Characterization of the Nuclear and Cytoplasmic Components of the Lymphoid-specific Nuclear Factor of Activated T Cells (NF-AT) Complex*

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The lymphoid-specific transcription complex, NF-AT, is involved in early gene activation in T cells and is assembled from a pre-existing, T cell restricted cytoplasmic factor and an inducible ubiquitous nuclear component within 30 min after activation through the antigen receptor. Recent studies have implicated the family of AP1 factors as components of the murine NF-AT complex. Evidence is provided here that the nuclear component of human NF-AT contains the phorbol ester-inducible transcription factor AP1 (Jun/Fos). We further characterize which AP1 family members can assume this role. Antisera to Fos inhibits NF-AT DNA binding as does an oligonucleotide containing a binding site for AP1. Constitutive expression in vivo of Fos, and to a lesser extent Fra-1, eliminates the requirement for phorbol 12-myristate 13-acetate (PMA) stimulation, leaving NF-AT-directed transcription responsive to calcium ionophore alone. Overexpression of c-Jun or JunD, but not JunB, also eliminates the requirement for PMA, indicating that many but not all Jun- and Fos-related proteins functionally activate NF-AT-dependent transcription in the presence of the cytoplasmic component. NF-AT DNA binding can be reconstituted in vitro using semi-purified AP1 proteins mixed with cytosol from T lymphocytes. Fos proteins are not needed for this reconstitution, and although JunB is not functional, it can participate in the NF-AT DNA binding complex. Finally, we have partially purified the cytoplasmic component of NF-AT and show by elution and renaturation from SDS-polyacrylamide gel electrophoresis gels that it has a molecular mass between 94 and 116 kDa and may have multiple differentially modified forms.

The specificity of the immune response is largely derived from the ability of the antigen receptor of T lymphocytes to rapidly induce the production of cytokines that in turn control cell fate decisions, proliferation, and differentiation of B cells, macrophages, granulocytes, and other cell types necessary for an effective immune response (1–3). A variety of evidence has implicated NF-AT1 as a specific nuclear target for the antigen receptor conveying its specificity to the early activation cytokines (4–6). Although the molecular makeup of NF-AT is at present unknown, recent studies (7) have shown that NF-AT can be reconstituted from a ubiquitous nuclear component that requires protein synthesis for induction and a T cell-specific constitutive cytoplasmic component (NF-ATc). This cytoplasmic component associates with the nucleus in response to calcium signaling in a manner that is inhibited by the immunosuppressive drugs cyclosporin A (CsA) and FK506. Like the nuclear component of NF-AT, AP1 activity can be induced in many different cell types. AP1 can contain any one of a group of related proto-oncogenes, the Jun family (8–12), which are capable of binding to specific responsive elements as homodimers (8–13) or as heterodimers with the product of the c-fos gene (13–17) or with other Fos-related proteins (18–20). The nuclear component of NF-AT can be induced with PMA, is not sensitive to CsA or FK506, and can be seen in cells of non-T cell origin such as HeLa (7) and Cos cells.2 Transcription of the proto-oncogene c-fos occurs within 30 min following treatment of T cells with PMA (21, 22) and is insensitive to CsA and FK506 (22). In light of these similarities, we have investigated whether Fos or a Fos-related antigen (Fra) might participate in the formation of the NF-AT complex.

Recent studies (23) have implicated Fos as a component of the murine NF-AT complex; however, the antisera used in this work recognizes the Fos M peptide which is essentially conserved in every Fos-related protein thus far identified including FosB, FosB2, Fra-1, and Fra-2 (18–20). The nuclear portion of the murine NF-AT complex binds to an oligonucleotide derived from the IL-2 enhancer containing a 6 of 7 base pair match with a consensus AP1 binding site and may contain one of the known Jun proteins (23).

