Molecular Analysis of the Interaction of Calcineurin with Drug-Immunophilin Complexes*

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The calcium/calmodulin-regulated phosphatase calcineurin (CN) is the site of action of the immunosuppressive drugs cyclosporin A (CsA) and FK506. CN has recently been established as a key signaling enzyme in the T cell signal transduction cascade and an important regulator of transcription factors such as NF-AT and OAP/Oct-1, which are involved in the expression of a number of important T cell early genes. CsA and FK506 act by forming complexes with their respective intracellular receptors cyclophilin and FKBP (immunophilins), which can then bind to CN, inhibiting its enzymatic activity and thereby preventing early gene expression. CN is comprised of two subunits: a 59-kDa catalytic subunit (CNA), which contains a calmodulin binding domain and autoinhibitory region, and a 19-kDa intrinsic calcium binding regulatory subunit (CNB). In this study, we have utilized a series of deletion mutants of the CNA subunit to investigate the subunit and molecular requirements that govern the interaction of CN with drug-immunophilin complexes. The calmodulin binding and autoinhibitory domains of the CNA subunit were found to be dispensable for the binding of CN to drug-immunophilin complexes. In contrast, we found that the regulatory CNB subunit appears to play an obligatory role in this interaction and have defined an amino acid sequence of the CNB subunit which forms the binding site for CNB. Although necessary, the CNB subunit per se is not sufficient to mediate an interaction with drug-immunophilin complexes; amino acid residues of the CNA subunit, specifically a region located within the putative catalytic domain, are also required for the interaction of CN with both FKBP-FK506 and cyclophilin A-CsA.

Cyclosporin A (CsA) and FK506 are microbial products that possess potent immunosuppressive properties and have proven highly effective clinically in the prevention both of allograft rejection and graft-versus-host disease (1–3). The immunosuppressive effects of CsA and FK506 can largely be ascribed to their potent inhibition of the T cell activation-dependent transcription of the T cell growth factor interleukin 2 and other immunologically important T lymphocyte-derived molecules (4–7).

The immunosuppressive properties of CsA and FK506 are manifested via interaction with their cognate intracellular receptors, cyclophilin and FKBP (8–10), respectively, collectively known as the immunophilins (11). The binding of each drug to its cognate receptor imposes a gain of function on the corresponding immunophilin, resulting in the formation of an inhibitory complex that directly interferes with a key Ca2+–sensitive step in the T cell signal transduction cascade (3, 8, 9, 11–13), thereby preventing the activation of specific transcription factors (such as NF-AT and OAP/Oct-1) involved in lymphokine gene expression (14).

Recent studies have afforded new insights into the mechanism of action of these two important immunosuppressant drugs. First, Schreiber and his colleagues used drug-immunophilin affinity chromatography to identify the major cellular target of the drug-immunophilin complex as the Ca2+/calmodulin-regulated serine/threonine phosphatase, CN (PP2B) (15, 16). Both FKBP-FK506 and cyclophilin-CsA complexes were found to completely inhibit the enzymatic activity of CN toward a well characterized model phosphoprotein substrate in vitro. Second, in vivo studies demonstrated that overexpression of CN and CNB subunits was able to render T cells markedly resistant to the inhibitory effects of CsA and FK506 and to augment significantly the activity of calcium-dependent promoters, such as NF-AT, OAP/Oct-1, and interleukin 2 (17–19). Moreover, a calcium-independent, constitutively active mutant of CN was able to synergize with phorbol ester and activate these latter promoters, replacing the normal requirement for an increase in intracellular calcium (18, 19). Taken together, these studies established CN as the in vivo target of the immunosuppressive drugs CsA and FK506 and as a key component of the T cell signal transduction cascade and an important downstream effector of the Ca2+ signal and regulator of T cell transcription factor activity.

CN is comprised of two subunits, a calmodulin-binding 59-kDa catalytic subunit (CNA) and a Ca2+-binding 19-kDa regulatory subunit (CNB) (20–22). In the present study, we have utilized a series of deletion mutants of the CNA subunit to investigate the molecular and subunit requirements that govern the interaction of drug-immunophilin complexes with CN. We have identified an obligate requirement for the CNB subunit in this interaction and have defined the sequences in the CNA subunit which represent the binding site for the CNB subunit. The CNB subunit, although necessary, is not sufficient to mediate binding to drug-immunophilin complexes. Thus, we have additionally identified sequences of the CNA subunit, within the conserved protein phosphatase catalytic domain, which are also essential for the CN/drug-immunophilin interaction.

