Purification and Characterization of Ceramide-Activated Protein Phosphatases†

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ABSTRACT: Ceramide has emerged as a potential regulator of diverse cellular functions, and a few direct targets have been identified for its action including protein kinases and phosphatases. In this study, we have purified the predominant ceramide-activated protein phosphatase (CAPP) from rat brain. Utilizing a novel chromatographic approach, CAPP was purified to near homogeneity using hydrophobic interaction chromatography on phenyl Sepharose followed by anion-exchange chromatography on MonoQ. The purified protein was composed of three major bands on SDS−polyacrylamide gel electrophoresis which comigrated with the three subunits of heterotrimeric PP2A. Immunologic studies further identified CAPP to be composed predominantly of heterotrimeric AB′C and ABαC as well as heterodimeric PP2A (AC), where C is the catalytic subunit, and A and B are regulatory subunits. These results were also supported by the coelution of CAPP with trimeric and dimeric PP2A on size-exclusion chromatography. These studies provide a convenient and efficient method for the isolation of trimeric and dimeric PP2A, and they allow the biochemical investigation of CAPP.

Attention has focused recently on the roles of sphingolipids in signal transduction, including roles for ceramide, sphingosine, and sphingosine-1-phosphate (*2*, *13*, *14*, *22*, *27*, *39*).

Sphingolipids constitute a diverse and ubiquitous group of eukaryotic membrane lipids with structural complexities and multiple biological effects, which are becoming very important for a mechanistic understanding of cell growth regulation. Investigation of regulated metabolism of sphingolipids has resulted in the elucidation of the sphingomyelin cycle and ceramide-mediated pathways, which have emerged as key mechanisms implicated in terminal differentiation, apoptosis, cell cycle arrest, and cell senescence (*12*, *17*, *34*, *36*, *42*). These pathways are activated following either ligand binding of receptors or the action of stress agents and the subsequent activation of sphingomyelinases, which in turn hydrolyze sphingomyelin to generate ceramide and phosphorylcholine (*12*, *22*).

The novel lipid second messenger, ceramide, mimics the cytotoxicity of compounds such as TNFα and Fas (APO-1, CD95). Ceramide causes an early, potent, and specific activation of caspases as well as internucleosomal DNA fragmentation (*16*, *33*, *38*), and it has been argued that ceramide may mediate, at least in part, the cytotoxic effects of TNFα and Fas (*12*, *22*, *40*, *41*). Ceramide also causes cell cycle arrest through the dephosphorylation of the retinoblastoma gene product (*6*). Other effects of ceramide include the down regulation of the c-myc proto oncogene (*44*) and inhibition of protein kinase C and phospholipase D.

The mechanisms by which ceramide induces its diverse biological effects remain to be elucidated. However, insight has been generated from a number of studies that address targets of ceramide action. One such direct target is a cytosolic serine/threonine protein phosphatase, termed ceramide-activated protein phosphatase (CAPP) (*11*, *21*, *25*, *31*, *42*, *43*, *45*). In addition, ceramide has been proposed to activate a serine/threonine kinase (KSR) which may mediate effects of ceramide on ras and raf (*29*, *47*). Ceramide has also been shown to interact with the protein kinase raf (*18*) and to activate protein kinase Cζ (*26*), although the physiologic significance of these interactions is not known.

CAPP is thought to be a member of the PP2A class of protein phosphatases (*8*). These phosphatases probably exist in vivo as dimeric (AC) or heterotrimeric complexes (ABC) possessing one structural (A), one regulatory (B), and one catalytic (C) subunit. The A subunit exists as a 60−65 kDa protein, whereas the C subunit is a 36−38 kDa protein. Based upon biochemical and molecular differences, several distinct B subunits, 55 kDa (B), 54 kDa (B′), 72 kDa (B″), and 130 kDa have been characterized (*5*, *15*, *30*, *44*).

Multiple studies now implicate CAPP as a mediator of at least some of the cellular effects of ceramide. For example, okadaic acid, a potent inhibitor of the PP2A family of phosphatases, inhibits the effects of ceramide on PKCα (*25*) and on c-myc (*44*). Also, it has been shown that c-jun may be a physiologic substrate for CAPP (*37*). CAPP has also been implicated in mediating the effects of ceramide on neuronal differentiation (*35*) and on tumor invasion (*28*). In *Saccharomyces cerevisiae*, CAPP has been identified and

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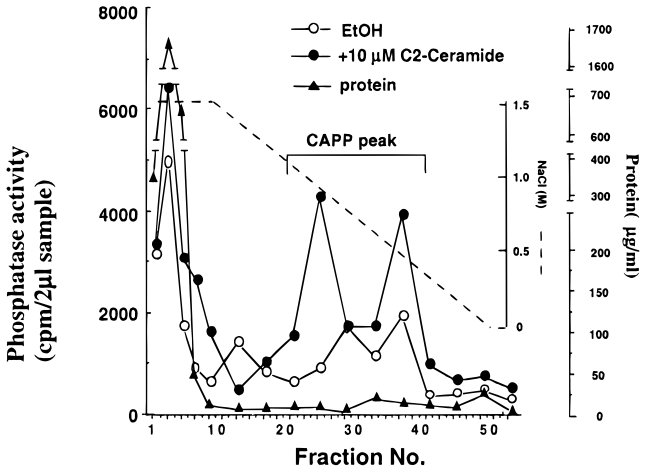

FIGURE 1: Chromatographic behavior of ceramide-activated protein phosphatase. The cytosol from rat brain (9.3 mg of protein) was initially loaded on phenyl Sepharose HP fast protein liquid chromatography column (HiTrapTM HIC Test Kit, Pharmacia Biotech). The column was washed with 10 mL of 1.5 M NaCl in Tris-HCl buffer, pH 7.4, 1 mM DTT, 1 mM benzamidine at 1.0 mL/min, and eluted with a linear gradient to 0 M NaCl over 40 min. Fractions (1 mL) were collected, and 2 μL of each fraction was assayed for phosphatase activity under the standard assay (50 μg/mL of [^{32}P]MBP, for 15 min at 30 °C) in the absence (○) or presence (●) of 10 μM C_{10}-ceramide. Protein concentration of each fraction was determined by Coomassie Blue binding using serum albumin as the standard. These profiles are representative of several (>10) experiments.

