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*J Immunol* 1999; 162:3316-3320; http://www.jimmunol.org/content/162/6/3316
Regulation of Lymphotoxin Production by the p21ras-raf-MEK-ERK Cascade in PHA/PMA-Stimulated Jurkat Cells

Yong Q. Li,* Charles S. T. Hii,2* Maurizio Costabile,* David Goh,* Channing J. Der,† and Antonio Ferrante*

Although the production of lymphotoxin (LT) from activated Th1 lymphocytes has been reported extensively, the intracellular signaling mechanisms that regulate this T cell function remain totally undefined. We have examined whether the p21ras-raf-1-mitogen-activated protein kinase/extracellular signal-regulated protein kinase (ERK) kinase (MEK)-ERK cascade plays a role in regulating the production of LT, because the activity of these signaling molecules is up-regulated in activated T lymphocytes. Transfection of Jurkat leukemic T cells with a dominant negative mutant of p21ras (ras17N or ras15A), raf-1 (raf 1–130), or ERK1 (Erk1-K71R) resulted in the suppression of the mitogen/phorbol ester-stimulated production/secretion of LT. This suppression was accompanied by a parallel inhibition of mitogen-stimulated ERK activation. The selective antagonist of MEK1 activation, PD98059, also attenuated the mitogen-stimulated or anti-CD3 Ab and phorbol ester-stimulated production of LT from Jurkat cells or peripheral blood T lymphocytes. This study provides, for the first time, direct evidence that the p21ras-raf-MEK-ERK cascade plays a vital role in regulating the production of LT. The Journal of Immunology, 1999, 162: 3316–3320.

Lymphotoxin (LT)1 is the first of the TNFs to be described. Like TNF-α, LT plays an important role in cell-mediated immunity and inflammation. Thus, LT has been shown to prime neutrophils for enhanced respiratory burst (1, 2), phagocytosis (2, 3), and the killing of microorganisms such as Plasmodium falciparum (4) and Haemophilus influenzae (5). LT has been implicated in stimulating the growth of B lymphocytes (6), in promoting proinflammatory reactions and tissue damage (7), and in causing cachexia and septic shock syndrome (8–10). The actions of LT and TNF-α are mediated by the p55 and p75 TNF receptors.

Although other cells also produce LT, activated T lymphocytes are a major source of LT (11). Whereas TNF-α can either be secreted or be displayed as a membrane-bound protein, LT is a secreted protein. Currently, the intracellular signaling mechanisms that regulate the production of LT are not understood. Engagement of the TCR leads to the activation of a number of intracellular signaling molecules, which can potentially lead to physiological responses such as cytokine production and proliferation. These signaling molecules include members of the p21ras-extracellular signal-regulated protein kinase (ERK) cascade (12). Activation of the p21ras-ERK cascade is achieved via the recruitment of raf-1 to the plasma membrane by GTP-loaded p21ras and its subsequent activation by p21ras in an as yet undefined mechanism (13), phosphorylation of mitogen-activated protein kinase/ERK kinase (MEK)1 and MEK2 by raf-1, and the subsequent dual phosphorylation of ERK1 and ERK2 by MEK1 and MEK2 (14). Studies in a number of cell types have demonstrated that activated ERK subsequently migrates into the nuclei, where it stimulates the activity of transcription factors such as elk-1 (14, 15). The aim of the present study was to determine whether the p21ras-ERK cascade plays a role in regulating the production of LT by activated T cells. By either transiently transfecting Jurkat cells with a plasmid that carried a dominant negative mutant of p21ras, raf-1, or ERK1 or using the recently characterized MEK inhibitor, PD98059, we report, for the first time, that members of the p21ras-ERK cascade play a direct role in regulating the production of LT by mitogen-stimulated Jurkat T leukemic cells or by anti-CD3 Ab and phorbol ester-stimulated purified peripheral blood T lymphocytes.

