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Induction of the Early Growth Response Gene 1 Promoter by TCR Agonists and Partial Agonists: Ligand Potency Is Related to Sustained Phosphorylation of Extracellular Signal-Related Kinase Substrates

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Responses to partial agonist TCR signals include positive selection of thymocytes, survival of naive T cells, and homeostatic proliferation. As part of an effort to understand the molecular basis of these processes, we have determined how agonist and partial agonist ligands act differently to induce a change in gene expression. We have found that the early growth response gene 1 (Egr1) promoter is activated by agonist and partial agonist ligands, but the partial agonist induces 10-fold lower promoter activity. Both agonist and partial agonist ligands require all six response elements in the Egr1 promoter to reach maximum induction. Although slightly fewer cells respond to the partial agonist, all of the responding cells have reduced activity compared with the cells responding to agonist. The factors binding to the serum response elements of the Egr1 promoter form a ternary complex (TC) consisting of serum response factor and either Elk-1 or serum response factor accessory protein-1a. Formation of a stable TC and inducible promoter activity are both dependent on extracellular signal-related kinase activation. Examination of TC formation over time reveals that this complex is induced well by partial agonist ligands, but it is not sustained, whereas agonist stimulation induces longer lived TCS. Therefore, the data suggest that both agonist and partial agonist ligands can induce formation of multiple TC on the Egr1 promoter, but the ability of the agonist ligand to maintain these complexes for an extended time results in the increased potency of the agonist. The Journal of Immunology, 2003, 170: 315–324.

A single TCR can recognize a variety of peptide/MHC ligands (1). Agonist ligands are peptide/MHC complexes that induce complete TCR signal transduction resulting in widespread changes in gene expression. Antagonist ligands have been made primarily by substituting amino acid side chains in agonist peptides such that the antagonist ligands will inhibit responses induced by the agonist (2). Although antagonist ligands are inhibitory for multiple cellular responses such as proliferation and cytokine production, these ligands can also act on their own to induce some alterations in gene expression and functional response in T cells. They have the characteristic of inducing a subset of the responses triggered by agonist ligands and therefore can be described as partial agonists (3).

Thymocytes and T cells must properly interpret partial agonist signals for positive selection and survival. Recognition of self-peptide/MHC as a strong agonist by immature thymocytes results in negative selection, but positive selection of thymocytes requires some interaction between the TCR and self-peptide/MHC. Therefore, positive selection requires recognition of self-peptide/MHC as a partial agonist (4). The requirement for thymocytes to recognize self-peptide/MHC to survive may also apply to naive T cells in the periphery. Several studies have demonstrated a reduced life span for naive T cells if they are unable to recognize self-peptide/MHC as a partial agonist (5–7). Although there is some controversy regarding the need for naive T cells to interact with self-peptide/MHC for survival (8, 9), it is clear that naive T cells must not respond to self-peptide/MHC as an agonist so that autoimmunity can be prevented (10). Thus, proper recognition of partial agonists is critical for normal T cell function at multiple stages.

The essential nature of partial agonist responses for the generation and maintenance of the mature T cell pool has led to numerous studies aimed at understanding how ligand binding and intracellular signaling differ between partial agonists and agonists. In general, the TCR binds to partial agonist ligands with a lower affinity, and this is primarily due to an increase in the TCR-ligand off-rate (11). The rapid dissociation of the TCR from partial agonist ligands results in an incomplete phosphorylation of the TCR-associated ζ-chain (12). This partially phosphorylated ζ-chain can be inhibitory for some T cell functions and may play a role in the ability of some ligands to act as TCR antagonists (13). The relationship between the partially phosphorylated ζ-chain and positive induction of T cell function is not fully understood.

To understand differences in signal transduction between an agonist and a partial agonist ligand, previous approaches have relied on following the path of signal transduction from the TCR as it travels toward the nucleus where gene expression can be influenced. This “top-down” approach for the study of partial agonist signaling has been limited by the low concentrations of activated signaling molecules induced by partial agonist ligands, therefore rendering the pursuit of signal transduction pathways by biochemical means difficult. An alternative approach has been referred to as “bottoms-up” (14). The idea is that analysis of the promoter elements of a gene responsive to partial agonist signals will provide information about the signaling pathways that lead to its induction.

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To determine differences in the ability of agonist and partial agonist ligands to induce changes in gene expression, we have taken a “bottoms-up” approach and examined promoter elements responsible for the induction of the early growth response gene-1 (Egr1) in T cells.

Egr1 is transiently induced in diverse cell types in response to growth and differentiation signals (15–17), suggesting that it is an important regulator coupling extracellular signals to rapid changes in gene expression (18). Egr1 is induced in both thymocytes and T cells by TCR signals, and this induction requires activation of the mitogen-activated protein kinase (MAPK) extracellular signal-related kinase (ERK) (19, 20). Our analysis of Egr1-deficient mice has found that Egr1 is required for efficient positive selection of thymocytes (21), and Egr1 has been implicated in the response to pre-TCR signals (22). In addition, we have found that mature T cells increase expression of Egr1 not only in response to agonist ligands, but lower levels of Egr1 are also induced in response to partial agonist ligands (M. Bettini and G. Kersh, manuscript in preparation). Therefore, an analysis of the Egr1 promoter may reveal important differences between agonist and partial agonist signaling.

cis-acting DNA sequences within the Egr1 promoter that are important for induction of Egr1 expression in response to growth factors, B cell receptor (BCR) stimulation, and various other stimuli have been analyzed previously in several cell types (23–39). A common theme in these studies is that ternary complex (TC) formation on six response sequence elements (SREs) of the Egr1 promoter is important for Egr1 expression. In some cases, all of the SREs contribute equally to expression (23, 26, 28, 35–37), but in some cell types specific SREs are critical (29, 31, 32, 38, 39). Analysis of Egr1 regulation in response to TCR stimulation has not been reported.

