Brief Definitive Report

INDUCTION OF c-*ets* AND c-*fos* GENE EXPRESSION UPON ANTIGENIC STIMULATION OF A T CELL HYBRIDOMA WITH INDUCIBLE CYTOLYTIC CAPACITY

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Upon mitogenic stimulation of normal lymphocytes, a number of protooncogenes are induced (1, 2), which led to the supposition that expression of some oncogenes is involved in cell division. Some of these oncogenes are, however, expressed by other stimuli that induce differentiation. Mitogenic and antigenic stimulation of T lymphocytes leads to cellular proliferation followed by maturation of effector cells. Thus, expression of some oncogenes may be linked to the functional activation of lymphocytes. In normal lymphocytes it is difficult to evaluate the contribution of protooncogene expression to lymphocyte maturation, because proliferation and differentiation are not readily separable. In addition, in normal lymphocytes, heterogeneous clones are stimulated, making it difficult to determine the population expressing the genes. The use of a T cell hybridoma with inducible cytolytic capacity (3) obviates these difficulties, since activation of cytolysis occurs without induction of proliferation. We examined expression of c-*fos* and c-*myc* genes, which are induced by mitogenic stimulation of lymphocytes (1, 2). We also studied expression of c-*ets* genes during hybridoma stimulation. The c-*ets* gene, originally reported in the chicken, is composed of two genes in mammalian species, c-*ets*-1 and c-*ets*-2, which are located on two different chromosomes (4, 5). Constitutive c-*ets* gene expression has been reported in human T and B lymphocytes (6). However, little is known as to the expression of the c-*ets* gene during lymphocyte activation.

Materials and Methods

CTL Hybridomas. Establishment, specificity, and the activation of the CTL hybridoma P47.21, the parental clone of PMM1, have been described elsewhere (3). For killing assays, PMM1 cells (3×10^5 cells/ml) were stimulated with irradiated (4,000 rad) EL4 cells at 3×10^4 cells/ml or with 1:200 dilution of ascites fluid of G7 anti-Thy-1 mAb (7) for 20 h. After preincubation, lysis of EL4 target cells was examined in a 6-h ^{51}Cr-release assay at an E/T ratio of 1.5:1. Proliferation of the hybridoma was monitored by [^{3}H]TdR incorporation during the last 30 min of the 6-h stimulation. To inhibit cytolytic activation,

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the hybridoma was preincubated with cyclosporin A (CsA) (Sandoz Laboratories, Basel, Switzerland) at a final concentration of 100 ng/ml or 1:10 with a dilution of culture supernatant of anti-LFA-1 mAb (8).

Oncogene Probes and Northern Blot Hybridization. An Nco I–Sph I fragment containing the 4th exon of the murine c-*fos* gene (9) was subcloned into M13 mp19. An Hind III–Xho I fragment of a mouse c-*myc* cDNA, pMc-*myc*-54, was subcloned into M13 mp19. ssDNA probes for c-*fos* and c-*myc* were prepared by primer extension using an M13 universal primer in the presence of α-[^{32}P]dATP and α-[^{32}P]dGTP. The plasmids pRD6K and pH33 (4, 5) containing human c-*ets*-1 and c-*ets*-2 were digested with Hind III–Eco RI and nick translated with a nick translation kit according to the manufacturer's instructions (Bethesda Research Laboratories, Gaithersburg, MD). Total RNA was prepared according to the method of Chirgwin et al. (10). For Northern blot hybridization, 20 μg of RNA from each sample was heated at 65°C in 50% formamide/20% formaldehyde in 4-morpholine-propane sulfonic acid buffer and electrophoresed in 0.9% agarose gel containing 15% formaldehyde. RNA was then transferred onto Nytran paper (Schleicher & Schuell, Inc., Keene, NH). Hybridization was carried out for 18 h at 42°C with probes containing radioactivity of 2–20 × 10^6 cpm. Blots were exposed to Kodak XAR film at −70°C for 1–4 d.

