Dissociation of Cyclic Inositol Phosphohydrolase Activity from Annexin III*

(Received for publication, August 25, 1995, and in revised form, January 29, 1996)

M. Chandra Sekar†, Vijaya Sambandam, William E. Grizzle, and Jay M. McDonald
From the Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Cyclic inositol phosphohydrolase is a phosphodiesterase that cleaves the cyclic bond of cyclic inositol monophosphate. In 1990, Ross et al. (Ross, T. S., Tait, J. F., and Majerus, P. W. (1990) Science 248, 605–607) purified this enzyme from human placenta and reported that cyclic inositol phosphohydrolase is identical to annexin III. Independent confirmation of this finding has not been provided. The relative distribution of annexin III and cyclic inositol phosphohydrolase activity in rat kidney and spleen indicated that annexin III can be dissociated from cyclic inositol phosphohydrolase activity. Rat spleen contains large quantities of annexin III, but has very little cyclic inositol phosphohydrolase activity. In contrast, rat kidney, one of the richest sources of cyclic inositol phosphohydrolase activity, possesses very little (immunohistochemistry) or no (Western blot) annexin III. Similar to cytosol of human placenta, cytosol of guinea pig kidney contains both annexin III and cyclic inositol phosphohydrolase. On SDS-gel electrophoresis, guinea pig kidney annexin III has a slightly different mobility than the human placental annexin III. Human placental annexin III co-migrates with cyclic inositol phosphohydrolase on ion exchange chromatography, while guinea pig kidney annexin III is clearly dissociated from cyclic inositol phosphohydrolase on ion exchange chromatography. Both guinea pig kidney annexin III and human placental annexin III pellet with the addition of calcium and centrifugation, while cyclic inositol phosphohydrolase activity in both of these tissues remains in the supernatant. Our studies clearly show that cyclic inositol phosphohydrolase and annexin III are two different proteins.

Phospholipase C hydrolysis of phosphoinositide results in the generation of both cyclic and noncyclic inositol phosphates (1). Cyclic inositol mono- and polyphosphate esters have been observed in intact tissue, and their accumulation in response to agonist activation has been described in mouse pancreas (2), kidney (3, 4), platelets (5–7), parotid gland (8), and SV40 trans- 

agonist activation has been described in mouse pancreas (2), observed in intact tissue, and their accumulation in response to (1). Cyclic inositol mono- and polyphosphate esters have been the generation of both cyclic and noncyclic inositol phosphates (15). Phospholipase C hydrolysis of phosphoinositide results in the generation of both cyclic and noncyclic inositol phosphates (1).

Cyclic inositol phosphohydrolase is a phosphodiesterase, and is first converted to cyclic inositol monophosphate by stepwise dephosphorylation, before the cyclic bond is cleaved by cyclic inositol phosphohydrolase (dPH). Kidney has the highest activity of dPH (10, 11). More recently, Ross and Majerus (12) have purified and characterized dPH from human placenta and later identified the placental dPH as identical with lipocortin III (13). They have further reported increased dPH activity following transfection of lipocortin III (14) and reported identification of another phosphodiesterase that converts dPH to inositol 2-phosphate (15).

Lipocortin III has also been referred to as placental anticoagulant protein III (16), calcimedin 35-kDa (17), and annexin III (18, 19). In accordance with recent agreement among 41 researchers working in this field (19), this protein will be referred to as annexin III. Annexin III is one of the 12 members of the annexin family of proteins that have been identified so far. Proteins belonging to this family have the following two characteristics: (a) Conserved 70-amino acid domain repeated either four or eight times in the overall structure and (b) ability to bind to phospholipids in a calcium-dependent manner. Annexins have been implicated in various cellular functions such as exocytosis (20), formation (21, 22) or modulation of ion channels (23), and membrane attachment of cytoskeletal elements (24–26).

During the course of investigating cyclic inositol phosphate metabolism, we reinvestigated the putative identity of dPH as annexin III (13). Evidence provided in this paper, when taken in conjunction with data already reported in the literature, clearly support our conclusion that annexin III and dPH are two different proteins.

MATERIALS AND METHODS

Inositol dehydrogenase, bathophenanthroline, inositol, cyclic inositol monophosphate, and NAD+ were obtained from Sigma; phenazine was from Aldrich; ferric chloride was from Mallinckrodt; rabbit polyclonal antibody to rat spleen annexin III was a gift from Dr. J ohn Dedman, University of Cincinnati, Cincinnati, OH; and rabbit polyclonal antibody raised against human neutrophil annexin III was a gift from Dr. Joel Ernst, University of California at San Francisco. All other reagents and chemicals used were of analytical grade. Kidney and spleen were obtained from Sprague-Dawley male rats (100–150 g), guinea pig tissues were obtained from male albino (350–450 g), and human placenta following normal delivery.

