The Regulatory Domain of Protein Kinase C Coordinates Four Atoms of Zinc*

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Protein kinase C (PKC) was found to be a zinc metallo-enzyme. Atomic absorption measurements on the intact enzyme indicated that four zinc atoms (4.2 ± 0.5) were bound per PKC α molecule. Similar stoichiometric ratios were determined for PKC βII and PKC γ, other PKC isoforms individually expressed in the baculovirus-insect cell expression system, as well as for purified rat brain PKC. By trypsin treatment of PKC α, a 32-kDa lipid binding regulatory and a 50-kDa catalytic domain were generated that were subsequently completely separated by gel filtration in the presence of Triton X-100/phosphatidylserine mixed micelles. Zinc was present at levels significantly above background in fractions that contained the 32-kDa fragment and displayed phorbol ester binding activity. Lipid association and phorbol ester binding did not lead to displacement of zinc from the protein. The stoichiometry determined for this fragment (4.7 ± 0.9) suggested that zinc was bound exclusively within the lipid binding regulatory domain of intact PKC. Furthermore, this stoichiometry is consistent with zinc being coordinated between 6 cysteine residues in a structural motif related to the Zn(II)2Cys, binuclear cluster identified in the GAL4 transcriptional factor (Pan, T., and Coleman, J. E. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2077–2081).

EXPERIMENTAL PROCEDURES

Materials—Histone III-S, phenylmethylsulfonyl fluoride, 1,10-phenanthroline, and phorbol 12,13-dibutyrate were purchased from Sigma; trypsin-L-tosylamide-2-phenyl ethyl chloromethyl ketone from Worthington; GF/C glass microfiber filters from Whatman International Ltd. Mardstone, Great Britain; [γ-32P]ATP and [3H]phorbol 12,13-dibutyrate were purchased from Du Pont-New England Nuclear; Triton X-100 from Pierce Chemical Co.; dioleyl phosphatidylserine and L-a-dioctanoylglycerol from Avanti Polar Lipids Inc., Birmingham, AL; Ultrogel AcA 44 from LKB-Reactifs IBF, Villeneuve-l’a-Garenne, France; Sephadex G-25 and the gel filtration calibration markers were from Pharmacia LKB Biotecnique Inc.; low molecular weight and prestained standards were from Bio-Rad; the zinc calibration standard solution (1 mg/ml) for atomic absorption spectroscopy was purchased from Perkin-Elmer Cetus; all other chemicals were of the highest grade available.

Purification of Protein Kinase C—Protein kinase C was purified from rat brain or infected Sf9 cells as described previously (16). The final preparations were essentially homogeneous as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and had a specific activity of 1 unit/mg of protein. After

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‡ The abbreviations used are: PKC, protein kinase C; DAG, sn-1,2-diaclylglycerol; PS, phosphatidylserine; PDBu, phorbol 12,13-dibutyrate; Sf9, Spodoptera frugiperda cells; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGT, ethylenebis(oxyethylenenitrito)tetracetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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purification the enzyme was stored at -70 °C in storage buffer (20 nM Tris-HCl pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA. 10 mM β-mercaptoethanol, 10% glycerol, and 0.05% Triton X-100). Rat brain PKC was purified following a similar procedure as described (6). To obtain enzyme of the quality available for the individual isoforms rat brain PKC (Catalytic Domain-Frozen) (Calciochrome BII-LKB Biotechnology Inc.) was additionally purified on an FPLC HR5/5 Mono Q column (Pharmacia LKB Biotechnology Inc.). Rat brain PKC bound to the Mono Q column equilibrated in storage buffer and eluted at as a pure PKC protein peak at 180 mM NaCl in a 34-ml linear salt gradient from 50 to 450 mM NaCl.

Sephadex G-25 Gel Filtration Analysis—To measure the zinc stoichiometry for PKC molecules, purified enzyme (5-10 µl in 100 µl) was analyzed on an FPLC HR5/5 column packed with about 1 ml of swelled Sephadex G-25, equilibrated in Buffer A (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM β-mercaptoethanol, 0.03% Triton X-100, 10% ethylene glycol, and 50 mM NaCl) at a flow rate of 1 ml/min. Fractions of 0.2 ml volume were collected. Zinc-free buffers were obtained by extraction with dithizone in chloroform (17), and zinc was determined by atomic absorption spectroscopy. Column void volume and inclusion volume were determined in calibration experiments, as the one indicated in Fig. 3B, trypsin-digested PKC was digested with trypsin essentially as described previously (6) with some modifications. A unit of PKC activity is defined as the incorporation of 1 pmol of [3H]PDBu. Unspecific binding was determined in the presence of 40 pM [3H]PDBu, and competitive binding was determined in the presence of 100 nM [3H]PDBu.

