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Brief Definitive Report

The Role of Raf-1 in the Regulation of Extracellular Signal-regulated Kinase 2 by the T Cell Antigen Receptor

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Summary

Triggering of the T cell antigen receptor (TCR) complex activates the serine/threonine kinase Raf-1 whose function is necessary for TCR induction of the interleukin 2 gene. Raf-1 has been identified as a candidate mitogen-activated protein (MAP) kinase kinase kinase (MKKK) and thus has the potential to couple the TCR to the activation of the MAP kinases such as ERK2. In the present study, the role of Raf-1 in ERK2 regulation of ERK2 in T cells has been explored. A constitutively active Raf-1 kinase, v-raf, or a dominant inhibitory Raf-1 mutant were expressed transiently from the pEF BOS vector in Jurkat cells and the effects of these Raf-1 mutants on a coexpressed ERK2 reporter was assessed. The action of the constitutively active Raf-1 was to stimulate the ERK2 kinase, whereas the dominant negative version of Raf-1 inhibited the ERK2 activation induced by triggering of the TCR. These data indicate a role for Raf-1 in the regulation of ERK2 in T cells.

T lymphocyte activation is controlled by the TCR for foreign antigen. The TCR is a multichain complex composed of a heterodimer of the idiotypic α and β chains noncovalently associated with the invariant γ, δ, ϵ chains of the CD3 antigen and the TCR-ζ subunit (1). Triggering of the TCR in conjunction with signals generated by accessory molecules such as CD2, CD4, CD8, and CD28 control T cell activation by regulating the expression of genes encoding cytokines and cytokine receptors (2).

The TCR is coupled via its cytoplasmic domain to a protein tyrosine kinase signaling cascade (3, 4) that couples the TCR to two important signal transduction molecules: the guanine nucleotide binding proteins p21ras (5) and an inositol lipid-specific phospholipase C, PLCγ1 (6). PLCγ1 controls the metabolism of inositol phospholipids thereby generating inositol polyphosphates that mobilize intracellular calcium and diacylglycerols that stimulate the serine/threonine kinase, protein kinase C (PKC) (7, 8).

Several other serine/threonine kinases are activated by the TCR, namely mitogen-activated protein kinases (MAP kinases) such as ERK1 and ERK2 and the kinase encoded by the protocogene Raf-1 (9–12). In fibroblasts and neuronal cells, Raf-1 appears to transmit signals important in gene expression, cell growth, and differentiation (13, 14). In T cells, moreover, it has been demonstrated that Raf-1 activity is necessary for TCR induction of IL-2 gene transcription and secretion (15). It is not known how Raf-1 contributes to cytokine gene regulation although recent studies of Raf-1 function have suggested that Raf-1 is an important component of the signaling pathways that stimulate MAP kinases such as ERK1 and 2 (16, 17). The MAP kinases are activated by a kinase cascade involving a MAP kinase kinase (MKK) that phosphorylates and stimulates the ERK1 and 2 kinases directly (18). The activity of the MKK is itself controlled by phosphorylation and hence a MAP kinase kinase kinase (MKKK) plays a crucial role in the regulation of ERK1 and 2 (19). In some cells Raf-1 has been identified as a MKKK that plays a key role in coupling p21ras and hence receptors that stimulate p21ras to the MAP kinases (20, 21). Raf-1 is equally important in PKC and G-protein coupled mechanisms for MAP kinase activation in certain cell systems (20, 22). The MAP kinase ERK2 can phosphorylate and regulate transcriptional factors such as c-*jun* and Elk1 (23, 24). Hence if Raf-1 was involved in coupling the TCR to ERK2 then the ability of ERK2 to translocate to the nucleus where it can directly modulate transcriptional factors could explain the Raf-1 role in TCR signal transduction.

Recently, we demonstrated that TCR triggering results in the activation of ERK2 by a p21ras-dependent mechanism (25). Raf-1 is one candidate for the MKK that couples p21ras and the TCR to the MKK and hence to ERK2. However, the role of Raf-1 in TCR stimulation of ERK2 has not yet been explored and it should be noted that Raf-1 is not the only candidate MKKK since a murine homologue of two yeast MKKKs (termed MEKK) has been isolated that can stimulate the MKK without activating Raf-1 (26). Furthermore, activation of the ERK2 pathway may not explain the stimulatory effect of expression of constitutively Raf-1 on

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IL-2 production since it has been reported that IκB, a regulator of the NFκB family of transcriptional factors, is a substrate for Raf-1 (27, 28) and this could allow Raf-1 to have a role in the control of gene transcription that is independent of its ability to regulate MAP kinases. In this context, activated Raf-1 mutants fail to stimulate the MAP kinase pathway in every cell system (29, 30), which supports the possibility that Raf-1 may have cellular functions in addition to its potential role as a MKK.

