Increased c-Fos/activator protein-1 confers resistance against anergy induction on antigen-specific T cell

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Abstract

We have studied the contribution of c-Fos/activator protein-1 (AP-1) to antigen-specific T cell response with reference to T cell anergy by increasing c-Fos/AP-1 in vivo and in vitro. First, after injection of a high dose of staphylococcus enterotoxin B (SEB), clonal deletion of SEB-reactive Vβ8⁺ CD4 T cells occurred both in control B6 and H2-c-fos transgenic (fos) mice, whereas proliferation of T cells against SEB was profoundly depressed in B6 but not in fos mice. Second, the keyhole limpet hemocyanin-specific CD4 Th1 cell clone produced decreasing amounts of IL-2 in response to increasing amounts of concanavalin A (Con A) in vitro, whereas the decrease was less significant in the Th1 clones stably transfected with c-fos gene. Electrophoretic mobility shift assay with nuclear protein from the transformants showed that overexpression of the c-fos gene compensated the amounts of AP-1 in the nuclei of Con A-treated Th1 clones. Thus, increased c-Fos/AP-1 confers resistance against anergy induction on antigen-specific T cells.

Introduction

T cells activated by receptor stimulation in the absence of accessory cell-derived co-stimulatory signals lose the capacity to produce IL-2 and to proliferate when re-stimulated with antigen-presenting cell (APC) plus antigen, a state called clonal anergy (1). As to the mechanism of clonal anergy, recent findings suggest that activator protein-1 (AP-1) transactivation is defective in anergic T cells. Anergic T cells show defective AP-1-dependent transcription and suboptimal binding of AP-1 to the element within the regulatory region of the IL-2 gene (1,2). Poor inducibility of c-Fos, FosB and JunB protein has also been demonstrated in T cells after induction of clonal anergy (1,3).

AP-1 is a heterodimer composed of Fos and Jun family members. During T cell activation, Fos and Jun are increased, and the Jun protein becomes phosphorylated (4). Two major kinase pathways have been implicated in the activation of AP-1 during T cell responses to antigen, i.e. mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK). The former pathway is downstream of p21ras. Ras localizes Raf to the membrane where it is phosphorylated and activated. Raf phosphorylates the dual-specific kinase MEK, which subsequently activates extracellular signal-regulated kinase (ERK) by direct phosphorylation on threonine/tyrosine residues. ERK then up-regulates c-fos through phosphorylation of Elk-1. The upstream component of the latter JNK pathway is as yet not clearly defined; however, it is thought that the serine/threonine kinase MEKK1 phosphorylates and stimulates the dual-specificity kinase MKK4/SEK1 that phosphorylates the MAPK family member, JNK (5). Activated JNK then transactivates c-Jun by phosphorylation on two N-terminal serine residues. The lack of AP-1 transactivation observed in anergic T cells could therefore be due to impaired signaling in the pathways leading to activation of c-Fos and c-Jun, such as ERK and JNK pathways (6). However, the role of c-Fos/AP-1 in the transcriptional activation of IL-2 has not been clearly defined, especially in relation to the contribution
of Jun and other regulatory elements such as the negative regulatory element (NRE-A) (7).

Previous studies in this and other laboratories have shown that features characteristic to rheumatoid joint destruction, including synovial overgrowth and bone resorption, are experimentally produced by specifically augmenting c-fos gene expression (8–13). Expression of c-Fos/AP-1 is markedly augmented in rheumatoid joints (14,15) and arthritic joint destruction can be ameliorated by selectively inhibiting transcription of c-Fos/AP-1 (16), suggesting that activation of c-Fos/AP-1 is essentially important in arthritic joint destruction. As to the question how c-Fos/AP-1 promoted joint destruction, previous studies indicate that c-Fos/AP-1 directly stimulates synovial mesenchymal cells to release inflammatory cytokines such as IL-1, tumor necrosis factor-α and others (17–19), and invade cartilageous substrates as pannus (9,10). c-Fos/AP-1 also stimulated osteoblasts and osteoclasts to enhance osteoclastic bone resorption (8,11,12). Nevertheless, antigen-specific T cell response should be critically important at least in the initiation of, and possibly in the perpetuation of, chronic arthritis (20).