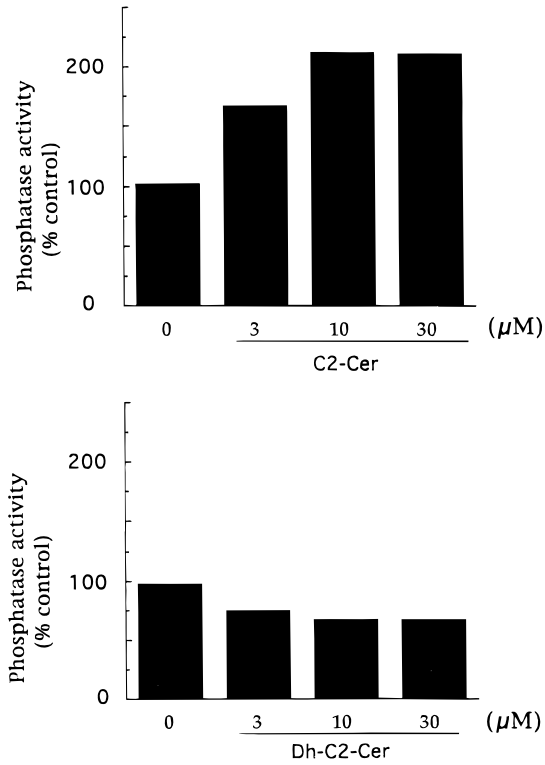

FIGURE 2: Effect of C_2-ceramide and dihydro-C_2-ceramide on CAPP. Fractions from the phenyl Sepharose column were assayed for phosphatase activity under the standard assay (50 μg/mL of [^{32}P]MBP, for 15 min at 30 °C) in the absence or presence of the indicated concentrations of C_2-ceramide. Phosphatase activity is presented as the activity relative to control in the absence of C_2-ceramide.

determined to be composed of the Sit 4 catalytic subunit, the Tpd3 A subunit, and CDC55 as the B subunit (*32*). These subunits are required for ceramide-induced growth suppression of *S. cerevisiae* since deletion of any of them imparts partial resistance to ceramide.

Although CAPP is a primary candidate for mediating ceramide signaling in mammalian cells, its identity and its connection to the downstream targets of ceramide activation remain poorly defined. Molecular characterization of CAPP is, therefore, a prelude to a better understanding of the mechanism by which ceramide causes its downstream effects. One factor which has impeded progress in the characterization of CAPP has been the inability to separate CAPP from other phosphatase activities. One recent study also suggests that special forms of AC and the C catalytic subunit are also responsive to ceramide (*24*). In this study we report for the first time on a rapid method for the purification of CAPP. These approaches have allowed us to characterize purified CAPP biochemically and immunologically.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma, unless otherwise stated. PP2A isoforms were purified as described (*19*, *20*) and antibodies to PP2A were raised as described (*20*).

Purification of CAPP from Rat Brain Cytosol. Frozen rat brains (Pel-Freez Biologicals) were homogenized in 4 mL/brain of Tris-HCl buffer, pH 7.4, 1 mM EDTA, and 1 mM EGTA. These were centrifuged at 1200 rpm, 4 °C, to clarify them from unhomogenized tissue. Cytosolic fractions were prepared from the low-speed supernatant by centrifugation at 100000*g* for 1 h at 4 °C. The cytosol, 9.3 mg of protein, was initially loaded on a phenyl Sepharose HP column (HiTrap HIC Test Kit, Pharmacia Biotech). The column was washed with 10 mL of 1.5 M NaCl in Tris-HCl buffer, pH 7.4, 1 mM DTT, and 1 mM benzamidine (buffer A) at 1.0 mL/min and eluted with a linear gradient to 0 M NaCl over 40 min. Ceramide-activated protein phosphatase activity was determined by assay of each fraction (1 mL) in the standard assay (2 μL of each fraction, 50 μg/mL of [^{32}P]-myelin basic protein (MBP), 15 min, 30 °C) using 10 μM of C_2-ceramide. Active fractions were pooled, diluted to approximately 50 mM NaCl, and loaded onto a 1 mL Mono-Q column (HR 5/5 Pharmacia Biotech). The column was washed for 30 min with 50 mM NaCl in buffer A at 0.5 mL/min and eluted with a linear gradient to 500 mM NaCl over 60 min.

To estimate the molecular mass of protein in each active fraction, active fractions were pooled and loaded onto a gel filtration column (Sephacryl S-200 column 16 × 50 in FPLC, Pharmacia Biotic). The column was eluted with 0.3 mL/min of buffer A.

