

p21^{ras} Couples the T Cell Antigen Receptor to Extracellular Signal-regulated Kinase 2 in T Lymphocytes

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Summary

It has previously been shown in T cells that stimulation of protein kinase C (PKC) or the T cell antigen receptor (TCR) induces the rapid accumulation of the active guanosine triphosphate-bound form of p21^{ras}. These stimuli also induce the activation of extracellular signal-regulated kinase 2 (ERK2), a serine/threonine kinase that is rapidly activated via a kinase cascade in response to a variety of growth factors in many cell types. In this study, we show that p21^{ras} is a component of the TCR signaling pathway that controls ERK2 activation. In the human Jurkat T cell line, transient expression of constitutively active p21^{ras} induces ERK2 activation, measured as an increase in the ability of an ERK2-tag reporter protein to phosphorylate myelin basic protein. Thus, constitutively active p21^{ras} bypasses the requirement for PKC activation or TCR triggering to induce ERK2 activation. In addition, activation of PKC or the TCR produces signals that cooperate with activated p21^{ras} to stimulate ERK2. Conversely, expression of a dominant negative mutant of ras, Ha-ras N17, blocks ERK2 activation after TCR stimulation, indicating that endogenous p21^{ras} function is necessary for the TCR-stimulated ERK2 activation. Taken together, these results demonstrate that the activation of p21^{ras} is both necessary and sufficient to induce ERK2 activation in T cells.

The TCR is the crucial receptor that controls the activation of T lymphocytes. One immediate consequence of triggering the TCR is the activation of cytosolic src family protein tyrosine kinases (PTKs)¹ and the resulting induction of phospholipase C γ 1-mediated hydrolysis of inositol phospholipids to generate diacylglycerol and inositol polyphosphates which induce protein kinase C (PKC) activation and elevate intracellular calcium, respectively (1). Recently, a second PTK-controlled signaling pathway originating from the TCR has been identified, one that couples the TCR to the guanine nucleotide binding proteins p21^{ras} (2, 3). The p21^{ras} proteins cycle between an "inactive" GDP-bound and an "active" GTP-bound form. In quiescent T cells, p21^{ras} exists in a complex with GDP but, after triggering of the TCR, there is a rapid and stable accumulation of p21^{ras} GTP complexes. In T lymphocytes p21^{ras} is stimulated not only in response to TCR triggering but also after the activation of PKC with phorbol esters and diacylglycerols (2). However, the role of PKC in

p21^{ras} regulation is poorly understood and, although the TCR activates PKC, the TCR does not regulate ras via PKC. Instead, a parallel PKC-independent, but PTK-dependent pathway seems to be responsible for TCR-stimulated p21^{ras} activation (3).

The importance of p21^{ras} in T cells has been examined by expressing transfected genes encoding either constitutively active or dominant inhibitory mutants of p21^{ras} (4, 5). An important function of the TCR is to control the expression and activation of transcription factors essential for the induction of the gene encoding the T cell growth factor IL-2 (6). Expression of a dominant inhibitory mutant of p21^{ras} prevents both TCR and PKC induction of IL-2 (4). Furthermore, expression of a constitutively active p21^{ras} initiates a signaling pathway that synergizes with signals generated by increasing cytosolic calcium to induce the IL-2 gene (4). These data indicate that cellular p21^{ras} has a vital role in TCR-stimulated IL-2 induction.

In fibroblasts and the pheochromocytoma PC12 cell line, the transmission of signals from p21^{ras} to the nucleus is proposed to involve the regulation of the activity of mitogen-activated protein (MAP) kinases or extracellular signal-regulated kinases (ERK) (7-10). T lymphocytes express at least

¹ Abbreviations used in this paper: ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MBP, myelin basic protein; PKC, protein kinase C.

two ERK or MAP kinases, ERK1 and ERK2, and the activity of ERK2 is stimulated in response to TCR and PKC activation (11, 12). Previous studies have suggested that the TCR either regulates the ERKs via PKC (11) or via an alternative PTK-dependent pathway involving the kinase p56^{lck} (13). However, any link between TCR-regulated p21^{ras} and the activation of MAP kinases has not yet been explored. The kinetics of TCR stimulation of p21^{ras} and the ERKs correlate (2, 12), but such a correlation does not prove that p21^{ras} couples the TCR to ERKs as active p21^{ras} does not always lead to ERK2 activation. For instance, it has been reported that the IL-2 receptor, which is as potent as the TCR in regulating the guanine nucleotide binding cycle of p21^{ras} (14), can activate ERK2 when expressed in myeloid cells (15), but IL-2 is not a potent activator of ERKs in T lymphocytes (16, 17). Furthermore, although p21^{ras} activates ERKs in several cultured cell lines (7–10), in Rat-1a fibroblasts, the expression of constitutively active p21^{ras} fails to activate ERKs (18). Also, in fibroblasts, phorbol ester-induced ERK activation is insensitive to expression of a dominant inhibitory ras mutant, and in these cells, PKC may function downstream of p21^{ras} (10, 19).

The purpose of the present study was to attempt to position p21^{ras} on the pathway via which the TCR induces ERK2 activation. Data obtained from transfection studies show that expression of a constitutively active p21 v-Ha-ras in T cells is a sufficient signal to stimulate the activity of the MAP kinase ERK2. Moreover, inhibition of endogenous p21^{ras} function by the dominant inhibitory mutant N17 ras suppresses TCR induction of ERK2 activity. These data demonstrate that p21^{ras} proteins are pivotal for the regulation of ERK2 in T cells and suggest that one mechanism whereby p21^{ras} regulates T cell activation is via the activation of the ERK cascade.

Material and Methods

Plasmids and Antibodies. A tagged ERK2 reporter construct was generated as described (19) using a COOH-terminal epitope tag from *c-myc* which is recognized by the mAb antibody 9E10. The ERK 2-tag encoding DNA was subcloned into the mammalian expression vector pEF-Bos (20). The pEF-Bos expression vector was also used to direct expression of a constitutively active p21^{ras}, p21 v-Ha-ras. The expression vector for a dominant inhibitory ras construct (N17 ras) has been described previously (21, 22): a 4.8-kb genomic fragment of *c-Ha-ras* which bears a mutant at codon 17 (Ser → Asn) expressed under the control of the RSV promoter. The RSV expression vector pB59W was used as an empty vector control as indicated. 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.