We have tested whether or not c-Fos/AP-1 plays an essential role in the induction of and resistance against anergy on antigen-specific T cell response in two ways. First, the T cell response against prior treatment with staphylococcus enterotoxin B (SEB) was studied in vivo in H2-c-transgenic (fos) mice. Second, the \textit{in vitro} response of keyhole limpet hemocyanin (KLH)-specific Tα1 clone stably transfected with the c-fos gene was studied in relation to prior treatment with concanavalin A (Con A), since Con A binds the TCR and does not activate co-stimulatory pathways (1). We present direct evidence indicating that increased c-Fos/AP-1 in the induction of and resistance against anergy, and also in disease conditions such as rheumatoid arthritis in which increased c-Fos/AP-1 has been shown to play an essential role in the pathogenesis of joint destruction.

\textbf{Methods}

\textbf{Mice}

C57BL/6 Cr Slc (B6) and C3H/HeJ mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). fos mice carried the exogenous mouse c-fos gene under the control of mouse MHG gene (H-2Kb) promoter (21).

\textbf{Tolerance induction}

Mice were i.p. injected with 100 µg of SEB (Makor Chemicals, Jerusalem, Israel) once or twice at an interval of 7 days, i.p. injection followed by i.p. injection. We administered 2 times higher doses of SEB than others to tolerize mice (22,23). Nine days after final inoculation, T cells were isolated from spleen by passing through a nylon wool column followed by cytotoxic treatment with mAb anti-CD8 (83.12.5) (24), mAb anti-HSA (J11d.2) (25) and Low-Tox-M rabbit C (Cedarlane, Hornby, Ontario, Canada) at 37°C for 50 min (21). Vβ8+CD4 T cells (2×10^6/well) in α-MEM supplemented with 10% FBS (Whittaker, Walkersville, MD), 50 µM 2-mercaptoethanol, 50 U/ml penicillin, 50 µg/ml streptomycin, 15 mM HEPES and 2 mM L-glutamine were stimulated with SEB in the presence of 30 Gy-irradiated syngeneic spleen cells (2×10^5/well) in a 96-well flat-bottomed culture plate for 4 days at 37°C in 5% CO2 in humidified air. At the end of culture, the T cells were pulsed with 37 kBq of [3H]thymidine (DuPont NEN, Boston, MA) and [3H]thymidine incorporation was determined by the scintillation technique. T cells were double labeled with biotin-conjugated anti-V68 mAb (F23.1) and FITC-streptavidin DCS (Vector, Burlingame, CA) and phycoerythrin (PE)-anti-TCRαβ mAb or PE-anti-CD4 mAb (PharMingen, San Diego, CA) for flow cytometry.

\textbf{Expression vector}

A 4 kb BamHI fragment containing the murine c-fos genomic DNA was excised from the p75/15 plasmid (26), and inserted into the BamHI site of the pH8 vector to construct the c-fos expression vector, 75/15pH8 (8).

\textbf{Tα1 clone}

KLH-specific CD4 Tα1 clone 28-4 (27), a gift of Dr Y. Asano, Ehime University, Japan, was maintained in RPMI 1640 medium supplemented with 10% FBS, 5% rat Con A supernatant (Collaborative Research, Bedford, MA), 50 µM 2-mercaptoethanol, 50 U/ml penicillin and 50 µg/ml streptomycin (complete RPMI). Cells (1×10^6/ml) were stimulated with 50 µg/ml of KLH (Calbiochem-Novabiochem, San Diego, CA) in the presence of 2×10^5/ml of mitomycin C-treated splenocytes (APC) (28) of C3H/HeJ mice every 14 days.

