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Anergic CD8$^+$ T Lymphocytes Have Impaired NF-κB Activation with Defects in p65 Phosphorylation and Acetylation

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Because of the cytotoxic potential of CD8$^+$ T cells, maintenance of CD8$^+$ peripheral tolerance is extremely important. A major peripheral tolerance mechanism is the induction of anergy, a refractory state in which proliferation and IL-2 production are inhibited. We used a TCR transgenic mouse model to investigate the signaling defects in CD8$^+$ T cells rendered anergic in vivo. In addition to a previously reported alteration in calcium/NFAT signaling, we also found a defect in NF-κB–mediated gene transcription. This was not due to blockade of early NF-κB activation events, including IκB degradation and NF-κB nuclear translocation, as these occurred normally in tolerant T cells. However, we discovered that anergic cells failed to phosphorylate the NF-κB p65 subunit at Ser^{311} and also failed to acetylate p65 at Lys^{310}. Both of these modifications have been implicated as critical for NF-κB transactivation capacity, and thus, our results suggest that defects in key phosphorylation and acetylation events are important for the inhibition of NF-κB activity (and subsequent T cell function) in anergic CD8$^+$ T cells. ***The Journal of Immunology*****, 2012, 188: 1213–1221.**

CD8$^+$ T cells are integral in host defense response against both intracellular pathogens and cells presenting abnormal cell surface molecules (1). The process of V(D)J somatic recombination generates the necessary diversity in the TCR to allow recognition of a tremendous range of foreign Ags, but also allows the generation of autoreactive T cells with receptors specific for self-Ags. Because of the cytotoxic nature of CD8$^+$ T effector cells, and the fact that their MHC class I/peptide ligands can be expressed on nearly all cells of the body, control of self-reactive CD8$^+$ T cells must be very stringent. Most self-reactive T cells are deleted during development in the thymus (central tolerance), but some are able to exit the thymus and enter the circulation. Autoreactive T cells in the periphery are regulated by a group of mechanisms collectively referred to as peripheral tolerance, including deletion, suppression by regulatory cells, and T cell anergy (2–5).

Anergy is a form of cellular hyporesponsiveness characterized by reduced proliferation and IL-2 production (6, 7). Anergic T cells have been reported to show defects in a variety of signaling pathways downstream of TCR stimulation. Although CD4$^+$ T cell tolerance has been studied extensively, far less is understood about the signaling alterations in anergic CD8$^+$ T cells. We and others have previously reported that anergic CD8$^+$ T cells have defective calcium signaling (8, 9), leading to altered regulation of NFAT family member activation (9). Other pathways that have been implicated in CD8$^+$ T cell anergy include the Ras/MAPK/AP-1 pathway (10, 11) and the activation of NF-κB (10). However, the use of different systems makes it unclear how these defects may cooperate to result in the anergic phenotype.

Activation of NF-κB is well established to be a critical step in the induction of IL-2 gene transcription by T cells. The NF-κB family is composed of at least five members, which can form hetero- and homodimers. The predominant form of NF-κB in T cells is a heterodimer of the p50 and p65 (RelA) subunits. In resting cells, the NF-κB dimers are sequestered in the cytosol by IκB proteins (12). Stimulation through the TCR and costimulatory receptors leads to IκB kinase (IKK)-mediated phosphorylation of IκBα (13–15), targeting it for ubiquitination and degradation (16). Degradation of IκBα exposes the nuclear localization sequence on NF-κB, allowing for translocation into the nucleus, where NF-κB can initiate gene transcription (17). However, it is becoming clear that release from IκB is insufficient to allow full activation of NF-κB. Notably, a series of posttranslational modifications of NF-κB proteins after IκB degradation appears to regulate nuclear localization, DNA binding, and transcriptional transactivation (18, 19). Phosphorylation of at least two serine residues (Ser^{276} and Ser^{311}) has also been shown to be important for interaction of NF-κB with the histone acetyltransferase (HAT) CREB-binding protein (CBP)/p300 (20, 21). This interaction allows the recruitment of HATs to the promoters of NF-κB target genes and facilitates the initiation of transcription (21, 22).

Using an in vivo TCR transgenic model that we have previously described (9, 23), we found that expression of NF-κB target genes is impaired in anergic CD8$^+$ T cells. Early events in NF-κB activation appear to be normal, including nuclear translocation, but anergic T cells show defects in phosphorylation of Ser^{311}. This correlates with the absence of Lys^{310} acetylation, which has been shown to be critical for NF-κB transactivation function. These results provide mechanistic underpinning for NF-κB defects seen in anergic T cells, and also suggest pathways that may be novel targets for the regulation of T cell tolerance.

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Abbreviations used in this article: CBP, CREB-binding protein; HAT, histone acetyltransferase; IKK, IκB kinase; PB, permeabilization buffer; PBS-T, PBS plus 0.1% Tween 20; PKC, protein kinase C; qPCR, quantitative real-time PCR.

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Materials and Methods

Abs and reagents

The H-2K^b–restricted 2C TCR-reactive peptide SIYRYYGL was purchased from NeoMPS (San Diego, CA). Anti-CD3 (mAb 145-2C11), anti-CD28 (mAb 37.51), control hamster IgG, PE-conjugated anti-Vβ8, PE conjugated anti-Thy 1.2, and FITC-conjugated anti-CD8α were purchased from eBioscience (San Diego, CA). Goat anti-hamster IgG was purchased from Pierce (Rockford, IL). Anti-IκBα, anti-actin, anti–NF-κB-p65, anti–phospho-p65 (Ser^{311}), and anti-lamin A/C Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–phospho-p65 (Ser^{536}) and anti–phospho-p65 (Ser^{276}) Abs were purchased from Cell Signaling Technologies (Danvers, MA). Anti–α-tubulin Ab was purchased from Sigma-Aldrich (St. Louis, MO). Anti–Ac-p65 (Lys^{310}) Ab was purchased from Abcam (Cambridge, MA). HRP-conjugated anti-mouse IgG and anti-rabbit IgG Abs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Mice

The 2C TCR transgenic/RAG$^{-/-}$ mice have been described previously (9). NF-κB-Luc transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and crossed with 2C TCR transgenic/RAG$^{+/+}$ mice. C57BL/6J mice (6–8 wk old) were purchased from The Jackson Laboratory. All mice were maintained in ventilated M.I.C.E. microisolator cages (Animal Care Systems, Littleton, CO) at the University of Maryland animal facility (College Park, MD). Animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (Bethesda, MD). All of the mice were euthanized by carbon dioxide inhalation, as recommended by the American Veterinary Medical Association Panel on Euthanasia.

