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SYNTHESIS AND CHARACTERIZATION OF THE SECOND CYSTEINE-RICH REGION OF MOUSE SKIN PKCη

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Abstract: The second cysteine-rich region of mouse skin PKCη, peptide D, was prepared by automated solid phase peptide synthesis. In the presence of zinc and phosphatidylserine, peptide D bound [^{3}H]phorbol 12,13-dibutyrate with high affinity (K_d = 0.91 nM). Peptide D serves as an effective surrogate for the mouse skin derived receptor, affording unique opportunities for the study of binding, affinity labeling, and solution structure related to the PKC regulatory domain.

Protein kinase C (PKC), a key enzyme family involved in cellular signal transduction, is the main target of the potent phorbol ester tumor promoters.[1] With the discovery of at least ten PKC isozymes in recent years,[2] increasing importance is placed on isozyme specific analysis of binding and function in order to elucidate at the molecular level the role of PKC in cellular signal transduction, tumor promotion, and carcinogenesis. Isozyme selective agonists represent powerful tools for such studies, but the rational development of such compounds has been hampered by limited information on the solution structure of the phorbol ester-PKC-phosphatidylserine aggregate. Because pure PKC isozyme is not easily obtainable from natural sources, cysteine-rich regions of the regulatory domain of PKC have been prepared by DNA recombination and solid phase synthesis.[3-11] These peptides have been shown to bind phorbol 12,13-dibutyrate (PDBu), allowing for further investigation of the phorbol ester binding site by NMR[8,9] and X-ray analyses.[10] We previously reported that such cysteine-rich regions of PKCγ could be obtained by automated solid phase synthesis using fluorenylmethoxycarbonyl (Fmoc) protected amino acids.[11] This approach is especially useful for the preparation of large quantities of highly pure PKC subunits. In our last paper, we described the synthesis of the first and the second cysteine-rich region of rat brain PKCγ, peptides B and C, respectively, and showed that peptide C, a model of the so called *conventional* PKC family (PKCα, β1, β2, γ),[2] bound PDBu to a greater degree than peptide B.[11] In this communication, we report the synthesis, characterization, and binding ability of the second cysteine-rich region of mouse skin PKCη, peptide D, a surrogate of the so called *novel* PKC's (PKCδ, ε, η, θ).[2]

Peptide D (mouse skin PKCη amino acids 246-296)[12] was synthesized with a Milligen/Biosearch 9050 automated peptide synthesizer using Fmoc amino acids. The crude peptide, which was obtained by cleavage and deprotection by trifluoroacetic acid (TFA) containing *m*-cresol, ethanedithiole and thioanisole, was purifed by gel filtration, followed by HPLC using μ-Bondasphere C_{18} (ODS, 19 mm i.d. x 150 mm) with elution at 8 ml/min by a 120-min linear gradient of 10-50% acetonitrile in 0.1% TFA. The peak of peptide D (t_R = 110 min) was collected and the solution was concentrated *in vacuo* under 30 °C to remove acetonitrile. Lyophilization of the residue gave pure peptide D, which exhibited satisfactory mass spectral analysis (MH^+, average molecular mass; obs. 5815.68, calc. for $C_{252}H_{397}N_{78}O_{65}S_8$, 5815.94) as shown in Figure 2.

Peptide C (rat brain PKCγ)

H_2N-HKFRLHSYSSPTFCDHCGSLLYGLVHQGMKCSCCEMNVHRRCVRSVPSLCG-COOH
101 151

Peptide D (mouse skin PKCη)

H_2N-HKFNVHNYKVPTFCDHCGSLLWGIMRQGLQCKICKMNVHIRCQANVAPNCG-COOH
246 296

Figure 1 Structure of peptides C and D.