\textbf{Stable c-fos transformant}

T cells (1×10^6) were isolated using lympholyte-M (Cedarlane) 9 days after stimulation with KLH and APC. They were suspended in 250 µl of saline G (137 mM NaCl, 5.4 mM KCl, 1.1 mM Na2HPO4, 0.86 mM KH2PO4, 0.49 mM MgCl2, 0.9 mM CaCl2 and 6.1 mM glucose, pH 7.1) (29) containing 10 µg of pH8 or 75/15pH8 plasmids linearized with a Scal restriction enzyme. After incubation on ice for 10 min, electroporation was carried out in a 2 mm gap cuvette at 300 V for 99 µs for 8 times (eight pulses) using an Electro Square Porator B820 (BTX, San Diego, CA). Cells were cultured in complete RPMI at 37°C for 2 days. Cells were stimulated with KLH and APC, and subsequently cultured in complete RPMI containing 800 µg/ml of genecin (Gibco/BRL, Grand Island, NY), and then maintained in complete RPMI containing 100 µg/ml of genecin.

\textbf{RT-PCR}

T cells were isolated at 2 h, and 3, 6 and 9 days after stimulation with KLH and APC. Total RNA was isolated using isogencendium thiocyanate (30) (Nippon Gene, Toyama, Japan). Integration of DNA in transformants was confirmed using RT-PCR (Perkin Elmer, Norwalk, CT). RNA was reversely transcribed in a 20 µl reaction containing 2.5 µM of oligo d(T)16, 5 mM MgCl2, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1 mM each of dATP, dCTP, dGTP and dTTP, 1 U/µl RNase inhibitor, and sample RNA for 10 min. After adding 2.5 U/µl murine leukemia virus reverse transcriptase, this was incubated at 42°C for 20 min, at 99°C for 5 min and at 5°C for 5 min (cDNA). Then 100 µl reaction containing cDNA, 2 mM
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MgCl₂, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.15 μM of upstream primer, 0.15 μM of downstream primer and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer) was incubated at 95°C for 1 min, at 53°C for 2 min and at 72°C for 2 min for 35 cycles. Aliquots of PCR products were electrophoresed in a 1.5% agarose gel. The primers were 5'-CTTTTTGCTTTTATTTCAG-3' (sense) and 5'-AGACAAAGGAGACGTTGAA-3' (antisense) for the exogenous c-fos (1.1 kb), and 5'-GAGAT-TGCAATCTGCTGAA-3' (sense) and 5'-AGACAAAGGAGAAGACGTGTA-3' (antisense) for the exo- and endogenous c-fos (488 bp). The β-actin primers were 5'-ACAAACGGCTCCG-GCATGTCGAA-3' (sense) and 5'-GCCTCAGGAGCAATGACTTG-3' (antisense) (969 bp).

Western blot

Purified T cells (1×10⁵) were dissolved in 50 μl of lysis buffer (10 mM Tris–HCl, pH 7.4, 3 mM MgCl₂, 0.1% NP-40, 0.5 mM DTT and 1 mM PMSF) on ice for 10 min (31). The nuclear fraction was precipitated by centrifugation at 5000 r.p.m. for 5 min and suspended in 50 μl of Laemmlı buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.72 M 2-mercaptoethanol and 0.00125% bromophenol blue). Proteins were separated using 7.5 or 10% SDS-PAGE and transferred to positively charged nylon membrane (Millipore, Bedford, MA). After blocking with 3% BSA, the membrane was incubated with rabbit IgG anti-c-Fos antibody (Ab-2; Oncogene Science, Uniondale, NY), rabbit IgG anti-c-Jun antibody (sc-45; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit IgG anti-JunB antibody (sc-46; Santa Cruz) or rabbit IgG anti-JunD antibody (sc-74; Santa Cruz) for 2 h. The membrane was incubated with horseradish peroxidase-conjugated anti-rabbit Ig antibody (NA934; Amersham, Little Chalfont, UK) and bound antibody was visualized using the ECL system (Amersham).