Protein Staining and Western Blotting. Samples were boiled in electrophoresis sample buffer and analyzed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE). The gel was stained by ISS Pro-Blue staining system, and the gel was then dried. For immunoblot analysis, following SDS−PAGE, proteins were electrophoretically transferred to a nitrocellulose membrane. The filter was blocked in 5% dried milk in PBST (0.1% Tween 20 in 1×PBS), incubated in rabbit antiserum against PP2A subunits (1:1000−5000 dilution), followed by horseradish peroxidase-labeled anti-rabbit IgG (BioRad, 1:5000 dilution), washed three times with 1×PBS, and then developed using the enhanced

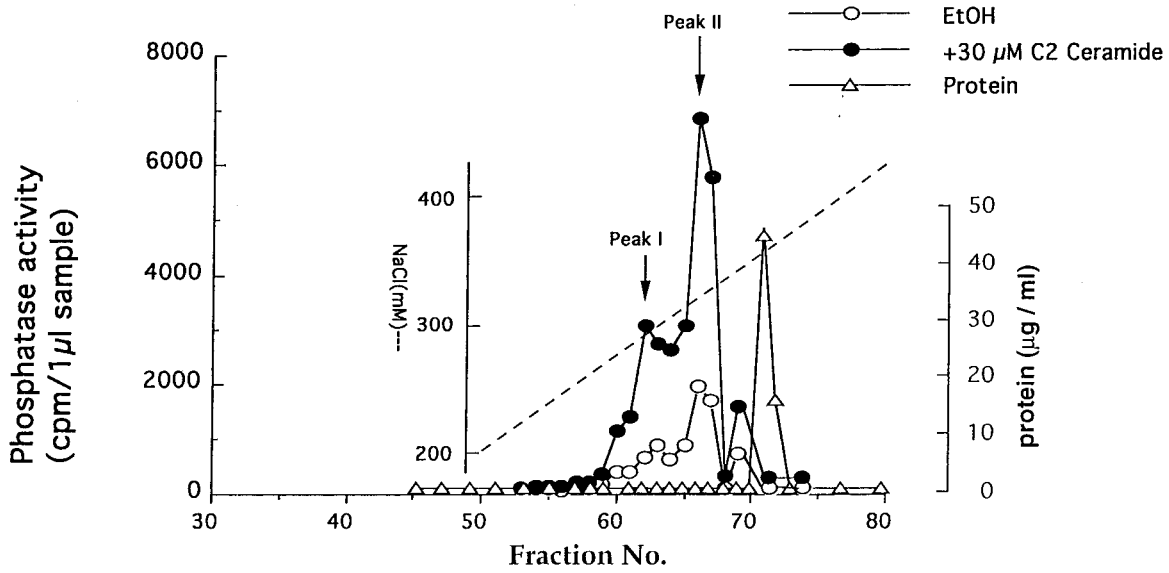

FIGURE 3: Chromatographic behavior of ceramide-activated protein phosphatase from rat brain on mono Q. Active fractions from the phenyl Sepharose column were pooled, diluted to approximately 50 mM NaCl and loaded onto a 1 mL Mono-Q column (HR 5/5 Pharmacia Biotech). The column was washed for 30 min with 50 mM Tris HCl buffer, pH 7.4, at 0.5 mL/min and eluted with a linear gradient to 500 mM NaCl over 60 min. One milliliter fractions were collected, and 2 μL of each fraction was assayed for phosphatase activity under the standard assay in the absence (○) or presence (●) of 30 μM C_2-ceramide. Protein concentrations of each fractions (△) were determined by Coomassie Blue binding using serum albumin as the standard.

chemiluminescence (ECL) detection system (Amersham Corp.).

Preparation of [^{32}P] *Phosphorylated MBP*. Phosphorylated MBP was prepared by phosphorylation with the catalytic subunit of cAMP-dependent kinase as previously described (*9*) with some modifications. Briefly, the reaction contained 50 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P]-ATP [(2−7) × 10^3 cpm/pmol], and 1 mg of MBP and 250 units/mL of the catalytic subunit of cAMP-dependent kinase. Reactions were incubated at 37 °C for at least 2 h, after which the reaction was stopped with the addition of 0.17 mL of ice-cold 100% TCA. Tubes were vortexed and left to stand on ice for at least 3 h and centrifuged at 15000*g* for 10 min at 4 °C. The supernatant was discarded, the pellet was washed with 2 × 1 mL aliquots of −20 °C acetone, and finally it was resuspended in 1 mL of 50 mM Tris-HCl, pH 7.4. The concentration of the labeled substrate was based on the specific activity of the [γ-^{32}P] ATP. Typical preparations of [^{32}P]MBP contained 3−9 nmol of P_i/mg. Assays were performed so that hydrolysis did not exceed 10% of total substrate added. Protein concentrations were determined by Coomassie Blue binding using bovine serum albumin as the standard (*3*).

Phosphatase Assay. This assay was performed as previously described (*9*) with some modifications. Briefly, the assay was performed in 50 mM Tris-HCl buffer, pH 7.4, containing 0.5−1.5 ng of protein and the indicated concentrations of radiolabeled substrate in a final volume of 0.1 mL. All lipid solutions were prepared fresh and dissolved in 100% ethanol to a concentration of 0−3 mM immediately prior to addition to the assays. Aliquots (1 μL) were added to reactions giving 0−30 μM lipid and a final ethanol concentration of 1%. Reactions were run for 15 min at 30 °C and were terminated by addition of 0.1 mL of 1 mM KH_2PO_4 in 1 N H_2SO_4. The released [$^{32}PO_4$]was chelated by the addition of 0.3 mL of 2% ammonium molybdate, and the phosphomolybdate complex was extracted with the addition of 1 mL of toluene:isobutanol (1:1, v/v). Quantitation was done by scintillation spectrometry of an aliquot

