16. D. L. Mueller, M. K. Jenkins, R. H. Schwartz, *Annu. Rev. Immunol.* **7**, 445 (1989).
17. M. K. Jenkins *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5409 (1987).
18. J. Markmann *et al.*, *Nature* **336**, 476 (1988).
19. M. A. Blackman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6682 (1991).
20. R. T. Kubo, W. Born, J. W. Kappler, P. Marrack, *J. Immunol.* **142**, 2736 (1989).
21. O. Kanagawa, E. Palmer, J. Bill, *Cell. Immunol.* **119**, 412 (1989).
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Transactivation by AP-1 Is a Molecular Target of T Cell Clonal Anergy

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Anergy is a mechanism of T lymphocyte tolerance induced by antigen receptor stimulation in the absence of co-stimulation. Anergic T cells were shown to have a defect in antigen-induced transcription of the interleukin-2 gene. Analysis of the promoter indicated that the transcription factor AP-1 and its corresponding cis element were specifically down-regulated. Exposure of anergic T cells to interleukin-2 restored both antigen responsiveness and activity of the AP-1 element.

In pursuing the observations of Billingham and colleagues (*1*) on acquired immunological tolerance, Dresser showed that immunization with deaggregated foreign proteins rendered adult mice unresponsive to subsequent antigenic challenge (*2*). In contrast, the same antigen preparation initially administered with adjuvant was immunogenic. Models that use two signals for lymphocyte activation can potentially explain these early observations (*3*, *4*). Experimental evidence shows that stimulation of the T cell receptor (TCR) together with a second, nonantigen-specific signal, termed co-stimulation (*3–7*), causes T lymphocyte activation. Antigen receptor stimulation in the absence of co-stimulation, however, causes functional inactivation or anergy of the T cells (*4–7*). Anergic T cells have a greatly reduced capacity to produce interleukin-2 (IL-2) and to proliferate when re-stimulated with antigen–major histocompatibility complex (MHC) and co-stimulation. They also produce less of other lymphokines such as IL-3 and γ-interferon (IFN-γ). Studies with transgenic mice have provided compelling evidence that T cell tolerance in vivo can be due to the induction of anergy (*8*).

The $CD4^+$ mouse T cell clone A.E7 can be stimulated to produce IL-2 by a pigeon cytochrome c peptide (amino acids 81 to 104) presented by the MHC class II molecule E^k in the presence of co-stimulation (*7*, *9*). A.E7 cells can be rendered anergic by depleting them of residual antigen-presenting cells (APCs) and activating them with agents that trigger the TCR or raise intracellular Ca^{2+} concentrations (*4*, *10*). We induced anergy with concanavalin A (Con A), a lectin that binds the TCR and does not activate co-stimulatory pathways (*10*, *11*). A.E7 cells were treated with Con A (5 μg/ml) for 24 hours, after which the blocking sugar α-methyl mannoside (10 mg/ml) was added (*12*). The cells were subsequently incubated for 4 to 6 days before use. The Con A–treated group (termed anergic) had nearly identical surface expression of CD3, CD4, CD45, and IL-2 receptor α chain, as compared to cells treated in parallel without Con A (termed normal) (*13*). Normal and anergic T cells were then restimulated with antigen and splenic APCs, which provide full co-stimulation. Anergic A.E7 cells typically showed a 70 to 90% reduction in maximal incorporation of ^3H-labeled thymidine and a requirement for 25- to 50-fold more antigen to obtain half-maximal proliferation, although anergic cells were capable of proliferating in response to exogenous IL-2 (Fig. 1A). In anergic cells, maximal production of IL-2 was significantly decreased (to between one-tenth and one-fiftieth of the normal value), whereas maximal IFN-γ production was only diminished from one-third to one-half at 10 μM antigen (Fig. 1, B and C).

Northern (RNA) blot analysis of a time course of induction revealed that steady-state mRNA for IL-2 peaked sharply at 4 hours after antigen stimulation, whereas steady-state mRNA for IFN-γ appeared to plateau by 6 hours (Fig. 2A, lanes 1 through 7) (*14*). Thus, critical regulatory events for IL-2 mRNA production occur at times earlier than 4 hours. Induction of both mRNAs depended on peptide antigen (Fig. 2A, lanes 8 and 9). As observed for lymphokine production, IL-2 mRNA amounts were dramatically decreased in anergic A.E7 cells, whereas IFN-γ mRNA amounts were only modestly attenuated (lanes 11 and 13).