Trypsin Digestion of PKC—Purified PKC α was digested with trypsin essentially as described previously (6) with some modifications. The final concentration of 200 µl/ml was used to digest PKC in 50% acetic acid (100 units/ml) using 100 units of trypsin/500 µl of 20 mM Tris-HCl, pH 8.2, with 10% ethylene glycol and stored at -70 °C. Replication was recorded at 30 min. PKC α (75 µg) was digested at room temperature with trypsin (0.5 units) in a final volume of 600 µl of storage buffer. Digestion was stopped with 20 µg of 20 mM DTT and 0.02% (w/v) Triton X-100, and 10% (w/v) glycerol. Buffers were adjusted to a final concentration of 200 µM calcium, 0.3% Triton X-100, 10 mM MgCl2, 200 µM CaCl2, 200 µM NaCl, 0.5 mM MEGTA, 10 mM 0-mercaptoethanol, and 200 pM CaCl2 in a final concentration of 1 mM. Digested samples were stored at -70 °C until further processed.

Separation of Mixed Membrane-bound PKC Regulatory Domain from the Catalytic Domain—Frozen aliquots (800 µl) of trypsin-digested PKC were placed on ice and 200 µl of cold 20 µM calcium was added (w/v) Triton X-100 mixed micelles containing 20 mol% PS in a final volume of 1 ml, and applied to an Ultrogel AcA 44 column (1.7 x 26 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 10 mM β-mercaptoethanol, 200 µM CaCl2, 200 µM NaCl, 0.02% (w/v) Triton X-100, and 10% (w/v) glycerol. Buffers were prepared in plastic containers using the highest quality water to reduce zinc levels. Flow rates were typically 0.22 ml/min. Column fractions (1 ml) were characterized by measuring zinc content, kinase activity, and PDBu binding activity. As a control for background zinc in these experiments, mixed micelles together with trypsin in the absence of zinc were analyzed in this fashion. In some experiments, as the one indicated in Fig. 3B, trypsin-digested PKC α was allowed to associate with mixed micelles as described above, but in the presence of 200 µM [3H]PDBu. Control experiments to determine background zinc were done as described above including PDBu binding activity and without PKC (Fig. 3A). In both types of column recovery experiments, as the one indicated in Fig. 3B, trypsin-digested PKC α was allowed to associate with mixed micelles as described above, but in the presence of 200 µM [3H]PDBu. Control experiments to determine background zinc were done as described above including PDBu binding activity and without PKC (Fig. 3A). In both types of column recovery experiments, as the one indicated in Fig. 3B, trypsin-digested PKC α was allowed to associate with mixed micelles as described above, but in the presence of 200 µM [3H]PDBu. Control experiments to determine background zinc were done as described above including PDBu binding activity and without PKC (Fig. 3A). In both types of column recovery experiments, as the one indicated in Fig. 3B, trypsin-digested PKC α was allowed to associate with mixed micelles as described above, but in the presence of 200 µM [3H]PDBu. Control experiments to determine background zinc were done as described above including PDBu binding activity and without PKC (Fig. 3A). In both types of column recovery experiments, as the one indicated in Fig. 3B, trypsin-digested PKC α was allowed to associate with mixed micelles as described above, but in the presence of 200 µM [3H]PDBu. Control experiments to determine background zinc were done as described above including PDBu binding activity and without PKC (Fig. 3A).
Purification step of PKC isozymes expressed in Sf9 insect cells (16). The three individual PKC isoforms peak fractions was essential. The Amidoschwarz dye binding protein at the low concentrations of protein present in such fractions together with the illustrated zinc measurements provided the basis with Coomassie Blue protein concentrations of PKC eluting from the phenyl-Superose column (Fig. 1), a protein-bound (fractions 5 and 6) and free (fractions 7–12) zinc were separated, thus allowing the determination of zinc bound to PKC. To determine the stoichiometric ratio between PKC and zinc, a simple, accurate, and sensitive method for determining protein at the low concentrations of protein present in such peak fractions was essential. The Amidoschwarz dye binding assay (20), with bovine serum albumin as a standard, was found to be the most reliable method. The protein concentration obtained by this method with a standard PKC α preparation (91 ± 8 μg/ml) was compared with those measured by two independent methods described in greater detail under “Experimental Procedures.” Both methods gave values for the protein concentration that lay within 20% of the value measured by the Amidoschwarz dye binding assay: Micro BCA (74.5 ± 6 μg/ml) and quantitative amino acid analysis (81 ± 5 μg/ml). Thus, in the experiments described all stoichiometries were calculated based on protein determinations by the Amidoschwarz dye binding assay. For PKC α an average ratio of 4.2 ± 0.5 zinc atoms were found per molecule (Table I). Following the same procedure similar stoichiometries of zinc were found in PKC βII, PKC γ, and rat brain PKC preparations (Table I).