In this study, we examined the transcription factors that regulate Egr1 induction in response to TCR stimulation by both agonist and partial agonist ligands. Although Egr1 promoter activity is much lower in response to the partial agonist ligand, all six of the SRE sites of the Egr1 promoter are required for a full response to both agonist and partial agonist ligands. Single-cell analysis of promoter activity demonstrates that all of the cells activated by partial agonist have reduced promoter activity. Thus, for a given T cell, a partial agonist ligand can use all six SREs of the Egr1 promoter, but is unable to induce high levels of activity. To understand why this is the case, we also investigated the factors binding to the SRE sites. A TC that forms on the SREs consists of serum response factor (SRF) and the Ets family members Elk-1 and SRF accessory protein-1a (SAP-1a). Recruitment of Elk-1 into the TC is inducible and depends on its phosphorylation by the ERK family of MAPK. Agonist ligands induce a stable TC that persists for >30 min whereas the TC induced by a partial agonist is present only for ~10 min. Therefore, the data suggest a model where the duration of ERK activation determines the magnitude of changes in Egr1 expression induced by a TCR ligand.

Materials and Methods

Luciferase reporter constructs

The Egr1 promoter luciferase construct p-701 was made by generating a PCR fragment spanning –701 to +38 region of the murine Egr1 using a plasmid containing the genomic Egr1 (pGL3neoTK, a gift from Dr. Jeffrey Milbrandt) as a template. The PCR primers were as follows: 5'-GGACGCCTTATATGGAGTGGCCGAATTCGGC and 3'-CGGAGCGCGTCCGCCTTAACAGACCTTATTTGGGCAGCGCCGAATTCGGCCCAATATGGCC (mSRE1), 5'-GGACGCCTTATATGGAGTGGCCGAATTCGGC and 3'-CGGAGCGCGTCCGCCTTAACAGACCTTATTTGGGCAGCGCCGAATTCGGCCCAATATGGCC (mSRE2), 5'-GGACGCCTTATATGGAGTGGCCGAATTCGGC and 3'-CGGAGCGCGTCCGCCTTAACAGACCTTATTTGGGCAGCGCCGAATTCGGCCCAATATGGCC (mSRE3), 5'-GGACGCCTTATATGGAGTGGCCGAATTCGGC and 3'-CGGAGCGCGTCCGCCTTAACAGACCTTATTTGGGCAGCGCCGAATTCGGCCCAATATGGCC (mSRE4), 5'-GGACGCCTTATATGGAGTGGCCGAATTCGGC and 3'-CGGAGCGCGTCCGCCTTAACAGACCTTATTTGGGCAGCGCCGAATTCGGCCCAATATGGCC (mSRE5), and 5'-GGACGCCTTATATGGAGTGGCCGAATTCGGC and 3'-CGGAGCGCGTCCGCCTTAACAGACCTTATTTGGGCAGCGCCGAATTCGGCCCAATATGGCC (mSRE6).

The Egr1 promoter luciferase construct p-382/mEs3 containing the mutated Es site was generated by PCR using p-382 as the template and the following primers: 5'-CCCCACCAGCTCGCTTTAACAGGTTATTTC and 3'-GCGTCCCTCCGCGCCGTCCCTCCGCTCTCCCATATATGGCC (mSRE7). Plasmids containing multiple copies of the Es/SRE element driving the luciferase gene were generated as follows. To generate pEsX2 and pEsX3 (two and three copies of the SRE/Ets motif, respectively), oligos containing the sequence of the EsX4/SRE motif were annealed and ligated into the SacI and MluI sites of the minimal Egr1 promoter luciferase construct p-4. Luciferase constructs containing four and five Es/SRE sites (pEsX4 and pEsX5) were generated by inserting an annealed DNA fragment containing an additional two copies of the Es/SRE element into the KpnI and SacI sites of the pEsX2 and pEsX3 constructs, respectively. For the construct that used the Egr1 promoter to drive green fluorescent protein (GFP) expression, base pairs –479 to +38 of the Egr1 promoter were amplified by PCR and cloned into the pMEGFP-1 vector (Clontech Laboratories, Palo Alto, CA). All the oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) and the plasmid constructs were verified by DNA sequencing.

Peptides

The hemoglobin (Hb) 64–76 peptide and altered peptide ligands for the 3.L2.12 T cell were described previously (40). The sequence of Hb64–76 peptide is GKKVITAENGLK. I72 and E72 represent the altered peptides of Hb64–76 in which the wild-type asparagine at position 72 is substituted by isoleucine or glutamic acid respectively. Peptides were purified by HPLC and their identity was confirmed by mass spectroscopy. Peptides were synthesized by the Emory University Microchemical Facility (Atlanta, GA) or were kindly provided by B. Evavold.

T cell stimulation

For T cell stimulation with peptide, 1 × 10^6 CH27 APCs in 0.5 ml of medium per well in a 24-well plate were pulsed with 100 μM peptide for 2 h at 37°C. One million 3.L2.12 T cell hybridoma cells in 0.5 ml of medium were added to the peptide-pulsed CH27 cells and incubated at 37°C for the indicated times. In experiments involving the MAPK kinase (MEK)-1 inhibitor, T cells were pretreated with either 10 μM U0126 (Promega) or DMSO for 30 min at 37°C followed by T cell activation and luciferase assay or nuclear extract isolation. To analyze GFP expression, cells were stained with PE-conjugated anti-CD4 Ab 18 h after stimulation, and GFP expression was determined by flow cytometry after gating on CD4-positive cells. For analysis of ERK activity, 5 × 10^5 3.L2.12 T cells were cultured with 5 × 10^6 CH27 cells that were preincubated with 100 μM peptide. After stimulation, CD4^+ T cells were isolated using CD4 Dynabeads and lysed on ice for at least 10 min (1% Nonidet P-40, 150 mM NaCl, 10 mM Tris (pH 7.6), 5 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Cell lysate was separated from the nuclei and beads by centrifugation at 15,000 × g for 10 min at 4°C. Lysates were analyzed by Western blotting for phosphorylated ERK and total ERK proteins using the PhosphoPlus p44/p42 MAPK Ab kit (Cell Signaling Technology, Beverly, MA).

