Synergism between the Calmodulin-binding and Autoinhibitory Domains on Calcineurin Is Essential for the Induction of Their Phosphatase Activity

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Elevation of the intracellular calcium concentration is necessary for cell growth and the activation of several lymphokine genes. The immunosuppressive drugs cyclosporin A and FK506 profoundly inhibit the calcium-dependent signaling pathway in T lymphocytes by interfering with the activity of the calcium/calmodulin (CaM)-dependent serine/threonine phosphatase, calcineurin (CN). Little is known, however, about how activation of CN enzyme activity or interaction with its substrate, nuclear factor of activated T cell (NF-AT), is regulated. We show here that the binding of CaM to CN may affect the conformation of CN at both the CaM-binding and the autoinhibitory (AI) domains and that this is critical for activation of CN to dephosphorylate NF-AT. Dissociation of the AI domain from the enzyme active site on CN leads to a conformational change at the AI domain from the cytoplasmic to the nuclear form by exposing the DNA-binding domain on NF-AT, and the nuclear form of NF-AT brings CN together into the nuclei. We therefore propose that the activation of CN by the CaM binding independently regulates the interaction with NF-AT and the dephosphorylation of NF-AT.

CN1 (also known as PP2B), a calcium- and CaM-dependent protein serine/threonine phosphatase, plays an important role in T cell receptor (TCR)-mediated signal transduction, leading to the transcriptional activation of cytokines such as IL-2, IL-4, interferon-γ, and granulocyte-macrophage colony-stimulating factor (1, 2). The function of CN in T cells has been identified as a common target for two immunosuppressive drugs, cyclosporin A and FK506 (3, 4). The cyclophilin-cyclosporin A and FKBP-FK506 complexes independently associate with calcineurin and inhibit its protein phosphatase activity. CN is a member of the serine/threonine protein phosphatase family. Unlike other members, however, the activation of CN is totally dependent on the elevation of intracellular calcium concentrations (5, 6). This phosphatase is a heterodimeric complex composed of a catalytic subunit (CN-A) and a calcium-binding regulatory subunit (CN-B) (6). The A subunit contains several distinct functional regions, including a catalytic domain (encoding amino acids 1–323), a B-subunit binding region (encoding amino acids 324–369), a CaM-binding domain (encoding amino acids 391–414), and an autoinhibitory (AI) domain (encoding amino acids 467–521) (6–10). At low calcium concentrations, the AI domain is thought to block the enzyme active site within the catalytic region, keeping CN inactive (10, 11). The TCR signal elevates the intracellular calcium concentration, leading to the activation of CaM, which interacts with the CaM-binding domain of the CN-A subunit. The binding of activated CaM may regulate the initiation of phosphatase activity through a conformational change at the AI domain (6). This notion is supported by the reports showing that a truncated mutant of the A subunit (residues 1–596) (ΔCaM-AI) (12–14) or the A subunit with a point mutation within the AI domain (15) possessed constitutive activity.

The best defined substrate for CN is NF-AT, which is a critical regulator of early gene transcription in the activation process induced by the TCR-mediated signal (2). The consensus motif for NF-AT has been identified in the promoter region of multiple cytokine genes, including IL-2, IL-4, IL-3, granulocyte-macrophage colony-stimulating factor, interferon-γ, and tumor necrosis factor-α (1). Four independent NF-AT genes, NF-AT1 (NF-ATp, NF-ATc2), NF-AT2 (NF-ATc, NF-ATc1), NF-AT3 (NF-ATc4), and NF-AT4 (NF-ATc3) have been identified, and recent studies have illustrated that each member of this family has a distinct physiological role (16–20). All molecules have the ability to transactivate the promoter containing the recognition site for NF-AT. The NF-AT molecules show striking sequence homology in the DNA-binding domain (DBD) containing the region responsible for nuclear transport and moderate sequence homology in the region NH2-terminal of DBD (20–22). This region is responsible for the interaction with CN and the masking of the nuclear localization signal. NF-ATs are localized in the cytoplasm of resting T cells, and TCR-mediated activation of CN through binding to CaM/Ca2+ results in the dephosphorylation of NF-AT, leading to its translocation into the nucleus.
the nucleus (23–27). Recent studies have shown that activated CN co-migrates with the cytoplasmic subunit of NF-AT into the nucleus, and the co-migration may be required for sustained activation of NF-AT in the nucleus (20, 27, 28). Moreover, it is possible that CN interacts with several other molecules, including the protein kinase A anchoring protein (AKAP79), the inositol 1,4,5-trisphosphate receptor, ryanodine receptors, and Cabin (Cain) (29–35). Previous studies have also provided evidence that CN plays an important role in the central nervous system. For example, it has been demonstrated that transgenic mice expressing the active form of CN show a suppression of the intermediate phase of long term potentiation (36).

