

# p21<sup>ras</sup> and Calcineurin Synergize to Regulate the Nuclear Factor of Activated T Cells

By M. Woodrow, Neil A. Clipstone,\* and D. Cantrell

From the Lymphocyte Activation Laboratory, Imperial Cancer Research Fund, London, WC2A 3PX, England and the \*Beckman Center for Molecular and Genetic Medicine, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, California 94305

## Summary

In T lymphocytes, triggering of the T cell receptor (TCR) induces several signaling cascades which ultimately synergize to induce the activity of the nuclear factor of activated T cells (NFAT), a DNA binding complex critical to the inducibility and T cell specificity of the T cell growth factor interleukin 2. One immediate consequence of T cell activation via the TCR is an increase in cytosolic calcium. Calcium signals are important for NFAT induction, and recent studies have identified calcineurin, a calcium-calmodulin dependent serine-threonine phosphatase, as a prominent component of the calcium signaling pathway in T cells. A second important molecule in TCR signal transduction is the guanine nucleotide binding protein, p21<sup>ras</sup>, which is coupled to the TCR by a protein tyrosine kinase dependent mechanism. The experiments presented here show that expression by transfection of mutationally activated calcineurin or activated p21<sup>ras</sup> alone is insufficient for NFAT transactivation. However, coexpression of the activated calcineurin with activated p21<sup>ras</sup> could mimic TCR signals in NFAT induction. These data identify calcineurin and p21<sup>ras</sup> as cooperative partners in T cell activation.

T lymphocyte growth is controlled by the cytokine interleukin 2 (IL-2). Quiescent T cells do not express the IL-2 gene (1). After T cell activation via the T cell receptor for antigen (TCR), there is transcriptional induction of IL-2 gene expression (2). The target for TCR-derived signals in the IL-2 gene is a T cell-specific transcriptional enhancer located in a region 275 bp 5' of the gene (3). The cooperative activity of at least eight proteins that interact with this enhancer appears to control IL-2 gene transcription (4). Several of these proteins, such as AP-1, NFκB, and Oct-1 are ubiquitous and regulate gene transcription in many cells. However, a protein complex termed the nuclear factor of activated T cells (NFAT)<sup>1</sup> has been identified that seems to determine both the T cell specificity and inducibility of IL-2 gene regulation (5). In particular, NFAT is a specific target for the TCR-derived signals that control IL-2 gene expression. NFAT is thought to be composed of two proteins: one is T cell-specific, cytoplasmic, and translocates to the nucleus upon T cell activation; the other is apparently a member of the AP1 family of transcriptional factors (i.e., Fos/Jun), predominantly nuclear, and present only in activated cells (6–10).

The expression of a functional NFAT complex in T cells

is controlled by synergistic signaling pathways initiated by the TCR (11). The immediate signaling consequences of TCR triggering include activation of protein tyrosine kinases (PTKs), which facilitates the rapid elevation of intracellular calcium concentration and activation of protein kinase C (PKC) (12). Calcium and PKC are important signals in T cell activation since pharmacological agents such as calcium ionophores and phorbol esters, that elevate intracellular calcium and activate PKC, respectively, can bypass the requirements for TCR triggering and synergize to induce a functional NFAT complex (13, 14).

A third signaling pathway that involves the guanine nucleotide binding proteins p21<sup>ras</sup> also originates from the TCR (15–18). The Ras proteins rapidly accumulate in an activated GTP-bound state upon TCR triggering and also in response to stimulation of PKC. However, although the TCR induces PKC activation, PKC does not appear to mediate TCR regulation of p21<sup>ras</sup> (19). Instead, the TCR couples to p21<sup>ras</sup> via a PKC-independent mechanism requiring the integrity of TCR-regulated PTKs.