Induction of T cell unresponsiveness

Unresponsiveness of T cells against re-challenge of antigen, so called anergy, was induced in T₁ cells by pretreatment with Con A (Wako Pure Chemicals, Osaka, Japan) (1). T₁ cells were cultured with Con A for 24 h. After adding 10 mg/ml of α-methyl mannoside (Wako), cells were cultured for 5 days. Cells (2×10⁵/200 μl/well) were isolated using lymphocyte-M and placed in a 96-well microtiter plate (Sumitomo Bakelite, Tokyo, Japan). They were cultured with KLH and mitomycin C-treated splenocytes (5×10⁵/2 ml/well), which were depleted of T cells by using anti-Thy 1 mAb (G7; Pharmingen), anti-CD4 mAb (H129.19; Pharmingen) and Lox-Tox-M rabbit C (32), in a 24-well plate (Sumitomo Bakelite). T₁ cells were collected by using Dynabeads Mouse CD4 (Dynal, Oslo, Norway). Cells were suspended with 1 ml of PBS/1% FBS and incubated with 100 μl of Dynabeads Mouse CD4 for 20 min at 4°C. CD4⁺ cells collected by a magnetic particle concentrator (Dynal) were resuspended in 500 μl of hypotonic buffer (10 mM HEPES/KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 μM leupeptin and 1 μM aprotinin) on ice for 10 min (32). After adding 5 μl of 10% NP-40, cells were incubated on ice for 20 min. The nuclear fraction was precipitated by centrifugation at 5000 r.p.m. for 3 min and resuspended in 50 μl of extraction buffer (20 mM HEPES/KOH, pH 7.9, 10% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 μM leupeptin and 1 μM aprotinin). This was incubated on ice for 30 min with agitation. Supernatant was obtained by centrifugation at 15,000 r.p.m. for 15 min and stored in aliquots at −80°C (nuclear extract).

Electrophoretic mobility shift assay (EMSA)

Nuclear extract (5 μg) was incubated for 30 min with 0.5 pmol of digoxigenin (DIG)-labeled oligonucleotide probes in a 20 μl volume containing 16 mM HEPES/KOH, pH 7.9, 8% glycerol, 8 mM KCl, 0.4 mM EDTA, 0.4 mM DTT, 0.5 mM PMSF, 1 μg of BSA and 0.5 μg of poly(dI–dC)poly(dI–dC) (Pharmacia Biotech, Uppsala, Sweden). Double-stranded DNA oligonucleotide of the AP-1 binding site of murine IL-2 gene (33) was 5’-aaATCCAGAGTCTACAGAAGA-3’ and 3’-TAAGGTCTCTAGTAGTCTTTCC-5’. Double-stranded DNA of the nuclear factor of activated T cell (NFAT) binding site (33) was 5’-ccCAAGAGGAAATTTGTTTTCATCAGAAAG-3’ and 3’-GGTTCTCCTTTTAAACAAATGTGCTTTC-5’. The probe was end-labeled with DIG-11-dUTP (Boehringer Mannheim, Mannheim, Germany). Non-labeled oligonucleotides (50 pmol) were added as competitor. The irrelevant double-stranded DNA oligonucleotides were 5’-GTTTACCCGCT-ATGGAAGGAA-3’ and 5’-GGGTTCTCTTCTACAGCCGGGTGAAA-3’ (μ-oligo) (16). Supershift was performed by adding 5 μg of anti-c-Fos antibody (Ab-2; oncogene) or anti-NFAT antibody (sc-1151; Santa Cruz) to the binding reaction. After electrophoresis in a 4% polyacrylamide gel, oligonucleotides were transferred to positively charged nylon membrane (Boehringer). DIG-labeled oligonucleotide was visualized by alkaline phosphatase-labeled Fab anti-DIG antibody, followed by chemiluminescence reaction using 100 μg/ml CSPD substrate (Tropix, Bedford, MA).

