Multiple NF-ATc Isoforms with Individual Transcriptional Properties Are Synthesized in T Lymphocytes

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*J Immunol* 1999; 162:7294-7301; 
http://www.jimmunol.org/content/162/12/7294

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Multiple NF-ATc Isoforms with Individual Transcriptional Properties Are Synthesized in T Lymphocytes

Sergei Chuvpilo,* Andris Avots,* Friederike Berberich-Siebelt,* Judith Glöckner,* Christian Fischer,* Andreas Kerstan,* Cornelia Escher,* Inna Inashkina,† Falk Hlubek,‡ Eriks Jankevics,§ Thomas Brabletz,† and Edgar Serfling‡

The transcription factor NF-ATc that controls gene expression in T lymphocytes and embryonic cardiac cells is expressed in three prominent isoforms. This is due to alternative splice/polyadenylation events that lead to the predominant synthesis of two long isoforms in naive T cells and a shorter NF-ATc isoform in effector T cells. Whereas the previously described isoform NF-ATc/A contains a relatively short C terminus, the longer isoforms, B and C, span extra C-terminal peptides of 128 and 246 aa, respectively. We show here that in addition to the strong N-terminal trans-activation domain, TAD-A, which is common to all three NF-ATc isoforms, NF-ATc/C contains a second trans-activation domain, TAD-B, in its C-terminal peptide. Various stimuli of T cells that induce the activity of TAD-A also enhance the activity of TAD-B, but, unlike TAD-A, TAD-B remains unphosphorylated by protein from 12-O-tetradecanoyl 12-phorbol 13-acetate-stimulated T cells. The shorter C-terminal peptide of isoform NF-ATc/B exerts a suppressive transcriptional effect. These properties of NF-ATc/B and -C might be of importance for gene regulation in naive T lymphocytes in which NF-ATc/B and -C are predominantly synthesized. The Journal of Immunology, 1999, 162: 7294–7301.
Like other NF-AT factors, NF-ATc is expressed in multiple isoforms (17). This is due to alternate splice/polyadenylation events leading to the predominant synthesis of the longer NF-ATc isoforms, B and C, in naive T cells and the shorter isoform, A, in effector T cells. Isoforms B and C mainly differ from isoform A in the length of their C termini. While isoform B spans an additional stretch of 128 aa, isoform C contains this peptide and an extra stretch of 118 aa (17). We will show here that the 246-aa C-terminal peptide of isoform C spans a trans-activation domain, TAD-B, which along with the common N-terminal TAD-A controls the transcriptional activity of NF-ATc/C. In contrast, the C-terminal peptide in NF-ATc/B appears to act like a transcriptional repressor. We assume that these properties of NF-ATc isoforms B and C modulate their transcriptional activity in naive and other T cells where they are expressed in high concentrations.

Materials and Methods

Cells, DNA transfections, and ELISAs

All lymphoid cells were grown to a density of 2 × 10^7 cells/ml in RPMI medium containing 5% FCS. In transient transfections of murine El4 T cells, PLC-e1, PAA, polyacrylamide; CsA, cyclosporin A. Rous sarcoma virus; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; PAA, polyacrylamide; CsA, cyclosporin A.

RNase protection assays

For RNase protection assays, total RNA was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD). The RNA was processed according to Pharningen’s RiboQuant protocol (San Diego, CA), using the human cytokine hCK-1 multiprobe template set.

Western blot assays and preparation of nuclear proteins

In Western blot assays, proteins were separated on 8 or 10% PAA SDS gels from electroblot transfer onto BA 85 nitrocellulose membranes (Schleicher & Schuell, Keene, NH) overnight and immunodetection with the NF-ATc-specific mAb 7A6 (19), designated mAb-A, or a polyclonal Ab, pAb-B, raised against the extra C-terminal peptide in NF-ATc/B (17).

Nuclear proteins from lymphoid cells were prepared as previously described (20), except that Nonidet P-40 was omitted for the preservation of cell nuclei. The swollen cells were disrupted by passing them 10 times through an injection needle (26 gauge, 0.375 in.). After centrifugation, the nuclear pellet was washed three times with large volumes of swelling buffer. Immunodetections were performed using the enhanced chemiluminescence detection system (Amersham, Aylesbury, U.K.) according to the instructions of the manufacturer.

