10-1992

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Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 9934–9938, October 1992
Immunology

A phorbol ester response element within the human T-cell receptor β-chain enhancer

(Ets/core-binding factor/T-cell activation)

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Communicated by George R. Stark, July 21, 1992

ABSTRACT **The activity of the T-cell receptor β-chain gene enhancer is increased by activators of the protein kinase C pathway during T-cell activation. Analysis of mutant enhancer constructs identified two elements, βE2 and βE3, conferring phorbol ester inducibility. Multimerized βE2 acted in isolation as a phorbol ester-responsive element. Both βE2 and βE3, which contain a consensus Ets-binding site, were shown to bind directly to the product of the c-*ets-1* protooncogene. Both regions also bound a second factor, core-binding factor. Mutation of the βE2 Ets site abolished the inducibility of the βE2 multimer. βE2 and βE3 Ets site mutations also profoundly affected activity and inducibility of the enhancer. In contrast, enhancer activity but not its inducibility was affected by mutation of the βE2 core-binding factor site. Cotransfection studies showed that Ets-1 specifically repressed activity of the multimerized βE2 element and the complete T-cell receptor β-chain enhancer. These data show that the T-cell receptor β-chain enhancer responds to protein kinase C-mediated activation signals via a functional domain, composed of two elements, which contains binding sites for Ets transcription factors and which is negatively regulated by Ets-1.**

The αβ T-cell receptor, expressed by most T cells, is encoded by genes that rearrange during thymocyte development (1, 2). The T-cell receptor β-chain (TCRβ) locus initiates recombination at around day 14 in murine fetal thymic ontogeny, and the α locus rearranges at about embryonic day 17 (3–5). The αβ TCR is first expressed at the thymocyte cell surface on embryonic day 18 at a low level together with CD4 and CD8 antigens (6). An increase in the surface level of the αβ TCR on the CD4 and CD8 double-positive thymocyte population has been observed and correlated with positive selection (7). TCR gene expression is further regulated in mature T cells, where antigen stimulation leads to an increase in transcription of TCR α and β genes (8). Thus, TCR gene expression is modulated both during thymic development and in mature T cells.

The transcription of the TCRβ gene is regulated by an enhancer of ≈350 base pairs (bp), located 6 kilobases (kb) 3′ to the TCRβ constant region locus, Cβ2 (9–12). This enhancer has been shown to be highly inducible by phorbol esters, which mimic the immune activation and differentiation of T cells (12).

We define two elements within the human TCRβ enhancer that confer phorbol ester inducibility and show that these elements bind factors belonging to the Ets family of transcription factors. The Ets-binding sites are adjacent to a conserved "core" site found also in the enhancers from the TCR γ- and δ-chain genes, the CD3 δ- and ε-chain enhancers, the polyoma virus enhancer, and the enhancers of mammalian C-type retroviruses (13–18). The core site in the mammalian C-type retroviral enhancers has been shown to be an important determinant of the T-cell disease specificity of the Moloney murine leukemia virus (19) and the leukemogenicity of the SL3-3 murine leukemia virus (20).

METHODS AND MATERIALS

Cells. Cell lines were cultured in RPMI 1640 medium/10% fetal calf serum. The cell lines used were J6, a subline of the human T-ALL cell line Jurkat, and K-562, an erythroleukemic line.

Plasmids. The plasmid KS7.8 containing the TCRβ enhancer was derived as described (12). Deletions were created by oligonucleotide-directed mutagenesis. The oligonucleotides used were as follows: ΔβE1, GGCTGCCGGGCTGAATTCACCAAGCCACAGCA; ΔβE2, TCTCTTACAGTCACAAGAATTCATTTACTGGGTCCTG; ΔβE3, CACCCACCCACCTGAATTCCACCCAACCCCCTC; and ΔβE4, CTGGGCCCCTGGTGAATTCGGAGGGGCAGGGTCT. The construct βE2x4 was produced by concatenating the double-stranded oligonucleotide βE2 and cloning into pBLCAT2. The sequence of the coding strand of βE2 was GATCCACAACAGGATGTGGTTTGACATTTA.