Cells and Transfections. The Jurkat T leukemia cells were maintained in RPMI 1640 supplemented with 10% heat inactivated FCS. Growth was at 37°C in a humidified 5% CO₂/95% air incubator. Cells were transfected via electroporation (Gene Pulser; Bio-Rad, Hemel Hempstead, UK), according to the manufacturer's instructions. Briefly, cells were incubated with the indicated concentration of plasmid DNA and then pulsed (1–1.5 × 10⁷ in 0.5 ml medium) at 960 μ F and 310V. 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 10⁶ cells was resolved in 12.5% SDS-PAGE minigels. Blots were probed as previously described (19) with 9E10 antibody to visualize the ERK2-tag protein. To detect transfected and endogenous ras proteins, Western blots were probed with a anti-ras antibody. To detect immunocomplexes, the Western blots were probed with horseradish peroxidase-coupled rabbit anti-mouse followed by enhanced chemiluminescence (Amersham Intl., Bucks, UK).

p21^{ras} Activation. p21^{ras} proteins were immunoprecipitated with antibody Y13-259 from cells in which guanine nucleotides were labeled biosynthetically with [³²P] orthophosphate as described (2). Labeled guanine nucleotides bound to ras were eluted, separated by thin-layer chromatography, and then quantitated by direct scanning for β radiation using a β scanner (Ambis, Inc., San Diego, CA). Results are expressed as proportion of p21^{ras} bound to GTP.

Analysis of ERK2-tag Kinase Activity. Jurkat cells were transfected with the indicated concentration of pEF-BOS ERK2-tag. After 24 h 1.5–1 × 10⁶ cells per 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 mAb. Briefly, cells were lysed in 0.5 ml lysis buffer (50 mM Hepes [pH, 7.4], 150 mM NaCl, 20 mM NaF, 20 mM iodoacetamide, 1 mM PMSF, and 100 μ M NaVO₄). After 20 min of preclearing with insoluble protein A-Sepharose suspension, lysates were incubated for 20 min with 2 μ g of 9E10 antibody, then for 1 h with 20 μ l of a 50% suspension of protein G-Sepharose beads (Sigma Chemical Co., St. Louis, MO). Immunoprecipitates were washed three times with lysis buffer and once with kinase assay buffer (30 mM Tris [pH, 8], 20 mM MgCl₂, and 2 mM MnCl₂). In vitro kinase assays were carried out for 30 min at room temperature in 20 μ l of kinase assay buffer supplemented with 10 μ M ATP, 5 μ Ci γ -[³²P]ATP and 10 μ g myelin basic protein (MBP) as a substrate. The kinase reaction was stopped with 20 μ l of 2 × SDS sample buffer and samples were run in 15% SDS-PAGE minigels. Quantitation of ³²P incorporated into the MBP protein band was done by β radiation scanning of dried gels using a scanner (Ambis, Inc.). 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.

Results

TCR Triggering and PKC Stimulation Activate p21^{ras} in Jurkat Cells. To confirm that p21^{ras} is regulated via the TCR and PKC in the Jurkat cells used in the present study, p21^{ras}-GTP loading experiments were performed. Endogenous guanine nucleotides were metabolically labeled with [³²P]orthophosphate and p21^{ras} protein was immunoprecipitated from Jurkat cells exposed either to the antibody UCHT1 that triggers the TCR or to the phorbol ester PDBu that directly activates PKC. The proportion of p21^{ras} bound to GTP (the ratio between GTP and GTP plus GDP labeled nucleotides on p21^{ras}) was evaluated by β emission scanning of TLC-resolved guanine nucleotides eluted from immunoprecipitated ras proteins. Fig. 1 shows that PKC stimulation with PDBu increased the level of GTP bound to ras from 6% in unstimulated Jurkat to 34% (five- to sixfold). TCR

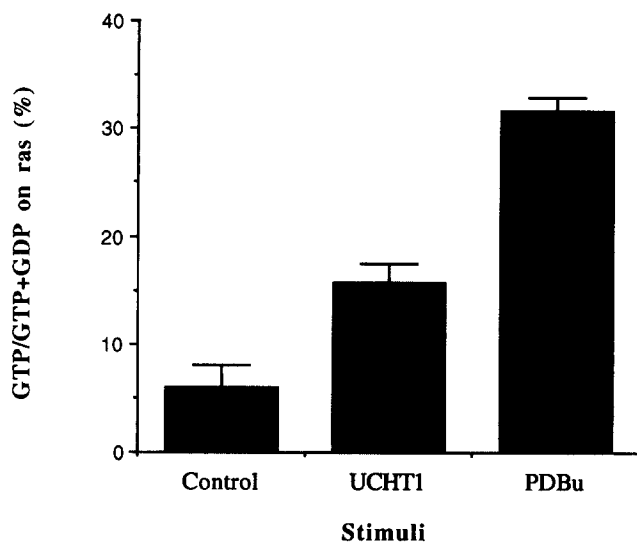


Figure 1. Effect of PDBu and TCR agonist UCHT1 on the activation state of p21^{ras} in Jurkat cells. Cells labeled with [³²P]orthophosphate as indicated in Materials and Methods were either unstimulated (*control*) or stimulated for 10 min with 100 ng/ml PDBu or 10 μ g/ml UCHT1. Immunoprecipitation of p21^{ras} was with mAb Y13-259 and nucleotides were separated by TLC. The figure shows the quantitation of guanine nucleotides on p21^{ras} by direct scanning of β radiation. Data are expressed as percentage of GTP bound to ras with respect to the total amount of guanine nucleotide on ras and are average (mean \pm SD) of the data obtained in four experiments.

triggering with UCHT1 resulted in a threefold increase in the proportion of GTP-bound ras (from 6 to 19%).