Materials and Methods

Materials

pSV β-galactosidase (β-gal) was obtained from Promega (Madison, WI). The construction of dominant negative p21ras (pZIPras17N and pZIPras15N), raf-1 (pCGNraf (1–130)), and ERK1 (pCMVErk1K71R) has been described previously (15–18). All plasmids were purified using the Qiagen maxi plasmid kit (Qiagen, Chatsworth, CA). Anti-ACTIVE ERK Ab and anti-ERK2 Ab were obtained from Promega and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. PD98059 was supplied by New England Biolabs (Beverly, MA). Anti-CD3 Ab was obtained from CLB (Amsterdam, The Netherlands). Anti-LT mAb was obtained from Boehringer Mannheim (Mannheim, Germany), and polyclonal anti-LT Ab was obtained from Serotec (Oxford, U.K.). Lymphoprep was prepared from Nycomed (Oslo, Norway). Myelin basic protein (MBP) and protein kinase A (PKA) peptide inhibitor (Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser) was purchased from Sigma. 2,2′-Azino-di(3-ethylbenzthiazoline sulfate) was purchased from Boehringer Mannheim.

Transfection of Jurkat cells

The Jurkat leukemic T cells were maintained in a humidified atmosphere of 5% CO2 in 95% air (37°C) in RPMI 1640 (Cell Image, Adelaide, Australia)
supplemented with penicillin, streptomycin, and 10% FCS. Cells were washed two times in serum-free RPMI 1640 before use and were transiently transfected using Lipofectamine (Life Technologies, Gaithersburg, MD) under serum-free conditions. Before transfection, plasmid DNA (10 μg in 300 μl of medium) was mixed with liposome (5 μl in 500 μl of medium), and the mixture was left to stand at room temperature for 2.5–3 h. The DNA-liposome complex was added to cells (4 × 10^6 to 1 × 10^7 in 200 μl of medium), and cells were incubated at 37°C for 8–12 h. Control cells received the pSV β-gal/liposome complex, and a portion of these cells was used to determine transfection efficiency. A total of 3–9 ml of fresh RPMI 1640 (20% FCS) was then added, and cells were further incubated for 36–40 h. The expression of β-gal did not affect either cytokine production or ERK activity (data not shown). We have consistently found that 60–70% of the pSV β-gal-transfected cells express the enzyme.

**Purification of peripheral blood T lymphocytes**

Mononuclear leukocytes were prepared by gradient centrifugation (600 × g for 35 min) on Ficoll-Hypaque (specific gravity 1.114, pH 7.1). After adherence of mononuclear cells on plastic surfaces, the lymphocyte fraction was applied onto columns that had been prepacked with nylon wool (19). After 30 min of incubation at 37°C in a humidified atmosphere of 5% CO2 in air, T lymphocytes were eluted with 10 ml of RPMI 1640 (10% FCS), washed once, and fractionated on Lymphoprep to remove non-T lymphocytes. Next, purified T lymphocytes (98% pure) were washed two times and resuspended in RPMI 1640 supplemented with 5% (v/v) heat-inactivated AB serum and 1.4 mM 2-ME.

**LT production**

**Jurkat cells.** At 48 h posttransfection, Jurkat cells (4 × 10^6 in 4 ml) were incubated with PMA (100 nM)/PHA (1 μg/ml) for 20 h; aliquots of the supernatant were taken for LT estimation as described below. To examine the role of MEK1, nontransfected Jurkat cells were preincubated with PD98059 (50 μM) for 45 min before being stimulated with PMA/PMA. Control cells received DMSO.

**Purified T cells.** Purified T lymphocytes (1 × 10^7/well) were preincubated with PD98059 for 45 min before being stimulated with anti-CD3 Ab (diluted 1/500) in the presence of PMA (32 nM) for 72 h. The supernatants were collected for LT determination.

**LT determination**

LT levels were determined by ELISA. Briefly, immobilized goat antiserum IgG was used to capture an anti-LT mAb. After the addition of supernatants containing LT, the solid phase-bound complex was incubated with a polyclonal rabbit anti-LT Ab. Detection was achieved with a horse- radish peroxidase-conjugated goat anti-rabbit IgG Ab, using hydrogen peroxide as a substrate and 2,2'-azino-di-3-ethylbenzthiazoline sulfate as chromagen.