Although the function of NF-AT has been well characterized, how NF-AT activity is regulated by the interaction with CN, and even how CN phosphatase activity itself is regulated still remain obscure. Thus, we assessed the role of the functional domains in CN-A subunit using a series of truncation derivatives. Truncation of the COOH-terminal region containing both the CaM-binding and the AI domains (CNΔCaM-AI) conferred constitutive phosphatase activity. Truncation mutant experiments clearly indicated that the deletion of the AI domain was sufficient for the interaction with NF-AT but not for activation of CN. The additional deletion at the CaM-binding domain was required for activation of CN to dephosphorylate NF-AT. We further discuss the activation process of CN and the interaction with NF-AT that alters the intracellular distribution of CN.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—The T cell hybridoma line 68-41 was established by fusion between the TCRα-β BW5147 and 2A-2 cloned cells as previously described (14). Anti-GFP mAb and anti-T7 mAb were purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA) and Novagen, Inc. (Madison, WI), respectively. Anti-mouse NF-ATc1 and NF-ATc2 mAb were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Plasmid Construction—Mouse CN Aα cDNA encoding amino acids 1–521 was kindly provided by Randall L. Kincaid (Veritas, Inc., Potomac, MD) (8). A series of truncation mutants was constructed by amplification with polymerase chain reaction based on a full-length cDNA (Figs. 1A and 2A), and those fragments encoding amino acids 1–396, 414, 437, or 460 (ΔCaM-AI, ΔAI-414, ΔAI-437, or ΔAI-460) were cloned into the Bgl II site of pEGFP-C1 (CLONTECH Laboratories, Inc.). The deletions of the CaM-binding domain at positions 397–404, 405–414, and 397–414 were introduced by the polymerase chain reaction method on CN or ΔAI-460 (ΔCaM M1, 2, 3 or ΔCaM M1-AI, ΔCaM M2-AI, ΔAI). The active site at the positions 148–152 was deleted from CN and ΔCaM-AI to construct CN IA and CN ΔCaM-AI IA. Mouse NF-AT1 cDNA was kindly provided by Dr. Anjana Rao (Harvard Medical School, Boston, MA) (37). The GFP-tagged NF-AT DNA-binding domain (DBD) was constructed by insertion of the NF-AT1 fragment encoding amino acids between 335 and 694 into the XhoI/Sall site of pEGFP-C1. T7-tagged NF-AT1 was constructed by insertion into the NotI/XbaI site of pRC/CMV double-T7.

Transfection and Luciferase Assay—Cells were co-transfected with 5 μg of IL-2 or IL-4 reporter construct, 2 μg of pSV2-neo plasmid, 10 μg of pEGFP-C1 CN constructs. The luciferase construct controlled by the mouse IL-2 or IL-4 promoter region was introduced into COS7 cells with pEGFP-C1 CN wild type or ΔCaM-AI, and after 36 h the cells were stimulated with ionomycin (1 μM). The GFP-tagged CN protein in the cell lysates was immunoprecipitated by anti-GFP mAb and protein A-Coupled Sepharose 4B. The supernatant was measured by an assay buffer (100 mM Tris-HCl (pH 8.0), 200 mM NaCl, 12 mM MgCl2, 1 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 0.2 mM CaCl2) containing 0.5 μM CaM, and the amount of free phosphate was measured by a RII phosphopeptide to estimate phosphatase activity.