The mutant construct βE2M1x4 was prepared by using the oligonucleotide GATCCACAACATTATGTGGTTTGACATTTA. The plasmid pRSV-luciferase was used as an internal standard for transfection efficiency (21). The 1.4-kb chicken ets-1 clone (22) was ligated into the *Xba* I site of expression plasmid pEF-BOS (23).

Chloramphenicol Acetyltransferase (CAT) Assays. Cells were transfected using DEAE-dextran as described (12). Induction with phorbol 12-myristate 13-acetate (PMA) was achieved by dividing cells into two and incubating one portion in medium containing PMA at 10 ng/ml. CAT assays were done with the method of Sleigh (24).

Gel-Retardation Assays. Complementary oligonucleotides were hybridized to create double-stranded probes and were radioactively labeled by filling in single-stranded ends by using avian myeloblastosis virus reverse transcriptase. Gel-retardation assays were done with 1 ng (10^4–2×10^4 dpm) of probe and ≈0.25 μg of insect cell extract containing recombinant Ets-1 (22) or 3 μl of purified core-binding factor (CBF) protein (25). Complexes were electrophoresed on 4% polyacrylamide gels in 0.5× TBE (1× TBE is 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3). Gels were dried and autoradiographed at −70°C on Kodak XAR-5 film. The

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Abbreviations: TCRβ, T-cell receptor β chain; PMA, phorbol 12-myristate 13-acetate; CBF, core-binding factor; HTLV-1, human T-lymphotropic virus type 1; CAT, chloramphenicol acetyltransferase.

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coding strands of the oligonucleotides used are shown: βE2, GATCCACAACAGGATGTGGTTTGACATTTA; βE2M1, GATCCACAACATTATGTGGTTTGACATTTA; βE2M2, GATCCACAACAGGATGTGAGATCTCATTTA; βE3, GATCCGCATCATGAGAACCACACTCACCGCATCCGGCACCCA; HTLV-1, CCCATTTCCTCCCCATGTT; and HTLV-1M, CCCATTTGGTCCCCATGTT.

RESULTS

Two Phorbol Ester Response Elements Lie Within the TCRβ Enhancer. The human TCRβ enhancer used in these studies was a 362-bp fragment. DNase I footprint analysis revealed four clear areas of protection by nuclear extracts of the human T-cell line Jurkat, designated βE1–βE4 (data not shown). These regions correspond broadly to those defined by Gottschalk and Leiden (10).

To assess whether the human TCRβ enhancer was inducible under conditions of T-cell activation, transient transfections using Jurkat cells were done with a CAT reporter gene under control of the herpes simplex virus thymidine kinase (tk) promoter and the TCRβ enhancer. Phorbol ester treatment of Jurkat cells resulted in an induction of the activity of the TCRβ enhancer construct by at least 10-fold (Table 1, KS7.8). To define the basis for this inducibility, each footprinting region was deleted, and the effect of the deletion on phorbol ester inducibility and basal activity of the enhancer was assayed (Table 1). Although the deletion of each footprinting region decreased activity of the enhancer, only deletion of βE2 and βE3 affected inducibility. Thus, deletion of βE2 reduced enhancer activity by at least 80% and completely abolished phorbol ester inducibility, and deletion of βE3 moderately impaired inducibility. These results show that βE2 and βE3 are responsible for phorbol ester inducibility of the TCRβ enhancer with βE2 apparently exerting the major effect.

