of the kind observed in this medfly experiment do not fit the simplistic formulation of the Lotka equation (*19*), and thus new equations will have to be developed to incorporate the observed plasticity of fertility and survival (*20*). (iv) The effects on longevity of dietary restriction may be mediated by gonadal activity or through the rate of ovarial depletion (*15*). The causal mechanism underlying the dietary restriction response that has been observed in a wide range of species (*21*) may be linked with physiological adaptations to nutritional stress in yeast (stationary phase) and nematodes (dauer stage) and to host and mate deprivation in insects (*12*, *22*).

References and Notes

1. T. Audesirk and G. Audesirk, *Biology: Life on Earth* (Prentice-Hall, Upper Saddle River, NJ, ed. 4, 1996).
2. C. E. Finch and R. E. Tanzi, *Science* **278**, 407 (1997); J. W. Vaupel *et al.*, *ibid.* **280**, 855 (1998); T. E. Johnson and W. B. Wood, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6603 (1982).
3. J. R. Carey, P. Liedo, D. Orozco, J. W. Vaupel, *Science* **258**, 457 (1992).
4. Pairs were housed in 6.5 cm by 6.5 cm by 12 cm clear plastic containers. Dead males were replaced with virgin males of the same age. The adult diet consisted of yeast hydrolysate and pure sucrose (1:3 ratio by volume). The yeast hydrolysate (ICN Biomedicals) contained 60% protein as well as vitamins and minerals.
5. Considering simultaneously all possible comparisons of the life expectancy for protein groups t = 0, 30, 60, and 90 days (the 95% confidence intervals using Tukey's method for the differences in the mean post-protein), lifetime differences between any two means are at most 9.21 days. For example, for the differences between protein groups t = 0 and t = 30, 60, and 90 we obtain the 95% confidence intervals 3.22 ± 4.72, 4.49 ± 4.72, and −0.70 ± 4.72 days, respectively.
6. J. R. Carey, P. Liedo, H.-G. Müller, J.-L. Wang, J. W. Vaupel, *Funct. Ecol.* **12**, 359 (1998).
7. Age-specific central death rates, defined as $m_x = 2d_x/(n_{x-1} + n_x)$, were smoothed to obtain hazard function estimates, applying case weights n_x. Here d_x is the number of observed deaths and n_x the number at risk at day x. The 95% pointwise confidence bands were calculated based on the variance of these estimates. We found that the lower bound of the 95% confidence band for the hazard function for the Control A (sugar-only) is above the upper bound of the 95% confidence band for the hazard function for the t = 30 cohort from day 33 through 53 (with a few days of minor overlapping). Similarly, the lower bound of the confidence band for Control A is consistently above the upper bound of the confidence band for the t = 60 cohort from day 63 through 80. For details of methods see H.-G. Müller, J.-L. Wang, W. B. Capra, *Biometrika* **84**, 881 (1997).
8. H.-G. Müller, J.-L. Wang, W. B. Capra, P. Liedo, J. R. Carey, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2762 (1997).
9. G. Bell and V. Koufopanou, in *Oxford Surveys in Evolutionary Biology*, R. Dawkins and M. Ridley, Eds. (Oxford Univ. Press, Oxford, 1986), pp. 83–131.
10. C. E. Finch, *Longevity, Senescence, and the Genome* (Univ. of Chicago Press, Chicago, IL, 1990); P. M. Wise, K. M. Krajnak, M. L. Kashon, *Science* **273**, 67 (1996).
11. L. Partridge and R. Andrews, *J. Insect Physiol.* **31**, 393 (1985); L. Partridge, in *Insect Aging*, K.-G. Collatz and R. S. Sohal, Eds. (Springer-Verlag, Berlin, 1986), pp. 45–54.
12. L. Partridge, L. A. Green, K. Fowler, *J. Insect Physiol.* **33**, 745 (1987); J. R. Carey, D. A. Krainacker, R. I. Vargas, *Entomol. Exp. Appl.* **42**, 159 (1986); T. Aigaki and S. Ohba, *Exp. Gerontol.* **19**, 267 (1984).
13. J. R. Carey and P. Liedo, *Gerontologist* **35**, 588 (1996); see also D. Reznick, *Oikos* **44**, 257 (1985); B. D. Roitberg, *Evol. Ecol.* **3**, 183 (1989); M. Tatar and J. R. Carey, *Ecology*, **76**, 2066 (1995).
14. Z. B. Ball, R. H. Barnes, M. B. Visscher, *Am. J. Physiol.* **150**, 511 (1947); M. B. Visscher, J. T. King, Y. C. P. Lee, *ibid.* **170**, 72 (1952); B. J. Merry and A. M. Holehan, *J. Reprod. Fertil.* **57**, 253 (1979); A. M. Holehan and B. J. Merry, *Mech. Ageing Dev.* **33**, 19 (1985); see also E. J. Masoro, *J. Gerontol.* **43**, B59 (1988).
15. L. V. DePaola, in *Modulation of Aging Processes by Dietary Restriction*, B. P. Yu, Ed. (CRC Press, Boca Raton, FL, 1994), chap. 10.
16. E. J. Masoro and S. N. Austad. *J. Gerontol. Biol. Sci.* **51A**, B387 (1996); R. Holliday, *Bioessays* **10**, 4 (1989).
17. J. R. Carey and C. Gruenfelder, in *Between Zeus and the Salmon: The Biodemography of Longevity*, K. W. Wachter and C. E. Finch, Eds. (National Academy Press, Washington, DC, 1997), pp. 127–160; K. W. Wachter, *ibid.*, pp. 1–16.
18. J. Bouletreau, *Oecologia* **35**, 319 (1978); J. Hendrichs and M. A. Hendrichs, *Ann. Entomol. Soc. Am.* **83**, 632 (1990); J. Hendrichs, C. R. Lauzon, S. S. Cooley, R. J. Prokopy, *ibid.* **86**, 250 (1993).
19. A. J. Lotka, *Science* **26**, 21 (1907).
20. S. Tuljapurkar, *Theor. Pop. Biol.* **35**, 227 (1989).
21. R. Weindruch, *Sci. Am.* **40**, 46 (January 1996).

