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In vivo anergized CD4$^+$ T cells express perturbed AP-1 and NF-κB transcription factors

(superantigen/T lymphocyte/interleukin 2 gene regulation/transcription factor)

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ABSTRACT **Anergy is a major mechanism to ensure antigen-specific tolerance in T lymphocytes in the adult. *In vivo*, anergy has mainly been studied at the cellular level. In this study, we used the T-cell-activating superantigen staphylococcal enterotoxin A (SEA) to investigate molecular mechanisms of T-lymphocyte anergy *in vivo*. Injection of SEA to adult mice activates CD4$^+$ T cells expressing certain T-cell receptor (TCR) variable region β-chain families and induces strong and rapid production of interleukin 2 (IL-2). In contrast, repeated injections of SEA cause CD4$^+$ T-cell deletion and anergy in the remaining CD4$^+$ T cells, characterized by reduced expression of IL-2 at mRNA and protein levels. We analyzed expression of AP-1, NF-κB, NF-AT, and octamer binding transcription factors, which are known to be involved in the regulation of IL-2 gene promoter activity. Large amounts of AP-1 and NF-κB and significant quantities of NF-AT were induced in SEA-activated CD4$^+$ spleen T cells, whereas Oct-1 and Oct-2 DNA binding activity was similar in both resting and activated T cells. In contrast, anergic CD4$^+$ T cells contained severely reduced levels of AP-1 and Fos/Jun-containing NF-AT complexes but expressed significant amounts of NF-κB and Oct binding proteins after SEA stimulation. Resolution of the NF-κB complex demonstrated predominant expression of p50–p65 heterodimers in activated CD4$^+$ T cells, while anergic cells mainly expressed the transcriptionally inactive p50 homodimer. These alterations of transcription factors are likely to be responsible for repression of IL-2 in anergic T cells.**

Staphylococcal enterotoxins (SEs) belong to a family of bacterial proteins denoted as superantigens (SAgs) because of their ability to activate a high frequency of both CD4$^+$ and CD8$^+$ T cells expressing certain T-cell receptor (TCR) variable region β chains (V_β) (1, 2). Administration of SE to adult mice induces rapid production of a panel of cytokines, including interleukin 2 (IL-2) and tumor necrosis factor (TNF), and subsequent expansion of reactive T-cell populations (3). After the initial phase of SE-induced activation *in vivo*, part of the reactive CD4$^+$ T cells are deleted and the remaining CD4$^+$ T-cell population fails to proliferate and secrete IL-2 in response to a subsequent SE challenge (4).

Production of IL-2 in CD4$^+$ T cells is strongly regulated at the transcriptional level (5). The IL-2 promoter/enhancer (6) consists of several DNA response elements, including proximal and distal AP-1 and NF-AT binding sites, NF-IL2A and NF-IL2D that bind octamer proteins, a single NF-κB binding site, and a NF-κB-like CD28 response element (CD28RE), which are all recognized by specific transacting proteins. Each one of these transcription factors is regulated specifically (7) and has been shown to contribute significantly to the activity of the IL-2 promoter (8). The AP-1 family of proteins includes a number of Fos and Jun proteins (9–11). The Rel family of proteins includes p50, p65, and c-Rel proteins, which may bind to the NF-κB element in a variety of protein complexes, such as the highly transcriptionally active p50–p65 heterodimer or the less active p50–p50 homodimer (12, 13). The Fos/Jun and Rel families of proteins seem to be of major importance in regulating the IL-2 promoter, since they may themselves bind to their AP-1 and NF-κB elements and further participate with other proteins to form an active NF-AT and CD28RE binding complex (14, 15).

Anergy was originally described by Schwartz and co-workers using an *in vitro* model with T-cell clones, which were rendered anergic by antigen presentation in the absence of appropriate costimulatory signals (16). Studies of these antigen-specific T-cell clones have shown that lack of IL-2 production, a hallmark of anergy, correlates with reduced levels of the AP-1 transcription factor (17). We now demonstrate that *in vivo* anergized T cells express diminished amounts of AP-1 and NF-AT/AP-1 (18) binding complexes compared to SAg-activated T cells. The anergic cells were further shown to retain the ability to mobilize NF-κB. However, while the induced NF-κB complex in activated T cells was mainly composed of the p50–p65 heterodimer, anergic T cells predominantly contained the p50 homodimer. This suggests that *in vivo* anergized CD4$^+$ T cells may have a more complex perturbation in IL-2 gene promoter-related transcription factors than was inferred from the *in vitro* anergy model (17).