Expression and Regulation of ERK2-tag in Jurkat Cells. Previous studies (4, 5) have established that transient transfection protocols provide a powerful system for the analysis of p21^{ras} function in T cells. Since the transfection efficiency in Jurkat cells is about 10–30%, attempts to examine the effects of transient expression of p21^{ras} mutants on ERK2 activity require the use of a reporter construct. Accordingly, a MAP kinase reporter construct (ERK2-tag) was generated to express ERK2 epitope tagged with a *c-myc* polypeptide that is recognized by the mAb 9E10. The 9E10 epitope allows both Western blot quantitation and immunoprecipitation of ERK2-tag in transfected cells so that the expression and activation of ERK2 in cells coexpressing mutant ras can be assessed (19).

In initial experiments, the expression and activation of ERK2-tag in response to TCR or PKC stimulation was examined. Fig. 2 *a* shows a Western blot developed with the 9E10 antibody of cell lysates isolated from Jurkat cells 24 h after the cells were transfected either with a control empty expression vector or the ERK2-tag expression vector. Cells transfected with the ERK2-tag construct expressed a 9E10-reactive 42-kD band corresponding to the predicted molecular weight of the ERK2-tag protein. The basal kinase activity of the transfected ERK2-tag was assessed by kinase assays on the 9E10 immunoprecipitates using MBP as a substrate. The basal kinase activity of the ERK2-tag isolated from un-

stimulated cells was weak (Fig. 2 *b*). However, if the transfected Jurkat cells were stimulated with PDBu or UCHT1 for 5 min there was a marked increase in the MBP kinase activity in the ERK2-tag immunoprecipitates (Fig. 2 *b*). The data in Fig. 2 *c* summarizes data from five experiments. A 5-min stimulation of Jurkat cells with PDBu induced a 15–20-fold increase in ERK2-tag kinase activity; whereas the stimulatory effect of the TCR agonist antibody UCHT1 was a 10–15-fold increase. No MBP kinase activity was ever detected in 9E10 immunoprecipitates isolated from either quiescent or activated nontransfected Jurkat cells (Fig. 2 *b*).

Kinetic analysis of ERK2 kinase activation (Fig. 2 *d*) showed that maximal stimulation occurs within 10 min of both UCHT1 and PDBu stimulation. ERK2-tag stimulation in response to UCHT1 decreased after 30 min, although a two-fold increase in activity was still detectable at 60 min. The PDBu response also declined after 30 min but was sustained at 50% of the maximal stimulation at 60 min. Increasing intracellular calcium in T cells does not activate p21^{ras} (2). The data in Fig. 2 *c* show that ERK2-tag kinase activity in Jurkat cells stimulated with the calcium ionophore Ionomycin was similar to the basal kinase activity present in nonactivated cells. Thus the ERK2-tag kinase activity is stimulated after TCR or PKC stimulation but not in response to increases in intracellular calcium. The kinetics of ERK2-tag activation as well as the relative magnitude of TCR versus PKC stimulation are exactly consistent with the regulation of endogenous ERK2 activity described in previous studies (11, 12).

p21^{ras} Activates ERK2. To determine whether p21^{ras} is involved in the signaling pathways that regulate ERK2, a mutationally activated ras protein (*v-Ha ras*) was coexpressed in Jurkat cells with ERK2-tag. *v-Ha-ras* is mutated at codon 12 (G \rightarrow R) and 59 (A \rightarrow T). These mutations decrease the intrinsic GTPase activity of p21^{ras} and render the protein insensitive to GTPase activating proteins so that the active GTP-bound form of p21^{ras} accumulates and has a positive dominant effect (23). Fig. 3 *a* shows a Western blot of cell lysates from Jurkat cells cotransfected with either ERK2-tag and a control empty vector or ERK2-tag plus *v-Ha-ras*. The Western blot was probed initially with 9E10 antibody to check expression of ERK2-tag (Fig. 3 *a*, *left*) and subsequently with a anti-pan ras antibody in order to assess the expression of the endogenous and transfected ras proteins (Fig. 3 *a*, *right*). These Western blots show that the level of ERK2-tag in *ras* transfected cells was comparable to the usual level of ERK2-tag in non *ras* transfected cells. The anti-pan ras antibody used in the present study cannot distinguish normal p21^{ras} from the mutated *v-Ha-ras* but the expression of *v-Ha-ras* in transfected cells can be judged by the overexpression of ras in the *v-Ha-ras* transfected cells compared to either untransfected or empty vector control transfectants (Fig. 3 *a*, *right*).

To examine the consequences of p21 *v-Ha-ras* expression on ERK2 activity, MBP kinase assays were carried out on ERK2-tag immunoprecipitates isolated from *v-Ha-ras* or control transfected cells. Fig. 3 *b* shows the SDS-PAGE analysis