Using a T cell line expressing the SV40 T antigen, we have characterized the nuclear and cytoplasmic components of human NF-AT. We find that any one of the Jun family members can complement T cell cytosol to form NF-AT binding activity in vitro but only c-Jun and JunD, not JunB, are able to functionally replace PMA induction of this component in vivo. Both Fos and Fra-1 can functionally replace PMA induction, and although Fos protein can participate in NF-AT DNA binding activity, it is not necessary; Jun alone can form

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1The abbreviations used are: NF-AT, nuclear factor of activated T cells; HNF-1α, hepatocyte nuclear factor-1α; PMA, phorbol 12-myristate 13-acetate; IL-2, interleukin-2; Oct-1, octamer factor-1; Fra, Fos-related antigen; CsA, cyclosporin A; Tag, T antigen; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase.
2J. Northrop and G. Crabtree, unpublished observations.
the nuclear component. We have partially purified the cytoplasmic component and find that it has a molecular mass between 94 and 116 kDa.

**EXPERIMENTAL PROCEDURES**

**Jurkat Cells Expressing SV40 T Antigen.—**The human T cell line, Jurkat, was grown in RPMI 1640 supplemented with 10% fetal bovine serum in a 5% CO₂ humidified atmosphere. A construct for expression of SV40 T antigen, RSVTag, was constructed by cloning a 2.6-kilbase pair HindIII/BamHI fragment of SV40 in place of the CAT gene in RSVCAT (24). A similar construct, RSVneo, was made by inserting the neomycin resistance gene (HindIII/BamHI fragment) from pSV2neo (25) in place of the CAT gene in RSVCAT. The RSVneo cassette was removed with NdeI/BamHI and blunted into the unique Apol site of RSVTag forming RSVTagneo. Twenty µg of this plasmid was linearized with BamHI and electroporated (240 V, 960 µF) into Cos cells and the NF-AT IacZ-positive cell line J.NFATZ.1 (26). Selections was done using G418.

Expression of T antigen in individual clones was assayed by ribonuclease protection and by immunofluorescence. Clone 15, TagC15, expresses ap-

**Components of NF-AT**

![Diagram](image-url)
Components of NF-AT

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and then bound proteins were eluted with three 150-µl aliquots of buffer B containing 80 mM KCl and 100 mM imidazole. Aliquots of the column load and flow-through (0.7% of total, 5 µl) and elutions (3.3% of total, 5 µl) were run on SDS-PAGE and blotted with the JunD-specific antisera described above. Binding of the primary antisera was detected with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (Zymed) and the enhanced chemiluminescence system (Amersham Corp.). Ni²⁺ column fractions (elution 2, 5 µl total), either alone or mixed with cytoplasmic extract (5 µg, prepared as described [7]), plus or minus competitor oligonucleotide (25 ng) were allowed to associate for 15 min in a buffer containing 10 mM Tris-HCL, pH 7.5, 40 mM NaCl, 0.5 mM EDTA, 5% glycerol, and 300 ng of poly(dI-dC) before addition of labeled oligonucleotide. Reactions were further processed as described above under gel mobility shifts.

**Denaturation and Renaturation of NF-AT**—Crude Jurkat cytosol was enriched approximately 50-fold in NF-AT activity by ammonium sulfate fractionation and ion exchange chromatography. This material (250 µg) was further fractionated by reducing SDS-PAGE. Gel slices were crushed and proteins eluted for 15 h at 25 °C in 1.5 ml of elution buffer (50 mM Tris-HCL, pH 7.9, 0.1 mM EDTA, 0.1% SDS, 5 mM DTT, 150 mM NaCl, and 40 µg/ml bovine serum albumin). After removal of gel, proteins were precipitated with 9 volumes of ice-cold acetone and recovered by centrifugation. Pellets were dissolved in 50 µl of denaturing buffer (50 mM HEPES, pH 7.5, 10% glycerol, 0.1 mM EDTA, 100 mM NaCl, 1 mM DTT, 0.03% lauryldimethylamine oxide, and 6 M guanidine HCl) and incubated 30 min at 25 °C. Samples were dialyzed against buffer B above containing 50 mM KCl and 0.1 mM EDTA in a system 100 microdialyzer (Pierce Chemical Co.). Aliquots of these samples (1 µl) and 5 µg of enriched starting material were analyzed by gel mobility shifts. NF-AT, was assayed in 10 µl of these fractions by mixing with 4 µg of nuclear extract prepared from HeLa cells stimulated for 2 h with 20 ng/ml PMA as described [7]. Gel shift conditions were as described above under “Gel Mobility Shifts.”