MATERIALS AND METHODS

Animals and Treatment. Transgenic (TG) C57BL/6 mice expressing a rearranged TCR $V_\beta3$ gene under the influence of an inserted immunoglobulin heavy-chain enhancer (19) were generously provided by M. Davis (Stanford, CA). The TG TCR $V_\beta3$ mice expressed 85–95% of TCR $V_\beta3$ in the CD3$^+$ T-cell population (3). Recombinant SEA was expressed in *Escherichia coli* and purified to homogeneity as described (3). Various doses of SEA in phosphate-buffered saline with 1% normal syngeneic serum (PBS) or in PBS alone were injected i.v. at 4-day intervals.

Reagents. Monoclonal antibodies (mAbs) directed to murine CD3, CD4, CD8, and TCR $V_\beta3$ were purchased from PharMingen. Recombinant murine IL-2 and mAbs to murine IL-2 (JES6-1A12 and JES6-5H4) were obtained from PharMingen. Polyclonal antisera directed to members of the AP-1 complex [c-Fos(sc-52), Fra-1(sc-183), Fra-2(sc-171), c-Jun(sc-45), JunB(sc-46), JunD(sc-75), pan-Fos members(sc-253)] and

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Abbreviations: SAg, superantigen; SEA, staphylococcal enterotoxin A; IL-2, interleukin 2; TNF, tumor necrosis factor; TG, transgenic; TCR, T-cell receptor; V_β, variable region β chain; mAb, monoclonal antibody; RT, reverse transcription.

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the NF-κB gene family [c-Rel(sc-70), p50(sc-114), p65(sc-109)] were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) for antibody supershift analysis. A polyclonal rabbit antiserum against recombinant NF-AT_p (20) was kindly provided by A. Rao (Dana–Farber Cancer Institute, Boston). A control extract for supershift experiments made from phorbol ester-stimulated mouse fibroblasts (sc-2125) was obtained from Santa Cruz Biotechnology.

IL-2 Serum Levels. Blood samples were drawn at various time points after i.v. injections of SEA or PBS and tested for IL-2 content by a specific ELISA using mAbs JES6-1A12 and JES6-5H4 according to instructions from the manufacturer. All groups contained pooled sera from at least two mice.

Reverse Transcription (RT)-PCR. Spleens were prepared from mice injected i.v. with SEA or PBS at different times before analyses of IL-2 and β-actin mRNA levels by a semiquantitative RT-PCR assay as described (4). The relative amount of IL-2 mRNA was calculated by the formula (cpm test − cpm no cDNA control)/(cpm β-actin test − cpm no cDNA control).

Cell Separation. Spleens were prepared from mice injected i.v. with SEA or PBS at different times before analysis. Purified $CD4^+$ T cells (>95% $CD4^+$ as determined by fluorescence-activated cell sorter analysis) were obtained by positive selection using magnetic beads coated with anti-CD4 mAb (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Preparation of Nuclear Extracts. Nuclear extracts were made according to the method described by Schreiber *et al.* (21); 5–10 × 10^6 purified $CD4^+$ T cells were used for extraction and the nuclear proteins were finally dissolved in 20 mM Hepes, pH 7.9/0.4 M NaCl/1 mM EDTA/1 mM EGTA/1 mM dithiothreitol (DTT)/1 mM phenylmethylsulfonyl fluoride/0.5 μg of leupeptin per ml/0.7 μg of pepstatin per ml/1 μg of trypsin inhibitor type S per ml. For NF-AT binding, nuclear extracts were prepared from 1–2 × 10^8 spleen cells using the method described by Choi et al. (22). Protein concentration of all extracts was measured with the Bio-Rad protein assay kit (Bio-Rad), and the extracts were stored in aliquots of −70°C until required.