Protein kinase C contains two cysteine-rich regions (Cys 1 and Cys 2) within the C1 domain. Both the Cys 1 and Cys 2 regions independently exhibit phorbol ester binding activity (10) and show homology to cysteine-rich regions present in transcription factors. Based on sequence comparisons to DNA binding motifs, two possible structures have been inferred for the cysteine-rich phorbol ester binding regions of PKC (10), a Cys4 “zinc finger” domain similar to those of the glucocorticoid receptor (26) and a Zn(II)4Cys6 binuclear cluster as identified in the GAL4 transcriptional factor (27). If a Cys4 zinc finger-like structure is involved in coordinating zinc, a stoichiometry of two per PKC molecule should result (one zinc per finger); alternatively, if a structure similar to a zinc binuclear cluster is involved, the expected stoichiometry should be four (two zinc per cluster). The results presented here favor the latter possibility.

If indeed the zinc is coordinated in the suggested fashion, the lipid binding domain of PKC should contain all the zinc bound to intact PKC. To test this prediction, PKC α was present in PKC α (Fig. 2). Void and inclusion volume of the column were determined in trial runs with Triton X-100 and tritiated water respectively (Fig. 2A). PKC α eluted with maximal kinase activity in fraction five corresponding to the column void volume (Fig. 2B). As observed previously with PKC eluting from the phenyl-Superose column (Fig. 1), a peak of zinc was measured in fractions displaying PKC activity. In this experiment, however, protein-bound (fractions 5 and 6) and free (fractions 7–12) zinc were separated, thus allowing the determination of zinc bound to PKC.

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The experimental data shown summarize the zinc stoichiometries (mol of zinc/mol of PKC) determined for rat brain and individual isozymes from baculovirus-infected Sf9 cells (PKC α, βII, γ) by gel filtration analysis with a 1-ml Sephadex G-25 column. Stoichiometry values for the regulatory domain of PKC α (α regulatory) were measured after association with mixed micelles (Triton X-100/PS) and separation on an Ultrogel AcA 44. Zinc concentrations were also measured in fractions from this column containing the catalytic domain (α catalytic). The results were obtained with a number of different enzyme preparations: rat brain PKC, 2; PKC α, 4; PKC βII, 1; PKC γ, 1; PKC α regulatory or catalytic domain, 2. All PKC preparations characterized possessed kinase and [3H]PDBu binding activities comparable with those described previously (16). [3H]PDBu binding activity of the isolated PKC α lipid binding domain was as reported (6). For each stoichiometry determination sample concentrations of zinc were measured four times and protein concentrations were measured in duplicate. The errors indicated for zinc stoichiometries are equivalent to the standard error calculated for the averaged stoichiometry determinations, except for the βII isozyme where the standard error of the respective measurements is indicated in brackets.

| PKC (source/isozyme/domain) | Stoichiometry | Determinations |
|----------------------------|--------------|----------------|
| Rat brain                  |              |                |
| α                          | 4.1 ± 0.4    | 3              |
| α regulatory               | 4.7 ± 0.9    | 4              |
| α catalytic                | Not significant | 2            |
| Bovaculovirus              |              |                |
| α                          | 4.2 ± 0.5    | 7              |
| βII                        | 3.4 ± (0.6)  | 1              |
| γ                          | 4.0 ± 0.4    | 3              |

