NF-AT–Driven Interleukin-4 Transcription Potentiated by NIP45

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The induction of cytokine gene transcription is mediated in part by the nuclear factor of activated T cells (NF-AT). Factors involved in the mechanisms of NF-AT–mediated transcription are not well understood. A nuclear factor that interacted with the Rel homology domain (RHD) of NF-ATp was identified with the use of a two-hybrid interaction trap. Designated NIP45 (NF-AT interacting protein), it has minimal similarity to any known genes. Transcripts encoding this factor were enriched in lymphoid tissues and testes. NIP45 synergized with NF-ATp and the proto-oncogene c-Maf to activate the interleukin-4 (IL-4) cytokine promoter; transient overexpression of NIP45 with NF-ATp and c-*maf* in B lymphoma cells induced measurable endogenous IL-4 protein production. The identification of NIP45 advances our understanding of gene activation of cytokines, critical mediators of the immune response.

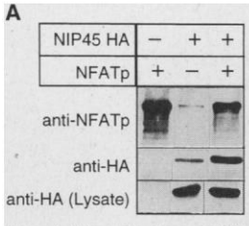
The molecular basis for cytokine gene expression in T helper cells is beginning to be elucidated. Analysis of the IL-4 promoter has revealed functionally critical sites for several transcription factors including members of the NF-AT and AP-1 families (*1*, *2*). The selective expression of the proto-oncogene c-*maf* in T_H2 cells is responsible for tissue-specific IL-4 expression (*3*). c-*Maf* acts in synergy with NF-AT proteins to transactivate the IL-4 promoter. The inducible expression of multiple cytokine genes and cell surface proteins after T cell receptor stimulation requires members of the NF-AT transcription factor family (*1*, *4*–*6*).

NF-AT is a multisubunit transcription complex that contains a cyclosporin A–sensitive cytoplasmic phosphoprotein and an inducible nuclear component composed of AP-1 family member proteins (*7*, *8*). NF-ATp (also called NF-ATc2 in the genomic database nomenclature) has a region of limited sequence identity to the Rel homology domain (RHD) of the NF-κB family of transcription factors (*9*). Subsequent cloning and sequencing of three related genes, NF-ATc (also called NF-ATc1), NF-AT4 (also called NF-ATx or NF-ATc3), and NF-AT3 (also called NF-ATc4) revealed similar domains. NF-AT family members share approximately 70% sequence similarity within this domain and approximately 18% similarity to the RHD of the NF-κB family of transcription factors, consistent with their behavioral differences with the NF-κB proteins. Because optimal reconstitution of endogenous IL-4 gene expression has not yet been achieved, it is likely that additional proteins that act in concert with known transcriptional activators such as NF-AT and c-Maf exist.

To identify such factors we sought to isolate proteins that could directly bind to the RHD of NF-ATp. Using a yeast two-hybrid interaction trap, we selected a T cell cDNA library for sequences encoding polypeptides that specifically interact with a NF-ATp(RHD)-Gal4 fusion protein (*10*). One class of interactors encoding a fusion protein with apparently high affinity for the NF-ATp(RHD)-Gal4 bait, as exhibited by high β-galactosidase activity and ability to confer leucine prototrophy, was isolated. The interaction with this factor was specific, because no interaction was detected with baits that encoded Max-Gal4 or CDK2-Gal4 fusion proteins, or only an epitope-tagged Gal4 protein (*11*).

The ability of this polypeptide to interact specifically with NF-ATp in vivo was tested in mammalian cells. The 1.9-kb insert was subcloned into a mammalian expression vector that fused the coding region to an epitope tag from an influenza hemagglutinin (HA) peptide. This tagged construct was cotransfected with an NF-ATp expression plasmid into HepG2 cells (which express low NF-ATp) and lysates were prepared for immunoprecipitation with an antibody to NF-ATp. Western immunoblot analysis of these samples with an HA-specific monoclonal antibody (mAb) showed that the antibody to NF-ATp co-immunoprecipitated the HA-tagged polypeptide, designated NIP45-HA (below). The amount of HA-tagged protein immunoprecipitated was further increased by co-transfection with the NF-ATp expression plasmid, demonstrating the specificity of this interaction (Fig. 1A). No immunoreactive material for either NF-ATp or the HA-tagged protein was detected when we immunoprecipitated with normal rabbit serum (*11*). Thus, NF-AT and NIP45 physically associate in vivo.