Transfection and dual luciferase assay

Ten million 3.L2 T cells in 0.5 ml of serum-free RPMI medium were transfected with 10 μg of various Egr1 luciferase reporter constructs and 1 μg of pRL-TK (Promega) by electroporation (240 V, 950 μF) using the gene pulser system (Bio-Rad, Hercules, CA). The transfected T cells were

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3 Abbreviations used in this paper: Egr1, early growth response gene 1; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; ERK, extracellular signal-related kinase; TC, ternary complex; TCF, TC factor; SRE, serum response element; SRF, serum response factor; NGF, nerve growth factor; EGF, epidermal growth factor; GFP, green fluorescent protein; LAT, linker for activation of T cells; RLU, relative light unit; Hb, hemoglobin; SAP-1a, SRF accessory protein-1a.
transferred to 10 ml of serum-containing medium and incubated at 37°C for
~24 h, followed by T cell stimulation. After stimulation, cells were har-
vested and lysed with 200 μl of 1× passive lysis buffer (Promega). Dual
luciferase assays were performed with 20 μl of the cell lysate using the
dual-luciferase reporter assay system (Promega) on a luminometer (Turner
Designs, Sunnyvale, CA) according to the manufacturer’s instructions.
The relative luciferase activity was measured by normalizing the firefly luci-
ferase units to the Renilla luciferase units. For stable transfection, 3.1.2.12
cells were electroporated as described above, followed by plating at lim-
iting dilution and selection using G418.

Nuclear extract preparation

For nuclear extract preparation, 5 × 10⁶ 3.1.2 T cells were serum starved
for 12 h and cultured with CH27 cells that were either unpulsed or pre-
pared with 100 μM peptide in serum-free medium. After harvest, CD4⁺
T cells were isolated using CD4 Dynabeads (Dynal, Great Neck, NY) and
lysed on ice for 10 min with 500 μl of hypotonic buffer (20 mM HEPES
(pH 7.9), 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM sodium vanadate,
1 mM sodium pyrophosphate, 1 mM DTT, 0.5 mM PMSF, 0.5 μg/ml
leupeptin, 50 μg/ml antipain, and 2 μg/ml aprotinin) containing 0.2%
Non-
idet P-40. Nuclei and beads were separated from cytosolic components by
centrifugation at 15,000 × g for 20 s at 4°C. The nuclei were then lysed
with high salt buffer (20 mM HEPES (pH 7.9), 420 mM NaCl, 20% glyc-
rol, 20 mM NaF, 1 mM sodium vanadate, 1 mM sodium pyrophosphate,
1 mM DTT, 0.5 mM PMSF, 0.5 μg/ml leupeptin, 50 μg/ml antipain, and 2
μg/ml aprotinin) and incubated at 4°C for 30 min. Debris was removed by
centrifugation at 15,000 × g at 4°C for 15 min.

EMSA

For preparation of the [³²P]labeled probe used in EMSA, two complement-
ary oligonucleotides were annealed and then labeled by the Klenow fill-in
reaction using [α-³²P]dCTP. EMSAs were performed as previously de-
scribed (41). Briefly, 5 μg of nuclear extract was incubated with 0.025
pmol of [³²P]-labeled probe for 30 min at 30°C in a 20-μl reaction contain-
ing 10 mM HEPES (pH 7.9), 50 mM KCl, 5% glycerol, 0.5 mM
DTT, 1 μg poly(dI-dC) (Sigma-Aldrich, St. Louis, MO), and 5 μg BSA
(Roche, Basel, Switzerland). For competition or Ab supershift assays, the
nuclear extract was preincubated with 100-fold molar excess unlabeled
double-stranded oligonucleotide or 2 μg of Ab, respectively, for 20 min at
room temperature, followed by incubation with the radioactive probe.
Binding reactions were analyzed by electrophoresis on a 5% nondenatur-
ing polyacrylamide gel in 0.25× Tris borate EDTA buffer. SRF (G-20), Elk-1
(I-20), p-Erk-1 (B-4), SAP-1a (C-20), Ets-1 (N-276), and Ets-2 (C-20) Abs
were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The
strand sequences of the oligonucleotides used were as follows (mutated nu-
icotides are underlined): ES1, GACCGCGAAACCCCATATAAGAGCAG
CAG; mES1, GACCGCTTAACCCCATATAAGAGCAG; EmS1, GAC
CGGGAAACCCCATATAAGAGCAG; mEmS1, GACCGCTTAACCC
CAG; CGGCGCGAAACCCCATATAAGAGCAG; ES2, CGCGCGAAACCCCATATAAGAGCAG;
mES2, CCGCCGAACAGCCGATCCGGCAG; SRE1, GAAACCGCCATATAAGGAGCAG;
mSRE1, GAAACCGCCATATAAGGAGCAG; SRE2, GAAACCCATATAAGGAGCAG;
mSRE2, GAAACCGCCATATAAGGAGCAG; SRE3, GCAACCGCCATATAAGGAGCAG; mSRE3,
GCACCGGCTTAACCCCATATAAGGAGCAG; SRE4, GATGTGGGCAGCACACCCCGCTGC;
mSRE4, GATGTGGGCAGCACACCCCGCTGC; SRE5, GTCCTTCATATAGGGCTGC;
mSRE5, GTCCTTCATATAGGGCTGC; SRE6, GCTTCCTCATATAGGGCTGC; and
mSRE6, GCTTCCTATATAGGGCTGC.