In recent studies, it has been shown that Ras function is essential for TCR signal transduction as judged by the demonstration that expression of a dominant inhibitory mutant of p21<sup>ras</sup>, N17 ras, prevents TCR induction of NFAT and the IL-2 gene (20, 21). Moreover, expression of a constitutively activated Ras protein, p21<sup>v-Ha-ras</sup>, has a dominant positive effect on NFAT induction and is able to generate a signal

<sup>1</sup> Abbreviations used in this paper: CAT, chloramphenicol acetyl transferase; CNA, wild type calcineurin A subunit; CNB, wild type calcineurin B subunit; CNM, calcineurin A mutant; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; PTK, protein tyrosine kinase.

that synergizes with a calcium signal to induce a level of NFAT that mimics the TCR response. The calcium-regulated signals that cooperate with p21<sup>ras</sup> to induce NFAT have not yet been identified. However, a component of the calcium signal that is essential for TCR induction of NFAT has recently been identified. The calcium-calmodulin dependent phosphatase calcineurin (22–25), which is the major cellular target for the immunosuppressive drugs cyclosporin A and FK506 *in vivo*, is activated in response to TCR triggering or stimulation of cells with calcium ionophore (26). The expression of a truncated constitutively active Ca<sup>2+</sup>-independent catalytic subunit of calcineurin generates a signal that can substitute for calcium signals and synergize with phorbol esters to induce the IL-2 gene (22). Overexpression of wild type calcineurin can also augment TCR-induced NFAT-dependent transcription (23).

The potential for p21<sup>ras</sup> to cooperate with calcineurin has not yet been explored, but activation of ras is a good candidate for the physiological partner for calcineurin in NFAT induction for at least two reasons. First, p21<sup>ras</sup>, like calcineurin, has an essential function in TCR regulation of NFAT (21). Second, p21<sup>ras</sup> can replace some requirements for phorbol ester activation of PKC in NFAT induction. Accordingly, the object of the present study was to examine the potential for p21<sup>ras</sup> and calcineurin to synergize during T cell activation and substitute for the TCR in the induction of NFAT. The present data show that in two T cell lines, the human leukemia Jurkat and the murine thymoma EL4, expression of either constitutively active p21<sup>ras</sup> or a constitutively active calcineurin is not alone sufficient for NFAT transcription. However, in combination, activated p21<sup>ras</sup> and calcineurin can synergize to give induction of NFAT that mimics the response induced by TCR. These data further define TCR signal transduction as involving cooperative interactions between signals by calcineurin and p21<sup>ras</sup> controlled signaling pathways.

## Materials and Methods

**Reagents.** Ionomycin (calcium salt) and phorbol-12, 13-dibutyrate were from Calbiochem Corp. (UK). UCHT1 (reactive for human CD3) was used at 10  $\mu$ g/ml in culture (27). [<sup>14</sup>C]-Acetyl coenzyme A (at 50–60 mCi/mmol) was from Amersham International (Buckinghamshire, UK).

**Cells and Transfections.** The human T leukemia, Jurkat, and the murine cells EL4 were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS. Growth was at 37°C and humidified in 5% CO<sub>2</sub>/95% air. Cells were transfected via electroporation (Gene Pulser; Bio-Rad Laboratories, UK), according to the manufacturer's instructions. Briefly, Jurkat cells were pulsed (2 × 10<sup>7</sup>/ml) in media + serum, at 960  $\mu$ F and 310 V. EL4 cells (supplemented with 10  $\mu$ M  $\beta$ -me for growth), at 10<sup>8</sup>/ml, were pulsed at 250 V and 960  $\mu$ F. Cultures were transfected and immediately stimulated, as necessary, for 24 h.

**Plasmids.** IL-2 chloramphenicol acetyl transferase (CAT) (11), and NFAT CAT (5) have been previously described. IL-2 CAT contains regulatory sequences upstream of the IL-2 gene (from –326 to +47), that include the IL-2 minimal promoter and enhancer, directing transcription of the reporter gene. NFAT CAT contains three copies of the sequence 5' GGAGGAAAACACTGTTT-