Gal4 fusion proteins and trans-activation assays

For determination of the trans-activating properties of NF-ATc peptides, expression vectors for the overexpression of Gal4/NF-ATc fusion proteins were constructed on the basis of the RSV-LTR-controlled Gal4 vector pABgal linker (21) encoding the 1–147 aa of the yeast transcription factor Gal4, which contains the DNA binding and dimerization domains of Gal4. The following vectors were constructed: Gal4/TAD-A containing 1–205 aa from NF-ATc/A, and a PCR product encoding the first 301 aa of NF-ATc, cut by NcoI and cloned as a blunt end fragment into the filled BamHI site of the vector. DNA in these plasmids was verified by restriction mapping. In a similar way pGal4/TAD-B constructs were obtained by cloning blunt end PCR products from NF-ATc/B into the filled BamHI site of pABgal linker plasmid, resulting in the constructs Gal4/TAD-B containing aa 99–930, Gal4/TAD-B_130 (aa 690–912), Gal4/TAD-B_130 (aa 690–912), Gal4/TAD-B_130 (aa 723–812), and Gal4/TAD-B_130 (aa 813–930). The structures of all plasmids were verified by sequencing. All DNA work was performed using enzymes from MBI Fermentas (Vilnius, Lithuania). Transient expression levels of Gal4/NF-ATc fusion proteins in 293 and El4 cells were determined in EMSAs using a Gal4 binding site as a probe (22).

To test the transacting properties of C-terminal peptides in yeast cells, the entire 3′ DNA fragment of NF-ATc/C and subfragments were inserted into the construct pAS2-1 (Matchmaker two-hybrid system 2, Clontech, Palo Alto, CA). Three separate yeast colonies (of strain Y190) transformed with the DNA constructs were used to inoculate 5 ml of YPD medium (Clontech). After overnight culture at 30°C, 2-ml portions were used to inoculate 5 ml of YPD medium (Clontech), and cells were grown for 3–5 h to an OD_600 of 0.5–0.8. After centrifugation of 1.5 ml, the pellet yeast cells were washed and resuspended in 300 μl in buffer Z (100 mM Na_2 HPO_4 , 40 mM NaH_2 PO_4 , 10 mM KCl, and 1 mM MgSO_4 pH 7). One hundred microliters of cells were lysed by freezing in liquid nitrogen and thawing. Following incubation at 30°C for 30 min, the supernatants were used for the OD_600 measurements. The β-galactosidase units were calculated according to the formula: β-galactosidase units = 1000 × OD_600 × t × V × OD_600, where t is the elapsed time of incubation in minutes, V is 0.1 ml × concentration factor, and OD_600 is A_600 of 1 ml of culture.
Results and Discussion

Lymphokine synthesis and the nuclear occurrence of NF-ATc isoforms in human T lymphocytes

IL-2 is the first lymphokine synthesized after stimulation of naive T lymphocytes from human CB (23). This is shown in Fig. 1, A and B, where the synthesis of IL-2 RNA and secretion of IL-2 were detected after induction of CB T cells by TPA and ionomycin (T+I) for 6 and 12 h, respectively. Apart from IFN-γ and minute amounts of IL-5, no further lymphokine RNAs were synthesized under these conditions. In contrast, treatment of resting human peripheral blood T lymphocytes with T+I led to a rapid synthesis of numerous lymphokine RNAs. In addition to the massive synthesis of IL-2 and IFN-γ RNAs the synthesis of Th2-type lymphokines IL-4, IL-5, and IL-10 was detected (Fig. 1A, lanes 4 –7). A similar, albeit much weaker, synthesis of lymphokine RNAs was also observed after stimulation by αCD3 and CD28 Abs (Fig. 1A, lanes 8 –10).

To correlate the induction of lymphokine synthesis with the appearance of nuclear NF-ATc, we performed Western blot assays using nuclear proteins from CB and PBL T cells and a mAb raised against NF-ATc for immunodetection. As shown in Fig. 1C, untreated CB and PBL T cells did not contain any detectable nuclear NF-ATc. Stimulation of CB T cells with T+I led to the nuclear appearance of NF-ATc isoforms B and C after 6 –12 h, whereas the same treatment of PBL T cells resulted in the detection of these large NF-ATc proteins after 2 h. After 6 h, NF-ATc isoform A of about 90 kDa became detectable. A moderate nuclear accumulation of NF-ATc proteins was observed when PBL T cells were treated with αCD3-CD28 Abs. Thus, the onset and extent of lymphokine synthesis and secretion correlate well with the nuclear appearance of NF-ATc proteins. However, while the concentrations of lymphokine RNAs decreased in PBL T cells after stimulation for 6 h, the concentrations of nuclear NF-ATc proteins increased, suggesting a role for NF-ATc in the control of promoters in addition to those of lymphokine genes.