**RESULTS**

As shown previously [23], an antisera against the conserved M peptide region of Fos (30) inhibited NF-AT binding activity from nuclear extracts of Jurkat T cells; however, there is no inhibition by an antisera which recognizes hepatocyte nuclear factor-1α (HNF-1α), a liver-enriched transcription factor not found in T cells [27] (Fig. 1A, lanes 1–3). As expected, similar results with the anti-M peptide antisera were found using an oligonucleotide to detect AP1 binding proteins (Fig. 1A, lanes 4–6). In contrast, no inhibition is seen of Oct-1 DNA binding (lanes 7–9) or of HNF-1α DNA binding (lanes 10–12). The HNF-1α antisera induces a supershift of HNF-1α of slower mobility (lane 12) as observed previously [27]. To investigate whether an entire AP1 binding activity forms a component of NF-AT, we performed competitions with the cognate binding sites for these two factors (Fig. 1B). As expected, AP1 binding activity was effectively competed by excess AP1 oligonucleotide but not by the binding site for NF-AT or the κB2 site of the immunoglobulin κ enhancer (lanes 5–8). This implies that the NF-AT binding site does not contain a cryptic AP1 binding site. Surprisingly, while the κB2 site does not compete for the NF-AT binding activity, the AP1 site competes effectively (lanes 1–4). These results suggest that NF-AT contains an AP1 binding activity and that Fos or a Fra can participate in the formation of the nuclear component of NF-AT.

To determine if AP1 binding proteins could replace the requirement for PMA stimulation in NF-AT-directed transcription, we cotransfected either murine or rat c-fos with a reporter expression vector for rat Fra-1, which was isolated by virtue of the fact that it is immunologically cross-reactive with Fos [32], also replaces the PMA signal, although less efficiently than rat Fos. There was no effect of

![FIG. 2. Expression of Fos, Fra, and Jun proteins eliminate the PMA requirement for NF-AT-dependent transcription.](image-url)
Fig. 3. Reconstitution of NF-AT binding activity. A, Western blot of Ni²⁺-agarose affinity column fractions. Jurkat cells expressing SV40 T antigen were transfected using either HisJunD expression vector plus SVFos vector or JunD expression vector plus SVFos vector and extracts made as described under “Experimental Procedures.” Aliquots of the Ni²⁺ column flow-through (FT) and each elution were run on SDS-PAGE and blotted with JunD-specific antisera. The top panel shows the elution profile for His-tagged JunD, and the bottom panel shows profile for nontagged JunD. B, reconstitution of NF-AT. Cytoplasmic extracts from Jurkat (J) and Cos (C) cells were used as indicated. Elution 2 from A was used as indicated. HisD, elution from His-tagged JunD transfection; D, elution from nontagged JunD transfection; Bf, column elution buffer. Oligonucleotide competitors were used as shown. The labeled NF-AT oligonucleotide was used as a probe. C, AP1 binding activity in Ni²⁺-agarose affinity column elutions. These five lanes contain identical components to lanes 1-5 in B except that a labeled AP1 oligonucleotide was used as a probe. The prominent lower band in B and C above is nonspecific as it competes with both oligonucleotides. The free probe is not shown. D, nickel column eluates (5 μl total, elution 2) from extracts of Jurkat cells transfected with the indicated expression vectors were analyzed for AP1 binding activity as in C. Lane 1, JunD alone; lane 2, Fos alone; lane 3, mix of JunD and Fos; and lane 4, JunD and Fos cotransfection. Arrows indicate Jun/Jun homodimers and Jun/Fos heterodimers.

c-fos cotransfection on NFκB- or IL-2-directed transcription (Fig. 2B), indicating that Fos cannot substitute for all PMA generated signals necessary for IL-2 expression. Since Fos does not bind to an AP1 site as a monomer or homodimer (14-17), it seemed likely that the competition results in Fig. 1B could best be explained if Fos or a Fra were complexed with a Jun protein capable of binding to an AP1 site. Cotransfection of expression vectors for either c-jun or junD resulted in partial independence from the PMA signal for induction of NF-AT transcriptional activity, whereas expression of junB had no effect (Fig. 2A).

