Biochemical Properties of Phosphatidylcholine-Binding Proteins That Share Common Antigenic Determinants with Fcγ_{2b} Receptor†

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ABSTRACT: Biochemical and immunological properties of biosynthetically radiolabeled phosphatidylcholine- (PC-) binding proteins were investigated. The PC-binding proteins were extracted from the detergent lysate of biosynthetically radiolabeled P388D$_1$ cells by affinity chromatography on PC–Sepharose and filtered through a Sephadex G-100 gel column in the presence of 6 M urea. Isoelectric focusing of the gel-filtered materials in the presence of 6 M urea revealed the presence of a major protein component of pI_e of 5.8 and minor heterogeneous cellular proteins. The yield of the electrofocused PC-binding proteins based on protein determination by Lowry's method ranged from 0.7 to 4 mg per 10^9 cells. The purified PC-binding proteins appeared to be tightly associated with Triton X-100 and phospholipids in the weight ratio of 0.57 and 0.05 g/g of proteins, respectively. The majority of lipids that could be extracted from the PC-binding proteins by chloroform/methanol (2:1 v/v) are free fatty acids, whereas lipids extracted from Pronase-treated PC-binding proteins contained phosphatidylethanolamine. By amino acid analysis, the purified PC-binding proteins were found to consist of a minimum of 417 amino acid residues, suggesting a minimum molecular weight of about 38 000 for this protein. Results of radiolabeling experiments with [^{3}H]glucosamine and amino acid analysis both showed the presence of a mole of glucosamine per a mole of the PC-binding proteins, suggesting their glycoprotein nature. About 40% of the purified PC-binding proteins coprecipitated with monoclonal anti-Fcγ_{2b}R antibody (2.4G2) in detergent-containing buffer, whereas only 6% of the isolated IgG binding proteins reacted with this antibody. About 50% of the purified PC-binding proteins are probably originated from the cell surface, since the yield of the PC-binding proteins from the lysate of 2.4G2 antibody-bound cells was about half of that obtained from the lysate to which this antibody was added.

Murine macrophages and macrophage-like cell lines such as P388D$_1$ carry on their surface at least two different types of Fcγ receptors, one specific for IgG$_{2a}$ (Fcγ_{2a}R)[1] and another for IgG$_{2b}$ (Fcγ_bR). They differ not only in IgG subclass specificity (Walker, 1976; Heusser et al., 1977; Unkeless, 1977) but also in molecular size (Anderson & Grey, 1978; Suzuki et al., 1982; Lane et al., 1980), charge properties (Suzuki et al., 1982; Lane et al., 1980), and susceptibility to trypsin at the cell surface (Heusser et al., 1977; Unkeless, 1977). These FcγRs should transmit, upon specific binding of an appropriate ligand, a signal that leads to the modulation of cellular functions, since the interaction of immune complexes with the cell surrface FcγRs has been shown to result in the suppression of the humoral immune response (Uhr & Möller, 1968) or B cell differentiation (Kölsch et al., 1980), to the destruction of target cells by antibody-dependent cell-mediated cytotoxicity (Perlman et al., 1972; Revillard et al., 1975), or to the activation of the arachidonic acid metabolite cascade (Bonney et al., 1979; Passwell et al., 1979, 1980; Rouzer et al., 1980). Our previous studies with P388D$_1$ cells (Suzuki et al., 1982) demonstrated that the materials isolated as phosphatidylcholine- (PC-) binding proteins from a detergent lysate bind specifically to murine IgG$_{2b}$ and exhibit phospholipase A$_2$ activity, a rate-limiting enzyme in protaglandin synthesis, whereas the materials isolated as IgG-binding proteins bind specifically to murine IgG$_{2a}$ and are devoid of phospholipase A$_2$ activity. Furthermore, the arachidonic acid metabolic cascade in intact P388D$_1$ cells was shown to be triggered by the binding of IgG$_{2b}$-immune complexes to cell surface Fcγ_{2b}R (Nitta & Suzuki, 1982). Our previous results thus suggested, when taken together, that a signal for the activation of the arachidonic acid metabolic cascade is transmitted by Fcγ_{2b}R, but not by Fcγ_{2a}R, on the surface of P388D$_1$ cells, probably through the initial generation of phospholipase A$_2$ activity associated with Fcγ_{2b}R. A question was raised, however, as to the homogeneity of the isolated PC/IgG$_{2b}$- and IgG$_{2a}$-binding proteins, since the yields of these materials exceeded the amounts estimated by the binding assay (Unkeless & Eisen, 1975; Segal & Hurwitz, 1977). We approached this problem by analyzing various cellular components and detergent potentially associated with the isolated PC-binding proteins.

