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Characterization of a novel zinc binding site of protein kinase C inhibitor-1

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The zinc-binding properties of an endogenous protein inhibitor of protein kinase C was studied. Equilibrium gel penetration revealed that 1 mol of this protein binds 0.97 mol of zinc with a dissociation constant of 4.3 μM. The site of zinc-binding, MVVNEGSDGGQSVYHVHLHVLGGR, was identified by a multi-step process consisting of tryptic digestion, fragment isolation, transfer to nitrocellulose, and hybridization with $^{65}ZnCl_2$. Binding of $^{65}ZnCl_2$ to selected synthetic fragments further localized the site of interaction to the sequence QSVYHVHLHVL. This region contains 3 closely positioned histidine residues and represents a novel zinc-binding site.

Protein kinase C inhibitor; Zinc ion

1. INTRODUCTION

Protein kinase C (PKC) contains a Cys-rich region analogous to the 'zinc finger' sequence which has been shown to enhance its binding to phorbol esters [1]. Biphasic effects of zinc ion on PKC activity have been reported; low concentrations being stimulatory and higher concentrations inhibitory [2,3]. These results are at variance with the findings of Speizer et al. [4], who reported that zinc is only inhibitory. Our laboratory became interested in determining whether or not zinc ion was bound by a bovine brain-derived protein inhibitor of PKC, protein kinase C inhibitor-1 (PKCI-1) [5], when inspection of its primary amino acid structure revealed the presence of a His-rich region similar to that of zinc-binding metalloproteinases. PKCI-1 was shown, by radioactive zinc overlays, to bind zinc ion specifically over calcium and magnesium ions [5]. The fact that PKC and its endogenous inhibitor, PKCI-1, both appeared to bind zinc ion suggested that this common property may play a role in their interaction. To probe this further, we have mapped the binding site of PKCI-1 to a His-rich region which binds one zinc ion per protein molecule with a K_d of 4.3 μM. This site represents a new zinc-binding sequence, His-X-His-X-His, which is discussed with respect to other well-characterized zinc-binding domains.

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Abbreviations: PKC, protein kinase C; PKCI-1, protein kinase C inhibitor-1; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; FAB MS, fast atom bombardment mass spectrometry

2. MATERIALS AND METHODS

2.1. *Removal of metal ions from equipment and reagents*

Care was taken during the course of these studies to eliminate adventitious metal ions from water, buffers and equipment. Water was distilled and filtered through a Milli-Q Type 1 Reagent-Grade Water System (Millipore, Bedford, MA) until its resistivity was 16–18 MΩ/cm at 25°C. Buffers made up in this water were rendered metal-free by passage over a 200 ml bed of Chelex-100 resin [6]. Plastic and glassware were soaked in 20% HNO_3 for several days and rinsed with demetalized water. Low-metal Spectra/Por molecular-porous membrane dialysis tubing (Spectrum Medical Industries, Los Angeles, CA) was used after soaking in demetalized water until the zinc ion concentration was sufficiently low (see below).

2.2. *Analysis of zinc ion*

Washes of the equipment and the Chelex-100-treated reagents were analyzed by graphite furnace atomic absorption spectrometry at 213.9 nM (Varian AA-875 and GTA-95) to ensure that they were sufficiently zinc-free. The instrument was calibrated with a zinc ion standard solution (Fisher Scientific, Fair Lawn, NJ). All washes and reagents were determined to be less than 1.0 ng/ml zinc ion before their use in binding experiments.

2.3. *Removal of zinc ion from proteins*

Carbonic anhydrase (Sigma, St. Louis, MO) and PKCI-1 were converted to apoprotein forms by incubation at 0.75 mg/ml in 0.1 M *N*-methylimidazole (Sigma) and 0.13 M dipicolinate (Aldrich Chemical, Milwaukee, WI) at pH 6.5 for 1 week at 4°C [7]. Protein solutions were then dialyzed against water (4°C) with 5 changes of 2 liters each in 72 h. Finally, the samples were prepared for zinc-binding studies by dialysis against 0.02 M 2-(*N*-morpholino)ethanesulfonic acid (Sigma) at pH 6.5 for 24 h.