**T lymphocyte proliferation**

Purified T lymphocytes (1 × 10^7/well) were preincubated with either DMSO or PD98059 for 45 min before being stimulated with anti-CD3 Ab and PMA for 72 h. At 6 h after stimulation, the cells were pulsed with [^3H]thymidine; incorporation of labeled thymidine into DNA was determined after harvesting the cells using a Titer-Tek cell harvester (Flow Laboratories, Irvine, U.K.).

**Partial purification of ERK**

ERK was partially purified and assayed according to the methods described by Anderson et al. (20) with some modifications (21, 22). Briefly, transfected cells (1 × 10^7) were incubated with PHA/PMA (37°C) for 5 min; incubations were terminated by centrifugation (3000 × g for 3 min at 4°C). The cell pellets were resuspended in 0.9 ml of 25 mM Tris/HCl (pH 7.5), 2 mM EGTA, 25 mM NaCl, 1 mM NaN_3 VO_4, 38 mM 5-nitrophenylphosphosphate, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml leupeptin, 2 mM PMF, and 1 mM DTT; sonicated (3 × 10 s, output of 2 units, Soni- fier, Branson Sonic Power Co., Danbury, CT); and centrifuged (100,000 × g for 15 min). Cytosolic fractions were adsorbed onto phenyl-Sepharose CL4B, and ERK was eluted with 200 μl of 60% ethylene glycol after the beads had been washed two times with 10% ethylene glycol and then two times with 35% ethylene glycol (v/v). Previous studies have demonstrated that ERK is eluted by ethylene glycol at concentrations between 35% and 60% (v/v) (20). The kinase activity in the samples was determined as described below. In some experiments, Laemmli buffer was added to the samples, which were then stored at ~20°C until Western blotting.

**ERK assay**

15 μl of partially purified ERK was added to 35 μl of assay mixture (25 mM Tris/HCl (pH 7.4), 50 mM β-glycerophosphate, 0.33 mg/ml MBP, 1.5 mM EGTA, 0.1 mM sodium orthovanadate, 10 μM MgCl_2, 10 μg/ml PKA peptide inhibitor, 40 μM ATP, and 0.1 μCi [γ-^32P]ATP), and the mixture was incubated for 20 min at 30°C. Assays were terminated by spotting aliquots of the reaction mixture onto P-81 filter paper followed by extensive washing with 75 mM orthophosphoric acid. Radioactivity was determined by liquid scintillation spectrometry. The validity of this method of ERK preparation and assay is verified in an assay in which one half of the soluble fractions was subjected to partial purification and the other half to immunoprecipitation, using an ERK2 Ab. The characteristics of the MBP kinase activity in partially purified fractions were found to be very similar to those in immunoprecipitated ERK fractions. Further evidence that partially purified ERK fractions contain ERK as the major, if not the only, kinase activity was strongly inhibited when cells were pretreated with PD98059 (Table I). Our other studies in a range of hemopoietic and nonhemopoietic cells have demonstrated a very close correlation in ERK activity between partially purified ERK fractions and ERK fractionated on Mono Q fast protein liquid chromatography or retardation in the electrophoretic mobility of ERK in SDS gels (21–23).

**Western blotting**

Denatured proteins were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes (100 V, 1.5 h). Immediately after transfer, blots were stained with Ponceau S (0.1% in 5% acetic acid) to confirm equal loading of all lanes of the gels. Affinity-purified polyclonal anti-ACTIVE ERK Ab was used to detect dual-phosphorylated ERK isofoms, and an anti-ERK2 Ab was used to compare loading between lanes. Immune complexes were detected by enhanced chemiluminescence as described previously (21).

**Statistical analysis**

Results were analyzed by the Student t test and were considered significant if p values were <0.05.