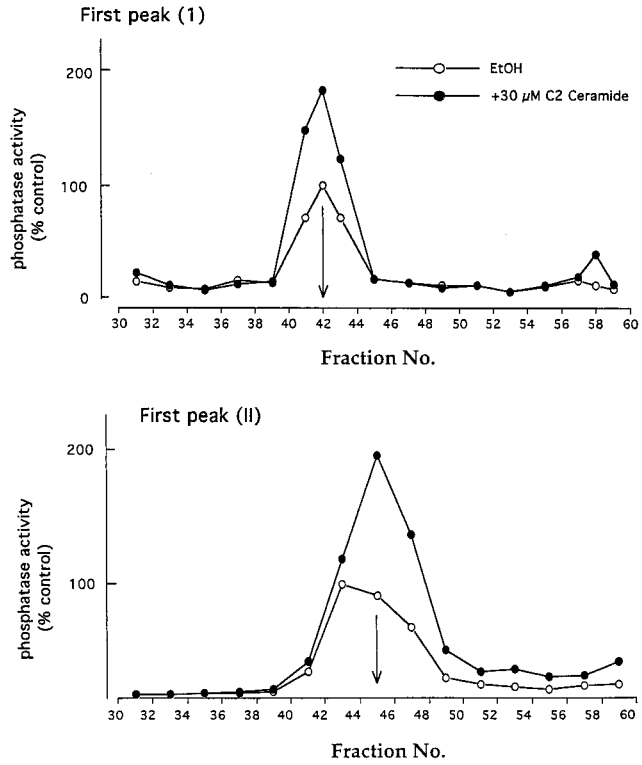

FIGURE 4: Elution of peaks I and II of CAPP on a Sephacryl S200 gel filtration column. Active fractions from Mono-Q column (upper panel, peak I; lower panel, peak II) were pooled and loaded onto a gel filtration column (Sephacryl S-200 column 16 × 50 in FPLC, Pharmacia Biotech). The column was eluted with 0.3 mL/min of 50 mM Tris-HCl buffer, pH 7.4. One milliter fractions were collected, and 2 mL of each fraction was assayed for phosphatase activity under the standard assay in the absence (○) or presence (●) of 30 μM C_2-ceramide. Phosphatase activity is presented as the activity relative to peak activity in the absence of C_2-ceramide.

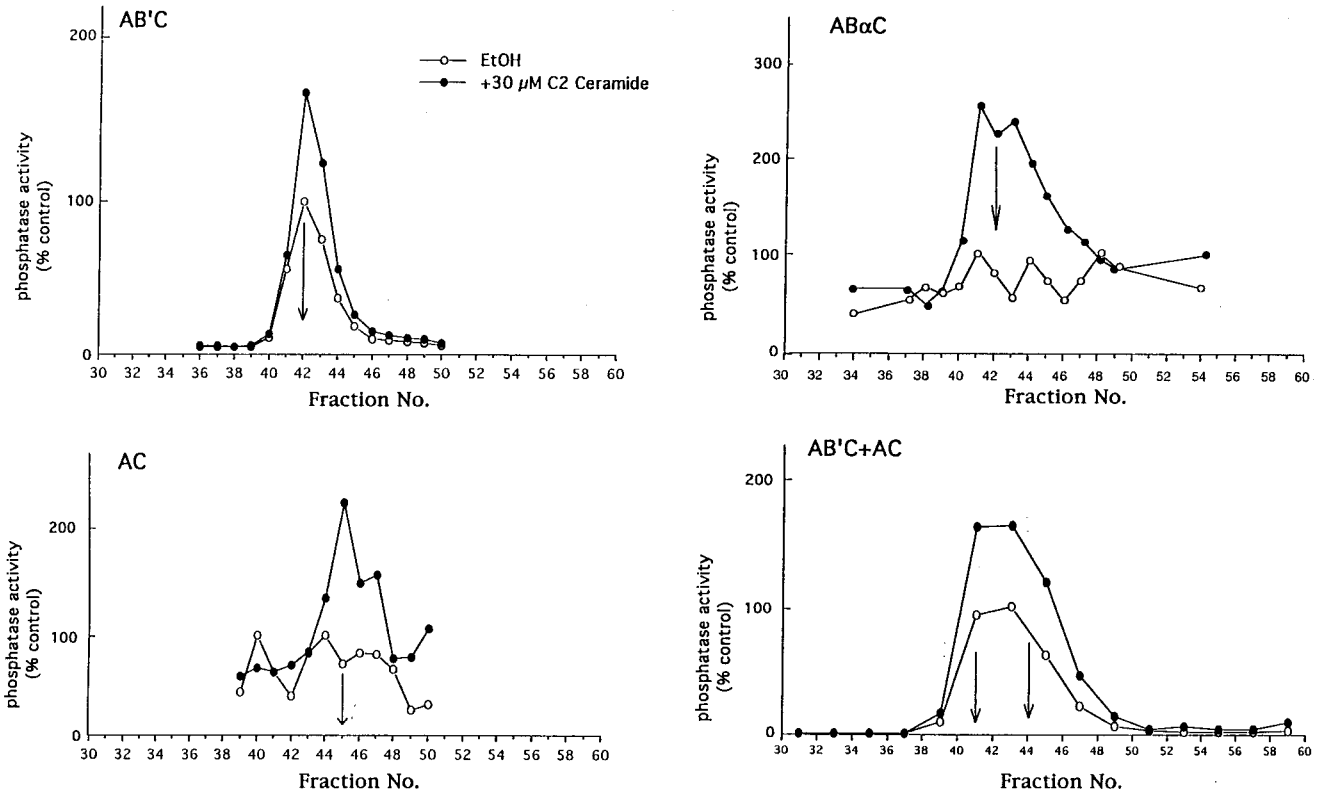

FIGURE 5: Elution of AB′C, AC, and ABαC on Sephacryl S200. Purified AB′C, ABαC, AC, and a combination of AB′C and AC were loaded onto the gel filtration column (Sephacryl S-200 column 16 × 50 in FPLC, Pharmacia Biotech). The column was eluted with 0.3 mL/min of 50 mM Tris HCl buffer. One milliliter fractions were collected, and 2 mL of each fraction was assayed for phosphatase activity under the standard assay in the absence (○) or presence (●) of 30 μM C_2-ceramide. Phosphatase activity is presented as the activity relative to peak activity in the absence of C_2-ceramide. Arrows indicated the fraction with peak activity in the presence of C_2-ceramide.

of the extracted phosphomolybdate complex. CAPP activity was quantitated as the difference in activity in the presence versus absence of ceramide.