To show directly that Jun proteins are capable of participating in NF-AT DNA binding activity, we overexpressed JunD tagged with histidine and nontagged Fos in Jurkat cells which constitutively express SV40 T antigen. Extracts from these cells were passed over Ni²⁺-nitrilotriacetic acid-agarose affinity columns and eluted with imidazole. A Western blot using a JunD-specific antisera shows that the His-tagged JunD is found predominantly in the second fraction eluted from the column, whereas the column flow-through is effectively depleted (Fig. 3A, upper panel). In contrast, wild-type JunD remains in the flow-through and is not eluted off the Ni²⁺ column (Fig. 3A, lower panel). To control for nontagged proteins in the extract which elute with the His-tagged JunD, the second elution fraction from each column was used in reconstitution experiments (Fig. 3B). Although neither Jurkat cytosol nor the HisJunD elution alone contain NF-AT binding activity (lanes 1 and 2), mixing these two fractions (lane 3) resulted in regeneration of NF-AT binding activity which is specifically competed by excess NF-AT oligonucleotide but not by a nonspecific oligonucleotide (lanes 8 and 9). The wild-type JunD does not appear in the column elution and, as expected, this elution is incapable of reconstituting an NF-AT gel shift (lanes 4 and 5). Thus the ability to regenerate NF-AT DNA binding activity correlates with the presence of JunD protein. Although not shown here, we assume that the
overexpressed Fos copurifies with the HisJunD in these experiments by virtue of their strong interaction through the leucine zipper motif (17, 33-35). The presence of JunD/Fos heterodimers is shown in Fig. 3, C and D (see below). Finally, Cos cell cytosol alone or when combined with the HisJunD elution (lanes 6 and 7) fails to regenerate NF-AT binding activity. This confirms the T cell-specific nature of the cytosolic component (7). We tested the same elution fractions alone or when mixed with Jurkat cytosol for AP1 binding (Fig. 3C). The column elution from the His-tagged JunD transfection contains AP1 binding activity even in the absence of cytosol, as expected (lane 2), whereas the corresponding elution for the nontagged JunD does not (lane 4).

Two specific AP1 DNA binding activities appear in Fig. 3C, an upper sharp band and a lower more diffuse band. Expression and purification of His-tagged JunD alone gives rise to the upper band (Fig. 3D, lane 1), whereas expression of His-tagged Fos alone gives rise to no detectable AP1 binding activity (lane 2). Mixing purified HisJunD with HisFos reconstitutes the lower band (lane 3) present when Jun and Fos
are coexpressed (lane 4). We interpret these results as indicating the presence of Jun/Jun homodimers (upper band) and Jun/Fos heterodimers (lower band).

Since expression of JunD alone replaces the PMA requirement for NF-AT-directed transcription, we tested whether JunD alone could reconstitute NF-AT DNA binding in the presence of the cytoplasmic component. Addition of cytosol to JunD homodimers (Fig. 4, lanes 7–9) in reconstituted NF-AT DNA binding with the correct specificity (lanes 10–12). This result is consistent with the functional data presented above. For comparison, reconstitution using coexpressed JunD and Fos is shown in Fig. 4A, lanes 1–6. Jurkat cells do not express Fos in the absence of PMA stimulation (22); however, JunD is expressed at low levels in nonstimulated Jurkat cells and therefore is available to pair with exogenously expressed Fos. This may explain why expression of Fos or Fra-1 alone can functionally replace the PMA signal. To investigate whether Fos alone could reconstitute NF-AT DNA binding, cytosol was added to the nickel column elution fraction 1 (lanes 22–24), and consequently a large increase in the amount of reconstituted NF-AT (lanes 22–24). As shown in Fig. 3, the reconstituted NF-AT DNA binding activity correlates with the presence of immunoreactive JunD. As expected, JunD is easily detected in fractions able to reconstitute appreciable NF-AT DNA binding (Fig. 4B) with the exception of HisFos/JunD (Fig. 4B, lane 12, E2). A longer exposure of this blot shows some JunD copurifying with the HisFos (data not shown), indicating that the Western blot is yet less sensitive than the gel shift assay. Since JunB is unable to transactivate the NF-ATLuc reporter (Fig. 2A), we determined whether it could participate in the NF-AT complex. Coexpression of JunB with HisFos enhances reconstituted NF-AT DNA binding as did JunD (Fig. 4A). This indicates that JunB can participate in a nonfunctional NF-AT complex.

