The interaction between the catalytic A subunit of calcineurin and its autoinhibitory domain, in the yeast two-hybrid system, is disrupted by cyclosporin A and FK506

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Abstract **The Ca^{2+}-calmodulin dependent protein phosphatase, calcineurin, is thought to mediate the action of the two immunosuppressants, cyclosporin A (CsA) and FK506. Calcineurin from all species consists of a catalytic A subunit and a regulatory peptide B, which plays an essential role in catalysis. The enzymatic function is probably also regulated by an autoinhibitory domain (AID) present in the catalytic subunit. We have used the yeast two-hybrid system to show that the putative AID of the yeast catalytic subunit Cna1 binds only to truncated Cna1, devoid of AID. Although deletion of the genes encoding the yeast catalytic subunits of calcineurin (*CNA1* and *CNA2*) maintain the interaction, absence of the regulatory subunit Cnb1 prevents binding. Interestingly, both CsA and FK506 disrupt this interaction, whereas binding of Cna1 to calmodulin remains unaffected. This indicates that a simple cellular system, developed in yeast, could provide further insight into an understanding of calcineurin inhibition.**

Key words: Autoinhibitory domain; Calcineurin; Cyclosporin A; FK506; Inhibitor; Yeast two-hybrid system

1. Introduction

The immunosuppressive drugs, cyclosporin A (CsA) and FK506, exert their major therapeutic effect by inhibiting T cell activation. CsA is widely used for prevention of graft rejection after organ or bone marrow transplantation. The more recently discovered FK506, which is 10- to 100-times as potent as CsA, can effectively address similar problems and has shown great promise in early clinical trials [1].

CsA is a cyclic undecapeptide whereas FK506 is a macrolide [1,2]. Despite their structural differences, the two drugs have almost indistinguishable effects in the cell. The immunosuppressants cause the down-regulation of certain cytokine genes, which is ascribed to the drugs' capacity to interfere in a Ca^{2+}-sensitive T cell signal transduction pathway. Interleukin-2 is one of the genes the transcription activation of which is severely compromised in the presence of CsA or FK506 [1,2].

Recent findings indicate that CsA and FK506 act in the cell by inhibiting the protein phosphatase, calcineurin [1–3]. It has been proposed that the two drugs target calcineurin for inhibition only after they are bound to their respective cytosolic receptors, cyclophilin or FK506 binding protein (FKBP) [4]. The ever- growing family of cyclophilins and the FKBPs are collectively known as the immunophilins. Not only do the immunophilins bind an immunosuppressant, they also share the property of catalyzing the isomerization of a *cis* prolyl moiety in a peptide bond to the *trans* form [3].

Calcineurin is the only known protein phosphatase that is regulated specifically by Ca^{2+} and calmodulin [5]. The calcineurin holoenzyme purified from mammalian cells is a heterodimer consisting of a large catalytic (A) subunit (~59 kDa) and a small regulatory B subunit (~19 kDa) [1,5]. The A subunit binds calmodulin whereas the B subunit attaches to four molecules of Ca^{2+}. Calcineurin is incapable of functioning as a phosphatase in vitro when calmodulin or the B subunit is absent. Although the sequences of the B subunit and calmodulin are related, neither of the two proteins can complement the other's absence [6]. In the presence of Ca^{2+}, calmodulin and the B subunit, the catalytic activity of the calcineurin holoenzyme is completely inhibited by a synthetic peptide which corresponds to a region in the C- terminus of the A subunit [7,8]. This suggests that the catalytic A subunit contains an autoinhibitory domain (AID) [7].

The budding yeast *Saccharomyces cerevisiae* encodes proteins with properties similar to the mammalian A and B subunits of calcineurin. In fact, yeast cells possess two genes, *CNA1* and *CNA2*, homologous to the mammalian A subunit [9,10]. Both the Cna1 and Cna2 proteins contain regions which have similarity to the putative AID sequence of the mammalian enzyme [9]. The B subunit in yeast, which is also conserved amongst different species, is encoded by the *CNB1* gene [11,12].

Inhibition of catalytic activity of an enzyme by an autoinhibitory peptide implies physical association of AID with the active site of the enzyme. We have investigated whether AID of Cna1p would interact with the catalytic part of the protein in a yeast two-hybrid system [13,14]. In this communication it is shown that the two regions bind to each other when a functional Cnb1 protein is present in the cell. Surprisingly, the specific interaction between AID and the catalytic domain is prevented by CsA and FK506. This opens up a unique possibility of identifying the natural ligands which specifically affect calcineurin activity in the cell.

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Abbreviations: AID, autoinhibitory domain; Cna1, protein coding for the catalytic A subunit of yeast calcineurin; Cna2, protein coding for the second A subunit of yeast calcineurin; Cnb1, protein coding for the regulatory B subunit of yeast calcineurin; CsA, cyclosporin A; FKBP, FK506-binding protein; PCR, polymerase chain reaction; *SUC2t*, transcription terminator from the yeast *SUC2* gene.

