Characterization of a New Isoform of the NFAT (Nuclear Factor of Activated T Cells) Gene Family Member NFATc

(Received for publication, April 22, 1996, and in revised form, June 6, 1996)

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The cyclosporin A (CsA)/FK506-sensitive nuclear factor of activated T cells (NFAT) plays a key role in the inducible expression of cytokine genes in T cells. Although NFAT has been recently shown to be inducible in several non-T immune cells, the NFAT gene family members characterized to date have been isolated only from T cells. To further characterize NFAT function in human B cells and to demonstrate cytokine gene specificity of NFAT proteins, we report here the isolation and characterization of a cDNA clone from the Raji B cell line. The cDNA clone encodes a new isoform, NFATc, of the NFAT gene family member NFATc (designated here as NFATc). The amino acid sequence of NFATc, β differs from that of NFATc, α in the first NH₂-terminal 29 residues and contains an additional region of 142 residues at the COOH terminus. Northern analysis using a probe encompassing a common region of both isoforms showed two mRNA species of 2.7 and 4.5 kilobase pairs, while an NFATc, β-specific probe detected only the 4.5-kilobase pair mRNA which was preferentially expressed in the spleen. Transient expression of NFATc, β was capable of activating an interleukin-2 NFAT-driven reporter gene in stimulated J urkat cells in a CsA-sensitive manner. However, NFATc, β neither bound to the κB element (an NFAT-binding site) in the tumor necrosis factor-κ promoter nor activated the tumor necrosis factor-κ promoter in cotransfection assays. These data suggest that different members or isoforms of NFAT gene family may regulate inducible expression of different cytokine genes.

Nuclear factor of activated T cells (NFAT)1 is a multicomponent transcription factor regulating expression of several cytokine genes in antigen-activated T cells and functions as a major molecular target for the immunosuppressive drugs cyclosporin A (CsA) and FK506 (reviewed in Refs. 1–3). It consists of a protein kinase C-inducible nuclear component and a pre-existing cytoplasmic component(s) whose translocation to the nucleus is dependent upon Ca²⁺ mobilization (4, 5). The protein kinase C-inducible nuclear component was found to be AP-1 proteins (6), and several members of the Fos and Jun families have been described to participate in NFAT complexes, contributing to NFAT-dependent transcription (7–10). Although an investigation of the inducible expression of the IL-2 gene led to the primary characterization of NFAT complex, functional NFAT binding sites have also been identified in the regulatory regions of other cytokine genes, including IL-3/granulocyte/macrophage colony-stimulating factor (11, 12), IL-4 (13–15), IL-5 (16), and TNF-α (17), whose expression has been shown to be CsA-sensitive.

Recently several cytoplasmic components of NFAT, collectively termed NFAT family proteins, have been cloned and characterized in murine and human T cells. They include NFATp (18), NFATc (19), NFATx/NFAT4/NFATc3 (20–22), and NFAT3 (21) and share a conserved region of ∼290 amino acids. This region exhibits DNA binding activity and shows a limited sequence similarity to the DNA binding and dimerization domains of dorsal/rel/NFκB transcription factors (reviewed in Ref. 23). Another common feature of these proteins is the presence of the repeated motifs of serine-proline residues (SPXXSPXXSPXXXX(D/E)(D/E)) in the NH₂-terminal region (20). Most (if not all) NFAT family proteins appear to undergo dephosphorylation in response to stimuli increasing intracellular Ca²⁺ concentration. It has been suggested that they are either direct or indirect substrates for the Ca²⁺/calmodulin-dependent phosphatase calcineurin which is sensitive to the immunosuppressive drugs CsA and FK506. For example, NFATp acts as an in vitro substrate of calcineurin (24, 25) and becomes dephosphorylated upon treatment with ionomycin prior to its nuclear translocation. This dephosphorylation process is blocked by CsA and FK506 (5). Moreover, phosphorylated NFATp fails to bind to the IL-2 NFAT binding site, suggesting that CsA not only affects nuclear translocation but also DNA binding ability of NFAT family proteins (26).