Immunoprecipitation and Western Blotting—pEGFP-C1 CN wild type or mutant constructs plus pRcCMV double-T7 NF-AT1 were transfected into COS7 cells with DOPSER Liposomal Transfection Reagent (Roche Molecular Biochemicals). The cells were stimulated with 1 μM ionomycin for various time periods and were extracted in a lysis buffer (100 mM potassium phosphate (pH 7.8), 0.2% Triton X-100, 1 mM dithiothreitol, 10 μg/ml leupeptin, and 100 μg/ml aprotinin). The GFP-tagged CN protein was immunoprecipitated with anti-GFP mAb and protein A-Coupled Sepharose 4B. After running a SDS-polyacrylamide gel electrophoresis, the association with NF-AT1 was detected by immunoblotting with anti-T7 mAb. The membrane was probed with horseradish peroxidase-conjugated anti-mouse Ig Ab (DAKO, Glostrup, Denmark).

RESULTS

The CaM-binding and Autoinhibitory Domains Mutually Control the Process of Calcineurin Activation—To assess the role of the CaM-binding and autoinhibitory domains on the activation of CN, a series of truncation derivatives of CN was established (Fig. 1A). The truncated forms were transfected into T cell hybridoma 68-41 cells with luciferase reporter constructs controlled by either the IL-2 or the IL-4 promoter. After stimulation with PMA, PMA plus ionomycin (P + I), or anti-TCR mAb, the activation of NF-AT by CN was assessed according to promoter activity. Each recombinant protein was tagged at its N terminus with GFP, and fluorescence-activated cell sorting analysis showed that 15–20% of the transfectants transiently expressed the recombinant CN molecules (data not shown). The expression of wild-type CN initiated significant enhancement of IL-2 and IL-4 promoter activity when cells were stimulated with either PMA plus ionomycin or TCR cross-linking. The expression of CNΔCaM-AI in the T cell line drastically increased the IL-2 and IL-4 promoter activity in the absence of a calcium signal (Fig. 1B). As we have previously described (14), the expression of CNΔCaM-AI was sufficient for the full activation of the IL-4 promoter even in the absence of the activation of the mitogen-activated protein kinase cascade. Immunoprecipitated CNΔCaM-AI molecule without calcium activation exerted the same level of phosphatase activity as the wild-type CN precipitated from the ionomycin-activated transfectant (Fig. 1C), suggesting that the constitutive activation of promoter activity could have resulted from the constitutive phosphatase activity of CNΔCaM-AI.

The calcium-independent phosphatase activity of CNΔCaM-AI could be due to the deletion of the autoinhibitory domain (AI domain). We therefore tested whether the induction of CN enzyme activity was simply regulated by the AI domain that occupied the active site. To clarify this, we generated plasmid containing CNΔAI-460, -437, and -414, which sequentially deleted the COOH-terminal side of the CaM-binding domain, and the phosphatase activity of these mutants was evaluated according to the IL-2 promoter activity. As shown in Fig. 1B, all mutants containing the CaM-binding domain required ionomycin stimulation for the activation of the IL-2 promoter activity (Fig. 1B), indicating that the
The deletion of the AI domain was insufficient for the induction of CN phosphatase activity. A, organization of functional domains CN-A and construction of CN-A deletion mutants. The catalytic subunit, CN-B binding helix (B), CaM-binding domain (CaM), and AI domain (AI) are indicated by shaded areas. B, the deletion of the AI domain was insufficient for the activation of IL-2 and IL-4 promoter activity in the absence of calcium signal. The GFP-tagged wild-type, ΔAI-414, ΔAI-437, or ΔAI-460 and ΔCaM-AI CN were co-transfected with the IL-2 or IL-4 promoter construct into 68-41 cells. The cells were stimulated with PMA, PMA plus ionomycin, or anti-TCR mAb for 12 h. Following the stimulation, the emitted light was measured, and the relative light unit was represented as promoter activity. The data represent the mean and S.D. of the promoter activity obtained from independent three experiments. C, the overexpression of ΔCaM-AI CN exerted a constitutive phosphatase activity. COS7 cells were transfected with pEGFP-C1 CN wild-type or ΔCaM-AI, and after 36 h the CN were stimulated with ionomycin (1 μM). The CN protein was immunoprecipitated by anti-GFP mAb and protein A-coupled Sepharose 4B. The amount of free phosphate was measured by a RII phosphopeptide to estimate phosphatase activity. The data represent the mean and S.D. of three independent experiments. WT, wild type.

