Regulation of Nuclear Factor-κB and Activator Protein-1 Activities After Stimulation of T Cells Via Glycosylphosphatidylinositol-Anchored Ly-6A/E[1]

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Cross-linking of glycosylphosphatidylinositol-anchored proteins, including mouse Ly-6A/E, leads to IL-2 secretion and T cell activation, whereas engagement of Ly-6A/E uniquely inhibits IL-2 production induced via TCR. However, little is known concerning the molecular mechanism by which glycosylphosphatidylinositol-anchored proteins regulate IL-2 expression. In this study, we have examined the ability of an anti-Ly-6A/E mAb to regulate transcription factors controlling IL-2 expression. Stimulation of EL4J(Ly-6E).A4 cells with anti-CD3ε or anti-Ly6A/E mAbs induced nuclear factor (NF)-κB p65-p50 (RelA/p50) and AP-1 (Fos/Jun) binding activities and increased nuclear factor of activated T cells (NF-AT) activity, whereas octamer-binding factor and NF-Y levels were stable. Cyclic AMP response element binding protein and T cell-specific factor-1(α) activities were selectively enhanced by anti-CD3ε, but not by anti-Ly6A/E, which suggests that signaling via the TCR and Ly-6 were not identical. Costimulation of these cells with both mAbs produced substantially reduced levels of AP-1, NF-AT, and, especially, NF-κB p65-p50 whereas cyclic AMP response element binding protein and T cell-specific factor-1(α) were induced to a level seen after stimulation by anti-CD3ε. The inducibility of the IL-2 enhancer in vivo and the contribution of individual transcription factors for this induction were assessed with use of reporter chloramphenicol acetyltransferase constructs containing the IL-2 enhancer or oligomerized binding sites for transcription factors. These experiments also demonstrated a key role for NF-κB and AP-1 in the transcriptional regulation of the IL-2 gene by TCR- and Ly6A/E-mediated signaling. By using the 2B4.11 T cell hybridoma and a mutated variant, we revealed a crucial role for the ζ-chain in Ly6A/E-mediated activation of NF-κB. *The Journal of Immunology,* 1994, 153: 2394.

T cell activation is accompanied by the coordinated transcription of many genes, including the lymphokines IL-2, IL-4, IFN-γ, and their respective receptors (1, 2). The structure of the regulatory region of the IL-2 gene has been well characterized (3–5). Transcription factors that interact with cognate motifs in this region are AP-1[4] (6), NF-κB (3, 7), NF-AT (3), OCT-OAP (8–10), and NF-κB/Rel-like factors, which serve as components of the CD28 response element-binding complex (11, 12).

Because the affinity of TCR binding to the MHC peptide is low, many co-receptors, including CD4 and CD8, are thought to function to increase and stabilize cell-cell interactions and to enhance the development of intracellular signals (13). In addition to receptor molecules having typical transmembrane and intracellular domains, glycosylphosphatidylinositol (GPI)-anchored proteins, such as human CD59, mouse Ly-6, and Thy-1, have been implicated in the regulation of T cell activation (14). mAb binding to these proteins readily affects T cell activity, including IL-2 expression and T cell proliferation. Recent studies have shown that signal transduction for these proteins is dependent on their GPI anchor (15, 16) and the expression of the ζ-chain of the TCR complex (17, 18). Src-family and other unidentified protein tyrosine kinases have been demonstrated in Ly-6 and Thy-1 immunoprecipitates, which suggests that these proteins may be linked to the interior of the cell by another transmembrane protein (19). Ly-6 proteins may have a broader role in the regulation of T cell activation, thus, resulting not only in

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[4] Abbreviations used in this paper: AP-1, activator protein-1; NF-κB, nuclear factor κB; CAT, chloramphenicol acetyltransferase; CREB, cyclic AMP-response-element-binding protein; EMSA, electrophoretic mobility shift assay; GPI, glycosylphosphatidylinositol; NF-AT, nuclear factor of activated T cells; OCT, octamer binding factor; TCF-1(α), T cell-specific factor-1(α).

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Table I. *Oligonucleotide probes with binding sites for transcription factors*

Sequences[a]	Sites	Factors	Enhancers
5′ 3′			
agctTGGGGACTTTCCAGCCG	(κB)	NF-κB	HIV
agctTGGGGATTCCCCATCTG	(κB)	NF-κB	MHCI
agcTGAGGGATTTCACCTCG	(κB)	NF-κB	IL-2
agcTGATGAGTCAGCCG	(TRE)	AP-1	collagenase
gatccAATTCCAGAGAGTCATCAGA	(IL-2, −150)	AP-1	IL-2
agctCCATGACGTCATGG	(CRE)	CREB	TCR-α
AAAGAGGAAAATTTGTTTCATACAGAA	(AT1)[b]	NF-AT	IL-2
GAAAATATGTGTAATATGTAAAACATTTTG	(IL-2A)[c]	OCT	IL-2
AAAAAAGAACAAAGGGCCTAGATT	(CD4 = 2)	TCF1 (α)	CD4
GTCTGAAACATTTTTCTGATTGGTTAAAAGT			
TGAGTGCT	(Y-BOX)	NF-Y	MHCII(Eα)

[a] Only one strand of a double-stranded oligonucleotide is shown. Known binding sites of transcription factors are underlined.
[b] $(NF\text{-}AT)_c$- and non-canonical AP1-binding sites are underlined.
[c] Non-canonical Oct-binding site is underlined.

up-regulation of cytokine production, but also in down-regulation of IL-2 secretion after simultaneous engagement of Ly-6A/E and the TCR complex in T cell lines and normal T lymphocytes (20).

Currently, little is known about the molecular mechanism by which GPI-anchored proteins regulate IL-2 expression. In this study, we have examined the ability of an anti-Ly-6A/E mAb to induce transcription factors controlling IL-2 expression, namely NF-κB, AP-1, NF-AT, and OCT, and several other factors important for regulation of gene expression in T cells, such as CREB, TCF-1(α)/LEF1 (21), and the ubiquitous CCAAT binding factor, NF-Y (22). We have revealed differential regulation of transcription factor binding activities by using anti-CD3ε and anti-Ly-6A/E mAb stimulation and have established a key role for NF-κB and AP-1 transcription factors in positive or negative signaling via Ly-6 proteins.

Materials and Methods

Cell lines and hybridomas

EL4J(Ly-6E).A4 cells were derived by transfection of the EL4J thymoma cells with the expression plasmid vector pEG-2, which contained exons 2, 3, and 4 of Ly-6A/E gene (18). 2B4.11 is a T cell hybridoma specific for pigeon cytochrome *c* and I-E^k (23). MA5.8 is a mutant of 2B4.11 characterized by the absence of TCR ζ- and η-chains expression and by low levels of surface TCR. 2A7.37 is a subclone of the 2A7 cell line, which was made by transfecting MA5.8 with full-length mouse ζ-chain cDNA (24). The B cell lymphoma A20 cells (25) were treated with mitomycin C (25 μg/ml; Sigma, St. Louis, MO) and used as accessory cells when indicated.

Abs

The following mAbs were used in this study to stimulate T cells and have been characterized previously: anti-Ly-6A/E, to a nonpolymorphic Ly-6A.2/E.1 (D7) (26); anti-CD3ε (145–2C11) (27); and anti-Thy-1 (G7) (28). A polyclonal rabbit antiserum to the p50 subunit of NF-κB (29) was kindly provided by Drs. K. Bruhn and M. J. Lenardo (National Institutes of Health). Polyclonal rabbit antiserum 567 to an N-terminal peptide of the p65 subunit of NF-κB and polyclonal rabbit antiserum 561 to a C-terminal peptide of p65 (30) were kindly provided by Dr. W. Greene (Gladstone Institute). Polyclonal antiserum 1226 to a C-terminal peptide of p65 subunit of NF-κB and polyclonal antiserum 265 to a C-terminal peptide of c-Rel (31) were kindly provided by Dr. N. Rice (National Institutes of Health). Rabbit antisera to full-length c-Fos (32) and to v-Jun (33) were kindly provided by Drs. T. Curran (Roche Institute of Molecular Biology) and H. Rahmsdorf, (University of Karlsruhe), respectively.

