

Adaptive Tolerance and Clonal Anergy Are Distinct Biochemical States¹

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Adaptive tolerance is a process by which T cells become desensitized when Ag stimulation persists following an initial immune response *in vivo*. To examine the biochemical changes in TCR signaling present in this state, we used a mouse model in which Rag2^{-/-} TCR-transgenic CD4⁺ T cells were transferred into CD3ε^{-/-} recipients expressing their cognate Ag. Compared with naive T cells, adaptively tolerant T cells had normal levels of TCR and slightly increased levels of CD4. Following activation with anti-TCR and anti-CD4 mAbs, the predominant signaling block in the tolerant cells was at the level of Zap70 kinase activity, which was decreased 75% *in vitro*. Phosphorylations of the Zap70 substrates (linker of activated T cells and phospholipase Cγ1) were also profoundly diminished. This proximal defect impacted mostly on the calcium/NFAT and NF-κB pathways, with only a modest decrease in ERK1/2 phosphorylation. This state was contrasted with T cell clonal anergy in which the RAS/MAPK pathway was preferentially impaired and there was much less inhibition of Zap70 kinase activity. Both hyporesponsive states manifested a block in IκB degradation. These results demonstrate that T cell adaptive tolerance and clonal anergy are distinct biochemical states, possibly providing T cells with two molecular mechanisms to curtail responsiveness in different biological circumstances. *The Journal of Immunology*, 2006, 176: 2279–2291.

The phenomenon of T cell anergy was first described in CD4⁺ Th1 clones and revealed an anti-proliferative state induced by TCR occupancy in the absence of costimulation (1). In this state the production of certain cytokines such as IL-2 and IL-3 were selectively down-regulated. Attempts to extend these observations to *in vivo* models generated what appeared to be similar anergic states following superantigen administration, either bacterial or viral (MIs) (2–4). In these models, IL-2 production and T cell proliferation were impaired upon superantigen rechallenge. More recently, the availability of TCR transgenic (Tg)⁵ mice has allowed the development of other *in vivo* models of anergy involving either administration of soluble peptide Ag (5, 6) or double-Tg adoptive transfer models (7–11). As these recent models have been more carefully examined, however, it has become clear that *in vivo* anergy has a number of characteristics that are distinct from those of T cell clonal anergy. One is the down-regulation of multiple effector cytokines such as IL-4 and IFN-γ in

addition to IL-2. Second is the dependence on Ag persistence for maintenance of the anergic state. The third characteristic is the failure of exogenous IL-2 to reverse the state. Because of these differences, we have recently proposed that the *in vivo* anergic state be given another name, adaptive tolerance, to clearly distinguish it from T cell clonal anergy (12). The biochemical experiments in the present report further solidify this distinction.

Signaling through the TCR with Ag begins with the phosphorylation of the TCRζ-chain on ITAMs by Src family kinases such as LCK or FYN (13). This phosphorylation is required to bring the Zap70 to the TCR, where it is also phosphorylated by the Src family kinases (14). Activated Zap70 phosphorylates LAT, the linker of activated T cells (15). This adapter is important for assembling multiple downstream pathways such as the RAS/MAPK and the calcium/calmodulin pathways (16–18). The phosphorylation of several tyrosines on LAT is necessary to activate the RAS/MAPK cascade via GRB2 and SOS. Subsequent signaling through the ERK and JNK pathways is important for the activation of the transcription factors Fos and Jun, respectively. The binding of these two proteins to the AP-1 sites in the promoter of the *IL-2* gene is critical for transcription of *IL-2*.

Phosphorylation of LAT at Tyr¹³⁶ in the mouse is essential to activate phospholipase Cγ1 (PLCγ1) and to release inositol triphosphate (IP₃), which mobilizes intracellular calcium and activates the transcription factor NFAT. The phosphorylated form of NFAT is kept in an inactive form in the cytoplasm. Upon stimulation of the TCR, the calcium/calmodulin-dependent phosphatase calcineurin dephosphorylates NFAT, which is then translocated into the nucleus where it can bind to its target sequences in the promoter of the *IL-2* gene (19). Also partially dependent on the calcium/calmodulin pathway for activation is the translocation of the transcription factor NF-κB to the nucleus (20). The primary stimulus for NF-κB is the diacylglycerol (DAG) released from phosphatidylinositol 4,5-bisphosphate cleavage by PLCγ1, which activates protein kinase C θ (PKCθ) and eventually the IκB kinase complex (21, 22). NF-κB is kept in the cytoplasm in an inactive form by complexing with the inhibitor IκBα. Upon TCR activation, IκBα is phosphorylated, ubiquitinated, and degraded, freeing

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⁵ Abbreviations used in this paper: Tg, transgenic; cdb3, cytoplasmic domain of human erythrocyte band 3; DAG, diacylglycerol; IP₃, inositol 1,4,5-triphosphate; LAT, linker of activated T cells; LN, lymph node; MBP, myelin basic protein; PKCθ, protein kinase Cθ; PLCγ1, phospholipase C γ1; SHP, Src homology region 2 domain-containing phosphatase; PCC; pigeon cytochrome *c*.

NF- κ B to move to the nucleus and participate in transcriptional activation (23). Calcineurin activation through the calcium pathway also enhances the degradation of I κ B. In addition, PKC θ is critical for AP-1 activation (24). In the *IL-2* promoter, there is an NF- κ B response element in the CD28-RE, which is preferentially dependent on c-Rel (25, 26). Costimulation through the CD28 molecule plays a key role in amplifying this pathway through PI3K and AKT activation. Thus, signaling through at least three different biochemical pathways is required to initiate *IL-2* gene transcription. The transcription factors induced by each of these signals must all be present at enhanced levels before transcription can commence (27).

We have developed an *in vivo* anergy model in which CD4⁺ TCR Tg T cells specific for a cytochrome *c* peptide (pigeon cytochrome *c* (PCC)) and I-E^k are transferred into a second, T cell-depleted Tg recipient that persistently expresses the cognate Ag under an MHC class I promoter and an Ig enhancer (11). The T cells initially go through a significant expansion (up to 100-fold) during the first 4 days. Over the next 3–4 days there is a small deletional phase in which ~50% of the cells are lost. This is followed by an adaptive tolerance phase in which 10–20 × 10⁶ cells remain but are hyporesponsive as measured by *in vitro* cytokine production and *in vivo* proliferation measurements. The availability of this large number of tolerant T cells made it possible to carry out biochemical experiments. Our goal was to determine where the biochemical block(s) occurs in TCR signal transduction in the adaptively tolerized T cells and to compare this with the block in clonally anergic T cells. Examination of the three major TCR signaling pathways revealed that all of them were attenuated in adaptively tolerant T cells following anti-TCR and anti-CD4 mAb stimulation. The primary impairment appeared to be at the level of Zap70 kinase activity, resulting in a profound block in the calcium/NFAT pathway. This result was in contrast to T cell clonal anergy, while the RAS/MAPK pathway was most strongly inhibited, whereas Zap70 activity and activation of the calcium/NFAT pathway were not greatly impaired.

Materials and Methods

Mice and adoptive transfers

All mice were on the B10.A (H-2^a) background. The recipient mice (named RO or PCC) were Tg for PCC under the control of an MHC class I promoter and an Ig enhancer and were also CD3 $\epsilon^{-/-}$ (11, 28, 29). The donor T cells were from CD4⁺, TCR-5CC7 Tg I, Rag 2^{-/-} mice that specifically recognize the PCC peptide 81–104 bound to I-E^a or I-E^k (30). Three million lymph node (LN) T cells (>90% CD4⁺) were transferred *i.v.* into each recipient mouse.

Preactivated cells (in vitro), primed cells (in vivo), and the Th1 clone A.E7

Populations of *in vitro*-preactivated TCR-5C.C7 Tg cells were made by activating naive LN T cells with 5 μ M PCC and a 10-fold excess of irradiated (3000 rad) B10.A splenocyte APCs. After 48 h, the activated T cells were expanded with 10 U/ml *IL-2*. The cells were then rested and used after 1 wk or more. *In vivo*-primed T cells were made by priming TCR-5C.C7 Tg, Rag2^{-/-} mice with the PCC peptide 81–104 (30 μ g) and LPS (10 μ g). LN and spleens were harvested after 3 wk. The T cell clone, A.E7 (31), is a CD4⁺ Th1 clone that recognizes PCC 81–104 and I-E^k. The clone was maintained by restimulation with Ag and APC and then *IL-2* as described above for *in vitro*-preactivated T cells. The A.E7 cells were used 2 wk after restimulation. A.E7 T cells were anergized by stimulating with plate-bound anti-TCR β (H57 at 2.5 or 10 μ g/ml) for 16 h, removing the cells from the stimulus, and using them after 7 days of rest. Before this, anergy was confirmed by restimulating an aliquot of the cells at day 5 and measuring *IL-2* production.

Abs and reagents

Ionomycin and PMA were purchased from Calbiochem. PCC peptide (aa 81–104) was synthesized through the National Institute of Allergy and

Infectious Diseases Peptide Facility (National Institutes of Health, Bethesda, MD). The following Abs were used for flow cytometry: anti-CD4-TC and anti-CD4-PE (Caltag Laboratories) and anti-V α 11-FITC, anti-V β 3-PE, anti-CD4-biotin, anti-TCR β -biotin, purified anti-CD3, and anti-TCR β (BD Pharmingen). Abs used for Western blots were from Santa Cruz Biotechnology (anti-Zap70, anti-phosphotyrosine 292 Zap70, anti-LAT, anti-PLC γ 1, anti-NFAT, anti-LCK, anti-FYN, anti-I κ B α , anti-mouse IgG-HRP, and anti-rabbit IgG-HRP), Cell Signaling Technology (anti-phosphotyrosine 493 Zap70, anti-phosphotyrosine 171 LAT, anti-phosphotyrosine 416 Src, anti-I κ B α , anti-phospho-ERK, and anti-ERK), Upstate (anti-phosphotyrosine, anti-phosphotyrosine 191 LAT, anti-phosphotyrosine 226 LAT, and anti-ERK), BioSource International (anti-phosphotyrosine 132 LAT, and anti-phosphotyrosine 783 PLC γ 1), or Bio-Rad (anti-mouse IgG-HRP and anti-rabbit IgG-HRP).