22. We thank J. Reyes (director of the Moscamed Program in Mexico) and J. Rull and J. Patino (assistant directors) for the use of the facilities in Metapa; D. Orozco, A. Oropeza, S. Salgado, R. Rincon, S. Rodriguez, and C. Fredersdorff for technical assistance; M. Tatar and L. Harshman for discussion; K. Brehmer for editorial suggestions; and B. Love for help with computing and graphs. Supported by grant AG-08761 from the National Institute on Aging and grants DMS-94-04906 and DMS-96-25984 from the National Science Foundation.

3 December 1997; accepted 30 June 1998

IEX-1L, an Apoptosis Inhibitor Involved in NF-κB–Mediated Cell Survival

Mei X. Wu,* Zhaohui Ao, K. V. S. Prasad, Ruilian Wu, Stuart F. Schlossman

Transcription factors of the nuclear factor–κB/rel (NF-κB) family may be important in cell survival by regulating unidentified, anti-apoptotic genes. One such gene that protects cells from apoptosis induced by Fas or tumor necrosis factor type α (TNF), *IEX*-1L, is described here. Its transcription induced by TNF was decreased in cells with defective NF-κB activation, rendering them sensitive to TNF-induced apoptosis, which was abolished by transfection with *IEX*-1L. In support, overexpression of antisense *IEX*-1L partially blocked TNF-induced expression of *IEX*-1L and sensitized normal cells to killing. This study demonstrates a key role of *IEX*-1L in cellular resistance to TNF-induced apoptosis.

Tumor necrosis factor type α (TNF), a major inflammatory cytokine, simultaneously activates a cell suicide program and an anti-death activity that results in resistance of many cancer cells to TNF-mediated killing, thus limiting its use in cancer therapy (*1*). TNF-stimulated anti-death activity, unlike TNF-induced cell death, depends on de novo protein synthesis and the genes involved appear to be transcriptionally activated by transcription factors of the nuclear factor–κB/rel (NF-κB) family (*2*, *3*). Hence, cells lacking NF-κB subunit RelA (p65) or overexpressing a mutated inhibitor IκBα gene showed enhanced susceptibility to TNF-mediated killing (*4*). Using the mRNA differential display technique (*5*), we cloned a gene that appeared to be the same as a previously reported immediate-early response gene *IEX*-1 (*6*), except that it had an in-frame insertion of 111 nucleotides at position 211 of the coding region for *IEX*-1, and it could encode a longer polypeptide with a 37–amino acid insertion relative to *IEX*-1 (*7*). The longer *IEX*-1 [referred to here as *IEX*-1L; the original *IEX*-1 is referred to as *IEX*-1S (short)] was found to be generated from *IEX*-1 in the absence of RNA splicing as it contained the entire intron sequence of *IEX*-1 (*8*).

