Regulation of Interleukin-2 Transcription by Inducible Stable Expression of Dominant Negative and Dominant Active Mitogen-activated Protein Kinase Kinase Kinase in Jurkat T Cells

EVIDENCE FOR THE IMPORTANCE OF Ras IN A PATHWAY THAT IS CONTROLLED BY DUAL RECEPTOR STIMULATION

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Engagement of the T cell receptor induces the activation of several mitogen-activated protein kinase modules, including the extracellular signal-regulated kinase and c-Jun N-terminal kinase (JNK) cascades. Whereas extracellular signal-regulated kinase is activated by T cell receptor/CD3 ligation alone, activation of JNK requires co-stimulation by the CD28 receptor. Activation of MEKK-1, which acts as a mitogen-activated protein kinase kinase kinase in the JNK pathway, was also induced by CD3 plus CD28 (CD3/CD28) ligation in Jurkat cells. To study the significance of the JNK cascade in T lymphocytes, we established stable Jurkat cell lines that inductively express dominant active (DA) or dominant negative (DN) MEKK-1. Whereas expression of DA-MEKK-1 resulted in the constitutive activation of JNK along with the transcriptional activation of the minimal interleukin-2 (IL-2) promoter, DN-MEKK-1 inhibited JNK responsiveness during CD3/CD28 co-stimulation. In addition to inhibiting CD3/CD28-induced IL-2 mRNA expression, DN-MEKK-1 abrogated the transcriptional activation of the IL-2 promoter and the distal nuclear factor of activated T cells (NFAT)-activating protein 1 (AP-1) response element in that promoter. A c-Jun mutant lacking activation sites for JNK also interfered with the activation of the distal NFAT/AP-1 complex, suggesting that the JNK pathway functions by controlling AP-1 response elements in the IL-2 promoter. Using inducible stable expression of DA- and DN-Ras in Jurkat cells, we found that Ras regulates JNK activation in these cells. Our results suggest that the dual ligation of CD3 and CD28 in T cells triggers a cascade of events that involve Ras, the JNK cascade, and one or more AP-1 response elements in the IL-2 promoter.

Ligation of the T cell antigen receptor (TCR) triggers multiple signaling pathways, including activation of the guanine nucleotide-binding protein, Ras (1–4). Recent studies have shown that Ras acts upstream of several effectors, including various mitogen-activated protein kinases (5–8). With regard to activation of mitogen-activated protein kinases, the TCR has been linked to the activation of the extracellular signal-regulated kinase (ERK) as well as the c-Jun N-terminal kinase (JNK) cascades (8–12). Whereas TCR ligation results in the activation of the ERK cascade (9), activation of the JNK cascade in T cells requires co-ligation of the CD28 receptor (10). CD28 is an accessory receptor that is involved in biological responses such as anergy, apoptosis, and cytokine production in T cells (13–16). The requirement for dual receptor ligation to activate the JNK cascade is unique for T cells and raises the important question of whether both receptors contribute to the JNK cascade, e.g., by activating a component that is shared by both receptor types. In this regard, it is known that both anti-CD3 and anti-CD28 mAbs induce GTP/GDP exchange on Ras (8, 17). Whereas Ras acts upstream of the JNK cascade in some receptor protein-tyrosine kinase signaling pathways, not all stimuli that activate JNKs in lymphocytes depend on Ras (18–22). It is important to determine whether Ras is involved in the T cell response, because it provides us with a potential target by which to study the synergy between the TCR/CD3 and CD28 receptors in this cascade.