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2.4. *Zinc-binding properties of carbonic anhydrase and PKCI-1*

Stoichiometric binding of zinc by carbonic anhydrase and PKCI-1 was performed by a modified equilibrium gel penetration method [8]. Proteins were diluted into 0.02 M Mes buffer (pH 6.5) to a concentration of 2.5 nmol/ml. Solutions of $ZnCl_2$ (Mallinckrodt, Paris, KY) in the same buffer were spiked with $^{65}ZnCl_2$ (New England Nuclear, Boston, MA) to a specific activity of 8800 cpm/nmol. Binding assays were carried out by incubating 2.5 nmol protein or buffer blank with 0.1–100 μM $ZnCl_2$ in a volume of 1 ml for 30 min with constant shaking at room temperature. The samples were then pipetted into Eppendorf tubes containing 200 mg of dry Sephadex G-25 superfine (Pharmacia, Piscataway, NJ). The tubes were capped, mixed well, and agitated overnight to allow equilibration of ligand with solvent. To measure binding, 100 μl of the supernatant was removed, diluted into 15 ml of scintillation cocktail, and counted 10 min for radioactivity. The excluded volume was determined by the change in absorbance (578 nm) of a 1 ml solution of Blue dextran which was treated in the same manner as the other samples.

The following equation [8] was used to calculate the pmol of zinc ion bound to protein:

$$\text{Bound} = \frac{[\text{ligand}][\text{excluded volume} \times \text{total volume}]}{[\text{total volume} - \text{excluded volume}]}$$

2.5. *Tryptic digest of PKCI-1*

600–800 μg of purified PKCI-1 [5] in 0.1 M ammonium bicarbonate (pH 7.8) was digested with 16 μg of TPCK-treated trypsin (Cooper Biomedical) for 4 h at room temperature. The reaction was terminated by dilution with 0.1% (v/v) TFA and directly injected onto a Varian 5000 liquid chromatograph equipped with a Hitachi diode-array detector (EM Science, Cherry Hill, NJ). The stationary phase was a C4 Vydac reversed-phase column (0.46 × 25 cm) from The Separations Group (Hesperia, CA), and the mobile phase was 0.1% TFA (v/v) with an 80 min linear gradient of 0–40% acetonitrile (HPLC-Grade, Burdick and Jackson, Muskegon, MI) in 0.1% TFA at 1 ml/min.

2.6. *Zinc overlay experiments*

Proteins, tryptic peptides and synthetic peptides were either spotted or applied with vacuum to sheets of nitrocellulose (0.2 μm, Schleicher and Schuell, Keene, NH). Samples applied by vacuum involved the use of a Bio-Dot apparatus (Bio-Rad, Richmond, CA) and washing with several volumes of buffer. The zinc-binding reaction was carried out as previously described [9]. Briefly, protein-impregnated nitrocellulose sheets were soaked for 30 min in 0.01 M Tris-HCl (pH 7.5) and then 15 min in the same buffer plus 0.1 M KCl and 5 μM $^{65}ZnCl_2$ (specific activity was 60 μCi/mol). The sheets were then washed 3 times for 15 min each in 0.01 M Tris-HCl (pH 7.5) containing 0.1 M KCl, blotted dry and exposed to X-ray film (X-OMAT AR, Kodak) for 1 h.

2.7. *Protein kinase C activity assay*

Rat brain PKC (isozymic, specific activity 1.9 μmol/min/mg) was purchased from Lipidex (Westfield, NJ) and stored in 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol, 0.05% Triton X-100 and 0.1 M NaCl. Phosphorylation of histone Type III-S (Sigma) was determined by mixed micelle assay [10] using 10 ng of PKC; since this mass was determined to be the minimum amount needed in the linear range of activity (data not shown). Inhibition of this reaction was analyzed by pre-incubation of PKC (15 min) with synthetic peptides (up to 250-fold molar excess peptide:PKC) corresponding to various segments of PKCI-1 in the presence or absence of 10 μM $ZnCl_2$. Reactions were performed in 50 μl at 37°C for 15 min and stopped by pipetting the solution onto phosphocellulose paper. The paper was then rinsed, dried, and immersed in 15 ml of scintillation cocktail (Ultima Gold, Packard Instruments, Downers Grove, IL) and counted 10 min for radioactivity.

2.8. *Synthesis and purification of peptides*

Peptides corresponding to specific regions of PKCI-1 were synthesized by solid-phase Boc chemistry on an Applied Biosystems 430A peptide synthesizer (Foster City, CA).

Purification was carried out on a Vydac C-18 reversed-phase column using a 0.1% (v/v) TFA/acetonitrile gradient. Peptides were analyzed for purity identity by amino acid analysis and FAB MS.

