Activation of CD4 T cells by Raf-independent effectors of Ras

Jan Czyzyk*†, Jennifer L. Brogdon*, Abdallah Badou*‡, Octavian Henegariu*, Paula Preston Hurlburt‡, Richard Flavell*‡, and Kim Bottomly*§

*Section of Immunobiology, †Department of Pathology, and ‡Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06510

Communicated by Vincent T. Marchesi, Yale University School of Medicine, New Haven, CT, March 14, 2003 (received for review December 12, 2002)

Small GTPase Ras is capable of mediating activation in T lymphocytes by using Raf kinase-dependent signaling pathway. Other effectors of Ras exist, however, suggesting that targets of Ras alternative to Raf may also contribute to T cell functions. Here we demonstrate that Ras^{V12G37} mutant that fails to bind Raf, potently increases intracellular calcium concentration and cytokine production in primary antigen-stimulated T cells. From three known effectors which retain the ability to interact with Ras^{V12G37}, overexpression of phospholipase C ε but not that of RIN1 or Ral guanine nucleotide exchange factors enhanced cytokine and nuclear factor-activated T cell reporter T cell responses. Hence T cell activation can be critically regulated by the Ras effector pathway independent from Raf that can be mimicked by phospholipase C ε.

Ras G proteins have been implicated in variety of biological processes including malignant transformation and tumor metastasis in mammals (1, 2), differentiation of photoreceptors in insects (3), and vulval induction in nematodes (4, 5). In immune system, both B cells (6) and T cells are highly dependent on active Ras. In T cells, Ras has been suggested mainly to promote early maturation of T cell in the thymus (7, 8), and positively control transcription of interelukin-2 (IL2) gene in peripheral lymphocytes (9, 10). More recently, constitutively active Ras has been shown to enhance primary T lymphocyte responsiveness to antigen (11). The importance of Ras to T cell functions is further highlighted by demonstrations that Ras activation is inhibited in functionally unresponsive T cells (12, 13). Such strong dependence of T cell functions on Ras can be due to the rapid and prominent activation of this GTPase on T cell receptor (TCR) stimulation (14), and has been attributed to the subsequent activation of a classic Raf/mitogen-activated protein kinase (MAPK) cascade (15).

Although binding between Ras and Raf is relatively well characterized (16, 17), recruitment of a number of other molecules by Ras, such as phosphoinositide 3-kinase (PI3K) (18) and Ral guanine nucleotide exchange factors (GEFs) (19, 20), has also been described. Nevertheless, the relative contributions of Raf-independent Ras effectors to the functioning of primary T lymphocytes remain totally unknown. Our approach to address this problem was to use effector CD4 T cells and express mutated Ras proteins that are selectively impaired for their ability to recruit individual effectors. In addition to the constitutively activating mutation at residue 12 (Ras^{V12}) these mutants carry a second substitution in the effector loop domain (amino acids 32–40 in H-Ras), which is essential for Ras interaction with variety of proteins. For example, Ras^{V12G37} mutant fails to bind Raf but retains its ability to recruit Ral-specific GEFs (Fig. 2*D Lower*). This is in sharp contrast to Ras^{V12S35} and Ras^{V12C40} that bind Raf and PI3K, respectively, but fail to physically interact with Ral exchangers (21). We hoped therefore, that Ras effector loop domain mutants can be used as potent skewing tools of Ras effector signaling in T cells.

Materials and Methods

Expression Plasmids. cDNAs encoding H-Ras, Ras interaction/interference (RIN1), Ral guanine dissociation stimulator (RalGDS), RalGDS-like factor (Rlf)-CAAX, and wild-type phospholipase C ε (PLCε) and its mutants (K1144L and K2150E) were obtained from other laboratories. Rab5A and RalA were generated by PCR amplification from the mouse cDNA library. Ras (G12V, T35S, E37G, and Y40C), Rab5A (Q79L and S34N) and RalA (G23V and S28N) mutants were created by oligonucleotides carrying the desire point mutation and PCR amplification using high-fidelity *Pfu* polymerase. Full-length RIN1 was used to generate its deletion mutants, RIN1 N (amino acids 1–295) and RIN1 C (amino acids 293–783). All signaling molecules were tagged with the flag sequence, and their expression in transduced T cells was confirmed by Western blotting. PCR-amplified constructs were verified by sequencing at Keck Facility at Yale University and subsequently subcloned into pMSCV that contains the cassette consisting of the GFP, and an internal ribosome entry site permitting the translation of two ORFs from one messenger RNA. For calcium measurements, Ras^{V12G37} was subcloned in pMIGR2 retroviral vector in which tailless human CD2 substitutes for GFP. Raf-N4 in pCGN encodes residues 23 to 284 of c-Raf-1. This dominant negative Raf mutant blocks Ras function by antagonizing Ras interaction with Raf and other Ras effectors (22).

Stimulation and Retroviral Transduction of CD4 T Cells. The supernatants obtained from cultures of Phoenix Ecotropic packaging cells transfected with retroviral constructs were used to spin-infect activated CD4 T cells. The supernatants were added to T cells isolated from 6- to 8-week-old AND TCR-transgenic [TCR specific for moth cytochrome *c* peptide (pMCC 81–103), VFAGLKKANERADLIAYLKQATK] B10.BR ($I\text{-}A^k$) mice and grown for 12 h in the presence of peptide (5 μg/ml) and T cell-depleted splenocytes.

Calcium Measurements. AND TCR transgenic CD4 T cells transduced with Ras^{V12G37} in pMIGR2 were stained with CyChrome-labeled anti-human CD2 monoclonal antibody (BD PharMingen). CyChrome positive T cells were sorted, loaded with 5 μM fluo-3/AM ester (Molecular Probes) and plated by centrifugation in 96-well plate at a concentration of 1.5×10^5 per 50 μl. The cells were scanned by using the ACAS 570 video laser cytometer (Meridian Instruments, Lansing, MI). After initiation of scanning, T cell-depleted splenocytes pulsed with pMCC were added to the CD4 T cells. The initial average fluorescence of each cell was digitized and normalized to 1, and the results are expressed as changes in normalized fluorescence intensity of individual cell over time.

