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Solid-phase Synthesis, Mass Spectrometric Analysis of the Zinc-folding, and Phorbol Ester-binding Studies of the 116-mer Peptide Containing the Tandem Cysteine-rich C1 Domains of Protein Kinase C Gamma

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Abstract—Tumor-promoting phorbol esters activate protein kinase C (PKC) isozymes by binding to the zinc-finger like cysteine-rich domains in the *N*-terminal regulatory region. Our recent studies have revealed that only PKCγ has two high affinity phorbol ester-binding domains, providing a structural blueprint for the rational design of PKCγ-selective modulators for the treatment of neuropathic pain. To extend this approach, the 116-mer peptide containing the double cysteine-rich motifs of PKCγ (γ-C1A-C1B) has been synthesized for the first time using an Fmoc-solid phase strategy with a stepwise chain elongation. This peptide was purified by the reversed phase HPLC to give satisfactory mass data (MALDI-TOF-MS and ESI-TOF-MS). The peptide was successfully folded by zinc treatment and the folded peptide was analyzed intact under neutral conditions by ESI-TOF-MS. The multiple charge mass envelopes shifted to those of the lower mass charge state by addition of 4 molar equiv. $ZnCl_2$, suggesting that γ-C1A-C1B preserves some higher order structure by the zinc folding. Moreover, the mass spectrum of the zinc-folded peptide in the presence of EDTA clearly showed that γ-C1A-C1B coordinates exactly four atoms of zinc. This zinc stoichiometry is identical to that of native PKCγ. Scatchard analysis of the zinc-folded peptide revealed two binding sites of distinctly different affinities ($K_d = 6.0 \pm 1.5$ and 47.0 ± 6.6 nM) comparable to those reported by Quest and Bell for the GST fusion protein of γ-C1A-C1B prepared by DNA recombination. These results indicate that γ-C1A-C1B serves as an effective surrogate for native PKCγ for the study of the structural characteristics of the binding–recognition event and the design, discovery, and development of new PKCγ-selective modulators.

Introduction

Protein kinase C (PKC)[1] isozymes play a central role in cellular signal transduction mediated by endogenous diacylglycerols and in tumor promotion induced by exogenous phorbol esters and other agents.[1] Conventional and novel PKC isozymes (PKCα, βI/βII, γ, δ, ε, η, and θ) consist of a catalytic domain for protein phosphorylation and a regulatory domain which binds diacylglycerols or phorbol esters (Fig. 1). Phorbol esters activate PKC at nanomolar concentrations by binding to the N-terminal cysteine-rich C1 domains (C1A and C1B)[2] consisting of ca. 50 amino acid residues.[3,4] Stimulated by the therapeutic significance of PKC signal modulation[5–7] and the interest in the rational development of novel PKC-targeted therapeutic agents, efforts to elucidate the structural and functional roles of the C1 domains of PKC isozymes have relied on NMR spectroscopy,[8–10] X-ray analysis,[11] point mutation,[12–14] and chemical synthesis.[15–20]

We have recently shown that only the C1B's of all conventional and novel PKC isozymes, except for PKCγ, bind [^{3}H]phorbol-12,13-dibutyrate (PDBu) with high affinities comparable to those of the native isozymes.[20] Of special significance, both C1A and C1B of PKCγ exhibit high affinity PDBu binding, providing a structural blueprint for the development of PKCγ-selective modulators, a finding of potential importance for the treatment of neuropathic pain.[6] To extend this approach, the preparation of native PKCγ or a suitable surrogate is indispensable. DNA recombination techniques have been used for this purpose,[21] but these procedures are time-consuming and often produce proteins of variable purity and low PDBu-binding activity.

Key words: Cysteine-rich domain; phorbol ester; protein kinase C; zinc finger.
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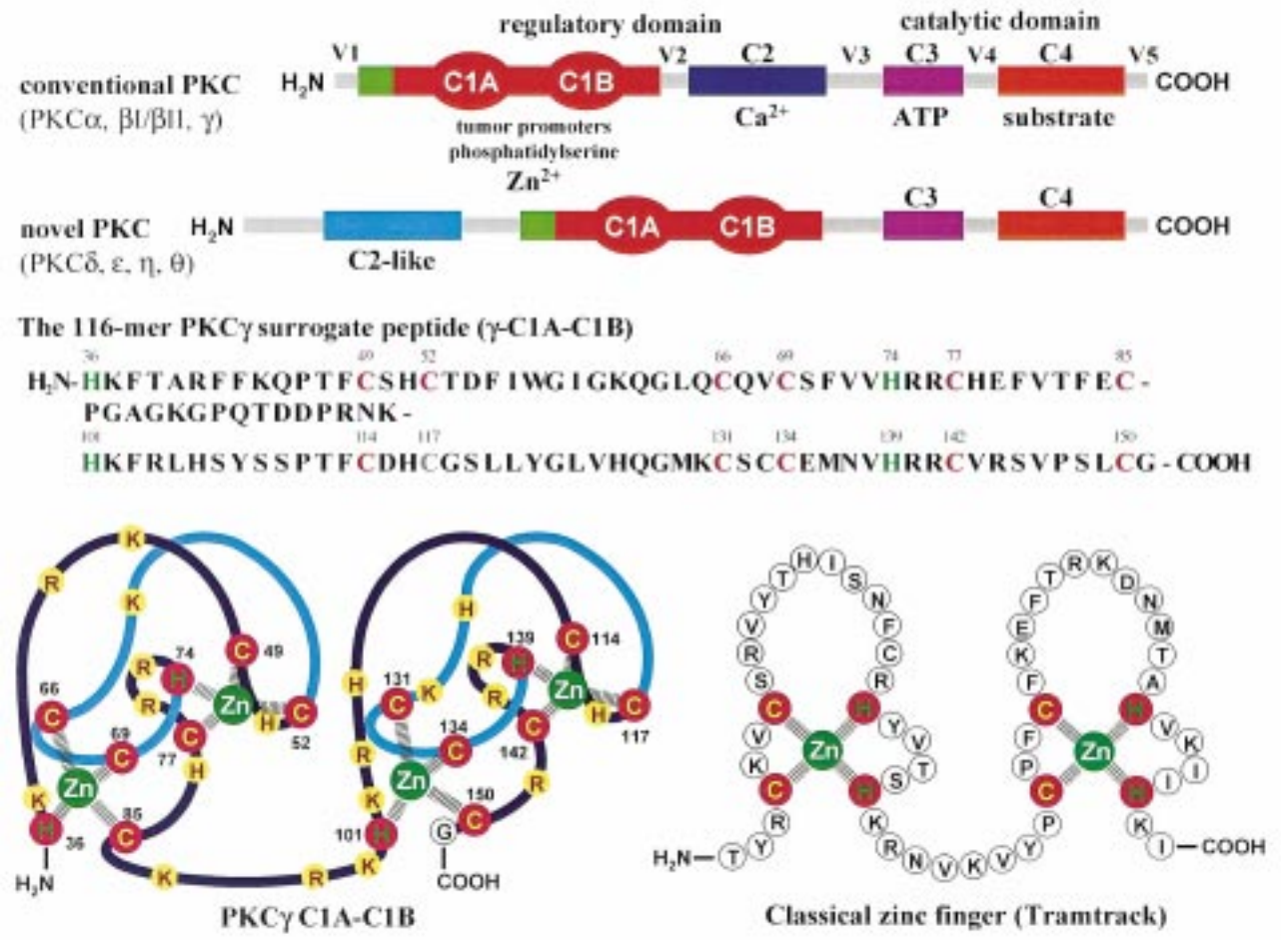

