Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 2, pp. 117-122, 1997
Copyright © 1997 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0960-894X/97 $17.00 + 0.00

PII: S0960-894X(96)00587-2

SYNTHESIS AND CHARACTERIZATION OF THE FIRST CYSTEINE-RICH DOMAIN OF NOVEL PROTEIN KINASE C

Yoshiaki Yanai, Kazuhiro Irie*, Hajime Ohigashi, and Paul A. Wender[a]*

Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan
[a]Department of Chemistry, Stanford University, Stanford, CA 94305, USA

Abstract: The first cysteine-rich domains (CRD's) of mouse PKCη and PKCδ (η-CRD1 and δ-CRD1) were prepared by automated solid phase peptide synthesis. In the presence of phosphatidylserine, zinc-folded η-CRD1 did not bind [^{3}H]phorbol 12,13-dibutyrate (PDBu) at all while the zinc-folded second CRD of mouse PKCη (η-CRD2) bound PDBu strongly with a K_d of 0.91 nM. Moreover, δ-CRD1 showed very weak PDBu binding affinity (K_d = *ca.*500 nM) while the K_d of the second CRD of mouse PKCδ was reported to be 1.9 nM. These results suggest that the second CRD's of PKCη and PKCδ play an important role in the PDBu binding.
© 1997, Elsevier Science Ltd. All rights reserved.

Protein kinase C (PKC), a key multi-enzyme family that mediates cellular signal transduction, is the main target of the potent phorbol ester tumor promoters and a receptor of interest for the development of new medicinal leads.[1] To date, at least eleven PKC isozymes have been identified and classified into three groups based on their structure and cofactor requirements (Figure 1).[2] The best characterized and first discovered are the so called *conventional* PKC's: PKCα, βI, βII, and γ. Cloning of these PKC's revealed four conserved domains (C_1-C_4) and five variable (V_1-V_5) regions. Conventional PKC's consist of a catalytic domain for protein phosphorylation (C_3 and C_4) and a regulatory domain (C_1 and C_2). The latter binds phorbol esters and calcium in the presence of phosphatidylserine. *Novel* PKC's (PKCδ, ε, η, and θ) are a second PKC subclass, structurally similar to conventional PKC's except for the absence of the C_2 domain and, consequently, of calcium independent binding. The least understood are *atypical* PKC's (PKCζ and λ/ι) which are not activated by phorbol esters.

There are tandem cysteine-rich domains (CRD's) at the N-terminal regulatory domain of conventional and novel PKC's. Since recent studies have indicated that a single CRD is sufficient for phorbol ester binding,[3-8] both CRD's in these PKC subclasses are candidates for the phorbol ester binding site. We have recently synthesized the second CRD of mouse PKCη (η-CRD2) as a model of novel PKC by automated solid phase synthesis using fluorenylmethoxycarbonyl (Fmoc) protected amino acids, and have shown that the binding affinity of η-CRD2 to [^{3}H]phorbol 12,13-dibutyrate (PDBu) was especially strong (K_d = 0.91 nM),[8] comparable to that reported for native mouse PKCη itself (K_d = 0.87 nM).[9] Kazanietz *et al.* have also reported that the second CRD of mouse PKCδ bound PDBu with high affinity, comparable to native mouse PKCδ.[7] These results underscore the importance of CRD peptide surrogates of novel PKC as useful molecular probes for the elucidation of the structural requirements for the recognition of phorbol esters. However, there is no evidence that the second CRD is the *only* phorbol ester binding site of novel PKC. In addition, the PDBu binding of any first CRD except for that of PKCγ has not yet been examined. In order to identify the binding site of phorbol esters in novel PKC, we have now synthesized the first CRD's of mouse PKCη and PKCδ (η-CRD1 and δ-CRD1) by the automated solid phase synthesis, and examined their phorbol ester binding ability.

