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Identification of a Putative Regulator of Early T Cell Activation Genes

JENG-PYNG SHAW, PAUL J. UTZ, DAVID B. DURAND, J. JAY TOOLE, ELIZABETH ANN EMMEL, GERALD R. CRABTREE

Molecules involved in the antigen receptor–dependent regulation of early T cell activation genes were investigated with the use of functional sequences of the T cell activation-specific enhancer of interleukin-2 (IL-2). One of these sequences forms a protein complex, NFAT-1, specifically with nuclear extracts of activated T cells. This complex appeared 10 to 25 minutes before the activation of the IL-2 gene. Studies with inhibitors of protein synthesis indicated that the time of synthesis of the activator of the IL-2 gene in Jurkat T cells corresponds to the time of appearance of NFAT-1. NFAT-1, or a very similar protein, bound to functional sequences of the long terminal repeat (LTR) of the human immunodeficiency virus type 1; the LTR of this virus is known to be stimulated during early T cell activation. The binding site for this complex activated a linked promoter after transfection into antigen receptor–activated T cells but not other cell types. These characteristics suggest that NFAT-1 transmits signals initiated at the T cell antigen receptor.

Interaction of antigen with the antigen receptor of T lymphocytes initiates an ordered series of phenotypic changes resulting in cell division and immunologic function. The role of the antigen receptor in this process appears similar to that of many hormone receptors: antigen binding leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (IP_{2}) (1), generation of diacylglycerol and inositol 1,4,5-trisphosphate (IP_{3}) (2), an increase in the concentration of intracellular calcium ion (3), and phosphorylation of membrane and intracellular proteins (4). These early events appear to be mediated through or at least require the T3 molecule, which is physically associated with the antigen receptor in the Ti-T3 complex (5). A second signal is provided by macrophages that can, in part, be replaced by tumor promoters that activate protein kinase C (6). Relative little is known about how these membrane and cytoplasmic signals result in the activation of genes essential for T cell proliferation and immunologic function.

The interleukin-2 (IL-2) gene is physiologically active only in T cells that have been stimulated through the antigen receptor or its associated molecules and plays a major role in T cell proliferation (7). This induction of IL-2 appears to be mediated by a transcriptional enhancer that extends from 52 to 319 bp upstream of the IL-2 gene (8). This sequence can activate a linked promoter in response to an antibody to the antigen receptor but not to antibodies to other cell surface molecules. By deletion analysis, four functional sequences have been found within the IL-2 enhancer (9). Oligonucleotides produced to two of these sites activate a linked promoter in response to signals from the antigen receptor but not to less specific stimuli such as tumor promoters. Hence they are named antigen receptor response elements, or ARRE-1 and -2 (9). One of these functional oligonucleotides bound a protein complex, NF-IL2-E, that was present only in activated cells. We have now found that this protein appears to be restricted to activated T cells and have renamed it nuclear factor of activated T cells, or NFAT-1. The oligonucleotide to which it binds activates a linked promoter in activated T cells, and the binding activity appears just before the activation of the IL-2 gene.

The two ARREs within the IL-2 enhancer are approximately 200 bp apart and are the binding sites for different protein complexes, NFAT-1 and NF-IL2-A. Binding sites for other proteins lie between these two functional elements but are not discussed here. The sequences on the coding and noncoding strands of the NFAT-1 site (ARRE-2) that are protected from deoxyribonuclease I (DNase I) digestion by nuclear extracts of activated Jurkat cells extend from -288 to -267 and -263 to -290, respectively (Fig. 1A and B).

To investigate the tissue distribution of NFAT-1 we used a double-stranded, blunt-ended oligonucleotide corresponding to the protected nucleotides (Fig. 1A). Since DNase I protection requires virtually complete occupancy of a site and, hence, is insensitive to low levels of DNA binding proteins, nuclear extracts of various cells and tissues were examined with the gel mobility shift assay (10). NFAT-1 was not detected in nonstimulated Jurkat cells, KB cells (a HeLa derivative), Hep G2 human hepatoma cells, TEPc murine B cells, Faza rat hematoma cells, EL-4 murine T cells, or C2C12 murine myoblast cells (Fig. 1C). A faint band, approximately 1/50 as intense as the NFAT-1 band, was present in extracts of nonstimulated Jurkat cells. The nature of this band is not clear since it does not comigrate precisely with the NFAT-1 band. Because several transcriptional factors, including NF kβ, can be activated by phorbol 12-myristate 13-acetate (PMA) in a variety of cell lines (11), we examined the nuclear extracts of cells stimulated with PMA (Fig. 1B).
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Neither the nuclear extracts of these stimulated cells or the extracts of rat brain, kidney, spleen, or liver formed a complex with ARRE-2, although the α-globin CAAT-binding protein and NF-IL-2-A could be detected. The inability to detect NFAT-1 in rodent tissues is not related to an inability of the rodent factor to bind the human sequence since NFAT-1 could be readily detected in PMA- and ionomycin-stimulated EL-4 and C5V1-B cells.