In vitro activation

LN and spleen cells were removed from mice on various days after transfer. Two rounds of negative selection were performed to purify the recovered T cells. The first round used sheep anti-mouse IgG Dynabeads (Dyna). In the second round, the cells were incubated with Ab against class II (M5114), CD11b (Mac-1), and CD45 (B220) (BD Pharmingen) and then incubated with sheep anti-rat IgG Dynabeads. T cells were routinely >90% pure. T cells (1 × 10⁴) were restimulated *in vitro* with Ag (PCC peptide 81–104) and 5 × 10⁵ irradiated B10.A splenocytes. After 48 h, supernatants were collected for cytokine determinations, and plates were pulsed with [³H]TdR for 18 h to measure proliferation. Supernatants from cell cultures were used to measure various cytokines produced during *in vitro* restimulation using ELISA kits from R&D Systems.

Westerns

Cells were coated on ice for 10 min with anti-CD4 biotin (10 μ g/ml) and anti-TCR β (H57) biotin (10 μ g/ml unless indicated otherwise) and washed once. Prewarmed streptavidin was added to the cells, and activation at 37°C took place for the indicated times. As shown in Fig. 4A, T cells were activated with Ag-pulsed DCEK cells following the procedure of Lucas et al. (32). DCEK cells are fibroblasts that had been transfected with MHC class II, B7-1, and ICAM and were kindly provided by Dr. R. N. Germain (National Institutes of Health). The cells were then washed once in ice-cold PBS and resuspended in lysis buffer containing 1% Nonidet P-40, 10 mM Tris-HCl (pH 7.2), 140 mM NaCl, 2 mM EDTA, 5 mM iodoacetamide, 1 mM Na₃VO₄, and complete protease inhibitor mixture (Roche). After incubating on ice for 30–45 min, lysates were spun at 14,000 rpm in the cold for 10 min. In a few experiments, total lysates were used as indicated in the figure legends. In all the other experiments, lysates were immunoprecipitated with specific Abs coupled to agarose beads, washed three times, resuspended with 2× SDS sample buffer, and boiled for 5 min. Samples were run on SDS-PAGE and transferred to polyvinylidene difluoride or nitrocellulose membranes. The membrane was blocked with buffer containing 5% milk for 1 h at room temperature. Membranes were incubated with the primary Ab overnight and then with a secondary Ab conjugated to HRP. Chemiluminescent substrates were used to reveal the bands (Pierce). Gel-Pro 32 software (Media Cybernetics) was used for quantitation of the bands.

Calcium measurements

T cells were harvested and purified as described above for *in vitro* activation. The standard protocol for labeling with Indo-1 was followed (33). Briefly, cells were washed with HBSS plus 1% FCS and incubated with 2 μ M Indo-1 (Molecular Probes) at 30°C for 30 min. Cells were washed and stained with anti-CD4-PE. Finally, the cells were activated with either ionomycin or anti-CD3. Flow cytometry was done on an LSR flow cytometer (BD Biosciences). Analysis was done using the kinetic package in FlowJo software (BD Biosciences).

In vitro kinase assay

The cells were lysed in 1% Nonidet P-40 lysis buffer containing 1% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EGTA, 5 mM β -glycerophosphate, 2 mM Na₃VO₄, 10 mM NaF, 1 mM DTT, 1 mM PMSF, and the complete protease inhibitor mixture. After incubating on ice for 30–45 min, lysates were spun at 14,000 rpm in the cold for 10 min. The supernatants were subjected to immunoprecipitation with protein A/G beads precoated with Abs to Zap70, LCK, or FYN, as indicated.

The immunoprecipitates were washed twice with ice-cold 1% Nonidet P-40 lysis buffer and once in kinase buffer (20 mM HEPES (pH 7.2), 5 mM MgCl₂, and 5 mM MnCl₂). The anti-LCK or anti-FYN immunoprecipitates were then suspended in 30 μ l of kinase buffer containing 5 μ Ci of

[γ - 32 P]ATP (Amersham Biosciences) and 4 μ g of myelin basic protein (MBP) from guinea pig brain or 10 μ g of enolase from yeast (Sigma-Aldrich) for LCK and FYN, respectively. The samples were incubated at room temperature for 20 min. Reactions were terminated by the addition of 30 μ l of 2 \times SDS sample buffer. The samples were heated for 5 min at 95°C and separated by SDS-PAGE. The gel was dried for 1 h at 50°C. Radiolabeled tyrosine-phosphorylated proteins were visualized with a Fujifilm FLA 5000 phosphorimaging device (Fujifilm Medical Systems), and the intensity of protein phosphorylation was quantitated with GelPro software. For Zap70, *in vitro* kinase assays were performed in a similar manner, but reactions were started by the addition of 30 μ l of kinase buffer containing 50 μ M ATP, 5 μ Ci of [γ - 32 P]ATP, and 10 μ g of GST fused to a 14-aa peptide (MEELQDYEDMMEEN) from the cytoplasmic domain of human erythrocyte band 3 (cdb3 peptide) as exogenous substrate (34). The plasmid containing the cdb3 peptide GST fusion protein was a gift from Dr. A. Weiss (University of California, San Francisco, CA). The samples were incubated for 5, 10, or 20 min at room temperature, and the reactions were terminated with 30 μ l of 2 \times SDS sample buffer. The sample was analyzed by SDS-PAGE and autoradiography, as described above.

Results

We demonstrated previously that peripheral CD4⁺ T cells persistently exposed to a low level of a transgenically expressed "self" Ag become hypo-responsive *in vitro* and adaptively tolerant *in vivo* (11). As seen in Table I, tolerant T cells produce approximately one-tenth of the amount of IL-2 as that made by naive T cells when stimulated with Ag and APC. In contrast, when activated with the calcium ionophore ionomycin, the phorbol ester PMA, and APC, the tolerant T cells were able to make equivalent amounts of IL-2. Because these pharmacologic reagents bypass the initial steps in TCR signaling, the results suggest that the tolerance process involves an early block. Because the levels of TCR are not different between naive and adaptively tolerized T cells (35) and because the level of CD4 is only slightly increased in tolerant cells (mean fluorescence intensity (MFI) tolerant/MFI naive = 1.41 \times / \pm 1.031; n = 22; p = 0.0001), we decided to examine the TCR signaling pathways that are critical for T cell activation after stimulation through the TCR, specifically the MAPK, calcium/NFAT, and NF- κ B pathways.

The phosphorylation of ERK1 and ERK2 are only modestly decreased in the adaptively tolerant T cells following activation

Because clonally anergic T cells had been shown previously to have a specific defect in the activation of the RAS/MAPK pathway, we first determined the amount of active (phosphorylated) ERK1 and ERK2 following TCR stimulation with cross-linked anti-TCR β and anti-CD4 mAbs. The ability of the adaptively tol-

erant T cells to phosphorylate these molecules in the MAPK pathway was reduced as compared with naive T cells (Fig. 1A). The magnitude of the impairment was 2- to 3-fold and was observed at all time points from 1 to 10 min after stimulation. This small block in ERK activation was first detected at 4 days after transfer of naive T cells into an Ag-bearing host and was continually observed as long as 100 days after transfer (35) (Fig. 1B).

The activation of NF- κ B is impaired in the adaptively tolerant T cells

Upon TCR activation, I κ B α is phosphorylated and then degraded, allowing NF- κ B to go to the nucleus and activate transcription. Over time, following activation by streptavidin cross-linking of anti-TCR β -biotin and anti-CD4-biotin mAbs, degradation of I κ B α was observed in naive T cells (Fig. 1C). In contrast, there was little if any degradation of I κ B α in the tolerant T cells. Thus, the nuclear translocation of the transcription factor NF- κ B is likely to be limited in the adaptively tolerant state.

Activation-induced rises in intracellular calcium are greatly curtailed in adaptively tolerant T cells

Because calcium mobilization has been shown to be critical for IL-2 production, we next investigated whether this pathway was impaired. As shown in Fig. 2A, the naive T cells mobilized calcium efficiently after activation with soluble anti-CD3. In contrast, there was little or no response by the adaptively tolerant T cells. This

Table I. The defect in IL-2 production in adaptively tolerant T cells is overcome by activation with PMA, ionomycin, and APC

	IL-2 Production (pg/ml)	
	Naive	Tolerant
APC + Ag ^a		
10.0 μ M	15,906	1,861
1.0 μ M	11,706	1,820
0.1 μ M	5,625	1,009
APC + PMA + Iono ^b		
1.0 μ M	9,604	10,604
0.5 μ M	8,302	7,375

^a Naive and tolerant cells (1×10^4) were stimulated *in vitro* with three different concentrations (10.0, 1.0, and 0.1 μ M) of Ag (PCC 81-104) and APC (5×10^5 irradiated B10.A splenocytes). Supernatants were collected at 48 h and ELISAs for IL-2 were carried out.

^b Naive and tolerant cells (1×10^4) were stimulated *in vitro* with APC, PMA (10 ng/ml), and two different concentrations (1.0 and 0.5 μ M) of ionomycin (Iono). Supernatants were collected at 48 h and ELISAs for IL-2 were carried out.