βE2 Acts in Isolation as a Phorbol Ester-Responsive Element. Four copies of βE2 were placed upstream of the tk promoter, and the activity of this construct (βE2x4) in Jurkat cells was measured. Table 2 shows that βE2x4 construct failed to enhance the promoter activity, in contrast with the TCRβ enhancer (KS7.8) but was functional on induction with PMA. This result showed that βE2 could function as a phorbol ester-responsive element without the requirement for interaction with other elements of the core enhancer. The behavior of the βE2x4 construct in the B-cell line Daudi was similar to that in Jurkat cells, being functional only upon PMA induction (data not shown).

Multimerized βE2 was also inducible in the erythroleukemic cell line K-562, although in this cell line the element was a potent transcriptional enhancer of the CAT reporter gene in the absence of PMA. The TCRβ enhancer also functioned well in K-562 cells and was inducible with PMA (Table 2).

βE2 and βE3 Contain an Ets-Binding Site. Comparison of the sequences of the βE2 and βE3 regions reveals several notable features (Fig. 1). (*i*) They are inverted repeats, sharing two areas of sequence homology; (*ii*) they contain sequences that correspond to motifs within other enhancers.

Table 1. Basal and inducible activity of deletion mutants of the TCRβ enhancer

Construct	CAT activity*	Inducibility†
KS7.8	100	11.3
ΔβE1	43	12.3
ΔβE2	19	0.8
ΔβE3	36	8.1
ΔβE4	68	15.3

*Activity in cpm × 10^{-3}, normalized for luciferase activity.
†Ratio of + PMA to − PMA.

Table 2. PMA inducibility of the TCRβ enhancer and the βE2 multimer

	CAT activity*			
	J6		K-562	
Construct	− PMA	+ PMA	− PMA	+ PMA
KS7.8	15	147	39	107
βE2x4	0	43	42	135

*Activity in cpm × 10^{-3}, normalized for luciferase activity.

One motif (Fig. 1, underlined) is related to the purine-rich Ets-binding sites in several viral and cellular promoter/enhancer sequences and to the recently defined consensus-binding site for Ets-1 (refs. 26 and 27). βE2 and βE3 also contain a second region of homology, the "core" site (Fig. 1, boxed), which is conserved in mammalian C-type retroviral enhancers (19). It is also found in the TCR γ- and δ-chain enhancers (15, 16), in the CD3 δ- and ε-chain enhancers (14, 15), and the polyoma virus enhancer (18). We have recently purified proteins, CBFs, that bind to this site in retroviral enhancers (25).

The ability of βE2 and βE3 to bind to Ets proteins was demonstrated by using recombinant Ets-1. When ^{32}P-labeled βE2 or βE3 oligonucleotide was added to baculovirus-infected insect cell extracts containing recombinant chicken Ets-1, a specifically retarded band was seen (Fig. 2*A*). The retarded band was inhibited by an oligonucleotide corresponding to the Ets-binding site described in the human T-lymphotropic virus type 1 (HTLV-1) long terminal repeat (22) but not with a mutated nonbinding version of this oligonucleotide. The βE2 and βE3 bands were also cross-inhibited by unlabeled βE3 or βE2, respectively. The affinity of the βE2 and βE3 Ets-1-binding sites appeared to be comparable with the HTLV-1 long terminal repeat Ets site. Similar experiments with infected insect cell extracts containing recombinant human Ets-2 showed that the βE2 and βE3 elements also bound to Ets-2 (data not shown).

The ability of βE2 to bind to CBF was also assessed by gel-retardation analysis. Labeled βE2 was retarded by purified CBF, and the retarded band was inhibited by unlabeled βE2 and by a βE2 oligonucleotide in which the Ets-binding site had been mutated (βE2M1) (Fig. 2*B*). However, a mutated βE2 oligonucleotide in which the core site was disrupted (βE2M2) failed to compete for binding. Unlabeled

