The Linker Region Joining the Catalytic and the Regulatory Domains of CnA Is Essential for Binding to NFAT*§

Received for publication, August 27, 2004, and in revised form, January 24, 2005 Published, JBC Papers in Press, January 25, 2005, DOI 10.1074/jbc.C400401200

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Calcineurin (CN), a well-known calcium/calmodulin-regulated serine/threonine phosphatase, is a major cellular sensor of calcium influx. CN is ubiquitously expressed and is highly conserved from yeast to human (1, 2). CN is a heterodimer composed of a catalytic/calmodulin-binding subunit, calcineurin A (CnA), which is tightly bound to a calcium-binding regulatory subunit, calcineurin B (CnB). Studies in gene-targeted mice have shown CN to be important in the regulation of developmental processes such as the patterning of the vascular system and the cardiac and skeletal muscle growth. In adults, CN plays an essential role in the activation of T cells. All these events are controlled by the tight binding and regulation of transcription factors of the NFAT family (3, 4). This family of transcription factors comprises five members, and four of them, named NFATc1–NFATc4 (HUGO nomenclature) are regulated by CN, which dephosphorylates them to promote nuclear translocation and subsequent NFAT-dependent gene expression (5). The CN/NFAT signaling pathway is the target of the immunosuppressive actions of cyclosporin A and FK506. These drugs, once bound to their cellular partners, block the active site of CN, inhibiting its phosphatase activity (6, 7). As a result, NFAT activation, essential for the immune response, is inhibited. However, treatment with these immunosuppressants is associated with severe side effects that are thought to be mediated through the inhibition of CN substrates other than NFATs (8). Although the motifs within the regulatory domain of NFAT mediate interaction with the catalytic subunit of CN (CnA) (4), the specific sequence(s) within CN involved in this interaction has not been described.

EXPERIMENTAL PROCEDURES

Reagents—Isopropyl β-D-thiogalactopyranoside was purchased from Apo-lo Scientific. Glutathione (GSH) beads were purchased from Amersham Biosciences. Anti-FLAG (M2 and M5) monoclonal antibodies and FLAG-agarose beads were purchased from Sigma-Aldrich. Anti-HA antibody-containing supernatant was generously provided by Dr. I Crespo. Oligonucleotides and linker peptide (NCSPHPYWLNPFF) were synthesized at Isogen. VIVIT (MAGHPVIVITGHPPE) and Control (MVGIPVAIHGTP-PHEE) peptides were obtained from the Centro de Biología Molecular “Severo Ochoa” protein synthesis facility.

Plasmid Constructs—The plasmid constructs are detailed in the supplemental data.

GST Fusion Protein Expression—All the GST fusion proteins were expressed in Escherichia coli strain BL-21. The overnight culture was diluted 1/20 and grown until the A550nm was 0.6–0.8. Production of fusion protein was induced by adding up to 1 mM isopropyl β-D-thiogalactopyranoside to the culture and incubating for 3 h at 37 °C with vigorous shaking. The cell pellet was collected by centrifugation, suspended in 10 mM EDTA containing PBS, and sonicated. After addition of Triton X-100 up to 1%, cell debris was discarded by centrifugation, and the soluble proteins were purified with GSH-Sepharose 4B beads (Amersham Biosciences), and the bound GST fusion proteins were stored at 4 °C. The amount of purified GST protein was confirmed by running an SDS-PAGE gel followed by Coomassie staining.

Cell Culture, Transient Transfections, and Cell Extracts—HEK-293 and HeLa cells were cultured in 10% fetal bovine serum containing Dulbecco’s modified Eagle’s medium medium supplemented with L-glutamine plus antibiotics (penicillin/streptomycin). The HEK transient transfections were performed by the modified calcium phosphate method (10). HeLa cells were transfected with Lipofectamine Plus reagents (Invitrogen) following the manufacturer’s recommendations.