Results

The Egr1 promoter is responsive to both agonist and partial agonist
tCR ligands

The Egr1 promoter contains multiple regulatory elements including
an AP-1 binding motif, an Egr binding site, and two cyclic-AMP response
elements (Fig. 1) (42). In several cell types, the SRE/ETS motifs have
been shown to be important regulatory elements for controlling
Egr1 expression. However, the elements important for Egr1
expression in response to TCR signaling have not been previously
reported. To analyze the mechanism by which agonist and partial
agonist TCR ligands can induce Egr1 expression, we generated a series of
luciferase reporter constructs containing sequences upstream of the
Egr1 transcription start site.

The activities of the various constructs were analyzed using the
3.1.2 T cell hybridoma (3.1.2.12), a cell bearing a TCR specific for
a peptide derived from residues 64 to 76 of the murine Hb β-chain
(Hb64–76) when presented by I-Ed. Numerous partial agonist ligands
have been characterized for the 3.1.2 TCR, most of them
created by substituting the P5 position of the peptide (residue 72)
In this study, we have used the I72 peptide as a partial agonist (with isoleucine at position 72 in place of the wild-type asparagine). In addition, we have also used E72 (glutamic acid at position 72) as a null peptide. The I72 peptide is a partial agonist because it can induce the cytolytic function of the 3.L2 T cell clone, without inducing IL-2 production or proliferation. When cytolyisis is used as a readout, the potency of the I72 ligand is >16,000-fold lower than Hb64–76 (40). I72 can also antagonize IL-2 production and proliferation induced by a low dose of the Hb64–76 peptide. The E72 peptide has no measurable effect on 3.L2 T cells in a variety of assays.

To determine whether the Egr1 promoter was responsive to agonist and partial agonist signals, 3.L2.12 T cells were transfected with a p-479 luciferase reporter construct (bearing base pairs −479 to +38 of the promoter). After transfection, the T cells were cultured for 8 h with the B cell lymphoma CH27 either unpulsed or prepulsed with 100 μM Hb64–76 I72, or E72 peptides. Stimulation of the T cells with APCs plus agonist peptide induced strong luciferase activity, usually a 40- to 60-fold induction compared with that of the transfected T cells alone (Fig. 1B). We also observed a small increase in luciferase activity (∼2- to 3-fold over T cells alone) when the T cells were cultured with APCs in the absence of added peptide or with E72 peptide. The partial agonist peptide I72 induced activity 6- to 7-fold over that of T cells alone and 2- to 3-fold over that of T cells stimulated with E72 or no peptide, and this activity reached a peak 8 h after stimulation (Fig. 1, B and C). The magnitude and kinetics of luciferase induction by the Egr1 promoter were similar to the induction of endogenous Egr1 mRNA in the 3.L2.12 hybridoma by Hb64–76 I72, and E72 ligands. Thus, the p-479 Egr1 promoter is responsive to both agonist and partial agonist TCR signals, but the magnitude of the response to partial agonist signaling is much lower.

The small increase in activity that we observed in T cells interacting with APCs alone probably represents a genuine induction of the Egr1 promoter. Although the increase is small, it is reproducible, dependent on ERK activity, and influenced by mutations in the Egr1 promoter (data not shown). We have also observed an increase in Egr1 mRNA in response to interactions of T cells with APCs, and this induction has both MHC-dependent and -independent components (M. Bettini and G. Kersh, manuscript in preparation). Because of the induction of Egr1 by interaction of T cells with APCs and no added peptide, we have expressed most of the luciferase data as fold induction over the activity observed in T cells alone.

SREs are critical for the response of the Egr1 promoter to agonist and partial agonist ligands

To understand why partial agonist signaling induces a much lower level of Egr1 promoter activity, we first wanted to define the elements of the Egr1 promoter required for maximum response to TCR signaling. This was first approached by making a series of constructs that had progressive deletions from the 5’ end of the promoter (Fig. 2). The longest construct (p-701) did not have any more activity than the p-479 construct, but truncations of p-479 reduced the promoter activity progressively. This suggests that all of the SRE/Ets motifs are playing a role in Egr1 promoter function in T cells, as the deletions remove each of the six SREs individually.

To address the role of the SREs more directly, we mutated each individual SRE using the p-479 construct as a template and transfected the 3.L2.12 hybridoma with the mutant constructs. As shown in Fig. 3A, mutation of each SRE reduced the promoter induction in response to Hb64–76 similarly (40–53%). These results demonstrate that each SRE makes a similar contribution to Egr1 induction in response to TCR stimulation by agonist peptide and that, for maximum induction, all six SREs are required. One possible explanation for the partial agonist peptide inducing such low levels of Egr1 is that the partial agonist is able to induce only a small amount of active transcription factors and can therefore involve only a subset of the SREs. However, when the partial agonist peptide was used to stimulate transfectants bearing the SRE mutant constructs, we also found that all six of the SREs played a similar role. Although the magnitude of the response was reduced, the mutation of each SRE reduced the activity similarly (44–53%). This result suggests that all of the SREs are required for maximum promoter activity in response to agonist or partial agonist ligands.

Previous studies of the Egr1 promoter have found that, in cell types where multiple SREs are important for induction of Egr1 by growth factors, intact adjacent Ets motifs are also critical (27, 32). To assess the role of Ets motifs in the induction of Egr1 by TCR stimulation, we generated a construct (p-382) that contained only

![FIGURE 2](http://www.jimmunol.org/)

Identification of upstream regulatory elements important for induction of Egr1 by TCR stimulation. A deletion analysis of the Egr1 promoter was performed by transfecting 3.L2.12 T cell hybridomas with the indicated constructs followed by stimulation with CH27 APCs that had been prepulsed with 100 μM Hb64–76 peptide. After 8 h of stimulation, luciferase activity was measured. The fold induction is expressed as the ratio of the RLU of stimulated T cells to that of T cells alone.
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SRE/Ets motifs are sufficient to drive luciferase expression in response to agonist and partial agonist ligands