The object of the present study was to examine the role of Raf-1 in MAP kinase regulation in T cells. ERK2 is the predominant TCR stimulated MAP kinase (10, 25) and the data demonstrate that expression of a constitutively active Raf-1 in T cells stimulates ERK2 whereas expression of a dominant negative Raf-1 suppresses TCR induction of ERK2. These studies implicate Raf-1 as an essential component of the MAP kinase cascade in T cells.

Materials and Methods

Plasmids and Antibodies. A tagged ERK2 reporter construct was generated as described (16) using a COOH-terminal epitope tag from c-*myc* that is recognized by the monoclonal antibody 9E10. The ERK2-tag encoding DNA was subcloned into the mammalian expression vector pEF-Bos (31). The pEF-Bos expression vector was also used to direct expression of v-raf, a gag-Raf fusion protein with constitutively high kinase activity (32) and a COOH terminus truncated Raf-1 (amino acids 1–257) that has a dominant negative phenotype (20, 21, 33). The antibodies UCHT1 and 9E10, reactive with the ε chain of the human TCR/CD3 antigen complex and the c-*myc* epitope tag, respectively, were purified from hybridoma supernatants by affinity chromatography with protein A–Sepharose. Rabbit antisera that recognizes ERK1 and ERK2 (antisera 124) (34) or Raf-1 were used to visualize endogenous ERK2 and Raf-1 by Western blotting (35).

Cell and Transfections. Jurkat cells were maintained in RPMI 1640 supplemented with 10% heat inactivated FCS. Growth was at 37°C in a humidified 5% CO_2/95% air incubator. Cells were transfected via electroporation (Gene Pulser; Bio-Rad Laboratories, UK), according to the manufacturer's instructions. Briefly, cells were incubated with the indicated concentration of plasmid DNA and then pulsed (1–1.5 × 10^7 in 0.5 ml medium) at 960 mF and 310 V. Transfected cultures were maintained in culture for 24 h and afterwards were harvested for subsequent procedures.

Western Blotting. Acetone-precipitated protein from cell lysates corresponding to 10^6 cells was resolved in SDS-PAGE gels. Blots were probed as previously described with 9E10 antibody to visualize the ERK2-tag protein (16). To detect endogenous ERK-2 or Raf-1, blots were probed with specific antisera as previously described (34, 35). To detect immunocomplexes the Western blots were probed with either horseradish peroxidase (HRP)-coupled rabbit anti–mouse or HRP-coupled goat anti–rabbit followed by electrochemiluminescence (ECL; Amersham International, Little Chalfort, UK).

Analysis of ERK-tag Kinase Activity. Jurkat cells were transfected with the indicated concentration of pEF-BOS ERK2-tag. After 24-h 1–1.5 × 10^6 cells/assay point were activated as indicated at 37°C in 1 ml of RPMI 1640. Thereafter immunoprecipitates of ERK2-tag were prepared using the 9E10 monoclonal antibody. Briefly, cells were lysed in 0.5 ml lysis buffer (1% Triton X 100, 50 mM Hepes [pH 7.4], 150 mM NaCl, 20 mM NaF, 20 mM iodacetamide, 1 mM PMSF, 100 μM Na_3VO_4). After 20 min of preclearing with insoluble protein A–Sepharose suspension, lysates were incubated for 20 min with 2 μg of 9E10 antibody, then for 1 h with 20 μl of a 50% suspension of protein G–Sepharose beads (Sigma Chemical Co., Poole, UK). Immunoprecipitates were washed three times with lysis buffer and once with kinase assay buffer (30 mM Tris [pH 8], 2 mM $MgCl_2$, 2 mM $MnCl_2$). In vitro kinase assays were carried out for 30 min at room temperature in 20 μl of kinase assay buffer supplemented with 10 μM ATP, 5 μCi γ-[^{32}P]ATP, and 10 μg myelin basic protein (MBP) as a substrate. The kinase reaction was stopped with 20 μl of 2× SDS sample buffer and samples were run in 15% SDS-PAGE minigels. Quantitation of ^{32}P incorporated into the MBP protein band was done by β-radiation scanning of dried gels using a scanner (AMBIS, Inc., San Diego, CA). The assay is linear with regard to time and amount of kinase activity. All MBP kinase activity in ERK2-tag immunoprecipitates was normalized to the relative amount of ERK2-tag protein expressed in different cell populations as assessed by Western blotting analysis (25).