Figure 1. Structure of conventional and novel PKC isozymes, and the 116-mer peptide, γ-C1A-C1B, along with the illustration of zinc-folded γ-C1A-C1B and the classical zinc finger (Tramtrack). The sequence of γ-C1A-C1B corresponds to mouse PKCγ.[22] The illustration of zinc-folded γ-C1A-C1B is based on the NMR solution structure of γ-C1B.[10]

We have recently synthesized C1A's and C1B's of all known PKC isozymes from mouse by a solid-phase Fmoc strategy. This has allowed us to identify uniquely the C1A and C1B peptides, which bind PDBu. Significantly, the observed affinities of those peptides that bind PDBu are comparable to those of the native PKC isozymes themselves.[20] We next applied this strategy to the synthesis of the 116-mer peptide (Fig. 1) containing both C1A and C1B of mouse PKCγ[22] (γ-C1A-C1B).[23] This is a full report on the synthesis and characterization of γ-C1A-C1B including the first mass spectrometric analysis of the zinc-folding of the PKCγ C1 domain. We determined the exact zinc stoichiometry of γ-C1A-C1B by electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) under neutral conditions.

Results and Discussion

Synthesis of the 116-mer peptide containing the tandem cysteine-rich C1 domains of PKCγ (γ-C1A-C1B)

The 116-mer peptide, γ-C1A-C1B, was synthesized with a PerSeptive Biosystems Model 9030 (Pioneer™ Peptide Synthesizer) using HATU[25] as an activator for Fmoc chemistry with a stepwise chain elongation procedure. To prevent the elongation of the unsuccessfully coupled peptide, the unreacted amino terminus was blocked with 5% acetic anhydride-pyridine in DMF. This step is necessary to facilitate the purification of the target peptide. After completion of the chain assembly, the peptide-resin was treated with trifluoroacetic acid (TFA) containing scavengers for final deprotection and cleavage from the resin. The resultant crude peptide was purified by gel filtration, followed by HPLC on preparative C4 and C18 reversed phase columns. The combination of these two columns was most effective in the purification of γ-C1A-C1B. Although Xu et al.[10] reported the effectiveness of the Mono-S column (Pharmacia) in the purification of the peptide containing C1B of PKCγ, γ-C1A-C1B was absorbed strongly to the column possibly because of high hydrophobicity and could not be eluted even by 2 M KCl or 1 M KCl containing 40% acetonitrile in phosphate buffer (pH 6.8). The purity of γ-C1A-C1B (>98%) was confirmed by HPLC (Fig. 2) and its molecular weight was determined by both matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and ESI-TOF-MS. Satisfactory mass data were observed as shown in Figures 3 and 4. Furthermore, enzymatic digestion of γ-C1A-C1B using trypsin gave expected peptide fragments as identified by MALDI-TOF-MS except for the C-terminal 10 amino acid residues (Table 1). The overall