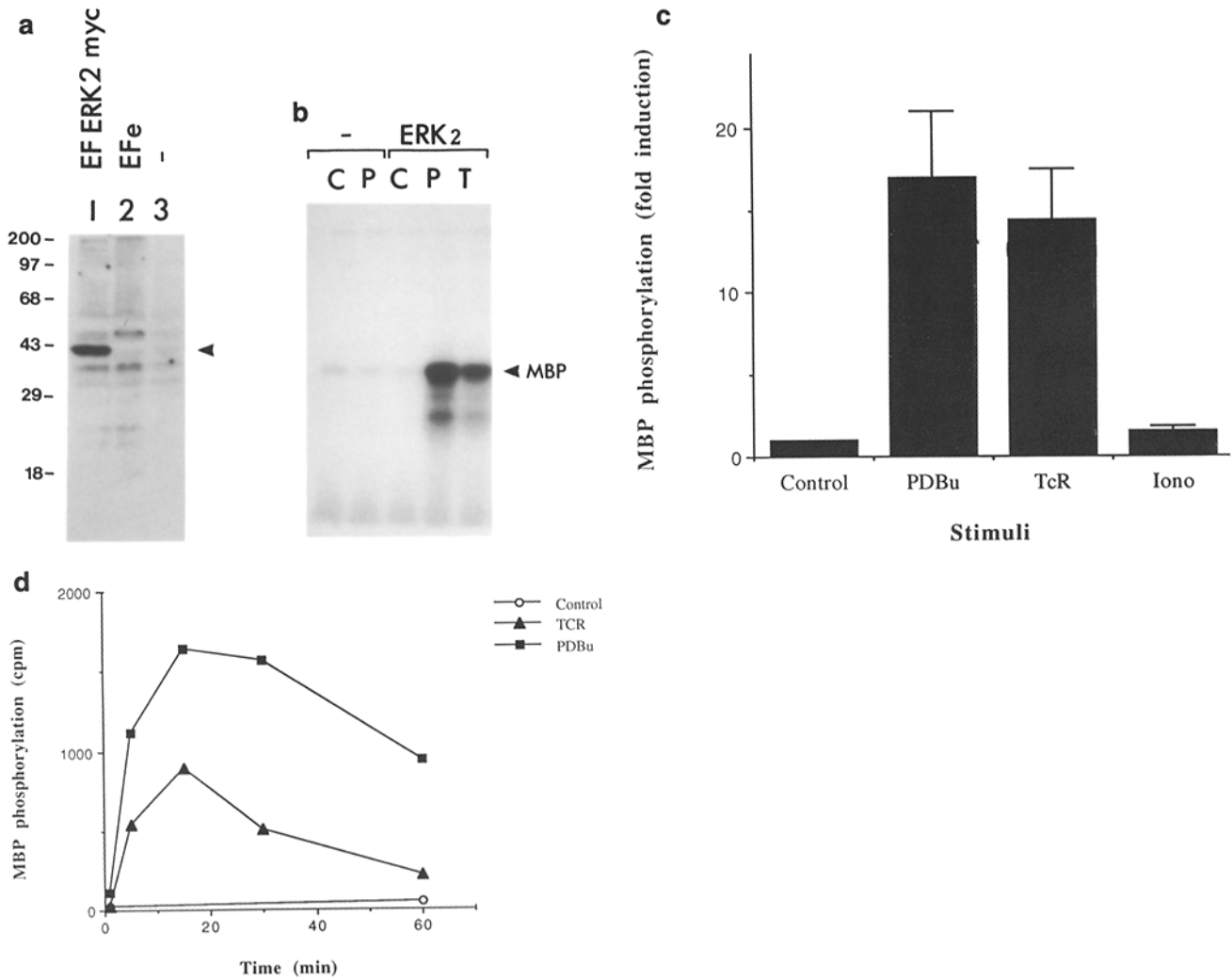


Figure 2. Transient expression and regulation of ERK2-tag in Jurkat cells. (a) Jurkat cells were transfected as indicated in Materials and Methods with either 30 μg of the pEF-ERK2-tag vector (lane 1) or the pEF empty vector (lane 2). After 24 h of transfection, Western blot of the lysates was developed with 9E10 antibody. Lane 3 corresponds to a lysate from untransfected cells. (b) Lysates from untransfected cells (-) or cells transfected with 30 μg of pEF-ERK2-tag (ERK2) and either unstimulated (C) or stimulated for 10 min with 100 ng/ml PDBu (P) or 10 $\mu\text{g}/\text{ml}$ TCR agonist (T) were immunoprecipitated with the 9E10 antibody. Kinase assays with MBP as a substrate were set up on the immunoprecipitates (IP) and MBP phosphorylation was visualized by autoradiography after 15% SDS-PAGE of the products of the kinase assay. (Arrowhead) The position of the MBP. (c) The figure quantitates the ERK2 activity data corresponding to several experiments similar to the one described in (b). Cells were either unstimulated (control) or treated with 100 ng/ml PDBu, 10 $\mu\text{g}/\text{ml}$ TCR agonist UCHT1, or 0.5 $\mu\text{g}/\text{ml}$ ionomycin. Data are expressed as fold induction in MBP phosphorylation as quantitated by β scanning of dried SDS-PAGE gels and are an average (mean \pm SD) of the results obtained in five experiments. (d) Time course of ERK2-tag stimulation. The experiment was performed as described above, but the stimulation was for the indicated times. Data are expressed as cpm of ^{32}P incorporated into MBP as assessed by Ambis β scanning.

of the MBP kinase assays of ERK2 isolated from the two populations. Since the level of ERK2-tag protein was similar in both populations (Fig. 3 a, left), the activity data are directly comparable. The data show a marked increase in the basal-specific ERK2-tag kinase activity isolated from v-Ha-ras transfected cells compared to control cells. (Fig. 3 b, lane C, right versus lane C, left). A further increase in ERK2 kinase activity was induced in the v-Ha-ras expressing cells by stimulating the cells with UCHT1 or PDBu. The data in Fig. 3 c quantitate the results of several experiments. All MBP kinase activity was normalized to the level of ERK2-tag pro-

teins assessed by 9E10 Western blot analyses. The data show that the expression of v-Ha-ras induced an approximate 10-fold increase in ERK2 kinase activity in T cells. This is somewhat lower than the level of ERK2 kinase activity stimulated by PDBu or UCHT1. However, a direct comparison of the relative effects of v-Ha-ras, PDBu and UCHT1 has to allow for the fact that in the ras experiments ERK2 kinase activity is assessed 24 h after v-Ha-ras transfection and thus the ERK2 kinase data from the v-Ha-ras transfected cells reflect a prolonged ERK2 kinase activation rather than the acute activation seen 5–10 min after cell stimulation via the TCR