In light of the above result, we next evaluated the functional significance of the CaM-binding domain in the activation of the IL-2 promoter activity using three mutants (CNΔCaM M1–3) that have deletions within the CaM-binding region (Fig. 2A). These mutants induced far below the level of IL-2 promoter activity induced by CNΔCaM-AI when stimulated with PMA alone (Fig. 2B). Thus, it is likely that the activation of CN was regulated not only by the CaM-binding domain but in conjunction with other elements, most likely the AI domain. To test
The interaction between CN-A and NF-AT1 was independent of the presence of the CN phosphatase activity. A, the ionomycin stimulation was required for the interaction between CN-A and NF-AT1. The pEGFP-C1 CN wild type or ΔCaM-AI was co-expressed with the pRec/CMV D-T7 NF-AT construct in COS7 cells. After stimulation with ionomycin for 10 min, 30 min, 1 h, and 4 h, the cell extracts were immunoprecipitated by anti-GFP mAb. The interaction with NF-AT1 was detected by immunoblotting with anti-T7 mAb. B, the deletion of the enzyme active site or the AI domain on CN was sufficient for the interaction with NF-AT. The pEGFP-C1 CN deletion mutant of the enzyme active site (CN IA), the AI domain (CN ΔAI-460), or the CaM-binding domain (CN ΔCaM M3) was co-expressed with NF-AT in COS7 cells. The interactions between CN-A and NF-AT1 with or without ionomycin stimulation were analyzed as described in the legend to Fig. 3A, C, the 13 amino acids from the N terminus side on CN were sufficient for the interaction with NF-AT1. The truncation mutants (1–13, 1–44, 1–226, 1–270, and ΔCaM-AI) were co-expressed with NF-AT1 in COS7 cells, and the interactions with NF-AT1 without ionomycin stimulation were analyzed as described in the legend to Fig. 3A.

In order to identify the region masked by the interaction between the AI domain and the active site, we investigated the target sequences in CN that interact with NF-AT1. A series of truncated mutants extending from the NH2 terminus of CN to the activity of the deletion mutants was estimated allowing it to release the masking. We further investigated the core sequence of the CaM-binding domain that regulated the CN phosphatase activity. Eight residues of the NH2-terminal half (CN CaM M2) and 10 residues of the COOH-terminal half (CN CaM M1) in the CaM-binding domain (residues 397–414) were deleted from CN M3-AI, and the CN activity of the deletion mutants was estimated following stimulation with PMA alone. Both mutants showed significant IL-2 promoter activity (Fig. 2C), indicating that the entire region of the CaM-binding domain was involved in the regulation of CN activity.

The Binding of CaM to CN Affects the Conformation of the AI Domain to Regulate the Interaction with NF-AT—Our data clearly illustrate the requirement of synergy between the CaM-binding and AI domains for the regulation of CN activation. We next examined whether the same synergism is necessary for the interaction of CN with its substrate, NF-AT. GFP-tagged CNs were co-transfected with T7-tagged NF-AT1 into COS7 cells, and the association of NF-AT1 with CN was detected by anti-T7 antibodies after immunoprecipitation with anti-GFP antibodies. As shown in Fig. 3A, lanes 1–5, NF-AT1 was coprecipitated with wild-type CN when cells were stimulated with ionomycin, indicating that the interaction between the two molecules was dependent on the activation of CN. Under these conditions, the activated CN caused a significant mobility shift in the NF-AT1 molecule due to dephosphorylation (Fig. 3A, white arrow). The active mutant, CN ΔCaM-AI, similarly showed a significant interaction with NF-AT1 (Fig. 3A, lane 6). However, NF-AT1 was co-precipitated with the inactive CN mutant lacking the enzyme active site (CN IA), indicating that the interaction between NF-AT1 and CN was irrespective of CN enzyme activity. NF-AT1 was co-precipitated with the mutant lacking the AI domain (CN ΔAI-460) (Fig. 3B, lanes 5 and 6), while the mutant lacking the CaM-binding domain (CN ΔCaM M3) failed to interact with NF-AT1 even after the calcium activation (Fig. 3B, lane 8). Therefore, these results suggest that the association between the active site and the AI domain masks the binding site for NF-AT1 and that the binding of CaM may modify a conformation of CN at the AI domain, allowing it to release the masking.