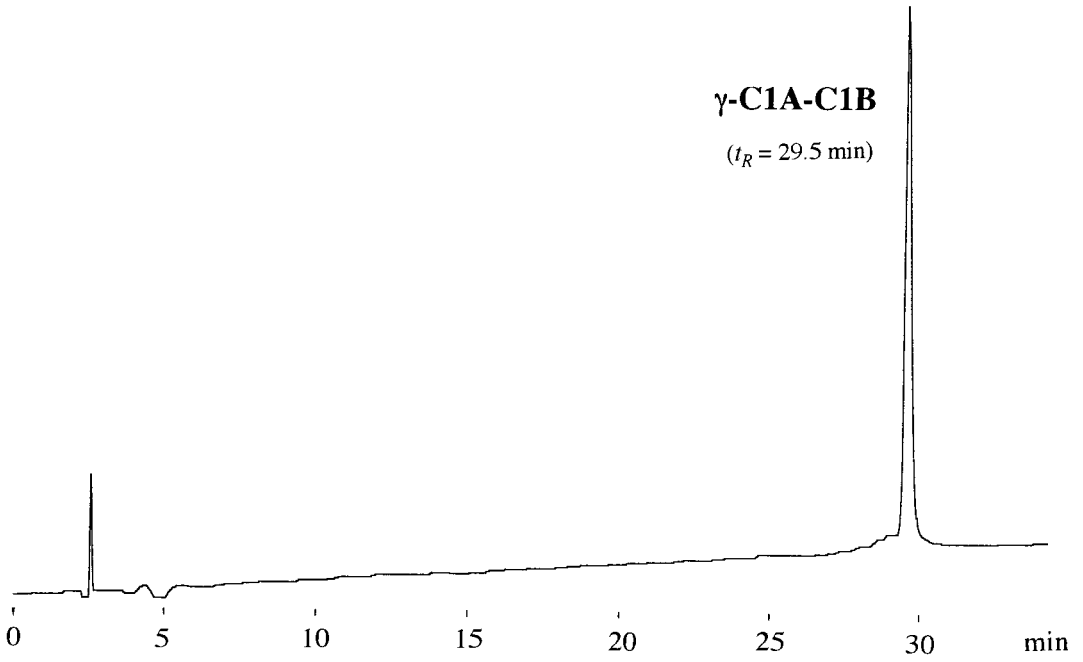

Figure 2. Chromatogram of pure γ-C1A-C1B after HPLC purification on the preparative C4 and C18 reversed-phase HPLC columns. The analytical conditions are as follows: YMC A-311 (ODS), 40 min linear gradient of 10–50% CH_3CN containing 0.1% TFA, 1.0 mL/min, UV 220 nm.

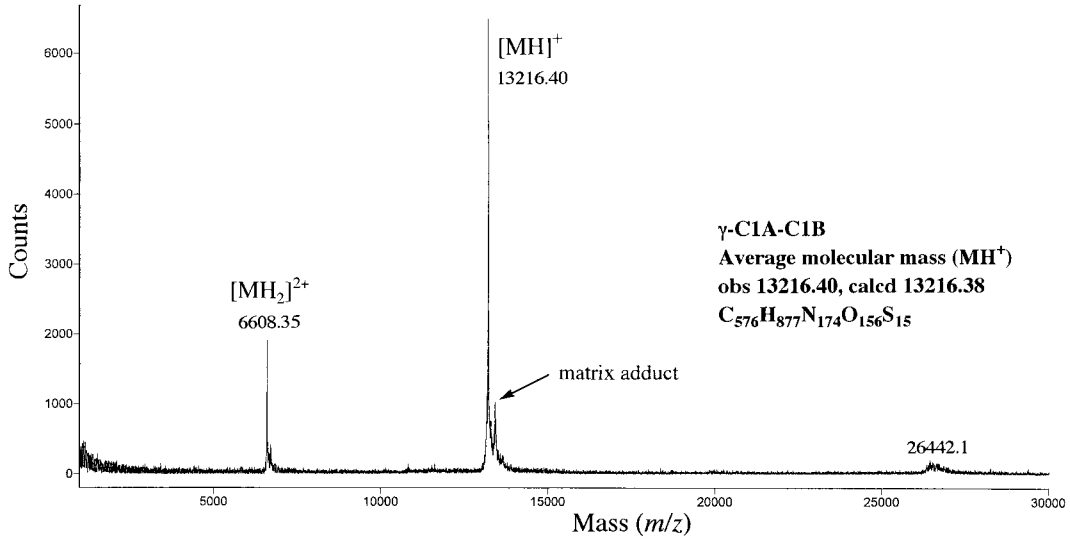

Figure 3. MALDI-TOF-MS of γ-C1A-C1B by Voyager-DE® STR (PerSeptive Biosystems). γ-C1A-C1B dissolved in 0.1% TFA aqueous solution (50 pmol/μL) was mixed with saturated sinapinic acid in 30% CH_3CN containing 0.1% TFA in the ratio of 1:1. One μL of the resultant solution was subjected to the measurement. Thioredoxin and bovine insulin were used as external references.

yield of γ-C1A-C1B was ca. 0.5%, requiring an average coupling yield of over 96.5%. This is the first synthesis of γ-C1A-C1B itself and one of the rare examples of the solid-phase peptide synthesis of over 100 amino acid residues without fragment condensation.[26] Quest and Bell[21] obtained this moiety as part of a GST fusion protein but were not able to liberate it by cleavage of the fusion protein.

Mass spectrometric analysis of the zinc-folding of γ-C1A-C1B under neutral conditions

The PKC C1 domains have six conserved cysteines and two histidines in the pattern $HX_{12}CX_2CX_nCX_2CX_4HX_2CX_7C$ ($n = 13$ or 14) where X is a variable amino acid residue. Each C1 domain is thought to coordinate two atoms of zinc with each metal bound by three sulfur atoms of cysteines and one nitrogen atom of histidine.[27,28] Differing from the classical zinc finger proteins, the C1 domain of PKC adopts a globular fold allowing two non-consecutive sets of zinc-binding residues to form two separate metal-binding sites (Fig. 1).[8–11] Because our synthetic γ-C1A-C1B was produced in the absence of zinc, zinc coordination was carried out by the method reported previously.[18–20]

The effects of zinc on the PDBu binding of γ-C1A-C1B are summarized in Figure 5. The PDBu binding was