It has recently become clear that NFAT also plays an important role in production of cytokines by non-T cells of the immune system. Although originally described as a T cell-specific transcriptional factor, NFAT proteins, in particular NFATp, have been detected in several other hematopoietic cells including B cells (27–29), natural killer cells (30), mast cells (31), and macrophages (5) as well as in neuronal cells (32) albeit with no well defined function. We have been characterizing NFAT function in human B cells and showed that B cell NFAT complex was indistinguishable from that detected in T cells (9, 27).

Furthermore, we have recently shown that CsA leads to phosphorilation of nuclear NFATp in transformed B and T cells and inhibits its DNA binding (26). In this regard, CsA has been shown to directly inhibit B cell activation elicited by anti-Ig, SAC, or ionomycin which induce Ca²⁺ mobilization during the signaling processes (reviewed in Ref. 33). Importantly, it also prevents TNF-α production by B cells stimulated through their surface Ig receptor (34).

Furthermore, in murine T cells, a
transcription factor similar or identical to NFATp has been shown to bind to the CSA-sensitive regulatory element (κ3 element) in the TNF-α promoter (17, 35). Thus, NFAT appears to mediate CSA-sensitive transcriptional regulation of the TNF-α and other yet unidentified, activation-associated genes in B cells by similar mechanisms operational in T cells.

To further understand the structural complexity and function of NFAT, we screened a cDNA library derived from Raji B cells to isolate a unique NFAT family protein(s). Here, we report cDNA cloning and initial characterization of a new isoform, designated NFATcβ of NFAT family member NFATcα.2 This molecule exhibits a unique expression pattern and likely plays a role in the mutually exclusive expression of cytokine genes.

MATERIALS AND METHODS
cDNA Cloning—A cDNA library constructed in λgt10 vector was purchased from Clontech Inc. (Palo Alto, CA) and was derived from a human Burkitt’s lymphoma cell line Raji using both random and oligo(dT) primers. The cDNA library was screened under low stringency conditions using a radiolabeled DNA fragment corresponding to nucleotides 1-1257 in the published murine NFATp sequence (38) as a probe. The DNA probe contained NH2-terminal of the NFAT superfamily domain and its upstream sequence. This was obtained from murine NFATp cDNA clone (kindly provided by Drs. A. Rao and J. Jain) by digestion with HindIII and AccI. Positive clones were isolated by three rounds of hybridization, and inserts were subcloned into Bluescript plasmids. The clone containing the largest cDNA insert was sequenced on both strands using the dideoxynucleotide chain termination (U. S. Biochemical Corp.) and polymerase chain reaction sequencing (Peri-mega) methods.

Northern Blots—A Northern blot with poly(A)+ RNAs isolated from multiple human tissues was purchased from Clontech Inc. For the common probe of NFATcα and NFATcβ, we used a 467-base pair NsiI-Ncol fragment of a NFATcβ cDNA clone. The nucleotide sequence of this fragment is identical between NFATcα and NFATcβ, and it respresents nucleotides 383-848 in the published NFATcα DNA sequence (19) and to nucleotides 180–646 in the NFATcβ DNA of this study (Fig. 1). For the NFATcβ unique probe, we used a 418-base pair polymerase chain reaction fragment encompassing the region encoding amino acids 676–814 located downstream of the rel similarity domain of NFATcβ. The human glyceraldehyde-3-phosphate dehydrogenase cDNA probe was used to quantitate the amounts of RNA loaded in each lane. DNA probes were labeled by random priming and hybridization with 5x SSC, 0.05% SDS at room temperature, and subsequently in 0.1x SSC, 0.1% SDS at 55°C. The same filter was used for all subsequent hybridizations.