Studies have shown that IL-2 mRNA

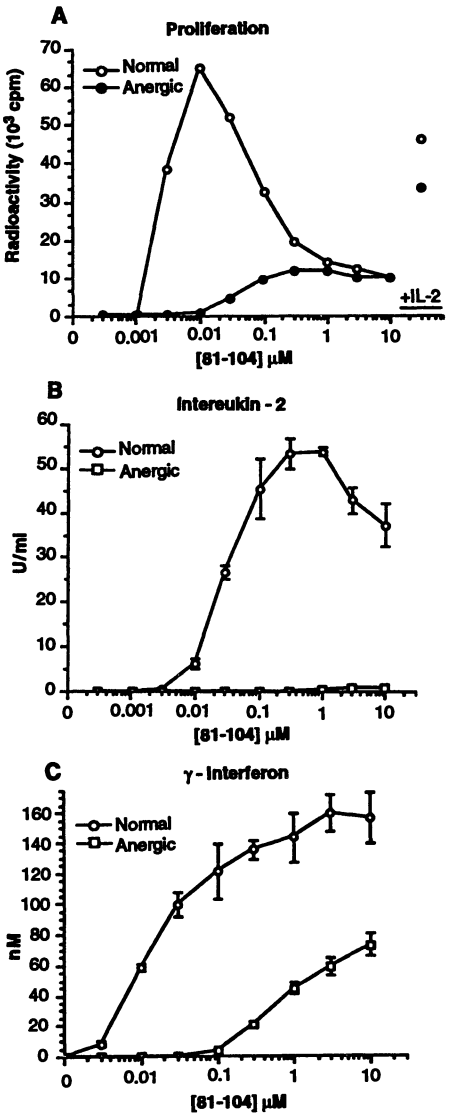

Fig. 1. Proliferation and lymphokine production assays of normal and anergic A.E7 cells. A.E7 cells induced into the anergic state or normal controls were rechallenged with B10.A spleen cells and increasing amounts of pigeon cytochrome c peptide (amino acids 81 to 104) (*12*). (**A**) Proliferation assay. [^3H]thymidine was added 24 hours after stimulation, and incorporation was measured after an additional 16 hours (*10*). Unconnected points above the "+IL-2" bar denote cells treated with IL-2 (30 units per milliliter) in the absence of antigen. (**B**) IL-2 bioassay on 24-hour culture supernatants. (**C**) IFN-γ assay on 24-hour culture supernatants. Error bars are for triplicate samples.

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amounts can be controlled at the level of either gene transcription or mRNA stability (*15*, *16*). To determine transcription rates for the IL-2 and IFN-γ genes, nuclear run-on assays were performed. For measuring lymphokine gene transcription, we needed to exclude homopolymeric nucleotide sequences and subtle repetitive elements from target DNAs (*15*). Using selected target sequences, we found that after a 4-hour stimulation with antigen and fibroblasts bearing E^k class II molecules (DCEK) cells, IL-2 gene transcription was induced in normal cells, but the induction in anergic cells was 1/7.4 that of normal (Fig. 2B). IFN-γ gene transcription was modestly reduced in anergic cells (Fig. 2B).

We next tested whether the activity of the 300-bp enhancer-promoter region of the IL-2 gene is affected in the anergic state. We used pools of stable transfectants of A.E7 cells containing this region linked to a chloramphenicol acetyltransferase (CAT) reporter gene (*16*–*19*). To analyze T cells without the presence of contaminating antigen-presenting cells, we stimulated A.E7 cells with magnetic bead–loaded DCEK cells as APCs (*19*). Either Con A–treated or normal A.E7 cells could then be isolated by a magnetic field from the APCs for CAT or nuclear extract analysis. In normal A.E7 cells with the IL-2 promoter construct, CAT activity was induced by 0.1 or 1 μM peptide antigen (Fig. 3). Induction of CAT activity by either dose of antigen was drastically reduced in anergic cells, concomitant with decreased endogenous IL-2 production (Fig. 3 and Table 1). Anergy also decreased the activity of the 300-bp human IL-2 gene enhancer linked to a minimal β-interferon promoter, but control constructs dependent solely on the TCR-α enhancer or the herpes virus thymidine kinase promoter were unaffected (*13*).

The 300-bp IL-2 promoter contains a number of DNA response elements that are bound by gene regulatory factors (*17*, *20*, *21*). Four well-characterized enhancer elements include those that bind NF-AT, NF-κB, and AP-1 and the NF-IL2A site that binds octamer proteins (*20*, *21*). We and others have shown that these DNA response elements contribute significantly to the activity of the mouse IL-2 promoter (*13*, *20*, *21*). We tested the activity of these elements in anergic cells by preparing pools of stably transfected A.E7 cells bearing CAT reporter constructs with multimerized copies of these elements. Analyses of normal and anergic pools of stable transfectants showed that a construct containing the AP-1 site from the IL-2 gene was the most affected (Fig. 3 and Table 1). In anergic cells, maximally induced CAT activity was decreased to one-twentieth of normal. In a second independent pool of stable transfectants of the AP-1 construct, the value was one-sixth of normal, and in a third pool, with a slightly different version of the IL-2 AP-1 site (*22*), the value was one-twelfth of normal. Control experiments showed that mutations in the AP-1 binding site of the IL-2 AP-1 construct eliminated antigen inducibility and left a low basal activity that was unaffected by anergy. By contrast, an IL-2 AP-1 construct with upstream mutations in a region that conferred CD28 responsiveness in Jurkat cells (*22*) retained antigen-induced activity that was decreased in anergic cells. We interpreted these effects on the AP-1 site to be significant because mutations in this site severely reduce or eliminate the activity of the 300-bp IL-2 promoter (*20*, *21*, *23*).

Induction of none of the other transcription element constructs was as significantly affected by anergy (Fig. 3 and Table 1). The NF-AT and NF-IL2A constructs were essentially unaffected. The maximal activity of a multimerized κB site from the mouse IL-2 gene (IL-2κB) was approximately one-half to one-fourth of normal, and the maximal activity of a multimerized κB site from the immunoglobulin gene (IgκB) was hardly affected in anergic cells. Endogenous IL-2 gene expression was decreased by the induction of anergy in all of the pools of stable transfectants as reflected by IL-2 bioactivity (Table 1).