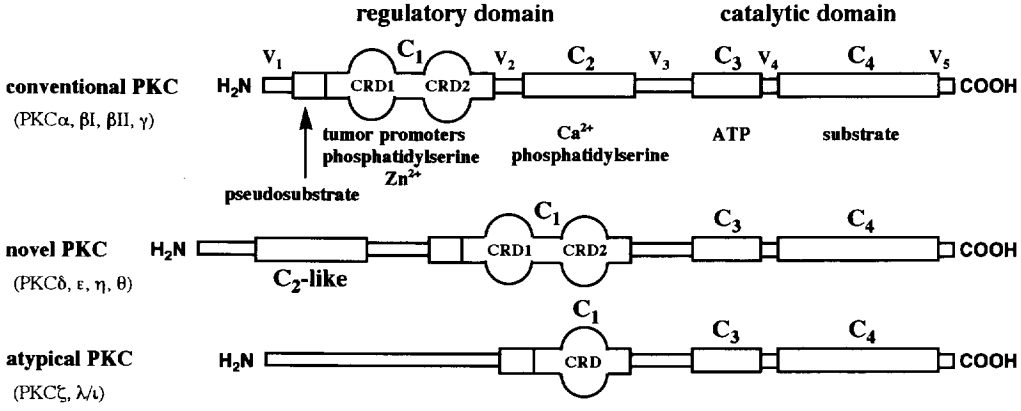

Figure 1 Structure of conventional, novel, and atypical PKC.

```
                1         10        20        30        40        50
η-CRD1 :  H2N-HKFMATYLRQPTYCSHCREFIWGVFGKQGYQCQVCTCVVHKRCHHLIVTACG-COOH
          172                                                   222
η-CRD2 :  H2N-HKFNVHNYKVPTFCDHCGSLLWG-IMRQGLQCKICKMNVHIRCQANVAPNCG-COOH
          246                                                   296
δ-CRD1 :  H2N-HEFIATFFGQPTFCSVCKEFVWG-LNKQGYKCRQCNAAIHKKCIDKIIGRCTG-COOH
          159                                                   210
γ-CRD1 :  H2N-HKFTARFFKQPTFCSHCTDFIWG-IGKQGLQCQVCSFVVHRRCHEFVTFECPG-COOH
          36                                                    87
γ-CRD2 :  H2N-HKFRLHSYSSPTFCDHCGSLLYG-LVHQGMKCSCCEMNVHRRCVRSVPSLCG-COOH
          101                                                   151
```

Figure 2 Structure of PKC model peptides prepared by solid phase synthesis.

η-CRD1 (mouse skin PKCη amino acids 172-222)[10] and δ-CRD1 (mouse hemopoietic cells PKCδ amino acids 159-210)[11] were synthesized with a PerSeptive Biosystems Model 9050 plus automated peptide synthesizer using Fmoc amino acids. To prevent racemization and oxidation during synthesis, the carboxyl-terminus was extended in each case from the final cysteine to a glycine. The crude peptides, which were obtained by cleavage and deprotection by trifluoroacetic acid (TFA) containing *m*-cresol, ethanedithiol, and thioanisole, were purified by gel filtration, followed by HPLC using SH-342-5 (ODS, 20 mm i.d. x 150 mm, Yamamura Chemical Laboratory) with elution at 8 ml/min by an 160-min linear gradient of 10-50% acetonitrile in 0.1% TFA. The peaks of η-CRD1 (t_R = 100 min) and δ-CRD1 (t_R = 115 min) were collected, and each fraction was concentrated *in vacuo* below 30 °C to remove acetonitrile. Lyophilization of each residue gave pure η-CRD1 and δ-CRD1, which exhibited satisfactory mass spectral analysis: for η-CRD1 (MH^+, average molecular mass: obs. 6077.25, calc. for $C_{269}H_{408}N_{79}O_{67}S_8$, 6077.23, and for δ-CRD1 (MH^+, average molecular mass: obs. 5927.98, calc. for $C_{265}H_{409}N_{74}O_{69}S_6$, 5928.02).