Cells transfected cells were washed with PBS, scraped from plates, and collected by centrifugation at 4 °C. Cells were then lysed by adding 100 μl of either binding buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 6 mM MgCl2, 1.5 mM CaCl2, 0.2% Triton X-100) for FLAG-tagged transfections or 1 mM CaCl2 supplemented 1% Triton X-100 buffer (50 mM Tris, 1 mM MgCl2, 1.5 mM CaCl2, 0.2% Triton X-100) for GST fusion proteins. The cell lysates were incubated overnight with 10 μg of either GST fusion protein or anti-FLAG antibody-containing supernatant at 4 °C. The antibody-containing supernatant was obtained from the 12CA5 clone. The antibody-containing supernatant was incubated overnight with 10 μg of wild-type GST or GST fusion proteins. The antibody-containing supernatant was obtained from the 12CA5 clone.
Calcineurin-NFAT Interactions

To identify the region of CN that interacts with NFAT, we generated deletion constructs of the α isoform of human CNA (Fig. 1A and supplemental Fig. 1) and expressed them in HEK-293 cells. Only the construct bearing residues 2–389, which conserves the structure of the full-length wild-type protein and encodes a constitutively active isoform (11), specifically interacted with the NFAT2-regulatory domain in pull-down experiments (Fig. 1B). This result suggests that amino acid residues 269–389 are necessary for interaction between these two molecules. To test whether this region mediates specific interaction (rather than simply maintaining conformation), we co-expressed residues 267–388 (CNA\(^{267-388}\)) with NFAT2 and confirmed their in vitro interaction in co-immunoprecipitation experiments (Fig. 1C). Constitutively active CN bound and dephosphorylated NFAT2, while full-length and CNA\(^{267-388}\) bound to phospho-NFAT2 to the same extent. CNA\(^{267-388}\) contains the carboxy end of the catalytic domain, the CNB binding domain, and the linker sequence joining them. The x-ray structure of the CN heterodimer (12, 13) indicated that the only accessible amino acids in the 269–389 region lie within the 13-amino acid linker sequence (residues 335–347). Mutational analysis in yeast has shown that three specific residues within the linker region of yeast CNA are necessary for stress-induced CN activity in vivo (14). Although yeasts lack NFATs, these residues are conserved in all known CN sequences, so we analyzed their potential involvement in the interaction between human CNA and NFAT2. We substituted each of these three amino acids (S337P, H339L, and L343S), alone or in combination, within the sequence encoding residues 2–389 of human CNA. Substituted CNA proteins were transiently expressed in HEK-293 cells, and cell extracts were pulled down with GST-NFAT2. Mutation of any of these residues abolished the interaction between CNA and the regulatory domain of NFAT2 (Fig. 2A). Equivalent amounts of wild-type and mutant CN proteins from HEK-293 transfected cell lysates were used in all these pull-down experiments (see supplemental Fig. 2). In addition, the ability of all mutant CNA proteins to bind CNB was similar to that of wild-type CNA protein (data not shown).

The only functional CN-interacting sequence present in NFAT2 is the PxIxIT motif (PxIxITC2), present in all CN-
protein mutated at position 343 was able to interact with the Cabin-1 and AKAP79 domains as efficiently as was the wild-type CnA sequence (Fig. 2D). The linker region of CN thus appears to be critical in its interaction with these two proteins, and single amino acid substitutions differentially affect the binding of CN to different PxIxIT-containing proteins.

To investigate the impact of these amino acid substitutions on the in vivo protein-protein interaction, we analyzed the dephosphorylation and nuclear import of NFAT. We performed Western blots on extracts of cells co-transfected with NFATc2 and the CnA-(2–389) constructs. Constitutively active wild-type CnA-(2–389) dephosphorylated NFATc2, and substitution of more than one of the identified CN-linker amino acids inhibited this (supplemental Fig. 3). This finding was confirmed and extended by immunostaining, which showed that proteins bearing more than one of the amino acid substitutions are unable to translocate NFATc2 to the nucleus (Fig. 2D). To rule out the possibility that double or triple substitutions were affecting the activity of the catalytic core of CNa, we tested the phosphatase activity of these mutant CnA proteins expressed in HEK-293 cells on the commonly used CN-specific substrate RII peptide (19). The phosphatase activities of lysates from these cells were even higher than those detected in lysates of cells expressing the wild-type protein (Fig. 2E). The failure of CnA mutants to interact with other CN inhibitors might underlie their high phosphatase activity toward the RII phosphopeptide.