**Results**

The stimulation of Jurkat cells with PMA (100 nM)/PHA (1 ng/ml) for 5 min resulted in increased ERK activity (Fig. 1). Western blotting of the samples with anti-ACTIVE ERK Ab, which detects dual-phosphorylated ERK, revealed that PMA/PMA caused the appearance of a band of immunoreactive material that migrated with a molecular mass of ~43 kDa (ERK2) (Fig. 2a, lanes 2, 4, and 6). Upon longer film exposure, a faint band that migrated with an apparent molecular mass of 44 kDa was also detected (data not shown). This finding demonstrates that ERK1 and ERK2 were dual phosphorylated, albeit to different degrees, in PMA/PHA-stimulated cells. The low level of ERK1 dual phosphorylation was most likely due to a preferential activation of ERK2 over ERK1. Such preferential activation of ERK2 over ERK1 has been reported previously in B cells following the engagement of the B cell Ag receptor (24). An anti-ERK2 probing of an identical blot demonstrates the presence of approximately equal amounts of ERK2 in all the lanes (Fig. 2b).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cpm/μg Protein/20 min</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1566 ± 200</td>
</tr>
<tr>
<td>PHA/PMA</td>
<td>5710 ± 1331^b</td>
</tr>
<tr>
<td>PD98059</td>
<td>1819 ± 160</td>
</tr>
<tr>
<td>PD98059/PHA/PMA</td>
<td>1345 ± 218</td>
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^a Cells were preincubated with DMSO (0.1% v/v) or PD98059 (50 μM) for 45 min prior to the addition of PHA/PMA. After 5 min ERK activity was determined as described in Materials and Methods. Results are the means ± SEM of three experiments.

^b Significance of difference from control, p < 0.05.
Role of p21ras

Transfection of cells with dominant negative p21ras, ras15A, or ras17N prevented PHA/PMA from stimulating the activity of ERK (Fig. 1). This was associated with a greatly reduced degree of ERK2 dual phosphorylation in PHA/PMA-stimulated cells (Fig. 2a). Consistent with the suppression of ERK activity and dual phosphorylation, the dominant negative p21ras mutants prevented PHA/PMA from stimulating the production of LT (Fig. 3).

Role of raf-1

Transfection of cells with an interfering raf-1 mutant (raf 1–130) also prevented PHA/PMA from stimulating the activity of ERK (Fig. 1). Consistent with a suppression of mitogen-stimulated ERK activity, expression of raf 1–130 suppressed the ability of PHA/PMA to stimulate LT production (Fig. 3).

Role of MEK

A role for MEK in LT production was demonstrated using the selective MEK1 antagonist, PD98059. Alessi et al. reported that PD98059, at the concentrations used in this study, did not affect the activity of 18 known kinases, including those that are involved in the c-Jun N-terminal kinase and p38 cascades, and that a >80% inhibition of MEK1 activity was achieved at 50 µM of PD98059 (25). Preincubating Jurkat cells with PD98059 (50 µM) for 45 min blocked the PHA/PMA-stimulated activation of ERK (Table I) and the production of LT (Fig. 4a). PD98059 also suppressed the production of LT by and the proliferation of anti-CD3 Ab and PMA-stimulated peripheral blood T lymphocytes (Fig. 4, b and c). The suppression of proliferation was associated with a lack of blastings cells as detected under light microscopy. PD98059 was not toxic to T cells, because >95% of purified T cells were found to exclude trypan blue, even after a 72-h incubation period with 50 µM of PD98059 (data not shown).

Role of ERK

Previous studies have demonstrated that transfecting cells with a dominant negative ERK1 construct (Erk1-K71R) blocked both ERK1- and ERK2-mediated responses and that cotransfecting cells with dominant negative ERK1 and ERK2 constructs did not result in a greater degree of inhibition than that observed when cells were transfected with the ERK1 mutant alone (15). This suggests that the expression of Erk1-K71R was sufficient to block the function of both ERK1 and ERK2. The results in Fig. 3 demonstrate that Erk1-K71R suppressed the ability of PHA/PMA to stimulate the production of LT. The phosphorylation of MBP by partially purified ERK was also blocked (Fig. 1). In contrast to transfection with the dominant negative p21ras, the transfection of cells with Erk1-K71R did not affect the ability of PHA/PMA to stimulate ERK2 dual phosphorylation (Fig. 2a).