The extra C-terminal peptides of NF-ATc isoform C contain an additional trans-activation domain

We have shown previously that in numerous types of T cells NF-ATc is expressed in the three isoforms, A, B, and C, which differ...
in the length of their C termini (17). While these investigations revealed the mode of NF-ATc isoform synthesis, they did not provide information on the function of extra C-terminal peptides. The schematic structure of the NF-ATc isoforms A and C is shown in Fig. 2A. Sequence comparison between the extra C-terminal peptides in NF-ATc/A and C with those of NF-ATp (isoform NF-AT1 C) (24). Identical residues between the C-terminal peptides in NF-ATc and NF-AT1 C (NF-ATp) are indicated by long vertical dashes; similar residues are indicated by short vertical dashes. Gaps are indicated by horizontal dashes. Note the identical decapeptide near the C termini of NF-ATc/C and NF-AT1 C, which is boxed. The peptides encoded by the various portions of TAD-B are indicated. C, induction of TAD-A and TAD-B in EL4 cells. One microgram of DNA of Gal4 expression vectors encoding TAD-A, TAD-B, TAD-B90, TAD-B120, or TAD-Bpro was transfected into EL4 cells, along with an Ela promoter luciferase construct driven by four Gal4 binding sites. Twenty hours later the cells were divided. One batch of cells was left uninduced, and one batch was induced by TPA for 20 h. The EMSAs shown in the insert were performed with nuclear proteins from EL4 cells transfected with the corresponding Gal4 vectors and induced for 3 h with TPA using a Gal4 DNA binding site as probe. One representative experiment from more than three transfections is shown. D, Induction of trans-acting domains TAD-A and TAD-B in 293 cells. The EMSAs of insert were performed as described in C using proteins from 293 cells transfected with Gal4 constructs. E, Activity of TAD-B in yeast cells. DNA segments encoding TAD-B, the TAD-B fragments, or the TAD of E12 were cloned in the yeast Gal4 vector pAS2–1 (Clontech) and used for transformation of yeast strain Y190. The activity of TAD-B proteins as determined in a quantitative lacZ (O-nitrophenyl β-D-galactopyranoside) assay is compared with the TAD-2 of human transcription factor E12 (spanning the C-terminal aa 378–654).
DNA segment encoding all 246 C-terminal amino acids of NF-ATc/C and several subfragments of them into a eukaryotic expression vector encoding the DNA binding and dimerization domains of the yeast transcription factor Gal4. The activity of chimeric Gal4/NF-ATc proteins expressed from these constructs was tested by cotransfection with a luciferase reporter gene bearing four Gal4 binding sites. In EL4 T lymphoma cells transfection resulted in a pronounced inducible activity of TAD-B, amounting to approximately 15% that of TAD-A, i.e., the strong N-terminal trans-activation domain spanning the amino acid residues from 113–205 in all NF-ATc isoforms (Fig. 2, A and C). The shorter C-terminal peptides, in particular TAD-B30 and TAD-B120, showed a measurable, albeit weak, activity in EL4 cells. In contrast, TAD-B90 was completely inactive (Fig. 2C). Similar results were obtained using 293 cells. While in these cells, TAD-B was as active as in EL4 cells; all shorter peptides were found to be uninducible and suppressed the weak activity of Gal4_{1–141} peptide (Fig. 2D).

We also tested the transcriptional activity of C-terminal peptides in yeast cells using a quantitative β-galactosidase assay and the TAD of the basic helix-loop-helix factor E12 for comparison (probably due to its dependence on the cofactor CBP/p300, TAD-A remained inactive in yeast cells). In these cells, TAD-B showed approximately 16% the activity of E12 TAD. Three shorter peptides, TAD-B_{90}, TAD-B_{120}, and TAD-B_{pro}, exhibited somewhat weaker activity, whereas TAD-B_{90} was again inactive, as in EL4 T cells and 293 cells (Fig. 2E). Taken together, these results indicate the existence of a second trans-acting domain in NF-ATc/C, TAD-B that appears to be composed of positively acting (present in TAD-B_{30}, TAD-B_{120}, and TAD-B_{pro}) and negatively acting elements (in TAD-B_{90}).