CATACAGAAGGCGT 3' (corresponding to the sequence from position –284 to –258 relative to the start of transcription of the IL-2 gene) upstream of the IL-2 minimal promoter driving the reporter gene CAT. This sequence has been identified as the binding site for NFAT, the ARRE-2 site of the human IL-2 enhancer (3). IL-2 mpCAT is a deletion mutant of NFAT CAT in which the binding sites for NFAT have been deleted, leaving the IL-2 minimal promoter to drive CAT activity. The sequence of the IL-2 minimal promoter was confirmed by ds sequencing (Sequenase, version 2.0; United States Biochem. Corp., Cleveland, OH). The construct used for the expression of activated Ras was created by inserting the 0.7-kb BamHI fragment of CMVras (21) into the BamHI site of pEF BOS (28). Orientation was determined by restriction analysis and activity was assessed in a focus forming assay. CNA and CNB have been described (22), and are expressed under the control of the SR $\alpha$  promoter of HTLV-1 (23). CNM is a truncation of CNA $\alpha$ 4, in which a stop codon has been introduced at aa397, resulting in a constitutively active, Ca<sup>2+</sup>-independent phosphatase (29).

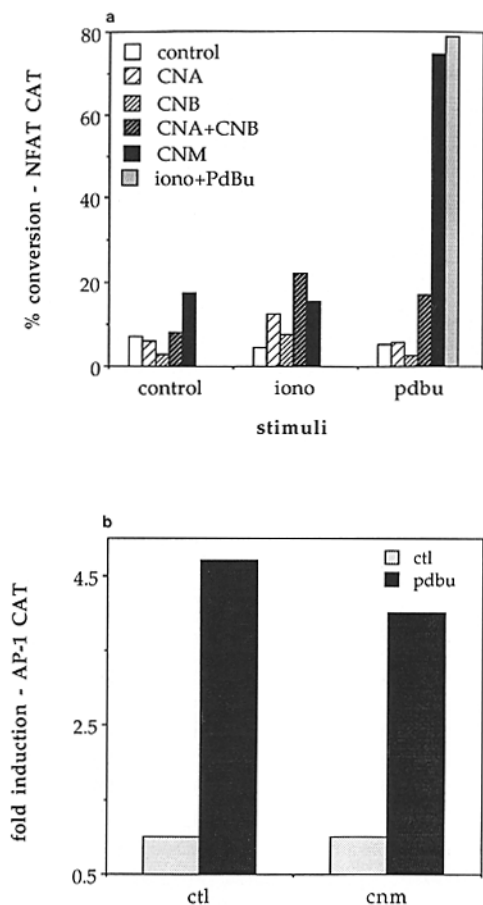
**CAT Assays.** Assays were performed according to the method of Sleigh et al. (30), with modifications. Briefly, cells were lysed in 100  $\mu$ l of 0.65% NP-40, 10 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl, for 10 min on ice. Cellular debris was pelleted, and the lysate heat was treated at 68°C for 10 min before use. Assay conditions were 150 mM Tris, pH 8.0, 0.05  $\mu$ Ci [<sup>14</sup>C]acetyl coenzyme A, and 2 mM chloramphenicol. Chloramphenicol was extracted with ethyl acetate, and the amount of radioactivity in the acetylated products and nonacetylated substrate of each reaction was determined by liquid scintillation counting of organic and aqueous phases, respectively.

## Results

**A Constitutively Active Mutant of Calcineurin Synergizes with Phorbol Esters to Regulate NFAT.** To monitor NFAT activity, we employed transient transfection and quantitation of the expression of a CAT reporter gene whose activity is regulated by a trimer of the NFAT binding site, located upstream of the IL-2 minimal promoter. The signaling requirements for expression of this transfected NFAT reporter gene in Jurkat cells have been described (3, 6, 13). Ionomycin, which elevates intracellular calcium, and Pdbu, which activates PKC, synergize for maximal NFAT induction (Fig. 1 a).

To examine the effect of calcineurin on NFAT, Jurkat cells were cotransfected with NFAT CAT plus one or a combination of calcineurin expression constructs: CNA $\alpha$  4, the wild type catalytic subunit of calcineurin (CNA); a wild type calcineurin B subunit (CNB), or the constitutively active calcineurin A mutant (CNM). The latter is a deletion mutant of the calcineurin catalytic subunit, calcineurin A $\alpha$ 4, that lacks functional calmodulin binding and autoinhibitory domains, and has Ca<sup>2+</sup>-independent constitutive phosphatase activity *in vitro* (29). The data show that expression of wild type CNA and CNB either alone or in combination does not induce a marked activation of NFAT CAT. As described previously, ionomycin alone has a slight stimulatory effect that is enhanced in cells transfected with the combination of CNA and CNB (23).

