Interchangeable Associations of Calcineurin Regulatory Subunit Isoforms with Mammalian and Fungal Catalytic Subunits*

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Two mammalian genes for the Ca\(^{2+}\)-binding regulatory subunit of the calmodulin-dependent protein phosphatase (calcineurin) were identified recently, suggesting unique associations between tissue-specific catalytic subunits. The murine brain (β1) and testis (β2) isoforms of the regulatory subunit were expressed with poly-histidine carboxyl termini and purified by Ni\(^{2+}\)-chelate chromatography, each exhibiting high affinity Ca\(^{2+}\) binding on nitrocellulose overlays. Using chromatographic methods to assess complex formation, the β1 and β2 isoforms appeared indistinguishable in their binding to bacterially expressed forms of the murine brain (α1) or testis (α3) catalytic subunits; this suggests that multiple heterodimeric forms may be present in some tissues. Furthermore, both β1 and β2 formed complexes with the recombinant catalytic subunit from Neurospora crassa. For this catalytically active fungal enzyme, stoichiometric amounts of mammalian regulatory subunit increased catalomluin-dependent activity without affecting that stimulated by Mn\(^{2+}\) alone. Maximal hydrolysis of p-nitrophenyl phosphate by the N. crassa catalytic subunit was stimulated 80–120% with the β1 isoform and 30–50% by β2. Significantly, the incubation time at 4 °C required for optimal activation (1 h) was much greater than that for association (1 h), indicating that the catalytic subunit undergoes a slow transition to an activated conformation after binding the regulatory subunit. The production of functional heterodimers of mammalian and fungal proteins implies highly conserved interaction domains on the catalytic and regulatory subunits of this phosphatase.

Calmodulin (CaM)-dependent protein phosphatases (type 2B) comprise a family of enzymes that include brain-specific (i.e., calcineurin) and non-neural isoforms (1–3). The holoenzyme is heterodimeric, consisting of a 60-kDa subunit that confers catalytic activity (4) and an intrinsic 19-kDa regulatory subunit that binds Ca\(^{2+}\) (5, 6). Electrophoretic characterization of the protein, both in crude tissue extracts and after its purification, indicates considerable isoenzyme heterogeneity (7–9). This diversity apparently results from different catalytic (A or α) subunits, based on immunological studies with monoclonal antibodies that distinguish between peptides expressed in brain and elsewhere (10). At a molecular level, three distinct mammalian genes for the catalytic subunit have now been identified which can produce additional forms by alternative splicing mechanisms (11–18).

Recently, cDNAs were characterized from rodent testis (19–21) that show homology to that of the regulatory (B or β) subunit, originally cloned from human brain (22). The so-called “brain” and “testis”-specific forms from mouse (denoted β1 and β2, respectively) are approximately 80% identical in amino acid sequence, with the β2 isoform possessing a nine amino acid extension on the carboxyl terminus. Our recent report of a testis-specific catalytic subunit (16) prompted us to examine whether or not the regulatory subunits can discriminate in their interactions with the corresponding catalytic subunits. This seemed an important question because both the β1 and β2 isoforms are likely to be present in some cell types, and the possibility of functional “hybrids” of phosphatase subunits may provide for additional enzyme diversity. To pursue such questions, we have expressed the regulatory and catalytic subunit isoforms in bacteria and have documented their ability to form complexes. In addition, by using a recombinant catalytic subunit from Neurospora crassa, we provide direct evidence for conserved regulatory features that enable activation of the fungal enzyme by the mammalian proteins.