To further confirm the role of the CnA linker region in the interaction with NFAT in vivo, we expressed the wild-type linker sequence in the presence of NFATc2 and the constitutively active CN wild-type protein and analyzed the phosphorylation state of NFATc2 by Western blotting. Although single substitutions of CnA had a dramatic impact on the in vitro binding to NFATs, double or triple substitutions were required to block dephosphorylation and nuclear translocation of NFATs in intact cells (Fig. 2D and supplemental data). This is likely to be related to in vivo low affinity interactions due to the higher level of the CnA mutants expressed in transfected cells.

In order to verify the role of the CnA linker region in the interaction with NFATc2, we generated two more constructs by inserting a stop codon right after either the linker region (construct expressing amino acids 2–346) or the catalytic domain (construct containing amino acids 2 to 336; Fig. 3A). These constructs were transfected into HEK-293 cells, and the corresponding cell extracts were employed in pull-down experiments using a GST fusion protein expressing the PxIxITc2 motif as bait (GST-PxIxITc2). Only those constructs containing the linker region were able to interact with the PxIxITc2 motif (Fig. 3B, lanes 1 and 3). Furthermore, these interactions were specifically inhibited by the addition of the VIVIT peptide (lanes 2 and 4). We further tested whether these constructs were able to interact with NFATc2 in vivo. To this end, HEK-293 cells were co-transfected with the new constructs described above and the NFATc2 expression plasmid. In accordance with the results obtained in vitro, we found that only the constructs containing the linker region (389 and 346) were able to translocate NFATc2 to the nucleus (Fig. 3C, left and middle panels). In contrast, the construct of amino acids 2–336, which does not contain the linker sequence, failed to induce the nuclear translocation of NFATc2 (Fig. 3C, right panels). These results demonstrate the involvement of the linker region of CnAα in the interaction with NFAT in vivo.

Finally, we found that the expression of the GFP-linker fusion protein, as well as that of the potent inhibitor GFP-VIVIT (20), blocked the phosphatase activity displayed by constitutively active CN, and hence, NFATc2 was maintained in a regulated NFAT proteins. A number of endogenous CN-binding proteins contain a PxIxIT-like motif within the region that binds to CN (15, 16). We analyzed the interaction of the CnA mutant proteins with the CN-interacting sequence from two of these, Cabin-1 (17) and AKAP79 (18) (Fig. 2C). Only the CnAα

![Figure 2](image-url)
Lysates were analyzed by immunoblotting with anti-HA (wild-type linker region (linker, lane 2) and dephospho-NFATc2. The binding of FLAG-tagged proteins was carried out in the presence of a 12.5 μm concentration of the control peptide (C) or the high affinity PxIxIT-derived peptide VIVIT that inhibits the CN/NFAT interaction. The protein bound to the GST-PxFxITc2 was evaluated by immunoblotting with an anti-FLAG antibody. C: NFATc2 is translocated to the nucleus by CN proteins that contain the linker region. HEK-293 cells were transfected with HA-NFATc2 and the indicated flag-tagged CnA proteins. Subcellular localization of transfected CnA (top) and NFATc2 (bottom) proteins are shown. D, HeLa cells were co-transfected with GFP constructs fused to the wild-type linker region (linker, lane 2) or the VIVIT peptide (lane 1) together with wild-type forms of NFATc2 and constitutively active (c.a.) CN. Lysates were analyzed by immunoblotting with anti-HA (top panel) and anti-GFP (bottom panel). Arrowheads indicate the positions of phospho- and dephospho-NFATc2.