RESULTS

Purification of CAPP on Phenyl Sepharose. In an effort to optimize the separation of CAPP from other phosphatases, we employed hydrophobic interaction chromatography with phenyl Sepharose. Figure 1 shows a typical profile for purification of CAPP obtained from rat brain. Cytosolic extracts from rat brain were loaded on a phenyl Sepharose column which was then washed with 1.5 M NaCl, following which the column was subjected to a linear decreasing salt gradient. Fractions were collected and tested for MBP-phosphatase activity in the absence or presence of C_{10}-ceramide. As can be seen from Figure 1, the bulk of the phosphatase activity eluted during the wash step. However, the ceramide-stimulated phosphatase (CAPP) eluted toward the end of the gradient, with peak elution at about 375 mM NaCl, hence, separating the CAPP activity from the total phosphatase activity. Notably, rat brain CAPP eluted in two distinct peaks that were resolved from other phosphatase activities.

When comparing the protein profile (Figure 1) to CAPP elution, the amount of protein retained by the column was almost negligible, indicating that this single column step provided a significant step in the purification of CAPP.

Next, the activation by ceramide of rat brain CAPP off the phenyl column was evaluated. C_2-ceramide activated the phosphatase in a dose-dependent manner with approximately 1.6-fold stimulation at 3 μM and 2.1-fold at 10 μM (Figure 2). On the other hand, dihydro-C_2-ceramide had almost no effect, consistent with previous studies on the specificity of activation of CAPP by ceramide (Figure 2).

Purification of CAPP on Mono-Q Chromatography. Next, CAPP was pooled from the two peaks of activity obtained from the phenyl Sepharose column and then subjected to ion-exchange chromatography on a Mono-Q column. As can be seen from Figure 3, CAPP activity eluted as two closely separated peaks, which eluted at 300 mM NaCl for peak I, and 320 mM NaCl for peak II, respectively. The protein profile showed little elution of proteins with the phosphatases (Figure 3), thus affording another high-resolution purification step.

Gel Filtration Chromatography of CAPP on Sephacryl S-200. The resolution of rat brain CAPP on Mono-Q reproducibly resulted in two peaks, suggesting the existence of more than one form of the enzyme, thus necessitating further investigation. Therefore, peaks I and II of the Mono-Q-purified CAPP fractions were independently subjected to gel filtration chromatography. Figure 4 illustrates a typical gel filtration chromatographic profile obtained on a Sephacryl S-200 column. The estimated molecular mass of the two fractions was 150 kDa and 100 kDa, respectively.

The estimated size of CAPP further corroborated the relationship of CAPP to PP2A as determined by previous pharmacological characterization. To compare the elution profiles of peaks I and II with those of PP2A, purified cardiac PP2A in different subunit composition (AB′C, ABαC, AC, and AB′C + AC) was subjected to S-200 gel filtration

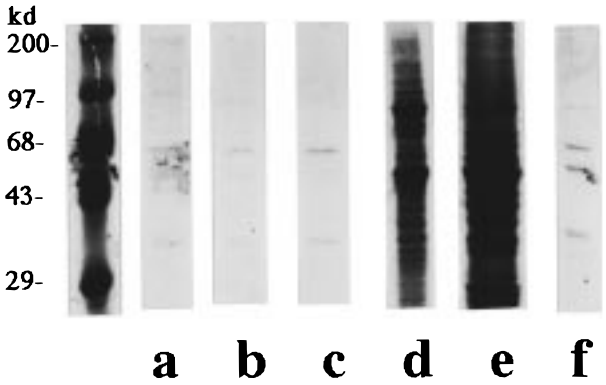

FIGURE 6: SDS−PAGE analysis of peaks I and II from Mono-Q column. Samples (boiled in electrophoresis sample buffer) were analyzed by SDS−polyacrylamide gel electrophoresis (SDS−Page). The gel was stained by the ISS Pro-Blue staining system, and the gel was then dried. First lane, protein markers; lane a, peak I from Mono-Q; lanes b and c, peak II from Mono-Q; lane d, fractions 20−40 (containing CAPP) from phenyl Sepharose; lane e, cytosol; lane f, purified AB′C.

chromatography. As can be seen from Figure 5, peak I eluted with a profile similar to that of AB′C whereas the elution of peak II resembled that of AC, suggesting that CAPP may be composed of both heterotrimeric and heterodimeric subunits.

SDS−PAGE of CAPP Fractions. To determine the relative purity of the CAPP fractions from the phenyl and the Mono-Q columns, equal CAPP activity from the two peaks was subjected to SDS−PAGE and protein bands were observed by using the sensitive ISS Pro-Blue staining system. From Figure 6, it can be observed that after the phenyl column, a few protein bands were still visible. However, following Mono-Q chromatography, an almost homogeneous preparation was obtained. As can be seen, three prominent bands were resolved with a molecular mass of about 68, 55, and 35 kDa, respectively. This estimated size is identical to that of the subunits of PP2A (AB′C and ABαC) which are also shown in Figure 6 for comparison.

Identification of CAPP by Western Blot Analysis. To identify and, hence, assign the major CAPP activity to a particular type of PP2A protein phosphatase, we carried out immunological characterization on the purified preparations. CAPP, purified by phenyl and Mono-Q columns, was blotted onto nitrocellulose, and the different blots were subjected to antibodies against the A, B′, Bα, Bβ, Bγ, and C subunits of PP2A as well as the C subunit of PP1 (Figure 7). The flow-through from the phenyl Sepharose column contained PP1 and PP2A catalytic subunits (data not shown). All fractions containing CAPP also contained the catalytic (C) and the structural (A) subunits of PP2A (Figure 7A). Peak I contained an equimolar amount of the regulatory (B′ and Bα) subunits (see Figure 6). However, peak II of the Mono-Q preparation contained the Bα regulatory subunit (Figure 7), which was present in sub-stoichiometric amounts as seen by the faint staining with Commassie (Figure 6). Neither peak contained PP1 reactivity (Figure 7B) or reactivity to antisera raised against Bβ or Bγ subunits (data not shown). Therefore, the Mono-Q peak I of CAPP consists of AB′C with some ABαC, whereas the Mono-Q peak II consists of AC and a minor component of ABαC.