To begin characterization of the cytoplasmic component of NF-AT, we fractionated Jurkat cytosol enriched in NF-ATc by SDS-PAGE and renatured proteins eluted form gel slices. These fractions are shown in Fig. 5A. Using HeLa nuclear extract as a source of the nuclear component (7), we were able to reconstitute NF-AT DNA binding activity specifically from three fractions (fractions 4–6, Fig. 5B) but mostly from fraction 5 (94–116 kDa). The reconstituted complexes migrate slower in the gel with higher molecular mass fractions, and each forms a subset of the migration of the NF-AT reconstituted from the starting material (L). This may indicate the presence of multiple forms of the cytoplasmic component.

**DISCUSSION**

Based on our results, we propose that human NF-AT contains both a PMA-inducible and relatively ubiquitous AP1 binding activity, forming the nuclear component, as well as a T cell-restricted cytosolic component. Recent work (23) has implicated AP1 in the formation of the inducible murine NF-AT complex. Our results confirm this notion for the human NF-AT complex. We further show that expression of Fos, Fra, and Jun proteins can eliminate the need for PMA induction of the nuclear component and characterize which Jun proteins are functional and which are able to reconstitute NF-AT binding in the presence of the cytosolic component.

The nuclear component of human NF-AT appears to contain both a Jun family member and Fos or a Fra. Functionally, both cJun and JunD operate to replace the PMA requirement as does Fos and Fra-1. Although the effect of cJun or JunD is not nearly as pronounced as that of Fos, this may reflect known differences between the transcriptional activation potential of cJun homodimers as compared with cJun/Fos heterodimers at a 12-O-tetradecanoylphorbol-13-acetate-responsive element (15, 36). Although JunB is able to participate in the NF-AT complex, it is not able to activate an NF-AT-dependent reporter. This finding is provocative given previous studies (37, 38) showing differences in the transactivation of NF-ATc.
potential and mutual antagonism between cJun and JunB on certain 12-O-tetradecanoylphorbol-13-acetate-responsive element constructs and by reconstitution of DNA binding activity and mutual antagonism between cJun and JunB on NF-ATc.

The Fos gene family contains a number of members already shown to functionally activate NF-AT-dependent transcription with regard to the activities of various Jun and Fos family members, NF-ATc may serve to couple AP1 to a new DNA binding site where Jun and Fos contribute all transcriptional activation functions.

Antibody inhibition and functional studies implicate Fos or a Fra as a participant in the NF-AT complex. Although the native NF-AT complex includes Fos or a Fra, we have shown both functionally and by reconstitution of DNA binding activity that Fos is not necessary and that cJun and JunD are sufficient to replace the nuclear component. Expression of Fos of Fra-1 functionally activate NF-AT-dependent transcription in the presence of NF-ATc, likely because of constitutive low level expression of Jun proteins in Jurkat cells.3 The Fos gene family contains a number of members already and is likely to grow; thus it remains a formal possibility that the native NF-AT complex in Jurkat cells contains an as yet unidentified Fas, pairing with Jun.

NF-ATc not only serves to couple AP1 to the NF-AT DNA binding site but also imparts dependence on a calcium flux and, thus, sensitivity to CsA and FK506. Therefore, with regard to calcium-mediated signal transduction in T cells and immunosuppressant action, NF-ATc is a key component. We have purified this protein and shown it to have a molecular mass between 94 and 116 kDa. NF-ATc is present in more than one molecular mass range, indicating some heterogeneity in this factor. This heterogeneity is unlikely due to proteolysis during the denaturation-renaturation steps as the mobility of the reconstituted complexes all fall within the range of the native NF-AT complex isolated from the nuclei of stimulated Jurkat cells. Purification and cloning of the gene for NF-ATc will be necessary to fully answer these questions. Although the full nature of the cytosolic component of NF-AT is not known, the complex formed with AP1 acquires the specificity of NF-AT, thereby explaining how a ubiquitous transcription factor like AP1 can contribute to the biologically specific pattern of early gene activation in T cells.

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