**The activity of TAD-B is induced by various stimulators of T cells**

The striking difference in the extent of TPA-mediated stimulation between TAD-A and TAD-B prompted us to investigate whether this is also true for other inducers of T cells. As shown in Fig. 3A (and Fig. 2, C and D) TPA is a quite strong inducer of TAD-A, leading to a 5- to 10-fold increase in its activity in EL4 cells, whereas a 2- to 3-fold increase was observed for TAD-B. In contrast, a rise in intracellular Ca^{2+}, as mediated by ionomycin treatment, increased neither TPA-mediated TAD-A nor TAD-B induction, and CsA, an inhibitor of Ca^{2+}-dependent calcineurin activity, was without effect on the induction of both TADs by T+I. On the other hand, irradiation of EL4 T cells by UV light or treatment with methyl methane sulfonate along with TPA markedly increased the induction of both TADs (Fig. 3A). These results illustrate remarkable similarities in the induction of both TADs from NF-ATc/A in vivo and in vitro at Ser^{177} (A. Avots et al., manuscript in preparation; see Fig. 4B), which overlaps with a binding site for calcineurin (28). To determine whether TAD-B is also phosphorylated after TPA treatment we performed solid phase protein kinase assays. In these assays whole protein extracts from EL4 cells induced by TPA, UV light irradiation, or both treatments were used to phosphorylate chimeric GST/TAD-A and -B proteins coupled to glutathione agarose beads. As shown in Fig. 3B, TPA treatment of cells led to a marked increase in constitutive TAD-A phosphorylation, whereas no phosphorylation could be detected for TAD-B. UV irradiation alone or in combination with TPA led to only a marginal, if any, increase in constitutive or TPA-mediated TAD-B phosphorylation. Both treatments were without effect on the phosphorylation state of TAD-B (Fig. 3B).

**NF-ATc isoforms differ in their transcriptional capacity**

To test the transcriptional activity of individual NF-ATc proteins we cotransfected expression vectors for NF-ATc/A, -B, or -C together with a variety of promoter/reporter gene constructs into 293 cells, which express only minor amounts of endogenous NF-ATc (not shown). As shown in Fig. 4, A and B, all three NF-ATc isoforms were properly expressed and translocated into the nuclei as proteins of approximately 90, 110, and 140 kDa, respectively. Dephosphorylation of nuclear proteins led to an increase in their electrophoretic mobility, indicating phosphorylation of all three isoforms in these cells (Fig. 4C). All three NF-ATc proteins stimulated the inducible activation of luciferase reporter genes driven by four copies of the distal NF-AT site (Pu-bd) from the murine IL-2 promoter (2) or three copies of Pu-bd from the murine IL-4 promoter (Fig. 4, D and E) (29). In all transfection assays, NF-ATc/B was the weakest trans-activator (Fig. 4D), whereas NF-ATc/C appeared to be the strongest NF-ATc, being 2-fold stronger than NF-ATc/A in the activation of Pu-bd and 3-fold stronger in Pu-bd activation (Fig. 4E).

**FIGURE 3.** Induction of TAD-A and TAD-B activities in T cells. A. One microgram of DNA of Gal4 expression vectors encoding TAD-A, TAD-B, or the Gal4 DNA binding domain (Gal4-vector) were transfected into EL4 cells along with an E1b promoter/luciferase construct driven by four Gal4 binding sites. Twenty hours later, the cells were treated as indicated. The irradiation by UV light (0.1 J/cm^{2}) and treatment with methyl methane sulfonate (MMS; 75 ng/ml in complete medium for 2 h) were performed to achieve complete stimulation of JNK kinases as tested by in vitro kinase assays (not shown). Twenty hours after the start of induction, the cells were harvested for luciferase assays. One typical experiment from three experiments is shown. B. Solid phase kinase assays for the detection of TAD phosphorylation in vitro. Five micrograms of GST/TAD-A and TAD-B fusion proteins were coupled to glutathione agarose beads and incubated with 2 μg whole cellular protein from uninduced EL4 cells (-), cells treated for 15 min with TPA (T), cells irradiated with UV light (UV), or cells induced by both treatments (T+UV). Autoradiographs were exposed for 4 h.

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We also tested the activities of individual NF-ATc isoforms on the induction of the IL-2, IL-4, and IL-5 promoters after transfection into 293 cells. To test the effect of NF-ATc proteins on the TPA-mediated induction of the murine IL-2 and IL-4 promoters, luciferase and CAT reporter genes controlled by four copies of the distal NF-AT site of murine IL-2 promoter (Pu-bd) (2) or three copies of an NF-AT site from the murine IL-4 promoter (Pu-b4) (29) by NF-ATc isoforms. DNA, 0.5 μg (left panel) or 0.25 μg (right panel), of luciferase reporter genes was cotransfected into 293 cells as indicated with 5 μg of RSV-NF-ATc/A or B expression plasmids (left) or RSV-NF-ATc/A or C plasmids (right) and the empty vector control as indicated. Twenty hours later, the cells were induced for 3–24 h as indicated and harvested for CAT and luciferase assays. n.d., Not determined.