MATERIALS AND METHODS

Preparation of Expression Vectors—Complementary DNAs corresponding to the mouse β1 and β2 forms of the regulatory subunit were isolated and characterized; their sequences are reported elsewhere (21). Briefly, mRNA from mouse brain (hippocampus) and testis was made into a complementary strand of DNA by using reverse transcriptase; this was then amplified by polymerase chain reaction using primers based on the published sequences of rat and human regulatory subunit cDNAs (19, 22). The DNA fragments obtained by this procedure were used for cloning of the corresponding murine cDNAs from a testis phage library. For expression, the open reading frame (ORF) of each protein was modified to add 6 histidine residues to the COOH terminus by using polymerase chain reaction amplification. The “sense” primers contained the first six amino acids of the ORF (β1S, 5′-ATGGGAAATGAGCCGAGGTAT-3′; β2S, 5′-ATGGGAAATGAGCCGAGGTAT-3′) and the “anti-sense” primers encoded six amino acids at the carboxyl terminus, followed by 6 histidines and a stop codon (β1AS, 5′-ATCGAATGAGCCGAGGTATATAC-3′; β2AS, 5′-ATCGAATGAGCCGAGGTATATAC-3′). After their purification by polyacrylamide gel electrophoresis, these fragments were inserted into the vector pUC-18, that had been digested with Smal. Subsequently, these plasmids were digested sequentially with KpnI and BamHI, which flank Smal in the multiple cloning site, and the coding region was inserted into the expression vector pYTQ-18 (23). After transformation of DH-5α bacteria with expression vectors and selection of recombinants, DNA sequencing was carried out to confirm the correct ligation and reading frame of all constructions. All recombinants

For the β2 expression vector, the last three amino acids of the murine ORF were deleted, in order to retain the same carboxyl sequence reported in rat (19, 20).
proteins used in this study contained seven amino acids on their amino terminus that are contributed by the vector.

**Purification of Recombinant Proteins**—Bacteria were grown in 1 liter of LB broth, containing 100 µg of ampicillin/ml and 1–2 mM isopropyl-1-thio-β-D-galactopyranoside, to an OD_{600} of 1.2–1.4, at which time they were harvested as described (14). Cell pellets were suspended in extraction buffer (10 mM Tris-HCl, pH 8.0, containing 1 mg of lysozyme/ml and 20 µg of soybean trypsin inhibitor/ml) and disrupted by a small Waring blender or by passage through narrow-gauge syringe needles. After centrifugation (20,000 × g, 40 min), the supernatant was mixed for 2–4 h (4 °C) with a Ni^{2+}-chelate gel (NTA-agarose, Qiagen) that was equilibrated in buffer A (50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl). After allowing the gel to settle by gravity, the supernatant was discarded and the gel transferred to a column for washing with 6 bed volumes of buffer A. The gel was washed with 3 bed volumes of buffer B (50 mM sodium MES, pH 6.0, plus 300 mM NaCl) followed by 2 bed volumes of buffer B containing 20 mM imidazole. The gel was then eluted with buffer C (250 mM imidazole, pH 7.0). Elution buffer also contained the protease inhibitors, soybean trypsin inhibitor, leupeptin, and antipain, each at a concentration of 10 µg/ml. Purification of recombinant α, α3, and Norfa catalytic subunits, expressed constitutively in pUC-18 vectors, was carried out using CaM-Sepharose, as described (24).

**Preparation and Use of Complexes Formation between Regulatory and Catalytic Subunits**—The supernatant from an extract containing a recombinant regulatory subunit was mixed with that expressing a specific catalytic subunit, usually overnight at 4 °C. The combined extract was used for batchwise chromatography using either NTA-agarose or CaM-Sepharose, essentially as described above. For small scale analytical experiments using 50 µl of affinity media, washings and elutions were carried out by repeated suspensions of the gel in 1 ml of wash buffer or 0.2 ml of elution buffer in 1.5-ml microfuge tubes. After elution of the affinity matrix, a portion of the eluate was concentrated by precipitation with trichloroacetic acid (final concentration 10%, w/v) and subjected to denaturing electrophoresis in the presence of sodium dodecyl sulfate. Proteins were analyzed by Western blot procedures as described, using affinity purified antibodies prepared against bovine brain calcineurin (25) or against peptides near the amino termini of the subunits (9).