Culture conditions

Culture conditions to induce IL-2 secretion have been described previously (18, 20). To prepare nuclear extracts, T cells (1×10^6 cells/ml) were cultured in 100-mm tissue plates, which contained 15 ml of media (20) with anti-CD3ε (10% hybridoma supernatant), anti-Thy-1 (10% hybridoma supernatant), or anti-Ly-6A/E (1:500 ascites fluid), for 1 to 20 h in either the presence or absence of A20 cells (2×10^5 cells/ml) as indicated.

Oligonucleotides and electrophoretic mobility shift assay (EMSA)

Sequences of double-stranded oligonucleotides used in this study are shown in Table I. These oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extracts were prepared from 10 to 15×10^6 T cells, as described by Schreiber et al. (34). Buffers for the extraction of nuclear proteins contained the protease inhibitors PMSF (0.5 μM) and leupeptin, aprotinin, pepstatin, chymostatin, and antipain (5 μg/ml each; Sigma). Proteins were quantified according to the method of Bradford (35). Binding reactions for all labeled probes were conducted by incubating the end-labeled DNA (50,000 cpm) with 5 μg of nuclear proteins and 2 μg of poly(dI-dC), as described previously (36, 37), except that quantities of poly(dI-dC), 1 μg/assay and 400 ng/assay, respectively, were used for the NF-AT and the NF-κB(IL-2) probes. For competition experiments, a 50- to 200-fold molar excess of unlabeled oligonucleotide was added to the reaction mixture before the labeled oligonucleotide probe. To identify transcription factors, nuclear extracts were preincubated with 1 μl of specific antiserum for 15 min at 20°C before the addition of the labeled oligonucleotide probes. Thereafter, labeled probes were added and the incubation was continued for an additional 30 min, followed by EMSA. Gels were dried and exposed overnight at −80°C with intensifying screens.

Transfection and CAT assay

T cells that were growing in log phase were transfected by the DEAE-dextran method, as described previously (38). Reporter CAT constructs are shown in Table II. Reference plasmid pRSVβgal was used as a control for transfection efficiency. For each transfection experiment, 5×10^7 cells were treated with 50 μg of reporter plasmid DNA, 10 μg pRSVβgal, and 250 μg/ml DEAE-dextran (Sigma). Twenty hours after transfection each cell culture was split into five 100-mm tissue plates and stimulated with PMA or mAbs as described above. After an additional 20 h of culture, transfected cells were harvested and used to isolate total cell extracts. Protein concentrations of cell extracts were normalized on the basis of β-galactosidase activity of transfected nonstimulated cells to correct for transfection

Table II. *Reporter CAT plasmids*

Plasmid	Regulatory Region	Binding Site	Reference
pILCAT2/1$^+$	−293/−7 mouse IL-2 with TK promoter	OCT, AP-1, NF-κB, and NF-AT	(4)
5 × TRE	5 × (−73/−65 collagenase) with TATAAAA box	5 × AP-1	(39)
5 × TCE_d	5 × (−215/−191 mouse IL-2) with minimal IL-2 promoter	5 × NF-κB	(4)
pILCAT2/1-M(3×)	3 × (−293/−200 mouse IL-2) with minimal IL-2 promoter	3 × (NF-AT, OCT, and NF-κB)	(40)
4 × Pub_d	4 × (−294/−264 mouse IL-2) with TK-promoter	4 × NF-AT	(40)
−121 HIV-CAT	−121/+232 HIV LTR	2 × NF-κB, 3 × SP1	(38)
−76 HIV-CAT	−76/+232 HIV LTR	3 × SP1	(38)

efficiency. Equal quantities of total protein extract (usually between 100 and 200 μg) for control and stimulated cells of each transfection experiment were assayed for CAT activity. Quantitation of CAT assays was performed by liquid scintillation counting of the appropriate area from chromatography plates. Relative CAT activity (or fold of induction) was determined as a ratio of the percentage of acetylated chloramphenicol for activated cells to that for control nonstimulated cells.

Results

Induction of transcription factor binding activities in EL4J(Ly-6E).A4 cells by anti-CD3ε and anti-Ly-6A/E mAb stimulation

Stimulation of various T cell lines by anti-CD3ε has been shown to induce binding activity of nuclear transcription factors NF-κB and AP-1, which regulate IL-2 transcription (41, 42, 6). To begin to determine the molecular basis by which T cell activation and IL-2 production is regulated via Ly6A/E signaling, we used EMSA to detect transcription factor binding activities in nuclear extracts that were prepared from untransfected or Ly-6E-transfected EL4J cells after stimulation with anti-Ly-6A/E or anti-CD3ε mAbs. As expected, anti-CD3ε, but not anti-Ly-6A/E, induced NF-κB (Fig. 1*A*, *lanes 1* through *3* and *5* through *7*) and AP-1 (*A*, *lanes 9* through *11*) nuclear transcription factor binding activities 6 h after stimulation of untransfected EL4J thymoma cells, which are Ly-6A/E negative (18). Similar results were obtained with use of two different NF-κB probes, one for the κB motif from the HIV enhancer (Fig. 1*A*, *lanes 1* through *3*), and another for the palindromic κB motif from the MHC class I enhancer (*A*, *lanes 5* through *7*). The induction of NF-κB was most notable in the upper complex I, whereas complex II was relatively stable. Previously, inducible complex I has been identified in various cell lines as a heterodimeric p65-p50 NF-κB DNA-binding complex and lower complex II has been identified as a homodimeric p50-p50 NF-κB DNA-binding complex (43). The palindromic NF-κB(MHC I) probe binds with high affinity to the p50-p50 homodimer, as well as to the p65-p50 heterodimer, whereas NF-κB(HIV) probe preferentially binds to the p65-p50 heterodimer (44). Our EMSAs performed with the two NF-κB probes are consistent with this finding. The induction of NF-κB and AP-1 was specific, inasmuch as the levels of the transcription factor NF-Y binding activity were stable after mAb stimulation of EL4J (Fig. 1*A*, *lanes 13* through *15*). Inducible NF-κB activity was readily detectable in EL4J cells after 30 to 60 min of PMA treatment, whereas anti-CD3ε stimulation induced detectable NF-κB only after 2 h. This activity became maximal after 6 to 8 h of stimulation (data not shown).

Similarly, EMSAs were performed by using nuclear extracts from EL4J cells transfected with the Ly-6.1 expression vector. The resulting EL4J(Ly-6E).A4 clone expressed relatively high levels of Ly-6E and secreted high levels of IL-2 after stimulation with anti-CD3ε or anti-Ly-6A/E. For these cells, anti-Ly-6A/E and anti-CD3ε induced NF-κB complex I (Fig. 1*B*, *lanes 1* through *3*) and AP-1 binding activities (*C*, *lanes 1* through *3*) 6 h after stimulation, although the level of induction of these activities by anti-Ly-6A/E was lower. In contrast, the level of NF-Y was relatively constant (Fig. 1*B*, *lanes 6* through *8*). PMA, an inducer of NF-κB and AP-1 binding activities and of high levels of IL-2 secretion in EL4 cells (45, 46), also dramatically increased NF-κB complex I (Fig.1*B*, *lane 5*) and AP-1 binding activities (*C*, *lane 5*). Furthermore, in the induction of AP-1 by anti-Ly-6A/E or anti-CD3ε, increased binding activity was noted with use of the canonical AP-1 probe, the TRE motif from the collagenase promoter (Fig. 1*C*, *lanes 1* through *3*), or a second AP-1 probe, the proximal noncanonical AP-1 binding motif from the IL-2 promoter at position −150 bp (*C*, *lanes 6* through *10*). Because this latter probe detected some additional nonspecific activities, the position of the specific complex is indicated by an arrow.