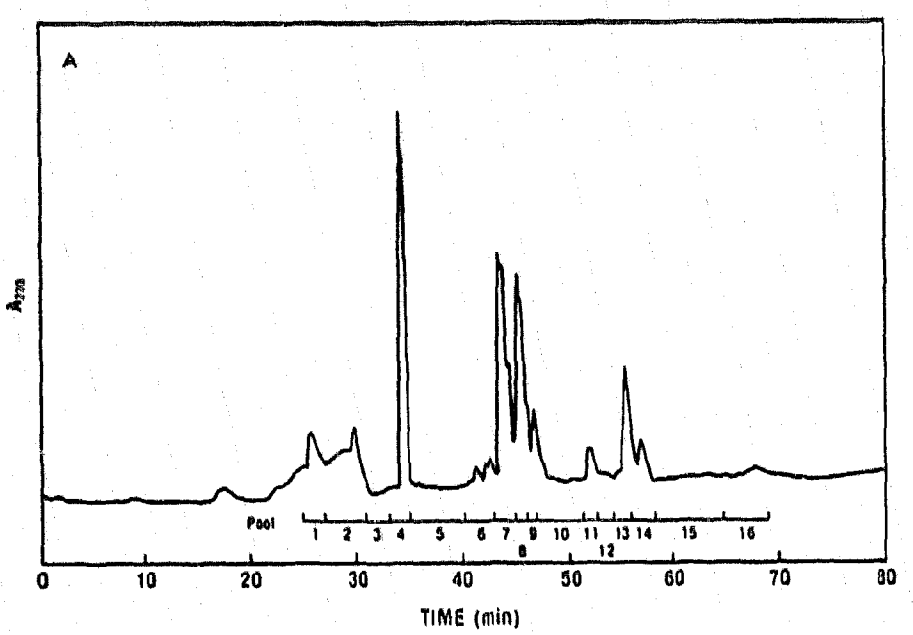
3. RESULTS

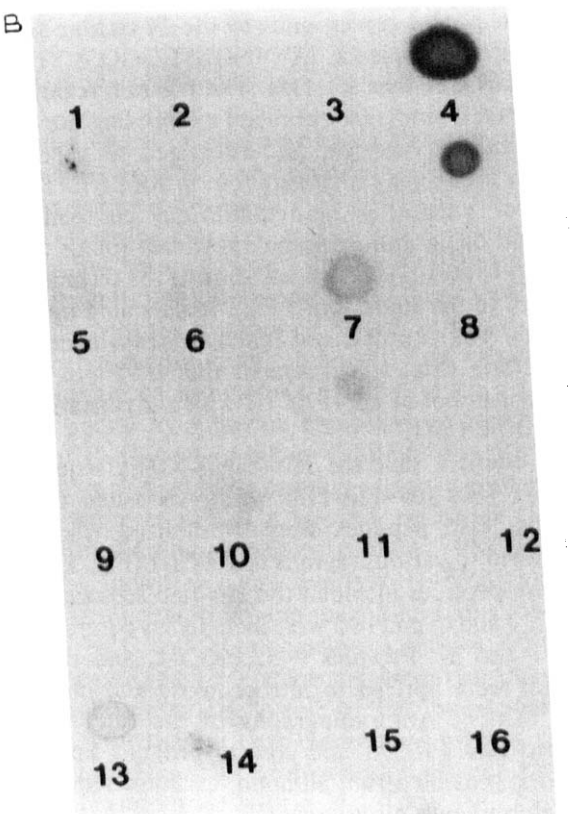
To determine the region responsible for zinc binding, tryptic fragments of PKCI-1 were separated by HPLC, pooled as discrete peaks (Fig. 1A), and applied onto nitrocellulose for hybridization with $^{65}ZnCl_2$ and subsequent autoradiography (Fig. 1B). Pool 4, the only sample which gave a strong signal, was analyzed by FAB MS [5] and shown to contain a single peptide having a mass of 2545 (data not shown). The weak signal in pool 7 probably represents a non-specific interaction, since previous work showed that this pool corresponds to the tryptic peptide containing EDDDE, five contiguous acidic residues [5]. According to the primary structure of PKCI-1 [5], the strong $^{65}Zn^{2+}$-binding tryptic peptide in pool 4 corresponds to the 24 residue fragment having the sequence MVVNEGSDGGQSVYHVHLH VLGGR (residues 95–118). The His-rich region in this sequence is similar to the metal ion binding site of other zinc-binding proteins, and suggested to us that this region might play a similar role in PKCI-1.