Gel Mobility-Shift Assay (Gel Shift). The oligonucleotides used contained the following sequences: Oct binding site, 5′-CGTCTCATGCGATGCAAATCACTTGAGATC-3′; AP-1 consensus, 5′-CTAGTGATGAGTCAGCCGGATC-3′; NF-κB consensus, 5′-GATCGAGGGGACTTTCCCTAGC-3′; distal NF-AT site of murine IL-2 promoter, 5′-GATCG-CCCAAAGAGGAAAATTTGTTTCATACAG-3′; NF-AT nonbinding M3 mutant, 5′-GATCGCCCAAAGACCTTA-ATTTGTTTCATACAG-3′ (23). The probes were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega) according to instructions from the manufacturer and purified on 5% polyacrylamide gels in 1× TBE (89 mM Tris/89 mM boric acid/2 mM EDTA). Binding reactions were performed with the same amount of protein in each mixture (0.5–1 μg) in 10 mM Tris·HCl, pH 7.5/50 mM NaCl/1 mM EDTA/1 mM DTT/5% (vol/vol) glycerol/2 μg of poly(dI-dC) (Pharmacia) for AP-1, NF-κB, and Oct binding. To detect NF-AT binding, 5 μg of protein was used in 20 mM Hepes, pH 7.9/40 mM KCl/2.5 mM $MgCl_2$/1 mM DTT/4% Ficoll/2 μg of poly(dI-dC). The reaction mixtures were incubated at 37°C for 30 min with 15,000 cpm of double-stranded ^{32}P-labeled oligonucleotides. The samples were electrophoresed on 5% polyacrylamide gels in 1× TBE. Unlabeled oligonucleotides, used for competition, were added to nuclear extracts and dI-dC before addition of the labeled probe. For supershift analyses, the mixture of nuclear extract and labeled oligonucleotide was incubated with 1–2 μg of Abs against various transcription factors for 1 hr on ice. No effect was observed with control IgG (data not shown). Control experiments performed with a mixture of Abs and DNA probe in the absence of protein extract demonstrated that none of the Abs used bound directly to the DNA target sequences (data not shown). For NF-AT supershift analysis, Abs were incubated with nuclear extracts for 15 min at room temperature before addition of labeled oligonucleotide.

RESULTS

SEA-Induced Anergy Corresponds to Reduced IL-2 Production *in Vivo*. In this study, we used TG TCR $V_\beta 3$ mice as a sensitive model (3) to investigate production and transcriptional regulation of IL-2 in SEA-activated and anergic $CD4^+$ T cells (4). High serum levels of IL-2 protein were recorded 1–4 hr after SEA priming of TG TCR $V_\beta 3$ mice and peak response was seen after 2 hr (Fig. 1*A*). However, when the mice were pretreated with two injections of SEA, the response to a subsequent SEA challenge was strongly reduced at all tested time points (Fig. 1*A*). RT-PCR analysis revealed that IL-2 mRNA was also decreased after three SEA injections (Fig. 1 *B* and *C*).

Different Patterns of Transcription Factors in Activated and Anergic T Cells. To evaluate whether the parallel decline of IL-2 mRNA and protein levels reflected a changed expression of transcription factors binding to regulatory elements in the IL-2 gene promoter, we used gel-shift analysis and oligo-

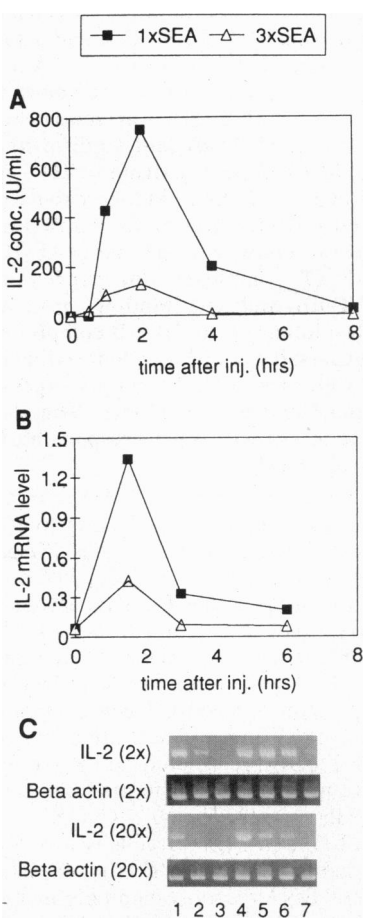

FIG. 1. Kinetics of IL-2 protein and mRNA levels after SEA treatment. Mice were treated with 10 μg of SEA one or three times, and spleens and sera were taken at the indicated time points after the last injection. (*A*) Protein levels were analyzed by specific ELISA. (*B*) Quantification of cytokine mRNA levels was performed by incorporation of [α-^{32}P]dCTP and expressed relative to β-actin. (*C*) PCR products at two dilutions are shown. Lanes: 1, 3× SEA (1.5 hr); 2, 3× SEA (3 hr); 3, 3× SEA (6 hr); 4, 1× SEA (1.5 hr); 5, 1× SEA (3 hr); 6, 1× SEA (6 hr); 7, PBS-treated control. One of three similar experiments is shown.