The data described above suggest that six SREs are necessary for Egr1 promoter activity in response to agonist and partial agonist ligands. We hypothesized that multiple copies of an SRE/Ets motif may be sufficient to drive TCR-mediated expression of the luciferase reporter gene in T cells and that the magnitude of the response would depend on the number of SRE/Ets motifs present. To test this idea, we generated luciferase reporter constructs containing two, three, four, or five copies of the SRE/Ets motif linked to the minimal Egr1 promoter p-45 construct. Each copy contains the sequences representing SRE2 with the adjacent Ets4 sequence from the Egr1 promoter. As shown in Fig. 4, the minimal Egr1 promoter containing only the TATA box had little response to TCR signals. However, the responsiveness of the promoter to agonist stimulation was increased in a dose-dependent manner by addition of multiple copies of the SRE/Ets motif to the minimal promoter (Fig. 4). With five copies of this motif, the levels of TCR-dependent luciferase activity were even greater than that achieved with the p-479 Egr1 promoter construct. The response of the Egr1 promoter to partial agonist signals also increased as copies of the SRE/Ets motif were added. The response of the promoter to the I72 ligand was increased to 13.3-fold when five copies of the SRE/Ets motif were used.

The data above suggest that both agonist and partial agonist signals can generate enough active transcription factors to use all of the SRE/Ets binding sites in the Egr1 promoter. However, the amount of promoter activity induced by I72 is still much less than that induced by Hb64–76. Two possible explanations for the lower activity induced by I72 are the following: 1) the I72 ligand induces high levels of promoter activity (similar to Hb64–76) but only in a small subset of the T cells in the culture; this results in a reduced amount of luciferase activity in the population due to the production of a smaller pool of mRNA. Fewer cells respond to a partial agonist ligand, but these cells all have a reduced response

To test the possibility that only a small number of cells respond to the I72 ligand, we wanted to measure Egr1 promoter activity on a single-cell level. To do this, we made a construct that used the

FIGURE 4. Induction of Egr1 in response to TCR stimulation by increasing copies of the SRE/Ets motif. Two to five copies of SRE/Ets motif were linked to the minimal promoter reporter construct containing −45 to +38 of the Egr1 promoter. The promoter constructs were transfected into the 3.1.2.12 T cell hybridoma followed by stimulation with CH27 cells plus Hb64–76, I72, or E72 peptide. Analysis of luciferase activity was performed as described in Fig. 1B. For comparison, the promoter construct p-479 and the minimal promoter p-45 were analyzed in parallel. The data shown represent the mean ± SEM of three experiments. The fold induction is expressed as the ratio of the RLU of stimulated T cells to that of T cells alone.

FIGURE 3. Mutagenesis analysis of SREs in the Egr1 promoter. A. The six SREs in the −479 region of the Egr1 promoter were individually mutated, and the effect of the mutations on Egr1 promoter induction by Hb64–76 agonist peptide (A) or I72 partial agonist peptide (B) was analyzed by transient transfection and luciferase assay. C. Effect of mutating an Ets site on the Egr1 promoter induction. The Ets element (Ets4) adjacent to the SRE2 was mutated in the p-382 construct. The TCR-mediated induction of the Ets element (Ets5) at position −319 without deleting any of the SREs. Loss of this region resulted in 23% reduction in promoter activity. Thus, both the SREs and Ets motifs are important for Egr1 induction in response to TCR stimulation.

SRE/Ets motifs are sufficient to drive luciferase expression in response to agonist and partial agonist ligands

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To test the possibility that only a small number of cells respond to the I72 ligand, we wanted to measure Egr1 promoter activity on a single-cell level. To do this, we made a construct that used the

one Ets motif (Ets 4) upstream of SRE2, along with a version of p-382 that contained a mutated Ets 4 motif (p-382/mEts4) and analyzed the effect of the mutation on luciferase activity. Compared with p-382, mutation of the Ets 4 motif reduced the promoter activity by −49% (Fig. 3C). In addition, we also compared luciferase induction between the p-325 and p-245 deletion constructs (Fig. 2). The deletion of sequence between −325 and −245 eliminates an Ets motif (Ets5) at position −319 without deleting any of the SREs. Loss of this region resulted in 23% reduction in promoter activity. Thus, both the SREs and Ets motifs are important for Egr1 induction in response to TCR stimulation.

SRE/Ets motifs are sufficient to drive luciferase expression in response to agonist and partial agonist ligands

The data described above suggest that six SREs are necessary for Egr1 promoter activity in response to agonist and partial agonist ligands. We hypothesized that multiple copies of an SRE/Ets motif may be sufficient to drive TCR-mediated expression of the luciferase reporter gene in T cells and that the magnitude of the response would depend on the number of SRE/Ets motifs present. To test this idea, we generated luciferase reporter constructs containing two, three, four, or five copies of the SRE/Ets motif linked to the minimal Egr1 promoter p-45 construct. Each copy contains the sequences representing SRE2 with the adjacent Ets4 sequence from the Egr1 promoter. As shown in Fig. 4, the minimal Egr1 promoter containing only the TATA box had little response to TCR signals. However, the responsiveness of the promoter to agonist stimulation was increased in a dose-dependent manner by addition of multiple copies of the SRE/Ets motif to the minimal promoter (Fig. 4). With five copies of this motif, the levels of TCR-dependent luciferase activity were even greater than that achieved with the p-479 Egr1 promoter construct. The response of the Egr1 promoter to partial agonist signals also increased as copies of the SRE/Ets motif were added. The response of the promoter to the I72 ligand was increased to 13.3-fold when five copies of the SRE/Ets motif were used.