Cyclic Inositol Phosphohydrolase Assay— Two cyclic inositol phosphohydrolase assays were performed: one using a radioactive substrate and the other a nonradioactive dPH substrate. Principles underlying the methods are similar to that described in Ref. 12, with some modifications. Tissue was homogenized in 10 volumes of 20 mM Tris, 20 mM MES, pH 7.8, containing 1 mM EDTA, 10 mM benzamidine, 3 mM sodium azide, 1 mg/liter aprotinin, and 0.25 mM diisopropyl fluorophosphate. Protein (20–100 μg) was incubated with dP and alkaline phosphatase (120 milliunits) in 20 mM Tris, 10 mM MgCl2 (pH 8.3) buffer in a Microfuge tube (total incubation volume 290 μl). dPH concentration in radioactive and nonradioactive assays were 55 μM and 135 μM, respectively. Incubation was terminated by the addition of 0.8 ml of Dowex-formate resin (radioactive assay) or 0.5 ml of 50% w/v Dowex-formate resin (nonradioactive assay). Tubes were centrifuged for 5 min at
Cyclic Inositol Phosphohydrolase

14,000 rpm in an Eppendorf centrifuge. Inositol present in the supernatant was determined either by counting radioactivity in the supernatant following addition of scintillation fluid or by the colorimetric method described below.

Inositol Determination—Inositol level in the supernatant was determined colorimetrically by a slight modification of the method described by Dolhofer and Wieland (27). Assay was carried out in a 96-well plate, and absorbances were read at 550 nm by Ceres 900 plate reader. Color reagent was freshly prepared by mixing 2:1:1 by volume of NAD+ (16.5 mg/ml in 1 N potassium phosphate buffer, pH 9.0), bathophenanthroline (9.8 mg/ml in ferric chloride 4 mM), and phenazine methosulfate (0.019 mg/ml in water). Inositol dehydrogenase was prepared by dissolving 20 units in 20 µl of 20 mM potassium phosphate buffer (pH 7.0) and aliquoted into 2-µl fractions and stored at −20°C. Standard (50 µl containing 0–3 nmol) or unknown inositol samples were added to wells of the 96-well plate, followed by water to give a total volume of 150 µl. After addition of 50 µl of color agent, reaction was started by the addition of inositol dehydrogenase (0.005 unit in 10 µl). Assay was carried out at room temperature and absorbance was read at 550 nm after 18 h.

SDS-Gel Electrophoresis and Immunoblotting—Protein samples were separated under reducing conditions by SDS-PAGE. Laemmli buffer (5×) was added to the protein sample and immersed into boiling water bath for 5 min. Proteins were separated on a 10% SDS-PAGE polyacrylamide gel with a 6% stacking gel, under constant current conditions. Proteins were transferred to Immobilon-P membranes. Immunodetection was performed with either polyclonal rabbit antibody to rat annexin III (1:1000 dilution) or polyclonal antibody to human annexin III at (1:5000 dilution). A nonimmune serum was used as control at appropriate dilution. Binding was detected by Bio-Rad alkaline phosphatase kit.

Immunohistochemistry—Immunohistochemical staining was done by methods similar to that described previously (28). Briefly, immunohistochemistry was performed on 4-µm thick paraffin sections of rat kidney and spleen. Sections were incubated with either 1% rabbit serum or annexin III antibody (1:400 dilution) for 30 min at 37°C in a humidity chamber. Following removal of the excess antibody by washing, antibody was localized using an anti-rabbit ABC peroxidase kit from Dako, Catalog No. 685.

Calcium Precipitation of Annexin III—Addition of calcium has been shown to precipitate phospholipid-binding proteins in several different tissues and cells (29) and has been used in the purification of annexins (30, 31). As annexin III precipitation has not been reported under similar conditions, therefore, we investigated the effect of calcium on the precipitability of annexin III and cIPH. To 250 ml of the cytosolic extract of guinea pig kidney and human placenta, calcium (3 mM final concentration) was added and stirred at 4°C for 30 min. The mixture was centrifuged at 100,000 × g for 15 min, and the pellet was resuspended in 5 ml of cytosolic buffer. cIPH activity and annexin III by Western blot were determined in cytosol, supernatant, and the pellet.

