Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 8, pp. 965-970, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00140-6

PROTEIN KINASE C REGULATORY DOMAIN SURROGATE PEPTIDES: EFFECTS OF METAL IONS ON FOLDING, PHORBOL ESTER-BINDING, AND SELECTIVITY

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Abstract: The effects of zinc and other metal ions on the folding and phorbol ester-binding of protein kinase C (PKC) surrogates have been investigated using the second cysteine-rich domain model peptides of rat PKCγ and mouse PKCη (γ-CRD2 and η-CRD2). The results clearly show that zinc plays an important role in the folding and phorbol ester-binding of these PKC surrogates. In addition, while treatment of these surrogates with various divalent first row transition metal ions other than zinc resulted in binding at background levels, treatment with copper, silver, gold, or mercury completely abolished binding. It is especially noteworthy that cadmium treated η-CRD2 showed a high level of binding while similarly treated γ-CRD2 exhibited no binding. These results suggest that recent reports on the inhibition of conventional PKC by heavy metal ions could be explained by their effects on the folding and binding of the CRD subunits. © 1997 Elsevier Science Ltd.

Protein kinase C (PKC) is a key enzyme family involved in the cellular signal transduction and tumor promotion.[1] It consists of a catalytic domain for protein phosphorylation and a regulatory domain which binds the endogenous messenger diacyl glycerol or exogenous agents such as the phorbol ester-type tumor promoters. Located within the N-terminal regulatory sequence of PKC are tandem cysteine-rich domains (CRD's). Synthetic CRD's consisting of *ca.* 50 amino acids have been shown to be exceptionally effective PKC regulatory domain surrogates, binding phorbol esters with affinities comparable to native PKC itself[2-6] and available in quantity and high purity through solid phase synthesis.[3,5,6] The CRD's of PKC have six conserved cysteines and two histidines in the pattern $\mathbf{H}X_{12}\mathbf{C}X_2\mathbf{C}X_n\mathbf{C}X_2\mathbf{C}X_4\mathbf{H}X_2\mathbf{C}X_7\mathbf{C}$ (n=13 or 14) where X is a variable amino acid residue. Each CRD coordinates two atoms of zinc with each metal bound by three sulfur atoms of cysteines and one nitrogen atom of histidine.[7,8] Differing from the classical zinc finger proteins, the CRD of PKC adopts a globular fold allowing two non-consecutive sets of zinc-binding residues to form two separate metal-binding sites.[9-11] Although this zinc coordination is required for folding and phorbol ester-binding, the effects of zinc and other metal ions on these processes have not been investigated. Such information is required for structural studies on PKC, elucidation of the role of metals in PKC signaling pathways, and the development of high throughput screens. We have recently synthesized the second CRD's of rat brain PKCγ (amino acids 101-151: γ-CRD2) and mouse skin PKCη (amino acids 246-296: η-CRD2), surrogates of conventional and novel PKC's, respectively, and have shown that these peptides serve as effective molecular probes for elucidation of the structural requirements for the recognition of phorbol ester-type tumor promoters (Figure 1).[3,5,12] This communication describes the initial study of the influence of zinc and other metal ions on the folding and phorbol ester-binding of these regulatory domain surrogate peptides, including the first observation of surrogate selective metal ion regulation of binding.

γ-CRD2 (rat brain PKCγ)

H_2N-HKFRLHSYSSPTFCDHCGSLLYGLVHQGMKCSCCEMNVHRRCVRSVPSLCG-COOH
101 151

η-CRD2 (mouse skin PKCη)

H_2N-HKFNVHNYKVPTFCDHCGSLLWGIMRQGLQCKICKMNVHIRCQANVAPNCG-COOH
246 296

Figure 1 Structure of γ-CRD2 and η-CRD2.