PdBU alone has no apparent stimulatory effect, but the most striking observation was the strong synergy between the CNM and Pdbu that resulted in a level of NFAT induction com-

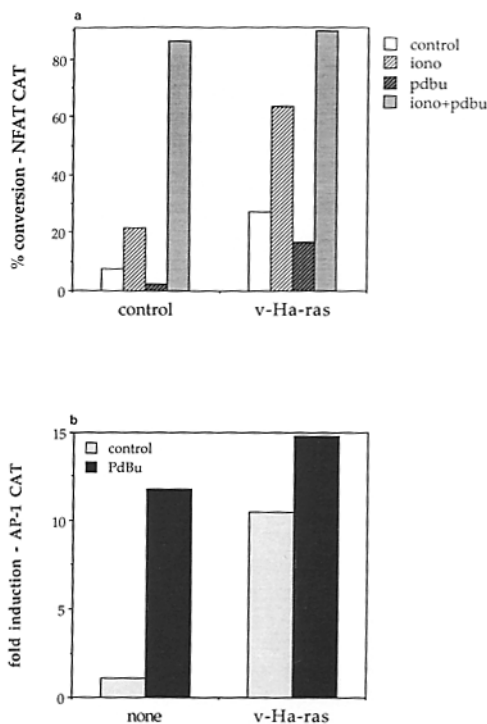


**Figure 1.** Constitutively activated calcineurin synergizes with phorbol ester to regulate NFAT transcriptional activity. (a) Jurkat cells were transfected with NFAT CAT and calcineurin expression vectors either alone, or in combination as described in Materials and Methods. Ionomycin was used at 0.5  $\mu\text{g}/\text{ml}$  and Pdbu at 50 ng/ml, as indicated. Here, 10  $\mu\text{g}$  of reporter construct was used in concert with 10  $\mu\text{g}$  of each calcineurin construct. Cells were electroporated and stimulated immediately after transfection for 24 h before cytosolic extracts were made and reporter gene activity was assessed. Percent conversion represents the ratio of radioactivity extracted in the organic phase vs. total radioactivity in organic plus aqueous phases. (□) control - NFAT CAT alone; (▨) CNA - NFAT CAT + 10  $\mu\text{g}$  wt CNA; (▩) CNB - NFAT CAT + 10  $\mu\text{g}$  wt CNB; (▧) CNA + CNB - NFAT CAT + 10  $\mu\text{g}$  wt CNA + 10  $\mu\text{g}$  wt CNB; (■) CNM - NFAT CAT + 10  $\mu\text{g}$  CNM. The data shown are representative of more than five experiments. Percent conversion in control unstimulated samples ranged from 2.4 to 7.7%. (b) Jurkat cells were transfected with 2  $\mu\text{g}$  of AP-1 CAT and 20  $\mu\text{g}$  of CNM or not (control). (□) = control unstimulated cells; (■) = Pdbu 50 ng/ml. Fold induction is the ratio of activity (% conversion) in stimulated cells vs. control unstimulated cell (no added CNM). The data shown are representative of three experiments. Percent conversion in control unstimulated cells ranged from 1.0 to 5.9%.

parable to that observed in response to ionomycin plus Pdbu (Fig. 1 a). These data indicate that the CNM but not wild type CNA or CNB can substitute for ionomycin-induced calcium signals in the regulation of NFAT. The effect of the CNM on NFAT activity was selective as judged by two independent observations. First, the CNM did not enhance expression of a CAT reporter gene driven by the IL-2 minimal promoter alone (data not shown). Second, the CNM had no

effect on the PKC inducibility of an AP-1 reporter construct (Fig. 1 b).