The role of JNK in T cell activation is not well understood. Several lines of evidence suggest, however, that JNKs may be involved in inducing the transcriptional activation of AP-1 response elements in TCR-responsive genes (10). JNKs play a role in the expression as well as transcriptional activation of AP-1 binding proteins (19–27). The JNK cascade leads to transcriptional activation of c-Jun by phosphorylation of serine residues (i.e. Ser63 and Ser73) in its transcriptional domain (18, 28–31). c-Jun, in turn, up-regulates its own expression by interacting with the c-Jun promoter (25, 31). In addition, JNKs up-regulate c-Fos expression by phosphorylation of the ternary complex factor, p62TCF (Elk-1), which binds the c-fos promoter (23, 25). Both c-Fos and c-Jun contribute to the expression of the IL-2 gene, which, as for JNK activation, is dependent on co-ligation of CD3 and CD28 (10, 16, 32, 33). This suggests that JNK may play a role in transcriptional activation of the IL-2 promoter. To this end, it is known that in the absence of CD28 co-stimulation, TCR ligation may lead to the induction of anearly response element. Therefore, the expression of JNKs in the absence of CD28 co-stimulation may be due to an early response element.
ergy and the inability to produce IL-2 (13, 14, 34, 35). Moreover, the defect in IL-2 production in anergic T cells has been correlated with the failure to activate AP-1 response elements in the IL-2 promoter (36).

The minimal IL-2 promoter, consisting of the first 326 base pairs immediately upstream of the start site of the IL-2 gene, is sufficient to confer TCR and CD28 responsiveness (32, 37, 38). The IL-2 promoter is composed of several regulatory elements (37–44), of which the distal response element for the nuclear factor of activated T cells (NFAT) has been best characterized (40–42). The distal NFAT is a composite element that consists of an NFAT site adjacent to a nonconsensus AP-1 site (39, 42). Occupancy of the distal NFAT/AP-1 site requires the cooperative interaction of NFAT proteins with the adjacent AP-1 complex (42–44). Suggestive evidence for the involvement of JNKs in regulation of the distal NFAT/AP-1 element is provided by the observation that a dominant negative (DN) c-Jun mutant lacking a transactivation domain, was able to block the activation of the distal NFAT site (45).

In light of the above, we were interested in determining whether the simultaneous ligation of TCR/CD3 and CD28 activates the JNK pathway in T lymphocytes in an MEKK-1-dependent fashion and whether this pathway contributes to activation of the IL-2 promoter. In order to accomplish our goals, we established stable transfected Jurkat cell lines with inducible expression of dominant active (DA)-MEKK-1, dominant negative (DN)-MEKK-1, DA-Ras, or DN-Ras under a tetracycline-controlled transactivator (tTA) promoter. We used these cell lines to study the effect of MEKK-1 on IL-2 mRNA expression, including the effect of this kinase on transcriptional activation of the IL-2 promoter and the distal NFAT response element. Our data show that DA-MEKK-1 expression leads to constitutive activation of the JNK cascade in Jurkat cells, whereas DN-MEKK-1 expression abrogated JNK activation during CD3/CD28 co-stimulation. Moreover, DN-MEKK-1 inhibited the induction of IL-2 mRNA expression along with inhibition of transcriptional activation of the IL-2 promoter and distal NFAT site. In contrast, DA-MEKK-1 enhanced transcriptional activation of these reporter constructs. DN-Ras interfered with activation of the JNK cascade during CD3/CD28 co-stimulation, whereas DA-Ras enhanced JNK activation by the same stimulatory combination, including treatment with anti-CD3 mAb alone.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The anti-CD3 mAb, OKT3, was from Ortho Pharmaceuticals (Raritan, NJ). Anti-CD28 (clone 9.3) was a generous gift from Brystol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA). Anti-MEKK-1 polyclonal antiserum was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and horseradish peroxidase-conjugated protein A was purchased from Vector Laboratories (Burlingame, CA). The latter combination also induced MEKK-1 activity in Jurkat BMS2 cells (Fig. 1, lane 6). These results suggest that MEKK-1 acts as a mitogen-activated protein kinase kinase kinase in the...
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**Fig. 1. In vitro kinase assay showing the induction of MEKK-1 activity by co-ligation of CD3 and CD28.** 5 × 10^6 Jurkat BM2S2 cells were either left untreated (lane 2) or stimulated with 10 μg/ml anti-CD3 (lane 3), 2 μg/ml anti-CD28 (lane 4), a combination of anti-CD3 and anti-CD28 (lane 5), or 100 nM PMA and 1 μg/ml ionomycin (lane 6) for 10 min at 37°C. The cells were lysed, and the cell lysates were precipitated with 5 μl of anti-MEK-K-1 antibody. After adsorption on 20 μl of protein A-Sepharose, precipitates were washed and resuspended in kinase buffer. The kinase assay was carried out for 25 min in the presence of 2 μg of kinase-inactive MAP or ERK kinase-1 and 10 μCi of [γ-32P]ATP. Lane 1 represents immune precipitation with nonimmune serum (NIS). The proteins were separated by 10% SDS-PAGE and gel-autoradiographed for 18 h. The experiment was repeated twice with identical results.

