Adaptive Tolerance and Clonal Anergy Are Distinct Biochemical States

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Adaptive Tolerance and Clonal Anergy Are Distinct Biochemical States

Lynda Chiodetti, Seeyoung Choi, Daniel L. Barber, and Ronald H. Schwartz

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 naïve 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 (Mls) (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) 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/calcineurin 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 Tyr135 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/calcineurin 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 (PKC) 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
NF-κB to move to the nucleus and participate in transcriptional activation (23). Calcineurin activation through the calcium pathway also enhances the degradation of IkB. In addition, PKCθ is critical for AP-1 activation (24). In the IL-2 promoter, there is an NF-κB 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-Eα 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-2b) 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 (11). The T cells were from CD4+ TCR Tg T cells with specific expression of the antigen 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.

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

Populations of in vitro-preactivated TCR-SC.C7 Tg cells were made by activating naive LN T cells with 5 μM 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-SC.C7 Tg, Rag2−/− mice with the PCC peptide 81–104 (30 μg) and LPS (10 μg). 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-Eα. 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-TCRβ (H57 at 2.5 or 10 μg/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-Vα11-FITC, anti-Vγ3-PE, anti-CD4-biotin, anti-TCRβ-biotin, purified anti-CD3, and anti-TCRγδ (BD Pharmingen). Abs used for Western blots were from Santa Cruz Biotechnology (anti-Zap70, anti-phosphorytose 292 Zap70, anti-LAT, anti-PLCγ1, anti-NFAT, anti-LCK, anti-FYN, anti-Ικβ, mouse IgG-HRP, and anti-rabbit IgG-HRP). Cell Signaling Technology (anti-phospho-tyrosine 493 ZAP70, anti-phospho-tyrosine 416 Src, anti-ικβα, anti-phospho-ERK, and anti-ERK), Upstate (anti-phospho-tyrosine, anti-phospho-tyrosine 191 LAT, anti-phospho-tyrosine 132 LAT, anti-phospho-tyrosine 783 PLCγ1), 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 × 10⁶) were restimulated in vitro with Ag (PCC peptide 81–104) and 5 × 10⁶ irradiated B10.A splenocytes. After 48 h, supernatants were collected for cytokine determination and transferred with [3H]ThDr for 18 h to measure proliferation. Supernatants from cytokine cells 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 biodot (10 μg/ml) and anti-TCRβ (H57) biodot (10 μg/ml unless indicated otherwise) and washed once. Perewarmed 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 and 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 membranes were 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). GelPro 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 μM 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).
anti-TCR have a specific defect in the activation of the RAS/MAPK pathway. The phosphorylation of ERK1 and ERK2 are only modestly impaired, as compared with naïve T cells. However, the tolerant T cells were able to make equivalent amounts of IL-2. The defect in IL-2 production in adaptively tolerant T cells 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 naïve 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 observation was verified by the inability of tolerant T cells to phosphorylate these molecules in the MAPK pathway was reduced as compared with naïve 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 naïve 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-κB is impaired in the adaptively tolerant T cells

Upon TCR activation, IκBα is phosphorylated and then degraded, allowing NF-κB to go to the nucleus and activate transcription. Over time, following activation by streptavidin cross-linking of anti-TCRβ-biotin and anti-CD4-biotin mAbs, degradation of IκBα was observed in naïve T cells (Fig. 1C). In contrast, there was little or no degradation of IκBα in the tolerant T cells. Thus, the nuclear translocation of the transcription factor NF-κB is likely to be limited in the adaptively tolerant state.

Results

We demonstrated previously that peripheral CD4+ T cells persistently exposed to a low level of a transgenically expressed “self” Ag become hyporesponsive 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 naïve 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 naïve 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 naïve = 1.41 × (± 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-κB 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-TCRβ and anti-CD4 mAbs. The ability of the adaptively tolerized T cells to phosphorylate these molecules in the MAPK pathway was reduced as compared with naïve 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 naïve T cells into an Ag-bearing host and was continually observed as long as 100 days after transfer (35) (Fig. 1B).