The effects of metal ions on phorbol ester-binding to γ-CRD2 and η-CRD2 are shown in Figure 2. In these studies, a distilled water solution[13] of each CRD was treated initially with the indicated metal salt (5-molar equivalent).[14] An aliquot of the resultant solution was then added to the assay mixture consisting of Tris-HCl (50 mM, pH 7.4), bovine γ-globulin (3 mg/mL), phosphatidylserine (50 μg/mL), and [^{3}H]phorbol 12,13-dibutyrate (PDBu, 30 or 20 nM). The binding of PDBu was measured by the procedure of Sharkey and Blumberg.[15] As reference standards, the specific binding of PDBu to zinc-treated (folded) γ-CRD2 (10 nM) and η-CRD2 (5 nM) were each fixed at 100 (entry 1). Different concentrations of the peptides and PDBu were employed since the K_d's for PDBu were significantly different from each other (44.1 nM for γ-CRD2, 0.91 nM for η-CRD2).[5] In accord with the importance of zinc in maintaining a properly folded peptide, only weak PDBu binding was observed when the peptides were initially *not* treated with zinc (entry 2). Moreover, even this binding was abolished (entry 3) when the *untreated* peptides were added to an assay mixture containing 2 mM EDTA, suggesting that chelatable ions in the assay mixture account for the background folding of untreated CRD2's. It is noteworthy that the binding of *zinc-treated* γ-CRD2 and η-CRD2 were not changed by exposure to 2 mM EDTA (entry 4), even for periods of up to 3 h, indicating that zinc binding to these peptides is too tight to be readily reversed by EDTA.

Initial treatment of the CRD's with divalent first row transition metal ions such as Cr^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+} in place of Zn^{2+} had no significant effect on binding relative to background levels (entries 5-9 vs entry 2). Mg^{2+} and Ca^{2+} were also ineffective (entries 10,11). On the other hand, when the CRD's were initially treated with ions of group Ib elements such as Cu^{2+}/Cu^{+}, Ag^{+}, and Au^{+}, specific binding was abolished (entries 12-14). This loss of specific binding was also found for $CuCl_2$ (2.5-molar equivalent) even when the CRD's were pretreated with zinc (data not shown). Cd^{2+} and Hg^{2+} which belong to the same group (IIb) as Zn^{2+} produced remarkably different effects. Hg^{2+} treatment of the CRD's eliminated binding (entry 16). *In contrast, Cd^{2+} treatment of γ-CRD2 abolished binding (entry 15) while Cd^{2+} treatment of η-CRD2 resulted in significant PDBu binding, approaching the maximum level observed for the Zn^{2+} treated peptide (entry 15).* Moreover, the addition of 2.5-molar equivalent of Cd^{2+} to Zn^{2+} pretreated γ-CRD2 caused complete disappearance of binding. The addition of a 100-fold excess of Zn^{2+} to Cd^{2+} pretreated γ-CRD2 did not restore binding, suggesting that binding of Cd^{2+} to γ-CRD2 is stronger than that of Zn^{2+}. With the discovery of at least eleven PKC isozymes (PKC α, βI, βII, γ, δ, ε, η/L, θ, μ, ζ, λ/ι),[16] increasing importance is placed on isozyme specific analysis of function in order to elucidate the role of PKC in cellular signal transduction and tumor promotion. Due to the limited information on the solution structure of the phorbol ester-PKC-phosphatidylserine aggregate,[17,18] *most efforts to generate isozyme selective agonists have focused thus far on variations in organic ligands.* The significant CRD selectivity observed for Cd^{2+} suggests that *metal ions might also become effective tools for controlling isozyme selective activation and inhibition of PKC.*