EXPERIMENTAL PROCEDURES

Materials—The murine CNAc (23) and CNB (24) cDNAs were gifts of Dr. R. L. Kincaid, NIAAA. The plasmid encoding the GST-FKBP-12 (15) was a gift of Dr. S. Schreiber, Department of Chemistry, Harvard University. The plasmid encoding GST-cyclophilin A (25) was obtained from Dr. R. Bram. Yeast strain Y153 and plasmids pS1 and pACT (26)

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were generous gifts of Dr. S. J. Eldridge, Baylor College of Medicine. The plasmid containing the Saccharomyces cerevisiae CNB subunit (27) was provided by Dr. M. Cyert, Stanford University. GAL4-E47 and ACT-C10L were gifts from Drs. L. Naumovski and M. Cleary, Stanford, CA. F5056 was a gift from Fujisawa USA Inc., Ca. was obtained from Sandoz Pharmaceuticals Corp. The VA1 mAb (28) was kindly provided by Dr. J. Wang, Genentech, South San Francisco, CA. Oligonucleotides were purchased from New England Biolabs.

**Plasmid Construction**—For carboxyl-terminal CN deletion constructs, the influenza virus hemagglutinin epitope (EFYFYDPDVYA) (29) was fused in-frame to the amino terminus of murine CN with the polymerase chain reaction (PCR). All CN carboxyl-terminal deletion constructs, except CA355, were generated by PCR using specific oligonucleotide primers and standard methodologies. Typically, reactions were carried out in 10 μl Tris, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 100 μM dNTPs, a 0.25 μM concentration of each primer, and 2.5 units of Taq polymerase (15–26 cycles; 1 min denaturation at 94 °C, 1.5-min anneal at 55 °C, 2-min extension at 72 °C). Amplified DNA fragments were digested with the appropriate restriction endonucleases and introduced into pBluescript II (pBluescript II) using standard molecular cloning techniques. pBluescript II is a derivative of pCDL-SRa296 (30) and contains translational termination codons in all three reading frames immediately downstream of the EcoRI site in the polylinker. pBluescript II is generated by utilizing the Pφaf site in murine CNA, which was rendered blunt with T4 DNA polymerase and fused to the EcoRI site of pBluescript II that had been blunt-ended with the Klenow fragment of DNA polymerase I. For N1-CA394, N1-32, N1-394, and N1-347-CA394, DNA fragments were amplified by PCR with specific oligonucleotide primers and inserted into pBEX1 (25) at the appropriate restriction sites. pBEX1 was derived from pBluescript II by the insertion of a synthetic oligonucleotide that allows open reading frames to be translationally generated by utilizing the existing restriction sites for plasmid reconstructions.

**Polymerase Chain Reaction (PCR)**—The VA1 mAb (28) was kindly provided by Dr. J. Wang, Genentech, South San Francisco, CA. Oligonucleotides were purchased from New England Biolabs. Plasmid Construction—For carboxyl-terminal CN deletion constructs, the influenza virus hemagglutinin epitope (EFYFYDPDVYA) (29) was fused in-frame to the amino terminus of murine CN with the polymerase chain reaction (PCR). All CN carboxyl-terminal deletion constructs, except CA355, were generated by PCR using specific oligonucleotide primers and standard methodologies. Typically, reactions were carried out in 10 μl Tris, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 100 μM dNTPs, a 0.25 μM concentration of each primer, and 2.5 units of Taq polymerase (15–26 cycles; 1 min denaturation at 94 °C, 1.5-min anneal at 55 °C, 2-min extension at 72 °C). Amplified DNA fragments were digested with the appropriate restriction endonucleases and introduced into pBluescript II (pBluescript II) using standard molecular cloning techniques. pBluescript II is a derivative of pCDL-SRa296 (30) and contains translational termination codons in all three reading frames immediately downstream of the EcoRI site in the polylinker. pBluescript II is generated by utilizing the Pφaf site in murine CNA, which was rendered blunt with T4 DNA polymerase and fused to the EcoRI site of pBluescript II that had been blunt-ended with the Klenow fragment of DNA polymerase I. For N1-CA394, N1-32, N1-394, and N1-347-CA394, DNA fragments were amplified by PCR with specific oligonucleotide primers and inserted into pBEX1 (25) at the appropriate restriction sites. pBEX1 was derived from pBluescript II by the insertion of a synthetic oligonucleotide that allows open reading frames to be translationally fused at their carboxyl terminus with the influenza virus hemagglutinin epitope (EFYFYDPDVYA). N1-3547 and N1-3554 were generated by PCR using specific oligonucleotide 5’ primers and a 3’ primer that adds the influenza virus hemagglutinin epitope as a carboxyl-terminal transla-