Generation of Recombinant Proteins and Purification—Escherichia coli expression plasmids for three deletion derivatives of NFATcβ proteins were constructed into pQE30 (Qiagen, CA) by subcloning the NFATcβ DNA fragments, which express recombinant proteins tagged with a hexahistidine at the NH2 terminus. Using the NFATcβ cDNA clone as template, a polymerase chain reaction fragment was generated with 5′- and 3′-primers containing restriction linker sites, BamH1 and Kpn1, respectively, and subsequently the restricted polymerase chain reaction products subcloned into pQE30 to generate the NFATcβ (amino acids 214-814) (Fig. 4) expression vector. The other two deletion mutants, NFATcβ (amino acids 214-721) and NFATcβ (amino acids 214–703) (Fig. 4), were derived from the NFATcβ (214–814) expression plasmid by blunt end ligation of the plasmid after restriction digestion with either SmaI and EcoRV or AgeI and EcoRV, respectively. Murine NFATp expression vector pNATpXS(1–297), constructed in pQE31 (Qiagen, CA), was kindly provided by Drs. A. Rao and J. Jain (36).

Proteins were expressed and purified as described previously with minor modifications (36). Expression was induced by addition of 2 mM isopropyl-β-D-thiogalactopyranoside to E. coli culture at OD600 0.7–0.9, and the cultures were harvested after 4 h incubation at 37°C. The cells were disrupted by three cycles of freeze-thawing in 8 mM urea, 5 mM 2-mercaptoethanol, 0.1 M sodium phosphate, 10 mM Tris-HCl, pH 8.0. The lysates were spun in microcentrifuge for 10 min to remove insoluble particulates. Proteins were purified from the soluble fractions of the extracts with nickel-chelate resin (Ni2+-agarose; Qagen). After washing the resin column with 10 mM imidazole in the same buffer, proteins were eluted with 100 mM imidazole. The eluates were dialyzed against a buffer (20 mM HEPES pH 7.4, 1 mM dithiothreitol, 100 mM KCl, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) containing 4 mM urea for 6 h, and overnight against an urea-free dialysis buffer. Proteins were stored in small aliquots at –70°C. Protein concentration was determined using the Bio-Rad protein assay kit with bovine serum albumin as standard. To verify the purification, 0.2 μg of each purified protein was separated in SDS-PAGE and stained with Coomassie Brilliant Blue. Purified recombinant c-Fos(1-321) and c-jun(199–334) proteins were kindly provided by Drs. T. Curran and T. Kerppola and have been described elsewhere (27).

Electrophoretic Mobility Shift Assay—Binding reactions (16 μl) were performed with 0.16 ng to 0.3 μg of purified NFATcβ and NFATp proteins, 300 ng of poly(dI)poly(dC), and 0.2–0.3 mg of radiolabeled oligonucleotides in a binding buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 1% Ficoll (M, 400,000), 0.125 mg/ml bovine serum albumin. For competition assays, 25–100-fold molar excess of unlabeled oligonucleotides were added to the binding reaction mixtures as indicated in the figure legends. Where indicated, c-Fos and c-jun proteins were included in the binding reaction mixtures to test their association with NFAT proteins. After 20-min incubation at room temperature, the reaction products were separated on a 4% non-denaturing polyacrylamide gel (27).

Oligonucleotide used in a DNA binding assay were as follows: 1) human NFAT, A 30-mer containing the distal NFAT site within the human IL-2 promoter (5′-GGAGGAAAAACTGTTTCTACAGAGGCGT-3′); 2) murine NFAT, A 33-mer containing the distal NFAT site in the human IL-2 promoter (5′-gatgGCCCCAAAGAAAGAACTTTTTTCATACAGG-3′); 3) mutant NFAT, A 30-mer containing 5 base changes at the 5′-end of the human IL-2 NFAT site (5′-GCCTCATAAAGCTTCTACAGAGGCGT-3′); 4) nonspecific oligonucleotide, A 33-mer (5′-AAGAGGAAAAATTTACCTTTTTTGCATTCA-3′); 5) AP-1, A 24-mer containing the AP-1 binding site (5′-CGCTGTGATGCTACCAAGGGA-3′); 6) x element, A 30-mer containing the NFAT-binding site within the human TNF-α promoter (5′-gatgGACGTCACTGTTTCTCCAG-3′) (35).