These studies demonstrated that CAPP is composed predominantly of heterotrimeric PP2A, but they also raised

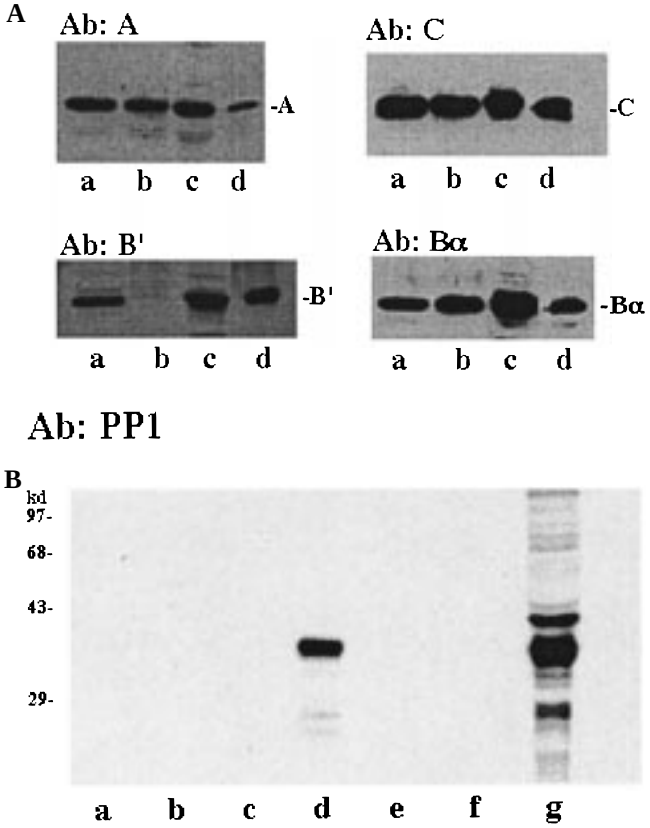

FIGURE 7: Western blot analysis of CAPP. For immunoblot analysis following SDS−PAGE, proteins were electrophoretically transferred to a nitrocellulose membrane. The filter was blocked in 5% dried milk in PBST (0.1% Tween 20 in 1×PBS), incubated in rabbit antiserum against PP2A subunits (1:1000−5000 dilution), followed by horseradish peroxidase-labeled anti-rabbit IgG (1:5000 dilution), washed three times with 1×PBS, and then developed using the enhanced chemiluminescence(ECL) detection system (Amersham Corp.). (A) Analysis of PP2A components. Lane a, peak I (Mono-Q); lane b, peak II (MonoQ); lane c, cytosol; lane d, purified AB′C or ABαC as shown on figure. The antibody used for each panel is indicated at the top. (B) Analysis of PP1. Fractions were immunoblotted with antisera directed against the catalytic subunit of PP1. Lane a, ABαC of PP2A; lane b, AB′C; lane c, C catalytic subunit of PP2A; lane d, recombinant Cα subunit of PP1; lane e, peak II of CAPP from Mono-Q; lane f, peak I of CAPP from Mono-Q; lane g, cytosolic fraction from rat brain.

the possibility that CAPP may also be composed of dimeric PP2A (AC). Therefore, additional studies were conducted in vitro to study the activation of purified AC by ceramide. As can be seen from Figure 8, C_2-ceramide activated AC approximately 2.5-fold at 30 μM; however, it appeared that C_2-ceramide was somewhat less effective in activation of AC since it showed little activation at 10 μM, a concentration that produces reproducible and significant activation of trimeric PP2A. As with AB′C, the effects of C_2-ceramide on AC were specific in that dihydro-C_2-ceramide had no effects (Figure 8).

DISCUSSION

In the past, the lack of separation of CAPP from other protein phosphatases made biochemical characterization very difficult. In this study we describe a rapid and easy method for the purification of CAPP using primarily two chromatography steps. This method has allowed us to identify the major CAPP in these fractions as heterotrimeric and het-