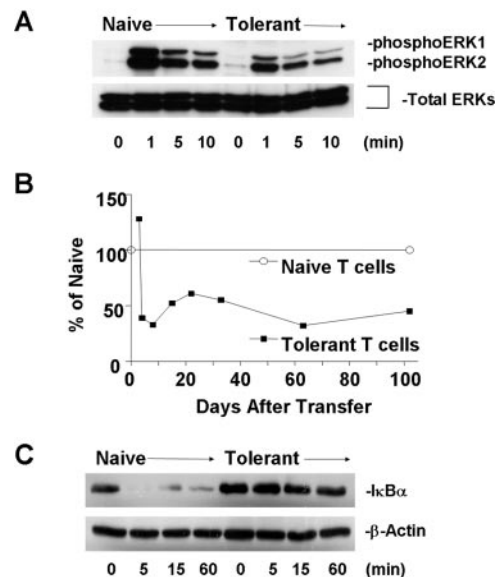


FIGURE 1. Both the activation of MAPK and the activation of NF- κ B are impaired in adaptively tolerant T cells. *A*, Naive or tolerant T cells were activated with anti-TCR β -biotin (10 μ g/ml) and anti-CD4-biotin (10 μ g/ml) and cross-linked with streptavidin for the indicated times. Cells were lysed, and a Western blot was done to detect phosphorylated ERKs with an Ab specifically recognizing phospho-ERK1 and 2. The blot was stripped and reprobed with an Ab that is specific for total ERK1 and ERK2. *B*, Naive and tolerant T cells were activated with plate-bound anti-CD3 (10 μ g/ml) on various days after T cell transfer. Whole cell extracts were prepared and blots were done as in *A*. Densitometry was performed, and the data were normalized to total ERK and then as a percentage of the naive T cell value. *C*, Naive and tolerant T cells were stimulated with anti-TCR β -biotin (10 μ g/ml) and anti-CD4-biotin (10 μ g/ml) and cross-linked with streptavidin for various times. Cells were lysed, and a Western blot was done for I κ B α . The membrane was stripped and reprobed for β -actin.

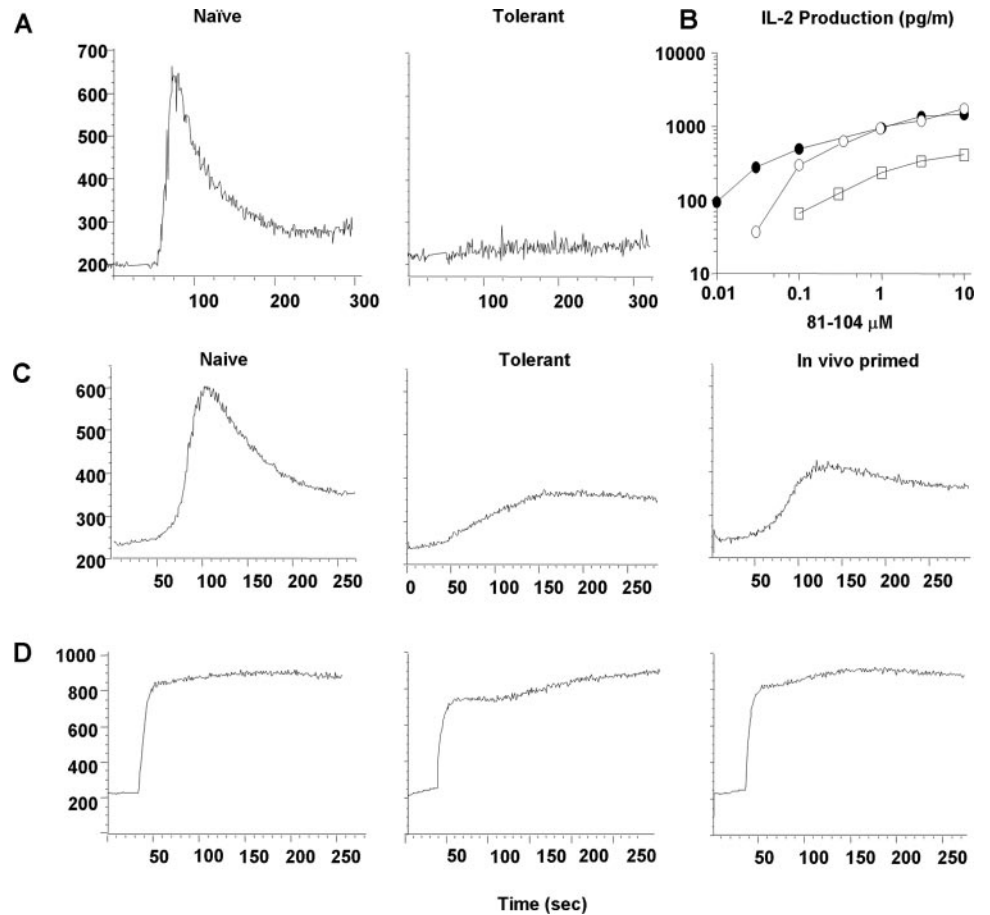


FIGURE 2. Calcium mobilization is impaired in adaptively tolerant T cells. *A*, *C*, and *D*, Naïve, tolerant, and in vivo-primed T cells were loaded with Indo-1 dye, and intracellular calcium levels were measured with a BD Biosciences LSR flow cytometer. Cells were activated with either 20 μ g/ml anti-CD3 (*A* and *C*) or 10 μ M ionomycin (*D*). *B*, Naïve (\circ), adaptively tolerant (\square), or in vivo-primed (\blacksquare) T cells (1×10^4) were restimulated in vitro with Ag (PCC 81-104) and APC (5×10^5 irradiated B10.A splenocytes). ELISAs were done with 48-h supernatants to measure IL-2 production.

was found in four experiments. In one experiment there was a limited but detectable response by the tolerant T cells. In this experiment we compared adaptively tolerized T cells to a 21-day-old, in vivo-primed T cell population (see *Materials and Methods*) in addition to a naïve T cell population. As shown in Fig. 2*B*, both the in vivo-primed and naïve T cell populations produced 10-fold higher amounts of IL-2 than the adaptively tolerant T cells under optimum Ag and APC activation conditions. However, the in vivo-primed T cells were more sensitive than the naïve T cells, because they could generate IL-2 when stimulated with a 10-fold lower concentration of Ag. The ability of these three cell populations to mobilize calcium was determined by flow cytometry. Again, the naïve T cells efficiently fluxed calcium when activated with anti-CD3 (Fig. 2*C*). At the peak of the response, ~85% of the T cells had responded. In contrast, the adaptively tolerized cells had a very blunted response with a maximum of only 15% of the cells responding above the unstimulated control, and this response was significantly delayed. The response was also less than that of the in vivo-primed T cell population, where ~65% mobilized calcium as quickly as the naïve cells. In all of the cell populations, equal mobilization of calcium was observed when ionomycin was used as a control (Fig. 2*D*). Finally, we found that the tolerant T cells also failed to mobilize calcium in response to Con A (data not shown). These results demonstrate that only the adaptively tolerant T cell population shows a large defect in its ability to increase intracellular calcium following TCR engagement. Thus, all three major signaling pathways downstream of TCR activation appear to be negatively affected in adaptive tolerance, indicating that the block was more proximal in the TCR signaling pathway.

Phosphorylation of PLC γ 1 is impaired in adaptively tolerant T cells following TCR activation

To further explore the biochemical block, we next examined the activation of PLC γ 1. This enzyme cleaves phosphatidylinositol 4,5-bisphosphate into IP $_3$ and DAG. IP $_3$ triggers the rise in intracellular calcium, and DAG contributes to the activation of RAS-GRP and PKC θ , the latter leading to I κ B α degradation. In Fig. 3*A*, PLC γ 1 was immunoprecipitated and blotted with anti-phosphotyrosine to determine the amount of phosphorylated PLC γ 1 induced (relative to total PLC γ 1) following activation by streptavidin cross-linking of anti-TCR β -biotin and anti-CD4-biotin mAbs. A clear reduction (90%) in the ability of the tolerant cells to phosphorylate PLC γ 1 was observed compared with that seen for the naïve cells. Similar results were observed in two other experiments. In addition, we examined a population of T cells previously activated with Ag in vitro (see *Materials and Methods*). These cells were able to phosphorylate PLC γ 1 almost as well as the naïve cells. There was an ~5-fold greater amount of phospho-PLC γ 1 in the previously activated T cells than in the adaptively tolerant T cells. Therefore, the block in PLC γ 1 activation is unique to the tolerant T cells. This result was confirmed with a second approach using an Ab directed against the tyrosine phosphorylation site (783), the phosphorylation of which is required for PLC γ 1 activation (Fig. 3*B*). In this experiment (one of three), the T cells were stimulated with four different concentrations of anti-TCR (plus anti-CD4) mAb for 1 min, and a Western blot was performed on total cell lysates. At the highest concentration, phosphorylation of site 783 in tolerant cells was only ~5% (normalized to total PLC γ 1) of that observed for extracts from