**RESULTS**

To investigate the molecular requirements for the interaction of CN with drug-immunophilin complexes, we initially generated a series of progressive carboxyl-terminal deletions of the CNA subunit (CNA₁, isozyme (23)) (see Fig. 7) and tested each of them for its ability to interact with either FKBP-FK506 or cyclophilin A-CaA in an in vitro binding assay. The recombinant CN molecules used for these studies were generated by transiently transfecting each of the CNA deletion mutants, together with an expression vector encoding the CNB subunit, into COS-7 cells. To facilitate detection of the ectopically expressed CN molecules, each of the CNA carboxyl-terminal deletion mutants was epitope tagged with a peptide epitope from influenza virus hemagglutinin (HA), which is recognized with high affinity by the 12C5 antibody (29). SDS-PAGE analysis of transiently transfected COS-7 cell extracts followed by immunoblotting with the 12C5 antibody revealed that each of the carboxyl-

**Drug-Immunophilin Binding and Immunoprecipitation**—Glutathione S-transferase immunophilin fusion proteins were prepared as described previously (25). Preformed immobilized GST-FKBP-12 fusion protein-FK506 or GST-cyclophilin A fusion protein CsA complexes (10 μg) were mixed with aliquots (50 μl) of extracts derived from transiently transfected COS-7 cells. CSAs were prepared by incubation with 200 μl of lysis buffer for 2 h at 4 °C. After extensive washing with ice-cold lysis buffer, immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with the VA1 mAb (28). For analysis of total cell extracts, 8 μl of the transfected cell extract was analyzed by SDS-PAGE and immunoblotting with the 12C5 antibody. Immunoblotting was performed as described in detail (31), using primary antibodies at a dilution of 1/1,000 in an acetic fluid. Immunoreactive bands were visualized using the ECL system (Amersham Corp.) according to the directions provided by the manufacturer.
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Identification of carboxyl-terminal CNA subunit sequences required for the interaction with drug-immunophilin complexes. Panel A, total cell extracts derived from COS-7 cells transiently cotransfected with pBJ5-CN and the indicated HA epitope-tagged carboxyl-terminal CNA deletion construct were analyzed by SDS-PAGE and immunoblotting with the 12CA5 mAb. Panels B and C, immunoblot analysis with the 12CA5 mAb of the carboxyl-terminal CNA deletion mutants specifically bound to either immobilized GST-FKBP.FK506 (panel B) or GST-cyclophilin A-CysA complexes (panel C).

is included in the binding buffer (Fig. 2). In contrast, we found that CΔ394, which lacks the carboxyl-terminal 127 amino acids including both the calmodulin binding site and the autoinhibitory domain (see Fig. 7), interacted with drug-immunophilin complexes in the presence of EGTA in an apparently calcium-independent fashion (Fig. 2). Hence the CN carboxyl-terminal domain containing the calmodulin binding site is required for the calcium-dependent interaction of wtCN with drug-immunophilin complexes.

The region of the CNA subunit between amino acids 355 and 376 is located carboxyl-terminal to the phosphatase homology domain and is contained within a region of CNA which is highly conserved between species (21, 23, 33-36). This high degree of conservation indicates that this region might represent the binding site for the regulatory CNB subunit (21). To test this hypothesis, each of the carboxyl-terminally truncated CN mutants was immunoprecipitated with the 12CA5 mAb, and the immunoprecipitates were analyzed by immunoblotting with the VA1 mAb (28), which is specific for the CNB subunit. As shown in Fig. 3, the CNB subunit was immunoprecipitated with wtCNA, CΔ394, and CΔ376, but not further carboxyl-terminal truncations of the CNA subunit. In addition, the CNB subunit was also found to be associated with wtCNA, CΔ394, and CΔ376 eluted from drug-immunophilin complexes (data not shown). Thus, it appears that sequences of the CNA subunit between amino acids 355 and 376 are required for the interaction of CNA with CNB. Interestingly, as shown above, this same region also appears to be required for the interaction of CN with drug-immunophilin complexes.