Cell culture

All cells were maintained in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% FBS (HyClone, Logan, UT), 2 mM glutamine, penicillin/streptomycin, 10 mM HEPES buffer, and 55 μM 2-ME at 37°C in a 5% CO_2 atmosphere.

In vivo anergy induction

Anergy was induced in 2C TCR transgenic mice, and splenic T cells were purified, as described previously (9). Anergy was confirmed by proliferation assay and/or IL-2 ELISA, as previously described (9).

T lymphocyte stimulation

Purified primary T lymphocytes were stimulated using soluble anti-mouse CD3 and anti-mouse CD28 Abs. Briefly, cells were incubated with 10 μg/ml each of anti-CD3 and anti-CD28 Abs on ice for 30 min and then incubated at 37°C for appropriate time points with 10 μg/ml goat anti-Syrian hamster IgG (Pierce) as secondary cross-linking Ab. Reactions were stopped by adding ice-cold PBS. For luciferase assays, purified T lymphocytes were stimulated using magnetic beads conjugated to anti-CD3 and anti-CD28 Abs, prepared as described previously (24).

ELISA

Culture supernatants were collected 36 h after T lymphocyte stimulation, and IL-2 and IFN-γ levels were determined by sandwich ELISA. Primary and biotin-conjugated secondary Abs and recombinant cytokine standards were purchased from eBioscience and used at the concentrations recommended by the manufacturer. Alkaline phosphatase-conjugated streptavidin was purchased from Jackson ImmunoResearch Laboratories and used at 1:3000 dilution. Colorimetric alkaline phosphatase substrate was purchased from Sigma-Aldrich and used at 1 mg/ml in 10% diethanolamine buffer. Quantification was performed on a Versamax spectrophotometer (Molecular Devices, Sunnyvale, CA), and data were analyzed using Softmax Pro software (Molecular Devices). Data points are presented as the mean of triplicate wells ± SD.

PCR

Total RNA was isolated from T cells using the NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA) as per the manufacturer's instructions, with the slight modification that samples were incubated with DNase for 45 min instead of 15 min. cDNA was generated using the iScript reverse-transcriptase kit (Bio-Rad, Hercules, CA). IκBα primers have been described previously by others (25), and 18S PCR primers were designed using Beacon Designer software (Premier Biosoft International, Palo Alto, CA). Primer sequences were as follows: IκBα, forward, 5′-GCTCTAGAGCAATCATCCACGAAGAGAA-3′ and reverse, 5′-CGGAATTCGCCCCACATTTCAACAAGAG-3′; 18S, forward, 5′-ATGCGGCGGCGTTATTCC-3′ and reverse, 5′-GCTATCAATCTGTCAATCCTGTCC-3′.

Quantitative real-time PCR (qPCR) was performed using the iCycler iQ system (Bio-Rad) with iQ SYBR Green Supermix reagents (Bio-Rad) or SensiMix SYBR and Fluorescein kit (Bioline, Taunton, MA). Data were analyzed using MyiQ software (Bio-Rad). The presence of a single PCR product was confirmed by melt curve analysis. Fold induction was obtained using the ΔΔCt method using 18S rRNA as the reference. Data points are presented as the means of triplicate wells ± SD.

Luciferase assay

Purified T lymphocytes (3×10^6/sample) were stimulated at 37°C for 48 h with anti-CD3/anti-CD28–conjugated magnetic beads at a bead:cell ratio of 3:1. Samples were washed twice with PBS, and cell pellets were resuspended in supplemented RPMI 1640 without phenol red at a density of 1×10^7 cells/ml. Luciferase activity was assessed by adding an equal volume of Bright-Glo Luciferase Assay System Reagent (Promega, Madison, WI) to each sample and incubating samples at 23°C for 15 min. Samples were loaded in triplicate in Optiplate 96-well plates (PerkinElmer, Shelton, CT). Luciferase activity was recorded using a 1450 Microbeta Trilux scintillation counter (Wallac, Turku, Finland) in luminometer mode. Data points are presented as the mean of triplicate wells ± SD.

Western blots

Cells were stimulated for the appropriate time points and then lysed with lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 1 mM Na_3VO_4 in PBS). Proteins were fractionated by SDS-PAGE on a 12% gel and electrotransferred onto nitrocellulose membranes. Approximately 1×10^6 cell equivalents were loaded per well. The membranes were blocked overnight with 5% nonfat dried milk in PBS plus 0.1% Tween 20 (PBS-T). The membranes were probed with primary Ab diluted in PBS-T and then incubated with HRP-conjugated secondary Abs (1:10,000 in PBS-T). For primary Abs, anti-IκBα, anti–NF-κB-p65, anti–phospho-p65 (Ser^{311}), and anti-lamin A/C were diluted 1:200 in PBS-T; anti–phospho-p65 (Ser^{536}) and anti–phospho-p65 (Ser^{276}) were diluted 1:1000 in PBS-T plus 5% BSA; anti–Ac-p65 (Lys^{310}) was diluted 1:1,000; anti-tubulin was diluted 1:10,000. Specific bands were visualized using SuperSignal West Pico Chemiluminiscent substrate (Pierce).