**Fig. 2. Western blot showing the inducible expression of DA- and DN-MEK-1 in stably transfected Jurkat-tTA cells.** Jurkat-tTA cells were transfected with 20 μg of cDNA encoding either DA-MEK-1 (lanes 2 and 3) or DN-MEK-1 (lanes 4 and 5). Cells in lane 1 were untransfected Jurkat-tTA cells. Following selection in 270 μg/ml hygromycin, the cells were grown in the presence (+) or absence (−) of 0.1 μg/ml tetracycline for 24 h. Total cell lysates from 5 × 10^6 cells were separated by 10% SDS-PAGE and transferred to Immobilon-P membrane. The membrane was overlaid with 0.1 μg/ml anti-MEK-1 antibody followed by horseradish peroxidase-conjugated protein A and developed by ECL.

TCR/CD28-inducible JNK cascade in T cells.

**Establishment of Jurkat Cell Lines with Stable, Inducible Expression of Dominant Negative and Dominant Active MEKK-1 through the Use of tTA—Jurkat BM2S2 cells were transfected with 10 μg of the pUHD15.1 neo vector, which encodes for the tTA protein (46, 47). After stable selection and subcloning in G418, we wished to ascertain that the selected clones express tTA protein. This was determined by co-transfecting the pUHC13.3 vector and performing luciferase assays on cells grown in the absence or presence of tetracycline. pUHC13.3 contains seven tet operator sequences upstream of a luciferase reporter gene. Whereas in the presence of tetracycline there was a minimal expression of luciferase activity, there was a 17-fold increase in luciferase activity in the cell clone designated as Jurkat-tTA in the absence of tetracycline.

We co-transfected Jurkat-tTA cells with pUHD10.3, which contains seven tet operator sequences upstream of a minimal promoter and a polylinker site. We used either empty vector or pUHD10.3, into which we subcloned DA-MEKK (MEKKΔ) or DN-MEKK (MEKKΔ K432M). A vector with a selectable hygromycin-resistant cassette was co-transfected and used to select hygromycin-resistant populations. When the cells were grown in the absence of tetracycline (tetr), abundant DA-MEKK-1 or DN-MEKK-1 protein could be seen to be expressed (Fig. 2). In contrast, no mutant protein was expressed in the same populations grown in the presence of tetracycline (tetr+) (Fig. 2).

**Stable Expression of DA-MEKK-1 in Jurkat Cells Leads to Constitutive Activation of the JNK Pathway, whereas Stable Expression of DN-MEK-1 Interferes with JNK Activation by CD3/CD28 Co-ligation—**To determine the possible effect of stable expression of DA-MEK-1 on Jun kinase activity, we employed an in vitro kinase assay, which utilizes glutathione S-transferase-c-Jun-(1–79) as substrate (Fig. 3A). In cells stably transfected with DA-MEK-1 and grown under tetr (+) conditions (Fig. 3A, lanes 6–10), treatment with anti-CD3 (lane 7) or anti-CD28 alone (lane 8) did not induce JNK activity compared with untreated cells (lane 6). Stimulation with a combination of anti-CD3 and anti-CD28 mAb stimulated JNK activity 14-fold (lane 9), whereas treatment with PMA and ionomycin stimulated JNK activity 12-fold (lane 10). Identical results were obtained in DN-MEK-1 or empty vector cells grown in the presence of tetr (data not shown). When tetracycline was withdrawn from the DA-MEK-1 population for 24 h, JNK activity in unstimulated cells was increased 10-fold compared with basal activity in tetr(+) cells (Fig. 3A, lanes 1 and 6). Whereas treatment with anti-CD3 alone (13-fold, lane 2), anti-CD28 alone (8-fold, lane 3), or a combination of anti-CD3 and anti-CD28 (11-fold, lane 4) showed little additional stimulation, PMA plus ionomycin treatment increased activation to 17-fold (Fig. 3A, lane 5). These results show that JNKs were constitutively active in DA-MEK-1-expressing cells and that receptor stimulation had very little additional effect. The specificity of DA-MEK-1 on JNK activation was confirmed by conducting in vitro ERK-2 assays (Fig. 3B). Whereas in vector-only cells, anti-CD3-induced ERK-2 activity compared with that in untreated cells (Fig. 3B, lanes 1 and 2), no increase above background level was seen in DA-MEK-1-expressing cells, which maintained their CD3 inducibility (lanes 3 and 4). These results show that stable expression of DA-MEK-1 selectively activates the JNK cascade.