**Table 1**

**Summary of zinc stoichiometries determined for different PKC isozymes and domains**

The experimental data shown summarize the zinc stoichiometries (mol of zinc/mol of PKC) determined for rat brain and individual isozymes from baculovirus-infected Sf9 cells (PKC α, βII, γ) by gel filtration analysis with a 1-ml Sephadex G-25 column. Stoichiometry values for the regulatory domain of PKC α (α regulatory) were measured after association with mixed micelles (Triton X-100/PS) and separation on an Ultrogel AcA 44. Zinc concentrations were also measured in fractions from this column containing the catalytic domain (α catalytic). The results were obtained with a number of different enzyme preparations: rat brain PKC, 2; PKC α, 4; PKC βII, 1; PKC γ, 1; PKC α regulatory or catalytic domain, 2. All PKC preparations characterized possessed kinase and [3H]PDBu binding activities comparable with those described previously (16). [3H]PDBu binding activity of the isolated PKC α lipid binding domain was as reported (6). For each stoichiometry determination sample concentrations of zinc were measured four times and protein concentrations were measured in duplicate. The errors indicated for zinc stoichiometries are equivalent to the standard error calculated for the averaged stoichiometry determinations, except for the βII isozyme where the standard error of the respective measurements is indicated in brackets.
digested with trypsin to generate regulatory and catalytic subunit fragments and analyzed by gel filtration as described previously (6). Under the conditions chosen for PKC digestion with trypsin, 65–90% of initial enzyme activity, quantitated both as kinase and phorbol ester binding activity, were typically maintained. Some 75–100% of residual kinase activity became lipid independent, indicating essentially complete cleavage of the PKC (data not shown). This was corroborated by gel analysis (Fig. 4A, lane 2). Two major breakdown products were routinely observed, migrating as expected at nominal values of 45,000–50,000 and 32,000; these most likely correspond to catalytic and lipid binding domains, respectively (6).

Trypsin-treated PKC α incubated with mixed micelles containing Triton X-100 and PS in the presence of [3H]PDBu was analyzed by gel filtration in zinc-free buffers (Fig. 3). A typical elution profile showing zinc background levels measured in trial runs with mixed micelles, trypsin, and [3H]PDBu is illustrated (Fig. 3A). When trypsin-digested PKC α was analyzed a peak of zinc was consistently found associated with fractions containing mixed micelles (Fig. 3B). Otherwise, zinc levels significantly above background were only detected in column inclusion volume fractions coinciding with free, non-protein-associated zinc (fractions 42–50). Error bars indicate the standard error of at least four or more measurements per fraction.

Assessment of the stoichiometry of zinc bound to the 32-kDa fragment in mixed micelle peak fractions of the Ultrogel AcA 44 gel filtration column revealed similar values to those found for the intact PKC isofrom (4.7 ± 0.9; see Table I). Essentially the same results were obtained when trypsin-treated PKC α associated with Triton X-100/PS mixed micelles in the absence of PDBu was analyzed (data not shown), suggesting that residues involved in the coordination of zinc need not directly interact with PDBu, since this event does not displace zinc from the lipid binding domain.

Previous work (6) and the data presented here strongly suggest that the 32-kDa fragment is indeed the lipid binding regulatory domain of PKC. To confirm this and assess precisely where cleavage occurred within PKC, fragments were analyzed a peak of zinc was consistently found associated with fractions containing mixed micelles (Fig. 3B). Otherwise, zinc levels significantly above background were only detected in column inclusion volume fractions coinciding with free, non-protein-associated zinc (fractions 42–50). Error bars indicate the standard error of at least four or more measurements per fraction.

The absorbance at 280 nm () and background zinc levels () are shown. Mixed micelles elute slightly after void volume fractions, as indicated by the 640-kDa calibration standard protein. Note the low levels and minimal fluctuations of zinc levels in these control runs. The elution profile of trypsin-treated PKC α incubated with mixed micelles in the presence of 200 nm [3H]PDBu is illustrated in B. [3H]PDBu binding (), lipid-dependent kinase activity ( ), as well as zinc concentrations (C) were measured as described. Protein associated zinc is essentially only present at levels significantly above background in the mixed micelle peak where PDBu binding was measured. High levels of zinc were consistently found in column inclusion volume fractions coinciding with free, non-protein-associated zinc (fractions 42–50). Error bars indicate the standard error of at least four or more measurements per fraction.