After validating the equilibrium gel penetration method using apo-carbonic anhydrase (binds zinc ion in a 1:1 ratio [11], data not shown) the technique was applied to the analysis of PKCI-1. Zinc binding to apo-PKCI-1 was saturable and Scatchard transformation of these data (Fig. 1C) indicated that 0.97 mol of zinc bound 1.0 mol of apo-PKCI-1 with a dissociation constant of 4.3 μM.

To confirm that the His-X-His-X-His sequence in PKCI-1 (His-109–His-113) was responsible for zinc binding, five peptides were synthesized which corresponded to various regions of PKCI-1 (Fig. 2). Three of these peptides included the His-rich sequence (peptides 2, 3 and 4) and two were included as controls (peptides 1 and 5). Peptides 1–5, PKCI-1, and standard proteins were applied to nitrocellulose and incubated with $^{65}ZnCl_2$. Autoradiography of the filter (Fig. 3) revealed that PKCI-1 and control proteins known to bind zinc (bovine serum albumin, carbonic anhydrase, hemoglobin) gave positive signals, whereas the negative controls (trypsin, chymotrypsin) did not. More importantly, Fig. 3 shows that peptides with the His-rich sequence (peptides 2, 3 and 4) all bound zinc and those without that sequence (peptides 1 and 5) did not.

Inclusion of synthetic peptides in the PKC assay at a 250-fold molar excess of PKC had no effect on PKC activity in the presence or absence of 10 μM $ZnCl_2$. Unpublished data from this laboratory have shown that 10 μM $ZnCl_2$ is slightly lower than that which is inhibitory in the mixed micelle assay [10].





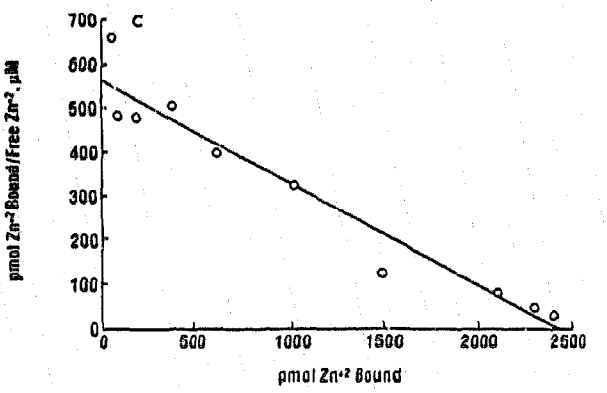


Fig. 1. Identification and stoichiometry of zinc-binding to PKCI-1. (A) Reversed-phase tryptic map, fractions pooled as indicated. (B) Pools from (A) were lyophilized and applied to nitrocellulose as aliquots of 2/3 and 1/3 (above and below the numbers, respectively) of the total sample, incubated with $^{65}ZnCl_2$, and subjected to autoradiography. (C) Scatchard plot of the binding data. Binding of Zn^{2+} (spec. act. = 8800 cpm/nmol) to apo-protein (2.5 nmol) was measured by equilibrium gel penetration. Linear regression analysis indicates that $r^2 = 0.95$, $y_{INT} = 2426$, and $m = 0.23$.

During the course of these experiments the removal of adventitious zinc proved to be vital since nearly all materials were contaminated with metals. The nature of the stoichiometry experiments demanded precise concentrations of zinc ion and this was possible only by scrupulous clean-up of reagents and equipment. Complete removal of zinc ion in all solutions was confirmed by atomic absorption spectroscopy before the experiments were performed.

4. DISCUSSION

The sequence His-Val-His-Leu-His in PKCI-1 represents a new site for zinc binding inasmuch as it has

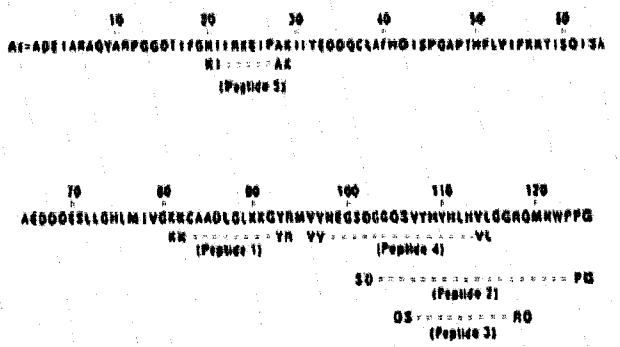


Fig. 2. Amino acid sequence of PKCI-1 and synthetic peptides. Peptide designations are indicated underneath the sequence by their first and last two residues.