In a similar fashion, we determined the effect of stable expression of DN-MEK-1 on activation of JNKs in Jurkat cells. Tetr(+) DN-MEK-1 cells (not shown) yielded results identical to those shown for tetr (+) DA-MEK-1 cells in Fig. 3A, lanes 6–10. In tetr(−) DN-MEK-1 cells, we could not induce JNK activity with any of the known stimuli (CD3 plus CD28, PMA plus ionomycin) that activate JNK in tetr(+) cells (Fig. 3A, lanes 6–10 versus lanes 11–15). In addition, basal JNK levels were depressed almost 10-fold in DN-MEK-1-expressing cells (Fig. 3A, lane 11). This was not due to an effect on cell viability (not shown) and likely represents interference with basal JNK activation by growth factors. The specificity of DN-MEK-1 on JNK activity was confirmed by studying ERK-2 activation in tetr(−) DN-MEK-1 cells. The results are shown in Fig. 3B (lanes 3 and 4) and confirm that DN-MEK-1 did not interfere with ERK-2 activation by anti-CD3 mAb.

**Inducible, Stable Expression of Wild-type (WT) Ras, DA-Ras, and DN-Ras Affects JNK Activation by the TCR, Including the Requirement for CD28 Co-stimulation—**T cells are unique in their requirement for dual receptor (CD3/CD28) ligation to activate the JNK pathway (10). It has been suggested that a postreceptor signaling component common to both the TCR/CD3 and CD28 pathways may contribute to this activation response (49). To this end, it has been shown that anti-CD3 as well as anti-CD28 mAb can activate Ras and that Ras acts upstream of the JNK cascade in non-T cells (19, 20). It should...
be mentioned, however, that JNKs are also activated in a Ras-independent fashion by certain cytokines (21, 22). In order to determine the possible role of Ras in our cells, we used the Jurkat-tTA cell clone to establish cell lines that stably express WT or DN-MEK-1. WT and mutant ras cDNAs were subcloned into the pUHD10.3 vector and transfected into Jurkat-tTA cells. After stable selection in hygromycin and subcloning of the resistant populations, we confirmed with an anti-pan-Ras antibody that there was increased expression of WT and mutant Ras proteins in tet(−) cells (not shown).