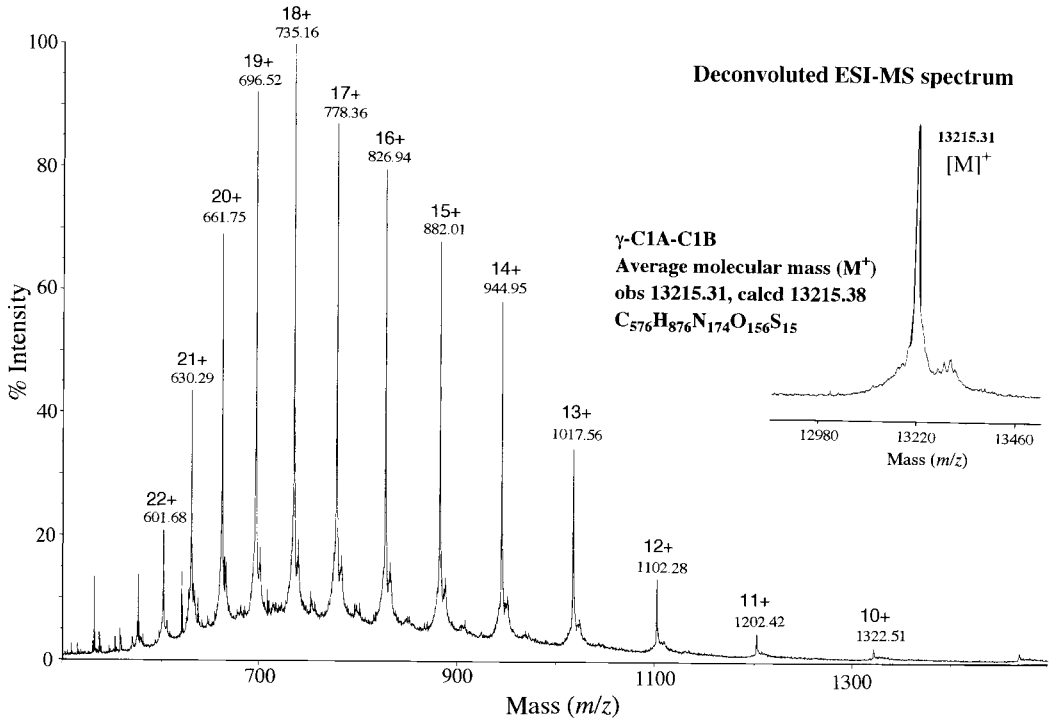

Figure 4. ESI-TOF-MS of γ-C1A-C1B by Marinar (PerSeptive Biosystems). γ-C1A-C1B dissolved in 50% CH_3CN containing 1% acetic acid (5 pmol/μL) was loop injected (5 μL/min). Neurotensin (3+: 558.311; 2+: 836.963) was used as an external reference.

Table 1. Identification of the peptide fragments of γ-C1A-C1B obtained from the trypsin digestion by MALDI-TOF-MS

Fragment name	Residue number	Obsd mass (MH^+)	Cald mass (MH^+)	Error
T1–T2	1–6	759.40	759.41	−0.01
T3–T4	7–26	2363.30	2363.72	−0.42
T3–T6	7–41	4104.82	4105.75	−0.93
T5–T6	27–41	1760.08	1761.04	−0.96
T5–T8	27–63	4133.81	4134.62	−0.81
T5–T11	27–69	4945.39	4945.58	−0.19
T6–T8	41–63	2548.26	2548.78	−0.52
T6–T9	41–65	2790.88	2791.05	−0.17
T6–T10	41–67	3055.70	3056.37	−0.67
T6–T11	41–69	3359.70	3359.73	−0.03
T7–T8	42–63	2391.70	2392.59	−0.89
T7–T9	42–65	2634.70	2634.87	−0.17
T7–T10	42–67	2899.99	2900.18	−0.19
T7–T11	42–69	3203.25	3203.55	−0.30
T9–T12	64–95	3693.25	3693.22	+0.03
T9–T14	64–106	5012.37	5012.75	−0.38
T11–T12	68–95	3185.80	3185.63	+0.17
T12	70–95	2882.02	2882.27	−0.25
T12–T14	70–106	4201.12	4201.80	−0.68
T13	96–105	1181.80	1181.40	+0.40

measured by the procedure of Sharkey and Blumberg.[24] As a reference standard, the specific binding of zinc-untreated γ-C1A-C1B at 10 nM was fixed at 100 (entry 1). The binding increased 4-fold when the peptide was pretreated with zinc (entries 2 and 3). Maximum binding was observed when the peptide was pretreated with 4 and 8 molar equiv. $ZnCl_2$. Moreover, the binding of zinc-untreated γ-C1A-C1B was abolished when the zinc-untreated peptide was added to the assay mixture containing 2 mM EDTA (entry 4), suggesting that chelatable ions in the assay mixture could account for the background folding of the untreated γ-C1A-C1B. It is especially noteworthy that the binding of zinc-treated γ-C1A-C1B did not change by exposure to 2 mM EDTA (entry 5), indicating that the zinc coordination is not readily reversed by EDTA. These results strongly suggest that zinc plays an important role in the folding and PDBu binding of γ-C1A-C1B.

In order to investigate the metal-induced conformational changes and to determine the stoichiometry of the zinc coordination, zinc-folded γ-C1A-C1B was analyzed

intact under neutral conditions by ESI-TOF-MS. The zinc stoichiometry of a GST fusion protein of γ-C1A-C1B has been examined by Quest and Bell[21] by atomic absorption spectroscopy. They showed that GST-γ-C1A bound 1.3 ± 0.4 zinc atoms and GST-γ-C1B bound 1.7 ± 0.1 zinc atoms. However, they obtained significantly lower values (2.2 ± 1.4) in the zinc stoichiometry of the GST fusion protein of γ-C1A-C1B even though intact PKCγ incorporates four zinc atoms.[21,28] They speculated that these differences might be attributable to the folding of the fusion protein.

High resolution ESI-MS equipped with time-of-flight mass analyser (ESI-TOF-MS) has recently been recognized as an effective method to analyze protein folding and to determine the metal-binding stoichiometry of proteins.[29] To determine the exact stoichiometry of the zinc coordination to γ-C1A-C1B by ESI-TOF-MS, however, it is indispensable to establish conditions that do not change the native conformation of the zinc-folded peptide. Although organic solvents such as acetonitrile are generally used for effective ionization as shown in Figure 4, they often induce significant conformational changes of the peptide. The use of a neutral aqueous solvent is more desirable for this purpose, but the ion intensity of ESI-TOF-MS is drastically suppressed in such solvent systems. We examined several buffer systems without organic solvent such as pyridinium acetate, triethylammonium acetate, *N*-methylmorpholinium acetate, and ammonium acetate to find the most suitable analytical condition using the C1A and C1B subunits of PKCγ (γ-C1A and γ-C1B). Among these buffers, only 10 mM pyridinium acetate (pH 6.8) produced sufficient ion intensity with 125 pmol of γ-C1A.