Given the concordance between the amino acid sequences of the CNA subunit required for the interaction with both drug-immunophilin complexes and the CNB subunit, we next attempted to determine whether CNB itself was required for the interaction between drug-immunophilin complexes and the CNA subunit. To this end we took advantage of the yeast two-hybrid system (26, 37). This system allows the analysis of protein-protein interactions, by virtue of the ability of GAL4 chimaeric fusion proteins to reconstitute GAL4-dependent transcription. Accordingly, the 1-147 amino acid residue DNA binding domain of GAL4 was fused in-frame to human FKBP-12 sequences (GAL-FKBP), whereas the GAL4 activation domain was fused with the CΔ394 fragment (ACT-CNA394) of CNA. We monitored the interaction between GAL-FKBP and ACT-CNA394 by utilizing the Y153 indicator yeast strain (26). This strain of yeast, which is a histidine auxotroph and harbors a disrupted endogenous GAL4 gene, contains the His-3 gene under the control of a GAL4-dependent promoter. Growth of this strain on media lacking histidine therefore re-
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Panel

Accordingly, we generated a CNB null in Y153 by targeteding GAL-FKBP and either ACT-CNA394 or ACT-CNA332. transformed with the indicated plasmids was plated onto selective media in the presence of FK506. As expected, since FK506 is required to promote the control, Y153 was also transformed with GAL4-E47 and ACT-IyI was readily observed (Fig. 4C). Thus, it
appears that the CNB regulatory subunit is necessary for the interaction between FKBP.FK506 and CN, whereas the control interaction between GAL-FKBP and ACT-CNA332 did not promote growth even in the presence of FK506 (Fig. 4B). Thus, in the yeast two-hybrid system, as in vitro, FKBP interacts with CN in a strictly FK506-dependent fashion and exhibits the same sequence requirements. Having established the integrity of the interaction between FKBP and CN in the two-hybrid system, we next investigated whether CNB was necessary for this interaction. Accordingly, we generated a CNB null in Y153 by targeted disruption of the endogenous yeast CNB locus and then transformed this mutant strain (Y153ΔCNB) with plasmids encoding the same chimaeric fusion proteins as those used above. In contrast to the wild type Y153 strain, we were unable to detect an FK506-dependent interaction between GAL-FKBP and ACT-CNA394 in the mutant strain (Y153ΔCNB) containing the disrupted CNB gene, whereas the control interaction between GAL4-E47 and ACT-IyI was readily observed (Fig. 4C). Thus, it appears that the CNB regulatory subunit is necessary for the interaction of CN with the FKBP-FK506 complex.

As a result of the clearly important role of the CNB regulatory subunit in the interaction between FKBP-FK506 and CN, we wished to define the CNA subunit sequences required for binding CNB. In preliminary experiments we had established that the CNB binding site was located in the carboxy-terminal half of the CNA subunit distal to the phosphatase homology domain. To define further the sequences required for CNB binding we generated further amino-terminal truncations of the CNA subunit, epitope-tagged at the carboxyl terminus with the HA epitope, and tested them for their ability to coprecipitate the CNB subunit from transiently transfected COS-7 extracts. The expression of these amino-terminal truncation mutants is shown in Fig. 5A. For unknown reasons the N364 fragment runs aberrantly on SDS-PAGE, with a slower mobility than the N347 mutant. Extracts from COS-7 cells cotransfected with the amino-terminal CNA mutants and a plasmid encoding the CNB subunit were immunoprecipitated with the 12CA5 mAb and the immunoprecipitates analyzed by immunoblotting with the CNB-specific mAb, VA1. As shown in Fig. 5B, CNB was coimmunoprecipitated with N347 but not N364. Furthermore, a fragment of CNA encompassing amino acid sequences 347–394 was sufficient to bind CNB (N347-C394; Fig. 5). Taken together with the results from Fig. 3, since C376 and not further carboxy-terminal truncations of CNB were able to bind CNB, it appears that the CNA subunit sequences between 347 and 376 form the minimal binding site for the regulatory CNB subunit.

