The role of loop 7 in mediating calcineurin regulation

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Calcineurin (CN) is a heterodimer protein consisting of a 61 kDa catalytic subunit A and a 19 kDa regulatory subunit B. It plays a critical role in T-cell activation and is involved in many cellular processes. Regulation of CN is rather complex, including a number of factors such as divalent metal ions (primarily Ca²⁺ and Mn²⁺), calmodulin (CaM) and autoinhibition (AI) segment. Previously, we reported that a loop 7 deletion mutant (V314) in subunit A exhibited high phosphatase activity, although the mechanism for the surprising activity enhancement and whether the activity change applies to other loop 7 residues were not known. In order to probe the role of loop 7, we have carried out extensive mutagenesis experiments, followed by systematic activity assays under a number of regulatory conditions. All mutants, including single deletion mutants Y315, N316 and double deletion mutant V314X315, showed increased phosphatase activity. Significantly, activities of the mutants containing the V314 deletion, namely V314 and V314Y315, were no longer regulated by regulatory subunit B. These results, along with the structure analysis, suggest that loop 7 as a whole plays an important role in mediating CN’s regulation through bridging the regulatory subunit and catalytic core and interaction with the AI segment of CN.

Keywords: autoinhibition/calcineurin/calmodulin/loop 7/ phosphatase activity

Introduction

Calcineurin (CN) plays a critical role in many cellular processes, such as in T-cell activation, in the cell cycle, and in apoptosis of certain cells. It has been implicated in diseases such as Alzheimer’s disease (AD) and cardiac disease. On the one hand, CN has been demonstrated to play a central role in the immediate, early activation of numerous lymphokines (such as interleukin-2) and in the regulation of cell surface receptors such as CD40L, CD95, and recently, CD25α, and then in the activation of the lymphocytes (Zhao et al., 1995). On the other hand, it has also been recognized for some time that CN is involved in programmed cell death of T and B lymphocytes (Bonnefoy-Berard et al., 1994). In addition, it has been shown that CN plays a role in apoptosis in neuronal cells via the cytochrome c/caspase-3 pathway (Asai et al., 1999). In brain, CN expression and activity are related with AD and brain aging. The hyperphosphorylation of tau protein (a microtubule-associated protein) could generate AD (Maho et al., 1995). In recent years, it has been recognized that CN provides a critical link between Ca²⁺ regulation, synaptic plasticity, cell survival and cognition (Perrino et al., 1998; Thomas et al., 2001). There are two subunits in CN, subunit A (CN-A, catalytic subunit, ~61 kDa in size) and subunit B (CN-B, regulatory subunit, ~19 kDa in size) (Frank and Pamela, 2000). CN-A consists of four domains: a phosphatase domain, a CN-B binding domain, a calmodulin (CaM) binding domain and an autoinhibitory (AI) segment. CN shows no activity in the absence of CN-B and CaM, and the phosphatase activity is enhanced greatly by CN-B and/or CaM. CN-B and CaM regulate the activity of CN through specific association with CN-A and binding to the CaM binding domain, respectively. However, at the molecular and structural level the exact mechanism through which CN-B and CaM affect CN-A and, subsequently, its phosphatase activity, remain to be fully understood.

As revealed by crystal structures (Griffith et al., 1995; Kissinger et al., 1995), the catalytic center in CN-A is formed by two β-sheets, sheet 1 and sheet 2, making a β-sandwich. In this β-sandwich, there is an important loop (loop 7) between β 12 (in sheet 1) and β 13 (in sheet 2). Loop 7 contains approximately eight amino acids, from Y311 to K318. Previously we reported that the preliminary result demonstrated that the loop 7 single deletion mutant (V314) in CN-A exhibited phosphatase activity 10 times greater than that of wild-type CN (Wei and Lee, 1997; Yan and Wei, 1999). This was a rather surprising and unexpected observation. In the current study, in addition to V314 deletion we have generated other deletion mutants in loop 7 (single deletion mutants Y315 and N316, double deletion mutant V314Y315) and carried out comprehensive functional assays in an attempt to clarify the role of this loop in the regulation of CN. Compared with wild-type CN-A, all mutants exhibited markedly higher activity. Furthermore, the experimental results revealed that the activity of the two mutants containing the V314 deletion was no longer regulated by CN-B.

