A second calcineurin binding site on the NFAT regulatory domain

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NFATc (a member of the family of nuclear factors of activated T cells) is a transcriptional factor responsible for the Ca2+-inducible activation of cytokine genes during the immune response. In resting T cells, NFATc is retained in the cytoplasm by a mechanism that depends on multiple phosphorylations in an N-terminal regulatory domain. Physical interaction with and dephosphorylation by Ca2+-activated calcineurin (Cn) allows the protein to enter the nucleus, where it binds to specific sites in cytokine gene promoters. Previous studies had identified a peptide segment in NFATc that binds Cn stably. Here we report the identification of a second Cn-binding element in NFATc, which synergizes with the previously identified element. Although these sequences are conserved in all isoforms of NFAT, we find that the two sites contribute differentially to the overall affinity for Cn in an isoform-dependent manner. The regulatory domain of NFAT also was found to be entirely devoid of structure, both in the phosphorylated and unphosphorylated state. This finding suggests that the NFAT regulatory domain does not undergo phosphorylation-induced conformational switching, but instead requires partner proteins to control accessibility of the NFAT nuclear localization and nuclear export signals.

The nuclear factor of activated T cells (NFAT) defines a family of Ca2+-inducible transcription factors discovered for their critical role in regulating the transcription of cytokine genes (1–3). Four individually encoded members of the NFAT family are known: NFATc (also known as NF-ATc1 or NFAT2) (4), NFATp (NF-ATc2/NFAT1) (5), NFAT3 (NF-ATc4), and NFAT4 (NFATx/NF-ATc3) (6, 7). [There is disagreement as to whether a fifth NFAT-related protein, NFAT5 (8), should be included within the family.] Each family member serves a distinct biological role, as revealed by their unique expression patterns, together with the markedly different phenotypes of mice carrying targeted disruptions of the NFATc, NFATp, and NFAT4 genes (9–12).

NFAT proteins share a conserved domain located toward the C terminus (13) that binds DNA and also participates in cooperative protein–protein interactions with other transcription factors (14–16). Immediately N terminal to this domain lies a second conserved module of ~300 residues known as the NFAT homology region (NFAT-h). The N terminus of NFAT-h, including the NFAT-h, regulates nuclear/cytoplasmic trafficking in response to changes in intracellular Ca2+ concentrations. This function is apparently self-contained, as Ca2+-dependent trafficking can be transferred to unrelated proteins through fusion to the NFAT regulatory domain (17–20). In resting T cells, the protein is retained in the cytoplasm and its NFAT-h is heavily phosphorylated. Engagement of the T cell antigen receptor or treatment of cells with Ca2+/ionophores activates calcineurin (Cn), a Ca2+/calmodulin-dependent Ser/Thr phosphatase, which dephosphorylates the NFAT-h, resulting in rapid translocation of the protein to the nucleus (21).

The clinically important immunosuppressive drugs FK506 and cyclosporin A (CsA) block this translocation process by inhibiting Cn activity in T cells (refs. 22 and 23, reviewed in ref. 24). These drugs also inhibit Cn activity in nonhemopoietic cells, a likely cause of side effects that seriously limit immunosuppressive therapy in a significant fraction of organ transplant patients.

Consequently, interest in new drugs that act by a more cell-specific mechanism runs high. Elucidating the detailed molecular mechanism of phosphorylation-dependent nuclear trafficking by the NFAT N-terminal domain would further this goal.

Previous studies have characterized signal sequences for both nuclear import (NLS) (17, 25, 26) and nuclear export (NES) (27, 28) in the NFAT-h and flanking regions. The prevailing model holds that the phosphorylation state of the NFAT-h controls switching between two alternative conformational states that are either import or export conducive. Direct structural evidence for or against this model has not been reported to date. As for the NFAT-h-Cn interaction, it has been shown that Cn stably associates with NFATc, NFATp, and NFAT4, even in the absence of substrate phosphate groups (18, 29). This interaction has been mapped to a conserved ~13-aa segment (Cn-binding peptide A, CnBP-A) within the N-terminal domain of NFATp (30). Substitution of key residues within this segment by alanine results in loss of binding to Cn in vitro and substantially reduces dephosphorylation and nuclear translocation after ionomycin treatment in vivo. Similar results were obtained for NFAT4 (26), suggesting that this peptide motif may be a common Cn binding site for all isoforms of NFAT.