IEX-1L protein was demonstrated in 293 cells transiently transfected with a pcDNA-HA·Tag–*IEX*-1L plasmid by using a monoclonal antibody (mAb) to influenza virus hemagglutinin (HA) (Fig. 1A, arrow L-HA) (*9*). The difference between the molecular mass of HA-IEX-1L (32 kD) and of HA–IEX-1S (28 kD) could be accounted for by a 37–amino acid insertion present in IEX-1L. Endogenous IEX-1L protein was also detected by using a polyclonal antibody (Ab) to IEX-1 (*10*) (Fig. 1B, arrow L), which was larger than the reported IEX-1S protein (*6*) (Fig. 1B, arrow S). When McF-7 cells expressed IEX-1L or IEX-1S fused to green fluorescence protein (GFP) (*9*), a typical pattern of fluorescence around the nuclear periphery and endoplasmic reticulum membrane was observed (Fig. 1C), which was distinct from the diffuse distribution of fluorescence visible throughout the entire cell when GFP alone was expressed (Fig. 1D).

Division of Tumor Immunology, Dana-Farber Cancer Institute, and the Department of Medicine, Harvard Medical School, Boston, MA 02115, USA.

*To whom correspondence should be addressed.

The localization of IEX-1L and IEX-1S in endoplasmic reticulum and on nuclear membrane was confirmed by immunoelectron microscopic study with a GFP-specific Ab (*8*). This observation is consistent with the presence of a putative transmembrane integrated region in IEX-1 proteins (*6*).

To investigate the function of *IEX*-1L and *IEX*-1S, we stably transfected Jurkat cells with the *IEX*-1L or *IEX*-1S coding frame inserted into a pRc/CMV plasmid. Easily detectable amounts of RNA of either *IEX*-1L or *IEX*-1S were observed in two of three randomly selected clones transfected with pRc/CMV–*IEX*-1L and in two of four clones carrying pRc/CMV–*IEX*-1S but in none of the clones receiving the parent control vector (Fig. 2B). When these cells were treated with mAb to Fas, the viability was more than 80% for clone 14/L and 70% for clone 15/L, both expressing *IEX*-1L after 4 hours of treatment (Fig. 2A). These percentages were significantly higher than the 35% observed in wild-type cells and in control plasmid-transfected clones (13/V and 11/V) and they were similar to that observed with Bcl-2–transfected Jurkat cells (*11*). In contrast, clone 9/L cells expressed undetectable *IEX*-1L RNA, showing little effect on cell susceptibility to Fas-mediated apoptosis compared with untransfected cells. Although *IEX*-1S transfectants, clones 2/S and 4/S, expressed amounts of *IEX*-1S mRNA similar to those transfected with *IEX*-1L (Fig. 2B), they offered no protection against Fas-induced killing, which strongly indicates a specific anti-death function of *IEX*-1L. Moreover, by using specific mAbs, we were able to show that all these clones expressed similar amounts of cell surface Fas and intracellular Bcl-2 and Bcl-x molecules, ruling out the possibility that the observed protection of *IEX*-1L was a result of effects of these key anti-apoptotic or apoptotic molecules (*8, 11*).

To avoid clonal variations, we used stably transfected bulk cultures of Jurkat cells to test *IEX*-1L–mediated protection (*12*). As indicated in Fig. 2C, Jurkat cells transfected with a control vector or *IEX*-1S–containing plasmid underwent Fas-induced apoptosis at degrees indistinguishable from wild-type cells (about 60%). In contrast, the Fas-mediated cell death of *IEX*-1L–bearing transfectants was significantly lower (about 23%).

IEX-1L–mediated protection appeared not to be restricted in Jurkat cells. McF-7 cells transiently transfected with an *IEX*-1S–GFP construct or GFP vector alone, along with a pRc/CMV-Fas plasmid, underwent Fas-mediated apoptosis by 35 to 40%, whereas the percentage of cells undergoing Fas-induced apoptosis was reduced by half if they expressed an IEX-1L–GFP protein (Fig. 2D).

Interestingly, *IEX*-1L restored the resistance to TNF-induced cell death of p65KO3T3 cells isolated from RelA−/− mice and Jurkat IκBαM cells generated by expression of a mutated IκBα protein (*4, 13*). As shown in Fig. 3A, TNF-induced apoptosis increased dramatically from 10 to 70% in p65KO3T3 cells bearing the parental control vector or in untransfected p65KO3T3 cells. In contrast, *IEX*-1L–transfected cells were markedly less