Flow Cytometry. The phenotype of CD4 T cells transduced with Ras^{V12G37} or empty vector control was determined by flow cytometry using PE-labeled antibodies against CD44, CD40L, CTLA4, and CD62L (BD PharMingen).

Abbreviations: TCR, T cell receptor; pMCC, moth cytochrome *c* peptide; APC, antigen-presenting cell; GEF, guanine nucleotide exchange factor; MAPK, mitogen-activated protein kinase; RIN1, Ras interaction/interference; PLCε, phospholipase C ε; RalGDS, Ral guanine dissociation stimulator; Rlf, RalGDS-like factor; NFAT, nuclear factor-activated T cell; Th*n*, T helper *n*; PI3K, phosphoinositide 3-kinase.

§To whom correspondence should be addressed. E-mail: kim.bottomly@yale.edu.

IMMUNOLOGY

Intracellular Cytokine Staining. GFP-sorted CD4 T cells were stimulated with the antigen for 12 h and cultured for additional 4 h in the presence of GolgiStop reagent (BD PharMingen) to trap cytokine in the endoplasmic reticulum. Cells were then processed for intracellular staining with phycoerythrin-labeled anti-IFNγ monoclonal antibody.

Proliferation Assay. T cells were stimulated with antigen-presenting cells (APCs) and increasing concentrations of pMCC in round 96-well plates for 48 h, and then pulsed for 16 h with [^{3}H]thymidine and harvested for counting.

RNA Analysis of the Primary Transcripts. Total cellular RNA was isolated by using TRIzol reagent (Life Technologies). Cytokine RNA levels were analyzed by RNase protection assay using the RiboQuant multiprobe kit with the mCK-1 template (BD PharMingen). Five micrograms of total RNA was used in each reaction. To ensure equal loading, transcripts levels of housekeeping gene (L32) were also analyzed. To analyze expression of PLCε, total RNA was reverse transcribed by using the Stratagene ProSTAR First Strand RT-PCR kit. For mouse PLCε, the forward primer was 5′-GCTTTGAAACGAGGATATCGACATCTTCAGCTGC-3′; and the reverse primer was 5′-GGCTTTGCATAAGGTCTGCT-GAATTACATCC-3′. For rat PLCε-flag, the forward primer was 5′-CATTGTCCACGATGACAGAGAGG-3′; and the reverse primer was 5′-TCATTTGTCATCATCGTCCTTGTAGTCC-3′. Gene expression was compared with the expression of GAPDH.

Microarray Analysis of PLCε Expression. Total RNA from primary CD4 T cells and the lung was subjected to reverse transcription with oligo(dT) and random hexamer primers, and aminoallyl-dUTP was incorporated during this reaction. In the next step, T cell and lung cDNA samples were conjugated with reactive dyes, Cy3 (green) or Cy5 (red), respectively. The two dye-labeled samples were mixed together and hybridized onto 70-mer "designer" lympho-array that included four different 70-mers specific for mouse PLCε. The array was printed onto poly(L-lysine) coated slides by using a GeneMachines Omnigrid arrayer in the intradepartmental facility. After overnight hybridization at 60°C in Genemachine hybridization chambers, slides were rinsed, dried, and scanned with an Axon GenePix 4000A scanner. The data were processed by using GENESPRING commercial software.

Transactivation Luciferase Reporter Assays. The AKR thymoma BW5147 reconstituted with the TCR from D10 T helper 2 (Th2) cell clone, wild-type murine CD4 and CD45RO were transfected by the standard DEAE-dextran method with various expression constructs, nuclear factor of activated T cell (NFAT)-luciferase reporter, and pRL-CMV (cytomegalovirus) plasmid containing the *Renilla* luciferase gene. Twenty-four hours after transfection, BW cells were stimulated with mitomycin C-treated B10BR T cell-depleted splenocytes and the CA37 conalbumin peptide (100 μg/ml). Cells were harvested 24 h after stimulation and analyzed for reporter gene expressions by the dual reporter assay system (Promega). In Fig. 2*D Upper*, PathDetect transreporting system (Elk1-GAL4 transactivator and 5xGAL4-luciferase reporter at ratio 1:10 were used as described (23) to confirm function of Ras^{V12S35}.

Pull-Down Assay. Aliquots of T cells transfected with single Ras mutants were lysed and incubated with GST-RBD of RalGDS coupled to the glutathione Sepharose 4B. After incubation the beads were washed, and the pulled down material as well as crude cell lysates were run on SDS/12% PAGE under reducing conditions, electroblotted, and stained with anti-flag antibody.

Western Immunoblotting. Proteins from precleared cell lysates of GFP+ CD4 T cells were fractionated under reducing conditions on a SDS 6–18% gradient polyacrylamide gel. After electrophoresis, proteins were electroblotted onto nitrocellulose membranes (Bio-Rad), probed with anti-flag M5 monoclonal antibody (Sigma) or rabbit serum raised against lck kinase, and developed with the enhanced chemiluminescence detection system (Amersham Pharmacia).

Results

Functional Phenotype of CD4 T Cells Transduced with Ras^{V12G37}. Initially, we wished to determine whether Ras influences generation of effector CD4 T cells. A common approach to analyze such event is to measure expression of the molecules which are expressed at relatively low levels in resting cells and strongly induced on TCR stimulation. Primary AND TCR transgenic CD4 T cells were obtained from B10.BR mice and stimulated with pMCC and T cell depleted splenocytes as APCs. After 12 h of antigen stimulation, the cells were transduced with individual Ras mutants (e.g., Ras^{V12}, Ras^{V12S35}, Ras^{V12G37}, or Ras^{V12C40}) or empty vector control (Fig. 1*A*). At 48 h after primary stimulation, a subpopulation of cells positive for GFP was analyzed by flow cytometry for the cell surface expression of CD40 ligand (CD40L), CD44, CD62L, CD69, and CTLA4 costimulation molecule. These experiments revealed that only Ras^{V12} (all effector functions intact) and Ras^{V12G37} [in addition to Ral GEFs (Fig. 2*D Lower*), also binds RIN1 and PLCε] caused significant changes: the levels of expression of CD40L and CD44 were elevated, and those of CTLA4 and CD62L were diminished in comparison to the cells carrying empty vector alone. However, no major shift in the expression of CD69 was noted, and the control fluorescence of GFP in both cell types was virtually identical (Fig. 1*B*). The above mentioned up-regulation of some early activation markers together with the down-regulation of CTLA4 expression, a well known suppressor of the immune response (24), may at least in part contribute to the elevated cytokine production and enhanced proliferation that are induced by Ras^{V12G37} (see below). On the other hand, a similar level of CD69 expression in Ras^{V12G37}-positive T cells and control cells could be related to the fact that CD69 is induced within the first hours of antigen stimulation (25), whereas the expression of retrovirally expressed Ras protein in T cells occurred at later time point.