Electrophoretic mobility shift analysis (EMSA) was then performed with nuclear extracts and defined factor binding sites (Fig. 4) (*24*). Control extracts from EL-4 mouse T lymphoma cells before and after stimulation with phorbol 12-myristate 13-acetate (PMA) revealed that Oct-1 and Oct-2 were constitutively expressed, whereas AP-1, NF-κB, and NF-AT were strongly induced. In nuclear extracts from A.E7 cells, Oct-1 was constitutive and did not change under the conditions tested. Oct-2, shown to activate the IL-2 octamer site (*20*, *21*), was present in unstimulated A.E7 cells and was induced moderately by antigen at 4.5 hours. Anergic cells had slightly higher basal amounts of Oct-2 that increased detectably on stimulation. For NF-AT, slight

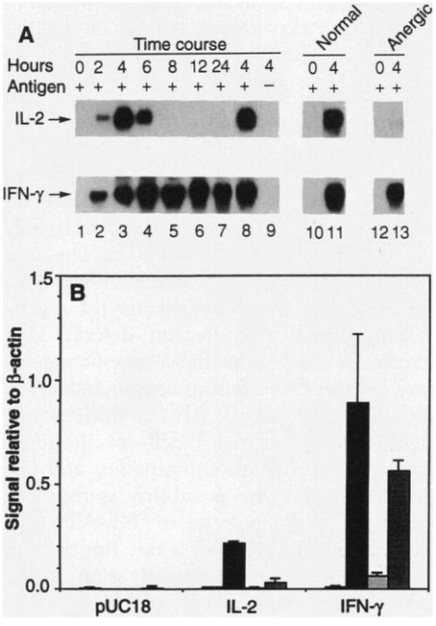

Fig. 2. Northern blot analysis of IL-2 and IFN-γ steady-state mRNA amounts and nuclear run-on analysis. (**A**) Total RNA was isolated from 2×10^7 A.E7 cells stimulated with 10 μM antigen and 1×10^8 irradiated B10.A spleen cells (depleted of T cells) after the times indicated above the panel (*14*). The same blots were sequentially hybridized with the IL-2 or IFN-γ probes as indicated. A.E7 cells in lane 9 were not stimulated with antigen. (**B**) Nuclear run-on assays. Bar graphs indicate the mean + SD of two determinations. In each set of four, first bar is normal, no antigen; second bar is normal 10 μM antigen; third bar is anergic, no antigen; fourth bar is anergic, 10 μM antigen. The pUC18 is a control for nonspecific hybridization. Results are representative of two separate experiments.

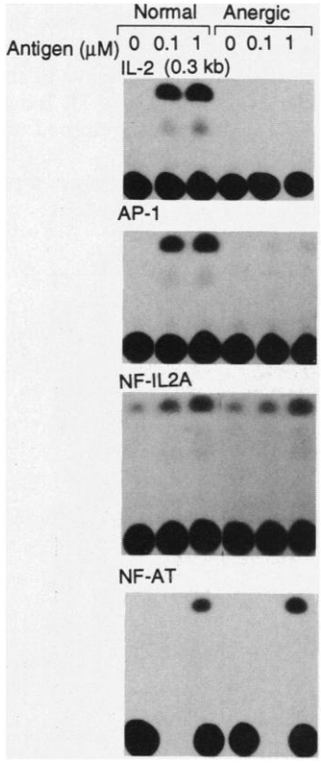

Fig. 3. CAT assays of A.E7 cells stably transfected with the constructs indicated to the left of the panel (*19*). Pools of stable transfectants were then treated with Con A or media as indicated and subsequently stimulated with the indicated concentrations of antigen and DCEK cells for 16 hours. A.E7 cells were magnetically separated from the DCEK cells before assay.

constitutive binding was detected in unstimulated anergic cells, but maximally induced NF-AT binding was nearly the same in normal and anergic cells. NF-κB was also induced to equivalent amounts in normal and anergic cells. The induced amounts of NF-κB in lanes 3 and 8, in which A.E7 cells were incubated 4.5 hours with DCEK cells in the absence of antigen, were due to the production of low amounts of tumor necrosis factor α (TNF-α) by DCEK cells. TNF-α induces NF-κB in T cells (*25*).

Unlike the other factors, AP-1 binding displayed a sharp peak in binding activity at 3 hours. This peak preceded the apogee of IL-2 mRNA accumulation at 4 hours and suggests that AP-1 may act as a limiting element for IL-2 gene transcription. AP-1 binding was reduced in anergic cells at the peak 3-hour time point (Fig. 4). At 4.5 hours, the low amount of induced AP-1 binding was comparable in normal and anergic cells (Fig. 4). Repeat experiments confirmed that AP-1 binding is decreased only at time points before the peak of IL-2 mRNA. Nonetheless, control experiments revealed no delay in the kinetics of either IL-2 mRNA accumulation or production of IL-2 bioactivity in anergic T cells (*13*). The binding complex from A.E7 cells comigrated with the AP-1 complex defined in HeLa cells with a human collagenase DNA site (*26*) and reacted with antiserum to either the c-*fos* or c-*jun* protein products (*13*). Thus, AP-1 induction is moderately decreased at early time points after antigen stimulation, and the low amount of AP-1 binding observed at 4.5 hours cannot support transcription at later times in anergic cells.