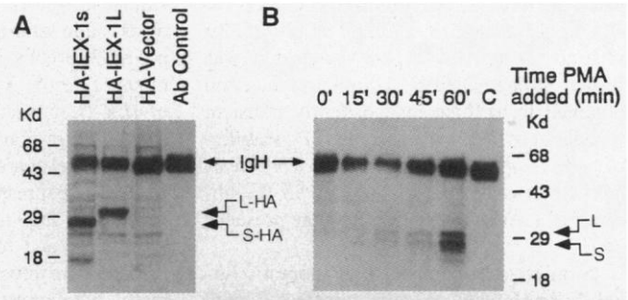

Fig. 1. Expression of transfected (A) and endogenous (B) IEX-1L proteins. (**A**) Immunoprecipitation followed by immunoblotting analysis was carried out as described (*16*) with cell lysates (50 μg) prepared from 293 cells transfected with the indicated HA·tag–containing constructs (*9*) using mAb 12CA5 to HA (Boehringer Mannheim). Lane Ab Control, an irrelevant mouse mAb used as control in immunoprecipitation. (**B**) Jurkat cell lysates prepared at various times after treatment with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) were analyzed as in (A) with an *IEX*-1L–specific Ab (*10*). Lane C, normal rabbit serum control. Molecular size markers (kD) are shown on the left (A) and right (B). IgH refers to the Ig heavy chain. (**C** and **D**) McF-7 cells were transfected with a plasmid pEGFP-IEX-1L (C) or pEGFP alone (D) and photomicrographed by inverted fluorescence microscopy after 40 hours. Bar = 20 μm. A similar pattern was also observed with cells expressing an IEX-1S–GFP fusion protein.

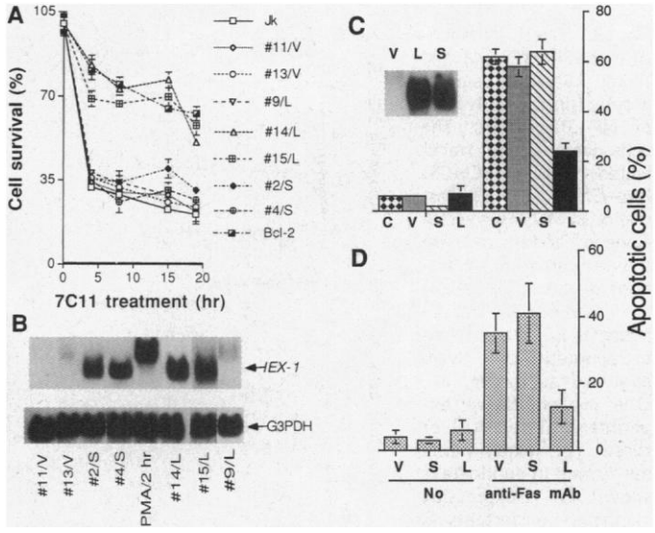

Fig. 2. Protective effect of *IEX*-1L against Fas-mediated apoptosis. (**A**) Viability of *IEX*-1L–transfected Jurkat cell clones. Jurkat cells were stably transfected with a plasmid pRc-CMV-*IEX*-1L (L), pRc-CMV-*IEX*-1S (S), or pRc-CMV control vector (V) and the subclones were selected by limiting dilution. Jurkat cells (JK), Bcl-2–transfected Jurkat cells (Bcl-2), and representative subclones of each transfection were treated with mAb 7C11 to Fas (1:10,000 ascites) for various times and then stained with propidium iodide (PI). Percentages of viable cells are shown as mean ± standard deviation (SD). One representative result of five independent experiments performed in triplicate is shown. (**B**) *IEX*-1 expression in Jurkat cell clones in (A) was analyzed by Northern blotting with an *IEX*-1 probe. Endogenous *IEX*-1 RNA (1.3 kb) in PMA-stimulated Jurkat cells (lane PMA/2 hr) is shown as a positive control, and transfected *IEX*-1 are about 0.6 kb for *IEX*-1L and 0.5 kb for *IEX*-1S, indistinguishable in size in the blot. The Pst fragment of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) is used as an equal RNA loading control. (**C**) Apoptotic cell death of bulk cultures of Jurkat cell transfectants. Jurkat cells were stably transfected with the same plasmids as in (A) (*12*). Untransfected control (C) and transfected Jurkat cells were either treated with mAb to Fas for 8 hours or left untreated. Percentages of apoptotic cells (SubG1 population) were determined by flow cytometry analysis of PI-stained cells. Data shown are means ± SD of three independent experiments using the same bulk transfectants. (Inset) *IEX*-1 RNA expression analyzed as in (B). (**D**) Protection of Fas-induced apoptosis in McF-7 cells transiently transfected with *IEX*-1L. McF-7 cells were cotransfected with a pEGFP–*IEX*-1L (L), pEGFP–*IEX*-1S (S), or pEGFP (V) plasmid (9) along with a pRc-Fas plasmid for 36 hours, after which the cells were treated with mAb 7C11 for 6 hours or were left untreated. Percentages (mean ± SD) of apoptotic cells (based on cell morphology) were obtained by averaging the results from triplicate wells of a six-well plate, with about 100 cells counted in each by inverted fluorescence microscopy. One representative result of three independent experiments is shown.