**v-Ha-ras Synergizes with Calcium Ionophore to Regulate NFAT CAT in Jurkat Cells.** p21<sup>v-Ha-ras</sup> is mutated at codon 12 and 59 (31). These mutations render the Ras proteins insensitive to the Ras-GTPase activating proteins such that the Ras accumulates in cells in an “active” GTP-bound state (32). When expressed in the murine thymoma EL4, signals generated by p21<sup>v-Ha-ras</sup> synergized with either the calcium ionophore ionomycin or the PKC activator Pdbu to induce NFAT (21). The Jurkat cells used in the present study differ from EL4 cells in their activation response to Pdbu (33). Hence in EL4 cells, PKC activation alone is sufficient for NFAT activity, whereas in Jurkat cells there is an apparent obligatory requirement for calcium signals for NFAT induction (11). The data in Fig. 2 a show the characteristics of NFAT regulation in Jurkat cells cotransfected with NFAT CAT and p21<sup>v-Ha-ras</sup>. The mutationally activated Ras alone had a stimulatory effect on NFAT that was not further enhanced by Pdbu treatment of the cells. However, p21<sup>v-Ha-ras</sup> generated a strong synergy with ionomycin to induce levels of NFAT comparable to that seen in ionomycin and Pdbu-treated cells. The fact that p21<sup>ras</sup> alone only elicits a weak stimulation of NFAT CAT

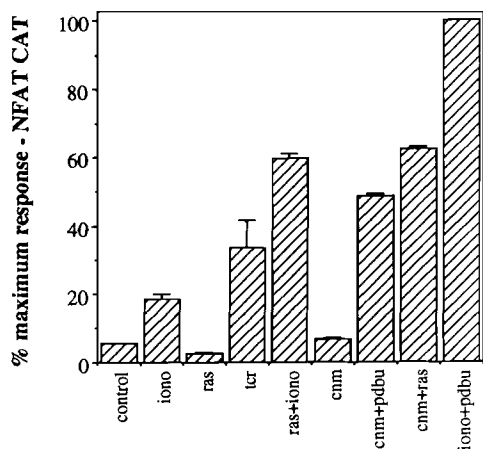


**Figure 2.** v-Ha-ras regulates NFAT CAT and AP-1 CAT in Jurkat cells. (a) Jurkat cells were transfected with 10  $\mu\text{g}$  NFAT CAT, and 40  $\mu\text{g}$  of EF empty vector (control) or EF v-Ha-ras (v-Ha-ras). Stimulation was as in Fig. 1: (□) unstimulated, (▨) iono, (▩) Pdbu, (▧) iono + Pdbu. Activity was as in Fig. 1. The data shown are representative of three experiments. Percent conversion in control unstimulated samples as per Fig. 1 a. (b) Jurkat cells were transfected with 2  $\mu\text{g}$  of AP-1 CAT and 20  $\mu\text{g}$  of EF empty (control) or 20  $\mu\text{g}$  EF v-Ha-ras (v-Ha-ras). Stimulation, (□) control, unstimulated or (■) Pdbu treated, and activity are as described in Fig. 1. The data shown are representative of four experiments. Percent conversion in control unstimulated samples ranged from 1.2 to 5.9%.

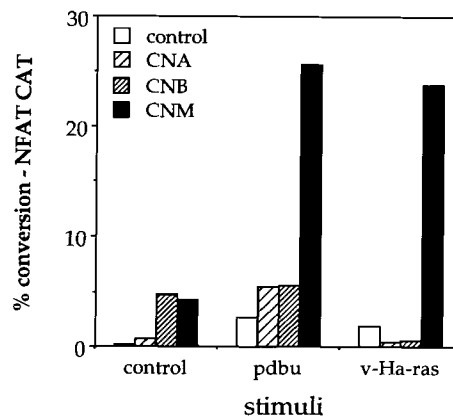
and also that Ras does not synergize with PdBu was not due to insufficient expression of Ras. Thus, the level of p21<sup>v-Ha-ras</sup> expressed in these experiments was alone sufficient to stimulate maximal levels of AP-1 activity, i.e., comparable to the level of AP-1 activity induced in response to PKC stimulation with phorbol ester (Fig. 2 b).