To examine the function of the binding site for NFAT-1 we prepared a plasmid in which three copies of ARRE-2 were placed upstream of a truncated γ-fibrinogen promoter (12). This promoter contains a TATA box and a functional Sp-1 site and initiates transcription correctly both in vivo and in vitro when truncated (13). This construct was cotransfected into the cell lines shown in Table 1 using Rous sarcoma virus luciferase (14) to control for efficiency of transfection (15). The ARRE-2 and the SV40 constructs function with similar efficiency in stimulated Jurkat cells (Table 1). In contrast, the pSV construct (15) functioned about 50- to 100-fold better than the ARRE-2 construct in PMA- and PHA (phytohemagglutinin)-stimulated KB cells, human Raji B cells, murine L cells, and Hep G2 cells (Table 1). Thus three copies of the ARRE-2 sequence can confer T cell-specific function upon the normally uninduced fibrinogen promoter.

A role of NFAT-1 in the activation of the IL-2 gene is further suggested by a comparison of the kinetics of appearance of binding activity for the NFAT-1 protein and the activation of the IL-2 gene. Jurkat cells were stimulated with PHA and PMA at the times shown in Fig. 2, and nuclear extracts as well as whole-cell RNA were prepared. The appearance of NFAT-1 was measured as the ability of the nuclear extract to form a complex with the ARRE-2 region on low ionic strength gels (10). By 20 min after stimulation, NFAT-1 was detectable and the amount produced steadily increased for 2 hours. The level of a CAAT-binding protein for the human α-globin promoter did not change during the time course shown in Fig. 2A; therefore the changes in NFAT-1 binding were not due to degradation or a nonspecific PHA effect on nuclear DNA-binding proteins. In Jurkat cells, IL-2 mRNA, measured by ribonuclease protection (16), was first detectable between 30 and 45 min after exposure to PHA and PMA (Fig. 2, B).

Table 1. Tandem repeats of the antigen receptor response element activate expression of a linked gene only in T cells activated through the antigen receptor. Values shown are percent conversion of chloramphenicol to the acetylated form. A cotransfected RSV-luciferase plasmid (15) was used to normalize for transfection efficiency. Results represent the means of two to three determinants.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Stimulus</th>
<th>Activity</th>
</tr>
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<tbody>
<tr>
<td>Jurkat</td>
<td>Anti-Ti + TPA</td>
<td>91.6</td>
</tr>
<tr>
<td></td>
<td>TPA alone</td>
<td>75.0</td>
</tr>
<tr>
<td></td>
<td>SV40</td>
<td>60.5</td>
</tr>
<tr>
<td></td>
<td>ARRE-2</td>
<td>2.3</td>
</tr>
<tr>
<td>KB</td>
<td>PHA + TPA</td>
<td>11.6</td>
</tr>
<tr>
<td>L cells</td>
<td>Iodo + TPA</td>
<td>89.0</td>
</tr>
<tr>
<td>Raji</td>
<td>Iodo + TPA</td>
<td>9.8</td>
</tr>
<tr>
<td>Hep G2</td>
<td>PHA + TPA</td>
<td>89.1</td>
</tr>
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</table>

Fig. 2. (A) Time course of the appearance of NFAT-1 binding activity. Nuclear extracts were prepared from Jurkat cells at the indicated times in minutes after PMA and PHA addition and the gel mobility shift assay performed with a probe derived from -93 to -63 (ARRE-1) to measure NF-IL-2-A and from -255 to -285 (ARRE-2) to measure NF-AT. (B) Time course of the activation of the IL-2 gene. Jurkat cells were stimulated with PHA (5 μg/ml) and PMA (50 ng/ml), and RNA was prepared at the indicated times (shown in minutes). IL-2 mRNA was measured with a uniformly labeled RNA probe (16) derived from the IL-2 gene. The 330-bp probe was hybridized to Jurkat RNA in 60% formamide at 47°C and then digested with ribonuclease as described (16). The positions of correctly initiated IL-2 transcripts (280 bp) are indicated at the right. The appearance of NFAT-1 10 to 25 min before IL-2 mRNA is consistent with a role of NFAT-1 in IL-2 gene activation.

We investigated the requirements for NFAT-1 binding activity in Jurkat cells using ansomycin, which inhibits ribosomal subunit association and blocks 98% of protein synthesis of Jurkat cells at 100 μM within 5 min (17). When nuclear extracts from Jurkat cells were stimulated with PHA and PMA in the presence or absence of 100 μM ansomycin, we found that ansomycin inhibited the NFAT-1 binding activity by about 95% without affecting NF-IL-2-A binding (Fig. 3A). This small induction in the presence of ansomycin may represent leakage of protein synthesis or may be due to residual protein synthesis from a superin- duced mRNA. When the same extracts were examined by DNase I protection, the sequences between -263 and -290 were not protected with the uninduced extracts or extracts from Jurkat cells activated in the presence of 100 μM ansomycin. Using DBR (5,6-dichloro-1-0-ribofuranosylbenzimidazole), a rapidly acting inhibitor of RNA synthesis, we found that RNA synthesis was also essential for the appearance of NFAT-1 (18), indicating that a gene must be activated to obtain NFAT-1 binding activity. Although this gene is likely to be the one encoding NFAT-1, another possibility includes a gene required for synthesis of a protein necessary for the development of NFAT-1 binding activity.