In order to identify the region masked by the interaction between the AI domain and the active site, we investigated the target sequences in CN that interact with NF-AT1. A series of truncated mutants extending from the NH2 terminus of CN were generated, and their association with NF-AT1 was studied by co-transfection of the mutants and NF-AT1 into COS7 cells. NF-AT1 again required ionomycin stimulation for association with the wild-type CN (Fig. 3C, lane 8), while CN ΔCaM-AI associated with NF-AT1 in the absence of iono-
mycin (Fig. 3C, lane 7; see also Fig. 3A). NF-AT1 was co-precipitated with the mutant CNs that deleted the downstream region from the B subunit-binding domain (CNβ-CaM-AI), whereas the interaction of these two molecules was independent of the presence of B subunit (Fig. 3C, lane 5). NF-AT1 was co-precipitated with all four CN truncation mutants (1–13, 1–44, 1–226, and 1–270), indicating that the N-terminal 13-amino acid region is sufficient for the interaction with NF-AT1 (Fig. 3C, lanes 2–6).

Role of the Interaction between CN and NF-AT in the Nuclear Transport of CN and NF-AT—It was reported that both CN and NF-AT are coincidentally translocated into the nucleus following the activation of CN (28). We speculated that the interaction between CN and NF-AT played an important role in determining the intracellular localization of these two molecules. The mechanisms regulating the nuclear transport of NF-AT have been well characterized, and the import and export of NF-AT between the cytoplasm and the nucleus are mutually regulated by the nuclear localization signal (NLS) and nuclear export signal on the NH2-terminal side of NF-AT (27). In fact, when GFP-tagged NF-AT1 was transfected into NIH3T3 and T cells, it was exclusively localized in the cytoplasm in the resting stage (Fig. 4A, a and c). Co-expression of the active form of CN resulted in the nuclear import of NF-AT1 (Fig. 4A, b and d), indicating that dephosphorylation by CN was essential for the nuclear translocation of NF-AT. However, the GFP-tagged DNA binding loop of NF-AT1 (NF-AT1 DBD) was restricted to the nucleus in the presence of activated CN (Fig. 4A, f and h). These results indicate that the NF-AT1 DBD itself has the ability to localize in the nucleus and that the remaining NH2-terminal part of NF-AT1 plays a regulatory role for the localization of NF-AT1.

We subsequently examined the subcellular localization of CN in NIH3T3 and 68-41 cells. Wild-type CN was exclusively localized in the cytoplasm of both cell lines (Fig. 4B, a and f) and T cell line showed the migration of CN into the nucleus following ionomycin stimulation (data not shown). The CN mutants lacking either the AI domain (CNΔAI-460 and ΔAI-414) or the CaM-binding domain (CNΔCaM M3) were localized in the cytoplasm (Fig. 4B, j–l), while the active CN mutants (CNΔCaM-M1 and ΔCaM M3-AI) were localized in the nucleus of 68-41 cells (Fig. 4B, g and m). These results indicate that the nuclear import is tightly correlated with their phosphatase activity. In contrast, fibroblast cell line NIH3T3 cells failed to show nuclear localization of CN even when the active form of CN was transfected (Fig. 4B, b). We speculated that the distinct localization of CN in NIH 3T3 cells may be due to a lack of NF-AT expression because the expression of NF-AT1 and -2 was below detectable levels in NIH3T3 cells (Fig. 4C). Indeed, the active form of CN localized in the nucleus when co-transfected with NF-AT1 (Fig. 4B, c), indicating that the presence of the cytoplasmic form of NF-AT is essential for the CN nuclear import. Therefore, NF-AT plays a role in the recruitment of CN into the nucleus, and the interaction between CN and the dephosphorylated NF-AT may be crucial for the recruitment of CN into the nucleus.