Cellular fractionation

A cellular fractionation protocol was modified from Park et al. (26). Briefly, stimulated cells were lysed on ice for 15 min using hypotonic lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, Complete Mini protease inhibitor mixture [Roche Diagnostics, Basel, Switzerland], 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4), at a concentration of 5×10^7 cells/ml. Triton X-100 was added to each tube to a final concentration of 1%, and cells were incubated on ice for 10 min. Lysates were centrifuged at $16,000 \times g$ for 1 min at 4°C, and supernatant was saved as cytosolic fraction. Pellets were washed one time with hypotonic lysis buffer for 5 min and then centrifuged at $16,000 \times g$ for 1 min at 4°C. The remaining pellet was resuspended using nuclear extraction buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, Complete Mini protease inhibitor mixture, 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4) at a concentration of 1×10^8 cells/ml, and incubated at 4°C for 30 min with constant agitation. Samples were centrifuged at $16,000 \times g$ for 5 min at 4°C, and supernatant was saved as nuclear fraction. For experiments measuring p65 acetylation, both hypotonic lysis buffer and nuclear extraction buffer were supplemented with 200 nM trichostatin A (Sigma-Aldrich). For Western blots, 1×10^6 cell equivalents of cytosolic extract or 2×10^6 cell equivalents of nuclear extract were loaded per lane.

Immunofluorescence microscopy

An immunofluorescence protocol was modified from Srinivasan and Frauwirth (9). Briefly, stimulated cells were mounted on poly–L-lysine microscope slides (Polysciences, Warrington, PA) and fixed for 15 min at 4°C with ice-cold methanol. Cells were then permeabilized and blocked by incubation on ice for 20 min with permeabilization buffer (PB), followed by 1-h incubation on ice with anti-p65 Ab at a concentration of 4 μg/ml. After incubation, cells were washed three times with PB and incubated on ice for a further hour with 2 μg/ml AlexaFluor 594-linked anti-IgG (Molecular Probes, Eugene, OR) as a secondary Ab. After washing the cells three times with PB, the samples were incubated at room temperature for 20 min with SYTO-13 (Molecular Probes) in the dark, washed twice with PB, and postfixed at room temperature for 10 min with 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA). Cells

were washed with PBS, and coverslips were fixed onto the slide using Aqueous Mounting Media (Biomeda, Foster City, CA). Cells were analyzed on a LSM 510 confocal microscope (Carl Zeiss Microimaging, Thornwood, NY) or a Leica SP5 X confocal microscope (Leica Microsystems, Bannockburn, IL). Confocal data were analyzed using Zeiss LSM image browser (Carl Zeiss Microimaging), and the degree of nuclear colocalization was ascertained using the colocalization tool in the Leica Application Suite AF software (Leica Microsystems).

Statistical analysis

All of the statistical analyses were performed using Prism software, version 5 (GraphPad, San Diego, CA). The minimal level of confidence at which experimental results were considered significant was $p < 0.05$. Statistical significance for time course data was determined by one-way ANOVA with Bonferroni posttest analysis. Statistical significance for ELISA and luciferase data were determined by unpaired two-tailed *t* test.

Results

Activation of NF-κB is defective in anergic CD8$^+$ T lymphocytes

As we have demonstrated previously (9, 23), injection of antigenic peptide into 2C TCR transgenic mice induces a tolerant state in CD8$^+$ T cells, inhibiting IL-2 production (Fig. 1*A*) and proliferation (data not shown) by 90% or more. In this same system, we have shown that the regulation of NFAT transcription factor activation is altered in anergic CD8$^+$ T cells (9). NF-κB activity has also been found to be defective in anergic CD8$^+$ T cells (10), although the precise mechanism has not been well defined. Notably, we have found that IFN-γ production is only modestly inhibited in anergic CD8$^+$ T cells (Fig. 1*B*) (9), suggesting that NF-κB targets may not be affected equally. To examine NF-κB activity in our previously established in vivo anergy model system, we crossed 2C TCR transgenic mice with NF-κB-Luc mice (27). The NF-κB-Luc mice contain a luciferase reporter transgene controlled by two NF-κB–responsive elements from the κB L chain enhancer upstream of a minimal Fos promoter (27). This allows quantification of NF-κB activity by measuring luciferase activity. Anergy was induced in 2C TCR/NF-κB-Luc mice by injection of antigenic peptide, and CD8$^+$ T cells were purified from naive and anergized mice. As shown in Fig. 2, anergic T cells showed substantially reduced luciferase activity after CD3/CD28 stimulation, indicating a defect in NF-κB function.

IκBα degradation is normal in anergic T cells, but its resynthesis is impaired

A critical early step in NF-κB activation is the degradation of the inhibitory protein IκB (28). To determine whether the NF-κB defect in anergic cells was due to a failure of this step, we stimulated naive and anergic T cells in vitro and examined IκBα levels. As seen in Fig. 3*A* (*left panel*), CD3/CD28 stimulation of naive T cells induced degradation of IκBα within 5 min. IκBα is resynthesized in response to NF-κB activation, providing a negative feedback loop (29, 30), and this can be seen by 30 min after stimulation. Anergic T cells show a pattern of IκB degradation that is similar to that of naive cells (Fig. 3*A*, *right panel*). However, we noticed that IκBα protein levels did not recover in anergic T cells. This is consistent with the NF-κB activity defect, as IκBα transcription is induced by NF-κB (29). To confirm that the failure to restore IκB protein was due to a defect in gene expression (as opposed to prolonged degradation, for example), we examined IκBα mRNA levels by standard PCR (Fig. 3*B*) and qPCR (Fig. 3*C*). Stimulation of naive T cells induced IκBα mRNA expression within 15 min, but this was significantly impaired in anergic T cells. Thus, NF-κB activity in anergic T cells is defective, despite normal degradation of IκBα.

p65 nuclear translocation is normal in anergic T cells

Degradation of IκB releases NF-κB from sequestration in the cytosol and allows translocation to the nucleus. Because IκBα was degraded normally in anergic T cells, we asked whether nuclear localization of NF-κB also occurred normally. The major form of NF-κB in T cells is a heterodimer of p50 and p65 (31), so we used cellular fractionation to analyze the location of p65. As shown in Fig. 4*A*, p65 was undetectable in the nuclear fraction of resting T cells, and it appeared in the nucleus with comparable kinetics in naive and anergic T cells. Similar results were found using immunofluorescence microscopy to track p65 localization in naive and anergic cells (Fig. 4*B*, quantified in Fig. 4*C*). Thus, despite a defect in NF-κB transcriptional activity, the early steps of NF-κB