Materials and methods

Mutagenesis and protein production

All chemicals were of reagent grade, unless otherwise stated. Single deletion mutants Y315, N316 and double deletion mutant V314Y315 were constructed using the Altered Sites mutagenesis system. The mutated cDNAs were cloned into the expression vector pET-21a and then transformed into Escherichia coli strain HMS174(DE3) cells. The sequences of all the mutated cDNAs were identified by DNA sequencing and confirmed by the expected pattern. Positive recombinant colonies were successfully demonstrated. The wild-type CN-A and all mutants were expressed and purified following the protocol previously established (Yan and Wei, 1999). Briefly, the transformed E.coli cells were cultured in TM media. IPTG...
(final concentration 16 μM) was added to the culture to induce expression. Four hours later, the cultures were centrifuged at 6000 g for 20 min at 4°C. The pellets were resuspended in buffer A (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 2 mM 2-mercaptoethanol, 0.4 mM PMSF) and then disrupted using ultrasonic waves. The subsequent steps were all done at 4°C. The homogenate was centrifuged at 20,000 g for 30 min; the pellet was discarded and the supernatant was made up to 45% in (NH₄)₂SO₄, stirred for 10 min and incubated on ice for 40 min. The precipitated proteins were collected by centrifugation at 11,000 g for 30 min and resuspended into 2 ml of buffer A, and combined with 10 ml of CaM–Sepharose resin (synthesized in our laboratory) equilibrated in buffer C (50 mM Tris–HCl, pH 7.4, 1 mM EGTA, 1 mM DTT, 0.4 mM PMSF). Wild-type CN-A and all mutant proteins were eluted with buffer C at a flow rate of 1 ml/min. Wild-type CN-A and all mutants were assessed using SDS gels.

**Assay of phosphatase activity**

The activities of wild-type CN-A and deletion mutants were assayed using p-nitrophenyl phosphate (pNPP) as the substrate, with Mn²⁺ as the activator. The experiment was carried out in assaying buffer (50 mM Tris–HCl, pH 7.4, 1 mM MnCl₂, 0.5 mM DTT, 0.2 mg/ml BSA and 20 mM pNPP. Reactions were performed in a 0.2 ml volume at 30°C for 20 min and terminated by the addition of 2.0 ml of 0.5 mM sodium carbonate. The absorbance was read at 410 nm. The units of activity were defined as nanomoles of pNPP hydrolyzed per minute. The CaM concentration was 2 μM and the CN-B versus CN-A ratio was 1:1 on a molar basis.

The assays were carried out with a number of variations, depending on the presence and absence of the two regulators, CN-B and CaM. Specifically, the assays were performed under four conditions: the absence of both regulators, the presence of CN-B, the presence of CaM, and finally, the presence of both CN-B and CaM.

**Structure analysis**

The structure of CN (Kissinger et al., 1995) (PDB code 1AIU) was used for structural analysis, which was carried out using graphics programs Turbo-Frodo (Jones, 1978) and Sybyl (Tripos, St. Louis, MO, USA) on an SGI Octane graphic workstation.

**Results**

All deletion mutants tested in loop 7 of CN exhibited markedly higher activity.

Single deletion mutants V314, Y315, N316 and double deletion mutant V314Y315 in loop 7 of CN were constructed using the Altered Sites mutagenesis system. The mutated cDNAs were cloned into the expression vector pET-21a and then transformed into *E. coli* strain HMS174(DE3) cells. The sequences of all the mutated cDNAs were identified by DNA sequencing and confirmed by the expected pattern. Positive recombinant colonies were successfully demonstrated. Wild-type CN-A and all mutants were expressed. As seen in Figure 1A, the deletion mutants were successfully prepared and purified, demonstrated by clear protein bands at the positions corresponding to their estimated molecular masses of 61 kDa. Yields of the proteins were up to 20 mg/l of culture.

Also, their activities were assayed using pNPP as the substrate, with Mn²⁺ as the activator (Figure 1B). The units of activity were defined as nanomoles of pNPP hydrolyzed per minute.

The activity of the two mutants containing the V314 deletion was no longer regulated by CN-B.

The results of comprehensive assays, in the absence or presence of CN-B, CaM or both, are listed in Table I. As is evident from the table, under all four conditions all mutants
exhibited considerably higher phosphatase activity than wild-type CN-A. The activity of the V314 deletion mutant was the highest among all the mutants tested. Depending on the presence and absence of the regulators, the activity of V314 is ~10–42 times higher, Y315 is ~7–26 times higher, N316 is ~5–15 times higher and V314Y315 is ~3.5–12.5 times higher than that of wild-type CN-A. Compared with wild-type CN-A, the activity enhancement of the mutants was highest in the absence of both regulators, the next highest was in the presence of CN-B, and the third highest was in the presence of CaM, followed by the condition in which both regulators were present.

As far as regulation is concerned, the regulatory effect of CN-B was less than CaM for both wild-type CN-A and mutants. The synergistic action of CN-B and CaM had the largest effect. Very importantly, the activities of both mutants containing the V314 deletion, namely V314 and V314Y315, were no longer affected by CN-B. For example, in the absence of CaM the activity of wild-type CN-A increased approximately two times due to CN-B, whereas both V314 and V314Y315 mutants showed almost no change upon addition of CN-B.