Results and Discussion

We examined protooncogene expression in a CTL hybridoma, PMM1, derived from BALB/c anti-EL4 (H-2^d anti-H-2^b) CTL (3). PMM1, when stimulated by cells presenting the H-2D^b antigen (Ag), becomes cytolytic to target cells expressing the H-2D^b antigen and secretes lymphokines. Stimulation by Ag does not induce hybridoma proliferation, rather it decreases thymidine incorporation slightly. We recently found that G7 anti-Thy-1 mAb, previously shown to induce T cell proliferation and IL-2 secretion in normal lymphocytes (7), also activates the killing function of PMM1 hybridoma (11). Several other anti-Thy-1 mAbs (HO-13-4, 31-11, and N-18-3), however, do not elicit this effect. Stimulation by G7 mAb also leads to a modest reduction in proliferation (11).

Expression of c-*fos*, c-*ets*-1, c-*ets*-2, and c-*myc* genes in PMM1 stimulated by Ag or by G7 anti-Thy-1 mAb is shown in Fig. 1. c-*fos* mRNA was not detected in the PMM1 hybridoma before stimulation. However, 30 min after activation with EL-4, high levels of c-*fos* mRNA appeared, peaked at 45 min, and decreased to an undetectable level by 8 h. Activation of the hybridoma by G7 anti-Thy-1 mAb (7) resulted in a similar rapid and transient induction of c-*fos* mRNA. Nonactivating anti-Thy-1 mAb, HO-13-4, did not induce c-*fos* mRNA, indicating that c-*fos* expression is coupled with active signal transduction (data not shown). c-*ets* gene expression was examined by using human c-*ets* probes (4, 5). A low constitutive expression of the c-*ets*-1 gene was found in the nonstimulated hybridoma. The mRNA band corresponded in size to the human c-*ets*-1 transcript of ~6.8 kb (4). After Ag stimulation, the levels of c-*ets*-1 mRNA were increased by fivefold at 4 h and declined by 8 h. Activation by G7 mAb also resulted in a small but significant increase in the level of c-*ets*-1 transcript by 4 h. The specificity of c-*ets*-1 induction by G7 has not been conclusively established. The c-*ets*-2 probe elicited high levels of mRNA in the nonstimulated hybridoma, which corresponded to the 4.7-kb human c-*ets*-2 mRNA in size (5). Activation of the hybridoma with either specific Ag or G7 mAb did not cause a major change in c-*ets*-2 mRNA levels. The c-*myc* expression was high in the nonstimulated hybridoma, and its levels remained unchanged during the first 4 h of stimulation.