**Measurement of Enzyme Activity**—Phosphatase activity was carried out essentially as described by King and Huang (26), except that 20 mM sodium p-nitrophenyl phosphate (pNPP) was used in the assays. All assay conditions used are those reported (24) except that Mn^{2+} was present at a final concentration of 0.1 mM. Unless otherwise noted, "basal" activity is that measured in the presence of 0.2 mM CaM. "Metal stimulated" activity is determined in the absence of CaM, which includes, in addition, 0.1 mM MnCl_{2} and "CaM-stimulated" activity, that containing 0.1 mM MnCl_{2} plus 2 µM CaM. In assays that measure the maximal effect of regulatory subunit, this was added at a molar stoichiometry of 4:1 with respect to catalytic subunit. All reactions were carried out at 26 °C for 10 min, and data are given as specific activities, assuming an optical density of 18 (405 nm) for a 1 mM solution of p-nitrophenol.

**Preparation and Use of Antibodies**—Polyclonal rabbit antibodies were prepared to peptides near the amino termini of murine β1 (ASYPLEMCSHFD) and α3 (MSVRPPQFST), essentially as outlined elsewhere (9). Briefly, peptides were modified to include a lysine in the first position and coupled to keyhole limpet hemocyanin; these peptides were prepared by Cambridge Research Biochemicals (Wilmington, DE). A portion of this peptide conjugate (0.75 mg) was emulsified with complete Freund's adjuvant and injected into rabbits, followed by two booster injections (0.25 mg each) at 2-week intervals. Sera were routinely purified on peptide-Sepharose affinity columns to determine the titer and specificity of the antibodies (9). Antibodies to bovine brain calcineurin were raised in rabbit and then purified by affinity chromatography on calcineurin-Sepharose, as described (25). For some experiments, this anti-calcineurin antibody was further fractionated by chromatography on NTA-agarose to which β1 or β2 was bound (~1.5 mg of regulatory subunit/ml gel). After washing with 6 bed volumes of 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl (buffer D), specific antibody to the regulatory subunit was eluted with 40 mM sodium acetate, pH 6.0, containing 6 M urea. After adjusting the pH to 7.5, the eluate was dialyzed versus buffer D plus 40% glycerol, and stored at −20 °C.

**RESULTS**

Expression and Properties of Recombinant Regulatory Subunits—Expression plasmids containing the ORF of the β1 and β2 isoforms were constructed to contain 6 histidine residues preceding the stop codon; this allows for facile purification and analysis of expressed proteins using Ni^{2+}-chelate chromatography (27). The β1 and β2 forms of the regulatory subunit were recovered in yields of 1–1.5 mg/liter of cells and displayed immunological and Ca^{2+}-binding properties on Western blot analysis that were essentially the same as those described (28). The apparent molecular mass of β2 was ~22 kDa, whereas that of β1 was ~21 kDa, the difference reflecting the presence of additional amino acids in the deduced sequence of murine β2 (21). For the bacterially expressed β1, low amounts of two smaller immunoreactive bands were also observed (lane 2, Fig. 1, A and B). These peptides did not react with antibodies prepared against the NH₂-terminus of β1 (lane 4, Fig. 1B), suggesting either that these were translation products derived from internal initiation sites or proteolytic cleavage products.

**Interactions of Recombinant Regulatory Subunits with Catalytic Subunit Isoforms**—Bacterial lysates containing recombinant murine brain (α1) or testis (α3) catalytic subunits were mixed with those expressing either the murine β1 or β2 regulatory subunits; to minimize proteolysis, incubations were routinely carried out at 4 °C. Western blot analysis of chelate chromatography eluates showed that both the α1 and α3 catalytic subunits can associate interchangeably with the β1 and β2 isoforms (Fig. 2A). The interactions were specific inasmuch as catalytic subunit was never observed in NTA-agarose eluates from extracts of α1 or α3, i.e. in the absence of expressed regulatory subunit. These data indicate that modification of the regulatory subunit carboxyl terminus is compatible with binding to the catalytic subunit, at least in

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3 Lower immunoreactivity was always seen for the β2 recombinant, when compared to apparently equal amounts of β1 protein; this may reflect fewer epitopes on β2 that are recognized by antibody raised against the bovine brain enzyme.
the absence of CaM. CaM-Sepharose chromatography of these extracts yielded similar results, showing that the poly-histidine terminus does not prevent formation of the ternary complex with CaM (data not shown and Fig. 2B).