nucleotides encoding AP-1, NF-κB and octamer consensus motifs, and the distal NF-AT site from the IL-2 promoter. Spleens were removed 1 hr after the last SEA injection, preceding the peak time point for IL-2 mRNA expression, and nuclear extracts were prepared from purified $CD4^+$ spleen cells. AP-1 binding activity was found to be markedly reduced in anergic $CD4^+$ T cells (3× SEA) compared to activated $CD4^+$ T cells (1× SEA) (Fig. 2*A*). Similar results were obtained using the AP-1 consensus sequence or an AP-1 element derived from the murine IL-2 promoter (17), although the latter element gave a generally weaker gel shift (data not shown), as reported in earlier studies (24). Extracts from PBS-treated mice contained no AP-1 binding (Fig. 2*A*). NF-κB, on the other hand, was induced in both anergic and activated cells (Fig. 2*A*). However, the migration pattern differed between the two groups. Anergic cells expressed mainly a faster-migrating band, while activated cells contained predominantly a slower-migrating band (Fig. 2*A*). Incubation of nuclear extracts from anergic, activated, and unstimulated cells with the octamer element showed constitutive expression of two bands, most likely corresponding to Oct-1 and Oct-2 (Fig. 2*A*). To investigate the presence of NF-AT binding proteins *in vivo*, nuclear extracts were prepared by a modified extraction method (22). In the activated cells (1× SEA), two complexes were induced (Fig. 2*A*), indicating the presence of NF-AT alone ($NF\text{-}AT_p$) and NF-AT/Fos-Jun as described (23). The anergic cells (3× SEA) expressed a slight reduction of the lower NF-AT band but a lack of the upper Fos/Jun-containing NF-AT complex (Fig. 2*A*). The identity of the NF-AT bands observed in activated cells was further defined by supershift analysis using Abs against $NF\text{-}AT_p$ (20) and Fos protein members (pan-Fos). All NF-AT binding activity was shifted by $NF\text{-}AT_p$-specific Abs, whereas only the upper band disappeared in the presence of Fos Abs (Fig. 2*B*), confirming the presence of a NF-AT/AP-1 complex in the activated cells. No effect was observed with control IgG (Fig. 2*B*). This finding was verified by oligonucleotide competition experiments with unlabeled NF-AT, AP-1, and a nonbinding NF-AT mutant [M3 (23)]. The NF-AT complexes were blocked by addition of the NF-AT probe but not by the NF-AT M3 mutant (data not shown). Furthermore, the upper but not the lower complex was inhibited by unlabeled AP-1 probe (data not shown).

To analyze the specificity of the shifted AP-1 and NF-κB bands, cross-competition experiments with unlabeled oligonucleotides were performed. The AP-1 and NF-κB bands were completely blocked with unlabeled AP-1 and NF-κB oligonucleotide, respectively, but not with the nonrelevant oligonucleotide (Fig. 2*C*). This indicates that the protein–DNA binding is specific and that no cross-reactivity exists between AP-1 and NF-κB binding proteins.

AP-1 and NF-κB Are Rapidly Induced *in Vivo*. To exclude the possibility that distinct expression of AP-1 and NF-κB in anergic and activated $CD4^+$ T cells reflected a change in kinetics, nuclear extracts were obtained at various time points