If protein synthesis is required for NFAT-1 binding activity and NFAT-1 is required for IL-2 gene activation, then protein synthesis should be required for IL-2 gene activation. We tested this prediction by examining the requirements for IL-2 gene activation using short periods of exposure to ansomycin (less than 1 hour) to avoid many secondary effects commonly encountered when longer periods of exposure are used. As shown in Fig. 3B, ansomycin added as early as 20 min after stimulation inhibited the appearance of nearly all IL-2 mRNA (Fig. 3B). In these same mRNA samples ansomycin did not inhibit c-myc gene activation or the constitutive expression of c-myb mRNA (19, 20). These controls indicate that the failure to induce IL-2 mRNA in the ansomycin-treated samples was not due to a nonspecific effect on mRNA production. Since ansomycin at 100 μM takes only a few minutes to inhibit protein synthesis by 8% (17), these results indicate that the protein required for IL-2 gene activation first appears at or before 20 min. This result is consistent with the first appearance of NFAT-1 at 20 min (Fig. 2) and strengthens the notion that NFAT-1 is involved in IL-2 gene activation.
We examined the regulatory sequences of the long terminal repeat (LTR) of the human immunodeficiency virus type 1 (HIV-1) for a binding site for NFAT-1, since previous workers had found that the lag time until appearance of the HIV-1 mRNA exceeds 30 min but is less than 2 hours (21, 22) after activation of HIV-1-infected T cells. Thus regulation of the HIV-1 LTR appears similar to that of an early T cell activation gene. A fragment from the HIV-1 LTR extending from $-342$ to $-154$ competed for binding of NFAT-1 to the ARRE-2 sequence at a 20-fold molar ratio (Fig. 4A). The increase in binding observed with a tenfold molar excess is typical of an effective competitor and may reflect quantitatively greater binding associated with a concentration of binding sites closer to the dissociation constant of the interactions. Similar concentrations of the HIV-1 LTR did not influence complex formation with NF-IL2-A. We localized the site of this protein’s interaction within the HIV-1 LTR using DNAse I protection (Fig. 4B). With stimulated nuclear extracts, we found a region of DNase I protection between $-216$ and $-254$ and a weak footprint between $-288$ and $-303$ (Fig. 4B, lane 2). The $-216$ to $-254$ region was not seen with extracts from nonstimulated cells or from cells stimulated in the presence of ansomycin (lanes 3 and 7). When the incubation was carried out in the presence of a 100-fold excess of several fragments of the IL-2 gene, only the $-285$ to $-255$ region was an effective competitor (lanes 4 to 6). The specificity of these interactions was internally controlled by a region of protection ($-303$ to $-288$) not affected by any of the treatments. The binding site between $-216$ and $-254$ in the HIV-1 LTR was not related to a region of similarity between IL-2 at $-254$ to $-275$ and HIV-1 at $-216$ to $-254$. These results indicate that either NFAT-1 or a protein with similar biologic characteristics and interacting with similar sequences binds to the HIV-1 LTR at a region outside of the enhancer that is required for optimum activation of the viral LTR (21). More definitive evidence for a role of NFAT-1 in the activation of the IL-2 gene and the HIV-1 LTR will require purification of NFAT-1 and examination of its function in vitro.

**REFERENCES AND NOTES**


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17. M. E. Greenberg, A. L. Hermanowki, E. B. Ziff, Mol. Cell. Biol. 6, 1050 (1986); D. Durand and G. R. Crabtree, unpublished studies. Jurkat cells were treated with 100 μM aminomycin for various periods in the presence of 14C-labeled, and TCA-precipitable radioactivity was monitored.
18. NFAT-1, CRP, and NF-IL2-A were measured by the gel shift assay in Jurkat cells stimulated with PHA and TPA in the presence and absence of 100 μM DRB, which inhibits 95% of RNA synthesis within 5 minutes. While no effect of DRB was observed on NF-IL2-A and CRP the appearance of NFAT-1 was inhibited by more than 90% [B. Schgdal and I. Tamir, Biochim. Biophys. Acta 27, 2478 (1979)].
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26. We thank K. Belagaj for the oligonucleotide, K. Ullman and G. Courtois for technical advice, and N. Legre for manuscript preparation. P.J.U. is a recipient of a Stanford University Medical Scholars Fellowship. These studies were supported by NIH grants CA 39612 and HL 33943 to G.R.C. and CA 01048 to D.R.D.

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