Two studies have proposed that a site centered around −180 of the IL-2 promoter is also an AP-1 site (*21*, *27*), although several studies have shown that deletion of this element has no effect on the inducibility of the IL-2 promoter (*17*, *20*, *21*). We detected no binding of AP-1 to this site (*13*). Moreover, antibodies to c-*jun* and c-*fos* protein products failed to react with complexes formed with this site, and neither the IL-2 AP-1 nor the human collagenase AP-1 oligonucleotides could compete with the complexes (*13*).

We and others have shown that IL-2 treatment of anergic cells can restore proliferation and lymphokine production to normal amounts (*6*, *28*). To determine whether this reversion was correlated with a renewed ability to induce the IL-2 AP-1 site in response to antigen, we treated pools of normal and anergic A.E7 stable transfectants with IL-2 for 7 days (Fig. 5). Five days after stimulation with Con A, proliferation of the anergic cells was one-twentieth the value for normal cells in response to stimulation by 1 μM antigen. After 7 days in IL-2, IL-2 production from the endogenous gene and inducibility of both the 300-bp IL-2–CAT and IL-2 AP-1–CAT constructs were restored to normal amounts in anergic A.E7 cells (Fig. 5 and Table 1). Induction of the AP-1 complex also returned to normal (*13*).

Our results show that anergy, a potentially important mechanism of extrathymic tolerance (*8*, *10*, *29*), results from a molecular change whereby an initial stimulation of a cell surface receptor diminishes gene activation in response to subsequent signaling through the same receptor. The fact that the NF-AT and octamer elements were unaffected by anergy argues against a generalized signal transduction defect. This reinforces the notion that a specific regulatory event occurs during anergy induction (*30*). Anergy affects AP-1 but does not significantly affect the T cell–specific regulator NF-AT, which contains Fos and Jun proteins (*31*). One possibility is that the cytoplasmic component of NF-AT (*31*, *32*), and not Fos or Jun, is rate limiting for NF-AT binding and transactivation. However, we have not detected significant differences in the induction of c-*fos* or c-*jun* mRNA between normal and anergic T cells (*13*). Thus, an alternative possibility is that specific posttranslational down-modulation of AP-1 transactivation occurs in anergic cells. This regulatory feature of the AP-1 transactivator may serve to modulate gene activation that is coupled to surface receptor signaling in other cell lineages.

Our experiments demonstrate that anergy and its gene regulatory phenotype can be reversed by IL-2 (*6*, *28*). In certain circumstances, anergy may down-regulate T cell responses immediately after antigen exposure and could later be reversed if the antigen does not persist. Immunomodula-

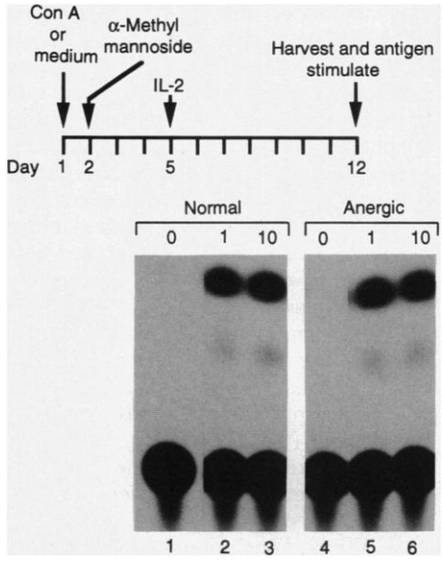

Fig. 5. Reversion of anergy by IL-2 treatment. (**Top**) A schematic of the protocol for the reversion of anergy. (**Bottom**) CAT assay of normal and anergic AP-1 stable transfectants treated with IL-2 (30 units per milliliter) for 7 days, then stimulated with antigen and DCEK cells for 16 hours before separation and harvest. Micromolar antigen concentrations are indicated at the top of the CAT assay.