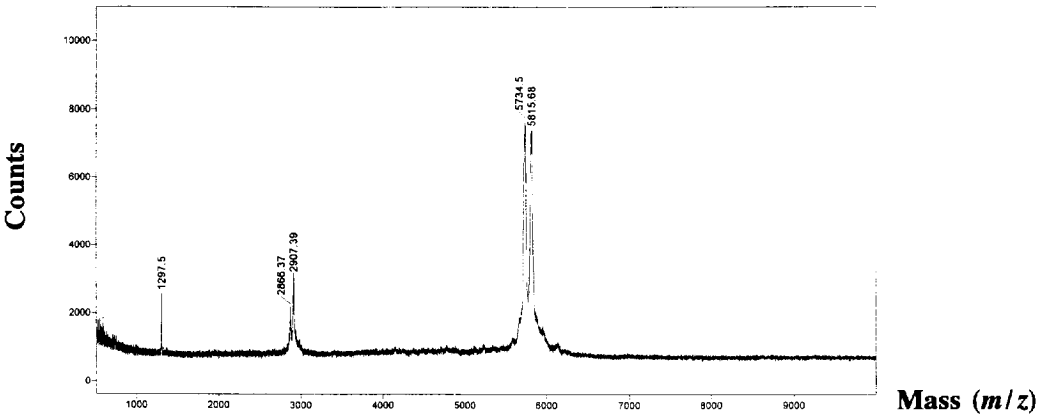

Figure 2 Matrix-assisted laser desorption time-of-flight mass spectrum (MALDI-TOF MS) of peptide D together with angiotensin I (MH^+, 1297.5) and bovine insulin (MH^+, 5734.5) as internal standards. Peptide D (2.5 pmol), angiotensin I (0.5 pmol), bovine insulin (2.5 pmol) were dissolved in saturated α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA-50% acetonitrile. Two peaks at 2866.37 and 2907.39 are doubly charged ions of bovine insulin and peptide D, respectively.

The regulatory domain of PKC coordinates four atoms of zinc,[13,14] and this zinc binding is believed to be indispensable to phorbol ester binding. The effect of zinc on the folding of peptides C and D is given in Figure 3. As reported previously,[11] significant specific binding between peptide C and PDBu was detected when peptide C dissolved in distilled water without $ZnCl_2$ was added to the reaction mixture containing 50 mM Tris-HCl, 50 μg/ml phosphatidylserine, 3 mg/ml bovine γ-globulin and 20 nM [^{3}H]PDBu at pH 7.4. However, the specific binding increased *ca.*4-fold when peptide C treated with 5-molar equivalents of $ZnCl_2$ in distilled water was added to the reaction mixture. A similar tendency was observed in the case of peptide D. A dramatic increase of the specific binding was detected by zinc treated peptide D. The use of zinc in 0.05% TFA, a general folding condition for zinc finger peptides,[15,16] also gave similar results. These data clearly indicate that zinc plays an important role in folding of these synthetic peptides.

Scatchard analyses of peptides C and D, folded by 5-molar equivalents of $ZnCl_2$ in distilled water, gave binding constants (K_d) of 44.1 and 0.91 nM, respectively (Figure 4). These values are in good agreement with K_d values reported for the PKCγ (59 nM)[4] and PKCδ (1.9 nM)[16] fragments obtained by DNA recombination. It is interesting that these *novel* PKC fragments bind PDBu with affinities comparable in potency to native PKCδ (K_d = 1.0 nM),[16] and about 50-fold stronger than that observed for the *conventional* PKC model peptide. To investigate in detail the effect of zinc, the K_d value of zinc-free peptide D was also determined (data not shown). The Scatchard plot revealed a K_d of 2.5 nM, which is comparable to the value for the zinc treated peptide D. However, the B_{max} was about 1/10 of that of the zinc treated peptide D. This indicates that the difference between entry 3 and 4 in Figure 3 reflects the total amount of properly folded peptide D.

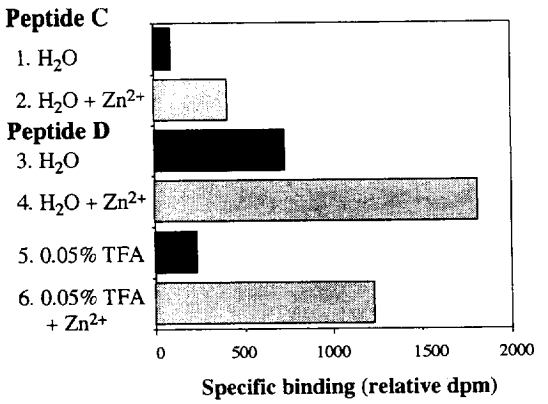

Figure 3 Effect of zinc on the folding of peptides C and D. The binding of PDBu was evaluated by the procedure of Blumberg with slight modification.[17,18] Peptides C or D in solution (2.9 μl) was added to the reaction mixture (247.1 μl) consisting of 50 mM Tris-HCl (pH 7.4), 3 mg/ml bovine γ-globulin, 50 μg/ml phosphatidylserine and 20 nM [^{3}H]PDBu. The final concentrations of peptides C and D were 10 nM. 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 peptide C dissolved in distilled water (entry 1) is fixed to 100. Entry 2: peptide C dissolved in distilled water containing 5-molar eq. $ZnCl_2$; entry 3: peptide D dissolved in distilled water; entry 4: peptide D dissolved in distilled water containing 5-molar eq. $ZnCl_2$; entry 5: peptide D dissolved in 0.05% TFA; entry 6: peptide D dissolved in 0.05% TFA containing 5-molar eq. $ZnCl_2$. Each point represents the mean of three experimental values with a standard deviation of <5%.