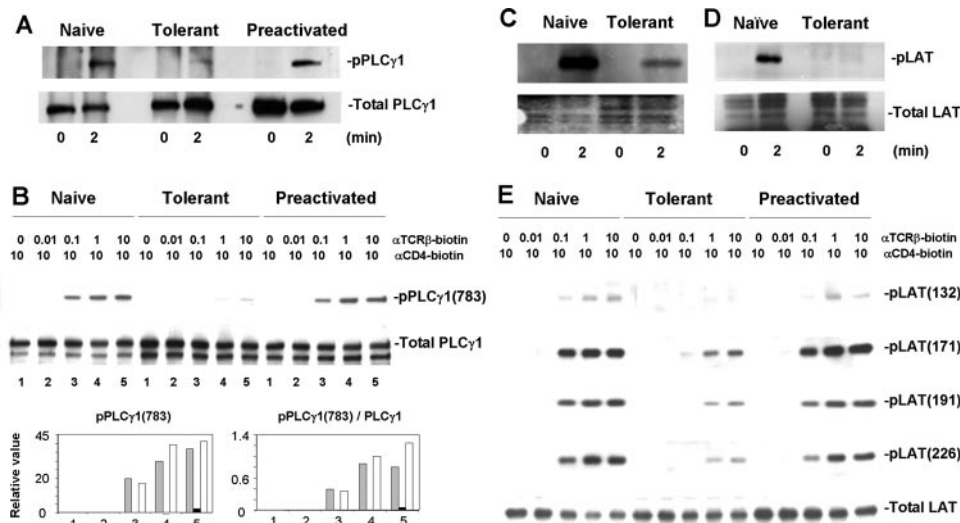


FIGURE 3. Activation of both PLC γ 1 and LAT are impaired in tolerant T cells compared with naive or in vitro preactivated T cells. T cells were activated with various concentrations of anti-TCR β -biotin plus anti-CD4-biotin (10 μ g/ml) and cross-linked with streptavidin for 2 (A, C, and D) or 1 min (B and E). After activation, each sample was lysed and immunoprecipitated with anti-PLC γ 1 (A) or anti-LAT (C and D), and Western blots were done with anti-phosphotyrosine. The blots were then stripped and reprobed for total PLC γ 1 (A) or LAT (C and D). In C and D there is a nonspecific band that runs directly above the total LAT band. B and E, naive, tolerant and in vitro preactivated T cells were stimulated with varying concentrations of anti-TCR β -biotin (0.01–10 μ g/ml) plus anti-CD4-biotin (10 μ g/ml), followed by streptavidin cross-linking. Whole cell extracts were then prepared, and a Western blot was performed with anti-phospho-PLC γ 1 Ab specific for site 783 (B) or with anti-phospho-LAT Abs specific for each of the four tyrosine phosphorylation sites on LAT (132, 171, 191, and 226, according to the human numbering) (E). A fifth blot was developed with an anti-LAT mAb to measure the total amount of LAT in each extract. For PLC γ 1 the blot was stripped and reprobed with an anti-PLC γ 1 Ab to measure the total amount (A and B). The relative values for PLC γ 1 tyrosine 783 phosphorylation are expressed in the box on the lower left of B, and the values were normalized to the total level of PLC γ 1 expression are shown in the box on the lower right. The gray bars are values for naive cells, the black bars are values for tolerant cells, and the white bars are values for preactivated cells.

either stimulated naive or preactivated T cells. Thus, we conclude that the phosphorylation of PLC γ 1, which is required for its function in TCR signal transduction, is greatly impaired in adaptively tolerant T cells.

Phosphorylation of the adapter protein LAT is impaired in the adaptively tolerant T cells

It has been shown both in vitro (16) and in vivo (17, 18) that tyrosine phosphorylation of LAT at position 136 in the mouse (132 in human, which is the numbering system used in *Materials and Methods* and Figs. 3 and 8, because the Abs were made against the human phosphopeptides) is critical for the activation of PLC γ 1. In addition, tyrosine phosphorylation of LAT at mouse positions 175, 195, and 235 (171, 191, and 226 in human) is important for activation of the RAS/MAPK pathway. To examine LAT activation, the protein was directly immunoprecipitated after stimulation of the T cells by streptavidin cross-linking of anti-TCR β -biotin and anti-CD4-biotin mAbs and blotted with anti-phosphotyrosine (Fig. 3C). LAT phosphorylation was greatly reduced in the adaptively tolerant T cells as compared with its activation in naive cells. A total of five experiments were performed and all showed a decrease in LAT phosphorylation, including several with a reduction similar to that shown in Fig. 3C. The most profound effect observed was in the experiment shown in Fig. 3D. In addition, we ran Western blots on total cell lysates and probed them with anti-phospho-LAT Abs specific for each of the four tyrosine phosphorylation sites. As shown in Fig. 3E, at four stimulating concentrations of anti-TCR all of these sites were significantly less phosphorylated in lysates from adaptively tolerant T cells than in lysates from either naive or previously activated T cells (one of two experiments is shown). Thus, the major adapter protein immediately downstream of TCR-activated tyrosine kinases is not properly phosphorylated in adaptively tolerant T cells.

Zap70 kinase activity is impaired in the adaptively tolerant T cells following TCR and CD4 activation

To examine the activation status of the tyrosine kinases involved in the initiation of TCR signaling, we first stimulated naive, tolerant, and in vitro preactivated T cells with Ag-pulsed APCs for 5 min. Cell lysates were then precipitated with anti-Zap70 and blotted with anti-phosphotyrosine (Fig. 4A). Both the naive and in vitro preactivated T cells phosphorylated Zap70 following activation. We were unable to detect Zap70 phosphorylation in the tolerant T cells, even though the APCs were pulsed with 50 μ M peptide. To examine the response to a more potent stimulus, we activated the T cells by streptavidin cross-linking of anti-TCR β -biotin and anti-CD4-biotin mAbs. Under these conditions, Zap70 was phosphorylated in all three cell populations (Fig. 4B). However, when normalized to the total amount of Zap70 immunoprecipitated, the tolerant cells showed slightly less tyrosine phosphorylation than did the naive or preactivated T cells, especially at lower doses of anti-TCR. Similar results were observed when total lysates were blotted with specific anti-phospho-Zap70 Abs (Fig. 4C). In this case, the small differences in the amounts of tyrosine phosphorylation at positions 292, 319, and 493 were substantially augmented by normalization to the total amount of Zap70, because Zap70 was selectively increased in the tolerant T cells (Fig. 4C). Finally, we measured the in vitro kinase activity of Zap70 on the known substrate cdb3 peptide. In this assay, as seen in Fig. 4D, immunoprecipitated Zap70 from tolerant cells was less active than when the enzyme was immunoprecipitated from either naive or preactivated T cells, even at high concentrations of anti-TCR mAb. In three experiments comparing naive and tolerant T cells stimulated with either 1 or 10 μ g/ml Ab, the normalized kinase activity of Zap70 from the tolerant T cells was diminished 76% ($p = 0.0004$; $n = 6$). We conclude that Zap70 kinase activity is significantly