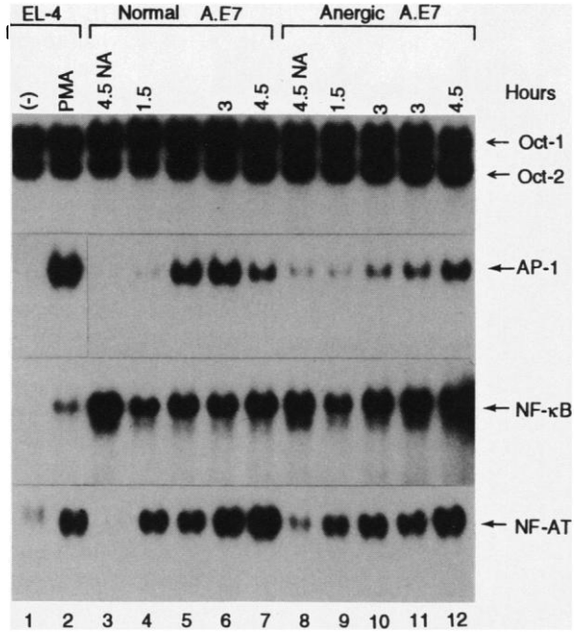

Fig. 4. Electrophoretic mobility shift assay. Nuclear extracts were prepared from normal and anergic A.E7 cells that had been stimulated with 10 μM antigen and DCEK cells and separated at the times indicated above the panel as described (*24*). The duplicate 3-hour time points are the result of two independent antigen stimulations and nuclear extract preparations. For each assay, 1 μg of nuclear extract, 0.5 μg of poly [dI, dC], and 20,000 cpm of end-labeled oligonucleotide probe were incubated in binding buffer for 20 min at ambient temperature in a 6-μl reaction (*20*, *21*, *24*). In lanes 3 and 8, A.E7 cells were incubated with DCEK cells in the absence of antigen (NA) for 4.5 hours before harvest. Only the shifted bands are shown. For AP-1 and NF-κB, the complexes shown were obtained with the AP-1 site from gibbon ape leukemia virus (gift of D. Levens, National Cancer Institute, Bethesda, Maryland) and the IgκB oligonucleotides, respectively. Similar results were obtained with the corresponding sites in the IL-2 gene, except for the IL-2κB site, which also detected a prominent homodimeric complex of the NF-κB p50 subunit (*13*, *20*, *21*).

Table 1. Transcriptional activity of IL-2 promoter elements in normal and anergic A.E7 T lymphocytes. Percent acetylation was calculated as the fraction of acetylated chloramphenicol to total chloramphenicol after thin-layer chromatographic separation and quantitation in a Phosphorimager. CAT assays were normalized to the amount of protein for all antigen doses and between anergic and normal groups of cells for the pool of transfectants for each DNA response element construct. Unstimulated control samples were incubated without antigen or APCs. For the IgκB site, CAT activity could be stimulated by APCs without antigen, but IL-2κB activation required both antigen and APCs (*20, 21*). Assay conditions may vary between pools of stable transfectants for individual constructs, which may contribute to variations in the total percentage of acetylated chloramphenicol observed. We measured IL-2 by testing the culture supernatants from restimulated pools of stable transfectants in a CTLL proliferation assay. One unit equals the amount of supernatant needed to achieve half-maximal proliferation under standard conditions. Assay conditions vary between pools of stable transfectants, leading to different total units although different antigen stimulations of normal and anergic cells were assayed together. We calculated CAT value by subtracting the background percent acetylation observed in unstimulated samples from the percent acetylation observed with the highest antigen dose and taking the ratio of the activity observed in normal cells to the activity observed in anergic cells.

Promoter element	Antigen (μM)	Normal		Anergic		CAT value
		Acetylation (%)	IL-2	Acetylation (%)	IL-2	
IL-2 promoter	0	0.38	0	0.47	0	
	0.1	11.00	97	1.30	0.3	
	1	11.00	120	1.60	0.6	9.4
IL-2 AP-1	0	0.08	0	0.11	0	
	0.1	1.5	79	0.20	0.2	
	1	2.3	126	0.22	0.8	20.2
NF-IL2A	0	0.18	0	0.21	0	
	0.1	0.54	61	0.34	0.6	
	1	1.31	136	1.10	2.8	1.3
IL-2κB	0	0.12	0	0.18	0	
	0.1	14.0	8	4.0	0	
	1	19.0	37	5.2	0.01	3.8
IgκB	0	0.04	0	0.09	0	
	0.1	0.30	1.8	0.31	<0.1	
	1	0.37	6.7	0.24	<0.1	2.2
NF-AT	0	0.03	0	0.04	0	
	1	1.30	117	1.40	11.5	0.9
IL-2 promoter (+ IL-2 treatment)	0	0.04	0	0.040	0	
	0.1	0.39	18	0.36	9	
	1	1.00	40	1.10	26	0.9
AP-1 (+ IL-2 treatment)	0	0.03	0	0.03	0	
	0.1	0.20	31	0.12	18	
	1	0.42	82	0.27	40	1.6

tion strategies based on anergy induction may require continuous antigen exposure and possibly blockade of IL-2 production to be effective. Because extrathymic immune tolerance in mature animals can be established in vivo by the induction of T cell anergy, it will be important to determine whether T cells induced into the anergic state in vivo display a defect in AP-1 transactivation. Also, the description of anergy in B cells (*33*) provides an opportunity to examine the possibility of a generalized mechanism for anergy in all lymphoid cells.