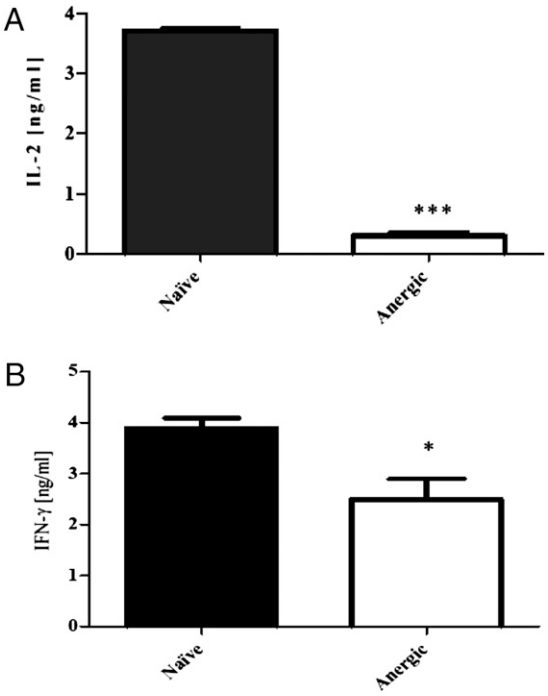

FIGURE 1. CD8$^+$ T lymphocytes from peptide-injected mice are anergic. Purified naive and anergic T lymphocytes were stimulated for 36 h, and supernatants were collected. IL-2 secretion (*A*) and IFN-γ secretion (*B*) were determined by ELISA. Data are representative of three independent experiments. *$p < 0.001$, different from naive, ***$p<0.001$, different from naive.

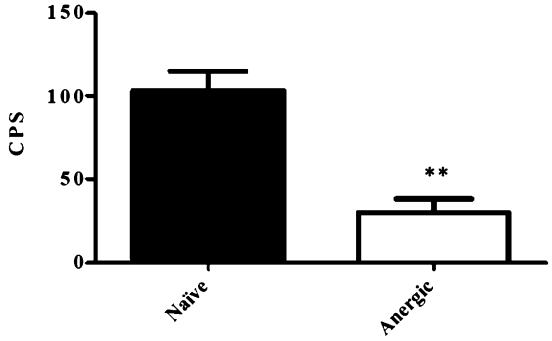

FIGURE 2. Anergic T lymphocytes display deficient NF-κB–dependent transcription. Purified naive and anergic 2C TCR/NF-κB-Luc transgenic T lymphocytes were stimulated for 48 h with anti-CD3– and anti-CD28–conjugated beads, and luciferase activity was analyzed. Data are representative of three independent experiments. **$p < 0.001$, different from naive.

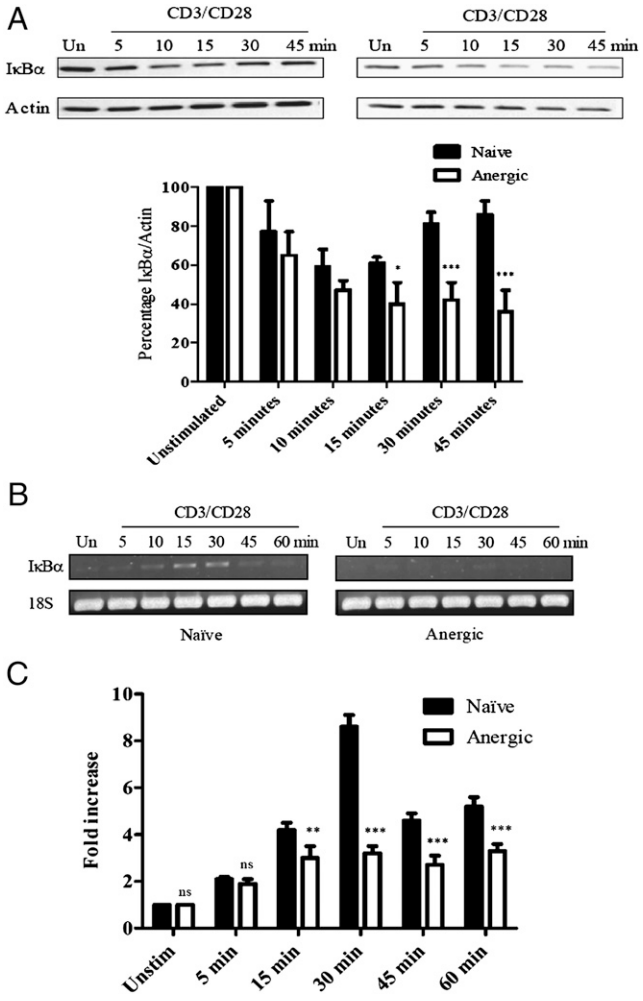

FIGURE 3. Anergic T cells show normal degradation, but impaired re-expression, of IκBα. *A*, Purified naive and anergic 2C TCR transgenic T cells were stimulated with anti-CD3 and anti-CD28 Abs for the indicated times, and IκBα levels were determined by Western blot. The relative intensity of the bands is defined as the ratio of intensity of IκBα to actin. Graphical data represent an average of three independent experiments. *B* and *C*, Purified naive and anergic cells were stimulated, as in *A*, for the indicated times, and IκBα mRNA levels were compared by RT-PCR (*B*) and reverse-transcription qPCR (*C*). All data are representative of three independent experiments. $*p < 0.001$, different from naive, $**p < 0.001$, different from naive, $***p < 0.0001$, different from naive. ns, Not significantly different from naive.

activation, including IκBα degradation and p65 nuclear translocation, are intact in anergic T cells.

Anergic T cells have defective phosphorylation of NF-κB p65 at Ser^{311}

It is becoming clear that NF-κB function is regulated not only by cellular localization, but also by a variety of posttranslational covalent modifications [reviewed in (18, 19)]. Phosphorylation of several serine residues in p65 has been shown to be particularly important for full activation of NF-κB. We therefore examined whether p65 was aberrantly phosphorylated in anergic T cells.