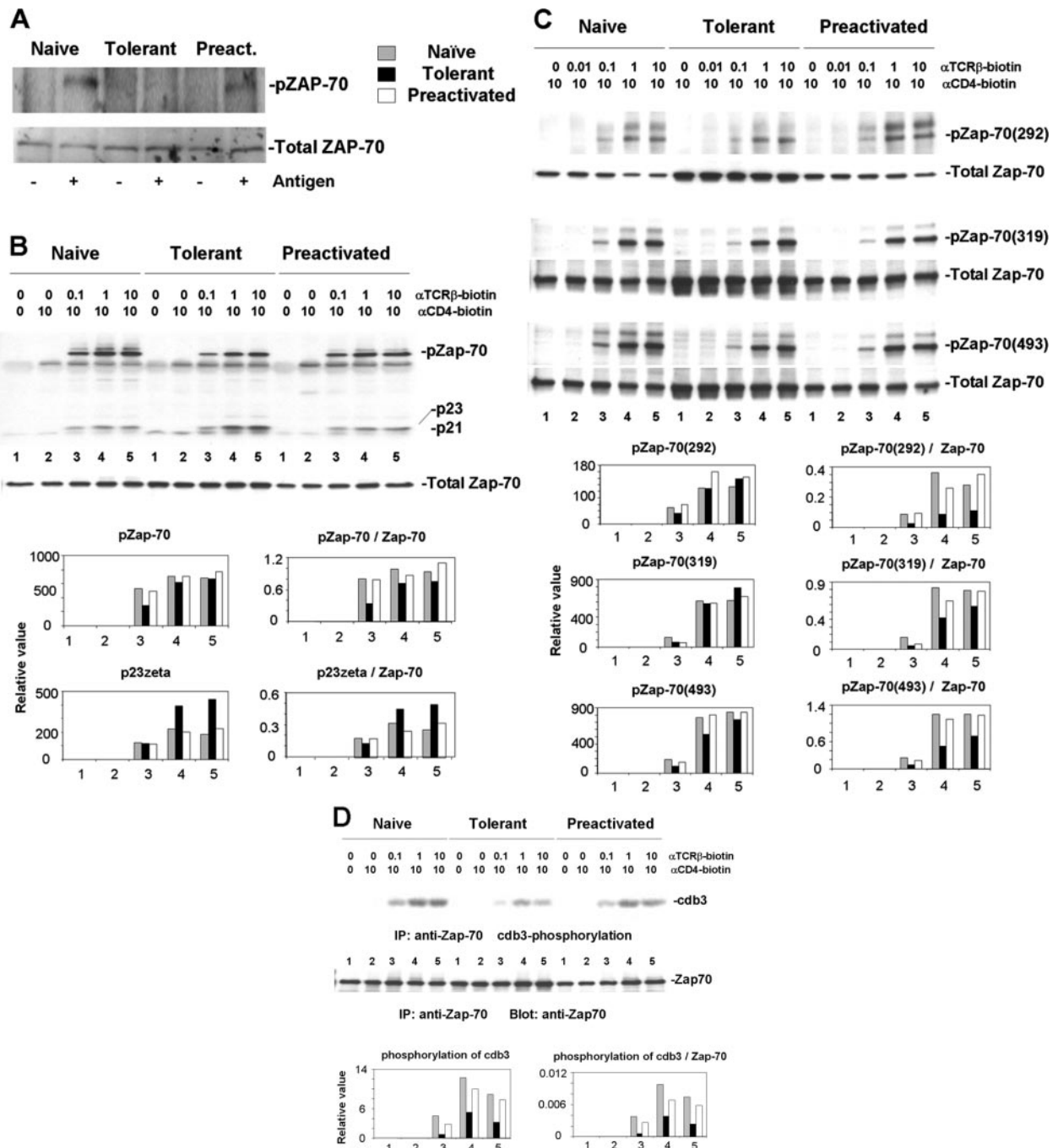


FIGURE 4. Zap70 kinase activity is reduced in adaptively tolerant T cells. **A**, Naive, tolerant and in vitro preactivated (Preact.) T cells were stimulated for 5 min with the Ia⁺ DCEK cell line that had been prepulsed with or without Ag (50 μM PCC 81–104). Cells were lysed and immunoprecipitated with anti-Zap70. A Western blot was done, and the blot was probed with anti-phosphotyrosine. The blot was later stripped and reprobed for total Zap70. **B**, Naive, adaptively tolerant, or preactivated T cells were stimulated for 1 min with varying concentrations of anti-TCRβ-biotin mAb (0, 0.1, 1, or 10 μg/ml) and anti-CD4-biotin mAb at either 0 or 10 μg/ml, followed by cross-linking with streptavidin. Samples were lysed and immunoprecipitated with anti-Zap70. The membrane was probed with anti-phosphotyrosine and then reprobed for total Zap70. The density of each band was determined using GelPro software. The relative values for Zap70 phosphorylation and p23 ζ phosphorylation are expressed in the boxes on the lower left, and the values normalized to the total level of Zap70 expression are shown in the boxes on the lower right. The gray bars are values for naive cells, the black bars are values for tolerant cells, and the white bars are values for preactivated cells. **C**, Naive, adaptively tolerant, or preactivated T cells were stimulated for 1 min with varying concentrations of anti-TCRβ-biotin mAb (0, 0.01, 0.1, 1, or 10 μg/ml) plus anti-CD4-biotin mAb at 10 μg/ml, followed by cross-linking with streptavidin for 1 min. Samples were lysed, and Western blots were done on the whole cell extracts with anti-phospho-Zap70 Abs specific for three of the tyrosine phosphorylation sites on Zap70: Y292, Y319, and Y493. Each blot was stripped and reprobed for total Zap70. The density of each pZap70 and total Zap70 band was determined using GelPro software. The relative values for Zap70 phosphorylation are expressed in the boxes on the lower left of **C**, and the values normalized to the total level of Zap70 expression are shown in the boxes on the lower right. **D**, The naive, tolerant, and preactivated T cells were stimulated and lysed as described in **B**. An in vitro kinase assay was performed on anti-Zap70 immunoprecipitates using the exogenous substrate GST-cdb3 peptide. The immunoprecipitates were separated by SDS-PAGE. Radiolabeled tyrosine-phosphorylated proteins were then visualized with a phosphorimaging device, and the intensity of protein phosphorylation was quantitated with GelPro software. Western blotting was also conducted with anti-Zap70 to determine the total amount of this enzyme present in each lane. The relative values for GST-cdb3 peptide phosphorylation are expressed in the box on the lower left, and the values normalized to the total level of Zap70 expression are shown in the box on the lower right. The gray bars are values for naive cells, the black bars are values for tolerant cells, and the white bars are values for preactivated cells.

inhibited in adaptively tolerant CD4⁺ T cells for both exogenous (cdb3 peptide) and endogenous (LAT and PLC γ 1) substrates, although the latter seem more profoundly affected (Fig. 3).

In Fig. 4B, one can also see the phosphorylation of TCR ζ -chain, because it coimmunoprecipitates with anti-Zap70 (36). Interestingly, phosphorylation of the activation-induced p23 form of TCR ζ was enhanced in adaptively tolerant T cells stimulated with high doses of anti-TCR, as compared with that of both naive and preactivated T cell populations, even when normalized to the total amount of Zap70 immunoprecipitated. The average normalized increase at 1 and 10 μ g/ml was 2.25-fold. To investigate this phenomenon, we examined the amounts and activities of the Src family kinases LCK and FYN, which are responsible for phosphorylation of TCR ζ . Phosphorylation of LCK at position 394 (which is required for its activation) was induced above a basal level by streptavidin-cross-linked anti-CD4-biotin alone or by anti-CD4-biotin plus anti-TCR β -biotin mAbs. No significant differences were observed in the amount of this phosphorylation between extracts from naive and tolerant T cells (Fig. 5A). However, in three immunoprecipitation experiments the *in vitro* enzymatic activity of LCK, using either mbp or LCK itself as substrates, was decreased by 35% (Fig. 5A). In contrast, FYN activity for the exogenous substrate enolase was elevated 3.9-fold in the adaptively tolerized T cells and 9.3-fold for autophosphorylation (Fig. 5B). This kinase was constitutively active in the tolerant T cells

and not further induced by TCR stimulation. The enhanced activity in tolerant T cells was accounted for by an increase in the amount of the enzyme (10.6-fold). Thus, very early signaling events involving activation of the Src family kinases appear to be quite complex in the adaptively tolerant T cells, with a slight decrease in LCK activity and a very significant increase in FYN activity. The enhanced TCR ζ phosphorylation may be related to the increased amount of active FYN in these cells. In addition, Zap70 recruitment to the TCR appears to be intact based on the coimmunoprecipitation of the TCR ζ -chain. Thus, the major inhibition appears to be beyond this point, at the level of full activation of Zap70 kinase activity and the consequent suboptimal steady state levels of phosphorylation of its substrates.

Early signaling events are marginally affected in clonally anergic T cells

Our observations suggest that adaptive tolerance is biochemically an inhibition in TCR-induced Zap70 kinase activity and the steady-state phosphorylation of its substrates, resulting in a large inhibition in the calcium/NFAT pathway and a small inhibition in the RAS/MAPK pathway. This pattern is distinct from the T cell clonal anergy model originally described for CD4⁺ T cell lines and clones. In that model, activation of the RAS/MAPK pathway was shown to be strongly impaired, but the calcium/NFAT pathway was not (37–39). As seen in Fig. 6A, we also observed a significant