![Fig. 3. Separation of lipid binding domain and catalytic domain by gel filtration.](image)

**Fig. 3.** Separation of lipid binding domain and catalytic domain by gel filtration. An Ultrogel AcA 44 column was used to separate proteolytic fragments of PKC as described. Migration positions of calibration markers are indicated at the top of the figure. The elution profile of Triton X-100/PS (20 mol%) mixed micelles with trypsin in the presence of PDBu is illustrated (A). The absorbance at 280 nm ([A], [3H]PDBu ([A]), and background zinc levels ([ ]) are shown. Mixed micelles elute slightly after void volume fractions, as indicated by the 640-kDa calibration standard protein. Note the low levels and minimal fluctuations of zinc levels in these control runs. The elution profile of trypsin-treated PKC α incubated with mixed micelles in the presence of 200 nm [3H]PDBu is illustrated in B. [3H]PDBu binding ([A]), lipid-dependent kinase activity ( [B]), as well as zinc concentrations ([C]) were measured as described. Protein associated zinc is essentially only present at levels significantly above background in the mixed micelle peak where PDBu binding was measured. High levels of zinc were consistently found in column inclusion volume fractions coinciding with free, non-protein-associated zinc (fractions 42–50). Error bars indicate the standard error of at least four or more measurements per fraction.

**Fig. 4. Analysis of PKC α tryptic fragments by SDS-PAGE and immunoblotting.** Samples resolved by SDS-PAGE on a 10% gel are shown after silver staining (A) or after transfer to nitrocellulose and visualization with specific polyclonal antisera (B). Lane 1, intact PKC α (2.4 μg); lane 2, PKC α after trypsin treatment (1.5 μg); lane 3, peak fraction 18 (0.8 μg) and lane 4, peak fraction 29 (0.5 μg), from the gel filtration elution profile shown in Fig. 3B. Lanes 1–4 in B were probed with antipeptide 102–115 antisera (similar results were obtained with antipeptide 19–36 antisera; not shown). B, lane 5 is the same as lane 4 except that antipeptide 313–326 antibodies were used to probe the nitrocellulose blot. Note that in peak fraction 18 only the regulatory lipid binding domain and in peak fraction 29 essentially only the catalytic domain are present. M, values of molecular weight standards (A, low molecular weight standards; B, prestained standards) are shown on the right (DF, dye front). The migration positions of intact PKC α, the catalytic domain (Cat), and the regulatory domain (LBD) are indicated on the left.
probed with polyclonal, antipeptide antisera directed against different segments of the regulatory PKC domain (Fig. 4B). Both the antisera against the pseudosubstrate domain (PKC α residues 19–36) and a peptide within the Cys 2 region (PKC γ residues 102–115) gave the same staining pattern and results for the latter antibody are illustrated (lanes 1–4). As expected the antisera recognized intact PKC (lanes 1 and 2; M, 80,000) and 32-kDa fragment (lanes 2 and 3) but not the protein bands at M, 45,000–50,000 (lanes 2 and 4). An antibody against the hinge region (PKC α residues 350–370) recognized intact PKC α (M, 80,000), two of the three bands at M, 50,000 (lane 5), and to a lesser extent the 32-kDa fragment (not shown). A similar array of protein bands (M, 45,000–50,000) representing the PKC catalytic domain, has also been observed previously after limited proteolysis of PKC α within the hinge region by calcium-dependent neutral proteases (28). Thus, all zinc bound to PKC is shown to be coordinated within the lipid binding PKC regulatory domain.

Attempts to demonstrate a physiological role for zinc in PKC by dialysis against chelators under nondenaturing conditions failed to remove the metal ion, indicating tight association between zinc and the protein (data not shown). The presence of urea and 1,10-phenanthroline, a high-affinity zinc chelator with low affinity for both calcium and magnesium, was equally ineffective, because PDBu binding activity could never be reconstituted after such exposures. In most cases the enzyme was lost during the dialysis of such dilute solutions containing 50–100 μg/ml protein. Experiments designed to unequivocally demonstrate a functional role for zinc coordinated within the regulatory domain of PKC will require far larger quantities of pure enzyme than currently available.