sensitive to the apoptotic effect, demonstrating an ability of *IEX*-1L to compensate the cells for a loss of RelA that results in them becoming sensitive to TNF-mediated apoptosis. The *IEX*-1L–mediated protection was highly reproducible with two independently transfected bulk cell cultures, p65KO3T3/*IEX*-1L(1) and p65KO3T3/*IEX*-1L(2), in four separate experiments. This conclusion was further strengthened by a similar protection obtained from three independently transient transfections. As shown in Fig. 3D, viability of p65KO3T3 cells in response to TNF treatment increased from 20 to 35% in the absence of *IEX*-1L to 65 to 72% in the presence of *IEX*-1L.

Similarly, the percentage of apoptotic Jurkat IκBαM cells or cells transfected with vector alone increased from 10% to more than 55% over the TNF concentrations used (Fig. 3B). However, Jurkat IκBαM cells transfected with *IEX*-1L showed a diminished capacity to undergo TNF-induced apoptosis; in fact, the TNF dose-response curve of *IEX*-1L–transfected Jurkat IκBαM cells was similar to that observed with Jurkat Lxsn cells (Jurkat cells transfected with a Lxsn vector alone) (*4*) (Fig. 3B).

We next examined whether TNF-induced expression of *IEX*-1L was defective in these cells. As shown in Fig. 3C, *IEX*-1L mRNA was about 14-fold lower in density in Jurkat IκBαM cells than in Jurkat Lxsn cells after 1 hour of treatment with TNF. A weak band similar to *IEX*-1L in size (about 1.3 kb) was observed in wild-type 3T3 (wt3T3) but not in p65KO3T3 cells 30 min after treatment with mouse TNF (*8*). The difference in the amount of *IEX*-1L transcript appeared to be diminished with prolonged TNF treatment.

These results suggested that an early defect in the expression of *IEX*-1L was likely a cause for the increased susceptibility of p65KO3T3 and Jurkat IκBαM cells to TNF-mediated apoptosis. To investigate this directly, we expressed *IEX*-1L in an antisense orientation in normal Jurkat cells. This partially blocked TNF-stimulated expression of *IEX*-1, as evidenced by a reduction in immunofluorescent staining by IEX-1–specific antibody in cells bearing antisense *IEX*-1L relative to that in control cells (Fig. 4A). In spite of having normal NF-κB proteins, the antisense *IEX*-1L–transfected cells underwent apoptosis at highly significant ($P < 0.01$) amounts at a TNF concentration of 200 units (U)/ml compared with those observed with untransfected cells or with cells transfected with vector alone (Fig. 4B), which suggests that NF-κB–mediated protection depends on expression of *IEX*-1L. The effect of antisense *IEX*-1L became less pronounced at a TNF concentration of 500 U/ml ($P < 0.05$), presumably due to a larger amount of induced *IEX*-1L transcript that overcame the neutralizing effect of antisense *IEX*-1L mRNA or additional anti-apoptotic genes being activated.

The data presented here demonstrate that cellular resistance to TNF-induced killing is directly related to the ability of cells to rapidly express *IEX*-1L in response to TNF stimulation. Thus, a rapid increase in the expression of *IEX*-1L after addition of