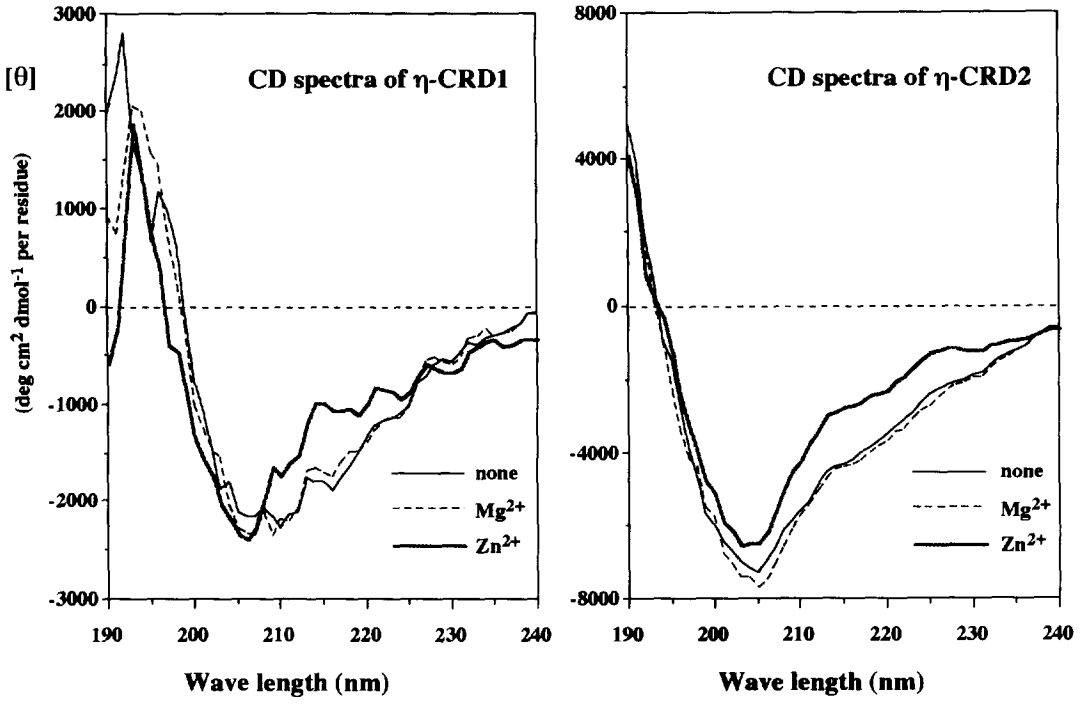

Figure 3 Left side: CD spectra of η-CRD1 itself and η-CRD1 treated separately with 2.5-molar equivalent of $ZnCl_2$ or $MgCl_2$ in helium purged distilled water. Right side: CD spectra of η-CRD2 itself and η-CRD2 treated separately with 2.5-molar equivalent of $ZnCl_2$ or $MgCl_2$ in helium purged distilled 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.

Each CRD of PKC coordinates two atoms of zinc,[12,13] and this zinc binding is believed to be required for phorbol ester binding. Since our synthetic CRD's did not contain zinc,[6] zinc coordination was carried out by addition of 5-molar equivalents of $ZnCl_2$ to a distilled water solution of each CRD. The specific PDBu binding increased *ca.*4-fold when γ-CRD2 or η-CRD2 treated with 5-molar equivalent of $ZnCl_2$ in distilled water 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 as reported previously.[8] However, there is no guarantee that each CRD1 properly folds like these CRD2's upon zinc treatment. In order to confirm the metal induced conformational change of each CRD1, the CD spectra of complexed and uncomplexed η-CRD1 and η-CRD2 were measured (Figure 3). A significant spectral change was detected for both η-CRD1 and η-CRD2 treated with $ZnCl_2$. In a control experiment, η-CRD1 and η-CRD2 treated with $MgCl_2$, which had no significant effect on the PDBu binding, gave a spectrum quite similar to η-CRD1 and η-CRD2 itself, respectively. The CD spectra of η-CRD1 and η-CRD2 were analyzed by the method of Yang *et al.*[14] In zinc-treated η-CRD1, the calculations are 55% β-sheets and 11% α-helix while in zinc-treated η-CRD2, the values are 67% β-sheets and 5% α-helix. These results strongly suggest that Zn^{2+} coordinates both η-CRD1 and η-CRD2 in a similar fashion, producing closely related peptide conformers. A significant spectral change similar to η-CRD1 was also observed in δ-CRD1 upon zinc treatment (data not shown).