To examine further the role of Ras^{V12G37} in T cells, a thymidine incorporation assay was used to compare proliferation between Ras mutant positive and control CD4 T cells. This analysis revealed that small doses of antigen caused proliferation that was several times stronger in T cells transduced with Ras^{V12G37} or Ras^{V12} (Fig. 1*C*) than in CD4 T cells transduced with other Ras variants or empty vector control. Moreover, higher mitotic activity of Ras^{V12G37} T cells correlated with a moderate up-regulation (50–100%) of IL2 production by these cells (Fig. 1*D*). These data, combined with the flow cytometry findings, suggest a model according to which Ras^{V12G37} promotes effector CD4 T cells toward stronger IL2 gene induction which in turn contributes to higher proliferative activity of the transduced lymphocytes.

An important paradigm of the immune response is differentiation of the primary CD4 T cells into T_H1 and T_H2 effector subsets (26). These two distinct cells types produce IFNγ and interleukin IL4, respectively, and play important roles in infectious, autoimmune, and allergic diseases. It has been therefore of central interest to us to investigate whether Ras induced signal transduction events influence cytokine production in CD4 T cells. To address this problem secondary cultures were set up with the sorted GFP-positive TCR transgenic CD4 T cells that were originally primed with the pMCC-pulsed APCs in the presence of cytokines, and retrovirally transduced with individual Ras mutants. Twenty-four hours after secondary stimulation, IFNγ and IL4 were measured by ELISA. The main conclusion from these experiments is that regardless of priming conditions, effector CD4 T cells transduced with Ras^{V12} or Ras^{V12G37} exhibited the strongest cytokine responses on secondary stimulation with the specific antigen (Fig. 2*A*, Table

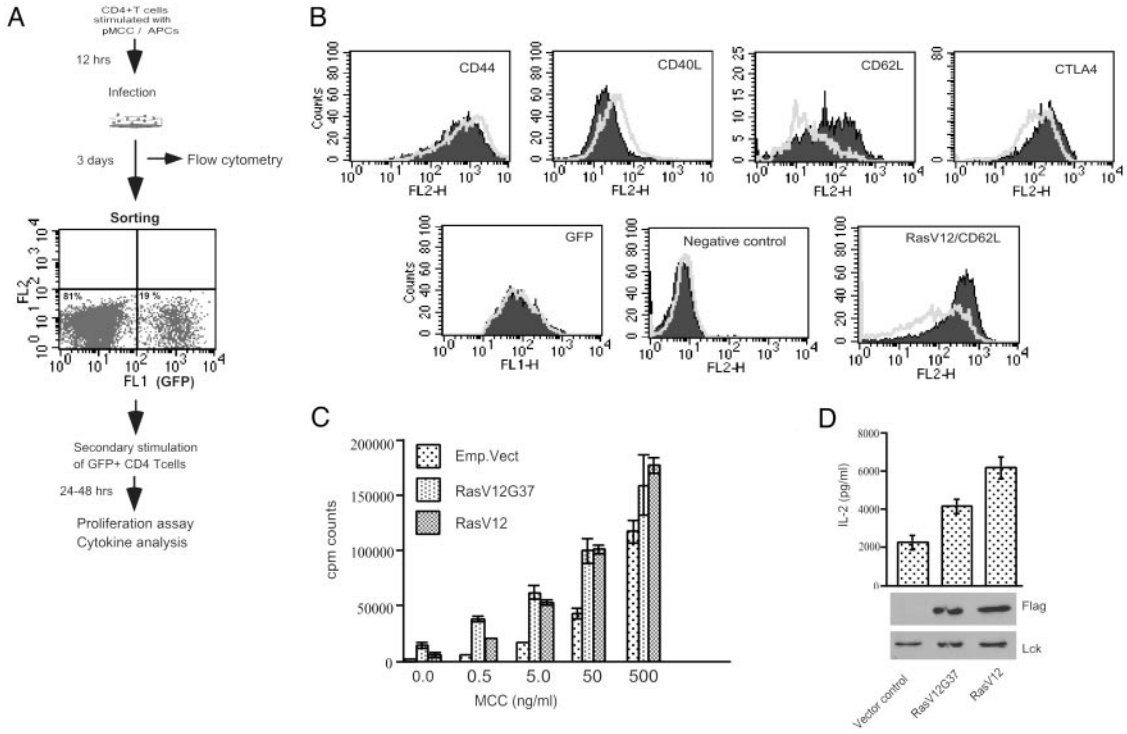

Fig. 1. Ras^{V12G37} augments activation of CD4 T cells. (*A*) Schematic outline of the experimental procedure. (*B*) Phenotype of CD4 T cells transduced with Ras^{V12G37} (solid) or empty vector control (filled) determined by flow cytometry using phycoerythrin-labeled anti-CD44, anti-CD40L, anti-CTLA4, and anti-CD62L monoclonal antibodies at 48 h after stimulation. The analysis was gated on CD4+GFP+ cells. Control staining of Ras^{V12} T cells with CD62L antibody was performed on a separate occasion. (*C*) Proliferation of Ras^{V12}+, Ras^{V12G37}+, and control CD4 T cells stimulated with APCs and increasing concentrations of pMCC. (*D*) IL2 response in Ras^{V12}+, Ras^{V12G37}+, and vector control CD4 T cells. Culture supernatants were obtained 24 h after secondary stimulation and analyzed by ELISA. Western blot staining with anti-flag mAb M5 (*Upper*) and anti-Lck rabbit serum (*Lower*) was performed to test expression of the tagged Ras mutants and confirm similar protein concentration in the cell lysates, respectively.