The data above suggest that both agonist and partial agonist signals can generate enough active transcription factors to use all of the SRE/Ets binding sites in the Egr1 promoter. However, the amount of promoter activity induced by I72 is still much less than that induced by Hb64–76. Two possible explanations for the lower activity induced by I72 are the following: 1) the I72 ligand induces high levels of promoter activity (similar to Hb64–76) but only in a small subset of the T cells in the culture; this results in a reduced amount of luciferase activity in the population due to the production of a smaller pool of mRNA. Fewer cells respond to a partial agonist ligand, but these cells all have a reduced response

To test the possibility that only a small number of cells respond to the I72 ligand, we wanted to measure Egr1 promoter activity on a single-cell level. To do this, we made a construct that used the
p-479 Egr1 promoter to drive the GFP gene. This construct was stably transfected into the 3.L2.12 T cell hybridoma, and several clones were analyzed for GFP expression in response to agonist and partial agonist ligands. Results obtained from a representative clone are displayed in Fig. 5. Incubation of the transfected T cells with APCs pulsed with the E72 null peptide results in more cells with a high fluorescence compared with the T cells alone (18.1% vs 9.3%), and similar results were seen after incubation of the transfected cells with APCs without the addition of exogenous peptide (data not shown). As stated above, this induction has both MHC-dependent and -independent components. Stimulation of the transfected T cells with APCs plus Hb(64–76) peptide induces green fluorescence in >30% of the T cells, and the mean fluorescence of these cells is ~900, or >4 times the fluorescence of cells responding to the APCs plus the null peptide. Stimulation with the I72 peptide results in both fewer cells responding than with Hb(64–76) stimulation (20.2%) as well as a lower amount of fluorescence in those cells that do respond (mean fluorescence intensity = 473.1). Therefore, a decreased number of responding cells is one reason that I72 induces a reduced amount of Egr1 promoter activity, but the cells that do respond all have less promoter activity than cells stimulated with agonist peptide.

We hypothesized that the lower amount of promoter activity in cells responding to partial agonist ligand was not the result of a failure to generate enough active transcription factor to bind all of the sites in the Egr1 promoter. This is supported by the data demonstrating that all six of the SRE sites are required for the promoter activity induced by I72 and by the fact that promoter activity induced by I72 increases as the number of SRE/Ets motifs are increased (Fig. 4). Therefore, we wanted to determine whether the length of time that transcription factors were induced to bind to the Egr1 promoter varied between agonist and partial agonist ligand. To accomplish this, we first determined the factors binding to the Egr1 promoter in response to TCR stimulation.

A TC that contains SRF plus Elk-1 or SAP-1a form on the Egr1 promoter in T cells

The core SRE of the c-fos promoter has been studied extensively, and it has been determined that SRF, a DNA binding protein, specifically recognizes the SRE (43). SRF, as a homodimer, can also form a TC with a member of the Ets family, with the Ets protein contacting both the SRF and an adjacent Ets motif (43). Although several studies have implicated TC formation in induction of Egr1 (27, 32), only a few of the proteins bound to the SRE/Ets motif of the Egr1 promoter have been identified. For example, binding of SRF, Elk-1, and SAP-1a to the SRE/Ets motif is associated with growth hormone induction of Egr1 in preadipocytes (27). Elk-1 and SAP-1a are two members of the Ets protein family that have the ability to participate in a TC (44, 45). However, not all cells that express Egr1 will contain Elk-1 or SAP-1a in the TC. For example, the Ets protein Fli-1 is present in the TC in myeloid cell lines stimulated with G-CSF (34).

To identify the cellular factors binding to the SRE/Ets motifs in response to TCR stimulation, an EMSA was performed using nuclear extracts from the 3.L2.12 T cell hybridoma stimulated for 15 min with CH27 APCs plus Hb(64–76) peptide. A 32P-labeled DNA fragment containing SRE2 with its adjacent Ets motif (ES2) was used as a probe. Two major complexes were apparent when the nuclear extract was incubated with the ES2 probe (Fig. 6B, lane 1). The slower migrating complex (upper arrow labeled TC) requires the ability to participate in a TC (44, 45). However, not all cells that express Egr1 will contain Elk-1 or SAP-1a in the TC. As shown in Fig. 6B, this upper complex could be completely supershifted by Abs specific for SRF (lane 6) and partially supershifted by Abs against phosphorylated Elk-1 (lane 7) and SAP-1a (lane 8). Addition of both phosphorylated Elk-1 Ab and SAP-1a Ab to the binding reaction also completely supershifted the upper complex (lane 9), indicating that the upper complex is the TC formed by an SRF homodimer associated with either phosphorylated Elk-1 or SAP-1a. Similar complexes containing Elk-1 and SAP-1a were observed in nuclear extracts from 3.L2.12 cells stimulated with CH27 APCs plus the I72 peptide (data not shown).

Detection of the faster migrating complex (lower arrow labeled TC) requires both the intact Ets and SRE sites to form. The addition of excess unlabeled oligonucleotide containing either the mutant Ets (mES2, Fig. 4B, lane 3) or the mutant SRE (EmS2, Fig. 6B, lane 4), but not the oligonucleotide in which both the Ets and the SRE sites were mutated (mEmS2, Fig. 4B, lane 5) abolished detection of the upper complex. Because Elk-1 and SAP-1a bind the Egr1 promoter in preadipocytes (27), we used Abs against these proteins to ascertain whether they were also bound to this promoter in T cells. As shown in Fig. 6B, this upper complex could be completely supershifted by Abs specific for SRF (lane 6) and partially supershifted by Abs against phosphorylated Elk-1 (lane 7) and SAP-1a (lane 8). Addition of both phosphorylated Elk-1 Ab and SAP-1a Ab to the binding reaction also completely supershifted the upper complex (lane 9), indicating that the upper complex is the TC formed by an SRF homodimer associated with either phosphorylated Elk-1 or SAP-1a. Similar complexes containing Elk-1 and SAP-1a were observed in nuclear extracts from 3.L2.12 cells stimulated with CH27 APCs plus the I72 peptide (data not shown).

Detection of the faster migrating complex (lower arrow labeled SRF, Fig. 6B) was abolished only by competitors containing the wild-type SRE motif (ES2 and mES2), but not by the competitors containing the mutant SRE (EmS2 and mEmS2). This lower complex was supershifted by SRF Ab, but not by Elk-1 or SAP-1a Ab. These results indicate that the lower complex is composed of an SRF homodimer binding to the SRE site. Neither the upper nor the lower complexes were affected by an Ab against Ets-1 (lane 11) or Ets-2 (lane 12). Similar results were obtained by EMSA using the ES1 probe containing the SRE1 with its adjacent Ets1 motif (data not shown).