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2. Materials and methods

2.1. Subcloning of yeast CNA1 and CNB1 gene fragments in the plasmid, pTM9, a vector used for integration of ACE1N fusions

Plasmid pTM9 [14] is a yeast integrating vector (containing the *S. cerevisiae TRP1* sequence as a selectable marker) which encodes the entire promoter of the *ACE1* gene and the segment encoding the DNA-binding domain of the yeast transcription activator Ace1 (i.e. Ace1N) [14]. The 3′ end of the *ACE1N* sequence contains two extra nucleotides (GC) just prior to the *Bgl*II site, which is used for cloning genes contiguous to the *ACE1N* fragment.

Two *Bgl*II–*Hin*dIII fragments of the *S. cerevisiae CNA1* gene, the complete coding sequence (1659 bp) and a truncated version *CNA1Δ* (1516 bp, devoid of *AID* and sequences C-terminal to *AID*) [9], were amplified by the polymerase chain reaction (PCR) [15] using genomic DNA from the wild-type yeast strain S288C (American Type Culture Collection #26108) as template. The primers used were 5′-ATAGATCTAATGTCGAAAGACTTGAATT-3′ (primer 1) and 5′-ATAAGCTTTCACAGTTGTGGCTTTTTCTCC-3′ (primer 2), for *CNA1*, and primer 1 and 5′-ATAAGCTTCTACAAACCTTCAGTCCCACGA-3′ for *CNA1Δ*.

The *Bgl*II–*Hin*dIII fragment of *CNA1* or *CNA1Δ* and a *Hin*dIII–*Sac*I transcription terminator fragment of the yeast *PHO5* gene (a 377 bp segment downstream of the stop codon) [16] were subcloned into pTM9 [14], and completely digested with *Bgl*II and *Sac*I. The resulting plasmids were named pMH6 and pMH7. The gene inserts in the plasmids were confirmed by sequencing.

The coding sequence of *CNB1* (435 bp) [11,12] was isolated as a *Bgl*II–*Eco*RI fragment by PCR, as detailed above, using the primers 5′-ATAGATCTCATGAAATTAGATAGAGATAG-3′ and 5′-ATGAATTCTTACACATCGTATTGCAATG-3′. The *Bgl*II–*Eco*RI fragment and an *Eco*RI–*Sac*I *SUC2* transcription terminator (*SUC2t*) fragment (a 300 bp segment downstream of the stop codon, isolated by PCR) [17] was subcloned into pTM9, digested with *Bgl*II and *Sac*I, for construction of plasmid pMH23.

2.2. Construction of CNA1, CNA2 and CNB disruption plasmids

The *CNA1* and *CNA2* genes (promoter and complete coding sequence) [9], were isolated as *Bam*HI–*Hin*dIII fragments by PCR from the strain S288C. The primers used were 5′-ATGGATCCCATCGCCTCTTGAAACATG-3′ and primer 2 (see section 2.1) for *CNA1*, and 5′-ATGGATCCATAGTCTATAATACGTTTG-3′ and 5′-ATAAGCTTCTATTTGCTATCATTCTTTGCA-3′ for *CNA2*.

The *Bam*HI–*Hin*dIII fragments were subcloned into pUC19 to obtain pMH1 and pMH2. Digestion with *Bst*EII removed 130 bp and 653 bp fragments from the coding sequences of *CNA1* and *CNA2*, respectively. The sticky ends in the purified large fragments from pMH1, pMH2 were flushed with T4 polymerase (Boehringer) and ligated to a blunt-ended *S. cerevisiae URA3* gene fragment [18] to yield the *CNA1* and *CNA2* gene disruption plasmids pMH3 and pMH4, respectively.

The disruption plasmid for *CNB1* was constructed as follows. An *Eco*RI–*Bam*HI fragment, containing the promoter and a part of the coding sequence, was isolated by PCR from strain S288C, using the primers 5′-ATGAATTCGGTAACTCAATGGTGATCAGA-3′ and 5′-ATGGATCCTTGGACCCACGCCCACTGAAA-3′. A *Bam*HI–*Hin*dIII fragment containing a part of the coding sequence and the transcription terminator was isolated by PCR (using again S288C as template) with the two primers 5′-ATGGATCCGACATTGACAAGGACGGTTTC-3′ and 5′-ATAAGCTTCTTATTGTTTGTTACATATAC-3′. The *Eco*RI–*Bam*HI and the *Bam*HI–*Hin*dIII fragments from *CNB1* were subcloned into pUC19 digested with *Eco*RI and *Hin*dIII. The resulting plasmid pUC19/ΔCNB1 has a 34 bp deletion in the coding sequence of *CNB1*. A *Bgl*II *URA3* gene fragment [18] was subcloned into pUC19/ΔCNB1 at the unique *Bam*HI site of ΔCNB1 to obtain the gene disruption plasmid pKO8.