MAPK activity

KLH-specific T cells (2×10⁵/2 ml/well) with or without pretreatment with Con A were cultured for 4 h with KLH and mitomycin C-treated, T cell-depleted, splenocytes (5×10⁵/2 ml/well) in a 24-well plate. KLH-specific T cells were collected by using Dynabeads Mouse CD4. They were suspended in 500 μl of MAPK lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na₂VO₄, 1 μg/ml leupeptin and 1 mM PMSF) on ice for 5 min.
After sonication using MICROSON (MS50; Heat Systems-Ultrasonics, Farmingdale, NY), the soluble fraction was obtained by centrifugation at 12,000 r.p.m. for 10 min. Then 200 µg of proteins in 200 µl MAPK lysis buffer was incubated with 2 µl anti-phospho-specific p44/42 MAPK (Thr202/Tyr204) mAb (9105; New England Biolabs, Beverly, MA) at 4°C overnight and then incubated with 50 µl Protein A–agarose beads (Boehringer) at 4°C overnight. The beads were washed twice with MAPK lysis buffer and twice with kinase buffer (25 mM Tris–HCl, pH 7.5, 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na3VO4 and 10 mM MgCl2), and then suspended in 25 µl kinase buffer supplemented with 200 µM ATP and 1 µg Elk1 fusion protein (9184; New England Biolabs). The reaction was carried out at 30°C for 30 min and terminated with 12.5 µl of 3×Laemmli buffer. Then 20 µl each was electrophoresed in 12% SDS–PAGE and transferred to Immobilon-P membrane. After blocking with 3% BSA (Amersham), the membrane was incubated with anti-phospho-specific Elk1(Ser 383) mAb (9181; New England Biolabs), followed by the reaction with horseradish peroxidase-conjugated anti-rabbit Ig antibody (Amersham). Bound antibody was visualized by the ECL system.

Results

fos mice are resistant to anergy induction in vivo

There are two major mechanisms by which a high dose of SEB induces antigen-specific T cell tolerance: activation-induced cell death (34) and anergy (34–36). The proportion of SEB-reactive Vαβ+ T cells in the TCRαβ+ CD4 T cell population was 24.6 ± 1.1% (n = 4) in control B6 mice. It decreased to 19.7 ± 1.1% (n = 4) 9 days after administration of 100 µg SEB once. The proportion of Vαβ+ T cells in the TCRαβ+ CD4 T cell population was basically higher at 28.2% (n = 2) in fos mice and it decreased to 20.2% (n = 2) after the inoculation of SEB. Thus, the activation-induced cell death occurred both in B6 and fos mice.

B6 and fos mice were next inoculated with 100 µg SEB twice at an interval of 7 days to tolerant SEB-reactive T cells in vivo (22, 23). Nine days after this treatment, the proportion of Vαβ+ T cells in the CD4 T cell population decreased to similar levels: 18.9 ± 2.6% (n = 3) in B6 mice and 20.2 ± 3.4% (n = 4) in fos mice. In SEB-treated B6 mice, T cell proliferation in vitro against SEB was reduced by 90% or more (Fig. 1a). In contrast, the reduction was less significant in SEB-treated fos mice as compared with those of B6 mice (Fig. 1b).

EMSA showed that the level of AP-1 was high in the T cells of fos mice and it was not decreased by the pretreatment with SEB in the fos mice (Fig. 2). Thus, the resistance of T cells against anergy in fos mice appeared to depend on the AP-1 level.

Antigen-specific Tαβ cells that overexpress c-fos are resistant to anergy induction in vitro

We studied the detailed mechanism of action of c-Fos/ AP-1 against anergy induction in in vitro experiments. The exog- enously introduced c-fos gene was expressed constitutively in 75/15pH8 T cells (Fig. 3a). Increased amounts of c-Fos protein were produced by 75/15pH8 cells, while pH8 T cells in a quiescent state, when cultured for 9 days after antigen stimulation, did not produce detectable amounts of c-Fos protein (Fig. 3b). The amount of c-Jun and JunB was similar between pH8 and 75/15pH8 clones, while JunD was undetectable 9 days after antigen stimulation (Fig. 3b). The amount of c-Jun, JunB and JunD was increased 20 h after antigen stimulation, whereas the amount of these proteins remained similar between pH8 and 75/15pH8 clones (Fig. 3c). At 20 h after antigen stimulation, increased amount of c-Fos protein
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Fig. 3. Characterization of 75/15pH8 T cell. (a) RT-PCR. Total RNA was extracted 2 h, and 3, 6 and 9 days after the stimulation with KLH and T cell-depleted, mitomycin C-treated splenocytes (APC). (b) Western blot for c-Fos, c-Jun, JunB and JunD protein. Nuclear fraction was extracted 9 days after the stimulation with KLH and APC, separated using 7.5 or 10% SDS–PAGE, transferred to membrane, probed with anti-c-Fos, anti-c-Jun, anti-JunB and anti-JunD antibodies, and detected by chemiluminescence. (c) Western blot for c-Fos, c-Jun, JunB and JunD protein in nuclear fraction extracted 20 h after the stimulation with KLH and APC. Duplicated bands were phosphorylated proteins. The 75/15pH8 T or pH8 T cells were either pretreated with (+) or without (−) 0.4 µg/ml of Con A.

