Identification of an Autoinhibitory Domain in Calcineurin*

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The hypothesis that calcineurin, the Ca\(^{2+}\)/calmodulin-dependent protein phosphatase, contains an autoinhibitory domain was tested using synthetic peptides corresponding to regions of the carboxyl-terminus of calcineurin. Of the several peptides analyzed, one, containing residues 1-T-S-F-E-E-A-K-G-L-D-R-I-N-E-R-, corresponding to regions of the carboxyl-terminus of calcineurin, was tested using synthetic peptides containing residues I-T-S-F-E-E-A-K-G-L-D-R-I-N-E-R-. The limited proteolysis which converts it to a fully active phosphatase when assayed in the presence of Ca\(^{2+}\)/calmodulin, or with calcineurin subjected to partial proteolysis which converts it to a fully active phosphatase, gave complete inhibition of its protein phosphatase activity. Using \([^{32}\text{P}]\)myosin light chain as substrate an IC\(_{50}\) of about 10 \(\mu\)M was obtained with either native calcineurin, assayed in the presence of Ca\(^{2+}\)/calmodulin, or with calcineurin subjected to partial proteolysis which converts it to a fully active phosphatase when assayed in the presence of Ca\(^{2+}\)/calmodulin. With 50 mM \(\mu\)-nitrophenylphosphate as substrate an IC\(_{50}\) of about 40 \(\mu\)M was observed. Studies with overlapping peptides suggested that the sequence P-P-R-R-D-A-M-P was essential but not sufficient for the observed inhibition. Kinetic analysis indicated that the inhibition of phosphatase activity was not competitive with respect to \([^{32}\text{P}]\)myosin light chain. This peptide did not show significant inhibition of the catalytic subunits of protein phosphatases type I or type II or of Ca\(^{2+}\)/calmodulin-dependent protein kinase II. These results indicate that amino acids within this sequence of calcineurin constitute a unique autoinhibitory domain which interacts with the active site and is responsible for the low basal phosphatase activity in the absence of Ca\(^{2+}\)/calmodulin.

Calcineurin (CaN)\(^4\) is a Ca\(^{2+}\)/CaM-dependent protein phosphatase which is highly enriched in brain but also present in other tissues. A heterodimeric enzyme, the 60–61-kDa A subunit contains the catalytic activity as well as the binding site for Ca\(^{2+}\)/CaM, whereas the 19-kDa B subunit has unknown regulatory function but does bind four equivalents of Ca\(^{2+}\) with high affinity (reviewed in Ref. 1). Recently, localization of functional domains in the catalytic subunit has been deduced by analyses of the partial amino acid sequence (2) as well as by limited proteolysis studies (3, 4). The published 307 amino acid COOH-terminal sequence of CaN (2) contains several of the highly conserved motifs which are presumed to subserve catalytic functions since they are present in all sequenced protein phosphatases (5). A putative CaM-binding domain was also identified (2) by homology to known CaM-binding domains in other proteins. At the COOH-terminal boundary of the CaM-binding domain is a site which can be phosphorylated by the autophosphorylated form of Ca\(^{2+}\)/CaM-dependent protein kinase II (CaM kinase II) and by protein kinase C (6). Phosphorylation of this site is blocked by binding of Ca\(^{2+}\)/CaM to CaN. Although phosphorylation of this site does not alter apparent binding of Ca\(^{2+}\)/CaM, it does partially inactivate CaN by increasing the \(K_c\) for protein substrate (7).

Studies using limited proteolysis of CaN are also of relevance for identifying functional domains. Proteolysis of CaN by clostripain in the absence of Ca\(^{2+}\)/CaM cleaves the 61-kDa A subunit to a 57-kDa polypeptide which still binds CaM, but the phosphatase activity is independent of Ca\(^{2+}\)/CaM (4). These results suggest an analogy to myosin light chain kinase and CaM kinase II. Both of these CaM-dependent protein kinases can also be converted to CaM-independent forms by limited proteolysis (8, 9). In both instances autoinhibitory domains have been identified which are adjacent to and partially overlap the CaM-binding domains (reviewed in Refs. 10, 11). In the absence of bound Ca\(^{2+}\)/CaM, these autoinhibitory domains interact with and inhibit elements of the catalytic sites that bind ATP and protein substrates (12). Binding of Ca\(^{2+}\)/CaM is thought to cause a conformational change in the autoinhibitory domain, thereby disrupting its inhibition of the catalytic site and accounting for the activation of the kinase.

Because of these similarities between CaN and the CaM-dependent protein kinases, we have searched for the existence of an autoinhibitory domain in CaN. The limited proteolysis experiments suggested that such a domain may exist within the COOH-terminal 40–50 residues (3, 4). Searches for autoinhibitory regions in the protein kinases were aided by the fact that these motifs often have homology to consensus phosphorylation site sequences for the kinase but lack the phosphorylatable serine or threonine (e.g. pseudosubstrates, Ref. 13). However, there does not appear to be well-defined primary sequence determinants in substrates of protein phosphatases such as calcineurin (see "Discussion"). Thus, we made synthetic peptides corresponding to regions of the COOH-terminus and tested their abilities to inhibit CaN.