2.3. Construction of plasmids that express Ace1C-AID, Ace1C-(AID)$_2$ and Ace1C

The AID sequences were expressed under the control of the yeast *GAPDH* promoter [14]. The plasmid pRH2 was obtained after subcloning a 393 bp *Bam*HI–*Eco*RI truncated version [14] of the *GAPDH* promoter downstream of the 275 bp *Sal*I–*Bam*HI fragment from pBR322 in pBluescriptII KS$^+$ vector (Stratagene). An *Eco*RI–*Spe*I double-stranded DNA linker (encoding 22 amino acids of the nuclear localization signal from the SV40 large T antigen) [19], was subcloned into pRH2 to yield pRH3. The *Sal*I–*Spe*I fragment from pRH3 and a ~540 bp *Xba*I–*Bgl*II fragment, encoding the Ace1C transcription activation domain [14], were ligated to the *Sal*I–*Bgl*II digested pUC19Bgl (obtained by modifying the *Bam*HI site of pUC19 to a *Bgl*II site) to yield the plasmid pRH10C. The ~540 bp *Xba*I–*Bgl*II *ACE1C* fragment was isolated by PCR from pTM4 [14] using the primers 5′-TATCTAGAGGACGTTCTTTTGGGCCT-3′ and 5′-TAAGATCTGCTTGTGAATGTGAGTTATGC-3′.

The DNA encoding the 42 amino acid peptide, which contains the putative AID sequence (see Fig. 1) was isolated as a *Bgl*II–*Xba*I (~130 bp) fragment by PCR using pMH1 (as a template) and the primers 5′-ATAGATCTGGGTTTGAATGAAACGCTAA-3′ 5′-TATCTAGACTTTAATATTTTTTCGTA-3′. This fragment bears an extra nucleotide at the 5′ end, following the *Bgl*II site, to make the *AID* sequence in-frame with the *ACE1C* domain.

The *Sal*I–*Bgl*II fragment from pRH10C, the *Bgl*II–*Xba*I *AID* sequence and a 300 bp *Xba*I–*Sac*I of the yeast *SUC2t* (see section 2.1) [17] were subcloned into pDP34 [14] to form the plasmid pMH10. This encodes a single copy of the *AID* sequence, which was confirmed by sequencing.

The *Sal*I–*Bgl*II fragment from pRH10C and the *Bgl*II–*Xba*I *AID* sequence were also subcloned into pUC19. The resulting plasmid was named pMH10-19.

pMH10-19 was digested with *Xba*I and a double-stranded linker encoding the peptide SS-GGGG-SS was ligated unidirectionally. A *Sal*I–*Xba*I fragment from the resulting plasmid pMH10-19-G4 and a *Spe*I–*Sac*I *AID-SUC2t* PCR fragment was subcloned into pDP34. The resulting plasmid pMH11 encodes two copies of the *AID* sequence. This has been confirmed by DNA sequencing.

The *Sal*I–*Bgl*II fragment from pRH10C and a 300 bp *Bgl*II–*Sac*I fragment of the yeast *SUC2t* (see section 2.1) [17] were subcloned into pDP34 [14] to yield pRH14C. This plasmid expresses only the Ace1C sequence and is used as a negative control for the study of protein–protein interactions described in this communication.

2.4. Construction of plasmids that express Ace1C-Cmd1

A *Sal*I– *Bgl*II fragment from pRH10C, a *Bgl*II–*Sal*I yeast calmodulin (*CMD1*) gene fragment (containing the 441 bp coding sequence) [20] and a 300 bp *Sal*I–*Sac*I *SUC1* fragment (see section 2.1) [17] were subcloned into pDP34 at the *Sal*I, *Sac*I sites. The resulting plasmid is referred to as pJC2. The authenticity of the *CMD1* fragment, isolated by PCR from the strain S288C using the primers 5′-ATAGATCTAATGTCCTCCAATCTTACCGA-3′ 5′-ATGTCGACCTATTTAGATAACAAAGCAG-3′ has been checked by sequencing.