DNA Transfections—The full-length coding region of NFATcβ was subcloned into the expression vector containing the SRα promoter (38). The NFATp expression vector, in which NFATp expression is under the control of the SV40 promoter, was kindly provided by Drs. A. Rao and P. Hogan (Harvard Medical School). The reporter plasmid SB3.1, a kind gift from Dr. G. Crabtree (Stanford University), expresses chloramphenicol acetyltransferase driven by the SV40 promoter up to the first four codons of a minimal β-galactosidase promoter (39). The human TNF-α promoter (−164/−20)-luciferase plasmid constructed in pGL2 basic vector was kindly provided by Dr. F. de la Brousse, Tularik, Inc. (40). The pTKGH plasmid which expresses human growth hormone (hGH) under the control of the herpes simplex virus thymidine kinase promoter served as an internal control for the efficiency of transfection. Exponentially growing J urkat or Raji cells (1 × 106 cells) were transfected with 5 μg of SB3.1 or the TNF-α promoter-luciferase plasmid, 2.5 μg of pTKGH, and 15 μg of either NFATcβ or NFATp expression vector by electroporation at 240 V, 960 microfarads. At 24 h after transfection, the cells were treated with 50 nm PMA and 1.5 μM ionomycin. After 8 h of treatment, the cells were harvested, and CAT and luciferase activities were determined using the established techniques. Transfection efficiencies were normalized by hGH assays using Allegro HGH kit (Nichols Institute Diagnostics, CA).

RESULTS

Cloning of NFATcβ—To isolate a cDNA clone(s) which potentially encodes an NFAT family protein from B cells, we screened a Burkitt’s lymphoma cell line Raji cDNA library (approximately 7×106 plaques) with a cDNA probe encoding NH2-terminal and the rec similarity domain of the murine NFATp at low stringency (18). Several positive clones were isolated and divided into two different groups closely related to NFATcα (5 clones) and NFATp (10 clones), respectively, by initial analysis using restriction digestion and partial sequencing. Interestingly, one clone related to the NFATcα gene showed nucleotide sequence differences in both 5′- and 3′-end
regions, and further sequencing analysis of this clone, designated NFATc.β, revealed an open reading frame encoding a new protein of 827 amino acids with deduced molecular mass of 89.5 kDa that is closely related to NFATc.α previously reported by Northrop et al. (19) (Fig. 1). At the amino acid level, this clone displayed perfect sequence identity with NFATc.α in the region corresponding to amino acids 30–684 with the exception of 2 amino acids at positions 219(Ser) and 222(Arg) in which the corresponding amino acids of NFATc.α are Gly and Gln, respectively. The identical region includes the rel similarity domain and NH2-terminal containing the SPXXSPXXSP (D/E)(D/E) repeat motifs that are well conserved in the NFAT family proteins, suggesting that the new NFAT family protein may play a similar biological role as other NFAT proteins (Fig. 2B). On the other hand, two unique regions were found in NFATc.β. The first NH2-terminal 29 amino acids of NFATc.β differ from that of NFATc.α and are rich in acidic amino acid content (8 of 29 amino acids), suggesting that this region may constitute a transactivation domain. Another unique region of NFATc.β is represented by an additional sequence of 142 amino acids at the COOH terminus, and this region, rich in proline residues (20%), shows no significant sequence similarity to the corresponding regions of other NFAT family members (Fig. 2A). Thus, the cDNA clone isolated here appears to encode a new isoform of NFATc.α that most likely resulted from alternative splicing and/or transcription from separate promoters.

Expression Pattern of NFATc.β mRNA in Human Tissues—Previous reports have shown that Northern analysis of NFATc using 5′-end fragments of NFATc cDNA as a probe displayed two hybridizing bands with apparent sizes of 2.7 and 4.5 kb (20, 21). To examine whether the new clone NFATc.β can be specifically assigned to one of these two bands, and to determine the tissue distribution of NFATc.β expression, we carried out Northern analysis of multiple human tissues using two separate probes. As expected, when a common sequence of NFATc.α and NFATc.β was used as a probe, two mRNA species with previously observed sizes were detected (Fig. 3). However, an NFATc.β unique probe corresponding to the region downstream from the rel similarity domain detected only the 4.5-kb band, indicating that this band represents NFATc.β mRNA. The 2.7-kb mRNA, on the other hand, appears to encode NFATc.α (19), as assessed by the reported cDNA size and its tissue distribution.