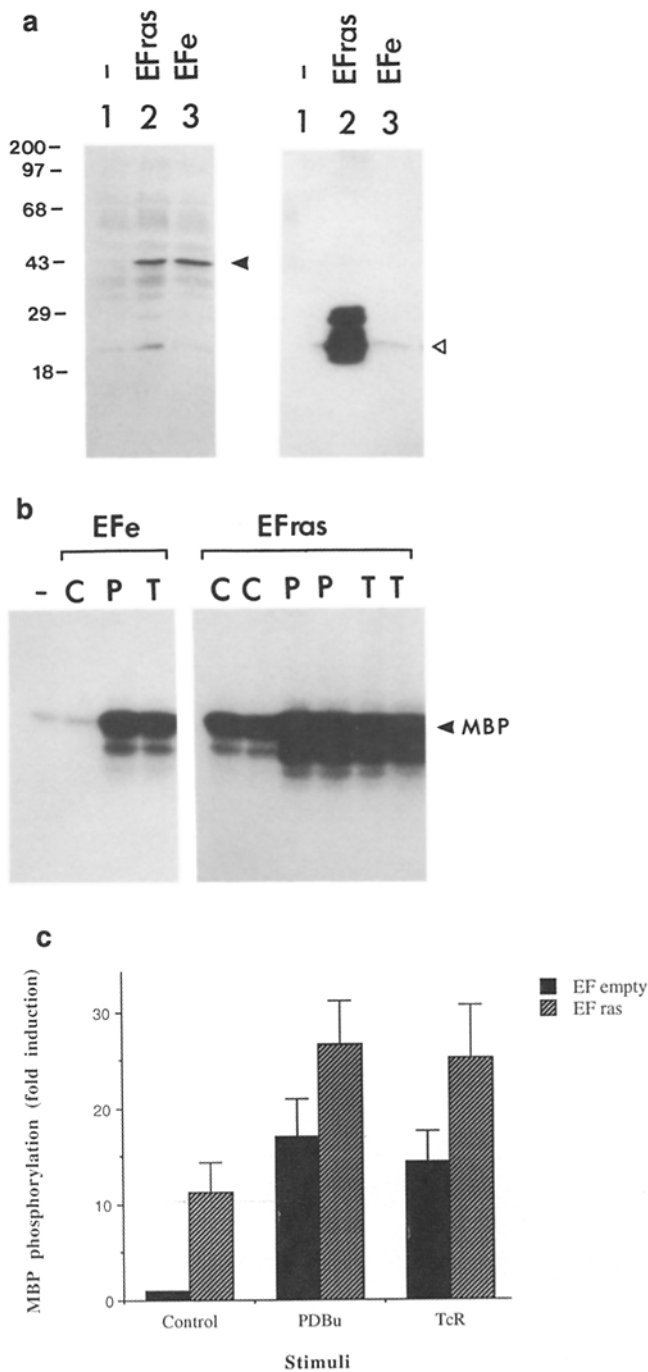


Figure 3. p21^{ras} activates ERK2-tag in Jurkat cells. (a) Jurkat cells were cotransfected with either 40 μ g of pEFempty vector and 30 μ g of pEF-ERK2-tag (lane 3), or 40 μ g of the pEFras vector and 10 μ g of pEF-ERK2-tag (lane 2). The figure shows the Western blots corresponding to the lysates obtained after 24 h of transfection and developed with 9E10 antibody to visualize ERK2-tag (left) and reprobbed with the pan-ras antibody (right). Arrowheads indicate the position corresponding to ERK2tag (\blacktriangleleft) and p21^{ras} (\blacktriangleleft). (b) Jurkat cells cotransfected as indicated above were either unstimulated (C) or stimulated for 10 min with PDBu (P) or TCR agonist (T). 9E10 IPs were carried out in the lysates and ERK2-tag activity was assessed using the MBP kinase assay. The tracks labeled with EFe correspond to cells cotransfected with the pEF empty vector. Tracks labeled with EFrAs are from cells cotransfected with the pEFras vector. The results of a representative experiment are shown. (c) The histogram

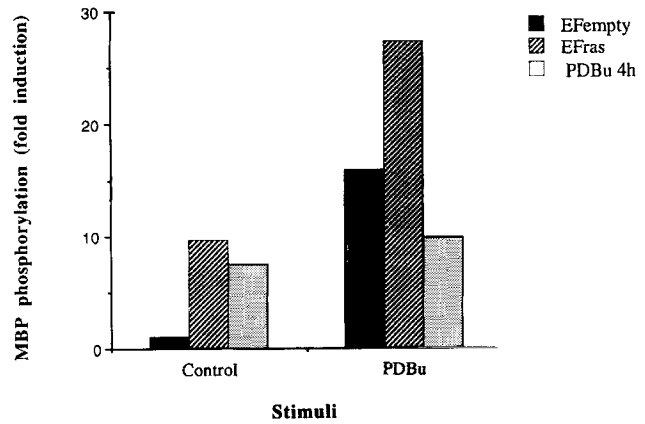


Figure 4. Effect of a sustained stimulation of Jurkat cells with PDBu on ERK2-tag. Jurkat cells were cotransfected with either 30 μ g of the pEF-ERK2-tag vector and 40 μ g of the pEF empty vector (\blacksquare), or 10 μ g of the pEF-ERK2-tag vector and 40 μ g of the pEFras vector (\boxtimes). In parallel, Jurkat cells were transfected with 30 μ g of the pEF-ERK2-tag, and after 20 h of transfection, then stimulated for 4 h with 100 ng/ml PDBu (\blacksquare). All the cultures were harvested after 24 h of the transfection, and subsequently were either unstimulated (control) or stimulated for 10 min with 100 ng/ml of PDBu. MBP kinase assays on the 9E10 IPs from the lysates were set up and data show MBP kinase activity on the IPs. All kinase data was normalized to the level of ERK2-tag expressed in the different populations. This was assessed by Western blot analysis with the 9E10 antibody.

or PKC. The data in Fig. 4 show that when the level of ERK2-tag kinase activity is assessed in cells expressing p21 v-Ha-ras or stimulated with PDBu for 4 h, then the level of ERK2 kinase activity is comparable and of similar magnitude.

A second explanation for the apparent failure of v-Ha-ras to maximally activate ERK2 kinase could be that the level of v-Ha-ras expression was suboptimal. The data in Fig. 5 address this point and show the regulation of ERK2 kinase activity in cells that express v-Ha-ras at a level of either 2- or 20-fold excess of endogenous p21^{ras} (Fig. 5 a). A 10-fold increase in ERK2 kinase activity occurred in populations of cells expressing 10-fold different levels of v-Ha-ras. Moreover, PKC triggering could induce an additional stimulation of ERK2 kinase activity in cells clearly expressing optimal levels of v-Ha-ras (Fig. 5 b).

Detailed dose-response curves are difficult to do in these transient transfection experiments because reducing the concentration of ras DNA can reduce the transfection efficiency. This makes it possible that subpopulations of cells could express the ERK2 reporter construct but not the v-Ha-ras. This is impossible to control completely in transient cotransfection studies. However, in the experiments in Figs. 3 and 4 the ratio of v-Ha-ras to ERK2-tag DNA was at least 4:1 to minimize this possibility.

Expression of p21 N17 ras Prevents Activation of ERK2. To determine whether p21^{ras} function is essential for TCR regulation of ERK2, the consequence of the expression of a dom-

quantitates the ERK-2 activation data obtained in four experiments performed as indicated above. Results are expressed as fold induction in MBP phosphorylation quantitated as indicated in Fig. 2 c.