1). Interestingly, Ras^{V12G37}-induced potentiation of IFNγ and IL4 production was much stronger than that of IL2. Moreover, both T_H1 and T_H2 cytokine responses were enhanced and no conversion of one T_H subset into the other has occurred. These observation suggest that the signaling pathway(s) that is (are) up-regulated by Ras^{V12G37} represent(s) a general mechanism by which Ras controls cytokine productions in T cells. Unexpectedly, Ras^{V12S35} which is known to up-regulate Raf/MAPK cascade (21), and can activate Ras/MAPK-dependent Elk transcription factor in T cells (Fig. 2*D Upper*) (23) induced only weak-to-moderate positive effect on T cell cytokine production. One interpretation of this finding is that TCR stimulation with the high-affinity peptide agonist may saturate regulation of MAPK and additional expression of Ras variant that selectively binds Raf may have relatively limited effect on T cell functions. Finally, PI3K activating Ras^{V12C40} did not influence the cytokine responses. This result is consistent with a previous study arguing that PI3K is an effector of Rac GTPase rather than Ras in T cells. Hence, an overexpression of Ras^{V12C40} may not necessarily mimic physiological conditions that control PI3K function in these cells (27).

High cytokine level in the culture of Ras^{V12G37}-transduced CD4 T cells may be caused by increased *de novo* cytokine protein synthesis, or alternatively, may be secondary to accelerated cytokine secretion. To distinguish between these two possibilities, an *in situ* staining of IFNγ was performed in the secondary cultures of the TCR-stimulated GFP-sorted CD4 cells that had been originally transduced with different Ras protein mutants. Ras^{V12} and Ras^{V12G37} induced dramatically higher accumulation of intracellular IFNγ than did Ras^{V12S35} (Fig. 2*B*). Similarly, ribonuclease protection assays showed much higher accumulation of T_H1 and T_H2 cytokine mRNAs in Ras^{V12G37}-positive than in control T cells (Fig. 2*C*). These data taken together are consistent with the idea that an enhancement of IFNγ and IL4 responses by Ras^{V12G37} in effector CD4 T cells results from augmented protein synthesis rather than accelerated release of these cytokines to the extracellular space.

Phospholipase C: A Putative Effector of Ras^{V12G37} in T Cells. In the next step we wanted to identify effector(s) of Ras which mediate Ras^{V12G37}-induced changes in T cells. This mutant can no longer bind Raf and activate PI3K; however, it still binds RalGEFs, RIN1 (28), and PLCε (29, 30). We therefore tested which of the above three Ras effectors contributes to the enhanced T cell activation.

Ras-dependent recruitment of RalGDS and subsequent stimulation of Ral was proposed to function parallel to the Ras-Raf-MAP kinase pathway (31). Our functional studies involving retroviral delivery of RalGEFs to the 12-h activated CD4 T cells, however, revealed that neither RalGDS nor a constitutively active form of another Ral GEF, termed Rlf, were capable of potentiating the cytokine responses (Fig. 3*A Left* and data not shown). Likewise, overexpression (confirmed by Western blotting) of the wild-type, inhibitory, and constitutively active variants of Ral had no effect on the cytokine level (data not shown). These data suggest that the Ras/RalGEF/Ral signaling cascade is not involved in connecting Ras to increased cytokine production in antigen-activated CD4 T cells.

Second, we analyzed the Rin1 molecule, which contains the GEF factor catalytic domain for Rab proteins, which are involved in the intercellular traffic (32), and proline-rich domain that mediates physical association of Rin1 with the hematopoietic specific kinase cAbl (33). Hence, Rin1 likely links Ras to multiple signal pathways such as Rab5-dependent receptor mediated endocytosis (32) and

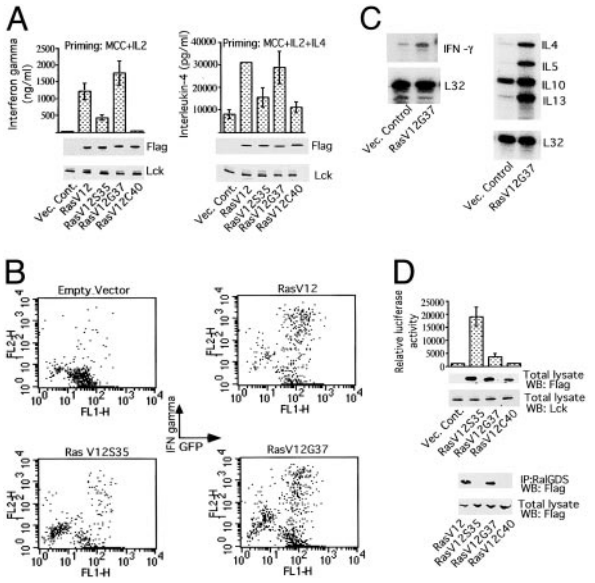

Fig. 2. Ras^{V12G37} strongly up-regulates productions of Th1 and Th2 cytokines. (*A*) IFNγ (*Left*) and IL4 (*Right*) production in Ras-transduced T cells. CD4 T cells were stimulated with the specific antigen in the presence of either IL2 (*Left*) or IL2+IL4 (*Right*), and transduced with individual Ras mutants in order as indicated. CD4 T cells expressing GFP were then sorted and restimulated with antigen for 24 h. After incubation cytokines were measured by ELISA, and cells were harvested to compare expression of the flag-Ras constructs and Lck by Western blotting analysis. (*B*) Intracellular staining of IFNγ in CD4 T cells transduced with single Ras mutants and control vector. At 3.5 days after primary stimulation, GFP-sorted CD4 cells were challenged with antigen for 12 h, cultured for additional 4 h in the presence of GolgiStop, and processed for intracellular staining with anti-IFNγ monoclonal antibody. (*C*) Analysis of the cytokine transcripts expressed in the GFP-sorted Ras^{V12G37}+ and vector control CD4 T cells stimulated with pMCC and APCs for 16 h. *Left* and *Right* correspond to CD4 T cells that were grown under Th1 (IL2) or Th2 (IL2 and IL4) polarizing conditions, respectively. Total RNA was analyzed by a multiprobe RNase protection assay. (*D*) Functional and biochemical characterization of Ras mutants used in this study. T cells transfected with individual flag-Ras constructs, as indicated, were analyzed for Elk reporter gene expression (*Upper*) or subjected to a pull-down assay with RalGDS binding domain as a probe (*Lower*).