2.5. Yeast strains and transformations

The *S. cerevisiae* strain TFY2 (*Matα* his ura3–52 trp1–285 ace1 LEU2::*YipCL* CUP1^r) [14] was used for integration of the fusion genes *ACE1N-CNA1* (pMH6), *ACE1N- CNA1Δ* (pMH7) and *ACE1N-CNB1* (pMH23) into the yeast genome. In order to disrupt *CNA1*, *CNA2* and *CNB1* from the yeast genome, the plasmids pMH3, pMH4 and pKO8 were utilized. Integrative transformation of the *ACE1N-CNA1*, *ACE1N-CNA1Δ* and *ACE1N- CNB1* fusions were targeted to the *ACE1* locus. The complete plasmids were thus integrated and transformants were selected as *TRP1* prototrophs [21]. However, after gene replacement at the *CNA1*, *CNA2* and *CNB1* loci, *URA3* prototrophs were selected. To obtain Ura3$^-$ mutants, Ura3$^+$ strains were transformed with *ura3Δ5* [22] and selected in 5-fluoro-orotic acid as described elsewhere [23]. The strain where both *CNA1* and *CNA2* genes are disrupted was constructed from a strain which initially carried Cna1$^-$ and Ura3$^-$ mutants.

For verification of correct integration and gene replacement events, PCRs were performed with primers flanking the insertion sites. The amplified fragments were analysed by agarose gel-electrophoresis and in some cases confirmed by sequencing. The resulting yeast strains, bearing the three integrations, are referred to as yMH6 (*ACE1N-CNA1*), yMH7 (*ACE1N-CNA1Δ*) and yMH23 (*ACE1N-CNB1*). The two strains with gene disruptions have been named TFY2*cna1cna2* (bearing Cna1$^-$ and Cna2$^-$ mutants) and TFY2*cnb1* (which harbors only a deletion in *CNB1*).

For transformation of the 2-micron based plasmids (i.e. the pDP34 derivatives) [14], which encode hybrid genes consisting of *ACE1C* and the gene of interest, the yeast strains yMH6, yMH7 and yMH23 were

used as hosts. All pDP34 derivatives contain the *URA3* gene [18] as the selectable marker for yeast. To substantiate that a plasmid was still maintained in the yeast cell, total DNA from yeast was isolated [24] and transformed into *E. coli* (see below). The DNA derived from the transformants were subjected to restriction enzyme analysis and sequencing [24].

All transformations were performed via electroporation [14,24] using the Bio-Rad gene pulser. The yeast transformants used in this study are described in Table 1.

2.6. Recombinant DNA techniques

Manipulation of DNA was carried out by using standard procedures [24]. *E. coli* HB101 was used for the construction and propagation of plasmids. PCR was performed using Pfu (Stratagene) or Vent (BioLabs) DNA polymerases. Double-stranded DNA was sequenced by the dideoxynucleotide chain-termination method, using the Applied Biosystems 370A automated DNA sequencer.

2.7. β-Galactosidase assay and induction of metallothionein expression

The assays were performed on yeast strains exactly in the way described previously [14]. All strains are TFY2 derivatives and are highly sensitive to Cu^{2+}. TFY2 bears a *CUP1-lacZ* fusion at the *LEU2* locus [14].

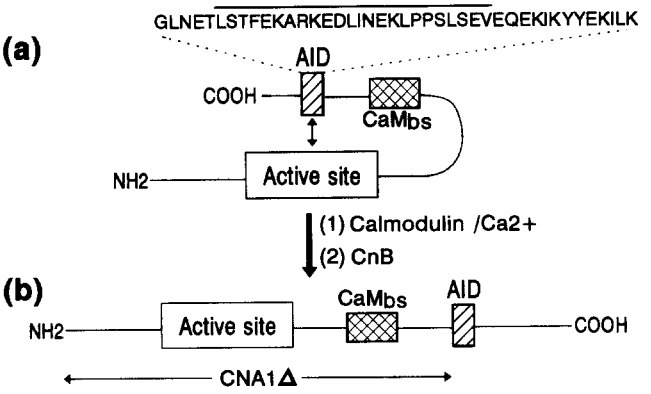

Fig. 1. The catalytic subunit A of calcineurin. The active site possibly interacts with AID in the inactive conformation (a). Ca^{2+}, calmodulin and the regulatory subunit B transforms it to an active state (b) [5,6]. The autoinhibitory domain (AID), the C-terminal truncated Cna1 without its AID sequence (CNA1Δ) and the calmodulin binding site (CaM_{bs}) are depicted. The amino acid sequence of AID from yeast Cna1, used in this work, is shown in (a). The region in this peptide which is similar to mammalian AID [9] is overlined.

3. Results and discussion

Two yeast genes, homologous to mammalian calcineurin A catalytic subunits, have been identified. They are *CNA1* and *CNA2*, also known as *CMP1* and *CMP2* [9,10]. Comparison of the amino acid sequences of Cna1p and Cna2p with that of a mammalian calcineurin A subunit reveals a presumptive calmodulin- binding site and an AID sequence in both the polypeptides [9] (Fig. 1). We have attempted to find out if two specific regions of Cna1p (i.e. AID and the catalytic domain of one of the two A subunits present in yeast) interact with one another, when in *trans*.