In this study we present evidence that NFATc has an additional Cn-binding site (CnBP-B), which synergizes with the previously identified docking site (CnBP-A) to increase the overall binding affinity for Cn. A peptide corresponding to CnBP-B from NFATp does not detectably bind Cn, whereas CnBP-B peptides from NFAT3 and NFAT4 bind somewhat more tightly than the NFATc counterpart. The existence of a second Cn binding site in only select isoforms of NFAT raises the possibility that a difference in the mode and strength of interaction with Cn may give rise to isoform-dependent differences in the activation of NFAT.

Materials and Methods

Preparation of Phosphorylated NFAT1–414/CD Spectroscopy. The N-terminal fragment of human NFATc containing the first 414 residues (NFAT1–414) and tagged with the hexa-his sequence at the C terminus was overexpressed in Escherichia coli BL21(DE3) cells and purified by using Ni-nitriotriacetic acid resin (Qiagen, Chatsworth, CA) according to the manufacturer’s recommended procedure. Priming phosphorylation by protein kinase A (PKA, a gift from S. Taylor, University of California, San Diego) was carried out at 30°C for 2 h in a buffer containing 5 μM NFAT1–414, 0.1 μM PKA, 50 mM potassium phosphate (pH 7.3), 125 mM NaCl, 1 mM DTT, 1 mM MgCl2, and 0.25 mM ATP. For full phosphorylation, recombinant rabbit glycogen synthase ki-

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Abbreviations: NFAT, nuclear factor of activated T cells; NFAT-h, NFAT homology region; Cn, calcineurin; NLS, nuclear localization signal; NES, nuclear export signal; CnBP-A, Cn binding peptide A; CnBP-B, Cn binding peptide B; PKA, protein kinase A; GSK-3β, glycogen synthase kinase-3β; GST, glutathione S-transferase; HSQC, heteronuclear single quantum correlation; SEAP, secreted alkaline phosphatase.

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nase-3β (GSK-3β) (a gift from P. Roach, Indiana University) then was added to a concentration of 0.3 μM, and the reaction was allowed to proceed for 15 h. CD spectra were measured on a Jasco J-710 spectrometer at 25°C, with a quartz cuvette of 1-mm path length. Eight scans were signal-averaged to obtain the final spectra for each sample and corrected for the buffer and the kinases, which introduced a 3–5% correction in the spectra.

Glutathione S-Transferase (GST) Binding Assay/Western Blot. Full-length NFAT<sub>1–414</sub> and various fragments of it were fused to GST, and the fusion proteins were purified by affinity chromatography with 300 μl glutathione resin per 500 ml of LB culture. GST resin containing bound GST-NFAT fusion polypeptide was equilibrated with 200 nM recombinant human Cn (purified to 95% homogeneity and high specific activity as described in ref. 31) in binding buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1.5 mM CaCl<sub>2</sub>, 6 mM MgCl<sub>2</sub>, 1 mM DTT, and 0.2% Triton X-100, for 30 min at 4°C. The resin then was washed five times in fresh binding buffer, and the bound fraction was analyzed by electrophoresis on a 12% SDS/PAGE gel. Bound Cn was detected by standard Western blot using monoclonal Cn antibody (PharMingen).