PMA treatment of EL4J(Ly-6E).A4 cells induced three specific NF-AT DNA-binding complexes, designated b1, b2, and b3 (Fig. 1*D*, *lane 5*). Anti-CD3ε primarily induced b1 and b2 (Fig. 1*D*, *lane 2*), whereas anti-Ly-6A/E minimally increased the binding activities represented in these two bands 6 h after treatment (*D*, *lane 3*). These bands represented a specific NF-AT complex on the basis of competition with unlabeled oligonucleotides, inhibition of induction of these bands by

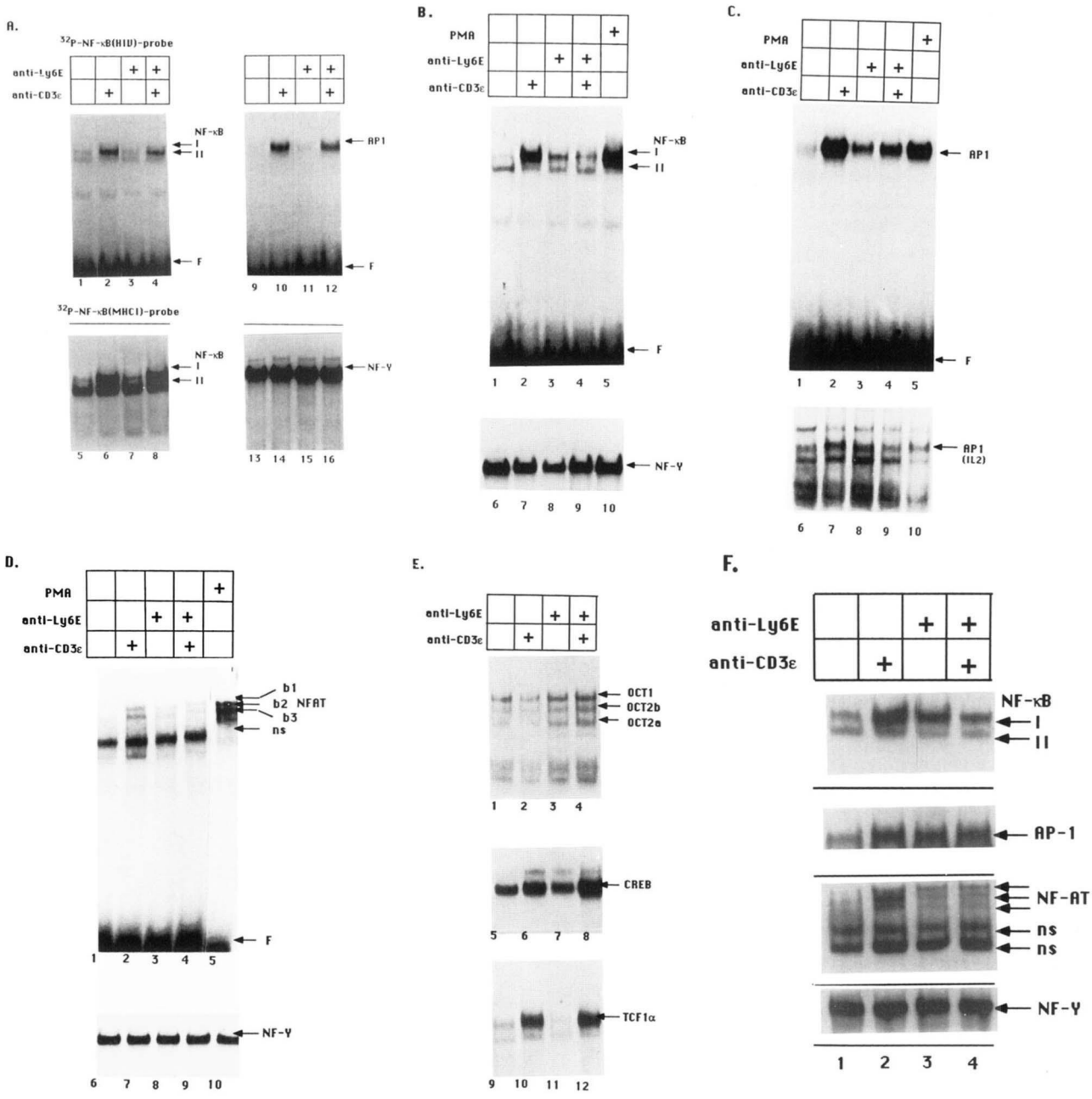


FIGURE 1. Induction of nuclear transcription factor binding activities in stimulated EL4J or EL4J(Ly-6E).A4 cells. EMSAs were performed with nuclear extract proteins from EL4J cells (*A*) and EL4J(Ly-6E).A4 cells (*B* through *F*) that either were not treated or were stimulated with mAbs and PMA (10 ng/ml), as indicated *above* each lane, for 6 h (*A* through *E*) or 20 h (*F*). The following labeled oligonucleotides were used for DNA-binding reactions: NF-κB(HIV) (*A, lanes 1* through *4*), NF-κB(MHC I) (*A, lanes 5* through *8*), AP-1(TRE) (*A, lanes 9* through *12*), and NF-Y probes (*A, lanes 13* through *16*); NF-κB(HIV) (*B, lanes 1* through *5*) and NF-Y probes (*B, lanes 6* through *10*); AP-1(TRE) (*C, lanes 1* through *5*) and AP-1(-150, IL-2) probes (*C, lanes 6* through *10*); NF-AT and NF-Y probes (*D*); OCT probe (IL-2A motif from IL-2 promoter), CREB probe (CRE motif from TCR-α enhancer), and TCF-1(α) probe (CD4 = 2 oligonucleotide sequence from CD4 enhancer) (*E*); NF-κB(HIV), AP-1(TRE), NF-AT, and NF-Y probes (*panel F*). In all panels, F refers to the free-labeled probes. Two main bands of retardation produced by NF-κB probes are designated as complex I and complex II NF-κB (*A* and *B*). Three bands of specific NF-AT binding activity are designated as b1, b2, and b3; ns, nonspecific activity (*D*).

treatment of cells with cyclosporin A (3), and partial inhibition of complex formation by pretreatment of nuclear extracts with antisera to v-Jun and c-Fos (47, 48) (data not shown). In contrast, OCT binding activities (OCT1, OCT2b, and OCT2a) were relatively stable after anti-CD3ε or anti-Ly-6A/E stimulation of EL4J(Ly-6E).A4

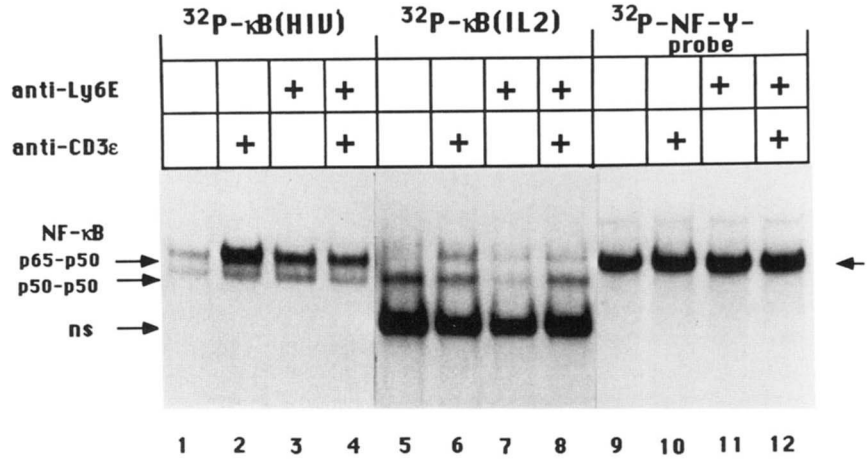


FIGURE 2. Induction of transcription factor binding activities in nuclear extracts of EL4(Ly-6E).A4 cells stimulated with anti-CD3ε and anti-Ly-6A/E mAbs in the presence of mitomycin-C-treated A20 accessory cells. EMSAs were performed with labeled NF-κ(HIV), (*lanes 1* through *4*), NF-κB(IL-2) (*lanes 5* through *8*), and NF-Y probes (*lanes 9* through *12*). The main bands of NF-κB activity are indicated as complexes I (p65-p50) and II (p50-p50).

cells (Fig. 1*E*, *lanes 1* through *3*). Collectively, these experiments indicated that signaling via CD3ε or Ly-6A/E up-regulated NF-κB, AP-1, and NF-AT binding activities, although the induction by anti-Ly-6A/E usually was weaker. TCR- and Ly-6A/E-mediated signals did not influence the levels of OCT and NF-Y; however, activation of EL4J(Ly-6E).A4 cells with anti-CD3ε, but not anti-Ly-6A/E, increased binding activity of CREB (Fig. 1*E*, *lanes 5* through *7*) and, especially, TCF-1(α)/LEF1 (*E*, *lanes 9* through *11*), further suggesting that specific signals emanate from CD3ε and Ly-6A/E.