There are a variety of in vitro methods with which interactions between proteins can be studied [14]. The yeast two-hybrid system [13,14] provides a convenient way in which the possible association between a peptide and a protein can be examined in a cellular environment. The system depends on reconstitution of a transcription factor from its two essential components, the DNA-binding domain and its activating sequence. The transcription factor, used in our experiments, is the yeast Ace1 protein, which activates transcription from the *CUP1* operon in the presence of divalent copper [14]. The DNA-binding domain of Ace1 is referred to as Ace1N and the activating sequence is known as Ace1C. When expressed as separate domains, the two regions are non-functional.

In our two-hybrid approach [14], two sets of fusion proteins are generated, each consisting of one of the two domains of Ace1. In the fusions, the domains are attached to the two unique proteins which are being tested for their ability to bind to each other. Only when the individual proteins interact can Ace1 be functional again. Depending on the strength of the protein–protein interactions, the activation of gene transcription would vary. In order to study the formation of a functional transcriptional activator, the activation of two genes (the yeast *CUP1* and the *E. coli lacZ*), are monitored in the same cell. While *CUP1* encodes metallothionein, a strong chelator of copper, the *lacZ* gene expresses the β-galactosidase enzyme. *CUP1* gene expression is determined by the levels of toxic copper which the yeast cells can endure during growth [14]. The β-galactosidase expression is measured colorometrically.

The complete *CNA1* gene and a deletion mutant (*CNA1Δ*),

Table 1
Interaction between proteins X and Y (encoded by the gene fusions *ACE1N-X* and *ACE1C-Y*) in the yeast two-hybrid system

Strain	Strain::*ACE1N-X* fusion (integrated in the genome)	Transformed with plasmid	Plasmids bearing *ACE1C* or *ACE1C-Y* fusion
yMH6	TFY2::*ACE1N-CNA1*	pMH10	*ACE1C-AID*
yMH6	TFY2::*ACE1N-CNA1*	pMH11	*ACE1C-(AID)$_2$*
yMH6	TFY2::*ACE1N-CNA1*	pRH14C[a]	*ACE1C*
yMH7	TFY2::*ACE1N-CAN1Δ*	pMH10	*ACE1C-AID*
yMH7	TFY2::*ACE1N-CNA1Δ*	pMH11	*ACE1C-(AID)$_2$*
yMH7	TFY2::*ACE1N-CNA1Δ*	pRH14C[a]	*ACE1C*
yMH6	TFY2::*ACE1N-CNA1*	pJC2	*ACE1C-CMD1*
yMH7	TFY2::*ACE1N-CNA1Δ*	pJC2	*ACE1C-CMD1*
yMH23	TFY2::*ACE1N-CNB1*	pJC2	*ACE1C-CMD1*
yMH23	TFY2::*ACE1N-CNB1*	pRH14C[a]	*ACE1C*
TFY2*cna1cna2*	TFY2*cna1cna2*::*ACE1N-CNA1Δ*	pMH11	*ACE1C-(AID)$_2$*
TFY2*cna1cna2*	TFY2*cna1cna2*::*ACE1N-CNA1Δ*	pRH14C[a]	*ACE1C*
TFY2*cnb1*	TFY2*cnb1*::*ACE1N-CNA1Δ*	pMH11	*ACE1C-(AID)$_2$*
TFY2*cnb1*	TFY2*cnb1*::*ACE1N-CNA1Δ*	pRH14C[a]	*ACE1C*

[a]The plasmid pRH14C was used as a negative control in all experiments where *CUP1* and *lacZ* transcription activation was monitored. pRH14C expresses only the transcription activation domain of Ace1 (i.e. Ace1C).