Stable overexpression of WT Ras exerted a dramatic effect on induction of JNK activity by antireceptor stimuli as well as treatment with PMA plus ionomycin (Fig. 4A). In unstimulated cells, overexpression of WT Ras increased JNK activity 4-fold compared with the basal activity in unstimulated tet(+) cells (Fig. 4A, lane 1 versus lane 6). Whereas anti-CD3 alone induced a 2-fold increase in JNK activity in tet(+) cells, the same stimulus induced an 18-fold increase in tet(−) cells (Fig. 4A, lane 2 versus lane 7). Overexpression of WT Ras also enhanced anti-CD3 plus anti-CD28 stimulation (26-fold increase above basal, lane 9) compared with tet(+) cells (14-fold response; Fig. 4A, lane 4). Moreover, during PMA plus ionomycin stimulation, the response in tet(+) cells (16-fold above basal) was elevated to 40-fold in tet(−) cells (Fig. 4A, lanes 5 and 10). Anti-CD28 stimulation induced a 6-fold response in tet(−) cells versus a 1.5-fold response in tet(+) cells (Fig. 4A, lanes 3 and 8). Tet(+) DA-Ras cells yielded identical results as tet(+) WT Ras cells (not shown). Stable expression of DA-Ras had an even more pronounced effect than WT Ras. V12Ras increased the response in anti-CD3 treated tet(+) cells from 2- to 26-fold in tet(−) cells (Fig. 4A, lane 12). Stimulation by anti-CD3 plus anti-CD28 (23-fold, lane 14) and PMA plus ionomycin (41-fold, lane 15) yielded about the same response as in WT Ras-overexpressing cells (Fig. 4A). Taken together, these data indicate that overexpression of WT Ras or DA-Ras affects both the magnitude as well as the requirement for dual receptor stimulation.

Experiments conducted in N17Ras-expressing cells showed that Ras is essential for activation of the JNK cascade during CD3/CD28 co-stimulation. Fig. 4B shows that, compared with nonexpressing cells (tet(−)), the response to CD3/CD28 co-stimulation was abrogated in DN-Ras-expressing cells (lane 4 versus lane 9). Weak JNK activation could be obtained in tet(−) cells during stimulation with PMA plus ionomycin (Fig. 4B, lane 10). This suggests that these pharmacological stimuli may utilize an additional Ras-independent pathway, which leads to JNK activation (Fig. 4B, lane 10).

DA-MEKK-1 Abrogates Induction of IL-2 mRNA Expression during CD3/CD28 Co-stimulation—Since CD3/CD28 co-stimulation induces MEKK-1 and JNK activities, an important question is whether this pathway plays a role in a biological effect mediated by co-ligation of these receptors. Jurkat cells have been instrumental in showing that CD28 co-ligation is essential for the induction of IL-2 secretion during CD3/TCR ligation (16, 32, 37). In order to determine whether MEKK-1 plays a role in generation of this response, we asked whether DN-MEKK-1 affects IL-2 mRNA expression as determined by a semiquantitative RT-PCR method (Fig. 5). Compared with basal or anti-CD3-induced mRNA expression, CD3/CD28 co-ligation clearly increased IL-2 mRNA expression in tet(+) cells (Fig. 5, top, lanes 1–4). PMA plus ionomycin also induced a prominent RT-PCR band in the same population (Fig. 5, top, lane 5). These responses are specific, since internal standardization with β-actin primers showed equal amounts of RT-PCR product in unstimulated and stimulated cells (Fig. 5, bottom, lanes 1–5). In DN-MEKK-1-expressing cells, basal IL-2 mRNA expression was reduced by 90% (Fig. 5, top, lanes 6–10). This agrees with the extent to which JNK activity levels were suppressed in DN-MEKK-1-expressing cells (Fig. 3A). Moreover, stimulation with a combination of anti-CD3 plus anti-CD28 mAb failed to induce any increase in IL-2 mRNA above background (Fig. 5, top, lane 9). In contrast, PMA plus ionomycin induced a small increase in IL-2 mRNA expression (Fig. 5, top, lane 10). These effects were specific, since DN-MEKK-1 expression had no effect on β-actin mRNA expression (Fig. 5, bottom, lanes 6–10). These results indicate that MEKK-1 plays a critical role in IL-2 gene expression.

DA- and DN-MEKK-1 Exert Stimulatory and Inhibitory Effects, Respectively, on the Minimal IL-2 Promoter—CD28 co-stimulation has two major effects on IL-2 mRNA. First, CD28 enhances the stability of IL-2 transients, leading to increased
IL-2 secretion (33). Second, CD28 contributes to transcriptional activation of the IL-2 gene as determined by a minimal IL-2 promoter linked to a luciferase reporter gene (IL-2p-Luc) (32). The minimal IL-2 promoter, consisting of 326 base pairs upstream of the transcription start site has stringent activation requirements (38, 45, 50). Minimally, the IL-2p-Luc reporter requires PMA plus ionomycin stimulation, which can be further increased by antireceptor mAb. In order to determine whether the JNK pathway contributes to the transcriptional activation of the IL-2 promoter, we used transient transfection of an IL-2p-Luc construct to determine the effect of stable DA- and DN-MEKK-1 expression on luciferase activity (Fig. 6).