RESULTS

Distribution of Annexin III and cIPH—Western blot with annexin III antibody to rat spleen shows a strong signal with rat spleen but no detectable band is observed in rat kidney and medulla (Fig. 1A). This is in complete contrast to the cPH activity in the same tissues (Fig. 1B). The specific activity of cPH in rat spleen was 1 nmol/mg of protein/min, and that in rat kidney medulla and cortex was 10- and 21-fold higher than that of spleen. Immunohistochemistry of annexin III with rat spleen antibody shows intense staining in rat spleen all across the red pulp (marked by arrows, Fig. 2, A and B). The red pulp is that area of the spleen that is composed of a network of stromal cords and vascular sinusoids lined with endothelial cells and containing numerous macrophages. In contrast in rat kidney, there is a very weak staining in the Bowman’s capsule of glomerulus (Fig. 2C, marked by arrows) and in the collecting duct region of renal medulla (Fig. 2D, marked by arrows).

Antibody raised against human neutrophil annexin III recognizes a specific band on the Western blot of both guinea pig spleen and kidney as well as in human placenta and neutrophil (Fig. 3A). Presence of annexin III on the Western blot is consistent with the earlier report indicating that 1% of the cytosolic protein in neutrophil is annexin III (32). Interestingly, while significant cPH activity is present in both guinea pig kidney and human placenta, no cPH activity is detected in human neutrophil (Fig. 3B). This observation further dissociates cPH activity from annexin III.

Annexin III from both guinea pig kidney and spleen, on SDS-gel electrophoresis consistently (five different experiments) migrate slightly slower than the annexin III obtained from human neutrophil and placenta (Fig. 3A). Interestingly, a slower migrating form of annexin III has also been observed in human monocytes (33). Guinea pig annexin III appears to be immunologically distinct from rat annexin III, as rat spleen annexin III antibody fails to recognize annexin III in either guinea pig spleen or kidney (data not shown).

Ion Exchange Chromatography of Placental Cytosol and Guinea Pig Kidney Cytosol—The first step in the purification of cPH by Ross et al. (13) employed separation on an HPLC anion exchange column. We injected 10 mg of crude cytosolic protein from human placenta (Fig. 4) and guinea pig kidney (Fig. 5) on perceptive anion exchange column. Fractions adjacent to the cPH activity fractions were probed on Western blot with annexin III antibody. Under these experimental conditions, placental annexin III and cPH activity appeared in the same fractions (Fig. 4), whereas in guinea pig kidney, fractions containing cPH activity did not show any annexin III binding on the Western blot (Fig. 5). This indicates that placental annexin III has ion exchange chromatographic characteristics similar to that of cPH, and, therefore, a different approach is required to dissociate these two proteins.

Calcium Precipitation of Annexin III—To further test...
whether annexin III and cIPH were identical in human placentas, we employed calcium precipitation which precipitates phospholipid-binding proteins and has been used in the purification of annexin I (30). Both guinea pig kidney cytosol and human placental cytosol contains annexin III (Fig. 6A, S) and cIPH (Fig. 6B, S). When 3 mM calcium was added to the cytosol and mixed at 4 °C for 30 min, followed by centrifugation at 100,000 × g, annexin III appears almost entirely in the pellet in both guinea pig kidney and human placenta (Fig. 6A, CaP), while cIPH activity remains in the supernatant (Fig. 6B, CaS). The annexin III-enriched pellet has no cIPH activity in both guinea pig kidney and human placenta (Fig. 6B, CaP). In contrast, cIPH-enriched supernatants have little or no annexin III by immunoblot (Fig. 6A, CaS).

**DISCUSSION**

Ross and Majerus (12) in 1986 purified cIPH from human placenta and reported in 1990 that cIPH is identical with...
observations (10, 11), renal homogenate possessed significant cPH activity (Fig. 1B), but to our surprise failed to bind rat annexin III antibody on a Western blot (Fig. 1A). The same antibody clearly recognized annexin III from rat spleen (Fig. 1A). Rat spleen, which is rich in annexin III and gives a strong positive signal both on Western blot and immunohistochemical staining with annexin III antibody, has 20-fold lower cPH activity compared to the kidney. The above results were fully consistent with what was reported in the literature. In an earlier study (17) (where annexin III has been referred to as calcimedin Ss–ss), a significant amount of annexin III could be detected on a Western blot of rat spleen, but none could be detected on the Western blot of rat kidney. The above discrepancy in the relative distribution of cPH and annexin III, as well as the fact that none of the groups working with annexin III have independently confirmed the cPH activity of annexin III, raised the possibility that cPH may have been misidentified as annexin III.