Data presented in this paper will demonstrate that biosynthetically radiolabeled PC-binding proteins copurify with heterogeneous minor cellular components throughout the affinity chromatography and gel filtration steps of purification. The separation of the contaminants from the major PC-binding proteins can be achieved by isoelectric focusing in the presence of 6 M urea but not in the presence of 0.5% Triton X-100. The major component of the PC-binding proteins focuses at pH 5.8, consists of 417 amino acids, is glycosylated with an oligosaccharide chain of M_r 8000, and is tightly associated with

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[1] Abbreviations: EDTA, ethylenediaminetetraacetate; Fcγ_{2a}R and Fcγ_{2b}R, receptors for IgG$_{2a}$ and IgG$_{2b}$, respectively; IAM, iodoacetamide; PBS, phosphate-buffered saline (0.15 M, pH 7.4); PC, phosphatidylcholine and its analogue *rac*-1-(9-carboxynonyl)-2-hexadecylglycero-3-phosphocholine; PMSF, phenylmethanesulfonyl fluoride; TBS, Tris-HCl-buffered saline (0.15 M, pH 8.0); Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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phospholipids (0.05 g/g of protein). The electrofocused major PC-binding proteins react with 2.4G2 antibody, which has been shown to react with macrophage surface Fcγ_{2b}R (Unkeless, 1979).

Materials and Methods

Cells. P388D$_1$ cells were a generous gift of Dr. H. Koren of Duke University and were usually cultured in a plastic flask (Falcon 3024) (Falcon Plastics, Oxnard, CA) at 37 °C in an atmosphere containing 5% CO_2 in the culture medium consisting of RPMI 1640, fetal calf serum (10%, heat-treated at 56 °C for 30 min), penicillin (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM) (all from KC Biologicals, Lenexa, KS). Cell density was maintained at approximately 5×10^5/mL.

Biosynthetic Radiolabeling. P388D$_1$ cells [$(1.1–6.8) \times 10^8$] were biosynthetically radiolabeled with [^{14}C]Leucine (0.33 μCi/mL) (Schwarz/Mann Spring Valley, NY) for 16 h in the RPMI 1640 culture medium from which leucine was deleted. In one experiment, cells (1.8×10^8) were biosynthetically radiolabeled with [^{3}H]tyrosine and [^{3}H]leucine (10 μCi each) in tyrosine- and leucine-depleted RPMI 1640 medium. In the experiments to examine possible association of the PC-binding proteins with RNA, glucosamine, or phospholipids, cells [$(0.6–1.9) \times 10^8$] were also biosynthetically radiolabeled with [^{3}H]uridine (40 μCi/mL), [^{3}H]glucosamine (2 μCi/mL), or [^{3}H]arachidonic acid (0.25 μCi/mL) (all from Amersham, Arlington Heights, IL), respectively, for various lengths of time as stated in the text.

Preparation of Lysate. Cells that were metabolically radiolabeled were washed 3 times with cold Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (Flow Laboratory, Rockville, MD) and were lysed at 0 °C with 0.2% Triton X-100 made in Tris-HCl-buffered saline (TBS; 0.15 M, pH 8) containing 1 mM each of phenylmethanesulfonyl fluoride (PMSF), ethylenediaminetetraacetate (EDTA) and iodoacetamide (IAM) (buffer I), with or without a trace amount (9 μCi) of [^{3}H]Triton X-100 (Amersham). After the mixture was stirred for 1 h at 0 °C, lysates were centrifuged for 60 min at 4 °C at 10000 rpm in a Beckman J21 refrigerated centrifuge to remove nuclei, unlysed cells, and other debris. Clear supernatants obtained were immediately subjected to affinity chromatography.