Discussion

Studies by Cantrell and others have previously reported that the transcriptional activity of NF of activated T cells and the IL-2 promoter are regulated by p21ras, ras-t, and MEK (12, 26–29). Thus, constitutively active and dominant negative mutants of these signaling molecules up-regulate and inhibit, respectively, the expression of reporter proteins under appropriate stimulatory conditions. Although these studies provide evidence that the production of IL-2 is regulated by p21ras, ras-t, and MEK, an involvement of ERK in the regulation of IL-2 production has not been directly demonstrated. MEK has also been reported to regulate the production of other cytokines. Thus, exposing mouse T cells that had been transfected with a constitutively active mutant of MEK1 to ionomycin was found to result in the production of IL-3, IL-4, IFN-γ, and granulocyte-macrophage CSF (30). Although these studies suggest that members of the p21ras-MEK cascade regulate the production of IL-2 and that MEK regulates the production of IL-3, IL-4, IFN-γ, and granulocyte-macrophage CSF, the intracellular signaling molecules that regulate the production of LT have remained totally undefined. Furthermore, direct evidence that ERK regulates the production of T lymphocyte cytokines is still lacking.
and MEK2, raf-1, protein kinase C (PKC), PKA, Ca²⁺ did not affect the activity of 18 known kinases, including MEK1 that PD98059, at concentrations similar to those used in this study, inhibitor appears to be specific, because Alessi et al. (25) reported MEK2 from being phosphorylated/activated by raf-1 (25). This PD98059 is currently believed to act by preventing MEK1 and mitogen-stimulated ERK activity. Inhibition of MEK1 using the LT from PHA/PMA-stimulated cells. The degree of suppression of that each member of the p21

ras

and the p38 cascades. Therefore, our data provide direct evidence that the effects of these mutants on cytokine production were mediated specifically via inhibition of the ERK cascade. Consistent with this possibility, the effects of the p21

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or raf mutants as well as PD98059 were found to be nearly identical in magnitude with each other. Furthermore, we have found that transfecting Jurkat cells with Erk1-K71R did not affect the expression of PKC α or β (our unpublished data), which are important in T cell responses (34). This observation is in agreement that of Sale et al., who reported that a loss of ERK function in preadipocytes did not affect the expression of PKC δ (35).

Previous studies have demonstrated that expression of Erk1-K71R alone is sufficient to block the biological functions of ERK1 and ERK2 in intact cells. Using an elk1 reporter construct, Westwick et al. demonstrated that the transfection of NIH 3T3 cells with Erk1-K71R blocked both ERK1- and ERK2-mediated increase in the transcriptional activity of elk1 and that the cotransfection of cells with Erk1-K71R and Erk2-K52R did not result in greater inhibition than that observed with Erk1-K71R alone (15). We have also demonstrated that the expression of Erk1-K71R in HeLa cells reduced the ability of PMA to stimulate p90

rsk

(our unpublished data), a kinase which is phosphorylated and activated by ERK (35). These observations, therefore, authenticate our finding that transfection of Jurkat cells with Erk1-K71R was sufficient to block ERK1-and ERK2-mediated signaling events.

Just how Erk1-K71R acts is currently not clear. Our data demonstrate that Erk-K71R did not affect the ability of MEK to phosphorylate ERK2 but prevented wild-type ERK from phosphorylating MBP in vitro, a finding that is consistent with the action of ERK1-K71R in intact cells. Thus, under the conditions in which cells were made to artificially express a kinase-dead ERK, the anti-ACTIVE ERK Ab was only able to provide information regarding the phosphorylation status of ERK and not the ability of ERK to phosphorylate a substrate. The lack of effect on ERK2 phosphorylation suggests that the main site of action of Erk-K71R is after ERK phosphorylation. We speculate that Erk-K71R, being a kinase-dead mutant, could act by stably binding to and sequestering relevant substrates, thereby preventing wild-type ERK from binding and phosphorylating relevant substrates without affecting the ability of MEK to phosphorylate the TEY motif of ERK.