A closer look at the original (12, 13) purification scheme suggests that the first ion exchange chromatography step may have had poor resolution capacity. In the study of Ross and Majerus (12), 816 mg of human placental cytosol protein was loaded on a Bio-Gel TSK DEAE 5 PW HPLC anion exchange column. Even though it is a preparative column, the total load was several fold higher than the maximum column capacity for that column. Direct loading of such a large amount of the crude preparation, without preliminary purification, on an HPLC will likely lead to poor resolution. When we loaded 10 mg of crude cytosolic placental protein (the maximum capacity for this column) onto a perceptive ion exchange column, to determine whether it is able to resolve annexin III and cPH, human placental annexin III and cPH appeared in the same fractions (Fig. 4). Interestingly, under identical conditions, in guinea pig kidney, annexin III cannot be detected in Western blot in fractions demonstrating cPH activity (Fig. 5).

In their original report, Ross et al. (13) identified the cPH activity as annexin III based on a single Western blot following final purification, with polyclonal antibody generated against a 12-amino acid amino-terminal peptide of annexin III. One of the well established properties of annexins is that they precipitate with calcium. When guinea pig kidney cytosol and human placental cytosol were treated with calcium, cPH activity remained in the supernatant, while annexin III is seen in the pellet (Fig. 6). But the pellet containing annexin III had no cPH activity. We suggest that misidentification of cPH as annexin III resulted because human placenta contains both cPH and annexin III in cytosol, which comigrated during purification (not unusual), and the purification procedure did not employ any specific step to deplete annexin III.

We have no logical explanation for the small cPH activity (0.02 nmol/min/mg of protein) reported with purified annexin III (13) or the small amount of activity detected following transfection of Swiss 3T3 cells with the cDNA of annexin III (14). As the assay conditions used in the transfections studies (14) were reported to be similar to their original paper (12), we can make the following calculations from their work. The total amount of substrate, based on a final substrate concentration of 75 μM and the incubation volume of 25 μl, was 1.87 nmol of cIP. In the only time course reported (14), the annexin III transfected cells were reported to generate 30 nmol of inositol monophosphate per mg of protein, over the entire incubation period of 120 min. If we assume they used 0.3 mg of protein (based on Fig. 1 of Ref. 15), this amounts to 9 nmol of cP being hydrolyzed during the 120-min incubation, which is 5-fold higher than the total cP present at the beginning of the incubation. These calculations make it highly unlikely that they
were measuring true cIPH activity.

Neutrophil provides an interesting test case. If annexin III indeed possessed dPH activity, one would expect a very high level of dPH activity in neutrophils, as 1% of the neutrophil cytosolic protein is annexin III (32). In a single observation, Ross and Majerus (34) reported that neutrophils have a dPH activity of 12,000 pmol/min/mg of protein, which would be consistent with the above hypothesis. However, the authors do not describe the exact conditions used for this single point measurement. If we assume that incubation conditions were similar to those described earlier (10-min incubation in the presence of 0.3 mg of protein), it would result in consumption of 36,000 pmol of substrate during the course of this incubation, 19-fold higher substrate than what was actually provided at the beginning of the assay.

In our hands, three different experiments with neutrophils (Fig. 2) as well as time course studies (data not shown) detected no dPH activity, which is consistent with our finding that annexin III and dPH are two different proteins. Further support for such a conclusion is obtained from the work of Riddle et al. (1985) where overexpression of the gene coding annexin III in Escherichia coli is not accompanied by enhanced dPH activity. Annexin III is much better characterized protein (36) than dPH, and this "presumed identity" between dPH and annexin III has developed progress in understanding the role of dPH and cyclic inositol phosphate metabolism. The importance of our finding is that it paves the way for purification, characterization, cloning, and determining the real sequence of dPH. Such studies should provide impetus to clarify the role of dPH in kidney and placenta as well as determine its significance in cell growth and proliferation.

Acknowledgments—We wish to acknowledge the technical assistance of Reginald Berry and Cecil Stockard.