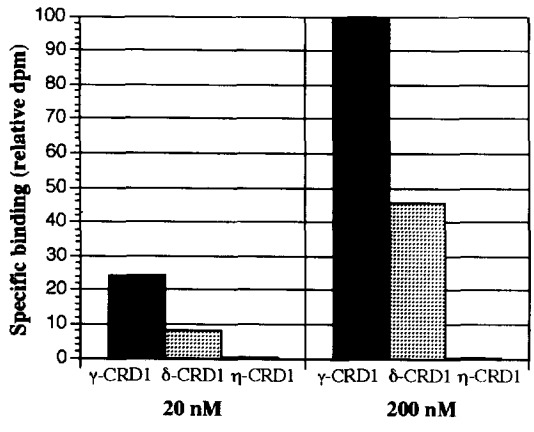

Figure 4 Specific PDBu binding of γ-CRD1, δ-CRD1, and η-CRD1. The binding was evaluated by the procedure of Sharkey and Blumberg.[15] Zinc coordination was carried out in a distilled water solution of each CRD1 (100 μg/ml) using 5-molar equivalents of $ZnCl_2$ at 4 °C for 10 min. After dilution with distilled water, an aliquot of the peptide 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 1,2-di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine, and 20 nM [^{3}H]PDBu (19.6 Ci/mmol). The final concentration of each CRD1 was 20 or 200 nM. The specific binding is the difference between total and nonspecific binding which was determined in the presence of 500-fold cold PDBu. The specific binding of 200 nM γ-CRD1 (7600 dpm) was fixed at 100. Each point represents the mean of three experimental values with a standard deviation of <5%.

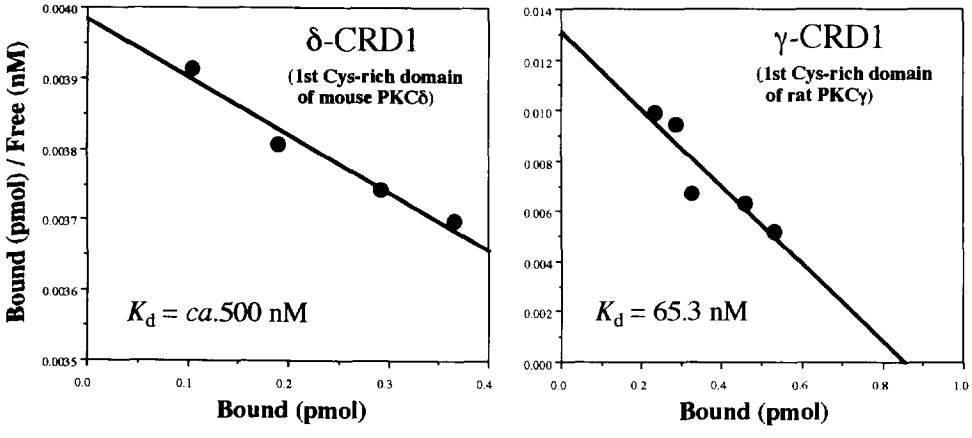

Figure 5 Scatchard analyses of [^{3}H]PDBu binding to δ-CRD1 and γ-CRD1. Zinc folded δ-CRD1 or γ-CRD1 was incubated with increasing concentrations of [^{3}H]PDBu in the presence of 50 μg/ml phosphatidylserine as described in Figure 4. Representative experiments for δ-CRD1 and γ-CRD1 are shown. Each point represents the mean of three experimental values with a standard deviation of <5%. Similar results were obtained in additional experiments.