Although the above data demonstrate that members of the p21

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-ERK cascade are required for LT production, activation of this cascade alone is insufficient to cause cytokine production. Thus, transfection with activated mutants of p21

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(H-ras 61L) or

PD98059. Cells were pretreated with PD98059 or DMSO and stimulated with PHA/PMA (Jurkat cells) or with anti-CD3 Ab and PMA (b and c, purified T lymphocytes) as described in Materials and Methods. The supernatants were harvested, and the amount of LT that was secreted was assayed by ELISA. Proliferation was assessed by determining the incorporation of [³H]thymidine into DNA. Results are the means ± SEM of at least three experiments (a) or the means ± SEM of three experiments (b and c).

* The significance of difference between control and stimulated cells, p < 0.01; **, p < 0.001.

Using PHA/PMA as stimuli, we provide direct evidence that members of the p21

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-ERK cascade regulate the production of LT. Thus, transfecting Jurkat cells with a dominant negative construct of p21

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, raf-1, or ERK1 greatly reduced the production of LT from PHA/PMA-stimulated cells. The degree of suppression of LT production was approximately the same as the suppression of mitogen-stimulated ERK activity. Inhibition of MEK1 using the selective MEK1 inhibitor, PD98059, also blocked LT production. PD98059 is currently believed to act by preventing MEK1 and MEK2 from being phosphorylated/activated by raf-1 (25). This inhibitor appears to be specific, because Alessi et al. (25) reported that PD98059, at concentrations similar to those used in this study, did not affect the activity of 18 known kinases, including MEK1 and MEK2, raf-1, protein kinase C (PKC), PKA, Ca²⁺/calmodulin-dependent kinases, and kinases in the c-Jun N-terminal kinase and the p38 cascades. Therefore, our data provide direct evidence that each member of the p21

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-ERK cascade participates in the regulation of LT production. This observation has been extended to the production of IL-2, because we have recently demonstrated that PMA/PHA-stimulated IL-2 production by Jurkat T cells was also inhibited by PD98059 and by Erk1-K71R (31).

The regulation of LT production by members of the ERK cascade was not restricted to PHA/PMA-stimulated Jurkat T cells. A role of MEK in LT production was also demonstrated in anti-CD3 Ab and PMA-stimulated purified peripheral blood T lymphocytes. Furthermore, PD98059 inhibited the ability of anti-CD3 Ab and PMA to stimulate T cell proliferation. These effects of PD98059 were not due to cytotoxicity, because >95% of the cells were capable of excluding Trypan blue, even after 72 h of incubation with the inhibitor.

Although dominant negative mutant constructs have been widely adopted as tools to examine the involvement of wild-type target proteins (including those in the ERK cascade) in biological responses, few studies have addressed the specificity the dominant negative mutant approach. In addition to the ERK cascade, a number of other effectors of p21

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and raf-1 have been characterized or proposed (28, 32, 33). It is conceivable that the effects of dominant negative p21

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or raf mutants could have been due to effects on these non-ERK cascade effectors. However, our data suggest that the effects of these mutants on cytokine production were mediated specifically via inhibition of the ERK cascade. Consistent with this possibility, the effects of the p21

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, raf-1, and ERK mutants as well as PD98059 were found to be nearly identical in magnitude with each other. Furthermore, we have found that transfecting Jurkat cells with Erk1-K71R did not affect the expression of PKC α or β (our unpublished data), which are important in T cell responses (34). This observation is in agreement that of Sale et al., who reported that a loss of ERK function in preadipocytes did not affect the expression of PKC δ (35).

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Although the above data demonstrate that members of the p21

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-ERK cascade are required for LT production, activation of this cascade alone is insufficient to cause cytokine production. Thus, transfection with activated mutants of p21

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(H-ras 61L) or
raf (raf22W) per se was insufficient to stimulate cytokine production despite a two- to threefold stimulation of ERK activity (our unpublished data). However, both mutants enhanced PHA/PMA-stimulated cytokine production and ERK activation in Jurkat cells (our unpublished data).

In summary, our data show that dominant negative mutants of p21ras, raf-1, and ERK1 as well as the MEK antagonist, PD98059, inhibited the production of LT from PHA/PMA-stimulated Jurkat cells. A role for MEK was demonstrated in anti-CD3 Ab and PMA-stimulated purified peripheral T lymphocytes. This study is the first to directly demonstrate that members of the p21ras-MEK-ERK cascade regulate the production of LT.

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