**DISCUSSION**

In this paper, we provide evidence that the binding of CaM to CN may affect the conformation of CN at both the CaM-binding and AI domains and that this process is critical for the induction of their phosphatase activities. The interaction between CN and NF-AT, however, occurs independently of the CN enzyme activity. The dissociation of the AI domain from the enzyme active site by the binding of CaM was sufficient for the enhancement of accessibility to NF-AT. Since mutant CN lacking the enzyme active site can associate with the cytoplasmic form of NF-AT, the association may occur prior to the process of dephosphorylation of NF-AT. After interaction with NF-AT, CN dephosphorylates NF-AT to alter its conformation from the cytoplasmic subunit to the nuclear form. The activated NF-AT plays an important role in the recruitment of CN associating with NF-AT into the nucleus.

Previous reports showed that synthetic peptides encoding the AI domain have the ability to neutralize CN enzyme activity in vitro (10). Thus, it has been hypothesized that the con-
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FIG. 5. Scheme of predicted mechanisms of activation of CN and the interaction with NF-AT. NES, Nuclear export signal.

constitutive activity in the truncation mutant, CNΔCaM-AI, was a consequence of the lack of the AI domain, which masks the enzyme active site (15). However, the deletion mutant experiments described in the present study provided evidence that the deletion of the AI domain was insufficient for the constitutive phosphatase activity and that further deletion of the CaM-binding domain was required. We speculate that, in the wild-type CN, the binding of activated CaM may cause a conformational change at both the CaM-binding and AI domains and generated a similar situation to that of the active form of CN. This possibility was supported by recent crystal structure analysis of the native CN molecule showing that the structure of the COOH-terminal side from the CaM-binding domain was not visible (38, 39). Thus, it is likely that the COOH-terminal side from the CaM-binding domain may be structurally unstable.

We showed that the binding site for NF-AT was blocked in wild-type CN, while the site was exposed in mutant CN lacking the AI domain. Recent crystal structure analysis has provided direct evidence that the polypeptide encoding the AI domain had the ability to directly interact with the enzyme active site (39). Thus, it is conceivable that occupation of the active site by the AI domain may create a loop structure (AI loop) that blocks the binding of NF-AT. The binding of the calcium-activated CaM may introduce a conformational change at the AI domain, which in turn releases the loop structure by the displacement of the AI domain from the active site. Since the release of the AI loop was essential for the binding of NF-AT, the displacement of the AI domain is a pivotal step in the interaction between NF-AT and CN.

Recently, two binding sites for CN on NF-AT1 have been identified besides the serine-rich and putative NLS masking domains. The first was independent of CN phosphatase activity (40), while the second required the presence of phosphatase activity (41). NF-AT4, however, showed independence of the phosphatase activity in its interaction with CN, and its binding site on NF-AT4 was at residues 104–114 (27). Our data are consistent with the latter observation, because the truncation mutant of CN lacking the enzyme active site could interact with NF-AT1. Although the conformational change of the AI domain by the binding of calcium-activated CaM may be crucial to release the masking of the NF-AT1 binding site, phosphatase activity is not essential for the interaction between NF-AT1 and CN. Thus, the cytoplasmic form of NF-AT1 can interact with the CN that is activated by the binding of CaM.

We identified the N-terminal 13 amino acids of the CN molecule as a region that interacts with NF-AT. However, the 13 residues are unique for the α isoform of the CN A-subunit used in our study, while the NH₂ terminus in other isoforms, β and γ, has a completely distinct amino acid sequence (42, 43). Thus, our results raise questions about whether different isoforms of CN can interact with NF-AT in different ways or whether each isoform interacts with distinct NF-AT family molecules. It also remains to be determined whether the dephosphorylation process of NF-AT is important for the interaction in CN-α, β or γ molecules. Further investigation of the interaction between CN-α, β or γ and NF-AT1 may provide a clearer answer to these questions. Moreover, the binding site for NF-AT1 on CN differs from that for of AKAP79, which has been identified at residues 30–98 and 311–336 of the CN-A (44), implying that CN may interact with different substrates by different binding mechanisms.