potentiation of the oncogenic potential of BCR-ABL (33). To this end we have tested wild type Rin1 and its N- and C-terminal truncated versions lacking proline-rich and nucleotide exchange activity domains, respectively (Fig. 3*A Left*, and data not shown). Moreover, we functionally examined both active and dominant negative variants of Rin1 effector, Rab5a. Altogether, no cytokine elevation was observed in the cultures of CD4 T cells transduced with the above constructs (expression of all cDNAs in CD4 T cells was positively verified by Western blotting, data not shown) suggesting that Rin1/Abl interaction and Ras/Rin/Rab5a pathway are not critical to the up-regulation of CD4 T cell functions by Ras^{V12G37}. These findings are supported by previous demonstrations that Rin1 suppresses rather than potentiates Ras signaling (34).

Finally, we wanted to examine whether the recently identified isoenzyme of PLC, termed PLCε, could mediate the action of Ras^{V12G37} in CD4 T cells. PLCε contains RasGEF and Ras association domains in addition to the conserved catalytic and regulatory domains common to other PLC enzymes, and it has been suggested to function as an effector of Ras. Indeed, Ras increases the activity of PLCε (29) and induces its translocation from the cytosol to the membrane in growth-factor stimulated cells (30). We evaluated the potential role of PLCε in lymphocyte activation by measuring cytokines in the cultures of effector CD4 T cells that were transduced with wild-type PLCε. In sharp contrast to Ral-GEFs and RIN1, transduction of CD4 T cells with PLCε resulted in dramatic increase in IFNγ production (Fig. 3*A Right*). Similarly, PLCε, but not RIN1, Rlf-CAAX, or hydrolytically inactive $PLC\varepsilon^{K1144L}$, potentiated transactivation of the NFAT-luciferase reporter in murine BW T cell line (Fig. 3 *B* and *C*). Importantly, the $PLC\varepsilon^{K2150E}$ mutant that is unable to bind Ras, failed to enhance NFAT reporter transactivation (Fig. 3*C*), and mutant RafN4 which binds to the Ras effector domain, significantly blocked PLCε-mediated induction of NFAT (Fig. 3*D*), confirming that physical interaction between Ras and PLCε is a key regulatory mechanism by which Ras controls involvement of this enzyme in antigen-stimulated T cells.

PLCε, similarly to other PLC isoenzymes, has been implicated in rapid phosphoinositol turnover and generation of intracellular free calcium ($[Ca^{2+}]_i$) (35). This second messenger has been well documented to be an activator of NFAT transcription factors and a positive regulator of the cytokine genes in T cells (36–38). Thus we wondered whether recruitment of PLCε via Ras^{V12G37}, which according to our observations up-regulates cytokine responses (see Figs. 1 and 2), also influences the relative level of $[Ca^{2+}]_i$ in CD4 T cells. Consistent with the postulate that PLCε is an effector of Ras in T cells, markedly higher number of Ras^{V12G37}-positive CD4 T cells fluxed calcium in response to antigen stimulation when compared with CD4 T cells carrying control empty vector (Fig. 4*A*). This difference was particularly pronounced in T cells stimulated with a suboptimal number of peptide-loaded APCs (Fig. 4*B*). Incubation with APCs alone, on the other hand, did not induce any substantial Ca^{2+}changes in either Ras^{V12G37} or control T cells (Fig. 4*C*). These data support the view that antigen stimulation is absolutely necessary for Ras in T cells to engage its effectors and initiate distal signaling events such as calcium mobilization.

Discussion

Our study demonstrates that an effector pathway that is relatively independent of Raf kinase markedly augments effector

Table 1. Cytokine induction by Ras^{V12G37}

	Stimulation		Transduction		
Cytokine	Primary	Secondary	Ras^{V12G37}	Ras^{V12}	Empty vector
IFNγ, ng/ml	pMCC + IL2	pMCC	1,758 ± 58	1,211 ± 248	12.7 ± 4.2
IFNγ	pMCC + IL12	pMCC	246 ± 51	281 ± 40	53 ± 27
IFNγ	T_H1 (*)	pMCC	2,072 ± 431	2,041 ± 49	473 ± 99
IL4, pg/ml	pMCC + IL4	pMCC	519 ± 109	940 ± 174	95 ± 17
IL4	pMCC + IL2/IL4	pMCC	28,791 ± 7,231	ND	7,877 ± 2,076
IL4	T_H2 (*)	pMCC	121,520 ± 8,033	160,572 ± 5,643	63,779 ± 15,150
IL2, pg/ml	pMCC	pMCC	4,162 ± 388	6,201 ± 578	2,251 ± 388

CD4 T cells were primed, as indicated, and retrovirally transduced with Ras^{V12G37}, Ras^{V12}, or empty vector control. Three and a half days later, GFP+ T cells were sorted and restimulated with pMCC/APCs. At 24 hours after secondary stimulation, culture supernatants were harvested and analyzed by ELISA. Th1 and Th2 cell lines were generated under skewing conditions (IL2 + IL12 and IL2 + IL4, respectively) and used for the analysis 2 weeks after primary stimulation. ND, not determined.