Because the transcript size detected by northern blot analysis (below) was approx-



B

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Fig. 1. Identification of NF-ATp Interacting Protein (NIP45). (**A**) Interaction of NIP45 and NF-ATp in HepG2 cells: cDNA from the NIP45 plasmid was cloned into the epitope tag expression vector pCEP4-HA (*16*). This construct results in the in-frame fusion of amino acids with the sequence YPYDVPDYA (*17*) of the influenza hemagglutinin protein to the open reading frame of the NIP45 coding region. Lysates were prepared from HepG2 cells transfected with NIP45-HA, NF-ATp, or both plasmids, as indicated. Samples indicated with (–) indicate cotransfection with corresponding expression vector (for NF-ATp) or an out of frame fusion with the epitope tag (NIP45-HA). Top and middle, lysates were immunoprecipitated with antibody to NF-ATp and Western blot analysis done with either antibodies to NF-ATp or to HA. Lanes showing transfection with only NIP45-HA reveal the low endogenous production of NF-ATp present in these cells. Bottom, Western blot analysis of untreated lysates to show equivalent expression of the NIP45-HA polypeptide. (**B**) Nucleotide and predicted amino acid sequence of the original NIP45 isolate (*17*). Coding region is shown from the first initiation codon through the first in-frame stop codon. Nucleotide and amino acid (bold) positions are indicated to the right of the primary sequence. Underlined portion indicates the basic region of the predicted polypeptide (GenBank accession number U76759).

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imately 3.1 kb, the 1.9-kb insert from this clone was used to screen a T cell cDNA library to identify full-length clones. Screening of a library containing approximately 8×10^5 clones yielded an isolate that contained an additional segment of 180 bp located 868 bp downstream from the 5′ end of the original clone. Junction sequences at the ends of this 180-nucleotide segment indicate it to be an unspliced intron, and conceptual translation of the nucleotide sequence within this region revealed an in-frame stop codon. Much of the additional sequence in this clone was at the 3′ end and represented an extensive 3′ untranslated region followed by a poly $(A)^+$ tail (*11*). Such extensive 3′ untranslated regions have been observed in many genes. Conceptual translation of the original isolate predicted a polypeptide of 412 amino acids with a molecular mass of 45 kD (Fig. 1B) and hence we have named it NF-AT Interacting Protein 45 (NIP45). Allowing for the splicing of the small intron and translation of the single, large open reading frame, the 3.1-kb cDNA clone is predicted to encode a polypeptide identical to that of the original isolate. A comparison of the sequence of NIP45 to the GenBank, European Molecular Biology Organization, and SwissProt databases using the BLAST algorithm revealed no significant sequence identities to known proteins. No similarities were observed with sequences in the Expression Sequencing Tag (EST) databases. Inspection of the amino acid sequence of NIP45 revealed a highly basic domain at the NH_2-terminus in which 13 of 32 amino acids are basic (Fig 1B), but in general the primary sequence had no striking features that could yield clues to the function of this gene.

Northern blot analysis of RNA from different murine tissues revealed a transcript of approximately 3.1 kb (Fig. 2). RNA from testis contained an additional 1.4-kb hybridizing species. The highest amounts of NIP45 transcripts were seen in spleen, thymus, and testis. The preferential expression in lymphoid organs may indicate a specific function for NIP45 in the immune system. The low intensity hybridization signal and the rare occurrence of NIP45 cDNA clones in the T cell cDNA library combined with its absence in the current EST databases indicate that the NIP45 RNA is a relatively rare message.