ESI-TOF-MS analysis of γ-C1A-C1B was performed under neutral aqueous conditions using 10 mM pyridinium acetate (pH 6.8) as shown in Figure 6. The multi-charged spectrum of γ-C1A-C1B without zinc treatment had the center of mass envelope at +9 and +8 charges with from +13 to +6 charge distribution (Fig. 6(A)). The deconvoluted molecular mass was 13215.5 for apo-γ-C1A-C1B (cald average mass: 13215.4; Fig. 6(B)). By addition of 4 molar equiv. of $ZnCl_2$, the mass envelope shifted to that of the lower charged state as shown in Figure 6(C): the +7 charge species was prominent, and those of +13 and +12 charges disappeared. This indicates that the zinc coordination induces significant conformational changes in γ-C1A-C1B to preserve higher order structure. Moreover, 8 molar equiv. of $ZnCl_2$ did not change the mass envelope any more (Fig. 6(E)), suggesting that 4 molar equiv. of zinc is sufficient for the folding of γ-C1A-C1B. These results coincide very well with those in Figure 5 (entries 2 and 3).

The deconvoluted spectrum of zinc-treated (4 molar equiv.) γ-C1A-C1B showed the predominant molecular mass of 13479.0 (Fig. 6(D)), corresponding to the 4 zinc coordination (cald average mass for M + 4Zn: 13476.9 and M + 4Zn–8H: 13468.9). The protonation states of the zinc-coordinated cysteine ligands in zinc-finger peptides

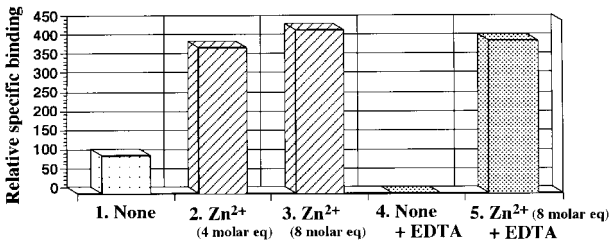

Figure 5. Effects of zinc ion on the PDBu binding of γ-C1A-C1B. Metal coordination was carried out in a distilled water solution containing γ-C1A-C1B (100 μg/100 μL) using 4 or 8 molar equiv. $ZnCl_2$ at 4 °C for 10 min. After dilution with the distilled water, an aliquot of the peptide solution (2.9 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 phosphatidylserine, and 40 nM [^{3}H]PDBu. The final peptide concentration was 10 nM. The specific binding of the zinc-untreated peptide was fixed at 100 (entry 1). Entry 2: the 4 molar equiv. zinc-treated peptide; entry 3: the 8 molar equiv. zinc-treated peptide; entry 4: the peptide without zinc-treatment in the presence of 2 mM EDTA in the assay mixture; entry 5: the 8 molar equiv. zinc-treated peptide in the presence of 2 mM EDTA in the assay mixture. Each point represents the mean of three experimental values with a standard deviation of less than 5%.

are not clearly understood.[30,31] There is a possibility that the thiol protons are retained in the zinc ion coordinate centers or in the neighboring basic amino acid residues. The NMR solution structure of zinc-folded γ-C1B indicated that basic amino acid residues are located close to each zinc atom (Fig. 1).[10] If all of the thiol protons were retained in the peptide, and if the charges of the 4 zinc atoms did not contribute to the net charge of γ-C1A-C1B by a 'cage-effect', the error would be less than 2 mass units. The error of 2 mass units might be partly due to the broadening and shape distortion of the isotopic distribution peak of the zinc-folded γ-C1A-C1B because three major isotopic species of zinc (^{64}Zn, ^{66}Zn, and ^{68}Zn) have almost similar relative abundances (48.9, 27.8, and 18.6%, respectively), resulting in a flattened isotopic distribution peak. Similar zinc stoichiometry has recently been reported by ESI-MS for the glucocorticoid receptor DNA binding domain (GR DBD).[32] In this case also, molecular mass of the zinc-coordinated peptide coincided with that of M + 2Zn, not M + 2Zn–2H.

It is noteworthy that the 5 and 6 atoms of zinc-coordinated ions were observed predominantly in the deconvoluted spectrum when the peptide was treated with 8 molar equiv. of $ZnCl_2$ (cald average mass for M + 5Zn: 13542.3 and for M + 6Zn: 13607.7; Fig. 6(F)). We added EDTA to zinc-treated (8 molar equiv.) γ-C1A-C1B to determine whether the zinc coordination was specific. As mentioned above, the PDBu binding of zinc-folded γ-C1A-C1B did not change by exposure to EDTA in the assay mixture (Fig. 5), suggesting that only tightly coordinated zinc is observed by the ESI-TOF-MS if the zinc-folded peptide is exposed to EDTA. As expected, peaks 5 and 6 coordinated zinc atoms almost completely disappeared when zinc-treated (8 molar equiv.) γ-C1A-C1B was exposed to 4 molar equiv. EDTA, instead the 4 coordinated zinc atom peak was the predominant species (Fig. 6(J)). Furthermore, the 4 coordinated zinc