Fig. 3. Requirement of the linker region for the in vitro and in vivo interaction between NFATc2 and CN. A, schematic diagram of the FLAG-tagged human CnA fusion proteins, generated in which the functional domains are underlined. B, the deletion of the linker sequence of CnA specifically impairs the in vitro interaction of CN with NFATc2. Lysates from HEK-293 cells expressing the indicated FLAG-tagged CnA proteins were analyzed by affinity binding to GST-PxFxITc2. The binding of FLAG-tagged proteins was carried out in the presence of a 12.5 μm concentration of the control peptide (C) or the high affinity PxIxIT-derived peptide VIVIT that inhibits the CN/NFAT interaction. The protein bound to the GST-PxFxITc2 was evaluated by immunoblotting with an anti-FLAG antibody. C: NFATc2 is translocated to the nucleus by CN proteins that contain the linker region. HEK-293 cells were transfected with HA-NFATc2 and the indicated flag-tagged CnA proteins. Subcellular localization of transfected CnA (top) and NFATc2 (bottom) proteins are shown. D, HeLa cells were co-transfected with GFP constructs fused to the wild-type linker region (linker, lane 2) or the VIVIT peptide (lane 1) together with wild-type forms of NFATc2 and constitutively active (c.a.) CN. Lysates were analyzed by immunoblotting with anti-HA (top panel) and anti-GFP (bottom panel). Arrowheads indicate the positions of phospho- and dephospho-NFATc2.

highly phosphorylated state (Fig. 3D). In this experimental system, the small amount of DNA encoding the constitutively active CN that can be employed was responsible for the incomplete dephosphorylation of NFATc2. This demonstrates that the linker peptide is able to disrupt the NFAT-CN interaction in vitro and suggests that the linker region could also act as a docking site for NFAT. Further experiments will be necessary to confirm this possibility. In this regard, a docking site for the exogenous VIVIT peptide has been recently described as a composite site that involves the β strands 11 and 14 of the catalytic domain of CN (21). Since the structure of activated CN remains to be defined, particularly in the linker region, this sequence could not be considered in the modeling of the complex performed by these authors. Thus, whether the residues within the linker region may also be involved in this interaction cannot be ruled out. However, our in vitro and in vivo experiments clearly demonstrate that the linker region is essential for the CN and NFAT proteins to interact. Since the linker region is required for NFAT binding (Fig. 3D) and the CN constructs employed by Li et al. (21) contained this region, the interaction of the PxIxIT sequence with the reported docking site is probably only functional in the presence of an intact linker sequence. Taken together, these results indicate that additional regions of CN, other than those reported previously (21), are also involved in the interaction with NFAT, generating a more complex picture for the CN-NFAT interaction.

Mutational analysis of the linker region allowed us to identify residues that affect interaction via this NFAT region. All three substitutions (S337P, H339L, and L343S) severely impaired the in vitro interaction with NFATc2, which only contains a PxIxIT site. The PxIxIT-derived VIVIT peptide is a high affinity NFAT inhibitor (20) and has been proposed as an investigative and therapeutic tool. But the discovery that most identified CN binding proteins contain a PxIxIT-like motif within the region that interacts with CN suggests that this peptide might not be highly selective for NFAT. The finding that a single amino acid substitution (L343S) selectively blocks interaction with NFAT without affecting the interaction of CN with other proteins (Cabin-1 or AKAP79) is of special significance. Reagents that exploit the selective interaction of CN with NFAT through residues of the linker region would be of use in dissecting important biological processes in which the CN/NFAT pathway plays a key role. These include the activation and development of the immune system, patterning of the vasculature, morphogenesis of the heart valves, and muscle and neural development.

Moreover, most side effects of the immunosuppressive drugs CsA and FK506 are thought to be mediated through the inhibition of CN substrates other than NFAT. Specific inhibitors that selectively target the linker region of CN would enable discrimination between binding of NFAT versus other substrates. This fine mapping may thus support the design of less toxic and more specific treatments for diseases where the CN/NFAT pathway is involved, including the pathological situations related to angiogenesis or cardiac and skeletal muscle growth.

Acknowledgments—We are very grateful to Dr. Simon Bartlett and Mark Sefton for excellent editorial assistance. We thank Drs. J. Heitman, I. Crespo, R. Treissman, and A. Rao for providing reagents and plasmids and R. Nieto for technical assistance.

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