It has been reported that nuclear transport of the activated CN occurred concomitantly with the nuclear translocation of NF-AT (27, 28). We demonstrated that the nuclear transport of active CN is strictly dependent on the presence of NF-AT. Dephosphorylation by CN is known to lead to a conformational change of NF-AT that releases the masking of the NLS region, which converts the cytoplasmic form of NF-AT into the nuclear form (1, 20). The truncated mutant of NF-AT that lacks the NH₂-terminal side automatically localizes in the nucleus independently of the dephosphorylation by CN, suggesting that the NH₂-terminal half of NF-AT plays a role in keeping NF-AT in the cytoplasm. This is consistent with previous reports that a NF-AT4 mutant with deletion of a NLS masking region constantly migrated into the nucleus without the addition of calcium activation (27). Since the localization of NF-AT influences the distribution of CN, it can be reasonably assumed that
nuclear form of NF-AT associated with CN translocates into the nucleus. Recently, Zhu and McKeon (45) demonstrated that the nuclear export signal on the NH2-terminal side of NF-AT4 regulates the export of NF-AT4. They found that the nuclear export of NF-AT4 is mediated by Crm1 and that the transfected constitutively active form of CN antagonizes the Crm1-mediated nuclear export of NF-AT4 (45). This suggests that the recruitment of the active form of CN by NF-AT is required for sustaining a transcriptional activity of NF-AT in the nucleus. We speculated that Crm1 also shares the same 13 residues that we identified in the interaction between Crm1 and NF-AT4 (45). This suggests that the nuclear export of NF-AT4 is mediated by Crm1 and that the NF-AT4 regulates the export of NF-AT4. They found that the nuclear export signal on the NH2-terminal side of NF-AT4 is converted from the cytoplasmic to the AI domains on CN, leading to activation of CN. Upon dephosphorylated NF-AT has a role in the recruitment of CN and NF-AT1

Finally, a summary of our model is provided in Fig. 5. In the resting stage of T cells, CN is negatively regulated by the AI of CN. As a consequence of the TCR-mediated elevation of the intracellular calcium concentration, activated CaM binds to CN and then cancels the negative regulation by AI. The binding of CaM may alter the conformation at both the CaM-binding and AI domains on CN, leading to activation of CN. Upon dephosphorylation, NF-AT is converted from the cytoplasmic to the nuclear form by releasing the masking its NLS region. The nuclear form of NF-AT associated with CN translocates into the nucleus and remains until it is exported from the nucleus.