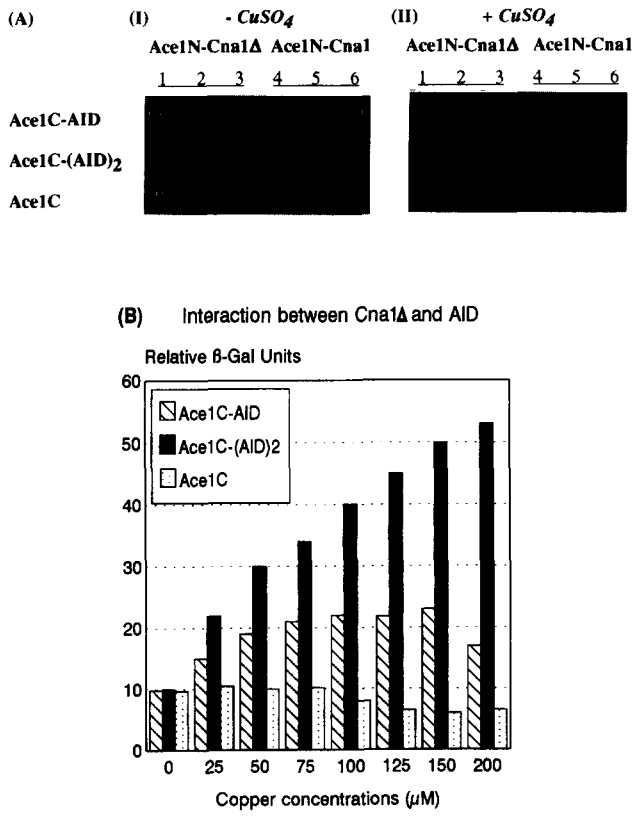

Fig. 2. Interaction between yeast calcineurin A subunit (Cna1 or Cna1Δ) and the autoinhibitory peptide AID (from Cna1), in the yeast 2-hybrid sytem. (A) Induction of metallothionein expression. Equal numbers of cells were spotted onto SD plates containing (I) no $CuSO_4$ and (II) 150 μM $CuSO_4$ ($CuSO_4 \cdot 5H_2O$; Fluka). Columns 1–3, yMH7 expressing Ace1N-Cna1Δ from the genome; columns 4–6, yMH6 expressing Ace1N-Cna1 from the genome. The strains (see Table 1) are transformed with the plasmids pMH10 (Ace1C-AID), pMH11 (Ace1C-$(AID)_2$) and pRH14C (Ace1C). (B) Induction of Cu^{2+}-dependent β-galactosidase expression [14]. Copper concentrations are that of $CuSO_4 \cdot 5H_2O$. Data are expresssed as means ± S.D. deviations from at least three independent transformants.

which lacks the autoinhibitory domain (see section 2) [9], were linked to the DNA-binding domain of *ACE1* (i.e. *ACE1N*). For expression, the hybrid genes (*ACE1N-CNA1* and *ACE1N-CNA1Δ*) are integrated into the genome of the yeast TFY2 (see section 2 and Table 1) [14]. The genome expresses single copies of the fusion proteins. The Ace1C-AID hybrid proteins were expressed from the 2-micron, multi-copy plasmid pDP34 [14]. Two different plasmids were constructed, one containing a single AID sequence and the other which encoded two AID elements. The two elements of AID were separated by DNA encoding a four-glycine spacer (see section 2) which could make the two domains structurally independent of each other. Fig. 2 depicts the interaction between Cna1Δ and AID sequences. The observed strength of interactions is probably dependent on the copies of AID sequences expressed. It should be noted that we have been unable to show any interaction between the complete Cna1 and the autoinhibitory peptides. It is very likely that the active site of Cna1, which binds the autoinhibitory peptide, is concealed when AID is still covalently attached to the calcineurin A subunit (see Fig. 1).

One could reasonably question the relevance of these interactions. In order to prove that the approach was valid, an attempt was made to establish an interaction between either Cna1 or Cna1Δ and one of the yeast calmodulins (viz. Cmd1) [20]. In parallel, we tried to create an interaction between Cmd1 and the regulatory B subunit (Cnb1) [11, 2] of yeast calcineurin. We have been able to substantiate published reports [5,25] which propose that calmodulin binds the catalytic A subunit and not the regulatory B polypeptide (Fig. 3). It seems that interaction of Cmd1 and Cna1Δ is relatively strong compared with its binding to Cna1. This might indicate that the site where Cmd1 binds Cna1 is probably completely unmasked with the removal of AID sequence (see Fig. 1).

It could be well imagined that, in the cell, AID normally covers the active site of calcineurin and prevents further catalysis by the enzyme. Only under specific conditions is an AID sequence thought to be displaced from the catalytic site of an enzyme [7,26,27]. The immunosuppressants CsA and FK506 inhibit the active phosphatase calcineurin after association with the immunophilins [1,2]. It is possible that the drugs and the autoinhibitory peptide target an identical site in the enzyme for inhibition. If so, then CsA or FK506 should prevent AID occupying the active site of calcineurin. Indeed, we do find that the ability of AID to associate with Cna1Δ, in the two-hybrid system, is dramatically impaired in the presence of CsA and FK506 (Fig. 4), and this occurs in a dose-responsive manner (Fig. 4B and C). It is noteworty that only after pre-incubation of the yeast cells with immunosuppressants is it possible to prevent the interaction of AID with CNA1Δ (see Fig. 4). Apparently, the drugs do not have any effect once the interaction is already established (unpublished observations), hinting that AID probably competes with the immunosuppressant–immunophilin complexes for the active site of calcineurin.

Remarkably, in this yeast system, too, FK506 is at least 10-times more potent than CsA. It appears that the effect of the drugs on this interaction is quite specific since the strength of the Cna1Δ–Cmd1 interaction is unperturbed at the same concentrations of CsA and FK506 which have an unmistakable influence on Cna1Δ–AID binding (Fig. 4D).