Phosphorylation of p65 Ser^{536} by the IKK complex occurs with timing similar to IκBα phosphorylation (32–35), and has been suggested to influence the kinetics of p65 nuclear import (33). As seen in Fig. 5*A*, CD3/CD28 stimulation of naive T cells rapidly induced phosphorylation of p65 Ser^{536} in the cytosol. We observed that p65 is constitutively phosphorylated at this serine residue in both naive and anergic cells, and that stimulation leads to hyperphosphorylation. The phosphorylation of Ser^{536} in anergic T cells was essentially identical to that in naive cells, consistent with the normal kinetics of p65 nuclear translocation. We next tested phosphorylation of p65 Ser^{276}, which has been reported to be controlled by protein kinase A in the cytosol (36) and mitogen- and stress-activated protein kinase-1 in the nucleus (37). Interference with this phosphorylation inhibits NF-κB gene transactivation without affecting nuclear localization (37), making it an attractive candidate for the defect in anergic T cells. However, induction of Ser^{276} phosphorylation in anergic cells was comparable to that in naive cells (Fig. 5*B*). Thus, phosphorylation of both Ser^{536} and Ser^{276} appears to be regulated normally in anergic T cells.

A third phosphorylation event that has been shown to be important for NF-κB activity involves Ser^{311}. This residue has been found to be phosphorylated by the protein kinase C (PKC) ζ isoform, and, like Ser^{276}, regulates gene transactivation without affecting nuclear localization (21). Unlike the phosphorylations at Ser^{536} and Ser^{276}, phosphorylation of Ser^{311} was clearly impaired in anergic T cells (Fig. 5*C*). Thus, the NF-κB defect in anergic cells correlates with a failure to phosphorylate p65 Ser^{311}.

Acetylation of NF-κB p65 is impaired in anergic cells

It has been shown that phosphorylation of p65 at Ser^{311} is required for the recruitment of the CBP/p300 transcriptional coactivators (21). These transcriptional coactivators have been observed to acetylate several transcription factors, including the p65 subunit of NF-κB (38–40). It has been suggested that acetylation plays an important role in regulating DNA binding and transcriptional activity (38). Because we observed a defect in the phosphorylation of p65 at Ser^{311} in anergic T cells, we decided to examine the acetylation of p65. We found that p65 is acetylated at Lys^{310} in nuclear fractions of naive cells after 30 min of CD3/CD28 stimulation (Fig. 6, *upper panel*). In contrast, p65 Lys^{310} was not acetylated in anergic cells (Fig. 6, *lower panel*). Taken together, these results suggest that the functional defect in the activation of NF-κB in anergic CD8$^+$ T cells is due to impaired phosphorylation of Ser^{311} and acetylation of Lys^{310} (Fig. 7).

Discussion

Using a TCR transgenic mouse model of in vivo tolerance, we previously showed that anergic CD8$^+$ T cells have defects in calcium signaling and altered NFAT regulation (9). Given the likelihood that multiple signal transduction pathways would be affected by tolerance induction, we extended these studies and examined the regulation of another transcription factor family known to be essential for IL-2 synthesis, namely NF-κB. Using a NF-κB–driven luciferase reporter gene, we found that in vivo tolerance induction of CD8$^+$ 2C TCR transgenic T cells led to strong inhibition of NF-κB transcriptional activity. Several other groups have also reported defects in NF-κB activation in anergic T cells (10, 41–43), but there is a lack of consensus on the mechanism behind the defects. Increased expression of p50-p50 homodimers (10, 41, 42), expression of noncanonical p65-containing dimers (e.g., p65-p65 and p65-c-Rel) (10), and defects in IκB degradation (41, 43) have all been suggested to be at least partly responsible for impaired NF-κB function. These differences may be due to the use of different systems (CD4$^+$ versus CD8$^+$ T cells, different modes of anergy induction, etc.) or may simply reflect small pieces of a larger whole. Indeed, we have found that production of IL-2 is decreased in anergic T lymphocytes, whereas production of IFN-γ shows only a moderate decrease (Fig. 1) (9). Because both IL-2 and IFN-γ are established