Down-regulation of transcription factor binding activities by costimulation of EL4J(Ly-6E).A4 cells with anti-CD3ε and anti-Ly-6A/E mAbs

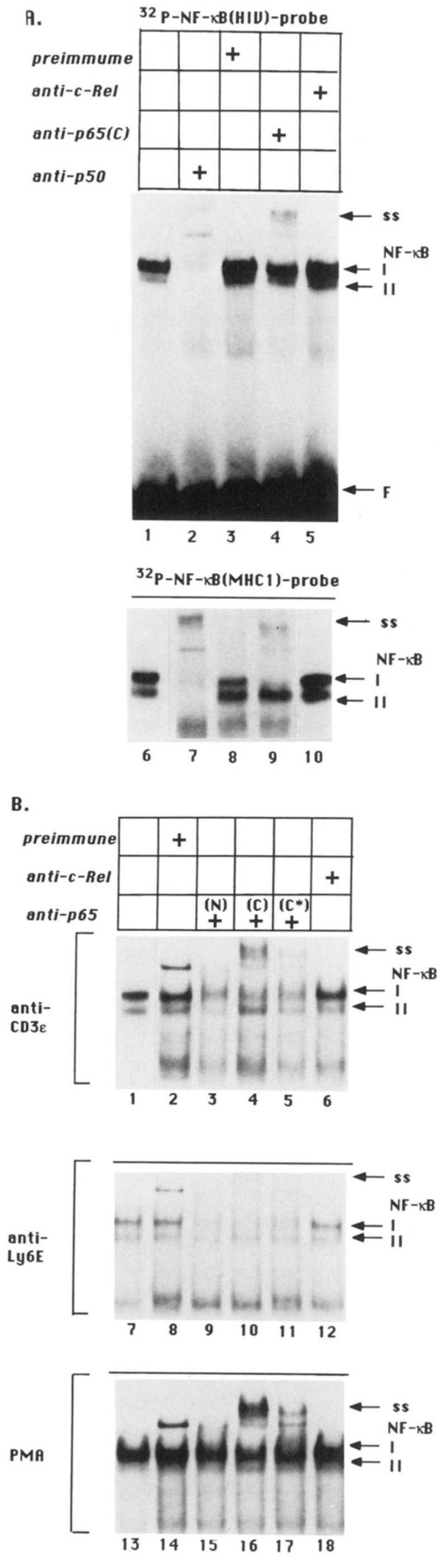
Although anti-Ly-6A/E directly induces IL-2 production, past studies have demonstrated that co-incubation of Ly-6A/E-positive T cells with anti-CD3ε and anti-Ly-6A/E resulted in substantial inhibition of IL-2 production (18, 20). To further characterize the basis of this phenomenon, EMSAs also were performed with use of nuclear extracts prepared from EL4J(Ly-6E).A4 cells 6 h after co-incubation with anti-CD3ε and anti-Ly-6A/E. The levels of NF-κB complex I (Fig. 1*B*, *lane 4*), AP-1 (*C*, *lanes 4* and *9*), and NF-AT complexes b1 and b2 (*D*, *lane 4*) were significantly less when compared with cells stimulated only with anti-CD3ε. This decrease was specific because the levels of NF-κB (Fig. 1*A*, *lanes 4* and *8*) and AP-1 (*A*, *lane 12*) after costimulation of untransfected EL4J cells by anti-CD3ε and anti-Ly-6A/E were comparable to cells only stimulated by anti-CD3ε. Furthermore, costimulation of EL4J(Ly-6E).A4 cells by these two mAbs did not result in reduced binding activity of CREB (Fig. 1*E*, *lane 8*) and TCF-1(α) (*E*, *lane 12*) when compared with cells stimulated solely by anti-CD3ε. This result is important because it indicated that anti-Ly-6A/E does not down-regulate all signaling through the TCR complex. Thus, these data suggest that the capacity of anti-Ly-6A/E to inhibit anti-CD3ε-induced IL-2 production by EL4J(Ly-6E).A4 cells is the result of a selective negative effect on NF-κB, AP-1, and NF-AT binding activities.

It should be stressed that although the data shown are from representative experiments, similar results were seen in more than 10 experiments. Furthermore, time-course studies revealed maximal levels of NF-κB and AP-1 activities induced by anti-CD3ε after 6 h of treatment. When nuclear extracts were prepared 20 h after culture initiation (Fig. 1*F*), the level of inducible AP-1 activity was already reduced after TCR and Ly-6A/E stimulation, whereas NF-κB and NF-AT activities reached the maximal level at this time then gradually declined during next 20 h (data not shown). In any case, the inhibition of anti-CD3ε-induced NF-κB and NF-AT by anti-Ly6A/E was more pronounced 20 h after mAb treatment, whereas down-regulation of AP-1 activity was already less pronounced at this time as compared with 6 h after culture initiation. (Fig. 1*F*).

Activation of IL-2 production in T cells by anti-CD3ε and/or anti-Ly-6A/E is dependent on cross-linking of cell surface bound mAbs by FcR on accessory cells or by a second Ab specific for Ig. Activation of EL4J cells does not require co-incubation with accessory cells because the former express sufficient FcR to cross-link the mAbs (data not shown). When A20 accessory cells were included in the culture, similar regulation of NF-κB activity by anti-CD3ε and anti-Ly-6A/E was seen after 6 h, although the level of induction of NF-κB complex I (p65-p50) binding activity by anti-Ly-6A/E was higher than in the absence of accessory cells (Fig. 2, *lanes 1* through *4*). There was less marked induction of NF-κB activity when the NF-κB(IL-2) probe for EMSA was used and this may be a result of the lower affinity of this probe to NF-κB complex 1 (p65-p50) (Fig. 2, *lanes 5* through *8*). NF-Y served as an internal standard for these nuclear extracts (Fig. 2, *lanes 9* through *12*).

Identification of transcription factor binding activities by using specific Ab pretreatment

EMSAs in which oligonucleotide competition was used confirmed the specificity of the nuclear NF-κB binding



activity in EL4 cells (data not shown). For precise identification of inducible and constitutive NF-κB DNA binding activities, 6 h after anti-CD3ε stimulation, nuclear extracts from EL4J(Ly-6E).A4 cells were pretreated with polyclonal Abs to distinct subunits of NF-κB before EMSA. As was expected, anti-p50 serum almost completely inhibited the formation of complexes I and II and produced a supershift that likely corresponded to complex I (Fig. 3*A*, *lanes 2* and *7*), whereas preimmune serum did not specifically influence NF-κB complexes (Fig. 3*A*, *lanes 3* and *8*). Similar results were seen when extracts from anti-Ly-6A/E activated cells were used (data not shown). Anti-p65(C) 561 serum specific for a C-terminal peptide of p65 partially inhibited the formation of complex I and produced a supershift of this complex that was most readily detected by using the NF-κB(MHC I) probe (Fig. 3*A*, *lane 4* vs *lane 9*). By using three different antisera to p65 NF-κB, the level of complex I was substantially inhibited for nuclear extracts from anti-Ly-6A/E stimulated cells (Fig. 3*B*, *lanes 7* through *11*); we also noted inhibition and/or supershifts for anti-CD3ε- (*B*, *lanes 1* through *5*) or PMA-activated cells (*B*, *lanes 13* through *17*). In contrast, anti-c-Rel did not inhibit formation of NF-κB complexes I and II (Fig. 3, *A*, *lanes 5* and *10*; and *B*, *lanes 6*, *12*, and *18*). Thus, these data indicated that PMA, anti-CD3ε, and anti-Ly-6A/E all induced canonical p65-p50 NF-κB represented in complex I, whereas complex II was homodimer p50-p50. However, the relatively high level of resting NF-κB activity after anti-p65 treatment of extracts, especially those from anti-CD3ε- or PMA-activated cells, raises the possibility that other NF-κB/Rel-related proteins might comprise complex I.