**Calcineurin and p21<sup>ras</sup> Synergize to Regulate NFAT.** p21<sup>v-Ha-ras</sup> and CNM can both generate signals that regulate NFAT, but these signals appear to differ. CNM synergizes with PKC, but not calcium controlled signals to regulate NFAT, whereas p21<sup>v-Ha-ras</sup> has the converse ability to synergize with calcium, but not PKC-controlled pathways. To examine the potential for p21<sup>ras</sup> and calcineurin to synergize in NFAT induction, Jurkat cells were cotransfected with NFAT CAT and p21<sup>v-Ha-ras</sup> and CNM, either alone or in combination. Data averaged from four experiments (Fig. 3) show that p21<sup>v-Ha-ras</sup> in combination with CNM induced a strong expression of NFAT comparable to that seen in response to either p21<sup>v-Ha-ras</sup> plus ionomycin or CNM plus Pdbu. Both p21<sup>ras</sup> and calcineurin are activated in response to triggering of the TCR (15, 24, 25). The data show that p21<sup>v-Ha-ras</sup> plus CNM also induced NFAT CAT to a level greater than that induced in response to TCR triggering with the CD3 antibody UCHT1 (Fig. 3).

The regulatory effect of p21<sup>v-Ha-ras</sup> on NFAT is not confined to Jurkat cells but also occurs in a murine T cell EL4. The effect of calcineurin mutants on NFAT expression in cells other than Jurkat has not been explored in previous studies. The data in Fig. 4 show that in EL4 cells expression of CNM but not wild type CNA or CNB can synergize with Pdbu or p21<sup>v-Ha-ras</sup> to induce NFAT. Expression of CNM alone induced only a weak NFAT response, but in combination with Pdbu or with the constitutively active Ras, the



**Figure 3.** Calcineurin synergizes with v-Ha-ras to regulate NFAT CAT in Jurkat cells. Jurkat cells were transfected with 10  $\mu$ g NFAT CAT plus 10  $\mu$ g of v-Ha-ras or CNM or both, as indicated. Stimuli are as indicated in Fig. 1; in addition, UCHT1 (10  $\mu$ g/ml in culture) was added as indicated. Percent maximum response represents the ratio of activity seen in samples vs. that in iono + Pdbu-treated cultures without added v-Ha-ras or CNM (mean iono + pdbu = 84.5  $\pm$  0.6%). Data are the mean from four experiments, error bars represent SEM. Percent conversion in control unstimulated samples as per Fig. 1 a.



**Figure 4.** Calcineurin synergizes with v-Ha-ras to regulate NFAT CAT in EL4 cells. EL4 cells were transfected with 20  $\mu$ g of NFAT CAT and 50  $\mu$ g of either (□) EF empty, (▨) CNA, (▩) CNB, or (■) CNM, as indicated. Stimuli were applied and data are represented as in Fig. 1. The figure is representative of two experiments.

active calcineurin mutant synergized to induce a level of NFAT activity similar to that induced in response to cell activation by ionomycin in combination with Pdbu (Fig. 4).

## Discussion

In T lymphocytes, p21<sup>ras</sup> is stimulated via the TCR in a mechanism involving TCR inhibition of ras GTPase-activating proteins (ras-GAPs) (15, 18). One important role for the TCR is to control the expression and function of transcription factors such as NFAT, that contribute to the control of IL-2 gene transcription (6). Expression of a mutated constitutively activated p21<sup>ras</sup> induces a response in T cells that can synergize with a calcium signal to modulate T cell activation as judged by NFAT induction (21, 34). Similarly, overexpression of two src-family kinases, p56<sup>lck</sup> and p59<sup>lyn</sup>, appear to be able to partially replace the calcium requirement for induction of NFAT-directed transcription (35, 36). The components of the calcium signaling pathway able to cooperate with p21<sup>ras</sup> in T cells to regulate NFAT have not been previously determined. However, recent studies have established that calcineurin, which is regulated by increases in cytosolic calcium, is essential for TCR signaling function (22, 23, 37). The data presented here show that expression of a truncated, constitutively active calcineurin in both a human leukemic T cell line, Jurkat, and in the murine thymoma, EL4, can substitute for calcium signals and, in synergy with an activated p21<sup>ras</sup>, stimulate T cells to induce NFAT-mediated transcription. Previous studies have indicated that calcineurin can synergize with phorbol esters to induce NFAT and hence the IL-2 gene (22, 23). The current study identifies p21<sup>ras</sup> as an alternative partner for calcineurin in T cell activation.