To gain further clues to the potential function of NIP45, subcellular localization of epitope-tagged protein was determined by indirect immunofluorescence (Fig. 3). The fluorescence pattern indicated that NIP45 is evenly distributed throughout the nucleus, matching the pattern seen for the same cells treated with the nuclear staining

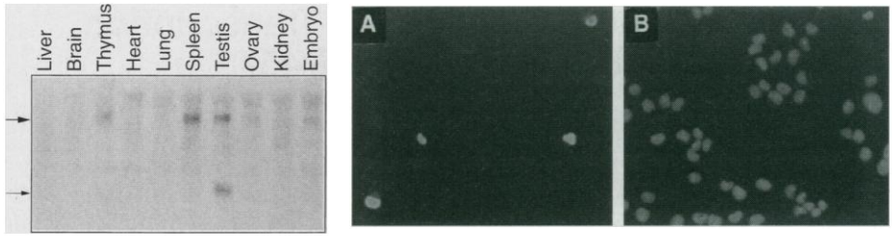

Fig. 2 (left). RNA blot analysis of NIP45 transcript. Total RNA (10 μg) from indicated tissues was separated on denaturing agarose gels, blotted, and hybridized with a radiolabeled 1.4-kb NIP45 cDNA fragment. Samples were controlled for equivalent loading of RNA by comparison of ethidium bromide fluorescence. **Fig. 3 (right).** Nuclear localization of NIP45. Immunofluorescence analysis of BHK cells transfected with an expression construct encoding an HA epitope–tagged NIP45 (pCEP4-HA). Transfected cells were fixed and probed with a monoclonal antibody specific for the HA peptide (*18*). Nuclear staining of NIP45 was observed with a indocarbocyanine-labeled goat anti-mouse secondary reagent (**A**) by comparison to the same cells counterstained with the DNA staining dye Hoechst 33258 (**B**). Indocarbocyanine fluorescence was not detected in control experiments of cells transfected with NIP45ΔHA.

dye Hoechst 33258 and that of cells transfected with NF-AT4 and stimulated with ionomycin (*11*). Stimulation with PMA, ionomycin, or both did not affect the subcellular localization of this NIP45 (*11*). Nuclear trafficking of NF-AT4 in HepG2 cells in response to changes in intracellular calcium concentrations was not affected by the overexpression of exogenous NIP45 (*11*).

We tested for a functional role of NIP45 in NF-AT–driven transcription by overexpressing NIP45 in HepG2 cells. HepG2 cells express low amounts of endogeneous NF-AT, and ectopic expression of NF-AT proteins transactivates NF-AT–driven transcription in this cell line in the absence of exogenous stimulation (*12*). The cDNA encoding NIP45 was cloned into an expression vector and cotransfected into HepG2 cells with NF-ATp and a reporter gene containing tandem copies of the NF-AT binding site (3XNF-AT-CAT). Transfection of NIP45 alone into HepG2 cells with the 3XNF-AT-CAT reporter did not lead to a significant increase in CAT expression (Fig. 4A), showing that NIP45 cannot act on its own to transactivate an NF-AT target sequence. Overexpression of NF-ATp alone resulted in substantial (six times greater than vector control) transactivation of the NF-AT-CAT reporter (Fig. 4A) (*12*). Cotransfection of NIP45 plus NF-ATp resulted in a four to five times increase in CAT activity relative to transfection with NF-ATp alone and a 25 to 30 times increase over that seen with vector alone (Fig. 4A). This increase was not observed when a mutant 3XNF-AT-CAT reporter or a control major histocompatability complex (MHC) class II promoter reporter was used, thus demonstrating its target site specificity (*11*).