Competition experiments with the canonical AP-1(TRE) probe or CREB probe confirmed the specificity of

FIGURE 3. Identification of NF-κB transcription factor DNA-binding activities in EL4J(Ly-6E).A4 cells. EMSAs were performed with nuclear extracts of cells stimulated by anti-CD3ε (*panel A*), or PMA, anti-CD3ε, or anti-Ly-6A/E 6 h after culture initiation (*panel B*). Nuclear extracts were preincubated with Abs to different NF-κB/Rel-related proteins for 15 min before the addition of the following labeled oligonucleotides: NF-κB(HIV) (*A*, *lanes 1* through *5*; *B*) and NF-κB(MHC I) probes (*A*, *lanes 6* through *10*). The following Abs were used: polyclonal rabbit antiserum to p50 subunit of NF-κB (anti-p50) (*A*, *lanes 2* and *7*); polyclonal rabbit antiserum 561 to a C-terminal peptide of p65 (anti-p65C) (*A*, *lanes 4* and *9*; *B*, *lanes 4*, *10*, and *16*); polyclonal rabbit antiserum 1226 to a C-terminal peptide of p65 (anti-p65*) (*B*, *lanes 5*, *11*, and *17*); polyclonal rabbit antiserum 567 to a N-terminal peptide of p65 (anti-p65N) (*B*, *lanes 3*, *9*, and *15*); polyclonal rabbit antiserum 265 to a C-terminal peptide of c-Rel (anti-c-Rel) (*A*, *lanes 5* and *10*; *B*, *lanes 6*, *12*, and *18*); and preimmune serum (*A*, *lanes 3* and *8*; *B*, *lanes 2*, *8*, and *14*). Control extracts were not treated with Abs (*A*, *lanes 1* and *6*; *B*, *lanes 1*, *7*, and *13*).

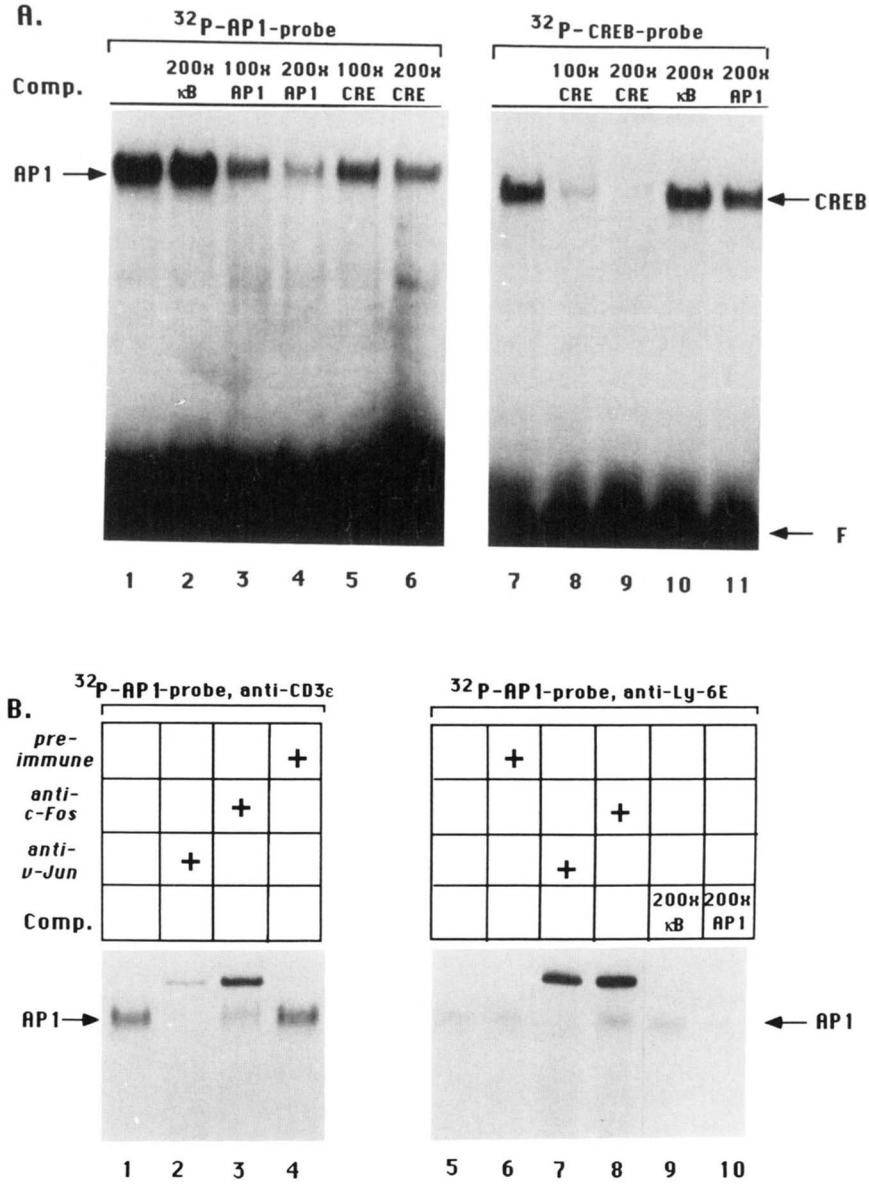


FIGURE 4. Identification of AP-1 and CREB transcription factor binding activities in nuclear extracts of EL4J(Ly-6E).A4 cells. EMSAs were performed under standard conditions with the indicated labeled oligonucleotide probes, except that nuclear extracts were preincubated with a 100- or 200-fold molar excess of the indicated unlabeled oligonucleotides as competitors (Comp.) (*A, lanes 2* through *6* and *8* through *11*; *B, lanes 9* and *10*) or with polyclonal rabbit antiserum to v-Jun (anti-v-Jun) (*B, lanes 2* and *7*), polyclonal rabbit antiserum to c-Fos (anti-c-Fos) (*B, lanes 3* and *8*), and preimmune serum (*B, lanes 4* and *6*). Control extracts were not treated by competitors or Abs (*A, lanes 1* and *7*; *B, lanes 1* and *5*). Nuclear extracts were isolated from cells stimulated for 6 h with anti-CD3ε (*A*) and (*B, lanes 1* through *4*), or anti-Ly-6A/E (*B, lanes 5* through *10*).

interaction of these labeled probes with transcription factors (Fig. 4*A*). The AP-1 transcription factor is comprised of different combinations of Jun and Fos protein subunits (49). Treatment of nuclear extracts from anti-CD3ε-stimulated EL4J(Ly-6E).A4 cells with broadly specific antisera to v-Jun and c-Fos, but not with preimmune serum, substantially inhibited formation of the AP-1-binding complexes (Fig. 4*B*, *lanes 1* through *4*). These antisera did not affect the detection of the CREB complex by EMSA, which suggests that CREB was a different transcription factor (data not shown). As the inhibition was complete with anti-v-Jun, but only partial with anti-c-Fos, these data suggest that the AP-1-binding complex seems to be a mixture of Jun-Jun homodimers and Jun-Fos heterodimers. The broad specificity of these antisera do not permit a more precise identification of these subunits. Similar results were obtained after analysis of nuclear extracts from anti-Ly-6A/E-stimulated EL4J(Ly-6E).A4 cells, although, in this case, anti-c-Fos only slightly inhibited the formation of specific complex (Fig. 4*B*, *lanes 5* through *10*).

Differential regulation of transcription factor binding activities in EL4(Ly-6A/E).A4 cells by anti-Thy-1 and anti-Ly-6A/E mAbs

Cross-linking of GPI-anchored Thy-1 leads to secretion of IL-2, but unlike anti-Ly-6A/E, does not inhibit anti-CD3ε-induced IL-2 production (20, 28). Therefore, the ability of anti-Thy-1 to affect the binding activity of several transcription factors was examined for EL4J(Ly-6E).A4 cells. An EMSA of nuclear extracts demonstrated that anti-Thy-1 did not induce NF-κB complex I (Fig. 5, *lanes 1* through *3*), but did increase the binding activity of NF-AT

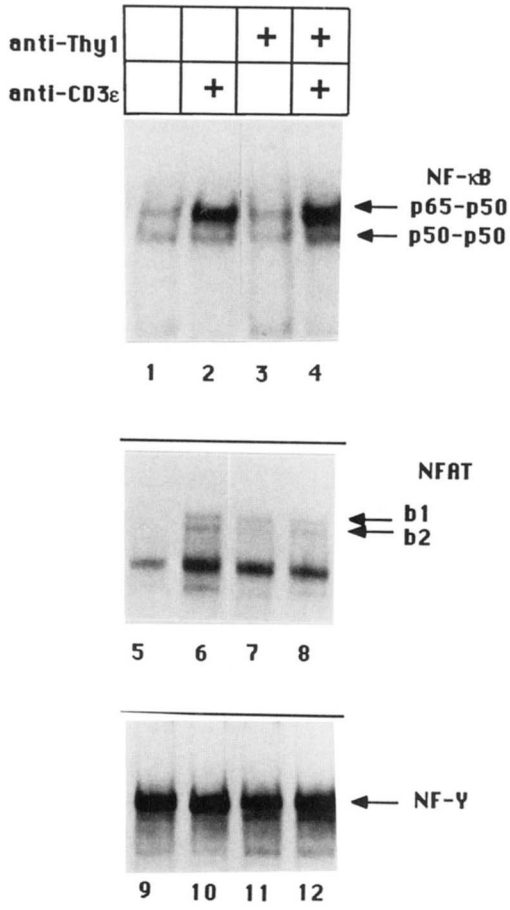


FIGURE 5. Comparison of effects of anti-Thy-1, anti-Ly-6A/E, and anti-CD3ε on the induction of nuclear transcription factor activities in EL4J(Ly-6E).A4 cells. Cells were stimulated for 6 h with mAbs, as indicated *above* the lanes. EMSAs were performed with labeled NF-κ (HIV) (*lanes 1* through *4*), NF-AT (*lanes 5* through *8*), and NF-Y probes (*lanes 9* through *12*).