Having established the essential role of the CNB subunit in the interaction between FKBP-FK506 and CN, we wished to establish whether the CNB subunit itself was sufficient to mediate binding to drug-immunophilin complexes. Accordingly, we tested the ability of the amino-terminal truncations of CNA from Fig. 5 to bind to either GST-FKBP-12-FK506 or GST-cyclophilin A-CsA complexes. As shown in Fig. 6, B and C, we were unable to detect any interaction between either N347 or N347-C394 and drug-immunophilin complexes even though both mutants interact with CNB. Thus, although necessary, CNB is not sufficient for binding to FKBP-FK506 and cyclophilin-CsA, indicating that sequences from the CNA subunit also play a major role in mediating the interaction of CN.
with drug-immunophilin complexes. To define those amino acid sequences of the CNA subunit required for binding, we tested the ability of a series of progressive amino-terminal truncations of CNA to bind to both FKBP-FK506 (Fig. 6B) and cyclophilin A-CsA (Fig. 6C). Thus, extracts from COS-7 cells transiently cotransfected with each of the HA epitope-tagged amino-terminal CNA deletion mutants specifically bound to either immobilized GST-FKBP-FK506 (panel B) or immobilized GST-cyclophilin A-CsA complexes (panel C).

**DISCUSSION**

The inhibitory effects of the FKBP-FK506 and cyclophilin A-CsA complexes on the enzymatic activity of CN underlie the molecular basis of action of these potent immunosuppressive drugs (15, 17–19). In the current study, we have investigated the subunit requirements and the molecular sequence determinants that govern the interaction of CN with drug-immunophilin complexes (Fig. 7). Analysis of progressive carboxy-terminal deletion mutants of CNA revealed that the autoinhibitory and calmodulin binding domains were completely dispensable for the interaction with drug-immunophilin complexes. Thus, CN mutants CA394 and CA376, which lack both the autoinhibitory and calmodulin binding domains, interacted strongly with both FKBP-FK506 and cyclophilin A-CsA complexes (Fig. 1, B and C). Further truncation of the CNA subunit to amino acid 355, however, completely abolished the ability of CN to bind to drug-immunophilin complexes.

The interaction of wtCN with drug-immunophilin complexes is strongly calcium-dependent and appears to be stimulated in the presence of calmodulin (15). In contrast, the interaction of drug-immunophilin complexes with CA394 occurs in the presence of 25 mM EGTA and consequently appears to be calcium-independent (Fig. 2). These results are therefore consistent with the notion that the CN carboxyl terminus normally precedes the interaction of drug-immunophilin complexes with CN and that in the presence of elevated calcium a conformational change occurs in the CN molecule which makes the drug-immunophilin binding site accessible. The role of either calmodulin or the calcium-binding regulatory CNB subunit in this putative conformational change remains to be fully explored.

The region of CNA between amino acids 328 and 375, which lies immediately distal to the protein phosphatase homology region, is highly conserved in CNB subunits from a wide variety of species (21, 23, 33–36). This has led to the speculation that this region is involved in the binding of the regulatory CNB subunit (21, 35). Consistent with this notion is the observation that CNB binds to CA376, but not further carboxy-terminal truncations of the CNA subunit (Fig. 3). Thus, CNA sequences located between amino acid 376 and 394 are sufficient to mediate CNB binding. Moreover, a polypeptide corresponding to the sequence between amino acids 347 and 394 of the CNA subunit is sufficient to mediate binding of CNB (Fig. 5). Taken together, these results indicate that a 29-amino acid region between amino acids 347 and 376 most likely forms the minimal CNB binding site, but do not rule out the possibility that other CNA amino acid sequences may influence the affinity of the CNB interaction with the CNA subunit.