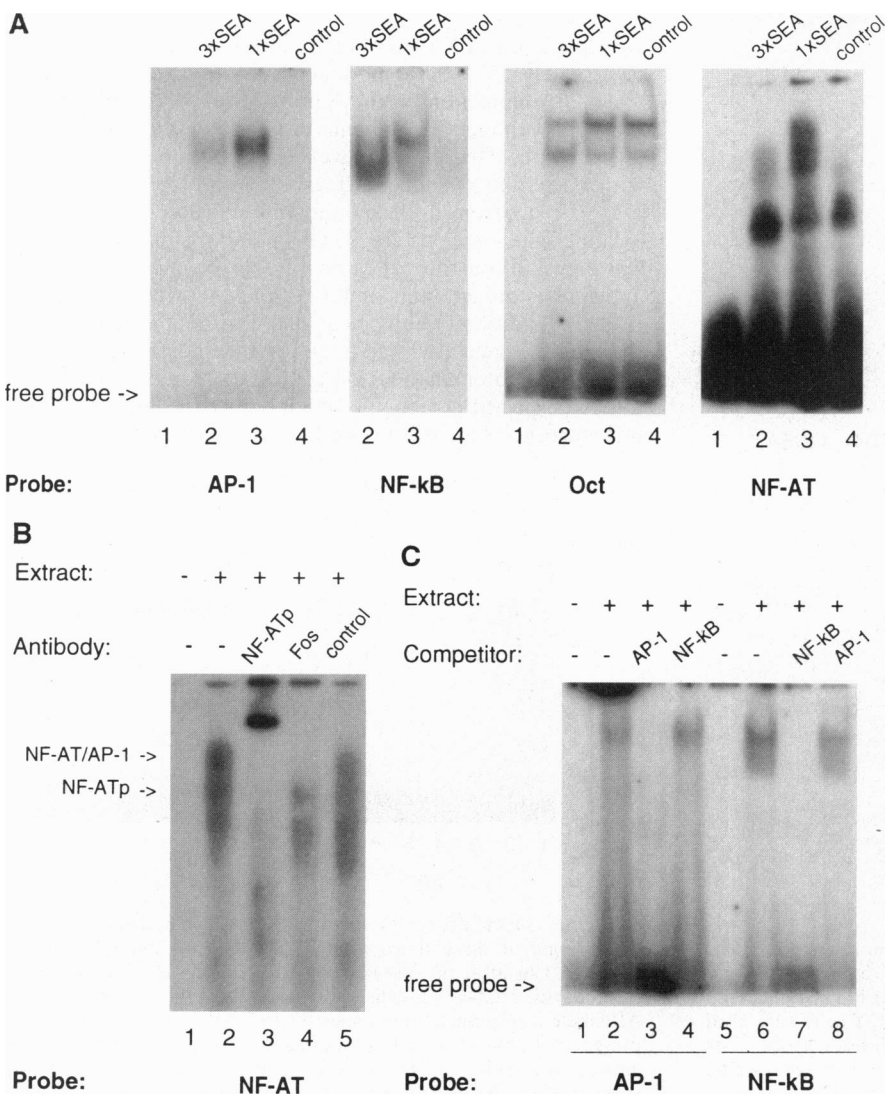

FIG. 2. Expression of AP-1, NF-κB, NF-AT, and Oct binding proteins after SEA treatment. (*A*) Mice were treated one or three times with 10 μg of SEA or vehicle (PBS), and spleens were removed 1 hr after the last injection. Nuclear protein extracts were prepared as described and used for gel-shift analysis with ^{32}P-labeled oligonucleotides specific for AP-1, NF-κB, Oct, and NF-AT sites. (*B*) Supershift analysis of the NF-AT binding complexes. Nuclear extract from spleen cells of mice treated once with 10 μg of SEA was incubated in the presence or absence of antisera directed against $NF\text{-}AT_p$, pan-Fos members (Fos), or control serum before addition of labeled NF-AT probe. (*C*) Cross-competition of AP-1 vs. NF-κB binding. Nuclear extract from $CD4^+$ T cells was incubated with 50-fold molar excess of unlabeled competitors for 15 min before addition of labeled DNA. One of three similar experiments is shown.

after *in vivo* SEA treatment. AP-1 activity was found to be induced after 15 min, increased to maximum at 1 hr, and remained detectable up to at least 4 hr after SEA activation (Fig. 3*A*). In contrast, AP-1 binding activity was strongly reduced in anergic cells at all time points studied (Fig. 3*A*). The upper NF-κB band (B1) was present in activated T cells after 15 min and remained the dominating band up to 4 hr after SEA injection (Fig. 3*B*). A minor component of a lower migrating band (B2) was seen in activated cells at 4 hr. In the anergic cells, the lower band became visible after 15 min and constituted the major band after 1–4 hr (Fig. 3*B*). A minor component of the upper band (B1) was indicated at 15 min and 1 hr in anergic T cells (Fig. 3*B*).

Low Expression of p50–p65 Heterodimers in Anergic CD4$^+$ T Cells. To identify which components of the Rel family bind as upper or lower bands to the NF-κB element, we used specific Abs to p50, p65, and c-Rel. Supershift analysis revealed that the lower NF-κB band, the main constituent in nuclear extracts of anergic CD4$^+$ T cells, was p50–p50 homodimers (Fig. 4). The upper band, which is the major NF-κB shift in nuclear extract of activated CD4$^+$ T cells, was shown to represent p50–p65 heterodimers, since it was shifted by both p50 and p65 Abs (Fig. 4). No c-Rel component was found in either of the extracts (Fig. 4). CD4$^+$ T cells from untreated mice contained very low levels of NF-κB binding, shown by supershift analysis to be solely p50–p50 homodimers (data not shown).