To assess SRF binding to other SRE sites of the Egr1 promoter, EMSAs were performed using the labeled ES1 as the probe and the six unlabeled SREs (SRE1–SRE6) as competitors. As shown in
Fig. 6C, detection of both the TC and SRF homodimer complex were abolished by all the unlabeled wild-type SRE competitors (SRE1-4 SRE6), but not by any of the mutant SRE competitors (mSRE1-4 mSRE6), indicating that SRF can bind all six of the SREs in the Egr1 promoter.

**TC formation is dependent on ERK activation**

The formation of the TC has been shown to be constitutive in various cell types (46, 47), yet it can also be induced by growth factor stimulation in cell types such as astrocytes and preadipocytes (27, 48). Previous studies indicated that signal-induced phosphorylation of a conserved C-terminal activation domain of TCF family members is implicated in the enhancement of TCF transcriptional activity. In addition, Elk-1 and SAP-1a have both been shown to be substrates of ERK and ERK-mediated phosphorylation of Elk-1 potentiates its DNA binding and transactivation activities (49, 50). As described above, the Elk-1 found in the TC of the Egr1 promoter in stimulated T cells is phosphorylated (Fig. 6, lane 7). Therefore, we asked whether Egr1 promoter activity, Elk-1 phosphorylation, and TC formation on the Egr1 promoter are dependent on ERK activation in T cells. MAPK-inhibition experiments were performed using the potent MEK-1 inhibitor U0126, which blocks the phosphorylation and activation of ERK1 and ERK2. 3.L2.12 T cells were transfected with the Egr1 reporter construct p-479 and then cultured with CH27 APCs with or without Hb64–76 peptide in the presence of U0126 or DMSO for 8 h at 37°C. Luciferase activity was measured as described in Fig. 1B. The data represent the mean ± SEM of three experiments.
U0126. The endogenous Egr1, the induction of the Egr1 promoter by TCR stimulation was strongly abrogated by U0126 (Fig. 7A). To determine whether the participation of Elk-1 and SAP-1a in the TC on the Egr1 promoter was inducible by TCR stimulation via the activation of ERK, EMSA was performed with the nuclear extracts from 3.L2.12 T cells stimulated with CH27 APCs plus Hb64–76 peptide in the presence or absence of U0126. The ES2 DNA fragment containing the Ets4/SRE2 motif was used as the probe. A basal level of TC was observed in unstimulated T cells, and this complex contained both phospho-Elk-1 and SAP-1a (Fig. 7B, lanes 1, 4, and 7). After stimulation, TC formation was dramatically increased, and this increase was blocked by the addition of U0126, which also inhibited the TC formation on the Ets1/SRE1 motif of the Egr1 promoter (data not shown). The p38 MAPK inhibitor SB203580 (25 μM) did not have a significant effect on Egr1 induction by agonist peptide (data not shown). Thus, ERK activation is critical for both participation of phosphorylated Elk-1 and SAP-1a in the TC, and induction of Egr1 by TCR stimulation.

**TC formation induced by the partial agonist is transient**

Having identified formation of a TC containing phospho-Elk-1 or SAP-1a as a critical event for activation of transcription from the Egr1 promoter, we could then lengthen the time of TCR stimulation from 3.L2.12 T cell hybridomas with CH27 APCs plus Hb64–76, I72, or E72 peptides. Nuclear extracts were prepared at various time points and EMSAs were performed using the Ets4/SRE2 motif as a probe. The results are displayed in Fig. 8A, and an anti-phospho-Elk-1 Ab was included in all of the lanes. After stimulation with Hb64–76, an increase in supershifted phospho-Elk-1 is observed at 5 min and the amount of phospho-Elk-1 peaks at 15 min and is still elevated at 30 min of stimulation. Stimulation with I72 induces similar amounts of phospho-Elk-1 as Hb64–76 after 10 min, but levels of phospho-Elk-1 rapidly decline in the I72-stimulated cells. The levels of phospho-Elk-1 induced by I72 are most likely sufficient to achieve binding at all of the sites on the Egr1 promoter, but elevated levels are not maintained. These results suggest that partial agonist ligands induce low amounts of Egr1 promoter activity because of an inability to sustain ERK activity, resulting in a transient TC formation.

To measure ERK activity directly after stimulation with agonist and partial agonist ligands, the 3.L2.12 T cell hybridoma was stimulated with CH27 APCs plus Hb64–76, I72, or E72 peptides. Total cell lysates were obtained and analyzed for the presence of phosphorylated ERK by Western blot. As displayed in Fig. 8C, agonist ligand induced the active form of ERK over a period of at least 60 min, whereas the I72 partial agonist induced phospho-ERK for only 5–10 min.

**Discussion**

In this report, we have established that an agonist and a partial agonist ligand for the 3.L2 TCR can both activate the Egr1 promoter, with the partial agonist being 10-fold less potent. This decreased potency is due to decreased promoter activity in all of the cells responding to partial agonist and to a slight decrease in the number of responding T cells. Although the partial agonist induces low promoter activity, it still requires all six SRE sites on the promoter to achieve its level of induction. We have also demonstrated that a TC consisting of an SRF homodimer and either Elk-1 or SAP-1a forms on the SRE sites in the promoter. Formation of this TC is dependent on ERK activation, and if TC formation is blocked by a MEK-1 inhibitor, then induction of the promoter is also blocked. Examination of the TC induced by agonist and partial agonist over time reveals that the agonist ligand induces a much longer lived TC, and this is most likely the critical difference.
between agonist and partial agonist signaling that leads to the high potency of the agonist.