In summary, the data presented show that protein kinase C is a metallo-enzyme in which zinc is bound within the lipid binding domain. Stoichiometry measurements of intact enzyme indicated that four zinc atoms are coordinated per PKC molecule. The zinc bond is associated within the segment of the protein possessing PDBu binding activity rather than in the segment encoding the kinase activity of the enzyme. Cysteine-rich motifs in both PDBu binding proteins PKC and n-Chimaerin are essential for that activity (8, 13). Similar or related sequence motifs present in a variety of transcription factors such as GALA, TFIIIA, and the glucocorticoid receptors are involved in the coordination of zinc in distinct DNA-binding structural motifs: (i) zinc clusters, (ii) zinc fingers, and (iii) zinc twists (29). Thus, the cysteine residues in the cysteine-rich PDBu binding motifs may be critically involved as ligands for zinc. The observed zinc stoichiometry for PKC corresponds to a value expected if it were coordinated by cysteine residues within the Cys 1 and Cys 2 regions (10) in a structural motif related to the Zn(II)Cys6 binuclear cluster of the GAL4 transcriptional factor (27). If this suggestion is supported by subsequent structural studies, then each of these zinc binuclear clusters may function as structural domains in the physiological activation mechanism of PKC by DAG (9). Furthermore, since PDBu binding did not lead to displacement of zinc from the enzyme, one may predict that residues other than the essential cysteines of the Cys 1 and Cys 2 regions interact directly with PDBu and also DAG, in contrast to previous suggestions (30).

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REFERENCES
1. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692–3695
2. Nishizuka, Y. (1984) Science 225, 1365–1370
3. Kikkawa, U., Kishimoto, A., and Nishizuka, Y. (1988) Annu. Rev. Biochem. 57, 31–44
4. Nishizuka, Y. (1986) Science 233, 305–312
5. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851
6. Lee, M.-H., and Bell, R. M. (1986) J. Biol. Chem. 261, 14867–14870
7. Ohno, S., Akita, Y., Konno, Y., Imajoh, S., and Suzuki, K. (1988) Cell 53, 731–741
8. Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U., and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4668–4671
9. Bell, R. M., and Burns, D. J. (1991) J. Biol. Chem. 266, 4661–4666
10. Burns, D. J., and Bell, R. M. (1991) J. Biol. Chem. 266, 18330–18338
11. Osada, S., Mizuno, K., Saito, T. C., Akita, Y., Suzuki, K., Kuroki, I., and Ohno, S. (1990) J. Biol. Chem. 265, 22434–22440
12. Clark, J. D., Lin, L.-I., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) Cell 65, 1043–1051
13. Ahmed, S., Kozma, R., Monfries, C., Hall, C., Lim, H. H., Smith, P., and Lim, L. (1990) Biochem. J. 272, 767–773
14. Hall, C., Monfries, C., Smith, P., Lim, H. H., Kozma, S., Vanniasingham, V., Leung, T., and Lim, L. (1990) J. Mol. Biol. 211, 11–16
15. Ahmed, S., Kozma, R., Lee, J., Monfries, C., Harden, N., and Lim, L. (1991) Biochem. J. 280, 233–241
16. Burns, D. J., Bloomenthal, J., Lee, M.-H., and Bell, R. M. (1990) J. Biol. Chem. 265, 12044–12051
17. Falchuk, K. H., Hilt, K. L., and Vallee, B. L. (1988) Methods Enzymol. 158, 422–434
18. Hannun, Y. A., Loomis, C. R., and Bell, R. M. (1985) J. Biol. Chem. 280, 10039–10043
19. Ebeling, J. G., Vandenbergh, G. R., Kuhn, L. J., Ganong, B. R., Bell, R. M., and Niedel, J. E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 815–819
20. Schaffner, W., and Weisssmann, C. (1973) Anal. Biochem. 46, 502–514
21. Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D., and Ullrich, A. (1986) Science 233, 853–859
22. Wessel, D., and Fluegge, U. I. (1984) Anal. Biochem. 138, 141–143
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Makowske, M., and Rosen, O. M. (1989) J. Biol. Chem. 264, 16155–16159
25. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
26. Haerd, T., Kellenbach, E., Boelens, R., Maler, B. A., Dahlman, K., Freedman, L. P., Carlstedt-Duke, J., Yamamoto, K. R., Gustafsson, J.-A., and Kapein, R. (1990) Science 249, 157–160
27. Pan, T., and Coleman, J. E. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2077–2081
28. Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S., Tomingana, M., Kuroda, T., and Nishizuka, Y. (1989) J. Biol. Chem. 2644, 4088–4092
29. Vallee, B. L., Coleman, J. E., and Auld, D. S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 999–1003
30. Geschwendt, M., Kittleen, W., and Marks, F. (1991) Trends Biochem. Sci. 16, 167–169