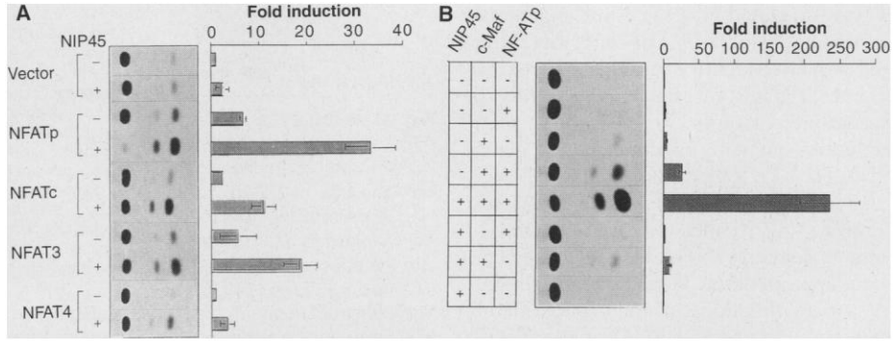

Fig. 4. NIP45 stimulates transcription with NF-AT. (**A**) NIP45 potentiated transactivation by NF-AT family member proteins. HepG2 cells were transfected with a 3XNF-AT–CAT reporter from the IL-2 gene (*19*) and control or expression plasmids for NIP45 and NF-AT family members (NF-ATp, NF-ATc, NF-AT3, and NF-AT4) (*9*) as indicated (*20*). One representative assay for each combination is shown adjacent to a bar graph representing relative CAT activity for each group. (**B**) NIP45 synergized with NF-ATp and c-*maf* to activate the IL-4 promoter. HepG2 cells were transfected with an IL-4–CAT reporter construct (extending to −732 bp of the IL-4 promoter) and expression vectors or controls for NIP45, NF-ATp, and c-*maf* as indicated. The control for NIP45 was a frame shift mutant at amino acid 13. Controls for NF-ATp and c-*maf* were the empty expression vectors pREP4 and pMEX, respectively (*3*). Representative CAT assays and bar graphs are depicted as in (A).

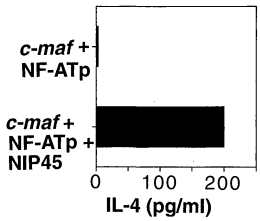

Fig. 5. Transient overexpression of NIP45 with NF-ATp and c-*maf* results in endogenous IL-4 production. M12 B lymphoma cells were transiently cotransfected with expression plasmids for NF-ATp and c-*maf* together with NIP45 or pCI vector control, and the concentration of IL-4 in supernatants harvested 72 hours later was measured by enzyme-linked immunosorbent assay (ELISA) (*21*).

To confirm that the polypeptide product encoded by the NIP45 cDNA was responsible for this enhanced transactivation, a frame shift mutation was introduced in the coding region that encoded a truncated NIP45 (NIP45Δ) (*13*). The NIP45Δ construct did not transactive the NF-AT reporter in the presence or absence of NF-ATp, thus confirming that the enhanced transactivation observed was due to the polypeptide expressed from NIP45 cDNA. Transactivation experiments were also done in the B cell line M12 and the T cell clone D10 with similar although less dramatic results (*11*), which may be due to higher endogenous NIP45 or NF-ATp expression in these latter cell lines. We conclude that NIP45 substantially and specifically potentiates transcription induced by NF-ATp, an activity that requires interaction with NF-ATp.

Because NF-AT proteins share approximately 70% identity within the RHD, we tested whether other NF-AT family members could interact with NIP45. NIP45 was cotransfected as above with expression constructs encoding either NF-ATc, NF-AT3 or NF-AT4 plus the NF-AT–CAT reporter plasmid. All NF-AT family members can transactivate a reporter gene containing three copies of an NF-AT–AP1 site when overexpressed in HepG2 cells, although to different extents (Fig. 4A). Although NF-ATp was the most potent transactivator of the NF-AT–CAT reporter, NIP45 potentiated both NF-ATc (fourfold increase) and NF-AT3 (threefold)–driven transactivation as well as NF-AT4 (twofold)–mediated transactivation (Fig. 4A). The ability of NIP45 to potentiate the activity of all NF-AT family members is not surprising given the high degree of sequence conservation of the RHD of the NF-AT family members.

We also determined if NIP45 was functional in the context of a native NF-AT–dependent promoter. IL-4 expression is tissue specific and restricted to the T_H2 subset of T cells and to mast cells. Thus, the IL-4 promoter is not active in the HepG2 cell line but could be activated by the introduction of NF-ATp and c-*maf* (Fig. 4B), consistent with the action of transfected c-*maf* with NF-ATp to transactivate the otherwise inert IL-4 promoter in T_H1 and B cells (*3*). Introduction of NIP45 together with NF-ATp and c-*maf* resulted in an additional ninefold increase in the activity of the IL-4 promoter relative to that seen for NF-ATp and c-*maf* alone (Fig. 4B). NIP45 also increased the activity of the IL-4 promoter in the absence of transfected NF-ATp, an effect likely due to interaction with endogenous NF-ATp (Fig. 4B).