Purification of CnBP-B Peptide and NMR Spectroscopy. A 21-aa <sup>15</sup>N-labeled peptide containing CnBP-B was biosynthesized by modification of a published protocol (32). The peptide was purified by reversed-phase chromatography on C18 column (Beckman) using a linear gradient (0.1% trifluoroacetic acid, 20–60% CH<sub>3</sub>COONa) over 20 min. A single major peak eluted at 8 min. An unlabeled sample prepared in parallel was found to contain a peptide of the expected molecular mass (2,491 Da), as determined by electrospray ionization MS (Harvard Chemistry Department Mass Spectrometry Facility).

All NMR spectra were acquired on a Bruker DMX500 spectrometer equipped with a 5-mm triple-resonance probe. The peptide was dissolved in NMR buffer (37.5 mM deuterated Tris-acetate, pH 6.5, 50 mM KCl, 37.5 mM MgSO<sub>4</sub>, 5 mM per-deuterated DTT) at a concentration of 0.5 mM. A <sup>1</sup>H-<sup>15</sup>N heteronuclear single quantum correlation experiment (HSQC) was performed with the sweep widths of 14 (F2) and 34 (F1) ppm, by using the enhanced sensitivity method with water suppression. Peptide assignments were derived from a nuclear Overhauser effect spectroscopy and a total correlation spectroscopy experiment. HSQC experiments were performed on two samples, one with the <sup>15</sup>N-labeled peptide alone, and the other with the <sup>15</sup>N-labeled peptide in the presence of 50 μM Cn. Data were analyzed with the FELIX software package (Molecular Simulations, San Diego).

Secreted Alkaline Phosphatase (SEAP) Assays. Wild-type and mutant versions of NFAT<sub>1–414</sub> were cloned into the mammalian expression vector pB5 (4) under the control of the SR-α promoter. Jurkat cells stably transfected with the adenovirus large-T antigen (Jurkat-TAg cells, ref. 33) were maintained in RPMI containing 10% FBS. The cells (5 × 10<sup>5</sup>) were transiently transfected by using 3 μl of cationic liposome DMRIE-C (GIBCO/BRL) with a reporter plasmid encoding the mammalian SEAP, together with varying amounts of the pB5 derivatives. The cells were allowed to recover for 24 h before stimulation with 1 μM ionomycin (Sigma) and 20 mg/ml phorbol myristate acetate (Sigma). The SEAP activity was measured a day later by using 600 μM methylumbelliferyl phosphate as the fluorescent substrate.

Results
To characterize the NFATc regulatory domain biochemically, we overexpressed and purified a fragment of human NFATc spanning the NH<sub>2</sub>-terminal 414 amino acid residues (NFAT<sub>1–414</sub>–Fig. 1a). The CD spectrum of unphosphorylated NFAT<sub>1–414</sub> (Fig. 1b) closely resembles that of an unfolded protein (34) and specifically lacks the spectral features characteristic of α-helical or β-sheet secondary structure (Fig. 1b). Consistent with the notion that unphosphorylated NFAT<sub>1–414</sub> possesses little, if any, folded structure, the protein is degraded rapidly on exposure to 500 ng/ml of trypsin or chymotrypsin, whereas the folded core domain of Cn A remained intact under these conditions (data not shown).

The kinases responsible for phosphorylating NFATc have been identified as PKA and GSK-3β (25). These enzymes act coordinately, with PKA catalyzing “priming” phosphorylations (35) that are necessary and sufficient for GSK-3β to further process the regulatory region and establish its fully phosphorylated state. In the case of NFAT4, MEK kinase 1 (MEKK1) and casein kinase 1α (CK1α) have been proposed as the intracellular kinases (26), but the phosphorylation sites for CK1α <i>in vitro</i> substantially overlap those for PKA plus GSK-3β. To test whether NFAT<sub>1–414</sub> undergoes conformational reorganization after phosphorylation, we reacted the protein <i>in vitro</i> with PKA and GSK-3β. Although SDS/PAGE analysis indicated that the protein underwent phosphorylation after treatment with each of the two kinases, no change is evident in the CD spectra obtained after phosphorylation with either PKA alone or PKA plus GSK-3β (Fig. 1b). Thus, phosphorylation of the NFAT regulatory domain does not induce a detectable change in its secondary structure.