The zinc-folded CRD's were subjected to the PDBu binding assay (Figure 4). γ-CRD1 which has a significant PDBu binding affinity[6] was selected as a positive control; the specific binding of 200 nM γ-CRD1 was fixed at 100. As shown in Figure 4, η-CRD1 did not show any PDBu binding even at 200 nM while the second CRD of mouse PKCη (η-CRD2) bound strongly PDBu with a K_d of 0.91 nM.[8] This result suggests that the binding site of PDBu in native PKCη is the second CRD. Recent crystallographic analysis of the second CRD of mouse PKCδ has indicated that phorbol esters fit into a narrow groove of the CRD created by amino acids 20-27.[16] Since η-CRD1 contains one excess valine in the corresponding region compared with other CRD's (Figure 2), this additional valine might interfere with the proper folding or PDBu binding of the CRD.

δ-CRD1 showed significant but weaker PDBu binding than γ-CRD1. Scatchard analyses of zinc-folded δ-CRD1 and γ-CRD1 gave dissociation constants (K_d) of *ca.* 500 and 65.3 nM, respectively (Figure 5). Since the K_d of the second CRD of mouse PKCδ was reported to be 1.9 nM,[7] the binding site of PDBu in native PKCδ is deduced to also be the second CRD. After completion of this work, Szallasi *et al.* have reported that the first and the second CRD's of mouse PKCδ are not equivalent and that the second CRD plays the predominant role in translocation of mouse PKCδ in response to 12-*O*-tetradecanoylphorbol 13-acetate.[17] The evolutionary dendrogram showing phylogenetic relationships among PKC isotypes indicates that PKCη is closely related to PKCε.[18] Since the first CRD of PKCε contains one excess valine like η-CRD1, the first CRD of PKCε would not have a PDBu binding ability like η-CRD1. It is interesting that most of the second CRD of novel PKC plays an important role in the PDBu binding.

The phorbol ester binding site of conventional PKC is, however, unclear at present since both CRD's of rat brain PKCγ showed similar K_d's for PDBu (65.3 nM for γ-CRD1, 44.1 nM for γ-CRD2).[8] Similar K_d's for PDBu (14.5 nM for GST-CRD1, 17.1 nM for GST-CRD2) have also been reported using carboxyl-terminal fusion protein of GST with the first and the second CRD of rat PKCγ.[19] Moreover, the PDBu binding of PKCγ was shown to be affected significantly by the calcium and phosphatidylserine binding domain (C_2 domain).[19] Slater *et al.* have recently reported that PKCα contains two activator binding sites that bind phorbol esters and diacylglycerols with opposite affinities.[20] Hitherto, only the second CRD's of PKC have been used for analyzing PDBu binding.[16,21-24] While the peptide surrogate of the second CRD of novel PKC might be a good model for native novel PKC itself, the extrapolation of results with conventional CRD surrogates to conventional native PKC's requires further attention, since the major binding site of phorbol esters in PKCγ could be the first CRD as Bell *et al.* proposed.[19] Further studies to determine the phorbol ester binding site in conventional PKC are in progress.

Acknowledgment: The authors thank Mr. Hiroyuki Fukuda for synthesis of η-CRD1, δ-CRD1 and γ-CRD1, and MALDI-TOF-MS measurement. They also thank Drs. Shogo Ozawa, Hiroshi Ueno, Rikimaru Hayashi, and Miss Asako Yamasaki at Department of Agricultural Chemistry, Kyoto University for CD measurements. This work was supported in part 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.).

