity for c-Jun (5, 6). On the other hand, different molecular weight isotypes of JNK1 and JNK2 were reported, suggesting that a simplified classification of JNK1 and JNK2 does not correlate with their biological counterpart. Originally, both JNK1 and JNK2 were isolated as kinases phosphorylating specific serine residues of c-Jun (23, 24) but the difference in the biological role of JNK1 and JNK2 was not clear. Recently, cytokine production of $CD4^+$ T cells in JNK1- or JNK2-deficient mice has been investigated (25, 26). Differentiation of T cells into Th1 but not Th2 is impaired in JNK2-deficient mice and augmentation of the production of Th2 cytokines in JNK1-deficient mice were observed. These results suggest that these pathways cooperatively potentiate the Th1 response but their functions are distinct.

We found that dBcAMP did not inhibit the activity of ERK2 in cloned human T cells. This result is consistent with the previous studies that no effect of dBcAMP or forskolin on ERK activation was detected in the mouse thymoma cell line EL4 or rat pheochromocytoma cell line PC12 (10, 27). However, it has also been reported that cAMP inhibited ERK2 activity in EGF-activated fibroblasts (15, 28). These results suggest that upstream regulators may vary within different the cell types.

We reported that the DNA-binding activity of AP-1 induced by PMA/A23187 stimulation was further enhanced by cAMP, although IL-2 gene expression was suppressed by cAMP treatment in human T cell clone (16, 17). Thus, it seemed that the binding activity of this factor to the target sequences may not reflect its activity regarding induction of IL-2 gene transcription. There is a possibility that inhibition of IL-2 gene activation by cAMP is achieved through the suppression of a transcription factor other than the AP-1 complex. It is possible that a component of AP-1 is changed to a transcriptionally inactive complex by the addition of cAMP even though it has a higher affinity to the AP-1 target site. Since our efforts to introduce exogenous DNA into human T cell clones has not succeeded (data not shown), it was not possible to determine whether the transcriptional activity of the AP-1 target site binding complex was modified or not by cAMP in these cells. It has been reported that dephosphorylation of C-terminal Thr-231, Ser-243 and Ser-249 residues of c-Jun by unidentified phosphatases induces DNA-binding activity (29). Therefore, it is also possible that cAMP treatment results in dephosphorylation of not only the N-terminus but also these residues at the C-terminus of c-Jun, leading to a slight enhancement of DNA-binding activity of AP-1.

How cAMP affects JNKp46 activity via signaling molecules upstream of JNKp46 remains to be elucidated. Various upstream activators for MAPK family members, ERK, JNK and p38, have been reported. These molecules have been shown to have specific as well as overlapped roles. For example ERK1 and ERK2 are activated by MEK1 and MEK2 (30), and JNKK1/SEK1/MKK4 activates JNK and p38 (31, 32, 33). Since JNKp55 and JNKp46 have different sensitivities to cAMP, it can be speculated that JNKs may be activated by distinct kinases which have different sensitivities to cAMP. Any specific activator which can distinguish between JNK1 and JNK2 has not been reported. Further studies may clarify specific functions and regulatory mechanisms of JNK isoforms.

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