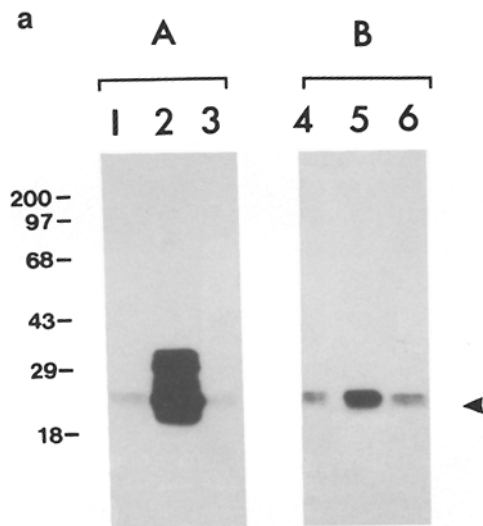
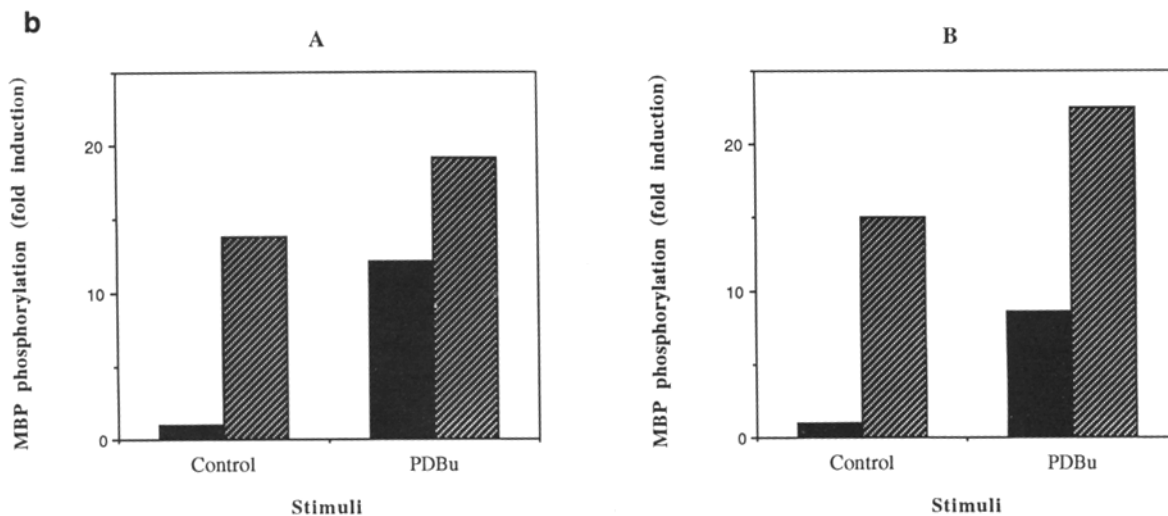


Figure 5. A low level of constitutively active ras activates ERK2-tag in Jurkat cells. (a) Western blot developed with pan-ras antibody of lysates from Jurkat cells that were cotransfected with 10 μ g of the pEF-ERK2-tag vector and either 40 μ g (A, lane 2) or 20 μ g (B, lane 5) of the pEF-ras vector. As a control, cells were cotransfected with 30 μ g of ERK2-tag construct and either 40 μ g (lane 3) or 20 μ g (lane 6) of the EF empty vector. Lysates from untransfected (lanes 1 and 4) are included as a reference for the levels of endogenous ras for each experiment. (b) Jurkat cells cotransfected as indicated above were either unstimulated (*control*) or stimulated for 10 min with 100 ng/ml PDBu. MBP kinase assays in the 9E10 IPs from cells cotransfected with either the pEF empty (■) or the pEFras vector (▨) were carried out, and the figure shows the quantitation of the normalized ERK2-tag activity. The data included in A correspond to the lysates from A in the Western blot (higher level of transfected ras) whereas the data in B corresponds to the lysates with lower levels of transfected ras.



inant inhibitory p21^{ras} mutant p21 N17 ras on the activation of ERK2-tag was assessed. In N17 ras the substitution of serine with asparagine at codon 17 generates a mutant that competes with endogenous p21^{ras} for guanine nucleotide exchange proteins and thus prevents accumulation of endogenous p21^{ras} into the active GTP-bound state (21). Previous studies (4, 5) have established the specificity of this mutant as an inhibitor of the ras pathway in T cells. Experiments examining the consequences of N17 ras expression for ERK2 regulation in Jurkat cells revealed a dose-dependent hierarchy of the sensitivity of ERK2 activation to the effects of the inhibitory ras mutant. To monitor the expression of N17 ras, Western blot analysis using the anti-pan ras antibody was carried out. The ras antibody does not distinguish the N17 mutant from endogenous ras but the relative ratio of N17 protein to endogenous ras can be inferred from the increase in the total levels of ras in the N17 ras transfected cells. In

cells expressing a two- to fourfold excess of N17 ras compared to endogenous ras (Fig. 6 a, panel A) the ability of ERK2 to be activated in response to PDBu was unchanged (Fig. 6 b). However, the responsiveness of the ERK2 kinase pathway to regulation via the TCR was strongly inhibited (Fig. 6 b). The MBP kinase activity present in the ERK2-tag immunoprecipitates was normalized to the level of ERK2-tag protein as determined by Western blot analysis of cell lysates with the 9E10 antibody. Quantitation of the data in Fig. 6 b by Ambis scanning, shows that the ERK2 kinase activity isolated from the TCR stimulated N17 ras transfected cells was 70–80% reduced compared to that found in TCR activated control cells. When N17 ras was expressed at levels equivalent to or lower than endogenous ras, no differences in either TCR or PKC activation of ERK2 were observed (data not shown). In Jurkat cells expressing higher levels of N17 ras, a 10–20-fold excess of N17 ras compared to endoge-