to a level almost comparable with that induced by anti-CD3ε (*lanes 5* through *7*). These effects were specific as judged by the binding activity of NF-Y (Fig. 5, *lanes 9* through *12*). Anti-Thy-1 also slightly induced AP-1 (data not shown). Furthermore, anti-Thy-1 did not inhibit anti-CD3ε-induced binding activity of NF-κB (Fig. 5, *lane 4*) and minimally decreased anti-CD3ε-induced NF-AT (*lane 8*). Thus, the regulation of several transcription factors, which are important for expression of IL-2 gene, is different after activation by anti-Thy-1 and anti-Ly-6A/E.

Inducibility of the IL-2 enhancer determined by CAT assay

To probe in vivo for transcription transactivator function of factors that were induced by mAbs or PMA treatment, EL4J(Ly-6E).A4 cells were transfected with reporter constructs carrying binding sites for different transcription factors (Table II). Each transfection experiment was repeated 3 or 4 times. Data from representative experiments are shown on Figure 6. Plasmid pILCAT2/1$^+$ contained the −293/−7-bp regulatory region of the mouse IL-2 gene with binding sites for OCT, NF-κB, AP-1, and NF-AT. Cells transfected with this construct (Fig. 6*A*) showed strong induction of CAT expression after anti-CD3ε or PMA stimulation, whereas anti-Ly6A/E induced a moderate level of CAT activity. Costimulation of transfected cells with both mAbs strongly inhibited CAT activity compared with that induced by anti-CD3ε alone (Fig. 6*A*, *lanes 1* through *4*). The enhancerless vector with the TK promoter, pBLCAT2, and the CAT construct with a minimal IL-2 promoter were not inducible (data not shown).

To assess the roles of individual transcription factors for TCR- or Ly-6A/E-dependent signaling, we used different plasmid constructs with oligomerized binding sites for transcription factors as artificial enhancers. Plasmid 5x TRE-CAT, which contained five AP-1 sites from the collagenase gene (Fig. 6*A*, *lanes 6* through *10*), and plasmid 5xTCE$_d$, which contained five NF-κB binding sites from the IL-2 enhancer (*C*, *lanes 6* through *10*), were induced with mAbs or PMA in a manner that was analogous to that detected with pILCAT2/1$^+$, although the magnitude of this response was lower. Furthermore, costimulation of cells with both mAbs also inhibited NF-κB- and AP-1-dependent CAT activity.

To determine whether NF-AT contributed to the induction of mouse IL-2 transcription in vivo, we transfected EL4J(Ly-6E).A4 cells with the 4xPub$_d$-CAT construct, which contained four repeats of the distal NF-AT binding site from the mouse IL-2 enhancer. Interestingly, this construct was induced substantially by PMA or PMA and ionomycin, whereas it was only induced slightly by anti-CD3ε and was not induced by anti-Ly-6A/E (Fig. 6*B*, *lanes 6* through *10*). The more complicated pILCAT-2/1-M(3x) construct, which contained a trimer of the murine NF-AT site, OCT site, and most of the NF-κB site (−293 to −200 bp), was characterized by a relatively high induction of CAT after treatment by anti-CD3ε and a low induction by anti-Ly-6A/E. Thus, NF-AT seems to contribute to the induction of CAT activity by anti-CD3ε only in the context of OCT and NF-κB, whereas a role for NF-AT was not obviously detected for stimulation by anti-Ly-6A/E.

We also used a second probe for NF-κB-directed transcription, the −121 HIV-CAT construct with two NF-κB and three SP-1 sites from the HIV-LTR. The −76 HIV-CAT construct with three SP-1 sites served as a negative control (Fig. 6*D*). For the −121 HIV-CAT construct, moderate levels of CAT induction, similar to those detected for the 5x TCE$_d$-CAT, were observed after stimulation either by anti-CD3ε or anti-Ly-6A/E.

Taken together, these data demonstrated that TCR- and Ly-6A/E-mediated regulation of IL-2 transcription in EL4J(Ly-6E).A4 cells primary is based on induction of NF-κB and AP-1 transcription factor activities that induced the IL-2 enhancer in the context of other transcription factors.