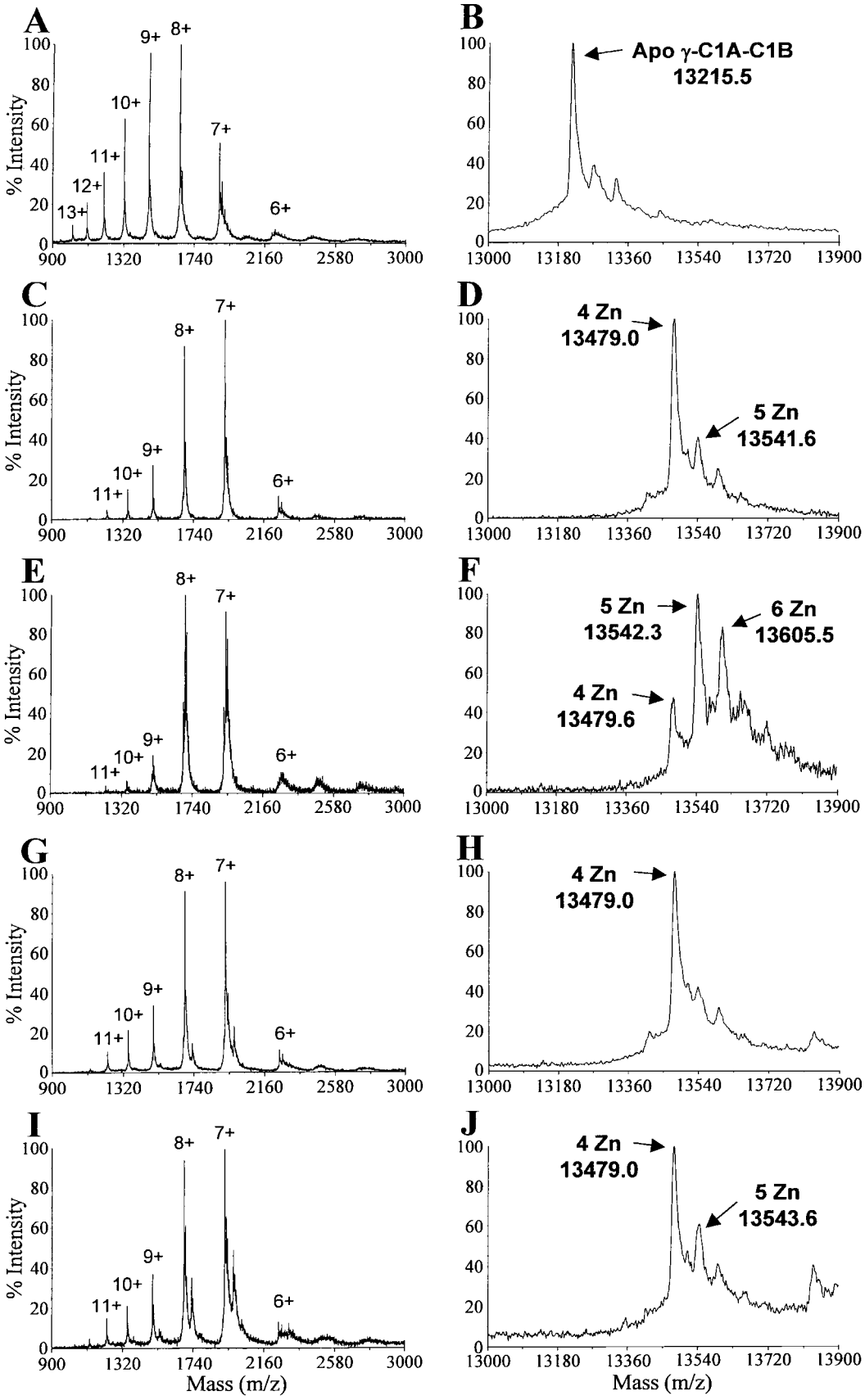

Figure 6. ESI-TOF-MS Spectra of zinc-folded γ-C1A-C1B under the neutral aqueous solution, 10 mM pyridinium acetate (pH 6.8). (A, B): The peptide without zinc treatment; (C, D): the 4 molar equiv. zinc-treated peptide; (E, F): the 8 molar equiv. zinc-treated peptide; (G, H): the 4 molar equiv. zinc-treated peptide followed by exposure to 2 molar equiv. EDTA in the buffer; (I, J): the 8 molar equiv. zinc-treated peptide followed by exposure to 4 molar equiv. EDTA in the buffer.

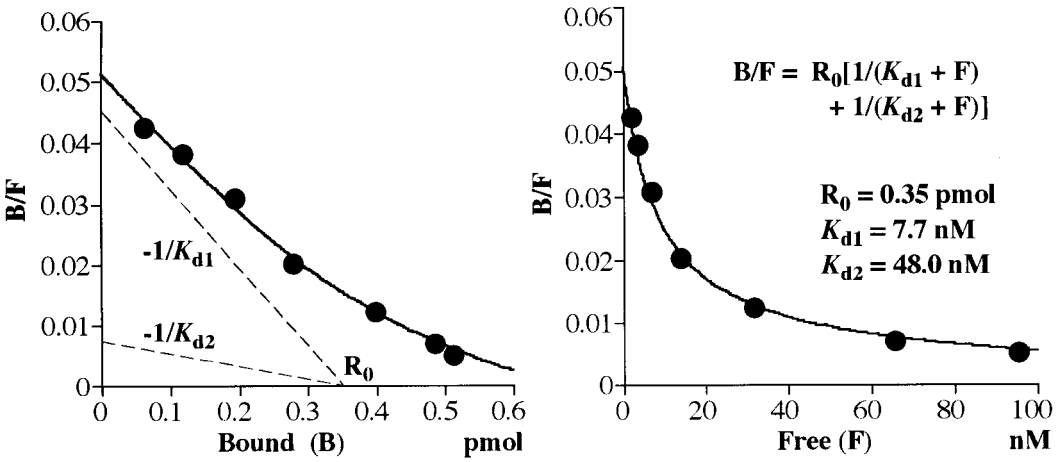

Figure 7. Scatchard analysis of the [^{3}H]PDBu binding to zinc-folded γ-C1A-C1B. A representative experiment of three independent ones is shown. Dissociation constants (K_{d1} and K_{d2}) and total effective concentration of γ-C1A-C1B (R_0) were calculated by a nonlinear least squares fit to the B/F equation shown above.

atom peak derived from the 4 molar equiv. zinc treatment (Fig. 6(D)) did not change significantly by exposure to 2 molar equiv. of EDTA (Fig. 6(H)). As a control experiment, magnesium-treated (4 or 8 molar equiv.) γ-C1A-C1B did not show any change in the multi-charged spectra, and only one peak ascribable to apo-γ-C1A-C1B was observed in the deconvoluted spectrum (data not shown). These results clearly indicate that 4 zinc atoms coordinate specifically to γ-C1A-C1B. This zinc stoichiometry coincided with that of native PKCγ.[21,28] The stoichiometry of the zinc coordination to each C1 peptide of PKCγ (γ-C1A and γ-C1B) was also analyzed similarly and determined to be two atoms each (data not shown).