Results

Activation of ERK2 and Hyperphosphorylation of Raf-1 in TCR and Phorbol Ester–treated T Cells. The activation of endogenous ERK2 can be monitored by Western blot analysis with an ERK2-specific antiserum because the activation of ERK2 requires its phosphorylation on threonine and tyrosine residues and the phosphorylated "active" ERK2 has a reduced mobility on SDS-PAGE gels compared with nonphosphorylated, "inactive" ERK2 (34). The experiment depicted in Fig. 1 *A* shows the reduced mobility of ERK2 in Jurkat cells treated with the PKC activator Pdbu and the CD3 antibody UCHT1 compared with control unstimulated cells. Stimulation with UCHT1 induced a transient mobility shift in the band corresponding to ERK2 that was readily detectable within 1 min of exposure to UCHT1, maximal at 10 min but had returned to basal by 30 min. Pdbu also induced a rapid shift in ERK2 mobility that was maintained even after 30 min. Previous studies have shown that Raf-1 is activated rapidly in TCR and phorbol ester–stimulated T cells (12). It is also well documented that Raf-1 is hyperphosphorylated in response to T cell activation (11) and this hyperphosphorylation of Raf-1 correlates with activation of the enzyme, although it is apparently not directly involved in mediating the increase in Raf-1 enzymatic activity (29). Hyperphosphorylated Raf-1 has reduced mobility on SDS-PAGE gels hence to determine whether Raf-1 is hyperphosphorylated in Jurkat cells, a Western blot of cell lysates prepared from cells treated with UCHT1 or Pdbu was probed with a Raf-1 antisera. The data in Fig. 1 *B* show that both UCHT1 and Pdbu induce a shift in Raf-1 mobility. The kinetics of the Raf-1 mobility shift are delayed in comparison with the ERK2 response. In both UCHT1- and Pdbu-treated cells the Raf-1 mobility shift was only discernable at the 10-min time-point and maintained at 30 min.

Stimulation of ERK2 by Expression of v-raf. The Raf-1 hyperphosphorylation in TCR-activated Jurkat cells indicates that Raf-1 is involved in a signal transduction pathway regulated by the TCR. However, to determine whether Raf-1 is in-