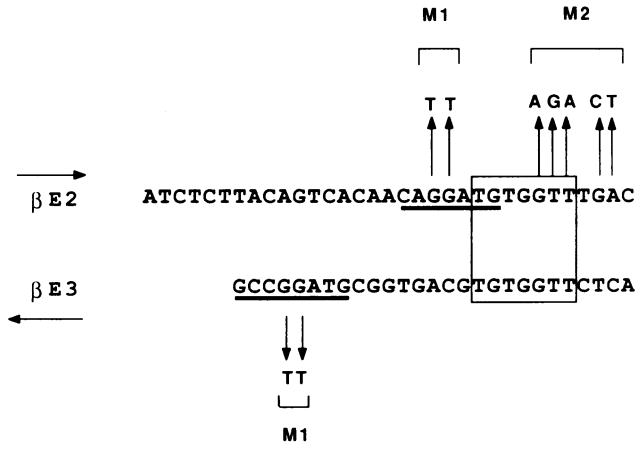

FIG. 1. Sequence comparison of βE2 and βE3 elements. Sequences of βE2 and βE3 footprinting regions are shown. Arrows represent orientation (→, upper strand; ←, lower strand). The two areas of homology between βE2 and βE3 are underlined and boxed. Mutations in the βE2 and βE3 sites used in functional assays are shown.

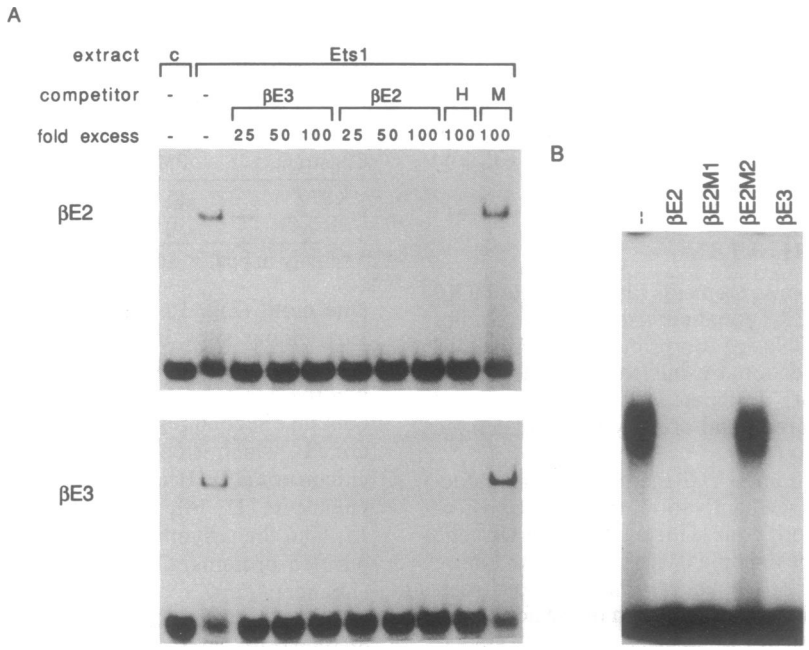

FIG. 2. Binding properties of βE2 and βE3. (*A*) Labeled βE2 or βE3 was incubated in the presence of baculovirus-infected insect cell extracts containing recombinant Ets-1 or in the presence of a control extract (lane c) containing no Ets protein. The retarded Ets-1 complex was inhibited with the following unlabeled oligonucleotides: βE2, βE3, HTLV-1 (H), HTLV-1M (M) (a mutant version of HTLV-1 that does not bind Ets proteins). (*B*) Labeled βE2 was incubated with CBF purified from calf thymus. The retarded complex was inhibited with unlabeled βE2, mutants βE2M1 and βE2M2 (see Fig. 1), or βE3.

βE3 completely inhibited binding of CBF by labeled βE2, showing that βE3 also binds CBF (Fig. 2*B*).