The observation that carboxy-terminal truncations of the CNA subunit which failed to bind the regulatory CNB subunit also failed to bind to drug-immunophilin complexes, prompted us to investigate the potential role of CNB in the interaction of CNA with the drug-immunophilin complex. In this regard, utilizing the yeast two-hybrid system, we were able to show that the regulatory CNB subunit was in fact necessary for the interaction of the CNA subunit with FKBP-FK506 (Fig. 4). This result is in accord with both the 1:1 stoichiometry of the CNA/CNB subunits isolated after drug-immunophilin chromatography (15) as well as recently published enzymatic (39), biochemical (40) and genetic evidence (41). Although necessary, however, it appears that the CNB subunit per se is apparently not sufficient to mediate an interaction with drug-immunophilin complexes, since the CN amino-terminal mutants N399, NΔ347, and NΔ347-NΔ394, which all bind to CNB (Fig. 5 and data not shown), fail to interact with FKBP-FK506 and cyclophilin A-CsA complexes (see Fig. 6, B and C). This notion is further supported by the observations that the isolated CNB subunit is not retained on a drug-immunophilin affinity column (38) and is incapable of being directly biochemically cross-linked to drug-immunophilin complexes (40, 44).
From the binding properties of the CN amino-terminally truncated mutants, it appears that sequences of the CN subunit between amino acids 32 and 99 are also required for the interaction of CN with the drug-immunophilin complex (Fig. 6). However, in apparent disagreement with this finding, Husi et al. (44) have recently demonstrated chemical cross-linking of FKBP-FK506 complexes to the CNB subunit in the presence of a minimal CNA fragment comprising only amino acids 332–390. It should be noted, however, that chemical cross-linking can potentially identify weak or transient interactions between molecules, whereas the affinity chromatography assay used in the present study is a more demanding test of intermolecular interactions. Whether our observed requirement for amino acids 32–99 indicates that this region is directly involved in the interaction with drug-immunophilin complexes or alternatively, is simply required for the structural integrity of the CN catalytic domain, thereby conferring an appropriate structural conformation that permits a high affinity interaction with drug-immunophilins, is not known.

Why is the CNB subunit required for the interaction of CN with drug-immunophilin complexes? Certainly, the requirement for the CNB subunit can in part explain why FKBP-FK506 and cyclophilin-CsA specifically interact with CN and not with the related serine/threonine phosphatases, PP1 and PP2A (15, 38). In this respect, although CN, PP1, and PP2A all share a common homologous protein phosphatase catalytic core (23, 33–36), only CN interacts with the CNB regulatory subunit (20–22). One possible explanation for the CNB requirement is that the primary interaction between CN and the drug-immunophilin complex may indeed be with the CNB subunit. However, it is clear from the analysis of the CNA amino-terminally truncated mutants that the CNB subunit per se is not sufficient to mediate an interaction with drug-immunophilin complexes. Thus, in this model the association of the CNA subunit with CNB would appear to be a prerequisite to confer an appropriate conformation on CNB to permit the interaction with drug-immunophilin complexes. However, since wtCN, NΔ32, NΔ99, NΔ347, and NΔ347-NΔ394 all bind CNB (Fig. 3, 5, and data not shown), yet only wtCN and NΔ32 bind drug-immunophilin complexes (Fig. 6), the conformation of CNB when bound to wtCN and NΔ32 would have to be fundamentally different than when bound to the other CN mutant proteins. A second possibility is that the CNB subunit is simply required to confer an appropriate conformation onto the catalytic CN subunit, which then allows CNA to interact directly with the drug-immunophilin complex. A third more parsimonious possibility, however, is that the CNA and CNB subunits both make direct contact with the drug-immunophilin complex.

Several recent studies have used cross-linking approaches in an attempt to identify the subunit of CN which directly contacts the drug-immunophilin complex (40, 42, 44). In two of the studies, drug-immunophilin complexes were selectively cross-linked to the CNB subunit (40, 44) but only in the presence of the CNA subunit; cross-linking to isolated CNB subunits was not observed. In the other case, the CNA, but not the CNB subunit, was selectively cross-linked (42). The apparent disparity between these studies may be explained by the different chemical nature of the cross-linking reagents used, which may bias for or against potentially accessible amino acid residues available for cross-linking. Despite this lack of consensus, these cross-linking data indicate that drug-immunophilin complexes are most likely in very close approximation to both the CNA and the CNB subunits, although the exact contact sites remain to be identified.

Interestingly, none of the CN mutants described in the current study was able to discriminate between FKBP-FK506 and cyclophilin-CsA, consistent with previous findings that FKBP-FK506 and cyclophilin-CsA appear to compete for the same binding site on CN (15, 40). Structure-function analysis of the FKBP-12-FK506 interaction with CN has revealed the importance of several charged residues in FKBP-12 which appear to be involved in making direct contact with CN. These residues have defined a 100 Å² surface patch that corresponds to the putative CN binding site (43). It will be interesting to identify the corresponding residues on CN which are involved in mediating the interaction with drug-immunophilin complexes. In this regard, the CN two-hybrid system described herein, combined with a random mutagenesis approach, should prove useful for rapidly identifying contact residues in CN. Ultimately, however, either x-ray crystallographic or NMR structural analysis will be required to resolve the precise three-dimensional relationship of CN with drug-immunophilin complexes.

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