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

<table>
<thead>
<tr>
<th>IL-2 Production (pg/ml)</th>
<th>Naive</th>
<th>Tolerant</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC + Ag^a</td>
<td>15,906</td>
<td>1,861</td>
</tr>
<tr>
<td>10.0 μM</td>
<td>11,706</td>
<td>1,820</td>
</tr>
<tr>
<td>1.0 μM</td>
<td>5,625</td>
<td>1,009</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>9,604</td>
<td>1,920</td>
</tr>
<tr>
<td>APC + PMA + Iono^b</td>
<td>8,302</td>
<td>1,004</td>
</tr>
</tbody>
</table>

^a Naive and tolerant cells (1 × 10^5) were stimulated in vitro with three different concentrations (10.0, 1.0, and 0.1 μM) of Ag (PCC 81-104) and APC (5 × 10^6 irradiated B10.A splenocytes). Supernatants were collected at 48 h and ELISAs for IL-2 were carried out.

^b Naive and tolerant cells (1 × 10^5) were stimulated in vitro with APC, PMA (10 ng/ml), and two different concentrations (1.0 and 0.5 μM) 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-κB are impaired in adaptively tolerant T cells. A. Naive or tolerant T cells were activated with anti-TCRβ-biotin (10 μg/ml) and anti-CD4-biotin (10 μg/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 μg/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 activated with anti-TCRβ-biotin (10 μg/ml) and anti-CD4-biotin (10 μg/ml) and cross-linked with streptavidin for various times. Cells were lysed, and a Western blot was done for IκBα. The membrane was stripped and reprobed for β-actin.

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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 naive T cell population. As shown in Fig. 2B, both the in vivo-primed and naive 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 naive 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 naive T cells efficiently fluxed calcium when activated with anti-CD3 (Fig. 2C). 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 naive cells. In all of the cell populations, equal mobilization of calcium was observed when ionomycin was used as a control (Fig. 2D). 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 PLCγ1 is impaired in adaptively tolerant T cells following TCR activation**

To further explore the biochemical block, we next examined the activation of PLCγ1. This enzyme cleaves phosphatidylinositol 4,5-bisphosphate into IP3 and DAG. IP3 triggers the rise in intracellular calcium, and DAG contributes to the activation of RAS-GRP and PKC, the latter leading to IκBα degradation. In Fig. 3A, PLCγ1 was immunoprecipitated and blotted with anti-phosphotyrosine to determine the amount of phosphorylated PLCγ1 induced (relative to total PLCγ1) 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 PLCγ1 was observed compared with that seen for the naive 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 PLCγ1 almost as well as the naive cells. There was an ~5-fold greater amount of phospho-PLCγ1 in the previously activated T cells than in the adaptively tolerant T cells. Therefore, the block in PLCγ1 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 PLCγ1 activation (Fig. 3B). 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 PLCγ1) of that observed for extracts from

**FIGURE 2.** Calcium mobilization is impaired in adaptively tolerant T cells. A, C, and D, Naive, 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 μg/ml anti-CD3 (A and C) or 10 μM ionomycin (D). B, Naive (□), adaptively tolerant (■), or in vivo-primed (■) T cells (1 × 10⁶) were restimulated in vitro with Ag (PCC 81–104) and APC (5 × 10⁵ irradiated B10.A splenocytes). ELISAs were done with 48-h supernatants to measure IL-2 production.
either stimulated naive or preactivated T cells. Thus, we conclude that the phosphorylation of PLCγ1, 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 Ab was made against the human phosphopeptides) is critical for the activation of PLCγ1. In addition, tyrosine phosphorylation of LAT at mouse positions 175, 195, and 235 (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 PLCγ1 the blot was stripped and reprobed with an anti-PLCγ1 Ab to measure the total amount (A and B). The relative values for PLCγ1 tyrosine 783 phosphorylation are expressed in the box on the lower left of B, and the values were normalized to the total level of PLCγ1 expression 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.

**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 μM peptide. To examine the response to a more potent stimulus, we activated the T cells by streptavidin cross-linking of anti-TCRβ-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 μg/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...
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 p23ξ 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 PLCγ1) substrates, although the latter seem more profoundly affected (Fig. 3).