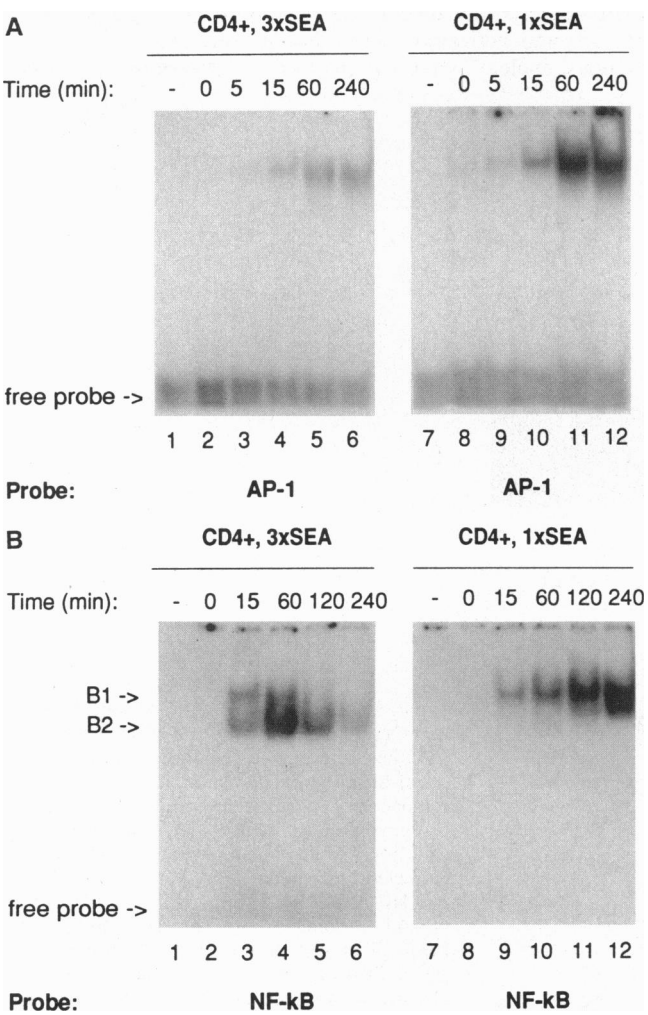

FIG. 3. Kinetics of AP-1 and NF-κB expression after SEA treatment. Mice were treated one or three times with 10 μg of SEA, and spleens were removed at different time points after the last injection. Nuclear protein extracts made from purified CD4$^+$ T cells were used for gel-shift analysis with ^{32}P-labeled oligonucleotides specific for AP-1 (*A*) and NF-κB (*B*). Arrows indicate positions of specific DNA–protein complexes. One of two similar experiments is shown.

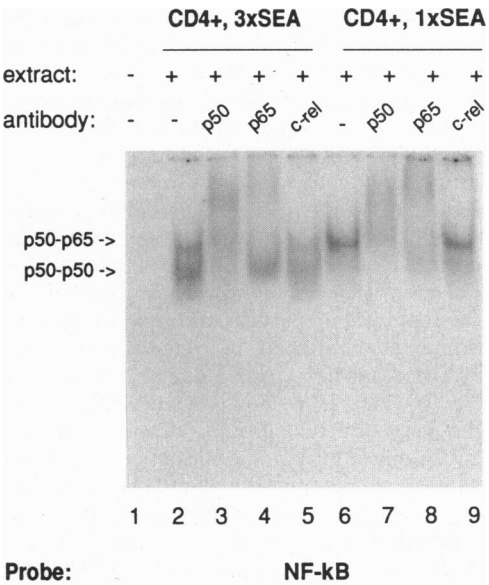

FIG. 4. Supershift analysis of NF-κB binding complexes. Mice were treated one or three times with 10 μg of SEA, and spleens were removed 1 hr after the last injection. Nuclear protein extracts made from purified CD4$^+$ T cells were used for gel-shift analysis with ^{32}P-labeled oligonucleotides specific for NF-κB in the absence or presence of anti-p50 (p50), anti-p65 (p65), or anti-c-Rel (c-Rel) subunit-specific Abs. One of three similar experiments is shown.