Affinity Chromatography. PC–Sepharose 4B used to extract PC-binding proteins was prepared by coupling *rac*-1-(9-carboxynonyl)-2-hexadecylglycero-3-phosphocholine (about 600 μmol) to AH-Sepharose 4B (Pharmacia) in the presence of carbodiimide as described in detail (Suzuki et al., 1980). Heat-aggregated IgG–Sepharose 4B was prepared as described (Suzuki et al., 1980, 1982). These were packed in glass columns connected in tandem and equilibrated against buffer I. Isolation and purification of PC- and IgG-binding proteins were carried out as described (Suzuki et al., 1980). Briefly, the detergent lysates of biosynthetically radiolabeled P388D$_1$ cells [$(1.8–8) \times 10^8$] were immediately subjected to affinity chromatography over the columns of PC– and IgG–Sepharose 4B connected in tandem in this order. After being thoroughly washed with buffer I, the columns were disconnected. The materials bound to the columns were separately eluted with 6 M deionized urea made in 0.2 ionic strength, pH 8 Tris-HCl buffer containing 1 mM each of PMSF, EDTA, and IAM (buffer II). The eluted materials were separately passed through a column (4.5 × 60 cm) of Sephadex G-100 which was previously equilibrated against buffer II. About 90% of PC-binding proteins and about 85% of IgG-binding proteins were usually excluded from Sephadex G-100 gel beads. These

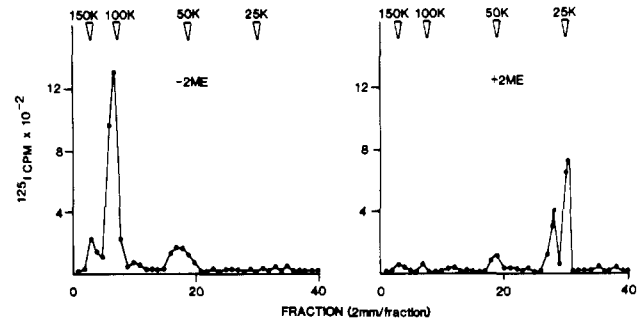

FIGURE 1: SDS–PAGE patterns of ^{125}I-labeled F(ab')$_2$ fragment of monoclonal antibody (2.4G2) under nonreducing (left) and reducing (right) conditions. F(ab')$_2$ fragment was produced by pepsin digestion of ^{125}I-labeled 2.4G2 antibody as described in the text. SDS–PAGE was carried out in 10% polyacrylamide gel as described by Laemmli (1970).

were then separately dialyzed against deionized water and subjected to isoelectric focusing in a pH gradient formed with carrier ampholyte, pH 5–10, in the presence of 6 M urea with the use of LKB ampholine columns (440 mL).

Monoclonal Antibody (2.4G2) and Its F(ab')$_2$ Fragment. Monoclonal antibody (2.4G2) was a gift of Dr. J. C. Unkeless of the Rockefeller University. It was supplied the first time as IgG purified by DEAE-cellulose chromatography and the second time as ascites fluid. They were radioiodinated with 1 mCi of ^{125}I (Amersham, Arlington Heights, IL) by a standard Chloramine-T method, dialyzed against TBS, and passed through a Sephadex G-25 column equilibrated against TBS to remove free ^{12}I. F(ab')$_2$ fragment of 2.4G2 antibody was prepared by pepsin digestion of ^{125}I-labeled 2.4G2 antibody mixed with 20 mg of normal rat IgG (Cappel Laboratory, Westchester, PA) in 0.2 M sodium acetate buffer (pH 4.5). The digests were then passed through a column (2.5 × 40 cm) of Sephadex G-150 previously equilibrated against TBS to remove undigested materials as well as small peptides. The protein peak containing F(ab')$_2$ fragments was then dialyzed against 0.02 ionic strength, pH 8 Tris-HCl buffer and was fractionated by DEAE-cellulose chromatography using 0–0.3M NaCl concentration gradient. The protein peak eluted at the salt concentration (0.09–0.12 M) was pooled and dialyzed against TBS. SDS–PAGE analysis (Laemmli, 1970) (Figure 1) of the purified materials showed a major band, in the absence of reducing reagent, with a mobility of the protein of M_r 100000 and two bands in the presence of reducing reagent with a mobility of the proteins of M_r 28000 and 25000, respectively. About 70–85% of F(ab')$_2$ fragment of 2.4G2 antibody thus prepared could be immunoprecipitated when incubated with goat anti-rat IgG (Cappel Laboratory, Westchester, PA).

Physicochemical Methods. Isoelectic focusing in the presence of 6 M urea was carried out as described (Suzuki et al., 1980, 1982). Protein concentration was measured by Lowry's method (Lowry et al., 1951). Glucosamine concentration and amino acid composition were determined by amino acid analysis using a JEOL automatic analyzer (Model 6AH) on samples which were hydrolyzed for 20 h at 110 °C in constant boiling HCl in an evacuated sealed Pyrex tube. The concentration of RNA was determined by the method of Schmidt-Thannhauser as described by Fleck & Munro (1962). The total carbohydrate contents were estimated by the Orcinol method (Williams & Chase, 1968).