The Ets-Binding Site Is Essential for Inducibility of βE2. The importance of the Ets- and CBF-binding motifs within βE2 for the activity and inducibility of the TCRβ enhancer was determined by mutation analysis. In Jurkat cells the basal activity of the TCRβ enhancer construct (βE2M1) in which the Ets-binding site in βE2 had been mutated was consistently higher (1.2- to 2-fold) than that of unmutated KS7.8; in contrast, the inducibility of βE2M1 was only ≈40% that of the wild-type enhancer (Table 3). The residual inducibility of the βE2M1 construct was accounted for by the activity of βE3. Mutation of both the βE2 and βE3 Ets-binding sites (βE2M1βE3M1) decreased the basal activity and abolished the PMA inducibility. Mutation of the βE3 Ets-binding site alone (βE3M1) decreased both activity and inducibility of the enhancer to levels similar to those seen when the entire βE3 region was deleted (ΔβE3). A construct in which the Ets sites within the βE2 multimer were mutated (βE2M1x4) showed no inducibility in Jurkat (data not shown).

In K-562 cells, mutation of the βE2 Ets-binding site drastically reduced both basal activity and inducibility (Table 3).

Table 3. Effect on activity and inducibility of mutations and deletions within the TCRβ enhancer

Construct	J6		K-562	
	CAT activity*	Inducibility†	CAT activity*	Inducibility†
KS7.8	5.25	13	7.41	5.9
ΔβE2	2.40	2.5	0.21	1.8
ΔβE3	2.75	9.8	2.30	4.5
βE2M1	7.65	5.6	0.40	1.3
βE3M1	3.60	7.9	3.33	2.4
βE2M1βE3M1	1.51	1.0	0.18	1.3
βE2M2	3.20	14.5	0.15	9.3
pBLCAT2	0.09	0.8	0.10	0.8

*Activity in cpm × 10^{-3}, normalized for luciferase activity.
†Ratio of + PMA to − PMA.

Mutation of the βE3 Ets site moderately reduced basal and inducible activity. Mutation of both Ets sites effectively abolished both basal and inducible activity.

Mutation of the 3′ region of βE2 that abolishes binding of CBF but not of Ets factors (βE2M2) reduced basal enhancer activity in both Jurkat and K-562 cells (Table 3). However, the inducibility of the TCRβ enhancer was not decreased by this mutation. Taken together, these data show that the Ets-binding sites in βE2 and βE3 are important for PMA inducibility, whereas the CBF-binding sites appear essential only for full activity of the TCRβ enhancer.

Coexpression of Ets-1 Represses TCRβ Enhancer Activity. To elucidate the functional consequences of the binding of Ets-1 to the βE2 and βE3 regions, cotransfection experiments were undertaken by using an expression plasmid encoding the chicken equivalent of the major 55-kDa form of Ets-1 found in lymphoid cells (22). In both K-562 and Jurkat cells Ets-1 expression repressed greatly the activity of KS7.8 and βE2x4 in the presence of PMA. Repression of basal activity was also seen in the absence of PMA (Table 4).

The specificity of the repression of TCRβ activity was demonstrated by several criteria. (*i*) Coexpression of Ets-2 had little effect on activity of the TCRβ enhancer, although Ets-1 and Ets-2 both transactivated the HTLV-1 enhancer in T cells. (*ii*) Ets-1 expression did not affect activities of the Moloney sarcoma virus long terminal repeat or the CD2 enhancer in T cells (data not shown).

Table 4. Effect of Ets-1 coexpression on activity of the TCRβ enhancer and the βE2 multimer

Reporter construct	Expression construct	CAT activity*			
		J6		K-562	
		− PMA	+ PMA	− PMA	+ PMA
KS7.8	Control	1.0	31.6	1.0	4.5
	Ets-1	0.6	3.2	0.2	1.2
βE2x4	Control	0	6.1	9.1	30.1
	Ets-1	0	0.6	0.6	4.1

*Activity in cpm × 10^{-3}, normalized for luciferase activity.