**EXPERIMENTAL PROCEDURES**

*Materials*—CaN purified from bovine brain was either a gift from Dr. Marita King (Ohio State University) or purified according to her procedure (14). The catalytic subunit of rabbit muscle protein phosphatase type I (15) and pig brain protein phosphatase type IIA (16) were kindly provided by Drs. Balwant Khatra (California State
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University, Long Beach) and Shaw-Der Yang (National Taiwan Hua University, Republic of China), respectively. Rabbit skeletal muscle myosin light chain (MLC) and myosin light chain kinase were gifts of Dr. Edwin Krebs (University of Washington). Rat brain Ca\(^{2+}/\) calmodulin-dependent protein kinase II was provided by Dr. Roger Colbran (Vanderbilt University). The \(\gamma\)-ATP was prepared using carrier-free \(\gamma\)-P\(_4\) from ICN Pharmaceutical. Trypsin and chymotrypsin were from Worthington Biochemical Corp. Other materials were purchased from commercial sources.

Methods—Peptides corresponding to COOH-terminal regions of CaN (see Table I) and syntide-2 (18) were synthesized using a Beckman 990 automatic synthesizer. Peptides were desalted on Triacryl GF05 in 0.1% trifluoroacetic acid and purified by reverse-phase HPLC using a Beckman C18 column equilibrated in 0.1% trifluoroacetic acid and developed with acetonitrile. Peptide 4 was derived from peptide 3 by cleavage with CNBr in 70% formic acid and subsequent purification by reverse-phase HPLC. The amino acid compositions of all peptides were verified and the concentrations determined by Waters picotag analysis. \([\gamma-P]\)MLC was prepared as described (7). The protein phosphatase activities of CaN and types I and IIA were assayed (7) using 2.8 mM \([\gamma-P]\)MLC but without Mn\(^{2+}\) or with PNPP as described in the figure legends. Limited proteolysis of CaN (3.25 \(\mu\)g) was performed using trypsin (0.0325 \(\mu\)g) in 50 mM HEPES, pH 7.5, 1 mg/ml bovine serum albumin, 0.4 mM CaCl\(_2\) at 30°C for 10 min. The reaction was stopped by addition of soybean trypsin inhibitor (1 \(\mu\)g) and cooling to 4°C. Autophosphorylated CaN kinase II (8.1 \(\mu\)g) was partially proteolyzed with chymotrypsin (0.08 \(\mu\)g) for 3 min, aprotinin (8 \(\mu\)g) was added, and the activity was assayed in the presence of EGTA using syntide-2 as substrate as described (9). Concentrations of CaN were determined spectrophotometrically using an absorbance index of 0.0 at 287 nm for a 1% protein solution (19). CaM concentration was determined by Waters picotag analysis.

RESULTS

Peptide 3

Fig. 1 lists the peptides corresponding to regions of the COOH terminus of CaN that we have tested for their abilities to inhibit the protein phosphatase activity of CaN. We initially synthesized the COOH-terminal 18-residue peptide (peptide 1, Fig. 1) and tested it as an inhibitor of CaN phosphatase activity using \([\gamma-P]\)myosin light chain as substrate. A concentration (2.8 \(\mu\)M) of \([\gamma-P]\)MLC was chosen for the assay which was well below the \(K_m\) of about 18 \(\mu\)M (see below, Fig. 3) to ensure that inhibition would be seen if the mechanism were competitive with substrate. Since peptide 1 was without effect on CaN activity (Fig. 2), the sequence was extended in the NH₂-terminal direction to make peptide 2. However, peptide 2 was also without inhibitory effect up to 100 \(\mu\)M (Fig. 2). Although not shown in Fig. 2, peptides 1 and 2 were tested using both intact as well as proteolyzed CaN. The limited proteolysis of CaN did not affect total phosphatase activity, assayed in the presence of Ca\(^{2+}/\)CaM, but it did convert the enzyme to a completely Ca\(^{2+}\)-independent species and reduced the subunit from 60 kDa to about 43 kDa (data not shown) in agreement with previous results (3, 4).

Next, we decided to test the sequence further NH₂-terminal in CaN and wanted to provide some overlap with the previous peptide, so peptide 3 was synthesized. As shown in Fig. 2, peptide 3 strongly inhibited CaN, using either proteolyzed or native enzyme, with an \(I_{50}\) of about 10 \(\mu\)M. With 50 mM PNPP as substrate an \(I_{50}\) of about 40 \(\mu\)M was estimated. To further define the inhibitory motif, the overlap region between peptides 2 and 3 was removed by treatment of peptide 3 with CNBr, and peptide 4 was then purified by reverse-phase HPLC and confirmed by amino acid composition. Fig. 2 shows that the overlap region between peptides 2 and 3 was required for the inhibition since peptide 4 was not inhibitory.