Surprisingly, NF-ATc/A exerted the same or even a stronger effect on the induction of both promoters as the longest isoform NF-ATc/C carrying two TADs (Fig. 5A). Very similar, albeit less detailed, data on the different transcriptional potencies of NF-ATc isoforms were also obtained after transfection into EL4 T cells (data not shown).

To determine whether these differences in the promoter induction depend on the type of promoter and/or the mode of stimulation we tested the effects of NF-ATc proteins on the IL-5 promoter whose activity is drastically stimulated by inducers of protein kinase A, e.g., forskolin (30), which, on the other hand, interfere with induction of the IL-2 promoter and only slightly increase IL-4 promoter induction (31). While NF-AT factors alone are poor activators of the IL-5 promoter, in cooperation with other transcription factors, such as GATA-3 and Ets-1, they strongly stimulate
the protein kinase A-mediated IL-5 promoter induction (S. Klein-Hessling et al., manuscript in preparation). As shown in Fig. 5B, cotransfections of an IL-5 promoter-driven luciferase gene with vectors expressing the three NF-ATc isoforms with GATA-3 and Ets-1 drastically enhance the T+I- and, in particular, the T+I- and forskolin-mediated induction of the IL-5 promoter. However, no marked differences among the three NF-ATc isoforms were detected when they were cotransfected with GATA-3 or Ets-1 alone or upon cotransfection with GATA-3 and Ets-1 together. These results indicate that the NF-ATc isoforms differ in their transcriptional activity depending on the promoter context.

**Concluding remarks**

All members of NF-AT transcription factor family are expressed in multiple isoforms. This has been shown in detail for NF-ATp and NF-AT4/x, which are expressed in several isoforms in T lymphocytes and other cells (24) (26). However, in contrast to the NF-ATc isoforms, all three NF-ATp isoforms are similar in length or even longer than NF-ATc/C, the longest NF-ATc isoform. A short isoform lacking an extra C-terminal peptide, as does NF-ATc/A, has also been described for NF-AT4 (32), but it remains to be shown whether it is inducibly synthesized like NF-ATc/A. The NF-ATp isoforms share a C-terminal QP-rich stretch of approximately 220 aa that shows >30% homology to the C-terminal NF-ATc/C peptide and is able to act as a TAD (33). Such a second TAD has also been identified within the C-terminal portion of the longest isoform of NF-AT4/x, designated NFATx1 (26). Thus, the existence of a C-terminal TAD is not a peculiarity of the long NF-ATc isoform C but is a typical component of numerous NF-AT proteins.

Using a panel of NF-ATc-specific Abs Lyakh et al. (34) observed in peripheral human T cells the expression of three prominent NF-ATc proteins very similar in length to the three NF-ATc isoforms we have cloned. An NF-ATc isoform similar to isoform B and designated NFATc/B, was isolated from a human Burkitt lymphoma cDNA library and described to possess a specific C-terminal peptide missing any homology to NF-ATp (18). However, introduction of 2 bp into the NF-ATc/B sequence (at positions 2334 (C) and 2451 (G); numbering according to Ref. 18) leads to a sequence identical to the NF-ATc/B peptide and, therefore, shares about 36% sequence homology to the C terminus of NF-ATp.

The identification of TAD-B in NF-ATC/B raises the question of whether these isoforms play in gene control in T lymphocytes and other cells where they are highly expressed at different relative levels. The conspicuous differences between the NF-ATc isoforms in activation of the IL-2 and IL-4 promoters, on the one hand, and of the IL-5 promoter, on the other, suggest important functional roles of individual isoforms in promoter control. Several lines of evidence indicate that threshold levels of NF-AT play a crucial role in the induction of promoters in T cells (17, 36). In addition, due to the different transcriptional capacities of NF-AT isoforms, changes in isoform composition will result in marked differences in specific transcriptional activity of nuclear NF-ATc. Apart from the analysis of NF-AT-driven promoters other than the typical lymphokine promoters, the establishment of mouse mutants defective in the synthesis of one or the other NF-ATc isoform will allow study of the function of individual NF-ATc proteins in detail.

**Acknowledgments**

We thank Ilona Pietrowski for excellent technical assistance and Dr. Wolfgang Haedicke for support in DNA sequencing. For critical reading of the manuscript, we thank Stefan Klein-Hessling and Anneliese Schimpl. For gifts of materials, we are indebted to Drs. P. Matthias (Basel, Switzerland), A. Rao (Boston, MA), L. Schmitz (Heidelberg, Germany), and Th. Wirth (Würzburg, Germany). A.A., E.J., and I.I. thank Prof. E. Grens (Riga, Latvia) for continuous support.

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