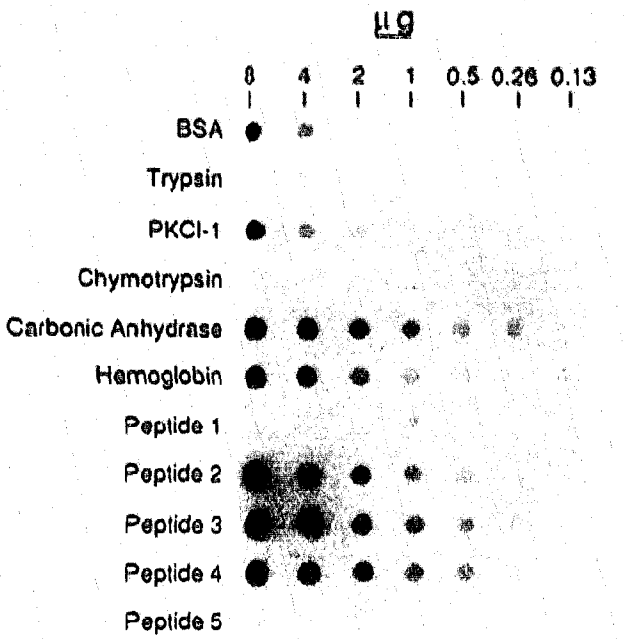


Fig. 3. Binding of $^{65}ZnCl_2$ to proteins and peptides immobilized on nitrocellulose.

not been seen in any other proteins which bind zinc. There is no similarity between this site and those of 'zinc-finger' proteins [1] since Cys residues do not appear to be involved. The site in PKCI-1 is distinct from the metal-binding motif (H-E-X-X-H) assigned to zinc-dependent metallopeptidases [12]. In a general sense, however, the zinc-binding site of PKCI-1 is similar to other His-rich zinc-binding sites which have been characterized by crystallographic analysis. For example, proteins having catalytic functions with zinc ion in their active sites (e.g. carboxypeptidase A, carbonic anhydrase) have a motif composed of two His ligands separated by a stretch of one to three amino acids, a third His ligand (sometimes Glu) 20 residues distal to the first two, and a fourth water ligand [13]. Comparing the PKCI-1 sequence to the above, any combination of two imidazole ligands in the His-X-His-X-His region matches the spacing requirement for the first two ligands. Our data indicate the third ligand is not a Glu or Asp since peptide 3 binds zinc as well as peptides 2 and 4 and does not contain any acidic residues. In fact, it can be seen in Fig. 3 that peptide 3 (QSVYHVHLH VLGGRQ) binds zinc at the same dilution as peptides 2 (SDGGQSVYHVHLHVLGGRQMNWPPG) and 4 (VVNEGSDGGQSVYHVHLHVL) and therefore contains all the structure necessary for binding. Since there are no other potential zinc binding amino acids (His, Cys, Glu, or Asp) in the sequence of peptide 3, it is likely that all three His residues participate in binding and the fourth ligand is water. This hypothesis is supported by the observation of Iyer et al. [14] that a synthetic cyclic heptapeptide, Gly-His-Gly-His-Gly-His-Gly, binds zinc ion in a 1:1 ratio. It was further shown by NMR spectroscopy that all three His residues in GHGHGHG participate in the binding.

PKCI-1 has previously been shown to bind zinc ion preferentially over magnesium or calcium ions [5]. A physiological role for this binding is suggested by the 1:1 stoichiometry of zinc:PKCI-1 and the K_d of 4.3 μM. Other zinc binding proteins are reported to contain micromolar K_d values [15–18] and the concentration of zinc in bovine brain is in the micromolar range [19]. Since PKC and PKCI-1 are both zinc-binding proteins, it is interesting to speculate that they may be co-regulated by an intracellular zinc ion flux. PKC was not inhibited by peptides of PKCI-1 that contained the zinc binding site (peptides 2, 3 and 4; data not shown), suggesting that other regions of the PKCI-1 molecule or its secondary structure are needed for activity. Further investigations into the details of this interaction will provide information about the role PKCI-1 plays in the modulation of PKC.

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