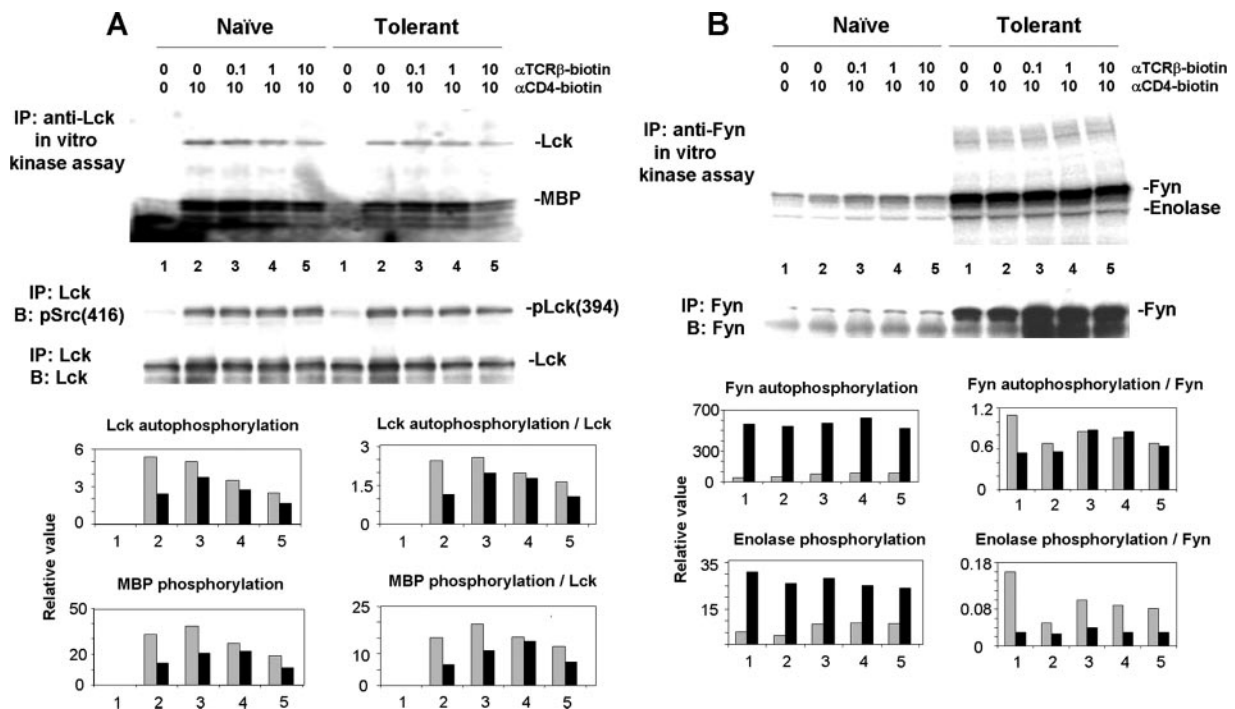


FIGURE 5. LCK kinase activity is slightly reduced and FYN kinase activity is significantly enhanced in adaptively tolerant T cells. *A*, Naive or adaptively tolerant T cells were activated with varying concentrations of anti-TCR β -biotin mAb (0, 0.1, 1, or 10 μ g/ml) and anti-CD4-biotin mAb at either 0 or 10 μ g/ml and cross-linked with streptavidin. Samples were lysed and immunoprecipitated (IP) with anti-LCK. The immunoprecipitates were separated by SDS-PAGE. A Western blot was performed with anti-phospho-Src (Y416) to detect mouse LCK phosphorylated on tyrosine 394, and the blot was stripped and reprobed for total LCK (*middle panels*). An *in vitro* kinase assay was also performed with the LCK immunoprecipitates using MBP as an exogenous substrate. The radiolabeled tyrosine-phosphorylated proteins were visualized with a phosphorimaging device (*upper panel*). The MBP phosphorylation and LCK autophosphorylation were quantitated using GelPro software. The relative values are expressed in the boxes on the *lower left*, and the values normalized to the total level of LCK are shown in the boxes on the *lower right*. The gray bars are the values from naive cells, and the black bars are the values from tolerant cells. *B*, The naive and tolerant T cells were stimulated and lysed as described in *A*. Samples were immunoprecipitated with anti-FYN. An *in vitro* kinase assay was performed using enolase as the exogenous substrate. The immunoprecipitates were separated by SDS-PAGE, and the radiolabeled tyrosine-phosphorylated proteins were visualized with a phosphorimaging device (*upper panel*). The enolase phosphorylation and the FYN autophosphorylation were quantitated using GelPro software. The relative values are expressed in the boxes on the *lower left* of *B*, and the values normalized to the total level of FYN (*middle panel*) are shown in the boxes on the *lower right* of *B*. The gray bars are the values from naive cells, and the black bars are the values from tolerant cells.

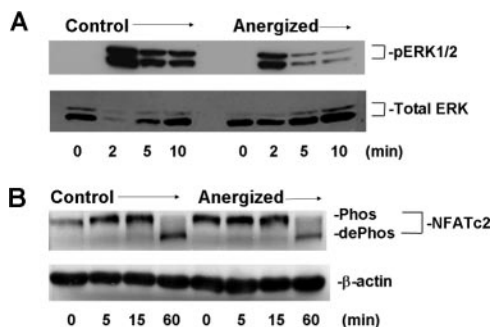


FIGURE 6. Clonally anergized A.E7 T cells are impaired in ERK phosphorylation (Phos) but have relatively normal NFAT dephosphorylation (dePhos). Control and anergized A.E7 CD4⁺ T cells were activated with anti-TCR β -biotin mAb (10 μ g/ml) and anti-CD4-biotin mAb (10 μ g/ml) and cross-linked with streptavidin for the indicated stimulation times. *A*, A Western blot was done using whole cell extracts, and the membrane was probed with anti-phospho-ERK1/2 and then stripped and reprobbed with an Ab that recognizes total ERKs. *B*, A Western blot was done with whole cell extracts, and the membrane was probed with anti-NFATc2 and then stripped and reprobbed for β -actin.

decrease in phospho-ERK activation in an anergized A.E7 CD4⁺ Th1 clonal population stimulated by streptavidin cross-linking of anti-TCR β -biotin and anti-CD4-biotin mAbs. The impairment in the experiment shown was 15-fold (normalized to total ERK) compared with that for unanergized control cells. Two other experiments gave similar results. Previously, Mueller and colleagues (39) had shown that clonally anergic A.E7 T cells can mobilize calcium and translocate NFAT into the nucleus. NFAT is kept in the cytoplasm in an inactive state and must first be dephosphorylated by calcineurin to translocate to the nucleus. To be certain that this pathway could be fully activated in our anergized T cells, we stimulated control and anergized cells with anti-TCR β and anti-CD4 mAbs and looked for the dephosphorylation of NFAT (Fig. 6B). Both control and anergized T cells equally dephosphorylated the majority of the NFAT by 1 h. Thus, the calcium/NFAT pathway is intact in the clonally anergized T cells, in contrast to the adaptively tolerant T cells.

To examine signaling events further upstream, we first turned to Zap70 activation. Stimulation with biotinylated anti-TCR and anti-CD4 for 1 min following streptavidin cross-linking led to comparable levels (<2-fold different in three experiments) of Zap70 phosphorylation as measured in Western blots with an anti-phosphotyrosine mAb of Zap70 immunoprecipitates from anergized and nonanergized T cells (Fig. 7A). Furthermore, Western blotting of whole cell extracts with specific anti-phospho-Zap70 Abs showed little difference in phosphorylation at site 319 (15%) and a small but significant decrease in phosphorylation at sites 292 (35%) and 493 (25%) (Fig. 7B shows one of five experiments). In five *in vitro* kinase assays using cdb3 peptide as the substrate, normalized Zap70 activity from anergized cells stimulated with either 1 or 10 μ g/ml anti-TCR was not significantly different (7%) from the Zap70 activity of control cells (Fig. 7C). Lastly, tyrosine phosphorylation of the *in vivo* endogenous substrates LAT and PLC γ 1 were examined. Sites 132, 171, 191, and 226 in LAT were diminished only 30–55% on Western blots of whole cell extracts from anergized T cells (Fig. 8A). A similar result was observed for PLC γ 1; the amount of phosphorylated protein on Western blots using an anti-phospho-PLC γ 1 Ab specific for phosphotyrosine 783 was decreased 40% ($n = 5$) (Fig. 8B). Also, in several experiments in which PLC γ 1 was immunoprecipitated from cell lysates and Western blots performed with an anti-phosphotyrosine mAb, only

a 2- to 3-fold decrease in phosphorylation for anergized cell precipitates was observed (Fig. 8C). However, these results should be contrasted with the 5- to 20-fold decreases observed with adaptively tolerant T cell populations (Fig. 3). We conclude that the induction of Zap70 kinase activity is only marginally decreased in anergized A.E7 T cells.

Clonally anergic T cells have an additional block in NF- κ B activation

We also looked at the NF- κ B pathway in clonally anergic T cells, because this had never been examined before at the level of I κ B degradation. Surprisingly, the clonally anergized T cells failed to degrade I κ B α , as compared with control cells, even 60 min after T cell activation (Fig. 8D). In the face of fairly normal PLC γ 1 activation, this observation suggests that clonal anergy interferes with NF- κ B activation in a different part of its signaling pathway, possibly at the level of PKC θ activation or downstream at the level of CARMA1, BCL-10, MALT1, TRAF6, or TAK1 (24, 40–42).

Discussion

In this work we have compared and contrasted the state of CD4⁺ T cell clonal anergy with that of *in vivo* adaptive tolerance. Although both states lead to an impairment of IL-2 production and proliferation, the biochemical blocks in TCR signaling appear to be in different places. Adaptive tolerance produces a deficit in Zap70 kinase activity, which propagates through LAT and PLC γ 1 to greatly impair mobilization of calcium and NF- κ B. ERK1/2 phosphorylation was also affected, but to a smaller degree. In contrast, clonal anergy had its major impact on MAPK activation, whereas the kinase activity of Zap70 was much less affected. These observations show that T cell clonal anergy and adaptive tolerance are biochemically distinct anergic states.

The mechanism of impairment of Zap70 kinase activity in adaptive tolerance is not known. Zap70 is phosphorylated by Src-family kinases following TCR occupancy. TCR levels were the same on naive and adaptively tolerant T cells. The *in vitro* LCK kinase activity from the adapted cells was only slightly decreased (35%), and the level of tyrosine 394 phosphorylation at the active site was normal (Fig. 5A). Furthermore, *in vivo* ITAM phosphorylation on the TCR ζ chain to produce the phosphorylated p23 isoform was actually enhanced, and recruitment of Zap70 to the receptor complex appeared to be intact based on coimmunoprecipitation. The enhanced ITAM phosphorylation may be because the CD4 levels are slightly elevated on the tolerant T cells (1.4-fold), bringing in more LCK on cross-linking, and/or because the amount of FYN activity is significantly enhanced (Fig. 5B). These results suggest that Src-family kinase activity should be adequate to phosphorylate and activate Zap70, and, indeed, a substantial amount of phosphorylation was observed at appropriate tyrosine residues (Fig. 4C). Nonetheless, the kinase activity was significantly impaired (Figs. 3 and 4D). Therefore, this impairment is more likely to be the consequence of negative feedback signals possibly involving phosphatases, serine/threonine kinases, ubiquitination, etc. Experiments published by Stefanova et al. (43) have demonstrated the rapid recruitment of Src homology region 2 domain-containing phosphatase-1 (SHP-1) to the TCR complex when partial agonist peptides are used for T cell stimulation. This is normally prevented during full agonist stimulation when ERK is rapidly activated and also recruited to the TCR complex. However, these phosphatase and kinase effects are on Src family kinases in the TCR complex, whereas our data suggest that the LCK and FYN kinase activities are relatively intact or even enhanced. As another possibility, recent papers have suggested that increased CD5 levels may be responsible for adaptive tolerance in CD8⁺ T cells (44) and for