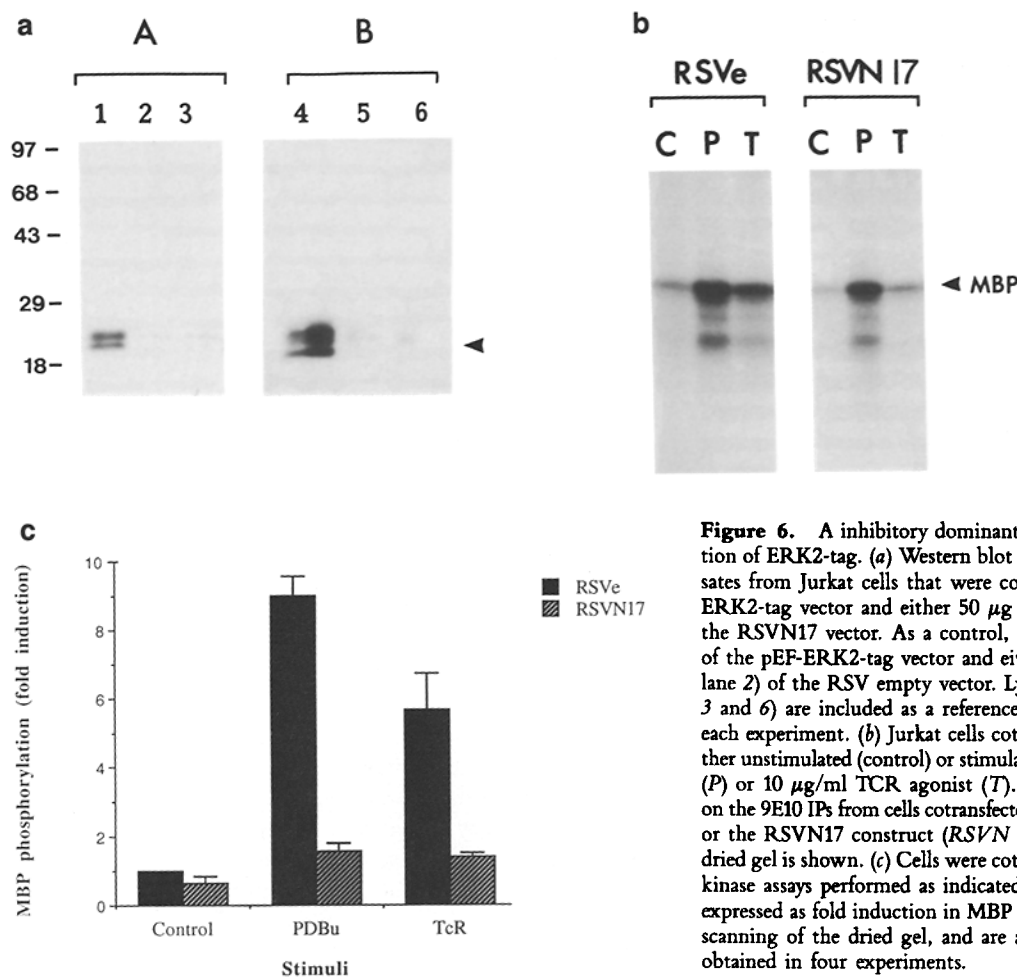


Figure 6. A inhibitory dominant mutant of ras blocks TCR stimulation of ERK2-tag. (a) Western blot developed with a pan ras mAb of lysates from Jurkat cells that were cotransfected with 40 μg of the pEF-ERK2-tag vector and either 50 μg (B, lane 4) or 40 μg (A, lane 1) of the RSVN17 vector. As a control, cells were cotransfected with 30 μg of the pEF-ERK2-tag vector and either 50 μg (B, lane 5) or 40 μg (A, lane 2) of the RSV empty vector. Lysates from untransfected cells (lanes 3 and 6) are included as a reference for the levels of endogenous ras in each experiment. (b) Jurkat cells cotransfected as indicated in A were either unstimulated (control) or stimulated for 10 min with 100 ng/ml PDBu (P) or 10 μg/ml TCR agonist (T). MBP kinase assays were performed on the 9E10 IPs from cells cotransfected with either the RSV empty (RSVe) or the RSVN17 construct (RSVN 17), and the autoradiography of the dried gel is shown. (c) Cells were cotransfected as indicated in B and MBP kinase assays performed as indicated in above. In this case, the data are expressed as fold induction in MBP phosphorylation as quantitated by β scanning of the dried gel, and are average (mean ± SD) of the results obtained in four experiments.

nous ras (Fig. 6 a, panel B) there was a similar 80% inhibition of the TCR response and a comparable suppression of PDBu stimulation of ERK2 (Fig. 6 c).

Discussion

Stimulation of the TCR activates p21^{ras} and initiates a ras controlled signaling cascade that is essential for T cell activation (2–5, 24). The effector pathway downstream of p21^{ras} has not previously been explored in T cells, whereas in other cell lineages, ras proteins have been shown to couple receptor tyrosine kinases to the ERK encoded family of MAP kinases (8–10). In the present study, an experimental system using transient transfection protocols was developed to explore the role of p21^{ras} in the regulation of the MAP kinase ERK2. The kinase activity of a tagged transfected ERK2 was assessed in immunoprecipitates using MBP as a substrate. TCR triggering induced a rapid increase in ERK2 activity, peaking after 10 min with a 10–15-fold elevation of ERK2 activity. PKC stimulation also rapidly activated ERK2, peaking with a 15–20-fold elevation of kinase activity. Increases in cytosolic calcium regulate vital signaling pathways in T cells (25). However, in T cells treated with the calcium ionophore

ionomycin, no ERK2 activation occurred indicating that calcium-sensitive signals do not regulate ERK2 kinase.

One immediate consequence of TCR and PKC stimulation of T cells is the regulation of the p21^{ras} guanine nucleotide binding cycle, resulting in increased levels of ras-GTP complexes (2). Mutationally activated p21 v-Ha-ras exists predominantly in the GTP-bound form in vivo (26). The activity of ERK2 isolated from Jurkat cells transfected with v-Ha-ras was elevated 10-fold compared to ERK2 isolated from control nonstimulated cells. The maximum ERK2 activity found in v-Ha-ras transfected Jurkat was a little lower than the peak of TCR response and 50% of the peak corresponding to the PKC response. However, the peak of TCR and PKC responses was maintained only transiently and a more appropriate gauge of the relative ability of v-Ha-ras or TCR and PKC activators to stimulate ERK2 is achieved by looking at ERK2 in cells activated over a prolonged period. These data (Fig. 4) show that the ERK2 activity present in Jurkat cells activated via PKC over a period of several hours was comparable to the ERK2 activity isolated from the v-Ha-ras expressing cells. This implies that the relative potency of v-Ha-ras with regard to MAP kinase regulation is similar to PKC and TCR activators.