REFERENCES

1. Wilson, D. B., Bross, T. E., Sherman, W. R., Berger, R. A., and Majerus, P. W. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4013–4017

2. Sekar, M. C., Dixon, J. F., and Hokin, L. E. (1987) J. Biol. Chem. 262, 340–344

3. Shayman, J. A., Auchus, R. J., and Morrison, A. R. (1986) Biochim. Biophys. Acta 888, 171–175

4. Wilson, K. M., and Minneman, K. P. (1990) J. Biol. Chem. 265, 17601–17606

5. Binder, H., Weber, P. C., and Sies, W. (1985) Anal. Biochem. 148, 220–227

6. Ishii, H., Connolly, T. M., Bross, T. E., and Majerus, P. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6397–6400

7. Taylor, A. P., King, W. G., and Rittenhouse, S. E. (1987) J. Biol. Chem. 262, 17268–17271

8. Dixon, J. F., and Hokin, L. E. (1987) Biochem. Biophys. Res. Commun. 149, 1208–1213

9. Koch, M. A., and Diringer, H. (1974) Biochem. Biophys. Res. Commun. 58, 361–367

10. Jungalwala, F. B., Freinkel, N., and Dawson, R. M. C. (1971) Biochem. J. 123, 19–26

11. Dawson, R. M. C., and Clarke, N. G. (1972) Biochem. J. 127, 113–118

12. Ross, T. S., and Majerus, P. W. (1986) J. Biol. Chem. 261, 11119–11123

13. Ross, T. S., Tait, J. F., and Majerus, P. W. (1990) Science 248, 605–607

14. Ross, T. S., Whitstey, B., Graham, R. A., and Majerus, P. W. (1991) J. Biol. Chem. 266, 9086–9092

15. Ross, T. S., and Majerus, P. W. (1992) J. Biol. Chem. 267, 19924–19928

16. Tait, J. F., Sakata, M., McMullen, B. A., Miao, C. H., Funakoshi, T., Hendrickson, L. E., and Fujikawa, K. (1988) Biochemistry 27, 6268–6276

17. Kaetzel, M. A., Hazariika, P., and Dedman, J. R. (1989) J. Biol. Chem. 264, 14463–14470

18. Ernst, J. D., Hoye, E., and Blackwood, R. A. (1989) Biochem. Biophys. Res. Commun. 161, 959–964

19. Crumpton, M. J., and Dedman, J. R. (1990) Nature 345, 212

20. Creutz, C. E. (1992) Science 258, 924–931

21. Pollard, H. B., and Rojas, E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2974–2978

22. Riddle, S., Pollard, H. B., Haigler, H. T., Parra, C., and Burns, A. L. (1990) J. Biol. Chem. 265, 21207–21215

23. Diaz-Munoz, M., Hamilton, S. L., Kaetzel, M. A., Hazaraika, P., and Dedman, J. R. (1990) J. Biol. Chem. 265, 15894–15899

24. Glenny, J. R., Tack, B., and Powell, M. A. (1987) J. Cell Biol. 104, 503–511

25. Gerke, V., and Weber, K. (1984) EMBO J. 3, 227–233

26. Ikebuchi, N. W., and Waisman, D. M. (1990) J. Biol. Chem. 265, 3392–3400

27. Dock, R., and Wieland, O. H. (1987) Clin. Chem. Clin. Biochem. 25, 733–736

28. Myers, R. B., Kudlow, J. E., and Grizzle, W. E. (1993) Mod. Pathol. 6, 733–737

29. Hulin, J., Raynal, P., Ragab-Thomas, J. M. F., Fauvel, J., and Chapi, H. (1989) J. Biol. Chem. 264, 3506–3513

30. Salies, J.-P., Gayral-Taminh, M., Fauvel, J., Delobbe, I., Mignon-Conite, M., Conte, J. J., and Chapi, H. (1993) J. Biol. Chem. 268, 12085–1211

31. Wirl, G., and Schwartz-Albiez, R. (1990) J. Cell. Physiol. 144, 511–522

32. Ernst, J. D., Hoye, E., Blackwood, R. A., and Jaye, D. (1990) J. Clin. Invest. 85, 1065–1071

33. Le Cabec, V., Russo-Marie, F., and Marionneau-Parini, I. (1992) Biochem. Biophys. Res. Commun. 189, 1471–1476

34. Ross, T. S., and Majerus, P. W. (1991) J. Biol. Chem. 266, 851–856

35. Majerus, P. W. (1992) Annu. Rev. Biochem. 61, 225–250

36. Pepinsky, R. B., Tizard, R., Mattaliano, R. J., Sinclair, L. K., Miller, G. T., Browning, J. L., Chow, E. P., Burne, C., Huang, K. S., Pratt, D., Wachter, L., Hession, C., Frey, A. Z., and Walner, B. P. (1988) J. Biol. Chem. 263, 10799–10811