B lymphoma cells stably transfected with NF-ATp and c-*maf* expression vectors produce low amounts of endogenous IL-4 protein (*3*). In contrast, endogenous IL-4 is not detected upon transient transfection, which presumably reflects the lower fraction of cells expressing the introduced genes. We investigated if providing the B lymphoma cells with NIP45 in addition to NF-ATp and c-*maf* might increase endogenous IL-4 production to levels detectable in a transient expression system. Therefore, M12 cells were transiently cotransfected with c-*maf* and NF-ATp and either NIP45 or control vector. Four independent sets of transient transfections were done and assayed for secretion of IL-4 into the culture supernatant. For each set of transfections, inclusion of NIP45 led to a 50 to 200 times increase in IL-4 production over cells that did not receive NIP45, in which IL-4 production was near the limit of detection (Fig. 5). The effect of c-*maf*, NF-ATp, and NIP45 was specific for IL-4 as no endogenous IL-5 protein was detected (*14*). Thus NIP45 substantially stimulates the ability of NF-AT to activate transcription of genes that contain binding sites for NF-AT. The mechanism of action for this effect is not known although some clues are provided by the localization of this protein to the nucleus. A tempting mechanism for the stimulation of NF-AT transactivation is through a coactivator function. This implies the existence of specific factors in the basic transcriptional complex that interact with NF-AT–bound NIP45. Further studies will be required to identify these putative factors. Electrophoretic mobility shift analysis did not provide evidence for cooperative binding of NIP45, c-*maf*, and NF-ATp in the presence of DNA (*15*). Although this does not rule out the possibility of a coactivator function, other mechanisms may explain the activity of this factor. This includes but is not limited to a posttranslational modifying enzyme of NF-AT, an accessory protein involved in the subcellular transport of NF-AT, or a factor involved in the assembly of an NF-AT transcription complex.

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10. A yeast two-hybrid bait was prepared by cloning a 900-bp fragment of murine NF-ATp (*9*) spanning amino acids 228 to 520 into the Bam HI site of vector pEG202 (*22*). In-frame fusion of the polypeptide sequence was confirmed by DNA sequence analysis. This bait was used to screen a D10 T cell cDNA library, constructed in the plasmid pJG4-5, for clones encoding interacting polypeptides as described (*22*).
11. M. Hodge and H. Chun, unpublished data.
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13. NIP45Δ was produced by creating a two-base deletion at nucleotide 50. This alteration results in the introduction of missense mutations at amino acid 13 and termination of the polypeptide after an additional 22 residues.
14. R. Lieberson, unpublished data.
15. M. Hodge, unpublished data.
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17. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
18. BHK cells were transfected with 1 μg of the indicated plasmids as described (*23*). Cells were incubated overnight in culture media and either fixed directly or first stimulated with 1 mM ionomycin for 10 min before fixation. Fixed cells were permeabilized (*23*) and probed with monoclonal antibody 12CA5 to HA (anti-HA) (Boehringer Mannheim) plus indocarbocyanine-labeled donkey anti-mouse (Jackson ImmunoResearch) and then counterstained with the dye Hoechst 33258.
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20. Fold induction was calculated by normalizing the CAT activity of cells transfected with the CAT reporter and each parental expression vector to one. Values represent the relative amount of CAT expression above this control transfection. All transfections were done at least three times with one representative autoradiograph shown. HepG2 cells were transfected by the DEAE-Dextran method (*12*).
21. A representative experiment from one of four independent transfections is shown. M12 cells were transiently transfected by electroporation (*3*) by incubating 3×10^6 cells in 0.4 ml of phosphate-buffered saline with 5 μg of each plasmid for 10 min at room temperature prior to to electroporation at 975 μF, 280 V. The expression vectors for c-*maf* (pMEX-Maf/pREP4), NF-AT (pREP4-NF-ATp), and NIP45 are those used in Fig 4. The control for NIP 45 is the empty vector, pCI. ELISA was done according to the instructions of Pharmingen except with modification as described (*3*).
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