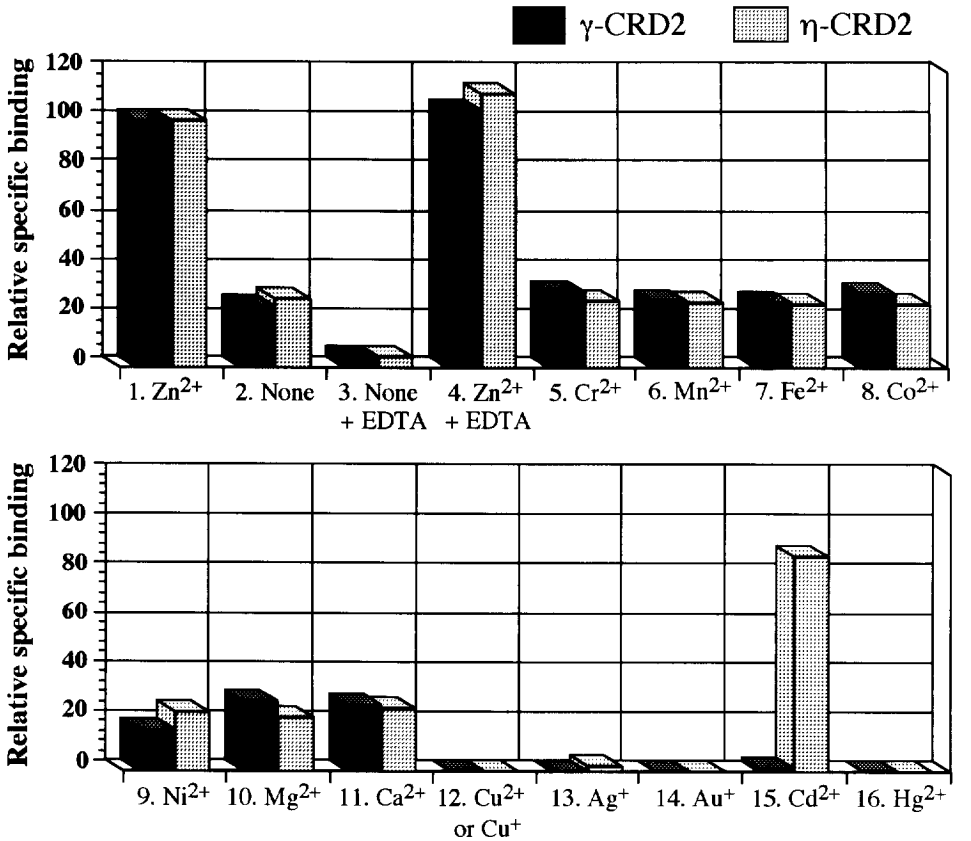

Figure 2 Effects of zinc and other metal ions on the PDBu binding of γ-CRD2 and η-CRD2. The binding was evaluated by the procedure of Sharkey and Blumberg.[15] Metal coordination was carried out in a distilled water solution of each model peptide (100 μg/mL) using 5-molar equivalent of each metal ion at 4 °C for 10 min. After dilution with distilled water, an aliquot of the peptide solution (2.9 μL) was added to the assay mixture (247.1 μL) consisting of 50 mM Tris-HCl (pH 7.4), 3 mg/mL bovine γ-globulin, 50 μg/mL phosphatidylserine, and 30 or 20 nM [^{3}H]PDBu (19.6 Ci/mmol). For the γ-CRD2 binding assay, 30 nM [^{3}H]PDBu and 10 nM γ-CRD2 were used; for the η-CRD2 assay, 20 nM [^{3}H]PDBu and 5 nM η-CRD2 were employed. Specific binding is the difference between total and non-specific binding which was determined in the presence of 500-fold cold PDBu. The specific binding of $ZnCl_2$-treated γ-CRD2 and η-CRD2 (16 600 dpm and 14 300 dpm) were each fixed at 100 (entry 1). Entry 2: the peptides without $ZnCl_2$ treatment; entry 3: the peptides without $ZnCl_2$ treatment in the presence of 2 mM EDTA in the assay mixture; entry 4: the $ZnCl_2$-treated peptides in the presence of 2 mM EDTA in the assay mixture; entry 5: the $CrCl_2$-treated peptides; entry 6: the $MnCl_2$-treated peptides; entry 7: the $FeSO_4(NH_4)_2SO_4$-treated peptides; entry 8: the $CoCl_2$-treated peptides; entry 9: the $NiCl_2$-treated peptides; entry 10: the $MgCl_2$-treated peptides; entry 11: the $CaCl_2$-treated peptides; entry 12: the $CuCl_2$ or CuCl-treated peptides; entry 13: the $AgNO_3$-treated peptides; entry 14: the AuCN-treated peptides; entry 15: the $CdCl_2$-treated peptides; entry 16: the $HgCl_2$-treated peptides. Each point represents the mean of three experimental values with a standard deviation of less than 5%.