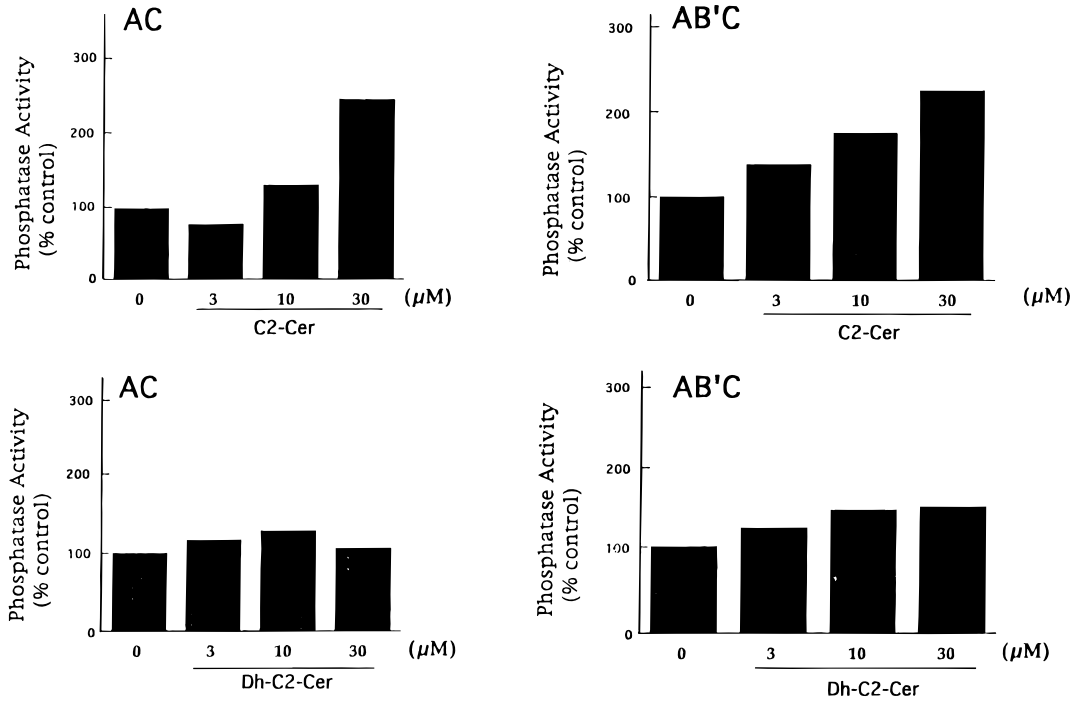

FIGURE 8: Effect of C_2-ceramide and dihydro-C_2-ceramide on AC and AB′C of PP2A. Purified dimeric (AC) or heterotrimeric (AB′C) of PP2A (purified from bovine cardiac tissue) was assayed for phosphatase activity under the standard assay in the absence or presence of the indicated concentrations of C_2-ceramide. Phosphatase activity is presented as the activity relative to control in the absence of C_2-ceramide.

erodimeric forms of PP2A as evidenced by chromatographic and immunologic criteria.

The employment of a phenyl Sepharose column as a first step in the separation of CAPP from other protein phosphatases leads to significant purification of CAPP as evidenced by the protein elution profile. This first step has for the first time led to the separation of the major CAPP activity peak from other protein phosphatases which elute much earlier. The CAPP activity off of the phenyl Sepharose column is activated dose-dependently by ceramide and in a specific manner, since dihydroceramide was unable to activate the phosphatase.

It is noteworthy to mention that when the phenyl Sepharose-resolved CAPP peak was subjected to ion-exchange chromatography by using the Mono-Q column, two peaks were produced, both of which showed CAPP activity. Moreover, gel filtration chromatography with Sephacryl S-200 of the Mono-Q peaks showed that the two peaks elute at 150 and 100 kDa, respectively, and consistent with immunologic studies showing that they are primarily composed of heterotrimeric and heterodimeric PP2A.

Previous pharmacological studies have shown that CAPP might belong to the PP2A family of protein phosphatases since (1) it was sensitive to inhibition by okadaic acid at low concentrations (*7*, *44*) and (2) ceramide was shown to activate purified heterotrimeric PP2A (*9*). In a recent study, Law and Rossie showed that ceramide stimulated the dimeric and the catalytic form of PP2A in addition to the trimeric form (*24*). Interestingly, in that study, ceramide activated AC and C only after they were resolved from partially purified ABC. In our hands, the activation of AC was lost after even short storage of the enzyme (<4 h), whereas activation of AB′C or ABαC was stable to storage. In addition, we have found on occasion very transient and moderate stimulation of the C subunit by ceramide. Taken together, these results raise the possibility that ceramide is capable of directly activating AC and C, and that perhaps a specific conformation is required which is stabilized by the B subunit. Studies in yeast support a role for the CDC55 B subunit in activation of CAPP by ceramide. Obviously, additional studies are required to investigate the precise mechanisms of activation of CAPP by ceramide.

Evidence is accumulating to indicate that some, but not all, of the effects of ceramide on cells require activation of CAPP. In *S. cerevisiae*, CAPP is required for ceramide-induced growth suppression based on studies with deletions of Sit4, Tpd3, and CDC55 (*32*). In mammalian cells, okadaic acid potently inhibits the effects of ceramide on c-myc down regulation and PKCα (*46*), but not on dephosphorylation of the retinoblastoma gene product (Rb) (*4*, *6*) or on phospholipase D (*1*, *43*). Therefore defining the biochemical composition of CAPP should allow investigation of its mechanism of regulation and specific substrates.

REFERENCES

1. Abousalham, A., Liossis, C., O'Brien, L., and Brindley, D. N. (1997) *J. Biol. Chem. 272*, 1069.
2. Ballou, L. R., Laulederkind, S. J. F., Rosloniec, E. F., and Raghow, R. (1996) *Biochim. Biophys. Acta 1301*, 273.
3. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
4. Chao, R., Khan, W., and Hannun, Y. A. (1992) *J. Biol. Chem. 267*, 23459.
5. Cohen, P., and Cohen, P. T. W. (1989) *J. Biol. Chem. 264*, 21435.
6. Dbaibo, G., Pushkareva, M. Y., Jayadev, S., Schwartz, J. K., Horowitz, J. M., Obeid, L. M., and Hannun, Y. A. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1347.
7. Dobrowsky, R. T., and Hannun, Y. A. (1992) *J. Biol. Chem. 267*, 5048.
8. Dobrowsky, R. T., and Hannun, Y. A. (1993) *Adv. Lipid Res. 25*, 91.
9. Dobrowsky, R. T., Kamibayashi, C., Mumby, M. C., and Hannun, Y. A. (1993) *J. Biol. Chem. 268*, 15523.