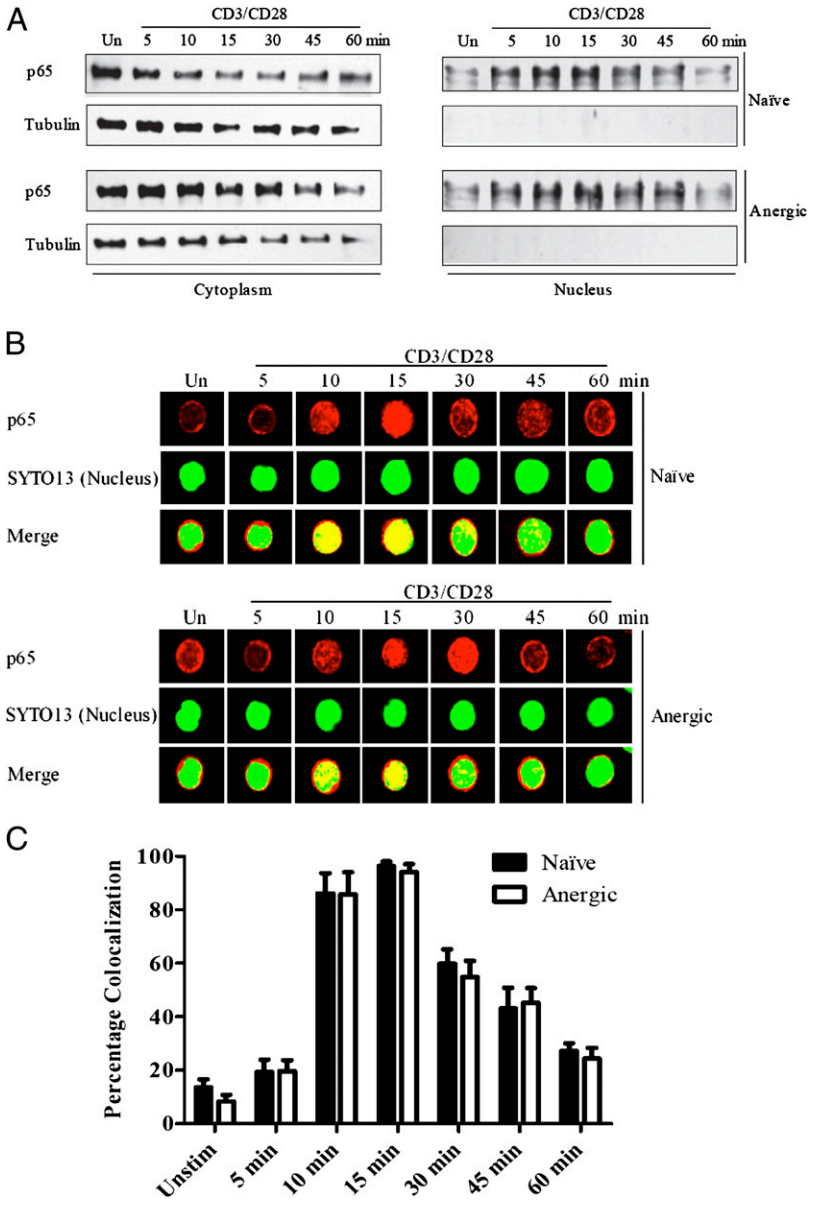

FIGURE 4. NF-κB p65 translocates to the nucleus normally in anergic cells. *A*, Purified T lymphocytes were stimulated for the indicated time points, and cytoplasmic and nuclear fractions were collected and resolved by SDS-PAGE. Proteins were then analyzed by Western blot using anti-p65 Ab. Blots were reprobed with anti-tubulin Ab as a loading control for cytosolic fractions and to confirm the purity of nuclear fractions. *B*, Naive (*upper panels*) and anergic (*lower panels*) T lymphocytes were stimulated for the indicated time points, and NF-κB p65 localization was determined by immunofluorescence microscopy. Cells were costained with the nuclear dye SYTO-13. *C*, Quantification of p65 nuclear localization shown in *B*. Data show mean percentage of p65/SYTO-13 colocalization of 15 individual cells ± SD and are representative of three independent experiments.

NF-κB targets, our data suggest that different NF-κB targets are differentially regulated. Current models of NF-κB gene regulation propose that NF-κB targets do not comprise a single uniform group, but rather show different patterns of regulation by NF-κB (44). Our data therefore indicate that anergy may inhibit NF-κB stimulation of certain targets, such as IL-2 and IκBα, whereas other targets, including IFN-γ, are relatively unaffected. We therefore asked what was responsible for the selective NF-κB activity defect in our model of $CD8^+$ T cell anergy.

A key event in the activation of NF-κB is the degradation of the inhibitory protein IκBα. We observed that IκBα was degraded with the same kinetics in naive and anergic T cells. This is distinct from the findings of Grundström et al. and Chiodetti et al. (41, 43), both of whom saw inhibition of IκB degradation in anergic T cells. It is notable that both of these groups analyzed anergy in $CD4^+$ T cells. It is therefore possible that blockade of IκB degradation is a feature of $CD4^+$ T cell anergy, but is less important for regulation of NF-κB in $CD8^+$ T cell anergy. We further found that anergic T cells failed to resynthesize IκBα mRNA or protein. This is consistent with the known role for NF-κB in regulating IκB gene transcription as part of a negative feedback loop (29, 30), and further demonstrates the defect in NF-κB transcriptional activity in anergic cells.

Degradation of IκB releases the NF-κB transcription factor from the cytosol and allows it to be imported into the nucleus. Because nuclear localization is critical for NF-κB function, we speculated that NF-κB might still fail to translocate into the nucleus, despite degradation of IκBα. NF-κB retention in the cytosol is mediated by multiple IκB family members (30), and so misregulation of one of these other inhibitory proteins might still block nuclear localization. However, we also found no defects in degradation of IκBβ in anergic cells (data not shown), and saw that TCR/CD28 stimulation induced nuclear localization of NF-κB p65 equivalently in naive and anergic cells. Thus, the early events of NF-κB activation, including IκB degradation and NF-κB nuclear translocation, are intact in anergic $CD8^+$ T cells. This is illustrated in Fig. 7, and indicates that there is another mode of regulation for NF-κB in anergic $CD8^+$ T cells.

It has been observed that coincident with or immediately following IκBα degradation, p65 is phosphorylated at multiple residues, and these phosphorylation events are necessary for proper regulation of NF-κB function (45). The phosphorylation patterns