REFERENCES AND NOTES

1. R. E. Billingham, L. Brent, P. B. Medawar, *Philos. Trans. R. Soc. Lond. (Biology)* **239**, 357 (1956).
2. D. Dresser, *Immunology* **5**, 378 (1962).
3. D. R. Forsdyke, *Lancet* i, 281 (1968). P. Bretscher and M. Cohen, *Science* **169**, 1042 (1970); K. J. Lafferty and A. J. Cunningham, *Aust. J. Exp. Biol. Med. Sci.* **53**, 27 (1975); K. J. Lafferty, S. J. Prowse, C. M. Simeonovic, H. S. Warren, *Annu. Rev. Immunol.* **1**, 143 (1983).
4. J. R. Lamb *et al., J. Exp. Med.* **157**, 1434 (1983); M. K. Jenkins and R. H. Schwartz, *ibid.* **165**, 302 (1987); H. Quill and R. H. Schwartz, *J. Immunol.* **138**, 3704 (1987); D. L. Mueller, M. K. Jenkins, R. H. Schwartz, *Annu. Rev. Immunol.* **7**, 445 (1989).
5. P. B. Martin *et al., J. Immunol.* **136**, 3282 (1986); P. S. Linsley, E. A. Clark, J. A. Ledbetter, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5031 (1990); H. Reiser *et al., ibid.* **89**, 271 (1992); F. A. Harding, J. McArthur, J. A. Gross, D. Raulet, J. P. Allison, *Nature* **356**, 607 (1992); L. Koulova, E. A. Clark, G. Shu, B. DuPont, *J. Exp. Med.* **173**, 759 (1991); Y. Liu *et al., ibid.* **175**, 437 (1992).
6. D. R. DeSilva, K. B. Urdahl, M. K. Jenkins, *J. Immunol.* **147**, 3261 (1991).
7. G. J. V. Nossal and B. L. Pike, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 16026 (1980); R. H. Schwartz, *Science* **248**, 1349 (1990); B. Beverly, L. Chiodetti, K. Brorson, R. H. Schwartz, D. Mueller, in *Molecular Mechanisms of Immunological Self-Recognition*, F. W. Alt and H. J. Vogel, Eds. (Academic Press, New York, in press).
8. L. C. Burkly, D. Lo, R. A. Flavell, *Science* **248**, 1364 (1990); D. Lo *et al., J. Exp. Med.* **170**, 87 (1989); M. Bonneville *et al., Nature* **344**, 163 (1990); G. Morahan, J. Allison, J. F. A. P. Miller, *ibid.* **339**, 622 (1989).
9. T. T. Hecht, D. L. Longo, L. A. Matis, *J. Immunol.* **131**, 1049 (1983).
10. M. K. Jenkins *et al., ibid.* **147**, 2461 (1991); D. L. Mueller, M. K. Jenkins, R. H. Schwartz, *ibid.* **142**, 2617 (1989).
11. S. C. Hubbard, D. M. Kranz, G. D. Longmore, M. V. Sitkovsky, H. N. Eisen, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1852 (1986); M. V. Sitkovsky, M. S. Pasternak, H. N. Eisen, *J. Immunol.* **129**, 1372 (1982).
12. Anergy induction was carried out on A.E7 cells purified over a Ficoll (Pharmacia) density gradient, treated with an antibody cocktail to Ia (10-2.16 and Y17) plus rabbit complement (Lo-Tox-M, Cedarlane, Hornby, Ontario) for 30 min at 37°C, and again isolated over Ficoll. Cells (4×10^7) were placed in 150 ml of medium with or without Con A (5 μg/ml). After 24 hours, α-methyl mannoside (10 mg/ml) was added. Cells were incubated for an additional 4 to 6 days, then isolated on Ficoll before restimulation. Proliferation assays used 2×10^4 cells and 5×10^5 irradiated B10.A splenic APCs depleted of T cells, as described (*10*). Supernatants were taken at 24 hours; IL-2 (Fig. 1B) was measured with the IL-2–dependent CTLL-2 line (American Type Culture Collection, Rockville, MD) as described [J. D. Ashwell, R. E. Cunningham, P. Noguchi, D. Hernandez, *J. Exp. Med.* **165**, 173 (1987)], and IFN-γ (Fig. 1C) was quantitated by enzyme-linked immunosorbent assay (ELISA) as described (*28*).
13. S.-M. Kang *et al.*, unpublished data.
14. We isolated mRNA from cells after stimulation using RNAzol (Cinnatex, Friendswood, TX) according to the manufacturer's instructions, and Northern blot analysis was performed with standard procedures [F. M. Ausubel *et al., Current Protocols in Molecular Biology* (Greene Publishing, New York, 1987) vols. 1 and 2]. RNA loading and integrity were determined to be equivalent by ethidium bromide staining or by hybridization with a rat α-actin probe (*13*). Nuclear run-on assays have been described (*15*). Autoradiographs of hybridized filters on pre-flashed film were quantitated on an optical gel scanner (LKB). Signal strengths for each group were normalized to that given by a mouse β-actin cDNA probe provided by M. Buckingham [S. Alonso, A. Minty, Y. Boulet, M. Buckingham, *J. Mol. Evol.* **23**, 11 (1986)].
15. K. A. Brorson *et al., J. Immunol.* **147**, 3601 (1991).
16. M. Kronke, W. J. Leonard, J. M. Depper, W. C. Greene, *J. Exp. Med.* **161**, 1593 (1985); T. Lindsten *et al., Science* **244**, 339 (1989).
17. T. Fugita *et al., Cell* **46**, 401 (1986); D. B. Durand *et al., J. Exp. Med.* **165**, 395 (1987); D. B. Durand *et al., Mol. Cell. Biol.* **8**, 1715 (1988); M. N. Brunvand, A. Schmidt, U. Siebenlist, *J. Biol. Chem.* **263**, 18904 (1988).
18. C. M. Gorman, *Mol. Cell. Biol.* **2**, 1044 (1982).
19. We performed stable transfections by placing A.E7 cells in IL-2 (30 units per milliliter) for 3 days. Then 5×10^6 cells were electroporated at 280 V and 960 μF on a Genepulser (BioRad) in RPMI 1640 with 20% fetal calf serum, 25 μg of linearized constructs, and 2.5 μg of pSV2Neo for selection. The electroporated cells were placed back in IL-2 (30 units per milliliter) for 2 days, after which G418 was added to a final concentration of 0.8 mg/ml. After 5 days, the cells were stimulated with antigen and carried normally in selection medium. After expansion, at least five separate transfections were pooled, and at least two different pools were tested for each construct, except for NF-IL2A, for which only one pool was tested. Typically, 1 to 2×10^6 stably transfected cells were stimulated by antigen for CAT analysis. The technique for antigen stimulation followed by cell separation is: Briefly, 2.5×10^7 trypsinized DCEK cells [F. Ronchese, R. H. Schwartz, R. Germain, *Nature* **329**, 254 (1987)] were mixed with 1×10^8 magnetic beads (Dynal) in 50 ml of DCEK medium and incubated for 2 days, during which time DCEK cells phagocytose the magnetic beads. DCEK cells have potent co-stimulatory activity. The cells that contained beads were isolated by five 5-min passes through a magnetic field. For antigen stimulation of A.E7 cells, the DCEK:A.E7 ratio was 1:2. After stimulation, the A.E7 cells were separated from the DCEK cells by vigorous pipetting and by three 5-min passes over a magnetic field. Stable transfectants were made with