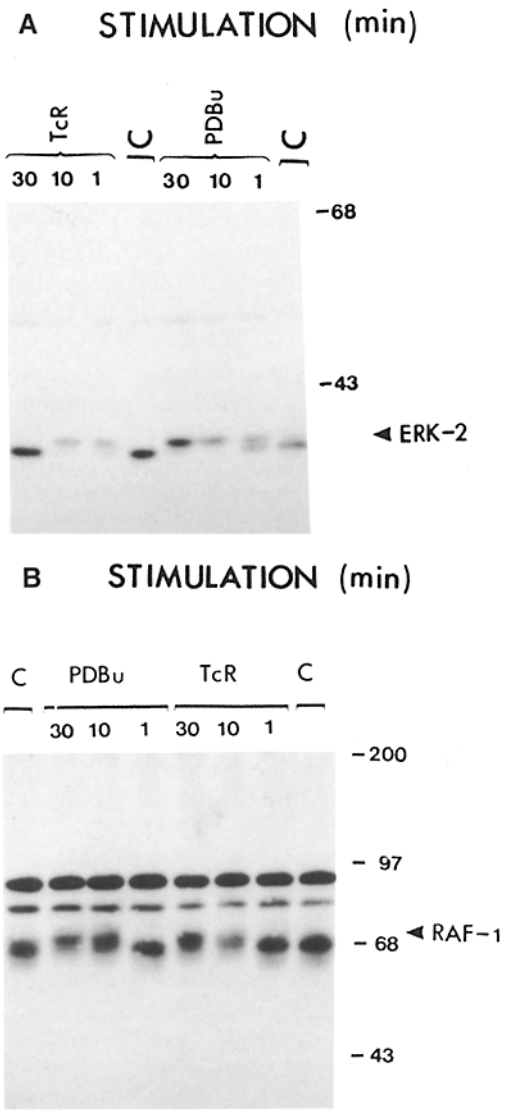

Figure 1. Triggering of the TCR hyperphosphorylates ERK2 and RAF-1. Lysates from Jurkat cells either unstimulated (labeled C) or stimulated for the indicated time points with 100 ng/ml PDBu, 10 mg/ml of the TCR agonist UCHT1 were resolved by SDS PAGE and subjected to Western blot analysis with 124 antiserum or Raf-1 antisera to visualize ERK2 (*A*) and Raf-1 (*B*).

volved in the signaling mechanism used by the TCR to stimulate ERK2 it is necessary to examine the requirement for Raf-1 in TCR stimulation of ERK2. Raf-1 is a 72-kD serine/threonine kinase composed of an NH_2-terminal regulatory domain and a COOH-terminal catalytic domain. Truncations of Raf-1 that remove the NH_2-terminal domain generate a constitutively active kinase (36). To examine the role of Raf-1 in ERK2 activation in T cells, the effect of a v-Raf, on ERK2 kinase activity was examined. The v-*raf* construct used is a gag Raf fusion protein with constitutively high Raf kinase activity, under the control of the pEF BOS expression vector. For these studies a sensitive assay for ERK2 kinase expression and function, developed previously to explore the role of $p21^{ras}$ in ERK2 regulation in T cells (25), was used. An epitope tagged ERK2 is transiently expressed in Jurkat cells. The epitope tag on ERK2 is a c-*myc* polypeptide that is recognized by the monoclonal antibody 9E10, thus allowing both Western blot quantitation and immunoprecipitation of ERK2-tag (16). The kinase activity of the transfected ERK2-tag can be quantitated by kinase assays on the 9E10 immunoprecipitates using MBP as a substrate. The advantage of this assay is that it can be readily applied to explore the regulatory effects of cotransfected, constitutively active or dominant negative Raf-1 mutants.

The consequences of the expression of the constitutively active v-Raf on ERK2 tag activity is shown in Fig. 2. MBP kinase assays were carried out on ERK2-tag immunoprecipitates isolated from v-Raf or control transfected cells. ERK2 isolated from unstimulated Jurkat cells has minimal activity that is rapidly stimulated when cells are treated with Pdbu or UCHT1 (Fig. 2). There was a marked increase in the basal-specific ERK2-tag kinase activity isolated from v-Raf–transfected cells compared with control cells (Fig. 2). The expression of v-Raf induced an increase in ERK2-tag kinase activity in Jurkat cells that was comparable to the ERK2 activity observed in T cells activated via the TCR but somewhat less than the ERK2 activity present in phorbol ester treated T cells. The stimulatory effect of v-raf on ERK2 activation could be further enhanced by exposure of cells to PDBu or UCHT1 (Fig. 2).

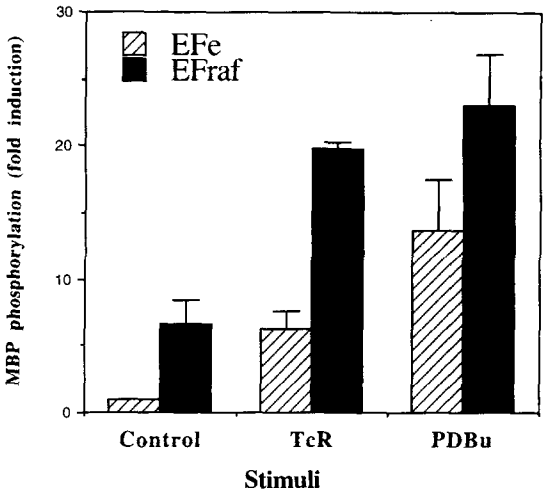

Figure 2. v-Raf activates ERK2-tag in Jurkat cells. Jurkat cells were cotransfected with either 40 μg of pEFempty vector and 40 μg of pEF-ERK2-tag (EFe) or 40 μg of the pEFv-RAF vector and 40 μg of pEF-ERK2-tag (EFraf). After 24 h of culture cells were either unstimulated (control) or stimulated for 10 min with PDBu (100 ng/ml) or UCHT1 (10 μg/ml). ERK2 tag was immunoprecipitated with the 9E10 antibody. Kinase assays with MBP as a substrate were set up on the immunoprecipitates, and the phosphorylated MBP was resolved by 15% SDS-PAGE of the products of the kinase assay. Data are expressed as cpm of ^{32}P incorporated into MBP as assessed by β-scanning of the band corresponding to MBP (AMBIS, Inc.). The ERK-2 activation data obtained in three experiments are expressed as fold induction in MBP phosphorylation. All kinase data was normalized to the level of ERK2-tag expressed in the different populations. This was assessed by Western blot analysis of ERK2-tag expression with the 9E10 antibody.