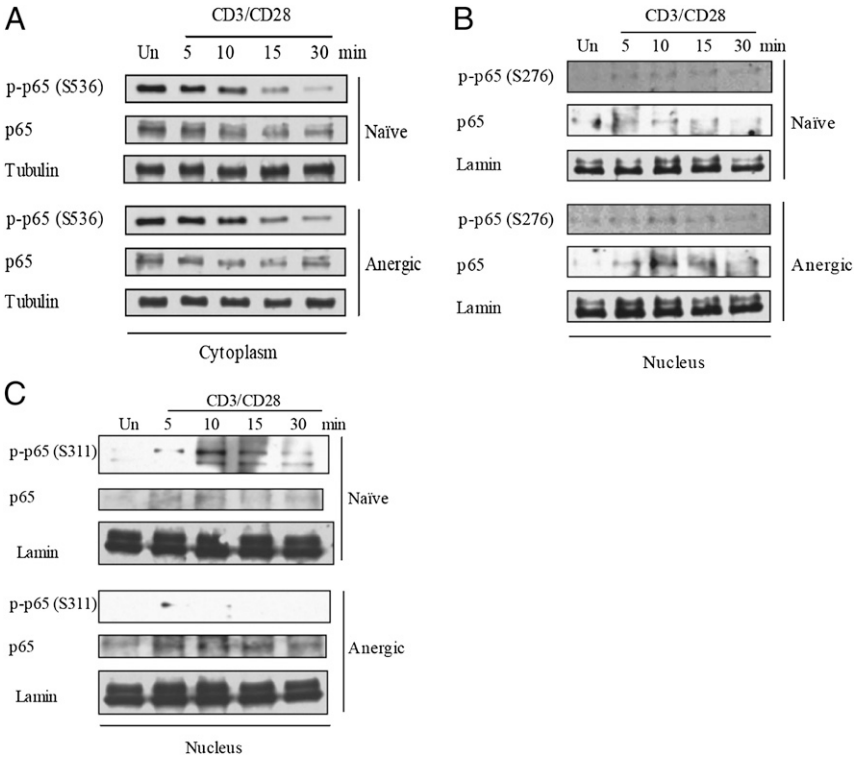

FIGURE 5. Phosphorylation of p65 at Ser^{311} is defective in anergic cells. Purified naive and anergic T lymphocytes were stimulated with anti-CD3 and anti-CD28 Abs for the indicated times. Cytosolic and nuclear fractions were purified and resolved by SDS-PAGE. Proteins were then analyzed by Western blot using anti–phospho-p65 (Ser^{536}) Ab (*A*), anti–phospho-p65 (Ser^{276}) Ab (*B*), or anti–phospho-p65 (Ser^{311}) Ab (*C*). Blots were stripped and reprobed with anti-total p65 and then with anti-tubulin Ab (*A*) or anti-lamin Ab (*B*, *C*) as a loading control for cytosolic fractions or nuclear fractions, respectively. All data are representative of three independent experiments.

of NF-κB proteins have not been characterized in T cell anergy, and so we asked whether aberrant phosphorylation was responsible for the defects in NF-κB function in anergic cells. An early step involves phosphorylation of p65 at Ser^{536} by the IKK complex (32–35), and it has been suggested that phosphorylation at this residue negatively regulates the kinetics of p65 nuclear translocation (33). We found that p65 is phosphorylated at Ser^{536} equivalently in both naive and anergic cells, which is consistent with our finding that p65 translocates to the nucleus with normal kinetics in anergic T cells. A second posttranslational modification important for NF-κB activity is phosphorylation at Ser^{276}. We found that, as with Ser^{536} phosphorylation, p65 is phosphorylated at Ser^{276} equivalently in both naive and anergic CD8$^+$ T lymphocytes after TCR/CD28 stimulation. Thus, although these two phosphorylation steps have previously been shown to be critical for proper activation of NF-κB, they are not differentially regulated in responsive and anergic CD8$^+$ T cells.

A third phosphorylation site that has been found to be important for NF-κB transcriptional activity is p65 Ser^{311} (21). We therefore examined p65 Ser^{311}, and found that it is rapidly phosphorylated after naive T cell stimulation. We only detected phospho-Ser^{311} in nuclear p65, and thus hypothesize that phosphorylation occurs after nuclear translocation. However, we cannot rule out the possibility that Ser^{311} can be phosphorylated in the cytosol, with the kinetics of p65 nuclear translocation preventing its detection. Strikingly, phosphorylation of Ser^{311} was abrogated in anergic T cells, suggesting that the NF-κB activation defect may be due to a loss in p65 Ser^{311} phosphorylation (see Fig. 7).

Ser^{311} is part of a conserved PKC target sequence, and has been identified as a target for the atypical PKC isoform ζ. PKCζ can phosphorylate this site directly in vitro (21), and overexpression of PKCζ in the Jurkat human T leukemia cell line enhances p65 transcriptional activity (46). Conversely, PKCζ deficiency inhibits p65 Ser^{311} phosphorylation and NF-κB function in embryonic fibroblasts. Surprisingly, however, PKCζ-deficient mice show normal thymic development (47), and T cells from these mice proliferate normally when stimulated in vitro (48). PKCζ-deficient T cells do show impaired Th2 differentiation, but this appears to be due to defects in IL-4R/JAK1/STAT6 signaling, rather than defects in TCR-induced NF-κB signaling (49). Together, these results suggest that PKCζ is not the physiological kinase for p65 Ser^{311} downstream of TCR/CD28 stimulation in T cells. We hypothesize instead that a different PKC isoform plays this role. Knockouts of PKCβ, ε, and λ have no defects in T cell activation (50–52), whereas PKCδ-deficient T cells show enhanced prolif-

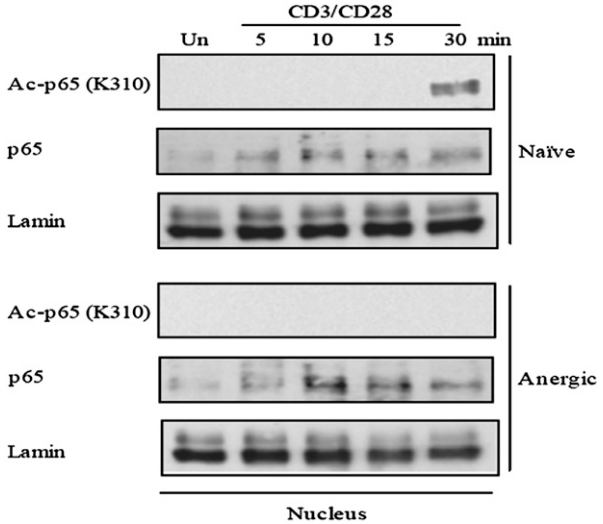

FIGURE 6. Acetylation of p65 at Lys^{310} is defective in anergic cells. After purification, T lymphocytes were stimulated for the indicated time points, and nuclear fractions were collected and resolved by SDS-PAGE. Proteins were analyzed by Western blot using anti–acetyl-p65 (Lys^{310}) Ab. Blots were stripped and reprobed with anti-total p65 and then with anti-lamin A/C Ab as a loading control for nuclear fractions. All data are representative of three independent experiments.