Partial agonist signaling can lead to cellular responses that are qualitatively different from those induced by agonist signaling. For example, agonist ligand signaling in double-positive thymocytes induces apoptosis, whereas partial agonist signaling induces positive selection and differentiation (4). Naive, mature T cells proliferate and differentiate in response to agonist, whereas in vivo, partial agonist signals lead to survival without significant proliferation or differentiation. T cells are somehow unique in that these different cellular responses are determined by signaling through a single receptor that interprets subtle differences in ligand structure. A more common scenario is for cells to undergo different cellular responses to different signals emanating from distinct receptor ligand pairs.

A classic example is the response of PC12 cells to epidermal growth factor (EGF) and nerve growth factor (NGF). The PC12 cell line is of neuronal origin and treatment of PC12 with NGF leads to differentiation: outgrowth of neurites and reduction in cell division (51). In contrast, treatment with EGF induces proliferation without differentiation in PC12 (52). It was initially thought that such distinct outcomes resulting from NGF and EGF signaling would require induction of distinct pathways by the two receptors. However, the pathways induced by the two receptors are largely overlapping, and a major difference in the signals induced by NGF vs EGF is that NGF induces a sustained activation of ERK whereas EGF stimulates a short burst of ERK activity (53). The sustained activation of ERK by NGF results in a sharp induction of Egr1 in PC12 cells, and it has been demonstrated that Egr1 activity is required for differentiation of PC12 in response to NGF (54). Although the different responses in PC12 cells are induced by distinct receptors, it is thought that sustained activation of ERK by NGF is the key signal that leads to differentiation (53).

Our data suggest that the duration of ERK activation in T cells is also an important parameter that determines the changes in gene expression induced by a TCR ligand. Previous studies have suggested that the duration of ERK activation can be dependent on the type of TCR ligand used. Chau et al. (55) demonstrated that stimulation of T cells with anti-CD3 Ab (a type of partial agonist) induced ERK activation for ~15 min, but stimulation with anti-CD3, anti-CD4 heterofunctional Abs (an agonist stimulation) activated ERK for at least 60 min. In thymocytes, Werlen et al. (56) reported that an agonist ligand could induce only transient ERK activation but that a partial agonist (that induced positive selection) could induce sustained ERK activation, the converse of what has been observed in mature T cells. This result in thymocytes highlights the fact that the nature of T cell responses to different ligands depends not only on the potency of the ligands, but also on the differentiation state of the T cell. Not only will the ability to sustain ERK activation by a particular ligand depend on the developmental stage of the cell, but the program of gene expression induced by sustained vs transient ERK activation will vary between cell types. Although the PC12 model has relevance for numerous cellular systems, the specific response to certain signals is not universal. However, we have found that Egr1 induction is important for positive selection of double-positive thymocytes, a cell type that has been shown to sustain ERK activation in response to positively selecting ligands. Similarly, PC12 cells undergo differentiation in response to sustained ERK activation, and also depend on Egr1 induction for this process. Thus, the two systems do share significant features on multiple levels.

How do some TCR ligands induce sustained TC formation, while others only induce a transient TC? A correlation has been established between TCR ligand off-rate and ligand potency, where low potency ligands have a much faster off-rate than agonist ligands (11, 57). For the 3.1.2 TCR, the Hb6a, 76/Ek ligand has a t1/2 of 10.8 s, whereas the 172/Ek ligand has a t1/2 of ~2.5 s (57). Although these measurements were done at 25°C using purified molecules, it is presumed that, on the cell surface, the partial agonist ligand will only induce short-lived complexes. Thus, ligand binding by TCR agonists is more sustained just as ERK activation is more sustained.

It is difficult to determine whether the differences in TCR-ligand half-life observed at 25°C can account for the differences in duration of ERK activation because the relationship between ligand binding and initiation of signaling has not been well established for the TCR. It is likely that some form of receptor multimerization is critical, but how this is induced by ligand binding is unclear (58). However, we can speculate that the TCR acts as an on/off switch, and that, if turned on for 2–3 s (by binding to 172/Ek), it is able to initiate enough intracellular phosphorylation to sustain ERK activation for 10–15 min, whereas if it is turned on for 10–12 s (by binding Hb6a, 76/Ek), then ERK activation can be sustained for 30–60 min. This model is clearly overly simplistic, but it provides a starting framework despite our lack of knowledge regarding the initial seconds of TCR-ligand binding on the cell membrane.

A more complex model for how different TCR ligands are able to maintain phosphorylated ERK substrates for different lengths of time can be postulated based on the differential phosphorylation of the TCR-associated ζ-chain. Partial agonist ligands with a short binding half-life induce phosphorylation on only a subset of the tyrosines in the ζ-chain (12). Completely phosphorylated ζ is thought to contribute to activation of ERK by binding and activating the ζ-associated protein of 70 kDa, which in turns phosphorylates the adapter protein linker for activation of T cells (LAT) (59). Phosphorylated LAT then serves as a docking site for several molecules including Grb-2 and the guanine nucleotide exchange factor SOS. ras is activated when brought into proximity with SOS, and activated ras initiates the classic cascade that results in ERK activation. Partial agonist signals only partially phosphorylate the ζ-chain, and as a result, are ineffective at inducing activation of ζ-associated protein of 70 kDa and LAT phosphorylation (60). Thus, it has been postulated that initiation of the ras/MAPK cascade by partial agonists involves a mechanism that does not include binding of Grb-2/SOS to phospho-LAT. One proposal is that partially phosphorylated ζ can initiate the MAPK cascade by direct binding of Grb-2/SOS to the partially phosphorylated ζ-chain (61). Alternative possibilities for ERK activation by partial agonists include the use of the GDP exchange factor rasGRP to activate the MAPK cascade (62), and ras-independent activation of ERK (63). Whatever the mechanism of ERK activation by partial agonists, it is certain to be different from the mechanism used by agonist ligands. Partial agonists may fail to sustain ERK activation because they activate it by a distinct mechanism that is inherently unable to induce prolonged activation.

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References