Tet(1) DN-MEKK-1 cells yielded identical results as tet(1) DA-MEKK-1 cells and are therefore not shown. Compared with tet(1) cells, expression of DA-MEKK-1, without the addition of any stimuli, had no effect on IL-2p-Luc activity (Fig. 6, Unstimulated). DA-MEKK-1 expression did, however, enhance IL-2p-Luc activation in stimulated tet(−) cells (Fig. 6). Compared with a 14.5-fold response during PMA plus ionomycin treatment in tet(+) cells, the response increased to 25.27-fold in tet(−) cells (Fig. 6). This represents an enhancement of 1.75 times by DA-MEKK-1 (Fig. 6).

Whereas DN-MEKK-1 expression exerted no effect on basal IL-2p-Luc activity in tet(+) cells (Unstimulated), it suppressed the induction of IL-2p-Luc activity by a respective amount of 47, 47, and 52% during stimulation with PMA/ionomycin, PMA/ionomycin/anti-CD3, and PMA/ionomycin/anti-CD28 (Fig. 6). Taken together, these results show that MEKK-1 plays an important role in the transcriptional activation of the IL-2 promoter.

**Mutant MEKK-1 Proteins Affect Transcriptional Activation of the Distal NFAT Complex without Affecting DNA-protein Binding**—The IL-2 promoter includes response elements for a large number of transcription factors, including NFAT, Oct proteins, AP-1, and NF-kB (37, 39). We focused on the distal NFAT site because (a) AP-1 proteins cooperate with NFAT proteins for binding to a composite response element (42); (b) JNKs control the expression and transcriptional activation of AP-1 proteins (10, 24, 25, 31); and (c) a dominant negative c-Jun protein, which lacks a transactivation domain, interferes with transcriptional activation of the distal NFAT site in the IL-2 promoter (45). A triplicate repeat of the distal NFAT element linked to luciferase reporter was transiently transfected into stable DA- and DN-MEKK-1 cells, and responses were compared in tet(−) and tet(+) cells. In unstimulated cells, DA-MEKK-1 induced a 1.5-fold increase in basal NFAT-Luc activity, whereas DN-MEKK-1 depressed basal luciferase activity by 50% (Fig. 6, Unstimulated). These differences were not due to differences in the efficacy of transfection, because co-transfection of a beta-galactosidase construct was used for correction. In tet(−) cells, DA-MEKK-1 expression enhanced PMA plus ionomycin reporter gene activity by a factor of 1.76 (27.6–48.7-fold stimulation) (Fig. 7A). Moreover, DA-MEKK-1 enhanced PMA/ionomycin/anti-CD3- and PMA/ionomycin/anti-
CD28-induced NFAT activity by factors of 1.76 (34.1–60.2-fold stimulation) and 1.57 (44.7–70.6-fold increase), respectively (Fig. 7A). Conversely, DN-MEKK-1 suppressed the induction of NFAT-luc activity by PMA plus ionomycin by 24% (27.6-fold stimulation down to 21.2-fold) (Fig. 7A). Also, DN-MEKK-1 decreased PMA/ionomycin/anti-CD3-induced stimulation by 44% (34.1-fold stimulation down to 15.7-fold) while decreasing PMA/ionomycin/anti-CD28-induced stimulation by 63% (44.7-fold stimulation down to 16.5-fold, Fig. 7A).