the following oligonucleotides; each oligonucleotide also has a 5′-TCGA-3′ Sal I extension that we added to the 5′ end to facilitate cloning, and mouse IL-2 sequences were used unless otherwise indicated: AP-1, 5′-GAAATTCCAGAGAGTCATCAGAAGA-3′; NF-IL2A, 5′-GAAAATATGTGTAATATGTAAAACATCGT-3′; IL-2κB, 5′-CCAAGAGGGATTTCACCTAAATCC-3′; NF-AT, 5′-AAGAGGAAAATTTGTTTCATACAGAAGGCG-3′; IgκB, 5′-CAGAGGGGACTTTCCGAGAGGC-3′ from the mouse κ light chain enhancer [J. W. Pierce, M. J. Lenardo, D. Baltimore, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1482 (1988)]; human collagenase AP-1, 5′-AAGCATGAGTCAGACAC-3′ from the human collagenase promoter (*26*); and 2m, an IL-2 AP-1 oligonucleotide with mutations underlined, 5′-GAAATTCCAGAGACGAATCAGAAGA-3′. Plasmid constructions were made as follows: IL-2 (0.3 kb), a Fok I–Pst I fragment of the IL-2 promoter was cloned into the Hind III/Pst I site of pBLCAT3 [B. Luckow and G. Schutz, *Nucleic Acids Res.* **15**, 5490 (1987)]; AP-1, two copies of the oligonucleotide containing three copies of the IL-2 AP-1 site were cloned into the Sal I site of pBLCAT2 in an AA orientation; octamer, four copies of the IL-2 octamer site were inserted into pBLCAT2 in the AAAA orientation (*20*, *21*); NF-AT, three copies of the NF-AT site were cloned into the Sal I site of pBLCAT2 in the BBA orientation; IL-2κB, six copies of the IL-2κB oligonucleotide were inserted into the Sal I site of △56 such that the IL-2κB sites were in the A orientation; and IgκB, two copies of the IgκB oligonucleotide were cloned into the Sal I site of △56 to make J16. A is the orientation of the sequence in the gene, B orientation is its inversion. All plasmids were constructed with standard techniques and confirmed by sequencing. Extract (5 to 25 mg) was used in CAT assays, and we used the same amounts of protein to compare normal and anergic cells at different antigen doses. CAT conversion (percent of acetylated forms over total chloramphenicol) was quantitated with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