A Dominant Negative Mutant of Raf-1 Inhibits ERK2 Activation in T Cells. The studies with v-*raf* suggest that Raf-1 induces ERK2 activation but to determine whether Raf-1 is involved in TCR signaling we examined the effect of a dominant inhibitory Raf-1 mutant on TCR activation of ERK2. A mutant of Raf-1 was generated that was composed of only the NH_2-terminal regulatory domain (amino acids 1–256) without the COOH-terminal catalytic domain (33). Previous studies have established that the expression of the regulatory domain without the kinase domain results in a protein that selectively inhibits endogenous Raf-1 activity with a dominant inhibitory effect (15, 21). To determine the effect of the inhibition of endogenous Raf-1 on ERK2 activation, Jurkat cells were transfected with the COOH-terminal truncated Raf-1 and the activity of cotransfected ERK2 tag was assessed. The data in Fig. 3 *A* show that unlike v-*raf*, expression of the dominant negative Raf-1 did not increase the basal levels of ERK2 tag activity but almost completely suppressed the stimulation of ERK2 induced by TCR triggering or activation of PKC by phorbol esters. This inhibition of ERK2 tag activity was not due to inhibition of ERK2 tag expression. In each experiment ERK2 tag expression was confirmed and quantitated by Western blot analysis with the 9E10 antibody (Fig. 3 *B*).

Discussion

The current studies examine the role of Raf-1 in ERK2 regulation in T lymphocytes. The data demonstrate that expression of a constitutively active Raf-1 kinase in T cells results in activation of ERK2 to a level comparable to that induced by TCR triggering. In addition the data demonstrate that expression of dominant inhibitory form of Raf-1 prevents ERK2 activation induced by the TCR. These results indicate that Raf-1 plays a critical role in the TCR regulation of the MAP kinase cascade. Previous studies have shown that Raf-1 plays an crucial role in T cell IL-2 production (15). The ability of Raf-1 to control the MAP kinase signaling pathway could explain the function of Raf-1 in the regulation of IL-2 gene transcription. This is because MAP kinases are important for controlling the activity of the AP-1 family of transcriptional factors (37) that are proposed to contribute to the regulation of IL-2 gene expression by interacting with several elements within the IL-2 promoter (38).

Although active Raf expression was sufficient to stimulate ERK2 it was observed that signals generated by the TCR and by phorbol esters were additive with v-*raf* for ERK2 induction. This phenomenon could reflect that the expression of the constitutively active v-*raf* construct was suboptimal. Alternatively the data may imply that the TCR and PKC can provide a signal not supplied by v-*raf* for MAP kinase activation. It is well established that MAP kinase activity is controlled by a phosphorylation cascade and the ability of v-*raf* to stimulate ERK2 in T cells is consistent with its ability to function as a MKKK and hence control the function of the MKK that phosphorylates and regulates ERK2. The TCR could contribute to ERK2 regulation by generating additional signals that positively regulate the MKK independently of