was detected in 75/15pH8 cells as compared with pH8 cells (Fig. 3c). IL-2 was produced from both pH8 and 75/15pH8 T cells after stimulation with KLH and APC (Fig. 4a). However, KLH-specific T$_{h}$1 cells produced decreasing amounts of IL-2 in response to KLH after the pretreatment of increasing amounts of Con A (Fig. 4b). Inhibition of IL-2 production after pretreatment with Con A was less significant in 75/15pH8 T cells as compared with those in control pH8 T cells. Surface expression of CD28 or IL-2 receptor α before and after treatment with Con A was similar between two types of T cell clones (data not shown).

EMSA showed that the level of transcription factor AP-1 increased in 75/15pH8 cells after stimulation with KLH as compared with pH8 cells (Fig. 5a, lane 1 versus lane 3). The AP-1 was super-shifted by the treatment with anti-Fos antibody (Fig. 5a, lane 7). The level of AP-1 was depressed in Con A-pretreated pH8 T cells but not in Con A-pretreated 75/15pH8 cells (Fig. 5a, lanes 1–4). The level of transcription factor NFAT in the nuclear extract, i.e. activated form of NFAT in nuclei combined with AP-1, decreased in Con A-pretreated pH8 T cells, whereas it remained constant in Con A-pretreated 75/15pH8 cells (Fig. 5b, lanes 1–4). Thus, overexpression of the c-fos gene compensated for the reduced translocation of AP-1 into the nucleus in anergized T$_{h}$1 clone.

Fig. 4. IL-2 production from CD4 T$_{h}$1 cells. (a) 75/15pH8 (open column) or pH8 (filled column) T cells ($2\times10^5$/200 µl/well) were cultured with KLH (0, 10, 50 or 100 µg/ml) and T cell-depleted, mitomycin C-treated splenocytes ($5\times10^5$/200 µl/well) for 24 h. IL-2 in the supernatant was measured using ELISA. Each column represented the mean ± SD of five experiments. (b) After induction of T cell unresponsiveness (anergy) by pretreatment with Con A, the T cells were stimulated with KLH (50 µg/ml) and APC for 24 h. The 75/15pH8 or pH8 T cells were pretreated with different concentrations of Con A. Each circle represented the mean ± SD of four experiments.
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Fig. 5. EMSA for transcription factor AP-1 and NFAT. The 75/15pH8 T or pH8 T cells (2×10^5/2 ml/well) with or without pretreatment with 0.4 µg/ml of Con A were stimulated with KLH (50 µg/ml) and T cell-depleted, mitomycin C-treated splenocytes (APC) (5×10^5/2 ml/well) for 20 h. Nuclear extracts were prepared from T cells collected by using Dynabeads Mouse CD4. Nuclear extracts 20 h after the stimulation with KLH and APC were reacted with DIG-labeled AP-1 oligonucleotides or NFAT oligonucleotides (a and b). Competition assay was done by adding unlabeled oligonucleotides (designated as comp-oligo) and unlabeled irrelevant oligonucleotides (g-oligo). Supershift was done for c-Fos and NFAT by adding specific antibodies. The data presented were representative of three independent experiments.

T cells (Fig. 6), indicating that increased c-Fos/AP-1 seems to have modified the upper pathway of cellular signal transduction rather than simply recruiting AP-1 onto the IL-2 promoter.