Sequence-specific DNA Binding of NFATc.β to the IL-2 NFAT Site—To characterize biochemical properties of NFATc.β, we generated three recombinant derivatives of NFATc.β that were tagged with a hexahistidine at the NH2 terminus. NFATc.β (214–814) contains 601 amino acids corresponding to residues 214 to 814 which include the rel similarity domain (290 amino acids) and most residues at the COOH terminus of NFATc.β. The other two polypeptides were derived from NFATc.β (214–814) by further truncation of the COOH terminus as shown in Fig. 4A. The NFATc.β (214–703) polypeptide is devoid of most COOH-terminal residues of NFATc.β, and thus resembles NFATc.α. A deletion derivative of NFATp, NFATpX5(1–297), which contains the rel similarity domain of NFATp (36), was also purified and used as a positive control in DNA binding assays. The deletion proteins were expressed in E. coli and purified by Ni-chelate affinity chromatography. The
Fos/Jun heterodimers to participate in the cooperative assembly with Fos/Jun and Jun/Jun dimers indicated that the ability of Fos/Jun heterodimers than that of Jun/Jun homodimers. Comparision of band intensities between NFAT complexes formed across a wide concentration range (Fig. 5). NFAT complex formation in the NFATc. family members. Rel similarity domains are indicated, and the SP repeat motifs are represented by small open rectangles. Amino acid sequence of NFATc.β is identical to that of NFATc.α from NH2 terminus to the Rel similarity domain, except for the first 29 amino acids, as indicated by solid bars.

Fig. 2. Structural similarities and dissimilarities in NFAT family members. A, amino acid composition of NFATc.β unique regions is compared with the corresponding regions of NFATc.α. Amino acid positions where sequence divergence starts and ends are indicated. B, schematic comparison of the NFAT family members. Rel similarity domains are indicated, and the SP repeat motifs are represented by small open rectangles. Amino acid positions where sequence diversion starts and ends are indicated.

same amounts (2 μg) of purified proteins were separated on SDS-PAGE and stained with Coomassie Brilliant Blue. As shown in Fig. 4B, the protein preparations exhibited highly purified forms although there was evidence of partial degradation in the NFATc.β(214–814) recombinant protein.

Using the purified NFATc.β(214–814), we tested sequence-specific DNA binding of NFATc.β to the distal NFAT site in the human IL-2 promoter. NFATc.β was unable to directly bind to the human IL-2 NFAT site (Fig. 4C, lane 1), but in the presence of Fos and Jun proteins, could form an NFAT complex (lane 3), consistent with previous observations of NFAT complex formation in T and B cells (9). This binding was sequence specific because it was competed away with excess unlabeled NFAT oligonucleotides, but not with nonspecific or mutant NFAT oligonucleotides. A diffuse band pattern is likely due to partial degradation of the purified NFATc.β(214–814) protein since the smaller polypeptide NFATc.β(214–703) which showed less degradation did not show a diffuse band pattern (Fig. 7, lanes 7 and 8).

Requirement of Fos and Jun for in Vitro NFAT Complex Assembly—We have previously shown that both Fos and Jun were required for efficient in vitro reconstitution of NFAT complex with pre-existing NFAT components in unstimulated B and T cells (9). To examine whether NFATc.β exhibits similar properties in cooperative binding with Fos and Jun, we compared the abilities of Fos/jun heterodimers and jun/jun homodimers to reconstitute an NFAT complex with NFATc.β across a wide concentration range (Fig. 5). NFAT complex assembly was efficiently detected at lower concentrations of Fos/jun heterodimers than that of jun/jun homodimers. Comparison of band intensities between NFAT complexes formed with Fos/jun and jun/jun dimers indicated that the ability of Fos/jun heterodimers to participate in the cooperative assembly of NFAT complexes was higher by at least 10-fold (data not shown).