**Structure analysis**

Loop 7 in CN is located in CN-A but is also close to CN-B. It is sandwiched in the concave area (Figure 2). There is a long linker between the two globular domains, i.e. CN-A on the right and CN-B together with the association segment of CN-A on the left. The linker covers both domains and contains approximately 20 amino acids, N327–M347. Residues of loop 7, for instance, D313, make a hydrogen-bonding interaction with H339 (2.96 Å), a residue in the middle of the linker. The CN structure containing part of the AI segment also shows that loop 7 is in direct contact with the AI segment. Among various contacts, for example, Y315 forms a strong hydrogen bond with D477 of AI segment (~2.70 Å) (Figure 2). In addition, there are van der Waals contacts between V314 and A473 (3.88 Å), between L312 and D477 (3.14 Å) and aromatic–aromatic interactions between Y315 and F470 (2.90 Å). Owing to the incomplete structure of the AI segment in the CN crystal structure determined (~30% missing), there are very likely to be other contacts that we cannot currently appreciate.

**Discussion**

All loop 7 mutants, including single deletion mutants Y315, N316 and double deletion mutant V314Y315, displayed increased phosphatase activity. It appears that the activity enhancement via manipulation of loop 7 is a general property of loop 7. More significantly, activities of the mutants containing the V314 deletion, namely V314 and V314Y315, were no longer regulated by regulatory CN-B. While it is common to observe most deletion/mutation mutants with reduced activity as a result of conformational change and/or deviation from optimal residues evolved for proper functioning of a given protein, seeking a molecular mechanism for activity enhancement is always challenging. Having discovered the functional importance of loop 7, through the demonstration of increased phosphatase activity, to be a general property of loop 7, we sought to identify a molecular mechanism from the structure of CN. The CN structure shows that loop 7 is in direct contact with two important regions. The first is a long connecting peptide segment starting from the core catalytic domain and ending at the region where CN-A directly interacts with regulatory CN-B. The second is the AI segment.

Fig. 2. Structure illustration of CN. (A) Overall ribbon diagram of CN. Dark blue and green colors represent CN-A and CN-B, respectively. The AI segment and loop 7 are colored in purple and red. The linker is also highlighted in light blue. Metal ions are shown as spheres (four in the CN-B calcium binding region and two in the active site of CN-A). The rectangular box represents the close-up area. (B) Close-up view including loop 7, linker and the AI segment in the same orientation as the overall structure in (A), highlighting the hydrogen bonds between H339 of the linker and D313 of loop 7, Y315 of loop 7 and D477 of the AI segment.
The regulation of CN is complex and involves multiple factors. Ca\(^{2+}\)-dependent CN-B is one of the most important factors. Although the exact structural basis by which Ca\(^{2+}\) binding CN-B regulates the activity of CN remains elusive, the peptide segment connecting the catalytic core and the region where CN-A is in direct association with CN-B is important as it is the only peptide that fully spans the two regions. Because linker residue H339 forms an interaction with loop 7 residue D313, it is plausible that the conformational changes resulting from loop 7 mutation could have an impact on the regulatory effect of CN-B, thereby altering the phosphatase activity of CN and its regulation.

The AI segment is also in close contact with loop 7. There are several hydrogen bonds, and van der Waals and aromatic–aromatic interactions. These interactions play a crucial role in stabilizing the AI segment in the binding pocket of CN. Disruption of these contacts would therefore facilitate the removal or release of the AI segment, producing less inhibition or higher activity. It appears that the suppression of CN activity is by, at least, a dual inhibition mechanism, i.e. the effects of the AI segment and the constrained state (inactive state) of the catalytic core regulated by Ca\(^{2+}\)-dependent CN-B. However, truncation of the C-terminus of CN-A, which completely removes the entire AI segment, does not lead to significant activation of the free CN-A (Wei and Lee, 1997; Perrino, 1999). Therefore, it is the latter control mechanism that has a much more profound effect on the whole process. In the light of this possibility, it is then perhaps not surprising that, in some cases where interaction between loop 7 and the linker may be severely impaired (such as mutations involving V314), the regulatory effect of CN-B could no longer be ‘felt’ by CN-A.

In conclusion, in this work we have shown the functional and regulatory significance of loop 7. Mutations of loop 7 residues show an increased phosphatase activity, which in some cases is no longer regulated by CN-B. Based on the structural analysis, we have proposed possible structure-based mechanisms through which loop 7 may mediate the regulation of CN.

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References