Discussion

The results directly show that antigen-specific T cells become resistant against anergy induction when c-fos gene is overexpressed. Previous studies have shown that anergic T cells stimulated with APC show defective AP-1-dependent transcription as well as suboptimal binding of AP-1 to the element within the regulatory region of the IL-2 gene (1,2). The amount of c-Fos, FosB and JunB was significantly depressed in anergized T cells (1,3). These findings suggest that T cell anergy may be controlled by the level of c-Fos/AP-1 in the cells.

The in vivo experiment using EMSA showed that the resistance against anergy induction was dependent on the level of transcription factor AP-1. The in vitro study also showed that the amount of AP-1 binding to the regulatory region of the IL-2 gene decreased in Con A-treated pH8 T cells, whereas it remained constant in Con A-treated 75/15pH8 T cells (Fig. 5). The amount of NFAT/AP-1 also did not decrease in Con A-treated 75/15pH8 T cells. AP-1 forms a complex with the activated form of NFAT which was in the nuclei and NFAT acts as a major transcription factor for IL-2 gene expression (37, 38). According to recent studies, it appears that NFAT complexed with AP-1 acts on the IL-2 promoter in energized T cells (39). These observations are consistent with the present result. We found that surface expression of accessory molecules, CD28 and IL-2 receptor,
which were also important for the T cell response against antigen, remained unchanged even when the c-fos gene was overexpressed. The level of c-Jun and JunB also did not change significantly. c-Jun is basically not inducible in lymphoid cells (40, 41). We may therefore assume that c-Fos/AP-1 plays a crucial role in the induction of and resistance against anergy.

The molecular target of increased c-Fos/AP-1 remains unclear. Previous studies have shown that activation of p21ras via CD3 is defective in anergized T cells (42). MAPK activity, immediately downstream to p21ras, is also impaired (6), while Shc, Grb-2 or Sos appears intact in anergized T cells (42). It has recently been shown that src family tyrosine kinase ZAP-70 and tyrosine phosphorylation of the TCRz chain are defective in anergized T cells (43–45), suggesting that the signaling pathway further upstream of c-Fos can be defective in anergized T cells. Li et al. have shown that activation of c-Jun is also blocked through impairment of ERK in anergic cells (6). Becker et al. have shown that transcription from one AP-1 binding sequence and two NRE-A is impaired in anergized T cells, whereas transcription through a single AP-1 binding sequence is normal in anergic T cells after anti-CD3 and anti-CD28 treatment, indicating that AP-1 is not the target of anergy (7). Nevertheless, the present study bypassing CD28 stimulation directly indicates that c-Fos/AP-1 is a target of T cell anergy. Continuous supply of c-Fos/AP-1 in fos mice or in antigen-specific T11 cells might have normalized the amount of c-Fos/AP-1 required for transcription of IL-2 in anergic T cells. Otherwise, as pointed out by the finding that induction of MAPK activity was maintained in 75/15pH8 cells but not in pH8 cells after anergy induction, overexpression of c-fos might have reconstituted the ability of anergic T cells to respond to antigen stimulation by affecting the pathway further upstream of c-Fos.

In summary, we have presented direct evidence that increased c-Fos/AP-1 confers resistance against anergy induction on antigen-specific T cells. The findings altogether would suggest that overexpression of c-Fos/AP-1 not only stimulates rheumatoid synovial cells to enhance joint destruction (9, 16), but also inhibits anergy induction of antigen-specific T cells and in this way may prolong chronic inflammation of RA.

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Abbreviations

AP-1 activator protein-1
APC antigen-presenting cell
Con A concanavalin A
DIG digoxigenin
EMSA electrophoretic mobility shift assay
ERK extracellular signal-regulated kinase
JNK c-Jun N-terminal kinase
KLH keyhole limpet hemocyanin
MAPK mitogen-activated protein kinase
NFAT nuclear factor of activated T cells
NRE-A negative regulatory element
PE phycoerythrin
SEB staphylococcos enterotoxin B

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