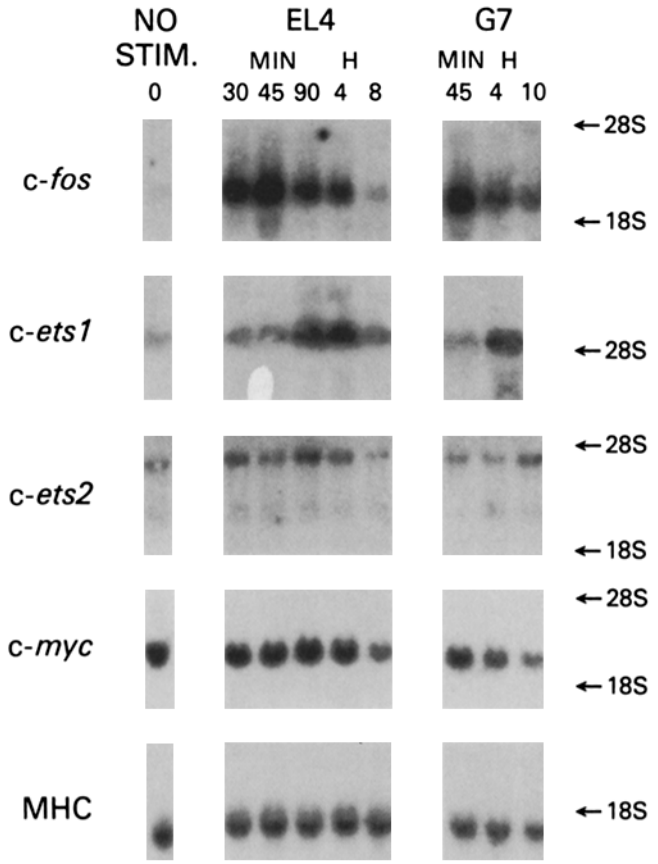

FIGURE 1. Induction of c-*fos* and c-*ets*-1 gene expression in PMM1 hybridoma stimulated with specific Ag or anti-Thy-1 mAb. Hybridoma cells were stimulated for the indicated times with EL4 or with G7 anti-Thy-1 mAb. Northern blot hybridization was carried out with 20 μg of total RNA using ^{32}P-labeled probes described in Materials and Methods.

The level of c-*myc* mRNA was reduced (~50 to 70%) 8 and 10 h after activation, which may correlate with the partial reduction of cell proliferation. Expression of MHC class I genes, tested as a control, was not affected by stimulation with either Ag or anti-Thy-1 mAb (Fig. 1).

To address whether the induction of c-*fos* and c-*ets* gene is related to the maturation of the CTL hybridoma, we tested the effect of CsA or anti-LFA-1 mAb, which block the activation of the hybridoma. CsA blocks CTL activation at an early phase of the induction and does not interfere with the killing (12). Anti-LFA-1 mAb inhibits both the induction and the effector phase of CTL function (8). Table I shows that Ag stimulation of the cytolytic activity in the PMM1 clone was completely inhibited by CsA and by anti-LFA-1. CsA had no effect on the rate of proliferation, whereas anti-LFA-1 inhibited proliferation by ~30%. Expression of the protooncogenes in the hybridomas treated with the inhibitors is shown in Fig. 2. CsA blocked induction of c-*fos* and c-*ets*-1 mRNA by >70%, 45 min and 2 h after stimulation (Fig. 2, lane *0* vs. lane *1*; control vs. CsA treated). Anti-LFA-1 mAb completely abrogated the induction of c-*fos* and c-*ets*-1 during the first 4 h of stimulation (see lane *2* in Fig. 2). In contrast, the constitutive expression of c-*myc* and c-*ets*-2 transcripts was not affected during this period. In the samples treated with CsA, a weak c-*fos* mRNA band was seen

TABLE I

Cyclosporin A and Anti-LFA-1 mAb Inhibit Induction of Cytolytic Activity of PMM1 Hybridoma

Stimulation by EL4	Inhibitor	Cytolytic activity (lysis)	Proliferation [^{3}H]TdR uptake
		%	*cpm* × 10^{-3}
−	—	1.5	154
+	—	33.6	137
+	CsA	1.2	144
+	Anti-LFA1	5.3	94

PMM1 hybridomas (2 × 10^6 cells/ml) were preincubated at 37°C for 30 min, with CsA or with anti-LFA1 mAb (8), before addition of irradiated EL4 cells (2 × 10^5 cells/ml). Cytolytic activity was tested 10 h after the onset of stimulation. Proliferation was tested after a 6-h incubation with EL4 cells in the presence of CsA or anti-LFA-1 mAb. Values represent mean of tetraplicate cultures.