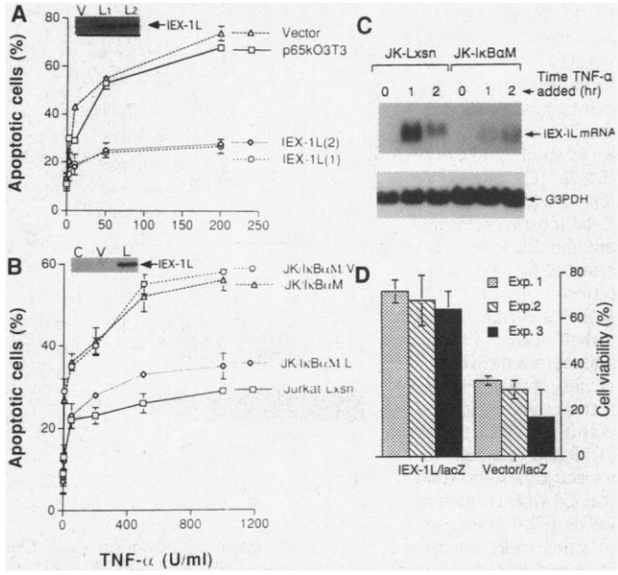

Fig. 3. *IEX*-1L compensates p65KO3T3 (**A**) and Jurkat IκBαM (**B**) cells for a defect in the activation of NF-κB proteins. The cells were stably transfected with a BCMGS-Hyg–*IEX*-1L (*IEX*-1L) plasmid or BCMGS-Hyg vector alone (V) and treated with the indicated concentrations of either mouse (A) or human (B) TNF for 40 hours (*9*, *12*). Percentages of apoptotic cells were analyzed as in Fig. 2C. One representative experiment of four (A) or three (B) experiments performed in duplicate is shown. *IEX*-1L expression in these transfectants is shown by immunoprecipitation, followed by immunoblotting analysis with *IEX*-1–specific Ab. V, L_1, and L_2 refer to vector alone- and *IEX*-1L–transfected p65KO3T3 cell cultures *IEX*-1L(1) and *IEX*-1(2), respectively (A, inset). C, V, and L represent untransfected and vector alone- or *IEX*-1L-transfected Jurkat IκBαM cells (B, inset). (**C**) Northern blotting analysis of *IEX*-1L expression in Jurkat IκBαM cells. Jurkat IκBαM cells and Jurkat Lxsn control cells were stimulated with TNF (1000 U/ml). Blot containing mRNA at 2 μg per lane purified at the indicated time points was hybridized with an *IEX*-1L–specific probe, consisting of a 111-nucleotide sequence present only in the *IEX*-1L gene (*7*). G3PDH was used as an equal mRNA loading control. (**D**) Transient expression of *IEX*-1L protects p65KO3T3 cells from TNF-induced cell death. p65KO3T3 cells were cotransfected with equal amounts of plasmid pRc–*IEX*-1L (*IEX*-1L) or pRc empty vector (Vector), along with pCMV-LacZ (lacZ) expressing vector (Stratagene) by the lipofectamine protocol. Mouse TNF-α (1000 U/ml) was added 40 hours later for 20 hours and then the cells were stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. Viable blue cells remaining after TNF treatment are shown as a percentage of untreated viable blue cells. Each represents the mean of triplicate wells of a six-well plate ± SD and cells are counted as described in Fig. 2D. Data of three independent experiments (Exp. 1, 2, and 3) performed are shown.

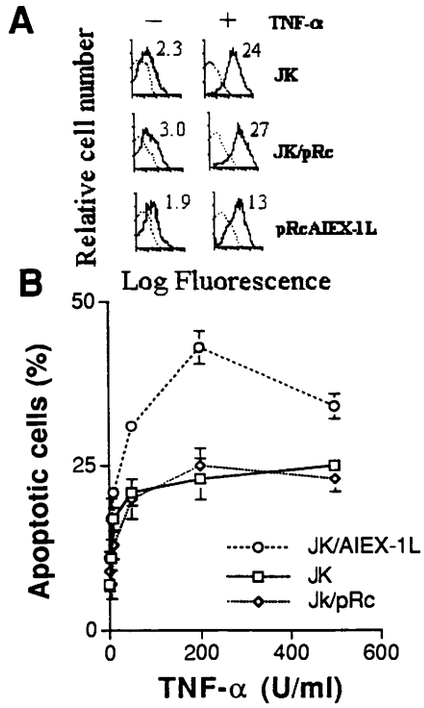

Fig. 4. Effect of antisense *IEX*-1L. (**A**) Jurkat cells were stably transfected with either a construct inserted with *IEX*-1L in an antisense orientation (JK/pRcA*IEX*-1L) or pRc/CMV vector alone (Jk/pRc) (*12*). The transfectants and untransfected cells (JK) were treated with or without TNF (500 U/ml) for 3 hours, fixed in 1% paraformaldehyde, permeabilized in 1% digitonin, and then stained with *IEX*-1–specific Ab, followed by phycoerythrin-conjugated goat immunoglobulin G to rabbit. Numbers represent mean fluorescence intensity for analysis of 5000 events; dashed line represents histograms of an irrelevant Ab. (**B**) Cells in (A) were treated for 40 hours with indicated concentrations of TNF. Apoptosis was analyzed as in Fig. 2C. Percentages represent mean ± SD of three independent experiments with one transfection. One representative experiment of three independent transfections performed is shown. Statistic significance was analyzed by Student's *t* test.