AP-1 Composition Is Not Significantly Altered. To identify the AP-1 binding components in nuclear extracts of anergic and activated T-helper cells, we performed supershift analyses with antisera to a panel of different Fos and Jun members. The 1-hr extracts from both anergic and activated CD4$^+$ T cells exhibited the same pattern of AP-1 components (Fig. 5). c-Fos was shown to constitute the major Fos protein in these extracts, and substantial amounts of JunB and JunD were also present (Fig. 5). Addition of antisera against Fra-2 somewhat reduced the bands in activated and anergic cells, indicating participation of this protein in the AP-1 complexes (Fig. 5). This was not due to unspecific proteolytic activity, since the antisera did not influence gel shift of an unrelated nuclear factor (data not

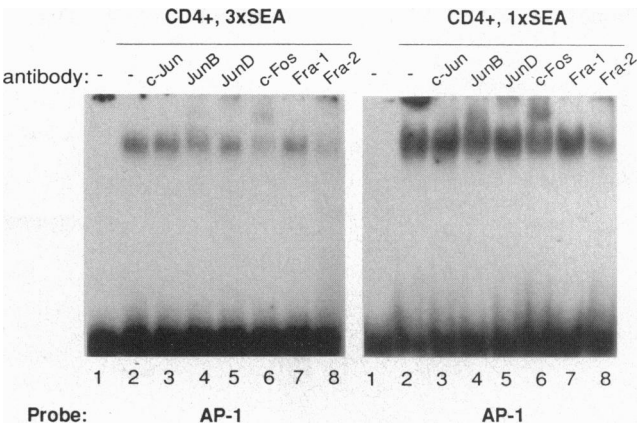

FIG. 5. Supershift analysis of AP-1 binding complexes. Mice were treated one or three times with 10 μg of SEA, and spleens were removed 1 hr after the last injection. Nuclear protein extracts made from purified CD4$^+$ T cells were used for gel-shift analysis with ^{32}P-labeled oligonucleotides specific for AP-1 in the absence or presence of anti-c-Jun (c-Jun), anti-JunB (JunB), anti-JunD (JunD), anti-c-Fos (c-Fos), anti-Fra-1 (Fra-1), or anti-Fra-2 (Fra-2) subunit-specific Abs. One of three similar experiments is shown.

shown). No effect of antisera against c-Jun and Fra-1 was observed (Fig. 5), even though these Abs were shown to be reactive in supershifts using a control extract from phorbol ester-stimulated mouse fibroblasts (data not shown).

DISCUSSION

In this study, we demonstrate that the failure of SAg anergized T-helper cells to produce IL-2 *in vivo* correlates with perturbed expression of AP-1, AP-1-containing NF-AT, and NF-κB transcription factors. Resting $CD4^+$ T cells contained low levels of p50–p50 NF-κB homodimer and did not express AP-1 proteins. In contrast, SEA-activated $CD4^+$ T cells were rapidly induced to express large amounts of p50–p65 NF-κB heterodimers, AP-1 proteins, and the two NF-AT binding complexes. These data are in accordance with earlier *in vitro* studies on murine T-cell clones and human peripheral blood T cells (25, 26). The *in vivo* induction of NF-κB heterodimer, NF-AT, and AP-1 was extremely rapid compared to earlier *in vitro* reported characteristics (27), implicating a coordinated and very rapid transition from induction of nuclear transcription factors, transcription, translation, and IL-2 secretion *in vivo*. A marked decrease of AP-1 binding proteins and a changed migration pattern of the NF-κB binding complex were seen in anergic $CD4^+$ T cells at 15 min to at least 4 hr after injection of SEA. The presence of a faster-migrating octamer binding complex, characterized as Oct-2 by supershift analysis (data not shown), was more pronounced in anergic compared to resting or activated T cells (Fig. 2*A*). The changed Oct-1/Oct-2 ratio might be important since functional differences between these factors have been demonstrated (28).