20. H. Shibuya, M. Yoneyama, T. Taniguchi, *Int. Immunol.* **1**, 143 (1989); J.-P. Shaw *et al.*, *Science* **241**, 202 (1988); E. A. Emmel *et al.*, *ibid.* **246**, 1617 (1989); T. Brabletz, I. Pietrowski, E. Serfling, *Nucleic Acids Res.* **19**, 61 (1991); M. J. Lenardo, A. Kuang, A. Gifford, D. Baltimore, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8825 (1988); D. W. Ballard *et al.*, *Science* **241**, 1652 (1988); A. Radler-Pohl, I. Pfeffer, M. Karin, E. Serfling, *New Biol.* **2**, 566 (1990); M. P. Kamps, L. Corcoran, J. H. Lebowitz, D. Baltimore *Mol. Cell. Biol.* **10**, 5464 (1990); K. S. Ullman, W. M. Flanagan, C. A. Edwards, G. R. Crabtree, *Science* **254**, 558 (1991); H. Shibuya and T. Taniguchi, *Nucleic Acids Res.* **17**, 9173 (1989); S.-M. Kang *et al.*, *Mol. Cell. Biol.* **12**, 3149 (1992); C. Randak *et al.*, *EMBO J.* **9**, 2529 (1990); S.-M. Kang, A.-C. Tran, M. Grilli, M. J. Lenardo, *Science* **256**, 1452 (1992).
21. E. Serfling *et al.*, *EMBO J.* **8**, 465 (1989).
22. J. D. Fraser, B. A. Irving, G. R. Crabtree, A. Weiss, *Science* **251**, 313 (1991).
23. T. M. Williams *et al.*, *J. Immunol.* **141**, 662 (1988); J. Jain, V. E. Valge-Archer, A. J. Sinsky, A. Rao, *J. Exp. Med.* **175**, 853 (1992).
24. Miniscale EMSA for NF-AT, octamer, and NF-κB was carried out with a custom-designed minigel (M. J. Lenardo, unpublished data; Owl Scientific, Cambridge, MA) as previously described by S.-M. Kang, A.-C. Tran, M. Grilli, and M. J. Lenardo (*20*). For AP-1, the binding was performed in buffer D [J. D. Dignam, R. M. Lebovitz, R. G. Roeder, *Nucleic Acids Res.* **11**, 1475 (1983)] without $MgCl_2$, and the assay was performed with 0.5× TBE buffer (1× is 45 mM tris base, 45 mM boric acid, and 0.5 mM EDTA) (pH 8.5). We found all bands shown to be specific, as judged by competition analysis, comigration with control extracts known to contain the various complexes, and by antibody supershift analysis for AP-1 and octamer (Fig. 5) (*13*).
25. M. Grilli, J. C. Chiu, M. J. Lenardo, *Int. Rev. Cytol.*, in press; (*13*).
26. W. Lee *et al.*, *Nature* **325**, 368 (1987); P. Angel *et al.*, *ibid.* **332**, 166 (1988); R. Chiu *et al.*, *Cell* **54**, 541 (1988).
27. K. Muegge *et al.*, *Science* **246**, 249 (1989).
28. K. Tomonari, *Cell. Immunol.* **96**, 147 (1985); G. Essery, M. Feldmann, J. R. Lamb, *Immunology* **64**, 413 (1988); B. Rocha and H. von Boehmer, *Science* **251**, 1225 (1991); B. Beverly, S.-M. Kang, M. J. Lenardo, R. H. Schwartz, *Int. Immunol.* **4**, 661 (1992).
29. H. Rammensee, R. Krosechewski, B. Frangoulis, *Nature* **339**, 541 (1989); F. Ramsdell, T. Lantz, B. J. Fowlkes, *Science* **246**, 1038 (1989); M. A. Blackman *et al.*, *Nature* **345**, 540 (1990).
30. G. R. Otten and R. N. Germain, *Science* **251**, 1228 (1991).
31. J. Jain, P. G. McCaffrey, V. E. Valge-Archer, A. Rao, *Nature* **356**, 801 (1992).
32. W. M. Flanagan, B. Corthesy, R. J. Bram, G. R. Crabtree, *ibid.* **352**, 803 (1991).
33. C. C. Goodnow, S. Adelstein, A. Basten, *Science* **248**, 1373 (1990).

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A Truncated Erythropoietin Receptor That Fails to Prevent Programmed Cell Death of Erythroid Cells

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A form of the human erythropoietin receptor (EPOR) was identified in which the cytoplasmic region is truncated by alternative splicing. The truncated form of the receptor (EPOR-T) is the most prevalent form of EPOR in early-stage erythroid progenitor cells, but the full-length EPOR (EPOR-F) becomes the most prevalent form in late-stage progenitors. EPOR-T can transduce a mitogenic signal. However, cells transfected with EPOR-T are more prone to programmed cell death than those expressing EPOR-F. EPOR-F may transduce a signal to prevent programmed cell death that is independent of the mitogenic signal, and alternative splicing of the EPOR gene may have an important role in erythropoiesis.

Erythropoietin (EPO) affects erythroid progenitors in the bone marrow by binding to the erythropoietin receptor (EPOR). The effects of EPO seem to be dependent on the stage of maturation of the cell on which it acts. EPO provides a proliferative signal to the erythroid burst-forming unit (BFU-E), early-stage progenitors, a differentiation signal to the erythroid colony-forming unit (CFU-E), late-stage progenitors (*1*), and a signal to maintain cellular viability to late-stage progenitors (*2*). EPO also inhibits DNA breakdown and prevents apoptosis in erythroid progenitor cells (*3*). Furthermore, EPO stimulates megakaryopoiesis (*4*). The mechanism by which these different signals are transmitted by one or more EPORs remains unknown.

We have detected three forms of the EPOR. Two of them were isolated by screening a cDNA library from an EPO-dependent megakaryoblastoid cell line, UT7 (*5*), with mouse EPOR cDNA as a probe (*6*). Of ten clones isolated, two had an open reading frame encoding full-length EPOR (EPOR-F) identical to the receptor isolated from liver or erythroid cell lines (*7*). Another cDNA clone had a 104-bp insert in the extracellular region (Fig. 1A). This insert introduces a stop codon before the transmembrane domain, such that this message encodes a soluble form of EPOR (EPOR-S) lacking both the transmembrane and cytoplasmic portions (Fig. 1B). The existence and the function of similar soluble receptor species have been reported for other members of the cytokine receptor superfamily (*8*).

A third form of EPOR was recovered by replicating mRNAs from normal human bone marrow (BM) mononuclear cells in the reverse transcriptase polymerase chain reaction (RT-PCR). After PCR with primers in exon I and IV (PCR 1-4) (Fig. 1A), a single 508–base pair (bp) band was observed as expected from the cDNA sequence (Fig. 1C). After PCR with the primers in exons IV and VIII (PCR 4-8) (Fig. 1A), three bands were detected. One was the 608-bp product predicted from EPOR-F; others were approximately 700 bp and 800 bp in size (Fig. 1C). We subcloned the 700-bp PCR products into pBluescript and sequenced several clones. There were two different 700-bp products with different inserts; one had a 104-bp insert (insert α) corresponding to that in EPOR-S and one contained a 95-bp insert in the cytoplasmic region (insert β) (Fig. 1A). The sequence in the 95-bp insert includes a stop codon. Thus, the message encodes an EPOR that

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