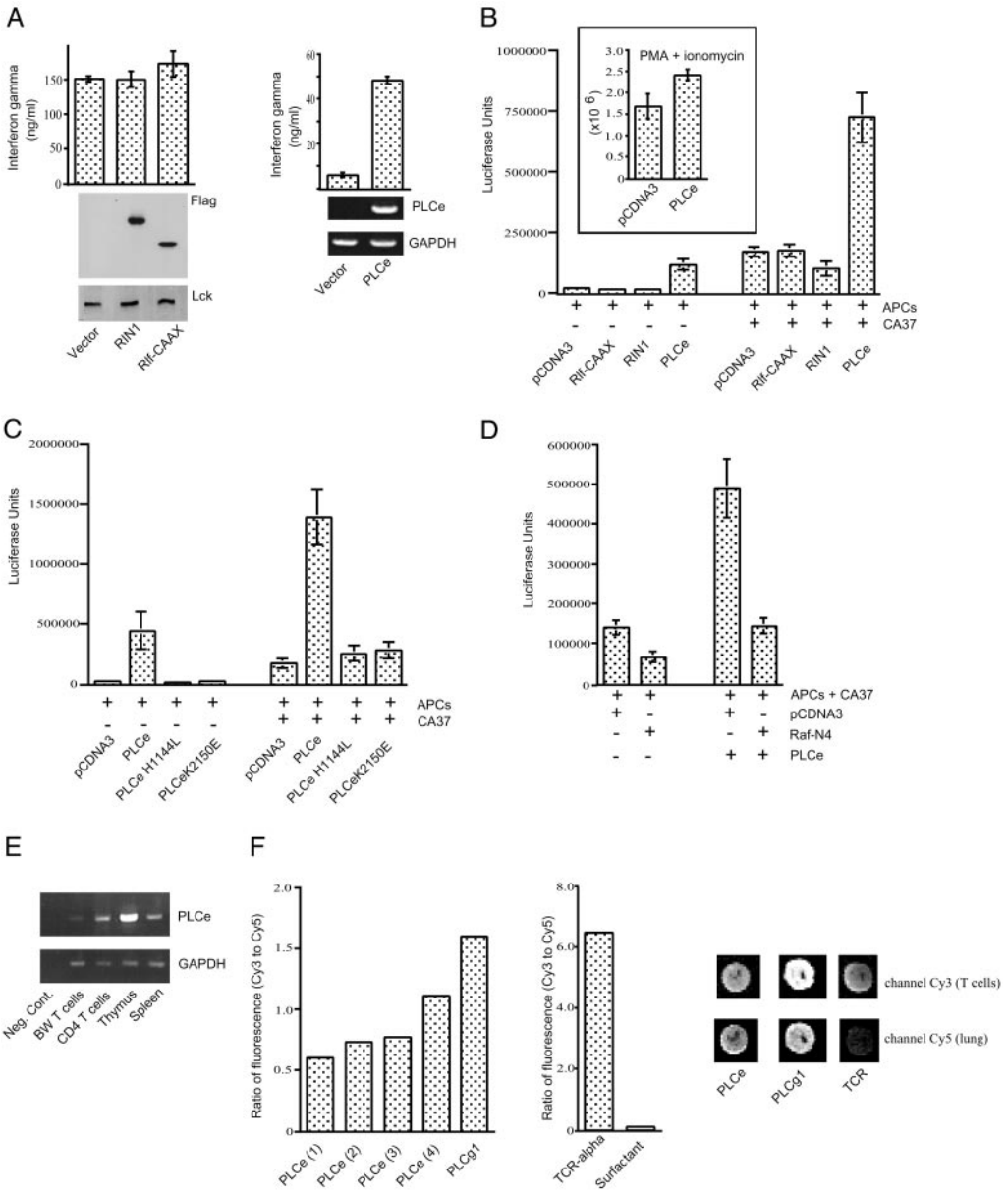

Fig. 3. Effector of Ras^{V12G37}, PLCε, but not RIN1 or RalGEFs, propagates activation of CD4 T cells. (*A*) IFNγ production in RIN1-, Rlf-CAAX-, and PLCε-transduced primary CD4 T cells. CD4 T cells were stimulated with specific antigen, transduced with individual constructs or empty vector, and sorted for GFP expression. Next, purified T cell populations were restimulated with antigen and analyzed by cytokine ELISA. A total of 1×10^5 CD4 cells transduced with RIN1 or Rlf-CAAX, and 2.5×10^4 CD4 cells transduced with PLCε were used for each stimulation. The protein level of PLCε-flag in the transduced T cells was below detection level by Western blot analysis employing anti-flag M2 mAb. Instead, the mRNA expression for PLCε-flag was tested in the transduced T cells by RT-PCR. In addition, the correct size of this construct (≈230- and 260-kDa bands) was verified by analysis of lysates of Phoenix cells that were originally transfected with PLCε cDNA and used for the generation of retrovirus. (*B–D*) Transactivation of the NFAT-luciferase reporter in T cells transfected with PLCε. BW T cells were transfected with various expression constructs or vector control (pcDNA3 or pCGN) as indicated, NFAT-luciferase reporter, and pRL-CMV internal control plasmid. Twenty-four hours after transfection, BW cells either were left unstimulated or stimulated and processed as described in *Materials and Methods*. (*B Insert*) Twelve-hour stimulation with PMA (50 ng/ml) and ionomycin (0.75 μg/ml). (*E*) The mRNA expression for PLCε. RNA isolated from BW T cells, murine primary CD4 T cells, the spleen, and thymus was subjected to RT-PCR using primers specific for mouse PLCε (*Upper*) and GAPDH (*Lower*). RNA that was not subjected to RT was added to a sample representing negative control (*F*) Relative expression of PLCε on microarray. The data for the genes of interest (PLCε and PLCγ1, *Left*), and controls (TCR alpha and surfactant, *Center*) are expressed as the ratio of fluorescence of Cy3 (T cells) to Cy5 (lung). In addition, three representative images are shown in split Cy3 and Cy5 channels (*Right*). Bars labeled PLCe (1–4) represent binding of RNA to 70-mers that correspond to four different sequences of mouse PLCε gene.

functions of CD4 T cells induced by Ras. Moreover, our data suggest that phospholipase activity may be a key direct mediator of these Raf-independent actions of Ras. Several pieces of information support this conclusion. First, wild-type PLCε, but not K2150E and H1144L mutants of PLCε, induced strong IFN-γ production and transactivation of the NFAT reporter in CD4 T cells. Second, Ras^{V12G37} mutant that binds PLCε, but not other Ras mutants failing to interact with PLCε, increases proliferation and *de novo* cytokine production in CD4 T cells. Third, other Ras effectors, in particular Ral-specific GEFs and RIN1 that are known interaction partners of Ras^{V12G37}, failed to enhance these responses. Finally, observations that intracellular Ca^{2+} is rapidly elevated in Ras^{V12G37}-transduced TCR-stimulated effector CD4 T cells further suggest that PLC activity positioned distal or parallel to Ras is involved in this process. Together, prominent elevation of the intracellular calcium and increased transcriptional activity of NFAT may represents key functional consequence of the putative Ras-PLCε interaction in antigen-stimulated CD4 T cells.