TNF may be key to the mechanism underlying TNF-mediated protection. Indeed, all TNF killing-resistant cell lines tested including HeLa, Jurkat, U937, Sw480, H9, NIH 3T3, and Hut78 cells express *IEX*-1L after TNF stimulation (*6, 8*). In contrast, a decrease or delay in TNF-induced expression of *IEX*-1L is likely to increase cell susceptibility to TNF-induced apoptosis, as was found in p65KO3T3, Jurkat-IκBαM, and Jurkat cells bearing an antisense *IEX*-1L (*4*). Our unpublished data also showed that *IEX*-1L was potentially regulated by the *RelA*/c-*rel* complex (*8*), in agreement with previous observations that overexpression of the c-*rel* gene protected cells from TNF-induced cell death (*2, 3*) and that *RelA* gene knockout mice died at 15 days of gestation (*14*). However, unlike RelA−/− mice, mice lacking the c-*rel* gene are developmentally healthy (*15*), which suggests that *IEX*-1L may be only one of the NF-κB/Rel protein-regulated survival genes.

References and Notes

1. R. Beyaert and W. Fiers, *FEBS Lett.* **340**, 9 (1994); D. Wallach, *Trends Biochem. Sci.* **22**, 107 (1997).
2. H. Hsu, H. Shu, M. Pan, D. V. Goeddel, *Cell* **84**, 299 (1996); Z. Liu, H. Hsu, D. V. Goeddel, M. Karin, *ibid.* **87**, 565 (1996).
3. P. A. Baeuerle and D. Baltimore, *ibid.* **87**, 13 (1996); M. Wu *et al.*, *EMBO J.* **15**, 4682 (1996); S. Nagata, *Cell* **88**, 355 (1997).
4. A. A. Beg and D. Baltimore, *Science* **274**, 782 (1996); C.-Y. Wang, M. W. Mayo, A. S. Baldwin Jr., *ibid.*, p. 784; D. J. Van Antwerp, S. J. Martin, T. Kafri, D. R. Green, I. M. Verma, *ibid.*, p. 787.
5. P. Liang and A. B. Pardee, *ibid.* **257**, 967 (1992).
6. A. D. Kondratyev, K. N. Chung, M. O. Jung, *Cancer Res.* **56**, 1498 (1996); C. H. Charles, J. K. Yoon, J. S. Simske, L. F. Lau, *Oncogene* **8**, 797 (1993); H. Schäfer, A. Trauzold, E. G. Siegel, U. R. Fölsch, W. E. Schmidt, *Cancer Res.* **56**, 2641 (1996).
7. GenBank accession number of *IEX*-1L cDNA, AF039067.
8. M. Wu, R. Wu, Z. Ao, S. F. Schlossman, unpublished data. GenBank accession number of *IEX*-1 genomic DNA, AF071596.
9. PCR products of the *IEX*-1 coding frame were in-frame inserted into the NH_2-terminus of GFP using the site Eco RI–Bam HI in a pEGFP-N1 plasmid (Clontech). The resulting pEGFP-*IEX*-1L and pEGFP-*IEX*-1S were digested and ligated to the site Hind III–Bam HI in a pcDNA plasmid (Invitrogen) containing HA epitope sequence at the COOH-terminus of expressing protein. *IEX*-1L excised from pBluescript II/KS(−)–*IEX*-1L plasmid was subcloned into the Sal I–Not I site in a hygromycin-resistant gene-containing plasmid BCMGS/Hyg. pRc-Fas plasmid was a kind gift from S. M. Lehar (ImmunoGen Inc. Cambridge, MA).
10. The Ab was produced in rabbit by injecting affinity-purified glutathione S-transferase–IEX-1L fusion protein and was purified on a protein A column followed by an IEX-1L protein column. It reacted with both IEX-1L and IEX-1S proteins.
11. S. Takayama *et al.*, *Cell* **80**, 279 (1995); T. Torigoe *et al.*, *Cancer Res.* **54**, 4851 (1994); E. Yang and S. J. Korsmeyer, *Blood* **88**, 386 (1996).
12. Jurkat cells or Jurkat IκBαM cells were transfected by electroporation (200 V, 960 μF) and p65KO3T3 cells were transfected by the lipofectamine protocol (Gibco/BRL). Two days after transfection with the indicated plasmids, the cells were selected for neomycin (1.5 mg/ml) or hygromycin (600 μg/ml for p65KO3T3 cells and 800 μg/ml for Jurkat IκBαM cells) resistance for 4 weeks and then their resistance to apoptosis induced by TNF or by mAb 7C11 to Fas [immunoglobulin M (IgM), 1:10,000 diluted ascites] was tested. In each transfection of these bulk cultures, we used 5×10^6 cells and the efficiency of transfection was 8 to 20% for Jurkat and Jurkat IκBαM cells and 15 to 25% for p65KO3T3 cells, as tested by the pEGFP-*IEX*-1L plasmid. Thus, the results obtained from each of these bulk cultures theoretically represent about 0.4 to 1.2×10^6 individual clones.
13. K. Brown, S. Gerstberger, L. Carlson, G. Franzoso, U. Siebenlist, *Science* **267**, 1485 (1995); J. A. S. Baldwin Jr., *Annu. Rev. Immunol.* **4**, 649 (1996).
14. A. A. Beg, W. C. Sha, R. T. Bronson, S. Ghosh, D. Baltimore, *Nature* **376**, 167 (1995).
15. F. Kontgen *et al.*, *Genes Dev.* **9**, 1965 (1995).
16. K. V. S. Prasad *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6346 (1997).