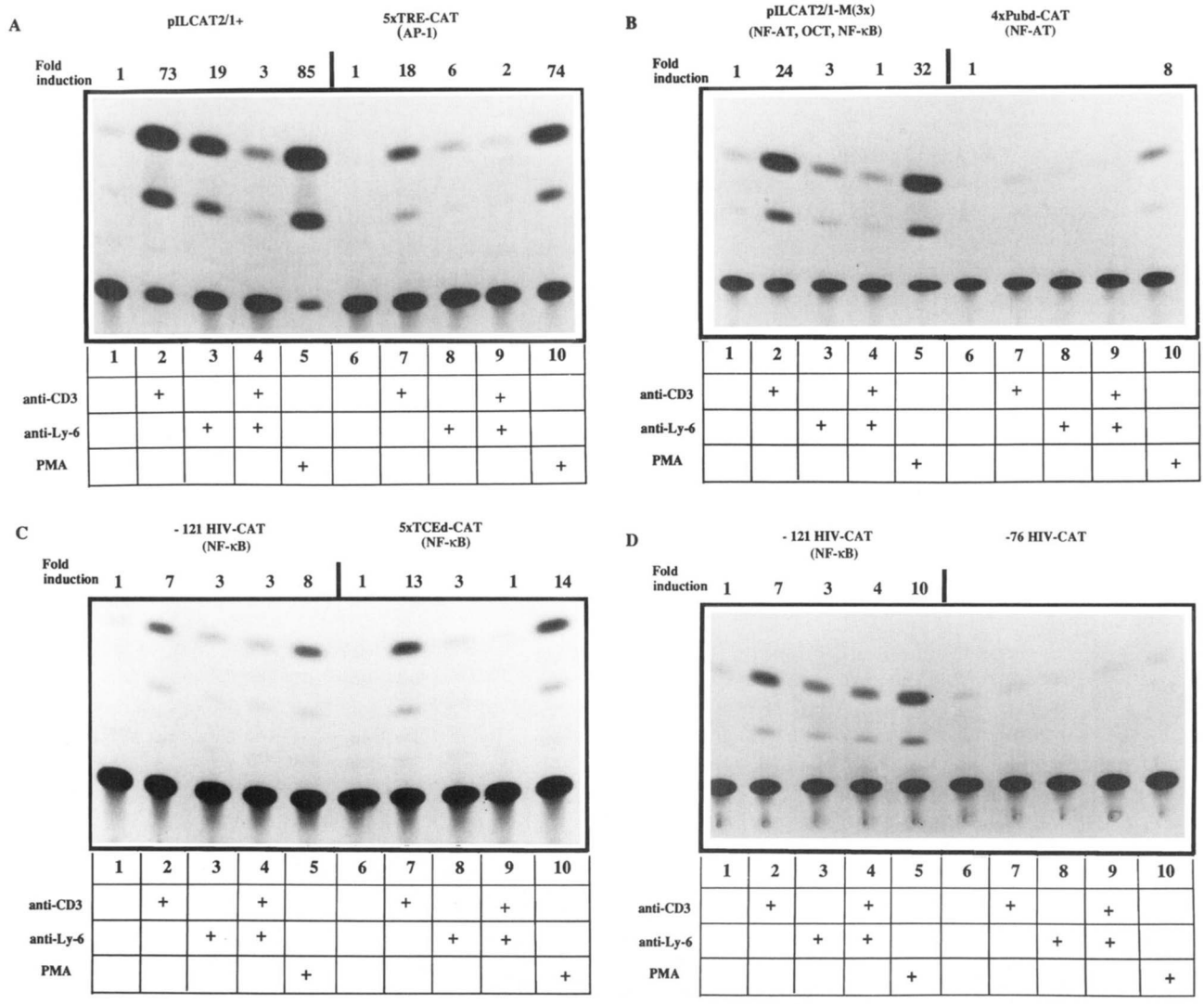


FIGURE 6. CAT activity directed by IL-2-enhancer CAT or AP-1- NF-κB- and NF-AT-dependent reporter CAT constructs in EL4J(Ly-6E).A4 cells stimulated with mAbs or PMA. Cells were transfected with reporter plasmids pILCAT2/1$^+$ (*A, lanes 1* through *5*), 5xTRE-CAT (*A, lanes 6* through *10*), pILCAT2/1-M(3x) (*B, lanes 1* through *5*), 4xPub$_d$-CAT (*B, lanes 6* through *10*), −121 HIV-CAT (*C, lanes 1* through *5*; *D, lanes 1* through *5*), −76 HIV-CAT (*D, lanes 6* through *10*), and 5xTCE$_d$-CAT (*C, lanes 6* through *10*) and were activated with mAbs or PMA, as indicated below the lanes. One experiment representative of four is shown. Average induction levels of CAT activity on the basis of the ratio of the percentage of conversion of chloramphenicol to the acetylated form are shown above the lanes.

In the case of stimulation with both mAbs, Ly-6A/E-mediated signals also inhibited anti-CD3ε induction of the IL-2 enhancer at the level of these two transcription factors.

Role of TCR ζ-chain for induction of nuclear p65-p50 NF-κB after anti-Ly-6A/E stimulation of 2B4.11 T cell hybridoma

Previous studies have demonstrated that anti-Thy-1- or anti-Ly-6A/E-induced IL-2 production in the 2B4.11 T cell hybridoma is dependent on expression of the TCR ζ-chain, whereas anti-Ly-6A/E-mediated inhibition of anti-CD3ε-induced IL-2 production seems to be independent of TCR-ζ (17, 18). To determine whether anti-Ly-6A/E similarly regulated transcription factor activities in a cell other than EL4J and to assess the role of TCR-ζ as a signal transduction molecule to induce transcription factors, EMSAs were performed with nuclear extracts of the 2B4.11 T cell hybridoma, its TCR-ζ-negative variant, MA 5.8, and the TCR-ζ retransfectant of MA5.8, 2A7.37. As was seen with EL4J(Ly-6E).A4 cells, the addition of anti-CD3ε or anti-Ly-6A/E 6 h after treatment substantially induced p65-p50 NF-κB binding activity in 2B4.11 cells (Fig. 7*A*, *lanes 1* through *3*). Anti-CD3ε, but not anti-Ly-6A/E, induced p65-p50 activity in TCR-ζ-negative MA5.8. (Fig. 7*A*, *lanes 5* through *7*); both Abs induced p65-p50 NF-κB in 2A7.37 cells (*A*, *lanes 9* through *11*). These effects were specific as

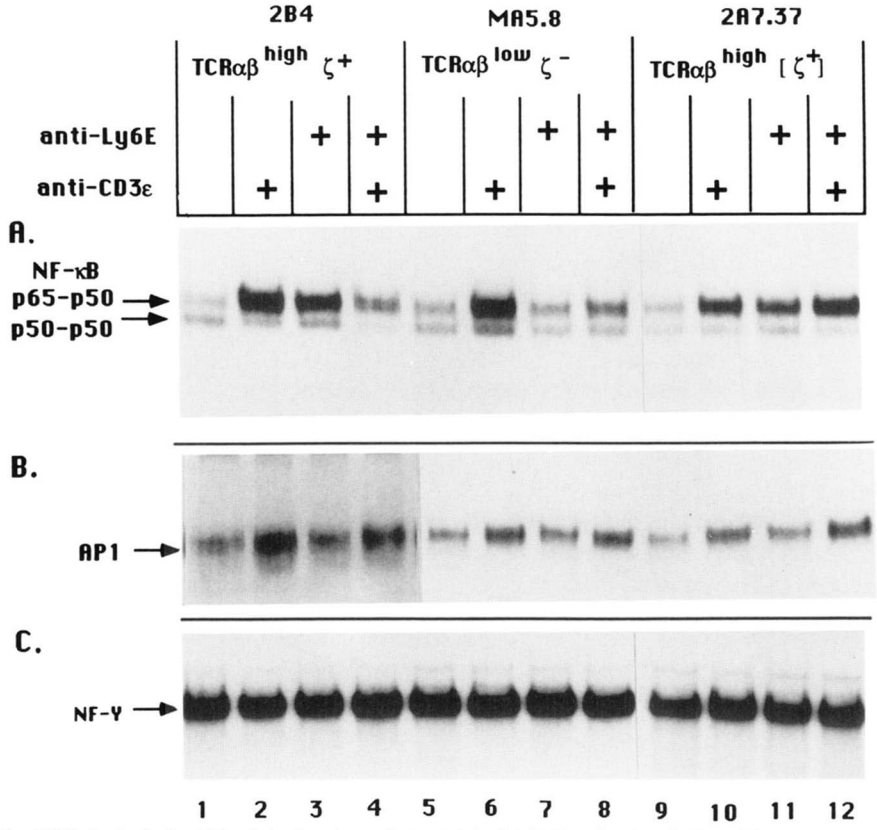


FIGURE 7. A role for TCR ζ-chain in NF-κB induction after anti-Ly6A/E T cell stimulation. Phenotypes of cells are shown on the *top* of the panel. 2B4.11, MA 5.8, and 2A7.37 cells were either untreated (*lanes 1, 5,* and *9*) or stimulated by different combinations of mAbs in presence of mitomycin-treated accessory A20 cells, as indicated above the lanes for 6 h. EMSA were performed with NF-κB(HIV) (*A*), AP-1(TRE) (*B*), and NF-Y probes (*C*).

judged by reference NF-Y activity (Fig. 7*C*). Thus, induction of NF-κB and IL-2 secretion in 2B4.11 cells by anti-Ly-6A/E is dependent on surface expression of TCR-ζ. However, anti-Ly-6A/E dramatically decreased anti-CD3ε-induced p65-p50 NF-κB for both 2B4.11 (Fig. 7*A*, *lane 4*) and MA5.8 cells (*A*, *lane 8*), which indicates that this inhibitory effect was independent of TCR-ζ expression. Somewhat surprisingly, cocultures of anti-Ly-6A/E and anti-CD3ε with 2A7.37 cells did not inhibit binding activity of p65-p50 NF-κB (Fig. 7*A*, *lane 12*). It is possible that overexpression of the ζ-chain in 2A7.37 cells enhanced TCR signaling via the ζ-chain pathway, thus, overcoming the inhibitory effects of anti-Ly-6A/E on TCR signaling. Additional experiments are required to confirm this hypothesis.

In the presence of accessory cells, these T cell hybridomas are characterized by relatively high background levels of AP-1 binding activity, which increased after anti-CD3ε stimulation. Anti-Ly-6A/E exerted minimal capacity to either directly induce AP-1 or inhibit anti-CD3ε-induced levels of AP-1 (Fig. 7*B*). EMSA performed with nuclear extracts of these cell lines revealed stable levels of OCT1 binding activity and trace levels of NF-AT binding activity, which were not induced by mAb treatment (data not shown). Control EMSA experiments were performed with nuclear extracts from mitomycin-treated A20 (Ly-6A/E-negative) cells and compared with nuclear extracts from 2B4.11. At the standard protein concentration used for EMSA, neither NF-κB nor AP-1 activities were detected in A20 cells, although these activities were detected after increasing the protein concentration 10-fold for the DNA binding reactions (data not shown). Thus, it is unlikely that nuclear factors from A20 cells contributed to the detection of NF-κB and AP-1 from nuclear extracts of 2B4.11 hybridoma and its variants.