In Fig. 4B, one can also see the phosphorylation of TCRζ-chain, because it coimmunoprecipitates with anti-Zap70 (36). Interestingly, phosphorylation of the activation-induced p23 form of TCRζ 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 μg/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 TCRζ. 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-TCRβ-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 tolerated 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 TCRζ 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 immunoprecipitation 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

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**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-TCRβ-biotin mAb (0, 0.1, 1, or 10 μg/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 (lower left). The gray bars are the values from naive cells, and the black bars are the values from tolerant cells. B, Naive or 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 MBP phosphorylation and LCK 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 LCK (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 anergic 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-TCRβ-biotin mAb (10 μg/ml) and anti-CD4-biotin mAb (10 μg/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 reprobed 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 reprobed for β-actin.

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-κB activation

We also looked at the NF-κB pathway in clonally anergic T cells, because this had never been examined before at the level of IkB degradation. Surprisingly, the clonally anergized T cells failed to degrade IκBα, as compared with control cells, even 60 min after T cell activation (Fig. 8D). In the face of fairly normal PLCγ1 activation, this observation suggests that clonal anergy interferes with NF-κB activation in a different part of its signaling pathway, possibly at the level of PKCθ 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 PLCγ1 to greatly impair mobilization of calcium and NF-κB. 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 TCRβ chain to produce the phosphorylated p23 isofrom was actually enhanced, and recruitment of Zap70 to the receptor complex appeared to be intact based on communoprecipitation. 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 FYX 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 FYX 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
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 expression. 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 7. Clonally anergic T cells can activate Zap70. A, Control and anergized T cells were stimulated for 1 min with varying concentrations of anti-TCRβ-biotin mAb (0, 1, or 10 μg/ml) and anti-CD4-biotin mAb at either 0 or 10 μg/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 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 anergized 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 anergized 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 anergized cells.
FIGURE 8. Clonally anergized T cells are fully impaired in their activation of NF-κB but have only a small impairment in LAT and PLCγ1 phosphorylation. A, Control and anergized T cells were activated for 1 min with varying concentrations of anti-TCRβ-biotin mAb (0, 1, or 10 μg/ml) and anti-CD4-biotin at either 0 or 10 μg/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-PLCγ1 Ab specific for tyrosine phosphorylation at site 783. The blot was then stripped and reprobed with anti-PLCγ1. The relative values for PLCγ1 783 phosphorylation are expressed in the box on the lower left, and the values normalized to the total level of PLCγ1 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-TCRβ-biotin mAb (10 μg/ml) and anti-CD4-biotin mAb (10 μg/ml) and cross-linked with streptavidin for 2 min. PLCγ1 was immunoprecipitated from cell lysates with anti-PLCγ1, and the membrane was probed with anti-phosphotyrosine and then reprobed for total PLCγ1. D, Control and anergized A.E7 T cells were activated with anti-TCRβ-biotin (10 μg/ml) and anti-CD4-biotin (10 μg/ml) for various times after streptavidin cross-linking. Whole cell extracts were run on a Western blot and probed with anti-λ5-actin. 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 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 developmental model in which 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 PLCγ1 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 PLCγ1 phosphorylation as the possible cause for this impairment. In ionomycin-induced anergy PLCγ1 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 PLCγ1 in resting cells. Its overexpression results in both decreased IL-2 production and diminished phospholipid mobilization (59). The PLCγ1 defect may also account for the impairment of the NF-κB pathway observed by us and others (60, 61) through a limited production of DAG required for PKCζ activation. Alternatively, an increase in the E3 ubiquitin ligase Itch may target PKCζ 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 PLCγ1 (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 TCRζ (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 IκBα and no activation of the NF-κB 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 PLCγ1. This, in turn, leads to a profound impairment in the activation of the NF-κB 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 TCR cross-linking and no impairment. This pattern is very different from what has been 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 PLCγ1/calcium pathway. In light of our results, we would suggest that the latter models represent a combination 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 IκBα 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-κB 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-TCRβ-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-κB activation by stimulating PKCθ 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 nonresponsiveness) (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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