CsA-sensitive Activation of IL-2 NFAT Site-dependent Transcription by NFATc.β—Since NFATc.β was capable of binding to the IL-2 promoter NFAT site, we investigated its transactivation potential in transient transfection assays. An NFATc.β, NFATp, or a control expression vector was cotransfected into J urkat cells with a reporter gene (CAT) construct containing three IL-2 NFAT sites upstream of a minimal G3PDH promoter, and the ability to activate NFAT-dependent transcription was tested with various combinations of PMA and ionomycin treatments (Fig. 6). In control transfection, treatment of the cells with PMA plus ionomycin strongly induced CAT expression by endogenous NFAT activity in J urkat cells, and the induction was inhibited by CsA. Transfection of the NFATc.β expression vector resulted in approximately 2-fold additional induction of CAT activity, and more importantly, the induction was also completely blocked by CsA. Treatment of the cells with either PMA or ionomycin alone failed to activate the IL-2 NFAT-dependent transcription. NFATp transfection, used here as a positive control, also led to additional induction of CAT activity by approximately 2.3-fold. Thus, these results indicate that transcriptional activation of the IL-2 NFAT/CAT reporter construct by NFATc.β in J urkat cells was strictly regulated by the mechanisms which control endogenous NFAT activity, and that NFATc.β was also sensitive to CsA.

Lack of DNA Binding to the TNF-α κ3 Element and Activation of the TNF-α Promoter by NFATc.β—It has been well established that TNF-α expression was induced in primary B cells as well as in numerous B cell lines by various cellular stimuli (34, 41, 42). Rapid induction of TNF-α transcription by anti-Ig was blocked by CsA and FK506 in B cells (34). The κ3 element of the TNF-α promoter is required for its transcriptional activation. Since NFATc.β was capable of binding to the TNF-α κ3 element, we investigated whether NFATc.β could activate transcription of the TNF-α promoter in the absence of PMA and ionomycin. A Northern blot with 2 μg of poly(A)+ RNA from different human tissues was hybridized with a probe derived from a common sequence of NFATc.α and NFATc.β (top panel). After hybridization, the same blot was rehybridized with an NFATc.β unique probe (middle panel), and subsequently with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe to quantify amounts of RNA loaded per lane (bottom panel). The positions of size markers are indicated on the left.
element in the TNF-α promoter was shown to be responsible for the CSA-sensitive induction of this gene, and NFATp was shown to bind to this element without AP-1 proteins, suggesting that NFATp is involved in TNF-α production (17, 35, 43). Since the NFATcβ rel similarity domain is closely related to that of NFATp and was cloned from B cells, we tested whether it could bind to the κ3 element (Fig. 7). Consistent with previous results (35), NFATp binding to the κ3 element gave rise to two strong NFATp-DNA complexes; binding of one or two NFATp molecules is thought to give rise to lower and upper complexes, respectively (top panel, lanes 10–12). Significantly, all three recombinant derivatives of NFATcβ were unable to bind to the same site (lanes 1–9). However, both NFATp and NFATcβ recombinant proteins showed relatively comparable binding affinities to the human IL-2 NFAT site (bottom panel). A wide range of poly(dI-dC) concentrations (0–1 μg/reaction) did not change binding specificities of the recombinant proteins to the κ3 element (data not shown), indicating that inability of NFATcβ deletion derivatives to bind to this site is not due to a high concentration of nonspecific competitor poly(dI-dC) used in this experiment. Thus, these results demonstrate that NFATcβ and NFATp exhibit differences in binding specificities to the TNF-α κ3 element.