Discussion

In eukaryotic cells, many different proteins are anchored to the external surface of the plasma membrane by covalently attached GPI (50). For mouse T lymphocytes, Thy-1 and Ly-6 proteins represent well-characterized examples whereby cross-linking of GPI-anchored proteins leads to T cell activation, especially as measured by IL-2 secretion. Little is known about the intracellular signals generated via GPI-anchored proteins. In the present study, we have examined the induction of nuclear transcription factors (51, 52) that regulate IL-2 expression after T cell stimulation via Ly-6A/E, Thy-1, and TCR to answer three general questions

about the mechanism by which GPI-anchored proteins regulate T cell activation. These questions are: 1) What transcription factors are the targets after signaling via TCR, Ly-6A/E, and Thy-1? 2) Does activation via Ly-6A/E and TCR proceed through a common signal transduction pathway? 3) What is the molecular mechanism by which cross-linking Ly-6A/E leads to inhibition of IL-2 production induced via the TCR?

By using Ly-6A/E-transfected EL4J cells as a model, we found a specificity in the pattern of induction of transcription factors after stimulation through CD3ε, Ly-6A/E, and Thy-1. Anti-CD3ε induced high levels of NF-κB, AP-1, NF-AT, CREB, and LEF1/-TCF-1(α) binding activities, whereas anti-Ly-6A/E induced moderate levels of NF-κB and AP-1, but only low levels of NF-AT. In contrast, anti-Thy-1 induced moderate levels of NF-AT and low levels of AP-1, but did not activate NF-κB in EL4J cells. Although mAb stimulation via CD3ε, Ly-6A/E, and Thy-1 leads to IL-2 secretion, the observation that the pattern of transcription factors is quite specific for each mAb provides strong suggestive data for the specificity of signals emanating through each of these protein.

Consistent with the results from EMSAs, CAT assays detected TCR- and Ly-6A/E-mediated induction of transactivator transcription function of NF-κB and AP-1 and a cooperative activating effect of these factors on the IL-2 enhancer in the context of other factors. Furthermore, co-incubation of these cells with both anti-CD3ε and anti-Ly-6A/E resulted in substantial inhibition of NF-κB- and AP-1-dependent CAT transcription, as well as inhibition of inducibility of the IL-2 enhancer. In cultures that were parallel to those used for nuclear extract preparation, we confirmed that IL-2 secretion was regulated by Ly-6A/E-mediated signals and that anti-CD3ε-induced IL-2 production was inhibited by anti-Ly-6A/E (data not shown). Thus, it seems that both the activating and inhibitory effects of Ly-6A/E-mediated signals on IL-2 expression occur at the level of NF-κB and AP-1 transcription factors.

In particular, regulation of NF-κB seems to be a key event in signaling via Ly6A/E in both the EL4J(Ly-6E) and the 2B4.11 model cell systems. In the presence of A20 accessory cells, NF-κB p65-p50 was the only transcription factor that was dramatically induced in these cell systems by anti-Ly6A/E. Inasmuch as AP-1 was also induced in EL4J(Ly-6E).A4 cells by anti-Ly-6A/E in cultures lacking accessory cells, it is highly likely that AP-1 also may be induced by adhesion and/or costimulatory molecular interaction between the T cell and accessory cell. By using Ab identification experiments, we established that anti-CD3ε and anti-Ly-6A/E induced canonical p65-p50 (RelA/p50) NF-κβ binding activity (53). For anti-Ly-6A/E-stimulated cells, this activity was almost completely inhibited by several different anti-p65 sera in EMSAs, thus, indicating that stimulation via Ly-6A/E primarily regulates RelA/p50 NF-κB. In contrast, these antisera only partially inhibited the p65-p50 NF-κB induced by anti-CD3ε in EMSAs, thus suggesting that other types of NF-κB, e.g., RelB/p50 (54), were also activated via CD3ε. The failure of anti-c-Rel to affect NF-κB in EMSAs suggests that c-Rel/p50 is not induced in EL4J cells.

EMSA revealed only a small fraction of nuclear p65-p50 NF-κB activity bound in vitro to a specific site in the IL-2 enhancer, using the NF-κB(IL-2) probe. However, CAT construct with multiple NF-κB(IL-2)-motif was readily transactivated in vivo. It is possible that actual binding of NF-κB to the IL-2 enhancer is also regulated by protein-protein interactions with other factors. This scenario has been shown for other genes that are regulated by NF-κB, such as the binding of NF-κB and serum response factor in the IL-2Rα enhancer (55), NF-κB and HMG I(Y) in the IFN-β enhancer (56), and NF-κB and C/EBP (57).

TCR recognizes antigenic peptides bound to MHC molecules on the APCs generating intracellular signals through at least two autonomous transduction modules from either the ε- or ζ-chain of the CD3/TCR complex (58, 59). Analysis of Ly-6-mediated signal transduction, which was performed with use of the 2B4.11 hybridoma and its TCR-ζ-negative mutated variant, indicated that the ability of anti-Ly6A/E to inhibit NF-κB p65-p50 binding activity was independent of ζ-chain expression, whereas the direct induction of NF-κB by anti-Ly6A/E required cell surface TCR ζ-chain. Furthermore, there is a good correlation between the ability of anti-Ly6A/E to regulate NF-κB p65-p50 and the capacity of 2B4.11 cells to secrete IL-2. However, this correlation was not linear, because the decrease in the level of anti-CD3ε-induced IL-2 secretion was often much greater than the reduction of NF-κB p65-p50. This finding suggests that some critical concentrations of nuclear transcription factors occur for effective integrative induction of IL-2 gene transcription.

Because there is considerable information about the regulation of NF-κB in T cells, we may be able to deduce levels at which triggering Ly-6A/E molecules affect intracellular signaling. Activation of NF-κB involves its release as a p65-p50 heterodimer from a complex with cytoplasmic inhibitor, IκB, and the subsequent translocation of this active form into the nucleus (60). It is likely that IκB is inactivated by phosphorylation, resulting in the release of NF-κB (61, 62). The protein kinase that inactivates IκB is unknown, but such a kinase must be linked to the signaling cascades. One candidate kinase is Raf-1, which seems to dissociate the NF-κB-IκB complex in vitro and in vivo (63). This observation correlates well with recent work demonstrating that Raf-1, which also regulates AP-1, is required by T cells to induce IL-2 production (64). Thus, Raf-1 also may be a good candidate for regulation via Ly-6A/E, given the ability of anti-Ly-6A/E to induce NF-κB and AP-1. A second pathway leading to nuclear NF-κB induction was described recently (31, 65), inasmuch as the p105 precursor of the p50 NF-κB subunit has an IκB-like function for p65 NF-κB (RelA) subunits. This latent p65-p105 form of NF-κB is activated by proteolytic processing of the p105 precursor, yielding an active p65-p50 NF-κB that can be translocated into the

nucleus. It is likely that activation of a specific protease for p105 is dependent on its modification as a result of signal transduction. EL4J(Ly-6).A4 cells contained high levels of a latent cytoplasmic precursor of NF-κB, which was activated in vitro by deoxycholate treatment that released NF-κB from IκB (data not shown). However, we have not as yet determined how T cell signaling affects cytoplasmic NF-κB and have not ruled out proteolytic processing as a potential mechanism of activation.

It is unlikely that blockade of IL-2 production via Ly-6A/E is analogous to T cell anergy. T cell anergy requires engagement of the TCR without any costimulatory signals, whereas Ly-6A/E-mediated inhibition of IL-2 production is the result of costimulation through the TCR and Ly-6A/E. Nevertheless, there is a similarity in distal signaling events for these two processes, because there is a selective inhibitory effect on factors that control IL-2 transcription. In two recent reports, the molecular mechanism of T cell anergy was studied; in one case, NF-κB and NF-AT were specifically inhibited (66) and, in the other, AP-1 was primarily down-regulated (67).

Targets for NF-κB include several cytokines and receptor genes, such as IL-2, IL-6, IL-8, TNF-α, granulocyte macrophage CSF, the IL-2R α-chain, MHC class I, and the MHC class II-associated invariant chain (53, 60). Inasmuch as NF-κB is a transcriptional regulator for a number of immunologically important genes, it is possible that triggering Ly-6, which regulates NF-κB, might affect the expression of many other genes in addition to IL-2; however, we have not as yet systematically examined this issue. Although the physiologic role of Ly-6A/E in vivo is still uncertain, engagement of these molecules might represent a mechanism to limit an ongoing immune response.

The question of the origin of Ly-6A/E-mediated negative signals for TCR signaling is now under investigation. The inhibition by cAMP of Ras-dependent activation of Raf (68) and the determination of NF-κB as a main target of cAMP-dependent inhibition of IL-2 transcription (69) may suggest a linkage of Ly-6A/E-generated negative signals with the cAMP-dependent signal pathway.

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