Modulation of PKC by neurotoxic heavy metals has recently been investigated since PKC is involved in neurotransmitter release[19-21] and signal transduction.[22] For example, Speizer *et al.*[23] and Rajanna *et al.*[24] have reported that heavy metal ions as Hg^{2+}, Cu^{2+}, and Cd^{2+} inhibited the enzyme activity of conventional PKC mixture and its PDBu binding. Thus far, it has been mainly considered that heavy metal ions interact with the catalytic domain of PKC to inhibit the PKC activation.[25,26] However, our present results strongly indicate that one of the target sites of these heavy metal ions is the CRD itself in the regulatory domain.

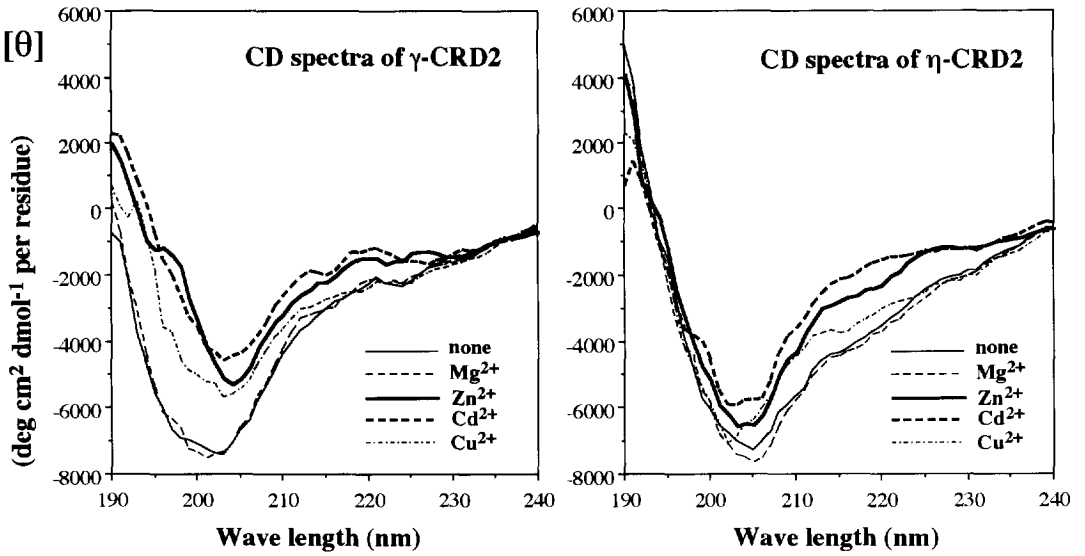

Figure 3 Left side: CD spectra of γ-CRD2 itself and γ-CRD2 treated separately with 2.5-molar equivalent of $ZnCl_2$, $CdCl_2$, $CuCl_2$, or $MgCl_2$ in helium purged distilled water. Right side: CD spectra of η-CRD2 itself and η-CRD2 treated separately with 2.5-molar equivalent of $ZnCl_2$, $CdCl_2$, $CuCl_2$, or $MgCl_2$ in helium purged distilled water. The spectra were obtained on a JASCO 700 CD spectrophotometer in a 0.5 mm cell using 275 μg/mL solutions in 10 mM Tris-HCl buffer (pH 7.4) at 24 °C.