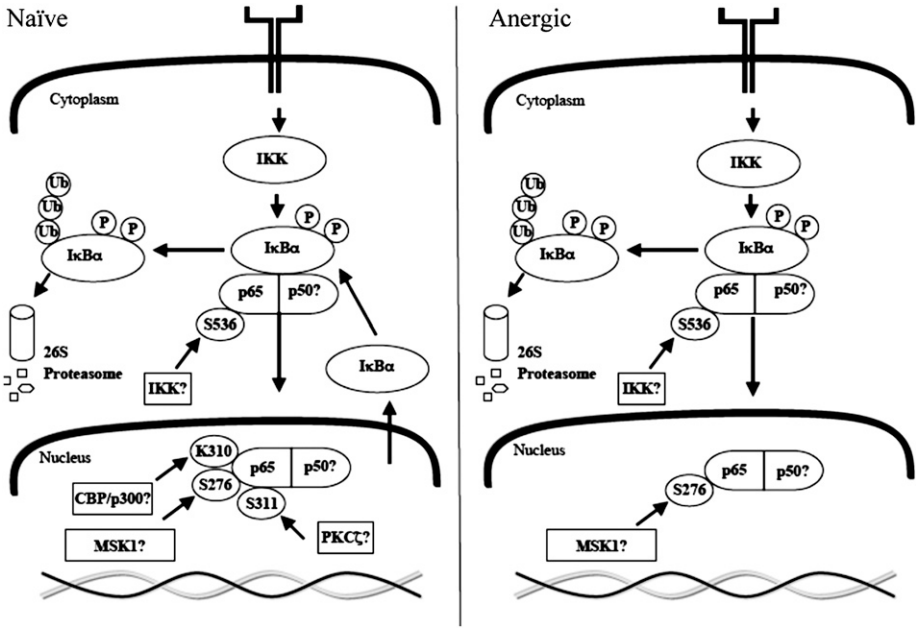

FIGURE 7. Schematic model of NF-κB signaling pathway in naive and anergic $CD8^+$ T lymphocytes. In naive cells (*left panel*), stimulation of the TCR causes the IKK-mediated phosphorylation of IκBα, which is subsequently ubiquitinated and degraded. This allows the phosphorylation of p65 at Ser^{536} and translocation into the nucleus. Once in the nucleus, p65 is phosphorylated at Ser^{276} and Ser^{311} and acetylated at Lys^{310}. In anergic cells (*right panel*), degradation of IκB, phosphorylation at Ser^{536} and Ser^{276}, and nuclear translocation occur normally. By contrast, p65 is not phosphorylated at Ser^{311} nor acetylated at Lys^{310} in anergic T cells.

eration and IL-2 production (53), making it unlikely that any of these isoforms is required for activation of NF-κB. PKCα-deficient T cells have reduced proliferation in response to various stimuli, but show normal IL-2 production (54), and so PKCα is also probably not the relevant kinase. One attractive candidate is PKCθ, which is known to be required for NF-κB activation in mature T cells (55, 56). However, PKCθ appears to be important for activation of the IKK complex, and thus degradation of IκB (57, 58), making it difficult to determine whether it is also important for later phosphorylation of p65 Ser^{311}. Identification of the p65 Ser^{311} kinase may therefore require the development of creative new approaches to separate the role of PKCθ in IKK activation from potential roles in downstream NF-κB activation events.

Understanding the mechanisms by which p65 phosphorylation regulates transcriptional activation remains a work in progress. Phosphorylation of Ser^{276} or Ser^{311} (see Fig. 7) enhances NF-κB–mediated gene transcription without affecting nuclear localization or DNA binding (21, 37), suggesting regulation of recruitment or assembly of the transcriptional initiation complex. Indeed, both phosphorylations have been shown separately to be important for association with HATs such as CBP and p300 (20, 22, 37), indicating a role in chromatin remodeling. It remains unclear whether either phosphorylation is sufficient, or if the two cooperate in HAT recruitment. Association of p65 with HATs appears to be required not only for histone modification, but also for acetylation of NF-κB itself. Nuclear p65 is regulated by multiple lysine acetylations, with both positive and negative effects (38, 39, 59). We were particularly interested in the acetylation of p65 Lys^{310} due to its proximity to Ser^{311}. Acetylation of Lys^{310} is required for full NF-κB transactivation function, but does not regulate nuclear localization or DNA-binding activity (38). It has been shown to be dependent on phosphorylation of Ser^{276} and Ser^{536} (40), but a role for Ser^{311} in induction of Lys^{310} acetylation has not been reported. We found that stimulation of naive T cells led to acetylation of Lys^{310} on nuclear p65, and that acetylation occurred substantially later than Ser^{311} phosphorylation (see Figs. 6, 7), consistent with a requirement for phosphorylation at Ser^{311} prior to acetylation. Conversely, acetylation of Lys^{310} was absent in anergic T cells, also supporting the hypothesis that Ser^{311} phosphorylation is required for Lys^{310} acetylation. Recent work by Levy et al. (60) showed that Lys^{310} is also a site of methylation, which suppresses NF-κB transcriptional activity, and that phosphorylation of Ser^{311} blocks the inhibitory effects of Lys^{310} methylation. The mechanism by which Ser^{311} phosphorylation antagonizes the inhibitory methylation is unclear, but it is tempting to speculate that there might be competition between methylation and acetylation at Lys^{310}, with phosphorylation of Ser^{311} favoring acetylation and transcriptional activation.

Taken together with the substantial body of work characterizing NF-κB activation, our results point to a model in which early events of NF-κB activation are intact in anergic T cells, but defective phosphorylation of p65 Ser^{311} inhibits association of NF-κB with HATs (Fig. 7). This in turn prevents acetylation of p65 Lys^{310}, inhibiting NF-κB transactivation, and may also inhibit chromatin remodeling at promoters of NF-κB target genes, such as IL-2. However, work remains to demonstrate a causal connection between the phosphorylation/acetylation defect and the blockade of IL-2 production in anergic $CD8^+$ T cells, and we are currently investigating this area. The cause of the impaired Ser^{311} phosphorylation also remains unknown. The simplest possibility is that the activity of the relevant kinase is inhibited in T cell anergy, and testing of this hypothesis will require identifying the kinase. Given the importance of NF-κB in a wide range of cellular functions, in both immune and nonimmune cells, we anticipate that a deeper understanding of the fine regulation of NF-κB activity will shed additional light on questions of how cell type-specific and stimulus-specific NF-κB responses are managed.

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Disclosures

The authors have no financial conflicts of interest.

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