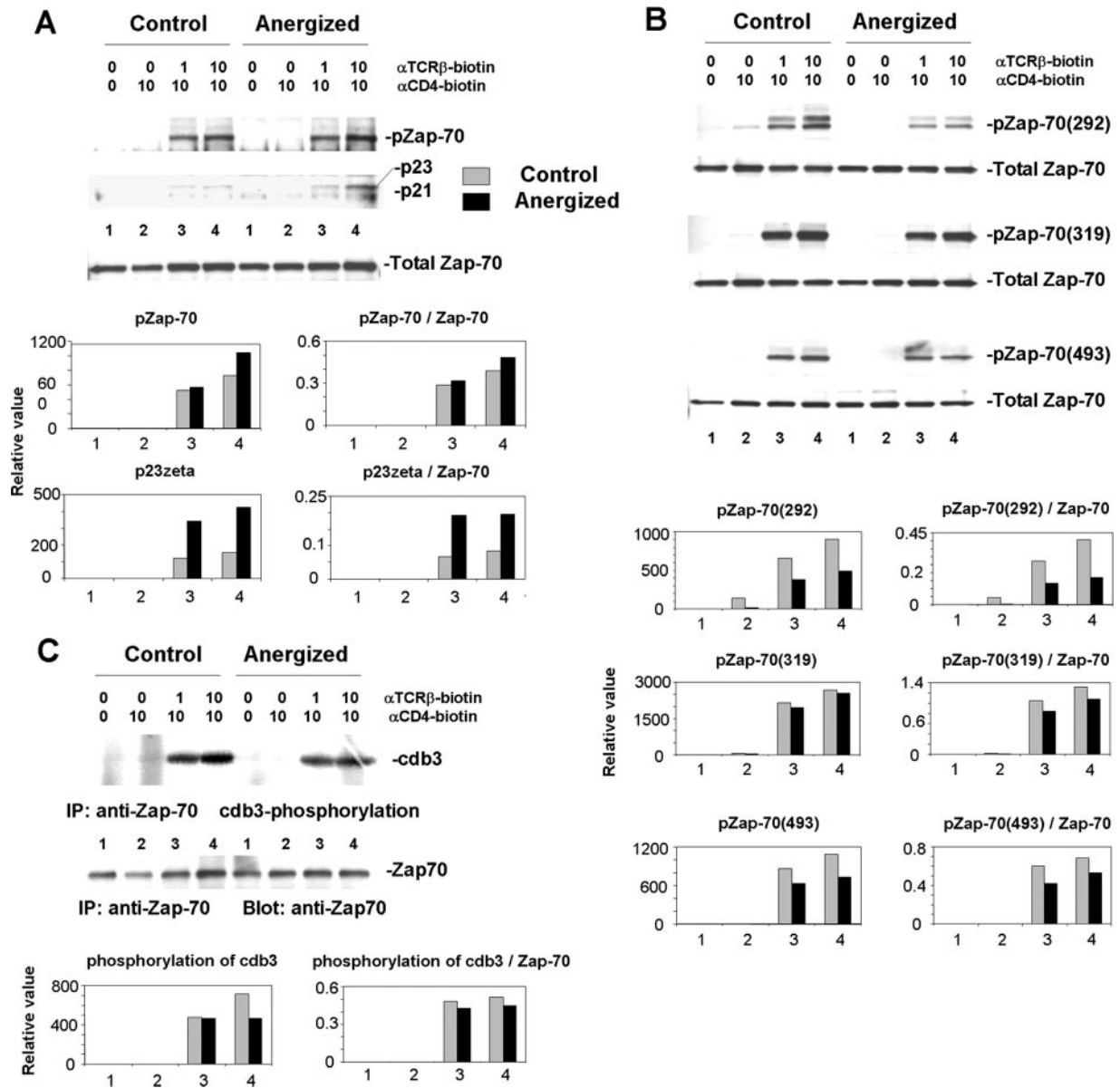


FIGURE 7. Clonally anergic T cells can activate Zap70. *A*, Control and energized T cells were stimulated for 1 min with varying concentrations of anti-TCR β -biotin mAb (0, 1, or 10 μ g/ml) and anti-CD4-biotin mAb at either 0 or 10 μ g/ml and cross-linked with streptavidin. Samples were lysed and immunoprecipitated with anti-Zap70. The membrane was probed with anti-phosphotyrosine and then reprobed for total Zap70 (*upper panels*). The density of each band was determined using GelPro software. The relative values for Zap70 phosphorylation and p23 ζ phosphorylation are expressed in the boxes on the *lower left*, and the values normalized to the total level of Zap70 expression are shown in the boxes on the *lower right*. The gray bars are values for control cells, and the black bars are values for energized cells. *B*, Whole extracts were prepared from cells stimulated as in *A*, and Westerns were done with anti-phospho-Zap70 Abs specific for three of the tyrosine phosphorylation sites on Zap70: Y292, Y319, and Y493. Each blot was stripped and reprobed for total Zap70. The density of each pZap70 and total Zap70 band in *B* was determined using GelPro software. The relative values for Zap70 phosphorylation are expressed in the boxes on the *lower left*, and the values normalized to the total level of Zap70 expression are shown in the boxes on the *lower right*. The gray bars are values for control cells, and the black bars are values for energized cells. *C*, The naive and anergic T cells were activated and lysed as described in *B*. An *in vitro* kinase assay was performed on anti-Zap70 immunoprecipitates (IP) using the exogenous substrate GST-cdb3 peptide. The immunoprecipitates were separated by SDS-PAGE. Radiolabeled tyrosine-phosphorylated proteins were visualized with a phosphorimaging device (*upper panel*), and the intensity of protein phosphorylation was quantitated with GelPro software. Western blotting was also conducted with anti-Zap70 to determine the total amount of this enzyme present in each lane (*middle panel*). The relative values for GST-cdb3 peptide phosphorylation are expressed in the box on the *lower left*, and the values normalized to the total level of Zap70 expression are shown in the box on the *lower right*. The gray bars are values for control cells, and the black bars are values for energized cells.

CD4⁺ T cell tolerance induced by Ag targeting to quiescent dendritic cells (45). We have observed a CD5 increase in our CD4⁺ T cells following tolerance induction, but again the phosphatase associated with CD5 is thought to be SHP-1. In addition, CD4⁺ memory T cells generated in our system by immunization with PCC peptide and LPS showed a similar enhancement in CD5 ex-

pression. In a third potential mechanism, the docking protein Gab2 has been shown to recruit the phosphatase SHP-2 to the TCR complex in activated cells. This requires Zap70 phosphorylation of LAT and Gab2 to initiate and, thus, could represent a negative feedback loop on the activity of Zap70 (46). By a different mechanism of negative feedback, the Cbl molecules c-Cbl and Cbl-b

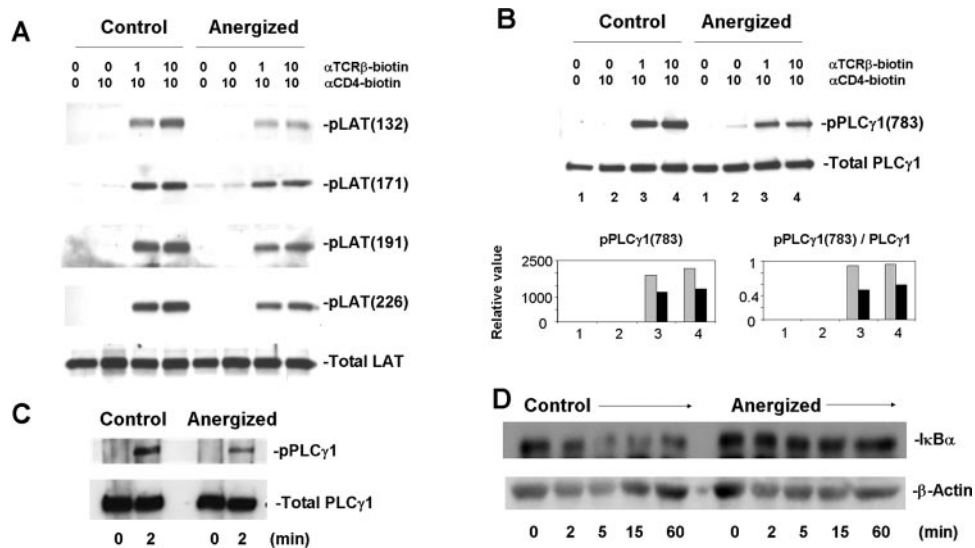


FIGURE 8. Clonally anergized T cells are fully impaired in their activation of NF- κ B but have only a small impairment in LAT and PLC γ 1 phosphorylation. *A*, Control and anergized T cells were activated for 1 min with varying concentrations of anti-TCR β -biotin mAb (0, 1, or 10 μ g/ml) and anti-CD4-biotin at either 0 or 10 μ g/ml followed by streptavidin cross-linking. Whole cell extracts were then prepared and a Western blot performed with anti-phospho-LAT Abs specific for each of the four tyrosine phosphorylation sites on LAT. A fifth blot was developed with an anti-LAT mAb to measure the total amount of LAT in each extract. *B*, Whole extracts were prepared from cells stimulated as in *A*, and a Western blot was done with anti-phospho-PLC γ 1 Ab specific for tyrosine phosphorylation at site 783. The blot was then stripped and reprobed with anti-PLC γ 1. The relative values for PLC γ 1 783 phosphorylation are expressed in the box on the *lower left*, and the values normalized to the total level of PLC γ 1 expression are shown in the box on the *lower right*. The gray bars are values for control cells, and the black bars are values for anergized cells. *C*, Control and anergized A.E7 CD4 $^{+}$ T cells were activated with anti-TCR β -biotin mAb (10 μ g/ml) and anti-CD4-biotin mAb (10 μ g/ml) and cross-linked with streptavidin for 2 min. PLC γ 1 was immunoprecipitated from cell lysates with anti-PLC γ 1, and the membrane was probed with anti-phosphotyrosine and then reprobed for total PLC γ 1. *D*, Control and anergized A.E7 T cells were activated with anti-TCR β -biotin (10 μ g/ml) and anti-CD4-biotin (10 μ g/ml) for various times after streptavidin cross-linking. Whole cell extracts were run on a Western blot and probed with anti-I κ B α . The blot was stripped and reprobed with anti- β -actin.

have been implicated in down-regulating TCR signaling through E3 ligase ubiquitination (47, 48). However, we have observed that phosphorylation of c-Cbl is actually diminished in adaptively tolerant T cells (data not shown). Furthermore, there was no detectable difference in the development of adaptive tolerance, as measured by cell number and cytokine production, when Cbl-b-deficient CD4 $^{+}$ TCR Tg cells were studied in our model (data not shown). Finally, the E3 ubiquitin ligase GRAIL (gene related to anergy in lymphocytes) has recently been postulated to be an anergic factor based on its ability to suppress IL-2 production when overexpressed in T cell hybridomas (49). Whether GRAIL plays a similar role in adaptive tolerance remains to be examined.