Scatchard analysis of the [^{3}H]PDBu binding to zinc-folded γ-C1A-C1B

Scatchard analysis of zinc-folded γ-C1A-C1B for [^{3}H]PDBu binding revealed two binding sites of distinctly different affinities ($K_d = 6.0 \pm 1.5$ and 47.0 ± 6.6 nM) comparable to those reported by Quest and Bell[21] using the GST fusion protein of γ-C1A-C1B ($K_d = 10.1$ and 42.0 nM) in the absence of calcium. A typical example of the three independent experiments is shown in Figure 7. The K_d values of γ-C1A-C1B are only slightly smaller than each K_d value of γ-C1A and γ-C1B (65.8 and 16.9 nM).[20] This indicates that each C1 subdomain binds essentially independent of the other C1 subdomain. There is a significant variation in the K_d values of PDBu in the absence of calcium for native PKCγ [$K_d = 18.0$,[33] 6.8,[19] and 0.37 nM[34]]. This variation is not unexpected since in conventional PKCs the calcium and phosphatidylserine binding domain (C2 domain) is proximate to the phorbol ester-binding domain (C1 domain), and changes in the assay conditions would be expected to result in some variation of the K_d values. The K_d value for the high affinity binding site of γ-C1A-C1B is within the reported range, indicating that γ-C1A-C1B serves as an effective surrogate for native PKCγ.

Conclusion

In summary, we have synthesized the 116-mer peptide, γ-C1A-C1B, using an Fmoc solid-phase peptide synthesis with a stepwise chain elongation procedure for the first time, and have shown that this peptide is an effective and preparatively available surrogate for native PKCγ in a phorbol ester binding assay. This study in conjunction with our previous work on all individual C1A's and C1B's[20] affords unique opportunities for the development of isozyme specific binding assays, for the design of high throughput screens, and for the study of the structural characteristics of the binding-recognition event. Synthetic studies on the PKCγ-selective activators and inhibitors, potentially new medicinal leads especially for neuropathic pain,[6] are currently in progress.

This study has also shown that ESI-TOF-MS allows for accurate determination of the zinc stoichiometry of the C1 domain of PKCγ. Of special importance, addition of EDTA to the zinc-folded peptides under neutral aqueous conditions represents an effective strategy to discriminate between specific and non-specific coordination. The present results suggest that ESI-TOF-MS can be used effectively for analyzing the folding and metal ion coordination of metal coordinated peptides under physiologically relevant conditions.

Experimental

General methods

The following spectroscopic and analytical instruments were used: MALDI-TOF-MS, PerSeptive Biosystems Voyager-DE™ STR; ESI-TOF-MS, PerSeptive Biosystems Mariner™; Peptide synthesizer, PerSeptive Biosystems Model 9030 (Pioneer™ Peptide Synthesizer); HPLC, Waters Model 600E with Model 484 UV detector and Waters Model 625LC with Model 486 UV

detector. HPLC was carried out on YMC packed PROTEIN-RP (C4, 20 mm i.d.×150 mm) and SH-342-5 (ODS, 20 mm i.d.×150 mm) columns for preparative purpose and on a YMC packed A-311 (ODS, 6 mm i.d.×100 mm) column for analysis of purity (Yamamura Chemical Laboratory). [^{3}H]PDBu (21.0 Ci/mmol) was purchased from NEN Research Products. Unless otherwise noted, reagents were obtained from Sigma, Wako Pure Chemical Industries, or Nacalai Tesque.

Synthesis of γ-C1A-C1B

γ-C1A-C1B was synthesized in a stepwise fashion on Fmoc-Gly-PEG-PS resin (PerSeptive Biosystems) by a Pioneer™ Peptide Synthesizer using Fmoc chemistry through slight modifications of the previously reported method.[20] Fmoc amino acids (PerSeptive Biosystems) were used with the following side chain protection: Cys(Trt), Asp(O*t*Bu), Glu(O*t*Bu), His(Trt), Lys(*t*Boc), Asn(Trt), Gln(Trt), Arg(Pbf), Ser(*t*Bu), Thr(*t*Bu), Tyr(*t*Bu). The Fmoc group was deblocked with 20% piperidine in DMF for 5 min (flow rate: 8.8 mL/min). During the deprotection of each Fmoc group, the amount of released Fmoc group was monitored by UV spectroscopy to confirm that the condensation had occurred successfully. The coupling reaction was carried out using each Fmoc amino acid (0.8 mmol), HATU (0.8 mmol), and *N*,*N*-diisopropylethylamine (DIPEA, 0.95 mmol) in DMF for 60 min (flow rate: 30 mL/min). Each Fmoc amino acid (0.8 mmol) and HATU (0.8 mmol) weighed in a test tube was dissolved in 3.8 mL of the base solution (0.25 M DIPEA in DMF). The mixture was purged with an N_2 stream and added within 2 min to the column containing the resin with a flow rate of 30 mL/min. Final amino acid and HATU concentrations in the coupling reaction were ca. 0.2 M. After each condensation step, the unreacted amino terminus was blocked with 5% acetic anhydride-pyridine in DMF for 5 min. DMF was used for the flow washes throughout the entire synthesis.