An initial assessment of the inhibitory mechanism by peptide 3 was made. When the concentration of \([\gamma-P]\)MLC was varied in the absence and presence of peptide 3, a double-reciprocal plot indicated that inhibition of CaN was not competitive with substrate (Fig. 3). This result, which is representative of three separate experiments, gave an average \(K_m\) for MLC of 18 \(\mu\)M. The inhibition of CaN using PNPP as substrate may also be noncompetitive as the degree of inhibition was similar using 20 \(\mu\)M peptide 3 and either 10 mM PNPP (35% inhibition) or 50 mM PNPP (26% inhibition).

To assess the specificity of peptide 3 for CaN, its ability to inhibit two other protein phosphatases was determined. Fig. 4 illustrates that neither of the catalytic subunits of protein phosphatases type I or type IIA was inhibited more than 25% by concentrations of peptide 3 up to 100 \(\mu\)M. Since alignment of the autoinhibitory domain of CaN showed some homology in positions of the basic residues with the autoinhibitory domains of CaM kinase II and myosin light chain kinase (not shown), we tested whether CaM kinase II was inhibited by peptide 3. Again, Fig. 4 shows that peptide 3 gave no inhibition of CaM kinase II. For this experiment the partially proteolyzed form of CaM kinase II, which is highly sensitive to its own autoinhibitory peptide (9), was utilized and assayed in the presence of EGTA.

DISCUSSION

Limited proteolysis experiments on CaN suggested the existence of an inhibitory sequence within 40–50 residues of the COOH terminus (4). The results of this paper confirm the existence of such an autoinhibitory domain within the sequence of peptide 3 of Fig. 1. This autoinhibitory domain in CaN is located 50–60 residues COOH-terminal of the putative CaM-binding domain (2). This is in contrast to the situation with CaM kinase II (9) and myosin light chain kinase (8) where the autoinhibitory domains are adjacent to and slightly overlap the CaM-binding domains. However, in cAMP kinase regulatory subunit and protein kinase C the inhibitory domains are also located some distance from the allosteric activator binding site (13). It will be of interest to determine the mechanism by which binding of Ca\(^{2+}/\)CaM to CaN disrupts the interaction of its autoinhibitory domain with elements of the catalytic site.

Removal of the COOH-terminal residues (M-P-P-R-R-D-A-M-P) of peptide 3 by CNBr cleavage rendered the peptide (peptide 4) impotent, thereby implicating these residues as critical for the inhibition. However, since peptide 2, which contained P-R-R-D-A-M-P as its NH₂-terminal was not inhibitory, this suggests that residues further NH₂-terminal are also important. The sequence -P-R-R-D-A-M-P- is of critical interest in that it shows strong homology to the phosphorylation site in many of the preferred substrates of CaN (Fig. 5). Although no consensus sequence for dephosphorylation sites in substrates of CaN is well documented, Fig. 5 illustrates...
some interesting possibilities. Phosphorylase kinase α subunit, DARP-32, and phosphatase inhibitor 1 as well as the regulatory subunit of cAMP kinase are all excellent substrates for CaN (1). The fact that all of them contain R-R-X-S/T- is probably more related to their phosphorylation by cAMP kinase, since this is the consensus phosphorylation site sequence for that kinase (20), than to their dephosphorylation by CaN. Nevertheless, since most autoinhibitory domains in the protein kinases show "pseudosubstrate" sequence (13) and are competitive inhibitors relative to protein or peptide substrates, it was important to determine the kinetic mechanism of inhibition of CaN by peptide 3. Using myosin light chain as substrate, the experimental data clearly demonstrated that inhibition was not competitive, and the results with PNPP as substrate tend to agree. However, since MLC does not contain the -R-R-X-S/T-sequence, it may be interesting to also determine the kinetic mechanism with another protein substrate.

Studies on recognition determinants for substrates of CaN using a synthetic peptide corresponding to the site in the regulatory subunit of cAMP kinase showed that addition of the amino acids D-L-D-V to the sequence P-I-P-G-R-F-D-R-R-V-S-V-A-A-F gave a dramatic decrease in the $K_v$ and increase in $V_{max}$ for dephosphorylation by CaN (21). This suggested that the presence of acidic residues NH$_2$-terminal to the phosphorylation site may be an important primary sequence recognition determinant. Again, most of the substrates of CaN listed in Fig. 4, except for M1C, do have multiple acidic residues NH$_2$-terminal of the phosphorylation site. This property is also shared by the autoinhibitory peptide 3. It will be important to determine if these acidic residues are important for the inhibitory property of this peptide. Many of the autoinhibitory domains of protein kinases have multiple basic residues that are important for their inhibitory potencies. Secondary structure predictions suggest that peptide 3 is quite hydrophilic and may contain a region of α-helix at its NH$_2$ terminus with random coil, due largely to the multiple prolines, at its COOH terminus. The NH$_2$ terminus is highly acidic whereas the COOH terminus is basic.
Peptide 3 appears to be inhibitory only for CaN and not for protein phosphatase I or type IIA using identical assay conditions. The fact that peptide 3 did not inhibit CaM kinase II further supports the conclusion that this sequence is quite specific for inhibition of CaN. Inhibition of CaN, however, was obtained using two very different phosphatase substrates and assay conditions. Experiments are currently in progress to further define the essential amino acids in the autoinhibitory domain of CaN.

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