In vitro models of anergy have clearly established an association between repressed IL-2 gene expression and the nonresponsive state (16). Recently, Kang *et al.* (17) reported diminished levels of AP-1 but normal levels of NF-κB, NF-IL2A (octamer element), and NF-AT in a T-cell clone rendered anergic by TCR stimulation in the absence of costimulation (17). The composition of NF-κB proteins was not examined in this study (17). However, transfection of the T-cell clone and reporter gene experiments indicated that an IL-2/NF-κB promoter was slightly less efficiently driven in the anergic T cells compared to normal T cells (17). Further experiments are required to evaluate the relative importance of NF-κB on the response of *in vitro* and *in vivo* anergized T cells. AP-1 and NF-κB transcription factors have been shown to be involved in several important aspects of T-cell development and activation (12, 29). Mutations in the AP-1 proximal site present in the IL-2 promoter greatly diminished or abolished induction of the IL-2 promoter (8, 24). In addition, overexpression of c-Fos in transgenic mice accelerated *in vitro* release of IL-2 by activated T cells (30), which underlines the central importance of AP-1 in regulating IL-2 production. Furthermore, regulatory AP-1 and NF-κB sites have been found in several cytokine gene promoters, including IL-6 and TNF-α (31, 32). Interestingly, we have shown that TNF production is also down-regulated in SEA anergized $CD4^+$ T cells *in vivo* (4). Supershift analyses of the AP-1 complexes demonstrated that activated T cells expressed substantial amounts of JunB, JunD, and c-Fos and minor amounts of Fra-2. Moreover, supershift with pan-Fos family antisera completely abrogated the shift (data not shown), indicating that all AP-1 DNA binding activity is related to Fos–Jun heterodimers, which have a notably higher DNA binding affinity than Jun–Jun homodimers (11). Recently, Rincón and Flavell (33) demonstrated that mouse T cells activated with anti-CD3 Ab *in vitro* expressed JunB, c-Jun, Fra-2, and FosB but not Fra-1 or JunD in the AP-1 complex. The discrepancy concerning JunD is unclear, but it should be noted that JunD has been reported to be a stronger transcriptional activator than JunB (10), suggesting that SAg-activated T cells express a favorable AP-1 composition for driving IL-2 gene expression. Using TG mice that expressed an AP-1 driven reporter gene, Rincón and Flavell (33) reported a lack of correlation between AP-1 DNA binding and IL-2 production. In our studies, however, there was a strong correlation between AP-1 expression and the ability to express IL-2 at the mRNA and protein levels. Indeed, reduced AP-1 levels were also found in *in vitro* anergized T cells (17). Whether these discrepancies reflect differences in the response to stimulation *in vitro* and *in vivo* or an abnormal regulation of the AP-1 promoter transgene needs to be further addressed.

NF-AT has been demonstrated to be induced in $CD4^+$ T-helper 1 clones stimulated through the TCR–CD3 complex and to be required for IL-2 gene induction in these cells (18). Factors binding to the NF-AT motif have previously been reported to consist of two components: $NF\text{-}AT_p$, which preexists in the cytosol, and a nuclear AP-1-associated complex, which contains translocated $NF\text{-}AT_p$ protein (18). We now demonstrate induction of both $NF\text{-}AT_p$ and NF-AT/AP-1 factors in activated cells, while the anergic cells predominantly expressed the $NF\text{-}AT_p$ component alone. These results imply that lack of the NF-AT/AP-1 complex in anergic cells might be secondary to modulation of Fos and Jun proteins. Interestingly, mutations in the NF-AT site that prevented association of Fos and Jun with $NF\text{-}AT_p$ abolished the function of this site in activated T cells (34), suggesting that the AP-1 component in the NF-AT complex is essential for transcription. This indicates that down-regulation of NF-AT/AP-1 in anergized T cells further contributes to defective IL-2 production.

In the present study, two different forms of NF-κB were detected in activated and anergic $CD4^+$ T cells at time points relevant for IL-2 mRNA and protein production. Kinetic analysis of the NF-κB response demonstrated that p50–p65 was induced in activated T cells after 5–15 min (Fig. 3*B*; data not shown), which most likely reflects release of preformed p50–p65 from IκB inhibitors (35). The presence of p50–p65 after 1–2 hr of activation may reflect *de novo* synthesized p65 protein. Anergized $CD4^+$ T cells expressed large amounts of p50–p50 homodimers throughout the 4-hr observation period, while activated $CD4^+$ T cells expressed the homodimer only after 4 hr of activation *in vivo*. The delayed induction of inhibitory p50–p50 protein may represent a normal regulatory feature of IL-2 production, since only low levels of IL-2 mRNA and protein were found at corresponding time points. In line with this finding, it has been demonstrated in several experimental systems that p50–p50 homodimers lack transcriptional activity and instead occupy cognate NF-κB binding sites and prevent transactivation (12, 13). A recent study showed a correlation between p50–p50 induction and lack of TNF production during LPS tolerance (36). Taken together, this suggests that the balance between NF-κB p50–p65 heterodimers and p50–p50 homodimers may be a central mechanism for regulating cytokine production *in vivo*.

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