Although PLCε can be detected in primary CD4 T cells (Fig. 3 *E* and *F*), we cannot rule out that additional members of PLCε subfamily exists and are expressed in these cells. It is equally possible, however, that RasGEF motif of the studied here PLCε activates Ras proteins, which then signal to PLCε again. If such self-amplifying mechanisms indeed exist in CD4 T cells, then it is not unlikely that even a low-stoichiometry physical interaction between Ras and PLCε could be sufficient to produce sustained functional engagement of PLCε on antigen stimulation.

Also we do not know whether functional changes observed in Ras-transduced CD4 T cells are specific to $H\text{-}Ras^{V12G37}$, studied here, or whether they also can be induced by analogous mutants of N- and K-Ras and Ras-related proteins termed Rap. Interestingly, Rap2B but not other Rap variants or Ras proteins has been implicated in the regulation of PLCε in HEK-293 cells and neu-

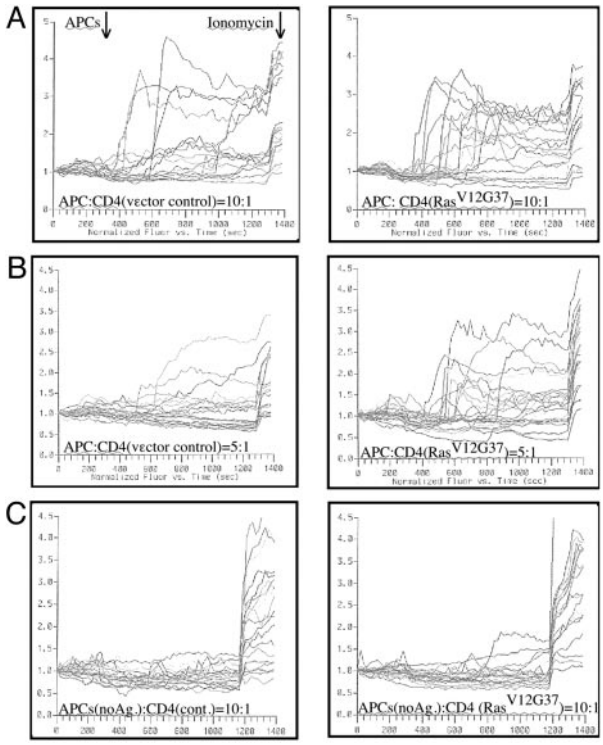

Fig. 4. Ras^{V12G37} potently increases calcium response in CD4 T cells. Primary CD4 T cells transduced with vector control or Ras^{V12G37} were sorted based on the expression of the tailless human CD2 and analyzed as described in *Materials and Methods*. After initiation of scanning, either unloaded APCs (1.5×10^6 cells per well in *C*), or pMCC-loaded APCs (1.5×10^6 cells per well in *A* or 7.5×10^5 cells per well in *B*) were added to the CD4 T cells. Each graph indicates the pattern of calcium mobilization in a field of 20 cells.

roblastoma cells (35), suggesting that at least in certain cell types Rap/PLCε pathway exists in parallel to Ras/Raf cascade. It is conceivable therefore that a similar regulation of PLCε by Rap in addition to Ras may occur in normal T cells.

Of interest are our data suggesting that Ras^{V12G37} influences so called "second signal" generated by costimulatory and/or adhesion molecules rather than the "first signal" induced by the TCR. This conclusion is supported by observations that a high dose of antigen-free APCs can induce substantial activation of NFAT in PLCε transfectants (Fig. 3*C*), and that stimulation of these cells with CD3 mAbs is less dependent on the overexpressed PLCε than a stimulation of T cells with the peptide-loaded APCs (data not shown). Importantly, effector CD4 T cells carrying Ras^{V12G37} responded to an antigen in a quantitative (increased percentage of cells fluxing calcium) rather than qualitative (increased amplitude of calcium flux per cell) manner (Fig. 4*A*), suggesting that formation of APC/T cell conjugates may have proceeded at a higher rate in Ras transfectants.

Efficient activation of PLCγ is by far the best documented mechanisms by which calcium signal is elicited following TCR stimulation with antigen. Our study now suggests that calcium response in T cells is also regulated by an additional pathway consisting of Ras GTPase and PLCε or another PLC isozyme that in a similar fashion to PLCε can physically associate with Ras. Although we do not fully understand the conditions of T cell simulation that control the propensity for Ras to associate with distinct downstream effectors, one may postulate that individual receptors including the TCR, integrins, and costimulatory molecules vary for their ability to engage Ras/PLCε pathway. According to this model, engagement of PLCγ by the TCR together with the recruitment of Ras-responsive PLC by other cell surface receptors on T cell may be necessary for sustained calcium signaling and productive induction of cytokine genes.

We thank D. Lu, P. Ranney, and M. Piecychna for technical assistance; G. Nolan for the Phoenix Ecotropic packaging cells; K. Murphy and G. Barton (with permission from A. Rudensky) for the retroviral vectors; G. Crabtree for NFAT-Luciferase reporter; K. L. Guan for H-Ras; J. Colicelli for Rin1; J. Bos for Rlf-CAAX; G. Kelley, T. Kataoka, and J. Lomasney for various PLCε constructs; C. J. Der for Raf-N4; and C. Fenwick and P. Fields for helpful discussion. This work was supported by National Institutes of Health Grants CA38350 (to K.B.) and AI01914 (to J.C.). J.C. is recipient of the Young Investigator Grant of the National Kidney Foundation.

1. Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W. & Winberg, R. A. (1999) *Nature* **400,** 464–468.
2. Webb, C. P., Van Aelst, L., Wigler, M. H. & Vande Woude, G. F. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 8773–8778.
3. Simon, M. A., Bowtell, D. D., Dodson, G. S., Laverty, T. R. & Rubin, G. M. (1991) *Cell* **67,** 701–716.
4. Beitel, G. J., Clark, S. G. & Horvitz, H. R. (1990) *Nature* **348,** 503–509.
5. Han, M., Aroian, R. V. & Sternberg, P. V. (1990) *Genetics* **126,** 899–913.
6. Harwood, A. E. & Cambier, J. C. (1993) *J. Immunol.* **151,** 4513–4522.
7. Swan, K. A., Alberola-Ila, J., Gross, J. A., Appleby, M. W., Forbush, K. A., Thomas, J. F. & Perlmutter, R. M. (1995) *EMBO J.* **14,** 276–285.
8. Swat, W., Shinkai, Y., Cheng, L., Davidson, L. & Alt, F. W. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4683–4687.
9. Baldari, C. T., Macchia, G. & Telford, J. L. (1992) *J. Biol. Chem.* **267,** 4289–4291.
10. Rayter, S. I., Woodrow, M., Lucas, S. C. & Cantrell, D. A. & Downward, J. (1992) *EMBO J.* **11,** 4549–4556.
11. Crespi, D., Massa, S., Basso, V., Colombetti, S., Mueller, D. L. & Mondino, M. (2002) *Eur. J. Immunol.* **32,** 2500–2509.
12. Fields, P. E., Gajewski, T. F. & Fitch, F. W. (1996) *Science* **271,** 1276–1234.
13. Li, W., Whaley, C. D., Mondino, A. & Mueller, D. L. (1996) *Science* **271,** 1272–1276.
14. Downward, J., Graves, J. D., Warne, P. H., Rayter, S. & Cantrell, D. A. (1990) *Nature* **346,** 719–723.
15. Franklin, R. A., Tordai, A., Patel, H., Gardner, A. M., Johnson, G. L. & Gelfand, E. W. (1994) *J. Clin. Invest.* **93,** 2134–2140.
16. Vojtek, A. B., Hollenberg, S. M. & Cooper, J. A. (1993) *Cell* **74,** 205–214.
17. Campbell, S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J. & Der, C. J. (1998) *Oncogene* **17,** 1395–1413.
18. Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D. & Downward, J. (1994) *Nature* **370,** 527–532.
19. Hofer, F., Fields, C., Schneider, C. & Martin, G. S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11089–11093.
20. Kikuchi, A., Demo, S. D., Ye, Z. H., Chen, Y. W. & Williams, L. (1994) *Mol. Cell. Biol.* **14,** 7483–7491.
21. Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A. & Downward, J. (1997) *Cell* **89,** 457–467.
22. Brtva, T. R., Drugan, J. K., Ghosh, S., Terrell, R. S., Campbell-Burk, S., Bell, R. M & Der, C. J. (1995) *J. Biol. Chem.* **270,** 9809–9812.
23. Czyzyk, J., Leitenberg, D., Taylor, T. & Bottomly, K. (2002) *Mol. Cell. Biol.* **20,** 8740–8747.
24. Walunas, T. L., Lenschow, D. J., Bakker, C. Y., Linsley, P. S., Freeman, G. J., Green, J. M., Thompson, C. B. & Bluestone, J. A. (1994) *Immunity* **1,** 405–413.
25. Ziegler, S. F., Ramsdell, F. & Alderson, M. R. (1994) *Stem Cells* **12,** 456–465.
26. Constant, S. L. & Bottomly, K. (1997) *Annu. Rev. Immunol.* **15,** 297–322.
27. Genot, E. M., Arrieumerlou, C., Ku, G., Burgering, B. M., Weiss, A. & Kramer, I. M. (2000) *Mol. Cell. Biol.* **20,** 5469–5478.
28. Han, L., Wong, D., Dhaka, A., Afar, D., White, M., Xie W., Herschman, H., White, O. & Colicelli, J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4954–4959.
29. Kelley, G. G., Reks, S. E., Ondrako, J. M. & Smrcka, A. V. (2001) *EMBO J.* **20,** 743–754.
30. Song, S., Hu, C. D., Masago, M., Kariyai, K., Yamawaki-Kataoka, Y., Shibatohge, M., Wu, D., Satoh, T. & Kataoka, T. (2001) *J. Biol. Chem.* **276,** 2752–2757.
31. Wolthuis, R. M. F., Zwartkruis, F., Moen, T. C. & Bos, J. (1998) *Curr. Biol.* **8,** 471–474.
32. Tall, G. G., Barbieri, M. A., Stahl., P. D. & Horazdovsky, B. F. (2001) *Dev. Cell* **1,** 73–82.
33. Afar, D. E. H., Han, L., McLaughlin, J., Wong, S., Dhaka, A., Parmar, K., Rosenberg, N., Witte, O. N. & Colicelli, J. (1997) *Immunity* **6,** 773–782.
34. Colicelli, J., Nicolette, C., Birchmeier, C., Rodgers, L., Riggs, M. & Wigler, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2913–2917.
35. Schmidt, M., Sandrine, E., Weernink, P. A., vom Dorp, F., Rehmann, H., Lomasney, J. W. & Jakobs, K. H. (2001) *Nat. Cell Biol.* **3,** 1020–1024.
36. Lewis, R. S. (2001) *Annu. Rev. Immunol.* **19,** 497–521.
37. Timmerman, L. A., Clipstone, N. A., Ho, S., N., Northrop, J. P. & Crabtree, G. R. (1996) *Nature* **383,** 837–840.
38. Rao, A., Luo, C. & Hogan, P. G. (1997) *Annu. Rev. Immunol.* **15,** 707–747.