After completion of the chain assembly, the peptide-resin (ca. 3.0 g) was treated with a cocktail containing TFA, *m*-cresol, thioanisole, and ethanedithiol (48, 1.2, 7.2, and 3.6 mL, respectively). After 2 h of shaking at room temperature, the resin was filtered and washed with a small amount of TFA. The filtrate was then distributed in eight tubes (ca. 10 mL each). Ether (35 mL) was added to each tube to precipitate the crude peptide. The mixture remained at 4 °C for 10 min and was then centrifuged (3000 rpm×5 min). The precipitate was washed with ether 5 times and dried under an argon stream.

The crude peptide was dissolved in 10% acetic acid (ca. 20 mL) and applied to a gel filtration column (Sephadex G-15, Pharmacia, 400 g) equilibrated with 10% acetic acid. Elution with 10% acetic acid gave several ninhydrin-positive fractions that were pooled and lyophilized. The gel filtered peptide was purified by HPLC on the PROTEIN-RP column with elution at 8 mL/min by a 100-min linear gradient of 25–45% CH_3CN in 0.1% TFA and then on the SH-342-5 column with elution at 8 ml/min by a 100-min linear gradient of 25–45% CH_3CN in 0.1% TFA. The main peak in each column was collected and concentrated in vacuo below 30 °C to remove CH_3CN. Lyophilization of the residue gave pure γ-C1A-C1B in ca. 0.5% yield.

Peptide mapping of γ-C1A-C1B using trypsin

Tryptic digestion was performed using Poroszyme™, an immobilized trypsin cartridge (PerSeptive Biosystems) at 40 °C. The digestion buffer consisted of 50 mM Tris-HCl, 10 mM $CaCl_2$, and 5% CH_3CN (pH 8.2). The solution of 50 μM γ-C1A-C1B and 3 M guanidine HCl (40 μL) was applied to the cartridge at a flow rate of 10 μL/min using a 500 μL syringe equipped with a Model 2000 syringe pump (Harvard Apparatus). After addition of 1 μM dithiothreitol (10 μL) to the eluted peptide fraction (50 μL), the resultant solution was treated with the $ZipTip_{C18}$ micro-adsorptive pipette tip (Millipore) to remove the buffer salt. The digested peptides were eluted with 10 and 30% CH_3CN in 0.1% TFA from the $ZipTip_{C18}$ tip. Each eluent was subjected to the MALDI-TOF-MS measurement. An aliquot of the peptide eluent (ca. 50 pmol/μL) was mixed with saturated α-cyano-4-hydroxycinnamic acid in 30% CH_3CN containing 0.1% TFA in the ratio of 1:1. One microliter of the resultant solution was subjected to the measurement at 20 kV. Angiotensin I and ACTH (7–38) were used as external references.

Zinc-folding and the [^{3}H]PDBu binding assay of γ-C1A-C1B

Metal coordination was carried out in a helium-purged distilled water solution (pH 5.5–6.0) of γ-C1A-C1B (100 μg/100 μL). Four or eight molar equiv. $ZnCl_2$ in helium-purged distilled water (50 mM) was added to the peptide solution, and the solution was allowed to stand at 4 °C for 10 min. Ten microliters of the solution was added to 990 μL of helium-purged distilled water, and an aliquot of the peptide solution (2.9 μL) was used in the [^{3}H]PDBu binding assay as described below.

The PDBu binding to the PKC surrogate peptides was evaluated using the procedure of Sharkey and Blumberg[24] with slight modifications. The standard assay mixture (250 μL) in a 1.5 mL Eppendorf tube contained 50 mM Tris-HCl (pH 7.4 at 25 °C), 50 μg/mL 1,2-di-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine, 3 mg/mL bovine γ-globulin, [^{3}H]PDBu (21.0 Ci/mmol), and 10 nM γ-C1A-C1B. Phosphatidylserine was suspended in 50 mM Tris-HCl (pH 7.4) by sonication (1 min), and added to the above reaction mixture. The assay solution was incubated at 30 °C for 20 min. After the mixture was cooled at 0 °C for 5 min, 187 μL of 35% (w/w) poly(ethyleneglycol) (average molecular weight, 8000) was added to the tubes, and the mixture was vigorously stirred. The tubes were incubated at 0 °C for 15 min and centrifuged for 20 min at 12000 rpm in an Eppendorf microcentrifuge at 4 °C. A 50 μL aliquot of the supernatant of each tube was removed, and its radioactivity was measured to determine the free [^{3}H]PDBu concentration. The remainder of the supernatant of each

tube was removed by aspiration. The tips of the tubes were cut off, and the radioactivity in the pellets was measured to determine the bound [^{3}H]PDBu. Specific binding represents the difference between the total and nonspecific binding where the nonspecific binding for each tube was calculated from its measured free [^{3}H]PDBu concentration and its partition coefficient. In each experiment, each point represents the average of at least triplicate determinations.

ESI-TOF-MS analysis of γ-C1A-C1B

ESI-TOF-MS analyses were performed on a Mariner mass spectrometer using 10 mM pyridinium acetate (pH 6.8) as a carrier buffer at a flow rate of 4 μL/min using a 100 μL syringe equipped with a Model 2000 syringe pump (Harvard Apparatus). Zinc coordination of γ-C1A-C1B for ESI-TOF-MS was carried out in a distilled water solution (1 nmol/20 μL). An aliquot of 1 mM $ZnCl_2$ solution (0–4 μL) was added to the peptide solution. After standing at 4 °C for 10 min, distilled water (0–16 μL), 4 μL of 100 mM pyridinium acetate buffer (pH 6.8), and 1 mM EDTA (0–8 μL) were added sequentially to the zinc-treated peptide solution. The total volume of the resultant solution was 40 μL.

The above peptide solution (5 μL) was applied to the carrier buffer by use of a 6-way injection valve (Rheodyne). All spectra were acquired in the positive-ion mode with the spray voltage of 4000 V, the nozzle potential of 130 V, and the nozzle temperature of 150 °C. The acceleration potential was 4000 V and the m/z range of 500–3000 was scanned. Neurotensine (3+:558.311; 2+:836.963) was used as an external reference. The deconvoluted molecular mass profiles were obtained by using the algorithms supplied with the instrument data system.

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