References

1. Nishizuka, Y. *Nature* **1984**, *308*, 693.
2. For reviews, see: Marks, F.; Gschwendt, M. *Mutation Res.* **1995**, *333*, 161; Newton, A. C. *J. Biol. Chem.* **1995**, *270*, 28495; Nishizuka, Y. *FASEB J.* **1995**, *9*, 484.
3. Ono, Y.; Fujii, T.; Igarashi, K.; Kuno, T.; Tanaka, C.; Kikkawa, U.; Nishizuka, Y. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 4868.
4. Burns, D. J.; Bell, R. M. *J. Biol. Chem.* **1991**, *266*, 18330.
5. Quest, A. F. G.; Bardes, E. S. G.; Bell, R. M. *J. Biol. Chem.* **1994**, *269*, 2961.
6. Wender, P. A.; Irie, K.; Miller, B. L. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 239.

7. Kazanietz, M. G.; Barchi, J. J.; Omichinski, J. G.; Blumberg, P. M. *J. Biol. Chem.* **1995**, *270*, 14679.
8. Irie, K.; Yanai, Y.; Ohigashi, H.; Wender, P. A.; Miller, B. L. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 353.
9. Kazanietz, M. G.; Areces, L. B.; Bahador, A.; Mischak, H.; Goodnight, J.; Mushinski, J. F.; Blumberg, P. M. *Mol. Pharmacol.* **1993**, *44*, 298.
10. Osada, S.; Mizuno, K.; Saido, T. C.; Akita, Y.; Suzuki, K.; Kuroki, T.; Ohno, S. *J. Biol. Chem.* **1990**, *265*, 22434.
11. Mischak, H.; Bodenteich, A.; Kolch, W.; Goodnight, J.; Hofer, F.; Mushinski, J. F. *Biochemistry* **1991**, *30*, 7925.
12. Hubbard, S. R.; Bishop, W. R.; Kirschmeier, P.; George, S. J.; Cramer, S. P.; Hendrickson, W. A. *Science* **1991**, *254*, 1776.
13. Quest, A. F. G.; Bloomenthal, J.; Bardes, E. S. G.; Bell, R. M. *J. Biol. Chem.* **1992**, *267*, 10193.
14. Yang, J. T.; Wu, C.-S. C.; Martinez, H. M. *Meth. Enzymol.* **1986**, *130*, 208.
15. Sharkey, N. A.; Blumberg, P. M. *Cancer Res.* **1985**, *45*, 19.
16. Zhang, G.; Kazanietz, M. G.; Blumberg, P. M.; Hurley, J. H. *Cell* **1995**, *81*, 917.
17. Szallasi, Z.; Bogi, K.; Gohari, S.; Biro, T.; Acs, P.; Blumberg, P. M. *J. Biol. Chem.* **1996**, *271*, 18299.
18. Dekker, L V.; Parker, P, J. *Trends Biochem. Sci.* **1994**, *19*, 73.
19. Quest, A. F. G.; Bell, R. M. *J. Biol. Chem.* **1994**, *269*, 20000.
20. Slater, S. J.; Ho, C.; Kelly, M. B.; Larkin, J. D.; Taddeo, F. J.; Yeager, M. D.; Stubbs, C. D. *J. Biol. Chem.* **1996**, *271*, 4627.
21. Hommel, U.; Zurini, M.; Luyten, M. *Nat. Struct. Biol.* **1994**, *1*, 383.
22. Ichikawa, S.; Hatanaka, H.; Takeuchi, Y.; Ohno, S.; Inagaki, F. *J. Biochem.* **1995**, *117*, 566.
23. Irie, K.; Ishii, T.; Ohigashi, H.; Wender, P. A.; Miller, B. L.; Takeda, N. *J. Org. Chem.* **1996**, *61*, 2164.
24. Irie, K.; Isaka, T.; Iwata, Y.; Yanai, Y.; Nakamura, Y.; Koizumi, F.; Ohigashi, H.; Wender, P. A.; Satomi, Y.; Nishino, H. *J. Am. Chem. Soc.* **1996**, *118*, 10733.

(*Received in Japan* 23 *October* 1996; *accepted* 2 *December* 1996)