To further confirm whether NFATcβ is indeed non-functional in vivo activation of the TNF-α promoter, we cotransfected either NFATcβ or NFATp expression plasmid with a TNF-α promoter-luciferase reporter plasmid into Jurkat T and Raji B cells, and tested their ability to activate the TNF-α promoter (Fig. 8). In control transfection of Jurkat T cells, luciferase activity was induced by about 25-fold upon stimulation with PMA plus ionomycin, and the induction was inhibited by CsA (Fig. 8A), indicating that activation of the TNF-α promoter is sensitive to CsA as previously shown (17). Cotransfection with an NFATp expression plasmid further increased luciferase activity up to 150-fold in a CsA-sensitive manner. However, NFATcβ transfection did not show any additional induction of luciferase activity (Fig. 8A). Similar results were observed in transient transfection assays with Raji B cells (Fig. 8B). Treatment of Raji cells with PMA and ionomycin led to 2-fold induction of TNF-α promoter-driven luciferase activity in the control transfection. It is important to point out that the low levels of net induction of luciferase activity by stimulation appears to be due to strong constitutive activity of the TNF-α promoter in Raji cells. In the absence of stimulation, TNF-α promoter-driven luciferase activity in Raji cells was higher by approximately 7-fold than that of Jurkat T cells (data not shown). These data agree with the observations of Goldfeld et al. (42) who showed that TNF-α mRNA was constitutively expressed in Raji cells which could be further enhanced by PMA treatment of cells. NFATp expression increased luciferase activity about 5-fold without stimulation, and simulation with PMA and ionomycin resulted in additional induction of luciferase activity which was blocked by CsA. Like Jurkat T cells, NFATcβ was unable to activate the TNF-α promoter in Raji B cells. It is unlikely that lack of additional induction of luciferase activity described under “Materials and Methods.” Purified proteins (2 μg) were separated on a 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue. Lane 1 contains molecular weight markers. The molecular sizes of the markers (kDa) are indicated on the left. C, electrophoretic mobility shift assay was performed with 0.1 μg of purified NFATcβ(214–814) with or without 100 nM of c-Fos/c-Jun heterodimers and a radiolabeled oligonucleotide corresponding to the distal NFAT site in the human IL-2 promoter. To assess binding specificity, unlabeled oligonucleotides bearing nonspecific sequence (NS), the NFAT site (NFAT), or a mutant NFAT site (mNFAT) were added to the assays as competitors at a 25-fold molar excess. Arrow indicates a reconstituted NFAT complex.
transfection of the NFATc.

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expression was confirmed by immunoblotting using antibodies raised against a common sequence of NFATc.a and NFATc.b (data not shown). Furthermore, transfection of the same expression plasmid activated IL-2 NFAT-dependent transcription in Jurkat T cells (Fig. 6). Taken together, these data indicate that NFATc.b is not involved in transcriptional induction of the TNF-α gene, and further suggest that distinct members or isoforms of NFAT gene family may regulate different NFAT cis-acting elements located in the promoters of cytokine genes.

DISCUSSION

Several studies have demonstrated existence of a single or multiple NFAT binding sites in the regulatory regions of several cytokine genes including IL-2, IL-3, IL-4, IL-5, granulocyte/macrophage colony-stimulating factor, and TNF-α (11–17; reviewed in Ref. 2). These cytokines are distinctly expressed by both Th1 and Th2 cells (15), and non-T immune cells also produce some of these cytokines in a CsA-sensitive manner (30, 31, 34). Several structurally related NFAT family proteins have recently been identified and characterized. This raises an intriguing question regarding the roles of multiple NFAT proteins in the expression of cytokine genes. In the present study, we report the existence of a new NFAT protein which might be a product of alternative splicing and/or differential promoter usage in the gene for the previously identified member NFATc (NFATc.a). Furthermore, our study provides evidence that this new NFAT protein does not participate in transcriptional induction of TNF-α, while it still activates the IL-2 NFAT-mediated transcription in a CsA-sensitive manner.

The new isoform NFATc.b resembles NFATc.a in that it has an identical rel similarity region and its entire NH2-terminal region except the first 29 amino acids which exhibit a high acidic amino acid content. On the other hand, the corresponding region of NFATc.a contains only 3 acidic amino acids out of 42 residues, and displays no significant sequence similarity to the NFATc.b NH2 terminus. In this regard, a deletion mutant of NFATc.a containing the NH2-terminal region (amino acids 1 to 418 upstream of rel similarity domain) has been previously shown to display a dominant negative effect on the IL-2 NFAT-
dependent transcription (19). Thus, it will be interesting to elucidate whether the 29-amino acid substitution with high acidic content will impart any functional differences.