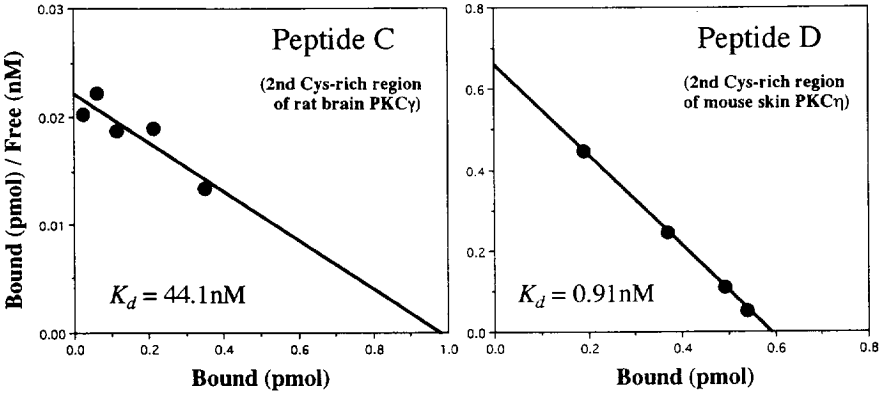

Figure 4 Scatchard analyses of [^{3}H]PDBu binding to peptides C and D, folded by 5-molar equivalents of $ZnCl_2$. Peptides C or D were incubated for 20 min at 30 °C with increasing concentrations of [^{3}H]PDBu in the presence of 50 μg/ml phosphatidylserine as described in Figure 3. Representative experiments for peptides C and D are shown. Each point represents the mean of three experimental values with a standard deviation of <5%. Similar results were obtained in another experiment.

Table 1. Inhibition of the specific [^{3}H]PDBu binding to peptides C and D [a]

Compound	K_i (nM) for peptide C	K_i (nM) for peptide D
Phorbol 12,13-dibutyrate (PDBu)	52.9	0.60
1,2-Dioctanoyl-*sn*-glycerol	2,930	14.50
(-)-Indolactam-V	1,030	3.36
Teleocidin B-4	2.0	0.10
4α-Phorbol 12,13-didecanoate	Inactive	Inactive

[a] This assay was carried out by the procedure of Blumberg with slight modification.[17,18] The assay solution (250 μl) consisted of 50 mM Tris-HCl (pH 7.4), 3 mg/ml bovine γ-globulin, 30 nM [^{3}H]PDBu, 50 μg/ml phosphatidylserine, 10 nM peptide C and various concentrations of an inhibitor. For peptide D binding assay, 20 nM [^{3}H]PDBu and 5 nM peptide D were used. Peptides C and D were folded by 5-molar equivalents of $ZnCl_2$.

The abilities of other compounds known to interact with the phorbol ester binding site on PKC to competitively inhibit the binding of [^{3}H]PDBu to peptides C and D were determined by varying the concentration of the compound in question. Dose-response curves were plotted for each compound, and the concentration at which 50% of [^{3}H]PDBu binding was inhibited (IC_{50}) was determined. The binding constant, K_i, was calculated by the method of Blumberg.[17] Table 1 gives the results of this analysis for PDBu, 1,2-dioctanoyl-*sn*-glycerol, (-)-indolactam-V, teleocidin B-4 and 4α-phorbol 12,13-dibutyrate. Significantly, the structure-binding relationship is close to those observed for PKC itself.[19,20] Furthermore, both peptides C and D exhibited the strong requirement for phosphatidylserine characteristic of PKC (data not shown).

In summary, we have synthesized the second cysteine-rich region of mouse skin PKCη, peptide D, and have shown that this peptide can be efficiently folded upon zinc treatment to produce a PKC regulatory domain surrogate that binds [^{3}H]PDBu with high affinity, comparable to native PKC itself. The similar binding behavior of peptide D and PKC suggests that peptide D could serve as a useful molecular probe for elucidation of the structural requirements for the recognition of phorbol ester-type tumor promoters and for photoaffinity labeling.

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