Impairment in TCR activation at the level of early tyrosine phosphorylation events has been observed in several other *in vivo* models of adaptive tolerance. The earliest biochemical studies were performed by Bhandoola et al. (50) in an Mls superantigen challenge model. They found in anergic T cells a significant decrease in tyrosine phosphorylation of a 38-kDa protein that most likely represented LAT. Subsequently, Migita et al. (51) used a staphylococcal enterotoxin B superantigen model to demonstrate an inhibition of both Zap70 and TCR ζ chain phosphorylation. More recently, in an adoptive transfer model involving TCR Tg CD8 $^{+}$ T cells, the Src family kinases were found to be inactive, leading to a failure to phosphorylate TCR ζ ; however, Zap70 was constitutively active and associated with CD3 ϵ , suggesting that a more complicated feed back loop was in play (52). In several other types of T cell unresponsiveness induced *in vivo*, a pattern of decreased Zap70 and LAT phosphorylation has been noted. One of these involved a high dose oral tolerance protocol (53) that may represent the equivalent of *i.v.* peptide injection. Another was a developmental model in which an $\alpha\beta$ double-negative anergic population was examined (54). The third was a transplant tolerance

model in which CD8 $^{+}$ TCR Tg cells were first exposed to F $_1$ cells before cardiac allografts (55). Thus, at least five possible models for adaptive tolerance suggest an impairment in the level of Zap70 kinase activity as a major biochemical event in the tolerance process. The involvement of Src family kinases is less clear, with some models showing no effect on LCK *in vitro* kinase activity (53) and others showing a decrease (55). Finally, one group has shown a decrease in the amount of LAT at the plasma membrane of anergic T cells as a result of oxidative stress found in the synovial fluid of rheumatoid arthritic joints (56). If such a mechanism were also operating in our model, it might explain why we often observe a greater quantitative inhibition of LAT and PLC γ 1 phosphorylation than inhibition of Zap70 kinase activity.

In many of these models, calcium mobilization was also shown to be significantly impaired. In one case this impairment was measured at the single cell level, and all of the cells tested failed to mobilize calcium (57). Our experiments demonstrate a profound defect in PLC γ 1 phosphorylation as the possible cause for this impairment. In ionomycin-induced anergy PLC γ 1 has also been identified as a target, but in this case it appears that Cbl-b activation is responsible for its down-regulation (48, 58). An additional level of regulation could be at the adapter molecule Sin, which was recently shown to bind PLC γ 1 in resting cells. Its overexpression results in both decreased IL-2 production and diminished phosphorylation of PLC γ 1 after TCR stimulation (59). The PLC γ 1 defect may also account for the impairment of the NF- κ B pathway observed by us and others (60, 61) through a limited production of DAG required for PKC θ activation. Alternatively, an increase in the E3 ubiquitin ligase Itch may target PKC θ for degradation as described for ionomycin-induced anergy (58).

Finally, only a small inhibition of ERK phosphorylation was observed in adaptive tolerance. This was highly reproducible and

observed from 4 days out to 3 mo after Ag exposure. A similar 2-fold inhibition was observed in vivo by intracellular staining following high dose peptide administration to the tolerant mice (data not shown; Ref. 62). The inhibition of ERK phosphorylation possibly stems from a decrease in RAS-GRP1 activity secondary to the impairment of PLC γ 1 (63). The reason ERK activation was not as profoundly affected as the other TCR-induced pathways, despite significant decreases in LAT phosphorylation at sites 171, 191, and 226, may relate to redundancy in the ability to activate RAS through GRB2/SOS by binding to phosphorylated TCR ζ (54, 64–66), which is elevated in our adaptively tolerant T cells (Fig. 4B). Other in vivo anergy models have also shown little or no effect on ERK phosphorylation (53, 54).

Double Tg models for B cell anergy induction were described by Goodnow (67) a number of years ago. In both the developmental model, where a BCR Tg was crossed to the Ag Tg, and the adoptive transfer model, where mature BCR Tg B cells were injected into an adult Ag Tg mouse, anergic Tg B cells emerged that had down-regulated their IgM receptors and showed impaired signaling through the remaining BCRs. There was a substantial decrease in tyrosine phosphorylation of all substrates, and later studies showed a profound defect in the disappearance of I κ B α and no activation of the NF- κ B and JNK-MAPK pathways (68). Anergic B cells also showed only low levels of calcium oscillations as compared with naive B cells, and this resulted in a lower level of NFAT activation. In contrast, ERK activation was normal. Anergic B cells have 10-fold lower IgM receptor levels, which could account for the impairment in signaling; but the cells have normal IgD levels, and culturing them for 2 days in the absence of Ag to re-establish normal surface IgM still yielded impaired tyrosine phosphorylation following anti-Ig stimulation (69). Hence, anergic B cells are likely to have additional mechanisms of proximal signaling impairment. As summarized above, this impairment is broadly similar to what we and others observe for T cell adaptive tolerance. Thus, the states may be functionally equivalent even if the exact molecular mechanisms inducing them are not identical. It would be interesting to look at Syk kinase activity in the B cell model to see if it parallels the decline in Zap70 kinase activity seen in T cell adaptive tolerance.

The biochemical pattern that emerges from all of these different in vivo model systems is that adaptive tolerance involves a primary impairment in TCR or BCR activation of the initial tyrosine phosphorylation of a variety of substrates such as LAT and PLC γ 1. This, in turn, leads to a profound impairment in the activation of the NF- κ B pathway and greatly impairs the mobilization of intracellular calcium. ERK phosphorylation, however, shows little or no impairment. This pattern is very different from what has been described for T cell clonal anergy in vitro. Initial studies from Kang et al. (70) showed an impairment in AP-1 binding in gel mobility shift assays, and Mondino et al. (39) showed that this was due to decreased expression of c-Fos, FosB, and JunB following T cell stimulation. The primary signaling pathway inhibited in clonal anergy was shown to be the RAS/MAPK pathway (37, 38). The block occurred at the level of RAS activation and affected both the ERK and JNK pathways. In one later report, impairment of the p38 pathway was also reported (71). In contrast, the calcium/NFAT activation pathway, from PLC γ 1 phosphorylation (Fig. 8, B and C) to the dephosphorylation of NFAT (Fig. 6B) and mobilization into the nucleus as well as binding to its DNA response element, was found to be mostly intact (39) or constitutively activated (72). In contrast, several other models of T cell clonal anergy (73, 74) do manifest a block in the PLC γ 1/calcium pathway. In light of our results, we would suggest that the latter models represent a com-

ination of T cell clonal anergy and adaptive tolerance or some other form of anergy (48, 58).

Interestingly, we also observed in the current experiments a block in the degradation of I κ B α in clonally anergic T cells (Fig. 8D). This was surprising, because our original gel mobility shift assays had detected at best only a 2-fold decrease in p65/p50 binding at 4 h after stimulation of such cells (70). However, those studies used a consensus NF- κ B site for the binding instead of the biologically relevant c-Rel site, which may bind with lower affinity. Furthermore, the stimulation in the original experiments was done with Ag and APC, whereas the current study used streptavidin cross-linking of anti-TCR β -biotin plus anti-CD4-biotin mAbs. APCs would have provided strong costimulation in the form of B7/CD28 interactions, which might have partially overcome the block in NF- κ B activation by stimulating PKC θ through PI3K/AKT activation (75).

Finally, and most importantly, clonally anergic T cells showed only a small impairment in Zap70 kinase activity, the primary site of impairment in adaptive tolerance. Previous studies have suggested alterations in Src family kinases in clonal anergy (76), but no effects on Zap70 have been reported. Thus, we would conclude that the two forms of anergy are distinct processes mediated by different biochemical mechanisms. It is also likely that they subserve different immunological functions (12). Adaptive tolerance sets in following an initial robust immune response if the Ag persists in the cell's environment. It appears to be a desensitization process to prevent immunopathology from an inappropriate autoimmune response. Clonal anergy, in contrast, is more of a growth arrest state. It might play a role in curtailing the expansion of T regulatory cells in vitro (77, 78) and CD8 T cells following activation (activation-induced non-responsiveness) (79).

The adaptive tolerance model used in our experiments involved the expansion of CD4⁺ T cells in a lymphopenic host. Recently, we have been able to cross the TCR Tg onto an Ly 5.1 background and transfer the T cells into an Ly 5.2 B10.A mouse expressing the mPCC Ag transgene. Although the level of expansion in this normal host was 20-fold less than that in the lymphopenic host, the recovered cells were still induced into an unresponsive state, as measured by proliferation and cytokine production (N. Singh and R. H. Schwartz, unpublished observations). Thus, we think that our biochemical studies in the lymphopenic model will be generalizable. Certainly, however, these studies will be applicable to lymphopenic clinical situations such as bone marrow transplantation, where, for example, T cells reconstituting an irradiated patient who is positive for cytomegalovirus will encounter chronic Ag stimulation in a lymphopenic environment. Understanding the conditions under which CMV-specific T cells do or do not become adaptively tolerized in these circumstances could be of great importance in achieving a successful clinical outcome.

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Disclosures

The authors have no financial conflict of interest.

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