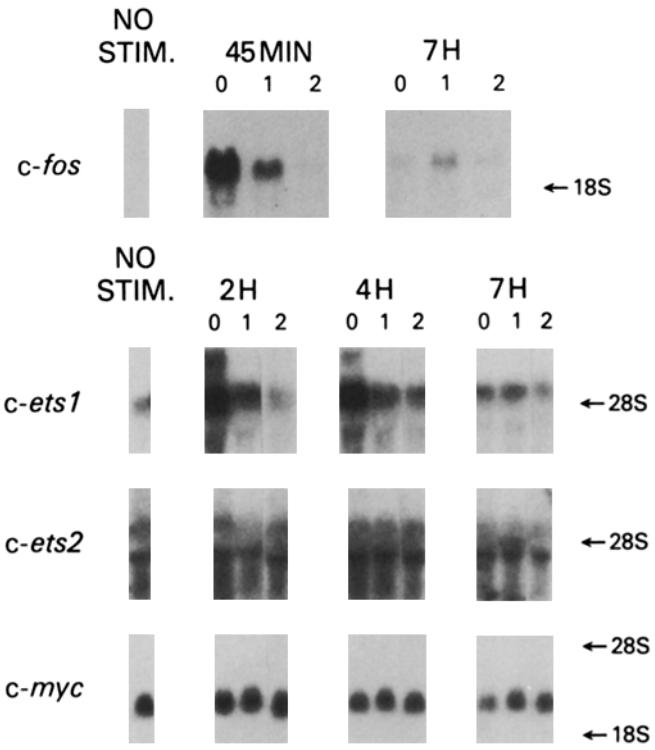

FIGURE 2. The induction of c-*fos* and c-*ets* expression is blocked by inhibitors of CTL activation. PMM1 hybridoma cells were preincubated with CsA or anti-LFA-1 mAb for 30 min at 37°C as in Table I. EL4-stimulating cells were then added, and cells were incubated for the indicated periods of time. Lane *0*, untreated control; lane *1*, treated with CsA; lane *2*, treated with anti-LFA-1 mAb. Northern blot hybridization was carried out as in Fig. 1.

7 h after stimulation (Fig. 2), which suggests stabilization of c-*fos* mRNA by CsA. Similarly, treatment of PMM1 cells with CsA and anti-LFA-1 appeared to block downregulation of c-*myc* seen 7–8 h after Ag stimulation (Fig. 2).

This study shows that cytolytic stimulation of the hybridoma occurs concomitantly with the induction of c-*fos* and c-*ets*-1 gene expression. Since the initiation of cytolysis occurs without induction of proliferation, expression of these oncogenes is likely to be associated with the events relevant to functional activation of the hybridoma, rather than those related to cell division. That induction of c-*fos* and c-*ets*-1 but not c-*myc* and c-*ets*-2 is abolished by the inhibitors of CTL activation, further indicates specific involvement of these oncogenes in the

functional activation of the hybridoma. The rapid induction of c-*fos* gene suggests its involvement in early steps of T cell activation, which may be pertinent to the recent report (13) showing that Ca^{2+} influx is one of the pathways of c-*fos* gene induction. Activation of T cells is known to cause immediate Ca^{2+} influx followed by a cascade of events that lead to elicitation of effector functions.

Even though the c-*ets*-1 mRNA appears relatively late during hybridoma stimulation, it is the result of early triggering events, as the induction is sensitive to the inhibitors of early lymphocyte activation. Reed et al. (2) reported that c-*ets* transcripts increased moderately 2–3 d after mitogenic stimulation of human peripheral blood cells. Although these authors have not determined whether the increase represents c-*ets*-1 or c-*ets*-2 mRNA, the results are consistent with concomitant induction of c-*ets*-1 and cytotoxic activity, since effector functions are manifest several days after polyclonal activation of lymphocytes.

Summary

Expression of cellular oncogenes was studied in a T cell hybridoma that undergoes cytolytic activation when stimulated by specific antigen or by anti-Thy-1 antibody. The activation occurs without induction of hybridoma proliferation, providing a model to examine oncogene expression during functional differentiation of lymphocytes. We found that c-*fos* and c-*ets*-1 mRNAs were transiently induced at high levels in the hybridoma 30 min and 4 h after stimulation, respectively. c-*myc* and c-*ets*-2 oncogenes were constitutively expressed in the hybridoma and their mRNA levels were unaffected during 4 h of stimulation, although c-*myc* expression was reduced in the later stage of stimulation. Inhibitors of T cell activation, cyclosporin A and anti-LFA-1 antibody, blocked the induction of c-*fos* and c-*ets*-1 mRNAs without reducing the levels of c-*myc* and c-*ets*-2. The results indicate that the functional activation of the CTL hybridoma is associated with induction of c-*fos* and c-*ets*-1 genes.

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