In binding studies conducted at equilibrium (12 h, 4°C) with a mixture of β1 and β2 in great excess over the catalytic subunit, comparable amounts of each regulatory subunit were recovered in the CaM-Sepharose eluate (Fig. 2B). These results, which indicate the lack of a strict interaction preference, were seen using either the α1 (Fig. 2B) or α3 catalytic subunits (data not shown). When the time courses of association were examined for the different combinations of isoforms, binding of the regulatory subunit was essentially complete after 1 h in all cases (Fig. 3, and data not shown), indicating rapid, high affinity interactions with the catalytic subunit. Thus, both the extent and rates of complex formation determined with the mammalian subunits appeared to be indistinguishable. Interestingly, both mammalian regulatory subunits also associated with the recombinant catalytic subunit of N. crassa in a similar manner, based on chromatography with CaM-Sepharose (Fig. 4) or Ni2+-chelate gels (data not shown).

Activation of Recombinant N. crassa Catalytic Subunit by β1 and β2—Recently, we reported that the bacterially expressed form of the Neurospora catalytic subunit exhibits activity with pNPP that is comparable to that of bovine brain calcineurin (24). When β1 and β2 were assessed for their abilities to bind regulatory subunits, the results, as shown in Fig. 3, were indistinguishable. Although many proteins are not completely removed by the washing procedure used for small scale experiments, nonspecific peptides are absent in the region of the regulatory subunits.
to stimulate pNPP hydrolysis after complex formation (4°C, 12-16 h), both forms increased the CaM-sensitive activity of the fungal enzyme, but showed little or no stimulation of the Mn⁺⁺-activated component (Table I). When assayed for basal activity, complexes formed with β1, but not β2, showed a 3-4-fold enhancement of phosphate hydrolysis (Table I), consistent with findings reported for reconstituted bovine brain enzyme (4). The β1 isoform was more effective in augmenting activity than was the testis isoform, with maximal stimulation by β1 being 100-120%, whereas that of the β2 form never exceeded 50%, even with a 10-fold molar excess of this subunit. The ability to increase the CaM-stimulated activity did not depend on the intrinsic CaM responsiveness of the recombinant enzyme, with preparations having 0-150% CaM stimulation yielding identical results (Table I and data not shown). The amount of recombinant regulatory subunit required for maximal stimulation was roughly stoichiometric with respect to fungal catalytic subunit (Fig. 5). Over six different preparations the average value was 1.3:1; the slight discrepancy in stoichiometry may reflect difficulties in estimating the concentration of recombinant subunits, especially for the fungal enzyme, which contains variable amounts of the bacterial protein groEL as a contaminant (24).

As observed with the mammalian catalytic subunits, the fungal protein rapidly formed complexes with β1 and β2 and appeared to be fully saturated with regulatory subunit after 1 h (Fig. 4B). However, when total calmodulin-stimulated enzyme activity was measured under similar conditions, maximal effects required at least 6-8 h for either regulatory subunit (Fig. 6A). In three experiments with different enzyme preparations, activities were relatively stable up to 10 h incubation at 4°C (>80% of the maximal activity observed during the time course). After this, activities declined gradually by 30-40% (at 24 h) suggesting some instability of the purified enzyme under incubation conditions. When the relative stimulation of activity by calmodulin was calculated, the apparent optimum incubation period was longer (Fig. 6B); however, this probably reflects the stabilization of CaM-dependent activity by the regulatory subunit. The presence or absence of CaM during complex formation appeared to make no difference in any activation property (data not shown). Although β1 and β2 differed in the extent of their maximal activation, the apparent rates at which this occurred were the same (Fig. 6C). Taken together, the data on interaction and activation indicate that the initial binding of the regulatory subunit takes place rapidly, whereas acquisition of full activity by the catalytic subunit is a much slower process.