17. We thank I. V. Verma for Jurkat-IκBαM, D. Baltimore for p65KO3T3 cells, J. C. Reed for Bcl-2-transfected Jurkat cells, P. Anderson and H. Saito for their critical reading of the manuscript, and H. Levine for flow cytometric analysis. Supported by National Institutes of Health grants p30AI28691 (to M.X.W) and AI12069 (to S.F.S).

17 March 1998; accepted 7 July 1998

Feedback Inhibition of Macrophage Tumor Necrosis Factor–α Production by Tristetraprolin

Ester Carballo,* Wi S. Lai,* Perry J. Blackshear†

Tumor necrosis factor–α (TNF-α) is a major mediator of both acute and chronic inflammatory responses in many diseases. Tristetraprolin (TTP), the prototype of a class of Cys-Cys-Cys-His (CCCH) zinc finger proteins, inhibited TNF-α production from macrophages by destabilizing its messenger RNA. This effect appeared to result from direct TTP binding to the AU-rich element of the TNF-α messenger RNA. TTP is a cytosolic protein in these cells, and its biosynthesis was induced by the same agents that stimulate TNF-α production, including TNF-α itself. These findings identify TTP as a component of a negative feedback loop that interferes with TNF-α production by destabilizing its messenger RNA. This pathway represents a potential target for anti–TNF-α therapies.

TNF-α is one of the principal mediators of the inflammatory response in mammals (*1*). In addition to its well-known role in acute septic shock, it has been implicated in the pathogenesis of chronic processes such as autoimmunity, graft-versus-host disease, rheumatoid arthritis, Crohn's disease, and the cachexia accompanying cancer and acquired immunodeficiency syndrome (*2*). Therapies such as neutralizing antibodies to TNF-α and chimeric soluble TNF-α receptors have demonstrated efficacy against some of these conditions in clinical trials (*3*).

We developed mice deficient in TTP, the prototype of a family of CCCH zinc finger proteins whose members have been identified in organisms ranging from humans to yeast (*4–7*). Although the TTP-deficient mice appear normal at birth, they soon develop a complex syndrome of inflammatory arthritis, dermatitis, cachexia, autoimmunity, and myeloid hyperplasia. Essentially all aspects of this syndrome can be prevented by repeated injections of antibodies to TNF-α (*8*). Macrophages derived from fetal liver of TTP-deficient mice, or from bone marrow precursors or resident peritoneal macrophages from adult mice, exhibited increased production of TNF-α, as well as increased amounts of TNF-α mRNA, after stimulation with lipopolysaccharide (LPS) (*9*). For example, relative to control macrophages, bone marrow–derived macrophages from the knockout mice secreted about five times as much TNF-α after incubation with LPS (1 μg/ml for 4 hours), and amounts of TNF-α mRNA were about twice as large in the knockout cells as in the controls (*9*).

To investigate the mechanism of this effect, we evaluated the potential influence of TTP on TNF-α gene transcription. We transfected a human TTP genomic construct, in which the instability-inducing 3′-untranslated region (UTR) of the TTP mRNA (*10*) was replaced by the 3′-UTR from the human growth hormone mRNA (*11*), with a TNF-α promoter–chloramphenicol acetyltransferase (CAT) reporter construct (Pro-CAT). This construct contained 2.3 kb of the mouse TNF-α promoter linked to the CAT coding sequence and a 3′-UTR from a human growth hormone cDNA (*12*). Transfection of several cell types (chick embryo fibroblasts, NIH 3T3 mouse fibroblasts, and Rat-1 fibroblasts) led to nonspecific "squelching" of several

Office of Clinical Research and Laboratory of Signal Transduction, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA, and Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, NC 27710, USA.

*These authors contributed equally to this report.
†To whom correspondence should be addressed. E-mail: black009@niehs.nih.gov