The pathway that couples the TCR to ERK2 is not apparent from these initial experiments with the activated ras. We have shown previously that the TCR is able to stimulate endogenous p21^{ras} and PKC in apparently independent pathways (3). There is, accordingly, the potential for the TCR to use either a PKC pathway to activate ERK2 or a ras controlled pathway. One strategy to explore the contribution of ras to TCR/ERK2 coupling is to examine the effect of a dominant inhibitory ras mutant N17 *ras* on TCR activation of ERK2.

GTP levels on ras are controlled by a balance of the activities of guanine nucleotide exchange proteins and ras GTPase activating proteins (27). The dominant inhibitory ras mutant N17 competes with endogenous ras for guanine nucleotide exchange proteins and this competitive interaction prevents ras activation (21). The data presented here show that removal of endogenous p21^{ras} function by expression of N17 *ras* prevents TCR-mediated activation of ERK2. This result indicates that the TCR is coupled to ERK2 via a p21^{ras} dependent pathway. Previous studies have suggested that a tyrosine kinase p56^{lck} may have a role in TCR regulation of the MAP kinases (13). We have shown that the TCR activates the ras proteins via a protein tyrosine kinase inhibitor sensitive pathway (3). Thus tyrosine kinases such as p56^{lck} may have an indirect regulatory effect on the MAP kinase via involvement in the mechanisms that couple the TCR to p21^{ras} (13).

Recent studies (11) have implicated PKC as a MAP kinase regulator in T cells. However, PKC does not mediate TCR activation of ras (3) and thus is unlikely to have a role in the ras pathway that couples the TCR to ERK2. The positioning of ras in the PKC signaling pathways is dependent on cell lineage (28, 29). For example, in fibroblasts and COS-1 cells, PKC-induced ERK2 activation is independent of p21^{ras} and PKC contributes to a parallel pathway for ERKs regulation (10, 19). In T cells in which the stimulation of ERK2 by p21^{ras} is maximal (Fig. 5), a further induction of ERK2 kinase activity could be achieved by PKC or TCR triggering (Fig. 3). Thus PKC and the TCR appear to contribute a signal additive with the p21^{ras}-mediated signal for ERK2 activation. Our previous studies have indicated that p21^{ras} may have a role downstream of PKC in T cells. This hypothesis was suggested by the stimulatory effect of PKC on the p21^{ras} guanine nucleotide binding cycle (2). In Jurkat cells, the expression of the inhibitory ras mutant, N17 *ras*, not only prevented TCR activation of ERK2 but could prevent the PKC-induced ERK2 response. However, there was a marked difference in the susceptibility of the TCR and PKC/ERK2 response for N17 inhibition (Fig. 6). Since PKC can induce a more potent stimulation of p21^{ras} than the TCR (Fig. 1), it is not unexpected that the PKC response is more resistant to inhibition. Nonetheless, the difference in sensitivity to N17 *ras* could reflect that in T cells, as in fibroblasts, PKC activates ERK2 via a ras-independent pathway.

In fibroblasts, the N17 *ras* mutant inhibits ras function when expressed at a level at least 20-fold lower than wild-type ras (21). However, from the present data it appears that in T cells, at least a two- to fourfold excess of N17 *ras* compared to endogenous ras is required to block TCR activation of ERK2. This discrepancy probably reflects the fundamental differences in ras guanine nucleotide mechanisms that apparently exist between T cells and other cell types. In fibroblasts, for example guanine nucleotide exchange on ras is regulated by triggering receptors such as the epidermal growth factor receptor (EGFR) (30). The ras exchange protein in fibroblasts is the mammalian homologue of the *Drosophila* SOS gene (31). EGF recruits SOS to the cell membrane by inducing association of SOS with the EGF receptor cytoplasmic domain. This EGFR/SOS association is mediated via the adaptor protein Grb2/Sem5 (32). In T cells there is no comparable tight coupling of receptor stimulation to the regulation of ras guanine nucleotide exchange. In T cells, guanine nucleotide exchange on p21^{ras} is constitutively high (2) and the increases in ras-GTP levels that occur in TCR- or PKC-activated T cells can be attributed to inactivation of ras GAP proteins (2, 33). The molecular basis for the constitutively high nucleotide exchange on ras in T cells is not clear. T cells may use a ras exchange protein distinct to the SOS exchange protein with different characteristics for regulation. Alternatively, levels of exchange proteins and hence basal exchange activity may be higher in T cells than fibroblasts. Differences in the level of ras exchange proteins or their regulation would explain differences in the sensitivity of endogenous ras function to the N17 *ras* mutant.

Ras does not directly stimulate MAP kinases but regulates a signaling cascade which has been well characterized in some cell lineages (34). Directly upstream of MAP kinases is an activator kinase, MAP kinase kinase (MAP KK). The link between p21^{ras} and the MAP KK is provided by at least one other kinase, MAP kinase kinase kinase (MAPKKK). Two candidates for the MAPKKK have been described, the protooncogene Raf-1 (19) and a kinase termed MEK1 (35). Raf-1 can be regulated via at least two pathways: one mediated by p21^{ras} and one controlled by PKC (36, 37). Raf-1 is activated in T cells by both TCR and PKC triggering (38, 39) and may provide the link between ras or PKC and ERK2. Accordingly, the positioning of p21^{ras} in the mechanisms that couple the TCR to ERK2 provides a framework for positioning other potentially important signaling molecules in the TCR signal transduction pathways. Further studies are required to establish both the nature and the contribution of the different components of the ERK activation cascade in T cells. Since it has been shown that ERKs can phosphorylate and regulate the activity of transcriptional factors such as *c-jun*, *c-myc*, and Elk1 (40–42), the ras-regulated ERK-mediated cascade could act to couple the TCR to the regulation of gene transcription in the nucleus.

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