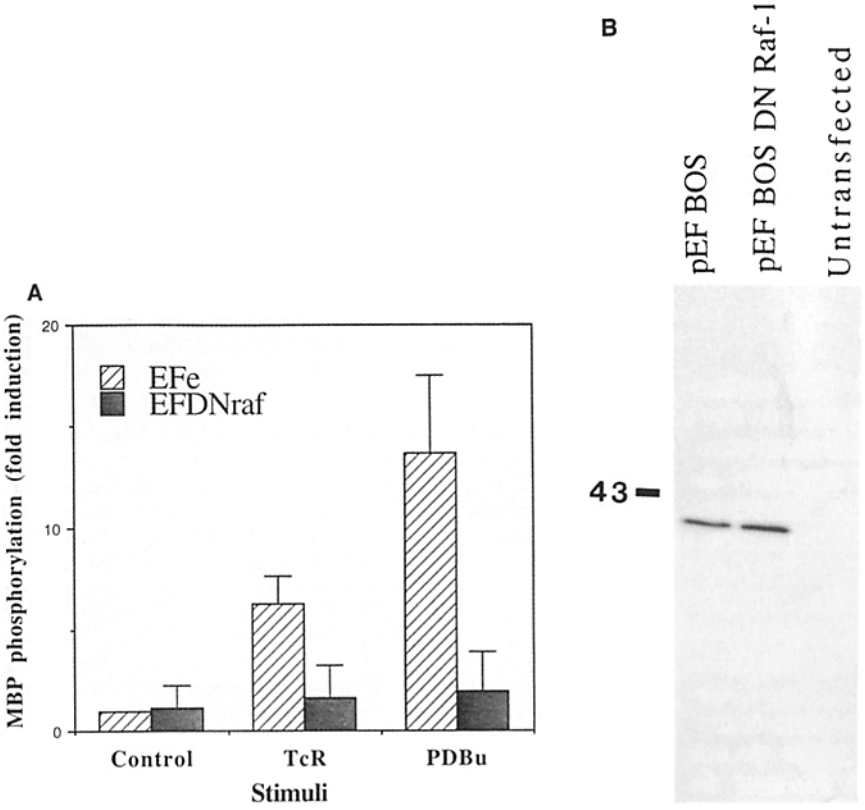

Figure 3. Effect of a dominant negative Raf-1 on ERK2-tag regulation in Jurkat cells. Jurkat cells were cotransfected with either 20 μg of the pER-ERK2-tag vector and 40 μg of the pEF empty vector (EFe) or 30 μg of the pEF-ERK2-tag vector and 40 μg of the pEF-vector expressing the COOH-terminal truncated Raf-1 (EFDNraf). After 20 h of transfection cells were either unstimulated (control) or stimulated for 10 min with Pdbu (20 ng/ml) or UCHT1 (10 μg/ml). (*A*) 9E10 IPs were carried out on the lysates and ERK2-tag activity was assessed using the MBP kinase assay as indicated above. All kinase data was normalized to the level of ERK2-tag expressed in the different populations as assessed by Western blot analysis of ERK2-tag expression with the 9E10 antibody. Results are expressed as fold induction ERK2-tag activity as an average (mean ± SD) of the data obtained in three experiments. (*B*) Western blot analysis with 9E10 of the *myc* tagged ERK2-tag in T cells expressing ERK2-tag alone or ERK2-tag together with dominant negative Raf-1.

Raf-1. However, it is also possible that the TCR could contribute to ERK2 activation via regulation of cellular phosphatases. In vivo phosphorylation levels always reflect the balance of the activities of kinases and phosphatases and it is thus noteworthy that it has been shown that cell phosphatase activity may be rate limiting for Raf-1 regulation of ERK2 (29). Recent studies have identified a dual specificity phosphatase, MKP-1, that dephosphorylates ERK2 in vivo. (39). A related dual specificity phosphatase, PAC-1, is expressed in mitogen activated T cells (40). It is thus possible that the TCR normally regulates ERK2 by controlling the activities of positive regulators of ERK2 such as Raf-1 and negative regulators such as PAC-1.

The effects of active and dominant negative Raf-1 on T cell ERK2 regulation are similar to the effects of active and dominant negative p21ras reported previously (25). It has been shown that the NH_2-terminal regulatory domain of Raf-1 can interact directly with activated GTP bound p21ras which supports the genetic evidence that Raf-1 is an effector molecule of p21ras (33, 41). Thus the fact that both Raf-1 and p21ras function are essential for T cell activation is consistent with the model that p21ras couples the TCR to Raf-1 and hence to the MAP kinase cascade. The current series of experiments has not addressed this issue directly. It is also not clear whether the physical association between GTP bound Ras and Raf-1 is sufficient to activate the kinase or whether additional regulatory signals are required. It has been suggested that PKC function is necessary for TCR regulation of Raf-1 activity (11). In contrast it appears that PKC does not play a major role in coupling the TCR to p21ras or ERK2 (5, 31). Accordingly future studies must investigate the mechanisms of Raf-1 regulation in T cells and determine whether Raf-1 is a convergence point for p21ras and PKC signals.

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