In a second experiment, the effect of DN-MEKK-1 was compared with the effect of a c-Jun mutant, which lacks the consensus serine sites (Ser63, Ser73) for JNK activation (Fig. 7B). As with the previous experiment, expression of DA-MEKK-1 enhanced the basal and stimulated activity of NFAT-luc. In addition, expression of DN-MEKK-1 inhibited PMA/ionomycin/anti-CD3-induced NFAT activity by >90% in this experiment (Fig. 7B). The same stimuli in the presence of co-transfected c-Jun (A63/73), inhibited NFAT-luc stimulation by >80% in tet(+) DA-MEKK-1 cells and 70% in tet(−) DA-MEKK-1 cells (Fig. 7B). This shows that a c-Jun mutant, which lacks the specific activation site for JNKs, has the same effect as DN-MEKK-1.

Since expression of DA-MEKK-1 enhanced the transcriptional activation of the distal NFAT response element in stable transfected cells (Fig. 7A), we were interested in determining whether this was due to altered quantities of the NFAT/AP-1 binding complexes. We therefore performed electrophoretic mobility shift assays using oligonucleotides corresponding to the distal NFAT/AP-1 response element. Our results show that expression of DA-MEKK-1 or DN-MEKK-1 did not appreciably alter the abundance of the shift complexes that could be induced by anti-CD3 treatment. These results suggest that the principle effect of the JNK pathway on the distal NFAT response element is transcriptional activation.

**DISCUSSION**

In this paper we show that the simultaneous ligation of CD3 and CD28 induces activation of the JNK pathway in T cells in a Ras- dependent fashion. Stable expression of DA-MEKK-1 resulted in the constitutive activation of JNKs and transcriptional activation of the minimal IL-2 promoter, including its distal NFAT response element. In contrast, expression of DN-MEKK-1 prevented JNK activation by CD3/CD28 co-ligation. DN-MEKK-1 also inhibited IL-2 mRNA expression by the same stimuli and interfered with transcriptional activation of the IL-2 promoter, including the function of the distal NFAT-site. Activation of the JNK pathway in T cells is unique insofar as it requires dual receptor ligation (Ref. 10 and Fig. 3). Whereas engagement of TCR/CD3 alone is sufficient to trigger the ERK cascade, activation of the JNK cascade requires that CD28 be co-ligated with TCR/CD3 (9, 10–12). This suggests that signaling components activated by each receptor may converge on MEKK-1 or some upstream effector in this cascade. A good candidate for the integration of signaling pathways by these receptors is the GTP-binding protein, Ras. Cross-linking of the TCR with antigen or with antibody has been shown to increase the GTP content of Ras (8). In contrast, anti-CD28 mAb, but not the physiological ligand, B7–1, induces Ras activation (17). One interpretation is that CD28 may be required to elevate Ras activation to a critical threshold for JNK activation. In favor of
this theory is the finding that overexpression of wild-type or DA-Ras reduced the dependence on dual receptor ligation, making it possible for CD3 alone to induce the JNK pathway (Fig. 4A). It remains to be shown, however, whether under physiological conditions CD3 plus CD28 can activate Ras in a cooperative fashion. Alternatively, Ras may act upstream of a pathway that also receives an independent input from CD28.

One possibility is Rac. Rac is a small GTP-binding protein that can be activated in a Ras-dependent fashion in fibroblasts (8, 51, 52). Rac, in turn, interacts with and activates the mammalian serine/threonine kinase, PAK65 (53). PAK65 is homologous to the Saccharomyces cerevisiae kinase Ste-20, which acts upstream of the yeast equivalent of MEKK-1, Ste-11. Little is known about the activation and role of Rac and PAK65 in T-lymphocytes, and these issues need to be further explored.