The observation that NFAT<sub>1–414</sub> apparently lacks a well-defined three-dimensional fold, even in the phosphorylated form, raises the possibility that its interaction with Cn may be localized to one or more short contiguous polypeptide stretches in the regulatory region of NFATc. Such a short Cn-BP sequence has been identified in NFATp (30, 36) and NFAT4 (26). Furthermore, other proteins that bind Cn, including AKAP79 (37) and Bcl-2 (38), also have been shown to do so by using short peptide segments. To localize the Cn-BP sequences in NFATc, we created a series of GST fusion proteins bearing systematically
truncated segments of NFAT1–414 (Fig. 2a). Their interaction with Cn was determined by pull-down assays using glutathione beads (Fig. 2a). Two polypeptide sequences, CnBP-A and CnBP-B, were located within the outermost motifs of the nine conserved in the NFAT homology region (39). SRR, serine-rich region; SP motif, serine-proline motif. Expansion brackets and parentheses show the amino acid sequences of certain key elements, in single-letter code. (c) Competition GST pull-down assays. CnBP-A or CnBP-B fused to GST (GST-CnBP-A or CnBP-B) was incubated with Cn in the presence of either the CnBP-A or CnBP-B peptide in solution. (d) Same as in c, except the full-length NFAT regulatory domain fused to GST was used as bait. (Left) The results of competition by either peptide alone; (Right) competition by both. Concentrations in c and d are as follows: lanes 1 and 8, no peptide added; lanes 2–4, CnBP-A at 50, 100, and 300 µM, respectively; lanes 5–7, CnBP-B at 50, 100, and 300 µM, respectively; lane 9, CnBP-A and CnBP-B at 25 µM each; Cn at 100 nM in all lanes. (e) Inhibition of NFAT-dependent reporter gene activity by intracellular overexpression of the NFAT regulatory domain. A reporter construct having the SEAP cDNA linked to three tandem copies of the human IL-2 promoter was transfected into Jurkat-TAg cells, along with an expression vector encoding the N-terminal regulatory domain of human NFATc. Both wild-type regulatory region (NFAT1–414) and mutants having three alanine substitutions (R119A, E121A, and T123A) in CnBP-A (NFAT1–414–mCnBP-A) and/or a deletion (residues 378–414) of CnBP-B (NFAT1–414–mCnBP-B) were analyzed. SEAP activity was measured with 4-methylumbelliferone phosphate as the substrate. All runs were normalized to 100% SEAP activity in the absence of NFAT plasmid.
motif from NFATc was most effective at competing against itself of the various NFAT isoforms (Fig. 4). We also used a CnBP-B from NFATc, NFAT3, and NFAT4 are effective at pulling down Cn from solution. As shown in Fig. 4, the CnBP-B motifs from NFATc, NFAT3, and NFAT4 are effective at pulling down Cn, whereas that from NFATp is not. The intracellular partitioning of NFAT between the cytoplasm and nucleus is controlled by the presence or absence of phos-

Discussion

The intracellular partitioning of NFAT between the cytoplasm and nucleus is controlled by the presence or absence of phos-
but also interacts with at least five known proteins: the phos-
tory domain of NFAT not only undergoes changes in its covalent
masking or unmasking of the NLS and NES (20, 26, 28). This
and 300 μM concentrations). (d) Quantification of the results in (c) analyzed by
scanning and MACBAS.

Fig. 4. Isoform dependence of the interaction of CnBP-B with Cn. (a) Amino
casein kinase I
other candidates include the reported NFAT kinases PKA,
and MEKK1 (26). Of these, only
casein kinase Iα has been shown thus far to associate stably with
the regulatory domain of NFAT4 (26).