Another distinct region of NFATc.β lies in the additional 142 amino acids downstream from the rel similarity domain which is missing in NFATc.α. A comparison of primary sequences of all NFAT family members except NFATc.α reveals that they form a tripartite structure consisting of the well conserved rel similarity domain, the moderately conserved NH2 terminus, and the COOH terminus with no significant sequence homology (Fig. 2B). Thus, NFATc.β appears to resemble more closely other members of the NFAT family in its primary structure than NFATc.α. The rel similarity domain is sufficient for DNA binding and cooperative interactions with AP-1 proteins (21, 36), and the NH2 terminus containing the conserved SP repeat motifs is thought to be involved in other common function(s) such as transcription activation and/or regulation of nuclear translocation (21). In contrast, the significance of COOH-terminal regions of these molecules remains poorly understood. The extended COOH-terminal region does not display any significant sequence similarity among the family members, and we could not discern any significant differences in DNA binding abilities of NFATc.β deletion derivatives in this region at least to NFAT-binding sites in the IL-2 and TNF-α promoters (Fig. 5). However, it is possible that this region may be involved in regulation of activity of NFAT proteins by interacting with other transcription factors or by influencing half-life of NFAT proteins. Further experiments will delineate the exact function of this region.

The two isoforms, NFATc.α and NFATc.β, could also be distinguished by their respective mRNAs. Northern blots containing poly(A)+ RNA from numerous human tissues always showed two mRNA species with the sizes of 2.7 and 4.5 kb when hybridized with a probe derived from common sequences of NFATc.α and NFATc.β. On the other hand, an NFATc.β-unique probe detected only the 4.5-kb mRNA (Fig. 3). Interestingly, the expression profile of both mRNAs in multiple human tissues was distinct. Although both mRNAs were expressed in various tissues, NFATc.α mRNA was predominantly expressed in the thymus and peripheral leukocytes enriched in T cells, whereas NFATc.β mRNA was a major species in the spleen and non-immune tissues. Since splenic cells consist of enriched B cell population, it is tempting to speculate that B cells are a major contributor to NFATc.β mRNA in the spleen. Failure to detect NFATc.β mRNA in peripheral leukocytes may be due to a low number of B cells (7–10%) contributing to a poor hybridizing signal. Further experiments to examine cell type-specific NFATc.β expression are currently under way.

Significantly, our present data demonstrate that NFATc.β was unable to bind to the TNF-α k3 element to which NFATp strongly bound (Fig. 7). Furthermore, transient transfection assays indicate that NFATc.β indeed was non-functional in vivo activation of the TNF-α promoter (Fig. 8). Thus, these findings strongly suggest that distinct NFAT proteins may play a role in regulation of different cytokine gene expression, although there is some functional redundancy in transcriptional activation of a certain cytokine gene such as IL-2. In fact, NFAT sites in the regulatory regions of different cytokine genes display some divergence in their sequence and properties to interact with other transcription factors (reviewed in Ref. 2). Many NFAT protein-binding sites are flanked by a cryptic AP-1 site, while some others such as those in the IL-4 and TNF-α promoters are adjacent to binding sites for other transcription factors such as Oct and ATF/Jun, respectively (13, 44). Particularly, TNF-α k3 element is considerably divergent in sequence from other NFAT sites and resembles an NF-κB site. This site is occupied by two molecules of NFATp that appear to form a functional dimer for the transcriptional activation of the TNF-α gene (35). Moreover, NFATp molecules bound to the k3 element have been shown to functionally cooperate with ATF/Jun proteins in immediate early induction of TNF-α gene transcription (44). Therefore, mutually exclusive regulation of cell type-specific lymphokine production may involve recruitment of distinct NFAT family members as well as sequential or combinatorial participation of different NFAT proteins and other transcription factors.

Acknowledgments—We thank Dr. Abby Maizel for thoughtful reading of the manuscript and for the continued support; Drs. Anjana Rao, Patrick Hogan, and Jugu Nain for providing NFATp reagents and a TNF-α promoter-luciferase construct; Drs. Tom Curran and Tom Kerppola for providing recombinant Fos and Jun proteins; Dr. Gerald Crabtree for providing an NFAT-CAT plasmid; Dr. Timothy Hoey for
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