It has been reported that the regulatory B subunit is required for the phosphatase activity of calcineurin [25]. If the binding of AID to Cna1Δ and the consequent actions of CsA and FK506 on this interaction are physiologically relevant for inhi-

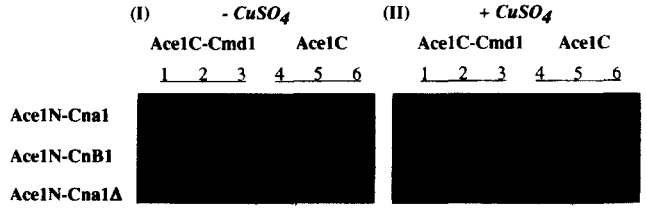

Fig. 3. Interaction of Cna1/Cna1Δ/CnB1 (as Ace1N fusions) with yeast calmodulin Cmd1 (as Ace1C fusion) (see section 2 and Table 1). Induction of metallothionein expression. Equal numbers of cells were spotted onto SD plates containing (I) no $CuSO_4$ and (II) 150 μM $CuSO_4$ (as in Fig. 2). The expressed fusion proteins Ace1N-Cna1 (from the genomic copy of yMH6), Ace1N-Cnb1 (from the genomic copy of yMH23) and Ace1N-Cna1Δ (from the genomic copy of yMH7) (see Table 1) are shown on the left (rows). Columns 1–3, individual integrants are transformed with the plasmid pJC2 expressing the fusion protein Ace1C-Cmd1; columns 4–6, individual integrants transformed with the plasmid which expresses Ace1C alone (pRH14C; see Table 1).