Whereas much remains to be learned about the afferent components of the JNK cascade, it is clear that MEKK-1 plays an important role in the JNK cascade controlled by CD3 plus CD28 ligation. This was demonstrated through the use of the tetracycline-repressible vector system that was developed by Dr. Bujard (Figs. 2 and 3) (46, 47). Whereas this system has been used for expressing mammalian genes in fibroblasts and HeLa cells, its application has not been investigated in lymphocytes (46, 47). In Jurkat cells, stable expression of these vectors has provided us with potent and tightly controlled expression of mutant MEKK-1 proteins (Fig. 2). DA-MEKK-1 induced specific and constitutive activation of the JNK pathway in Jurkat cells, which is in agreement with results in Swiss 3T3 cells that were stably transfected with DA-MEKK-1 subcloned into an IPTG-inducible vector (54). Constitutive activation of JNK in fibroblasts induced transcriptional activation of the transcription factor p62TCF/Elk-1 (54). Similar to DA-MEKK-1, expression of DN-MEKK-1 exerted potent effects on Jurkat cells. Not only did DN-MEKK-1 abrogate activation of JNK activity by CD3/CD28 co-stimulation, but it also suppressed IL-2 mRNA expression by the same stimuli. We foresee that, in addition to studying the effect of mutant MEKK-1 proteins on the IL-2 gene, the tetracycline vector system will be of use for looking at the effect of the JNK cascade in apoptosis, anergy, and Th2 cytokine production.

Whereas previous studies have shown that CD28 co-ligation is required for TCR-induced IL-2 gene expression, the mechanism by which these receptors combine to induce IL-2 secretion is not fully understood (10, 33). Our results show that one mechanism of cooperativity between these receptors is through the effect of the JNK cascade on the IL-2 promoter. To this end, DA-MEKK-1 enhanced the transcriptional activation of the minimal IL-2 promoter, whereas DN-MEKK-1 abrogated the responsiveness of the IL-2 promoter to CD3/CD28 co-stimulation (Fig. 6). Similar results were obtained when an AP-1 dependent response element in the IL-2 promoter was used, namely the distal NFAT site (Fig. 7A). Moreover, the fact that a mutant c-Jun protein, which lacks the transcriptional activity, was calculated based on this value. Identical results were obtained when tet(+) DN-MEKK-1 cells were used (not shown). B, luciferase assay showing that mutant c-Jun(A63/73) inhibits the activation of the distal NFAT site. 107 Jurkat-tTA cells, stably transfected with either DA- or DN-MEKK-1, were electroporated with 10 μg of NFAT-Luc construct corresponding to positions -287 to -269 with respect to the start site. The cells were rested for 16 h in the presence or absence of tetracycline as indicated. The cells were stimulated for 8 h as in Fig. 6. The base-line amount of luciferase activity in unstimulated cells was 5468 light units for tet(+) DA-MEKK-1 cells. The fold increase in luciferase activity was calculated based on this value. Identical results were obtained when tet(+) DN-MEKK-1 cells were used (not shown).
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viation site for JNK, exerts similar effects as DN-MEKK-1, suggests that an important target for MEKK-1 is AP-1 response elements in the IL-2 promoter. To this end, it is known that the IL-2 promoter contains four composite NFAT/AP-1 response elements and that activation of AP-1 proteins are required for binding of NFAT proteins at the adjacent NFAT site (42). We propose that the expression as well as the transcriptional activation of AP-1 proteins by the JNK pathway plays a critical role in the function of NFAT sites in the IL-2 promoter.

Future studies on the IL-2 promoter will look at the effect of the JNK pathway on NFAT sites other than the distal NFAT element. In particular, we will determine whether the JNK pathway plays a role in the activation of the NFAT element that partially overlaps with the CD28 response element (CD28RE) (48). To this end, it has been shown that the inducibility of the NFAT site at the CD28RE is absolutely dependent on the activation of its adjacent AP-1 response element also needs to be considered, because it has been shown that in anergized T cells there is a comitant defect in Ras, ERK, and JNK activation (55). The combinatorial effect of the ERK and JNK pathways on AP-1 response elements in the IL-2 promoter may determine the outcome of the response.

In conclusion, these data have increased our understanding of the mechanism by which a critical co-receptor for the TCR leads to T cell activation. Our study indicates that CD28 acts synergistically with TCR to induce the activation of MEKK-1 and the JNK pathway. The finding that this signaling pathway is also dependent on Ras provides an important target by which to interfere with T cell activation. In addition, this will allow further studies geared toward understanding the effects of AP-1 modifications on IL-2 production and ensuing biological outcomes.

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