Interactions Between Cn and the NFAT Signaling Domain. It is now
well established that the hypophosphorylated form of NFAT
remains stably associated with Cn in the nucleus of Ca2+-
stimulated cells. This association serves not only to provide a
high local concentration of phosphatase activity in the sustained
presence of the Ca2+ stimulus, but is also essential for the
physical masking of the NES (28) (see above for discussion).

Two NFAT peptide stretches that bind CnBP-B were required, along with
mutations in CnBP-B, to abolish the interaction of the NFATc regulatory
domain with Cn in vivo. Evidence for a second Cn binding site
in NFAT is also apparent in coinmunoprecipitation studies on
NFAT4, which localized the interaction to residues 310–407, a
region containing CnBP-B (residues 390–403) (39); however, a
second study on NFAT4 failed to identify CnBP-B, because the
analysis did not extend to the C-terminal end of the regulatory
region (26). In the case of NFAT4, the CnBP-A Cn docking site lies close to the reported
control nuclear export of NFAT4 and presumably for the other
NFAT isoforms as well. Just as Cn is required to mask the NES
from access to CRM-1, we reason it likely that a cytoplasmic
partner protein is required to mask the NLS from access to
importin-α. Cn would seem to be an obvious candidate, although
the weakness of its binding to NFAT in the absence of Ca2+ (44)
renders this scenario less likely. CRM1 also must be considered
a candidate, because it is an established interaction partner and
because it translocates to the cytoplasm along with NFAT (45).

Structural Implications. The prevailing model for regulation of
NFAT nuclear trafficking (20, 26, 28) predicts that the phos-
phosphorylation-dependent conformational switching, to which
potential interacting partners respond. Proteins that lack defined
tertiary structure are inherently dynamical and therefore are
incapable of being frozen in discrete states. Second, the absence
of folding makes intramolecular masking of the NLS by the
nonadjacent Ser/Thr-phosphates unlikely. In a folded protein,
elements widely separated in sequence space may end up close
in three-dimensional space, thus favoring their interaction
temporally. However, because the NFAT regulatory
domain does not fold on phosphorylation, intramolecular
contacts between the NLS and Ser/Thr phosphates are strongly
disfavored by a large entropic penalty.

The present analysis applies equally well to intramolecular
masking of the NES and indeed provides a rationale for the
recent observation that Cn is required to prevent nuclear export
of NFAT4 mediated by CRM-1 (28). In the regulatory domain of
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NFAT (39). Previous work has mapped the Cn interaction region
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and this cross-isoform competition has not been analyzed in vivo (30, 44). We find that neither the CnbP-A nor CnbP-B peptide alone from NFATc blocks the Cn/NFATc interaction, but both peptides together block effectively (Fig. 2c). These data are consistent with a model wherein Cn binds cooperatively to both CnbP-A and CnbP-B, but the strength of the interaction at each site varies among the individual isoforms. For instance, the CnbP-B sequence on the surface of Cnbp, resulting in unique thermodynamic and kinetic behavior for each isoform. These fundamental variations in binding strength at the individual CnbP sites are integrated on the surface of Cn, resulting in unique thermodynamic and kinetic behavior for each isoform. These fundamental physical differences create a mechanism for differential responses to Ca2+ stimulation among NFAT isoforms.

There exists a great deal of interest in small-molecule agents that selectively target the Cn/NFAT interaction, while leaving unaffected the interaction of Cn with its other substrates. The wild-type NFATp CnbP-A peptide and an affinity-optimized derivative of it potentially inhibit the Cn-dependent functions of NFAT, but the Cn activity on other proteins and non-NFAT-dependent promoters (30, 36). These results have raised the prospect that drugs targeting the CnbP-B docking site on Cn might act as selective immunosuppressants. In a similar vein, it is to be expected that effective competition at the CnbP-B docking site also will result in immunosuppression. However, given that CnbP-B and CnbP-A docking sites on Cn are distinct, targeting of each should produce distinct biological and pharmacologic responses.

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