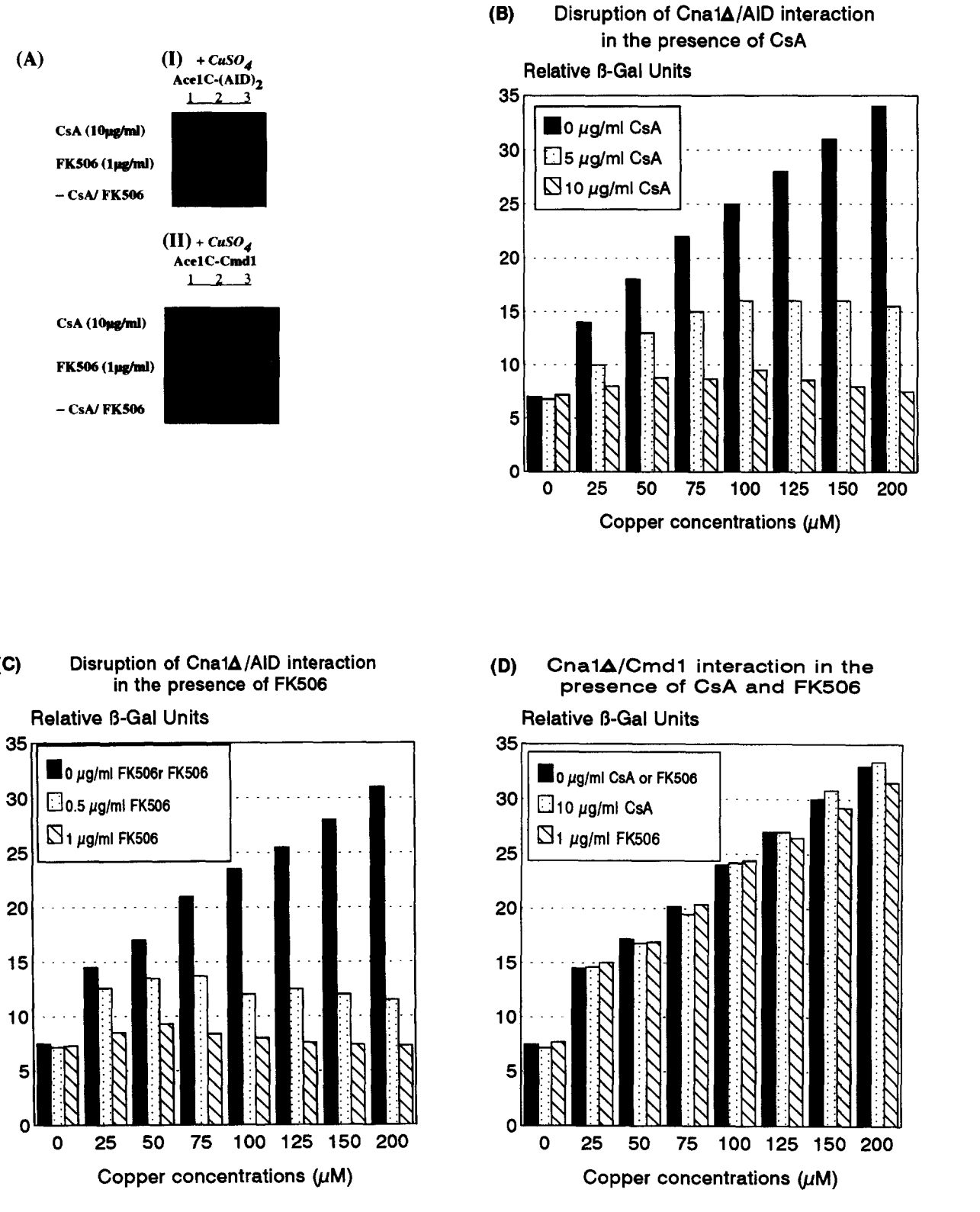

Fig. 4. Induction of metallothionein and Cu^{2+}-dependent β-galactosidase expression in the presence/absence of CsA and FK506. Cells were grown in SD minimal medium (0.67% yeast nitrogen base without any amino acids, 2% glucose) in the presence of CsA/FK506 at 30°C for 16 h. Cells were harvested, washed with SD and the expression for metallothionein and β-galactosidase (at different concentrations of $CuSO_4 \cdot 5H_2O$) was conducted as described previously [14]. β-Galactosidase units are obtained from an average of three independent transformants and are depicted as in Fig. 2. (A) The binding of (I) Cna1Δ to AID and (II) Cna1Δ to Cmd1, in the presence/absence of CsA and FK506 as visualized via metallothionein expression. (B–D) The effect of CsA and FK506 on the interactions of Cna1Δ-AID and Cna1Δ-Cmd1 as monitored by the β-galactosidase assay. The association of Cna1Δ to AID is measured at two different concentrations of CsA (5 and 10 μg/ml) and FK506 (0.5 and 1 μg/ml).

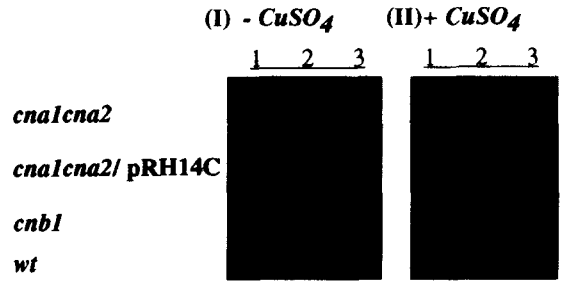

Fig. 5. The Cna1Δ-AID interaction in yeast strains disrupted of the regulatory B subunit and catalytic A subunits (see section 2 and Table 1). The assay for metallothionein expression was performed as in Fig. 2. The three strains TFY2 (*wt*), TFY2*cna1cna2* (*cna1cna2*) and TFY2*cnb1* (*cnb1*), bearing the integrated gene Ace1N-Cna1Δ (see Table 1), are transformed with the plasmid expressing Ace1C-$(AID)_2$ (pMH11). As a control, the TFY2*cna1cna2*::*ACE1N-CNA1Δ* integrant was transformed with pRH14C (which expresses Ace1C only). This row of transformants are depicted as *cna1cna2*/pRH14C.

bition of calcineurin (presupposing that Cna1Δ is enzymatically active in the fusion protein Ace1N-Cna1Δ), then inactivation of yeast Cnb1 should have a profound effect. We have inquired whether, in the absence of *CNB1*, AID interacts with Cna1Δ in the yeast two-hybrid system. By rendering the genomic copy of *CNB1* [11,12] non-functional (see section 2), we find that AID fails to bind Cna1Δ (Fig. 5). This suggests that a C-terminally truncated A subunit, although devoid of its AID sequence, still needs a functional B subunit. Interestingly, this result seems to parallel earlier evidence that inhibition of the catalytic A subunit by cyclophilin–CsA complex requires the B subunit [28]. Our observation strengthens the argument that, if the B subunit is essential for catalysis, its presence should also be necessary for permitting inhibition of the enzyme.

The pertinence of the result with an inactive B subunit was further investigated with a yeast strain which harbors disrupted copies of the two catalytic A subunits, Cna1 and Cna2 [9]. Predictably, the gene disruptions do not prevent binding of the two domains, AID and Cna1Δ (Fig. 5). It has also been shown that the Cna1Δ–AID interaction, developed in the strain TFY2*cna1cna2* (see section 2 and Table 1), can be destroyed by CsA and FK506 and that the disruption is also dose-responsive (data not shown).

The actual establishment of the interaction between an autoinhibitory peptide and the catalytic domain of an enzyme, by the two-hybrid system, could prove to be an interesting sideline. We believe that our studies have opened up a new avenue in which possible associations of autoinhibitory peptides and the corresponding catalytic domains of various protein kinases [26,27] would be amenable to further investigations. It has been postulated that nature has devised the autoinhibitory peptides to be the ideal inhibitors (or substrates) of an enzyme [27]. The approach taken in this work could prove valuable in the search for agonists and antagonists of any enzyme which contains an AID sequence.

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