DISCUSSION

A number of studies indicate that the transcription rate of the TCRβ gene varies during differentiation and in mature T cells on T-cell activation. Transcription of TCRβ genes is upregulated by protein kinase C activation (8), which can be mimicked with PMA (28). The basis for the inducibility of human TCRβ enhancer activity resides in a functional domain containing the βE2- and βE3-binding sites.

βE2 and βE3 each bind to Ets-1 and Ets-2 and to the CBF transcription factor. Mutational analysis reveals that the integrity of Ets-binding sites is essential for inducibility, whereas the CBF-binding site is important only for basal activity. Mutations within the CBF-binding site in the TCRδ enhancer also substantially decrease its activity (16). Removal of the entire βE2 element from the TCRβ enhancer severely reduces the phorbol ester response, suggesting that synergistic interactions of proteins binding to βE2 and βE3 are required for optimal phorbol ester induction. Interestingly, the Moloney virus enhancer also has a PMA-inducible element that contains a binding site for CBF (TGTGGTAA) and two Ets-binding sites (the leukemia virus factor b and c sites) (refs. 25 and 29; Barbara Graves, personal communication). The importance of the Ets-binding sites within the TCRβ enhancer for its phorbol ester inducibility is consistent with the observation that the activity of the stromelysin promoter that contains an AP-1 site and two Ets-binding sites is increased by PMA but that the PMA response does not require the integrity of the AP-1 site (30, 31).

The observed repression by Ets-1 of TCRβ enhancer activity contrasts with its transactivation of other enhancers (see, for example, ref. 22). This repression may, however, be of physiological significance in modulation of the transcriptional activity of the TCRβ enhancer in T-cell activation. It has been postulated that Ets-1 is involved in maintaining T cells in a quiescent state, whereas Ets-2 is required for cellular activation and proliferation (32). Thus, modulation of the activity of the TCRβ enhancer could be achieved by varying the occupancy of the βE2 and βE3 sites by Ets-1 by competition for binding with an additional Ets protein. There are several mechanisms by which the level of Ets-1 can be varied in T cells, depending on the activation stimulus received, including decreasing transcription of *ets-1* on exposure to phorbol esters (32) and reducing the binding of Ets-1 to DNA by calcium-dependent phosphorylation (33) induced by mitogens or calcium ionophores. This latter mechanism may operate on activation of quiescent T cells that express a very high level of Ets-1 (33).

Ets-binding sites are present in enhancers of other T-cell-specific genes including sites within the interleukin 2 enhancer that mediate activation in response to phytohemagglutinin stimulation and that bind the inducible nuclear factors NF-AT and NF-IL-2B (34). Recently, the Elf-1 Ets-related factor has been shown to bind to two sites within the interleukin 2 enhancer (35). Thus, it is possible that the Ets family of transcription factors perform a general regulatory function within genes that are transcriptionally up-regulated in T-cell differentiation and activation. Variations in the precise response of different genes during activation would depend on the interaction of distinct Ets factors with other transcriptional activators and coactivators. In this context, it is interesting to note that Ets proteins have been implicated as components of the signal-transduction pathway mediating the inducibility of enhancers in response to serum growth factors, phorbol esters, and nonnuclear oncogenes (31, 36–38).

We thank Ms. Vivienne Weller for her help with preparation of the manuscript and Dr. Nic Jones for critical comments on the manuscript. This work was funded by the Imperial Cancer Research Fund (H.M.P., D.W., and M.J.O.), the Centre National de la Recherche Scientifique (CNRS), Agence Nationale de Recherche sur le Cancer (ARC), and Institut Curie (A.G. and J.G.), and by the Leukemia Research Foundation, and Public Health Service Grant CA51065-01A1 from the National Cancer Institute awarded to N.A.S. N.A.S. is a recipient of a Junior Faculty Research Award from the American Cancer Society, and S.W. is an awardee of a Predoctoral Fellowship from the Norris Cotton Cancer Center at the Mary Hitchcock Hospital.

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