**DISCUSSION**

The regulatory subunit of the CaM-dependent protein phosphatase shows the "EF-hand" structure characteristic of Ca⁺⁺-binding proteins in the superfamily that includes CaM and troponin C (28). However, protein sequencing of the bovine brain protein indicated that its amino terminus can be myristoylated (29), making it unique among the members of this family. The significance of this modification, either for interaction with the catalytic subunit or for "targeting" of the holoenzyme to membrane environments has not been established. In order to pursue this and other questions related to its specificity for the catalytic subunit, we cloned the full-length murine brain regulatory subunit in hopes of producing functional recombinant proteins to facilitate such studies. Because the only methods for preparing "native" regulatory subunit require dissociation of brain holoenzyme under denaturing conditions (4), we felt that expression of the cloned cDNAs might be preferable. The properties of the bacterially expressed form of this protein reported here suggest that limited modification of the carboxyl terminus is compatible with Ca⁺⁺ binding and association with the catalytic subunit. Furthermore, myristoylation of the regulatory subunit does not seem to be required for functional interaction or regula-

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**Table I**

*Activation of the catalytic subunit of* N. crassa *by mammalian regulatory subunits*

<table>
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<th>Phosphatase activity</th>
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<th>Plus β2</th>
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**Fig. 6. Stoichiometry of Neurospora catalytic subunit activation by β1 or β2 regulatory subunits.** Purified recombinant proteins were prepared as described under "Materials and Methods." Samples of Norfa (3-5 μg) were incubated for 16 h at 4°C with increasing amounts of β1 (circles) or β2 (squares) and 5 μg of CaM in a final volume of 130 μl of incubation buffer (60 mM Tris-HCl buffer, pH 8.6, containing 0.3 mM Ca⁺⁺, 15 mM MgCl₂, 0.1% MnCl₂, 0.15 mM EGTA, 0.3 mg of bovine serum albumin/ml, and 30 μg of soybean trypsin inhibitor/ml). Assays were initiated by the addition of pNPP to a final concentration of 20 mM and the incubation continued for 10 min at 26°C in a final volume of 200 μl. The data are presented as the percent stimulation of the activity of control samples incubated with CaM, but without added regulatory subunit.
Interactions between Calcineurin Subunits

Fig. 6. Time-dependent activation of N. crassa catalytic subunit by β1 or β2 regulatory subunits. Samples of Nprf α (3–5 μg) were incubated at 4 °C for the indicated times, either alone (open circles) or in the presence of a 4–5-fold molar excess of β1 (closed circles) or β2 (squares) in incubation buffer containing 5 μg of calmodulin. Assays were carried out for 10 min at 26 °C in a final volume of 200 μl as described in the legend to Fig. 5. Panel A, time-dependent changes in the specific activity of the N. crassa enzyme complexes. Panel B, percent stimulation of activity at each timepoint, defined as the activity with regulatory subunit compared to that without regulatory subunit. Panel C, relative rate of change in stimulation, comparing the percent stimulation at each time point (panel B) to the maximal degree of stimulation at 24 h, set to 100%.

...tion of enzyme activity, per se, based on studies carried out with the N. crassa catalytic subunit.