10. Fishbein, J. D., Dobrowsky, R. T., Bielawska, A., Garrett, S., and Hannun, Y. A. (1993) *J. Biol. Chem. 268*, 9255.
11. Gomez-Muñoz, A., Martin, A., O'Brien, L., and Brindley, D. N. (1994) *J. Biol. Chem. 269*, 8937.
12. Hannun, Y. A. (1996) *Science 274*, 1855.
13. Hannun, Y. A., and Linardic, C. M. (1993) *Biochim. Biophys. Acta 1154*, 223.
14. Hannun, Y. A., and Obeid, L. M. (1997) *Biochem. Soc. Trans. 25*, 1171.
15. Hendrix, P., Mayer-Jaeke, R. E., Cron, P., Hofsteenge, J., Merlevede, W., and Hemmings, B. A. (1993) *J. Biol. Chem. 268*, 15267.
16. Jarvis, W. D., Kolesnick, R. N., Fornari, F. A., Traylor, R. S., Gewirtz, D. A., and Grant, S. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 73.
17. Jayadev, S., Liu, B., Bielawska, A. E., Lee, J. Y., Nazaire, F., Pushkareva, M. Y., Obeid, L. M., and Hannun, Y. A. (1995) *J. Biol. Chem. 270*, 2047.
18. Jirousek, M. R., Gillig, J. R., Gonzalez, C. M., Heath, W. F., McDonald, J. H., III, Neel, D. A., Rito, C. J., Singh, U., Stramm, L. E., Melikian-Badalian, A., Baevsky, M., Ballas, L. M., Hall, S. E., Winneroski, L. L., and Faul, M. M. (1996) *J. Med. Chem. 39*, 2664.
19. Kamibayashi, C., Lickteig, R. L., Estes, R., Walter, G., and Mumby, M. C. (1992) *J. Biol. Chem. 267*, 21864.
20. Kamibayashi, C., Estes, R., Lickteig, R. L., Yang, S. I., Craft, C., and Mumby, M. C. (1994) *J. Biol. Chem. 269*, 20139.
21. Kiss, Z., and Deli, E. (1995) *FEBS Lett. 365*, 146.
22. Kolesnick, R., and Golde, D. W. (1994) *Cell 77*, 325.
23. Kowluru, A., and Metz, S. A. (1997) *FEBS Lett. 418*, 179.
24. Law, B., and Rossie, S. (1995) *J. Biol. Chem. 270*, 12808.
25. Lee, J. Y., Hannun, Y. A., and Obeid, L. M. (1996) *J. Biol. Chem. 271*, 13169.
26. Lozano, J., Berra, E., Municio, M. M., Diaz-Meco, M. T., Dominguez, I., Sanz, L., and Moscat, J. (1994) *J. Biol. Chem. 269*, 19200.
27. Merrill, A. H., Jr. (1991) *J. Bioenerg. Biomembr. 23*, 83.
28. Metz, R. J., Vellody, K., Patel, S., Bergstrom, R., Meisinger, J., Jackson, J., Wright, M. A., and Young, M. R. I. (1997) *Invasion Metastasis 16*, 280.
29. Michaud, N. R., Therrien, M., Cacace, A., Edsall, L. C., Spiegel, S., Rubin, G. M., and Morrison, D. K. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 12792.
30. Mumby, M. C., and Walter, G. (1991) *Cell Regul. 2*, 589.
31. Nakamura, T., Abe, A., Balazovich, K. J., Wu, D., Suchard, S. J., Boxer, L. A., and Shayman, J. A. (1994) *J. Biol. Chem. 269*, 18384.
32. Nickels, J. T., and Broach, J. R. (1996) *Genes Dev. 10*, 382.
33. Obeid, L. M., Linardic, C. M., Karolak, L. A., and Hannun, Y. A. (1993) *Science 259*, 1769.
34. Obeid, L. M., and Hannun, Y. A. (1995) *J. Cell. Biochem. 58*, 191.
35. Prinetti, A., Bassi, R., Riboni, L., and Tettamanti, G. (1997) *FEBS Lett. 414*, 475.
36. Quintans, J., Kilkus, J., McShan, C. L., Gottschalk, A. R., and Dawson, G. (1994) *Biochem. Biophys. Res. Commun. 202*, 710.
37. Reyes, J. G., Robayna, I. G., Delgado, P. S., González, I. H., Aguiar, J. Q., Rosas, F. E., Fanjul, L. F., and De Galarreta, C. M. R. (1996) *J. Biol. Chem. 271*, 21375.
38. Smyth, M. J., Perry, D. K., Zhang, J., Poirier, G. G., Hannun, Y. A., and Obeid, L. M. (1996) *Biochem. J. 316*, 25.
39. Spiegel, S., and Merrill, A. H., Jr. (1996) *FASEB J. 10*, 1388.
40. Tepper, A. D., Boesen-de Cock, J. G. R., De Vries, E., Borst, J., and Van Blitterswijk, W. J. (1997) *J. Biol. Chem. 272*, 24308.
41. Tepper, C. G., Jayadev, S., Liu, B., Bielawska, A., Wolff, R. A., Yonehara, S., Hannun, Y. A., and Seldin, M. F. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 8443.
42. Venable, M. E., Lee, J. Y., Smyth, M. J., Bielawska, A., and Obeid, L. M. (1995) *J. Biol. Chem. 270*, 30701.
43. Venable, M. E., Bielawska, A., and Obeid, L. M. (1996) *J. Biol. Chem. 271*, 24800.
44. Wolff, R. A., Dobrowsky, R. T., Bielawska, A., Obeid, L. M., and Hannun, Y. A. (1994) *J. Biol. Chem. 269*, 19605.
45. Wong, K., Li, X.-B., and Hunchuk, N. (1995) *J. Biol. Chem. 270*, 3056.
46. Wright, S. C., Wei, Q. S., Kinder, D. H., and Larrick, J. W. (1996) *J. Exp. Med. 183*, 463.
47. Zhang, Y. H., Yao, B., Delikat, S., Bayoumy, S., Lin, X. H., Basu, S., McGinley, M., Chan-Hui, P. Y., Lichenstein, H., and Kolesnick, R. (1997) *Cell 89*, 63.

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