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REFERENCES
1. Rao, A., Luo, C., and Hogan, P. G. (1997) Annu. Rev. Immunol. 15, 707–747
2. Crabtree, G. R., and Clipstone, N. A. (1994) Annu. Rev. Biochem. 63, 1045–1083
3. Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., and Snyder, S. H. (1995) Cell 83, 463–472
4. Clipstone, N. A., and Crabtree, G. R. (1993) Annu. N. Y. Acad. Sci. 696, 20–30
5. Shendili, S. (1994) Annu. Rev. Cell Biol. 10, 55–86
6. Klee, C. B., Ren, H., and Wang, X. (1998) J. Biol. Chem. 273, 13567–13570
7. Watanabe, Y., Perrino, B. A., Chang, B. H., and Soderling, T. R. (1995) J. Biol. Chem. 270, 456–460
8. Kinecaid, R. L., Giri, P. R., Higuchi, S., Tamura, J., Dixon, S. C., Marietta, C. A., Amorese, D. A., and Martin, B. M. (1990) J. Biol. Chem. 265, 11312–11319
9. Srikumar, R., Haddy, A., MacKevie, S., Mertz, P., Litwiller, R., and Rusnak, F. (1995) Biochemistry 34, 8348–8356
10. Hashimoto, Y., Perrino, B. A., and Soderling, T. R. (1990) J. Biol. Chem. 265, 1924–1927
11. Parsons, J. N., Wiederrecht, G. J., Salowe, S., Burbaum, J. J., Rokosz, L. L., Kincade, R. L., and O’Keefe, S. J. (1994) J. Biol. Chem. 269, 19610–19616
12. Clipstone, N. A., and Crabtree, G. R. (1992) Nature 357, 695–697
13. O’Keefe, S. J., Tamura, J., Kincade, R. L., Tocci, M. J., and O’Neill, E. A. (1992) Nature 357, 692–694
14. Kubo, M., Kincade, R. L., Webb, D. R., and Ransom, J. T. (1994) Int. Immunol. 6, 179–188
15. Fruman, D. A., Pai, S. Y., Burakoff, S. J., and Bierer, B. E. (1995) Mol. Cell. Biol. 15, 3857–3863
16. McCaffrey, P. G., Luo, C., Kerpapla, T. K., Jain, J., Badalain, T. M., Ho, A. M., Burgeon, E., Lane, W. S., Lambert, J. N., Curran, T., Verdin, G. L., Rao, A., and Hogan, P. G. (1993) Science 262, 750–754
17. Northrop, J. P., Ho, S. N., Chen, L., Thomas, D. J., Timmerman, L. A., Nolan, G. P., Adron, A., and Crabtree, G. R. (1994) Nature 369, 497–502
18. Hoey, T., Sun, Y. L., Williamson, K., and Xu, X. (1995) Immunity 2, 461–472
19. Shibasaki, J., Shibasaki, F., Price, E. R., Guillemot, J. C., Yano, T., Dotsch, V., Wagner, G., Ferrara, P., and McKeon, F. (1998) Cell 93, 851–861
20. Shibasaki, F., Price, E. R., Milan, D., and McKeon, F. (1996) Nature 382, 379–373
21. Jayaraman, T., Brillantes, A. M., Timmerman, A. P., Fleischer, S., Erdjument-Bromage, H., Tempst, P., and Marks, A. R. (1992) J. Biol. Chem. 267, 9474–9477
22. Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnett, G. V., and Snyder, S. H. (1995) Cell 83, 463–472
23. Cameron, A. M., Nucifora, F. C., Jr., Fung, E. T., Livingston, D. J., Aldape, R. A., Rose, C. A., and Snyder, S. H. (1997) J. Biol. Chem. 272, 25732–25738
24. Brilliante, A. B., Ondrias, R., Scott, A., Kobrinsky, E., Ondriasova, E., Moschella, M. C., Jayaraman, T., Landers, M., Ehrlich, B. E., and Marks, A. R. (1994) Cell 77, 513–523
25. Coglian, V. M., Perrino, B. A., Howard, M., Langeberg, L. K., Hicks, J. B., Gallatin, W. M., and Scott, J. D. (1995) Science 267, 108–111
26. Sun, L., Youn, H. D., Loh, C., Stolow, M., He, W., and Liu, J. O. (1998) Immunity 8, 703–711
27. Lai, M. M., Burnett, P. E., Wolosker, H., Blackshaw, S., and Snyder, S. H. (1998) J. Biol. Chem. 273, 18325–18331
28. Winder, D. G., Mansuy, I. M., Osmann, M., Moallem, T. M., and Kandel, E. R. (1998) Cell 92, 25–37
29. Jain, J., McCaffrey, P. G., Miner, Z., Kerpapla, T. K., Lambert, J. N., Verdin, G. L., Curran, T., and Rao, A. (1993) Nature 365, 352–355
30. Griffith, J. P., Kim, J. L., Kim, E. E., Sintchak, M. D., Thomson, J. A., Fitzgibbon, M. J., Fleming, M. A., Caron, P. R., Hsiao, R., and Navia, M. A. (1995) Cell 82, 507–522
31. Kissing, C. R., Parbe, H. E., Knighton, D. R., Lewis, C. J., Pelletier, L. A., Tempczyk, A., Kalish, V. J., Tucker, K. D., Showalter, K. E., Moeznaw, E. W., Gastin, L. N., Habuka, N., Chen, X., Maldonado, F., Barker, J. E., Ibaquet, R., and Villafranca, J. E. (1995) Nature 378, 641–644
32. Aramburu, J., Garcia-Cozar, F., Baghavan, A., Okamura, H., Rao, A., and Hogan, P. G. (1998) Mol. Cell. 1, 627–637
33. Garcia-Cozar, F., Okamura, H., Aramburu, J. F., Shaw, K. T. Y., Pelletier, G., Ferrara, P., and McKeon, F. (1998) J. Biol. Chem. 273, 23877–23883
34. Giri, P. R., Higuchi, S., and Kincade, R. L. (1991) Biochemistry Res. Commun. 181, 252–258
35. Muramatsu, T., Giri, P. R., Higuchi, S., and Kincade, R. L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 529–533
36. Kashishian, A., Howard, M., Loh, C., Gallatin, W. M., Hoekstra, M. F., and Lai, Y. (1998) J. Biol. Chem. 273, 27412–27419
37. Zhu, J., and McKeon, F. (1999) Nature 398, 256–260
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