In order to investigate the metal induced conformational change of the model peptides, CD spectra of the complexed and uncomplexed CRD's were measured (Figure 3). In accord with the binding data above, each CRD treated with $MnCl_2$, $MgCl_2$, or $CaCl_2$, gave a spectrum quite similar to each untreated CRD itself; only the $MgCl_2$-treated spectra are shown in Figure 3. However, a significant spectral change was detected for both CRD's upon treatment with $ZnCl_2$. A quite similar spectral change to the $ZnCl_2$-treated peptides occurred in each CRD upon treatment with $CdCl_2$. Treatment with $CuCl_2$ also resulted in a similar spectral change, suggesting that Cu^{2+} coordinates each CRD in a similar fashion to Zn^{2+} and Cd^{2+} to produce closely related peptide conformers. Lack of the PDBu binding of Cd^{2+} or Cu^{2+} treated peptides would reflect the finely different conformation from that of the Zn^{2+} treated peptides though it is unclear at present why such selectivity arises. In the classical zinc finger peptides, Cu^{2+} is found to oxidize the peptide presumably to disulfide-linked species.[27] However, no significant difference in the PDBu binding and CD spectra of the $CuCl_2$-treated peptides was detected between aerobic and anaerobic conditions, indicating that such oxidation did not occur significantly in our condition.

The CD spectra of γ-CRD2 were analyzed by the method of Yang *et al.*[28] This calculation indicated that 0.5% and 58.3% of the residues of untreated γ-CRD2 are in α-helix and β-sheet, respectively. In $ZnCl_2$-treated γ-CRD2, these estimates were 9.2% α-helix and 56.3% β-sheet while in $CuCl_2$-treated γ-CRD2 the values were 5.2% α-helix and 58.5% β-sheet. These results suggest that coordination of Zn^{2+} and Cu^{2+} to γ-CRD2 significantly increases the α-helix content while the β-sheet content changes only slightly. On the contrary, the

spectral change by zinc coordination to η-CRD2 was less drastic than γ-CRD2; the calculation did not show any significant change of both α-helix and β-sheet content.

In summary, this study demonstrates that *metal ions*, not unlike the more extensively explored *organic ligands* for PKC, have a significant effect on the folding and PDBu binding of the PKC regulatory domain surrogate peptides, γ-CRD2 and η-CRD2. Zinc coordination is crucial for CRD folding and binding, while most divalent first row transition metals are ineffective. In contrast, exposure of the CRD's to Ib group element ions such as Cu^{2+}/Cu^{+}, Ag^{+}, and Au^{+} abolishes PDBu binding. It is especially significant that Cd^{2+} exhibits CRD selective effects, folding one and inactivating the other. Further studies on this novel approach to isozyme control are focused on the elucidation of the solution structure of γ-CRD2 and η-CRD2 coordinated with Cu^{2+}, Ag^{+}, Cd^{2+}, and Hg^{2+} and on the role of these ions in PKC function.

Acknowledgments

The authors thank Mr. Hiroyuki Fukuda at Nihon PerSeptive Ltd. for synthesis of η-CRD2 and MALDI-TOF-MS measurements. The authors are also grateful to Professors Edward Solomon and Daniel Stack (Stanford University) for valuable input. This research was partly supported by a Grant-in-Aid for Scientific Research on Priority Areas (No. 08219224) and Scientific Research (C) (No. 08660137) from the Ministry of Education, Science and Culture, Japan (K. I.), and by a grant (CA31841) from the National Institutes of Health (P. A. W.).

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13. Folding in distilled (pH 5.5) water gave *ca.*2-fold higher specific binding than that in 0.01% trifluoroacetic acid (pH 3.0) followed by neutralization, a general folding condition for the zinc finger peptides.[4] Careful

examination on the pH dependence of the PDBu binding indicated that the optimal pH for maximum binding is between 5.0 and 6.5 (data not shown). Folding studies conducted at pH values greater than 8 did not show any PDBu binding.

14. Each CRD2 coordinates two atoms of zinc.[7,8] Since 2.5-molar equivalents of zinc gave maximum binding, 5-molar equivalents of zinc or other metal salts were used in the folding of the peptides. An almost linear concentration dependence on the binding was observed at less than 2-molar equivalents of zinc or cadmium.
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(*Received in Japan* 30 *January* 1997; *accepted* 5 *March* 1997)