A recent study described a novel rat testis “calcineurin B-like protein,” and suggested that it may interact with components other than the catalytic subunit of calcineurin (19). Another group reported a cDNA for the same protein but noted a different 5’ coding region (20), this suggesting that variants of this testis protein may arise via alternative splicing at the amino terminus. Both rat testis cDNAs show a six-amino-acid COOH extension, relative to the human brain form (22), and antipeptide antibodies made against this deduced carboxyl terminus sequence could distinguish the subunit variants (30). Thus, these studies suggested that several testicular regulatory subunits may exist. Our cloning studies confirmed the presence of a murine testis form that is essentially the same as one of the rat forms (19), although it shows a nine-amino-acid extension at the carboxyl terminus (21). Because we had identified a testis-specific isoform (α3) of the catalytic subunit recently, we speculated that this tissue may contain an isoencezyme composed of these two subunits (18).

Thus, the primary objective of this work was to determine whether or not there are selective associations between different mammalian CaM-dependent protein phosphatase subunit isoforms. We had cloned both β1 and β2 murine forms from a germ-cell-enriched testis library and had detected mRNA for both regulatory subunit forms in whole testis. This, along with data indicating low amounts of β2 and α3 mRNA in tissues other than testis (21) suggested to us that multiple holoenzymes could arise if subunits were interchangeable and this might have important regulatory significance. However, because of the striking coincidence in expression of mRNA for the testis-specific forms of the catalytic and regulatory subunits (21), we expected that an exclusive, or at least preferred, association of these two might exist. The data presented here do not support that hypothesis; instead, they suggest that the two regulatory subunits are capable of equivalent interactions with several mammalian catalytic subunit isoforms.

In some cell types, this may yield isoenzymes that are differentially regulated. At present, it is impossible to assess subtle distinctions in these heterodimeric forms (e.g. in modulation of their activities) using the bacterially expressed catalytic subunits, and ongoing efforts to produce these isoforms in eukaryotic expression systems are directed toward addressing such issues. Nonetheless, these recombinant fusion proteins appear to be satisfactory for establishing the physical interactions reported here and for mapping the structural domains needed for regulatory subunit interaction.

As reported earlier, a primary difference between the fungal and mammalian catalytic subunit activities is that the Neurospora enzyme exhibits constitutive, CaM-sensitive activity in the absence of the regulatory subunit (24). For this reason, it was rather unexpected that the fungal catalytic subunit could interact with, and be activated by, mammalian regulatory subunits. Indeed, the stoichiometry and time course of activation are similar to properties reported for mammalian subunit reconstitution (4). Thus, in spite of exhibiting much higher intrinsic activity in the absence of regulatory subunit, the Neurospora catalytic subunit appears to have fundamentally the same properties as the mammalian form. The ability to monitor both the activation of the fungal protein and its interaction with a poly-histidine-modified regulatory subunit has allowed us to compare these events in detail. The data presented here suggest that two stages can be distinguished that represent discrete conformational states of the catalytic subunit. The initial interaction of subunits in crude extracts is rapid, with a half-time of 20–30 min at 4 °C for all isoforms, indicating that the initial association does not require extensive refolding or rearrangements. Subsequently, there is a rather slow transition (t1/2 = 4–5 h) to an activated state, whereupon the enzyme is substantially more sensitive to activation by CaM. Because the Mn2+-stimulated activity is not greatly affected, this suggests some selective enhancement of allosteric control, rather than a generalized effect on catalytic efficacy. Although the interaction with regulatory subunit may, in fact, alter the affinity of the catalytic subunit for CaM, this cannot account for the stimulation since CaM was present in great excess under assay conditions.

The finding that hybrid holoenzymes can be formed using mammalian and fungal subunits argues for interaction domains on both proteins that have retained many structural and functional similarities throughout evolution. These conserved properties have been mapped to a region of 140 amino...
acids between the "catalytic core" region and CaM-binding domain of the catalytic subunit\(^2\) that is essentially identical between fungal and mammalian proteins (24). Although the enzymological function of the regulatory subunit has not been elucidated completely, the fidelity with which these structural elements have been preserved argues for a biological role of key importance. Ongoing studies to characterize the interaction domains and the influence of subunit association on biochemical properties may provide further insights into such questions.

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REFERENCES


\(^2\) K. Ueki, T. Muramatsu, and R. L. Kincaid, submitted for publication.