NFAT is known to have a critical function in the regulation of the IL-2 gene (11, 21). However, the exact composition of this transcriptional factor is as yet unresolved. It is proposed that NFAT is a complex of at least two components: a T cell-specific cytosolic component that translocates to the nucleus in response to increases in cytosolic calcium, and a nuclear component, expressed only in activated cells,

that appears to be a member of the AP-1 family of transcriptional factors (6, 7, 9, 10). p21<sup>ras</sup> is well established as an inducer and regulator of AP-1 in fibroblasts (38). The present data confirm that p21<sup>ras</sup> is also able to induce AP-1-mediated transcription in T cells, whereas calcineurin has no apparent regulatory effect. It is most probable, therefore, that in the cooperation between p21<sup>ras</sup> and calcineurin, Ras would contribute to NFAT induction via control of AP-1 proteins, while calcineurin would regulate NFAT by control of the subcellular localization of the calcium-responsive NFAT subunit. This model for the cooperative interaction between calcineurin and Ras in NFAT induction warrants further exploration. One complication lies in the fact that the AP-1 proteins are a large heterogeneous family of transcriptional factors, which come together in various combinations of Fos and Jun homo- and heterodimers to form functional complexes (39). When T cells are activated, they express multiple Fos and Jun proteins (40), and the identity of the particular partners present in the AP-1 complex that contribute to NFAT induction is not yet clear (7, 9, 10).

The downstream effectors of both p21<sup>ras</sup> and calcineurin are not known. One potential Ras regulated pathway that could contribute to NFAT regulation is the MAP kinase signaling cascade (41). p21<sup>ras</sup> can regulate the activity of the MAP kinase Erk2 in Jurkat cells (42). Substrates for Erk2 include transcriptional factors such as the AP1 member *c-jun*, and Elk-1, protein that complexes with serum response factor to transactivate the *c-fos* promoter, whose function is controlled by phosphorylation at target sites for the MAP kinase family (43–45). Thus, the MAP kinases could be integral to the signaling pathways that couple the TCR and p21<sup>ras</sup> to the cell nucleus.

The potential for cooperation between p21<sup>ras</sup> and cal-

calcineurin has not been observed in previous studies of Ras function in other cell types. However, the idea that Ras controlled signaling pathways are not alone sufficient for cell activation is not novel. For example, oncogenic transformation in fibroblasts requires a cooperative interaction between Ras and other oncogenes (46). The demonstration herein that Ras and calcineurin can cooperate to activate T cells raises the possibility that calcineurin could be a protooncogene. Thus, mutations in calcineurin or mutations that perturbed the regulation of calcineurin could be oncogenic if they occurred in cells expressing oncogenic Ras.

Defects in NFAT regulation have profound functional consequences for T cells, as judged by the immunosuppressive effects of the drugs cyclosporin A and FK506 that inhibit the action of calcineurin on NFAT induction (33). The realization that p21<sup>ras</sup> is an important partner with calcineurin in NFAT regulation suggests that Ras could also be an important target for immunosuppression. Alternatively, any defects in Ras regulation would have severe immune consequences. This is supported by a recent study in nonobese diabetic mice, in which mature (CD4<sup>+</sup>8<sup>-</sup>, CD4<sup>-</sup>8<sup>+</sup>) T cells were not only unable to proliferate in response to TCR cross-linking, but could not demonstrate activation of p21<sup>ras</sup> or p42<sup>mapk</sup> after ligation of the TCR (47). Direct stimulation of PKC was able to activate p21<sup>ras</sup>, and also to restore the cells' ability to grow in response to TCR signaling. This suggests that a defect must exist between the TCR and the p21<sup>ras</sup> proteins in these anergic cells, supporting the hypothesis that p21<sup>ras</sup> may play an important role in the generation of an immune response. However, it remains to be seen if Ras defects exist in other anergic T cell models or autoimmune diseases.

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Address correspondence to Dr. Melissa A. Woodrow, Lymphocyte Activation Laboratory, Imperial Cancer Research Fund, Room 613, Lincoln's Inn Fields, London, WC2A 3PX, UK.

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