Lipid Analysis. Partial delipidation was performed with chloroform/methanol (2:1 v/v) extraction as described (Suzuki et al., 1981). Phospholipid contents were estimated by analyzing inorganic phosphate contents by the method of Bartlett

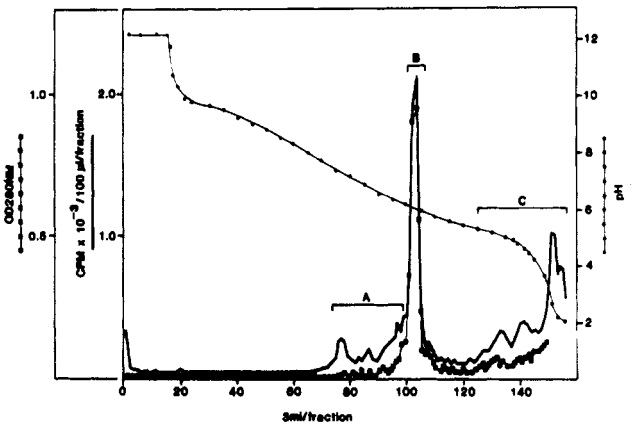

FIGURE 2: Electrofocusing pattern of the PC-binding proteins obtained from P388D$_1$ cells (1.8×10^8) which were cultured in the presence of 10 μCi each of [^{3}H]leucine and -tyrosine as described in the text. Electrofocusing was performed for 16 h at 10 °C at 6 *w* in an LKB 440-mL column in the presence of 6 M deionized urea. At the end of the run, the materials were recovered from the bottom of a column (3 mL/fraction). The radioactivity was measured by a scintillation counter with a 100-μL aliquot taken from each tube. Each fraction was also monitored by the absorbancy at 280 nm.

(1959) and also by incorporation of [^{3}H]arachidonic acid as described in the text. Qualitative analysis of the radioactive phospholipids extracted from the cell lysate or from purified PC-binding proteins was carried out by ascending thin-layer chromatography on a silica gel G coated glass plate which was previously heat activated (100 °C, 30 min) with the solvent system consisting of chloroform/methanol/acetic acid/water (25:15:4:1 v/v) as described (Suzuki et al., 1981). Components separated were identified as fatty acid, phosphatidylethanolamine, phosphatidylinositol or -serine, and phosphatidylcholine by comparison with the standards. They were scraped off from the plates and counted with a scintillation counter (Searle).

RESULTS

Heterogeneity of PC-Binding Proteins. The PC-binding proteins isolated from the detergent lysate of the surface-radioiodinated P388D$_1$ cells focus sharply at pH near 5.8 essentially as a single peak, when monitored by the radioactivity and optical density at 280 nm, during electrofocusing in the presence of 6 M urea (Suzuki et al., 1982). Although the surface radioiodination by the lactoperoxidase-catalyzed method was carried out in that study at 0 °C in the presence of cytochalasin D (1 μg/mL), the focused PC-binding proteins may still contain some cytoplasmic components which originated from nonviable cells (inevitably present) and copurifed. In order to exclude such a possibility, attempts were made to isolate PC-binding proteins from biosynthetically radiolabeled, therefore, viable P388D$_1$ cells as follows. First, 1.8×10^8 P388D$_1$ cells were cultured for 16 h in the presence of 10 μCi each of [^{3}H]leucine and -tyrosine in RPMI 1640 medium from which leucine and tyrosine were depleted. PC-binding proteins were extracted and filtered through a column of Sephadex G-100 gel in the presence of 6 M urea as described (Suzuki et al., 1982). An electrofocusing pattern of the gel-filtered materials is shown by Figure 2. On the basis of the radioactivity, about 68% of the materials subjected to electrofocusing were recovered in the zones denoted as A–C. About 28% of the recovered materials focused sharply at pH near 5.8, where the PC-binding proteins isolated from the surface-radioiodinated cells usually focus (Suzuki et al., 1982). In addition, the measurement of absorbancy at 280 nm revealed only one peak which coincides with the radioactive peak focused at pH 5.8. Thus, it appears that the heterogeneous

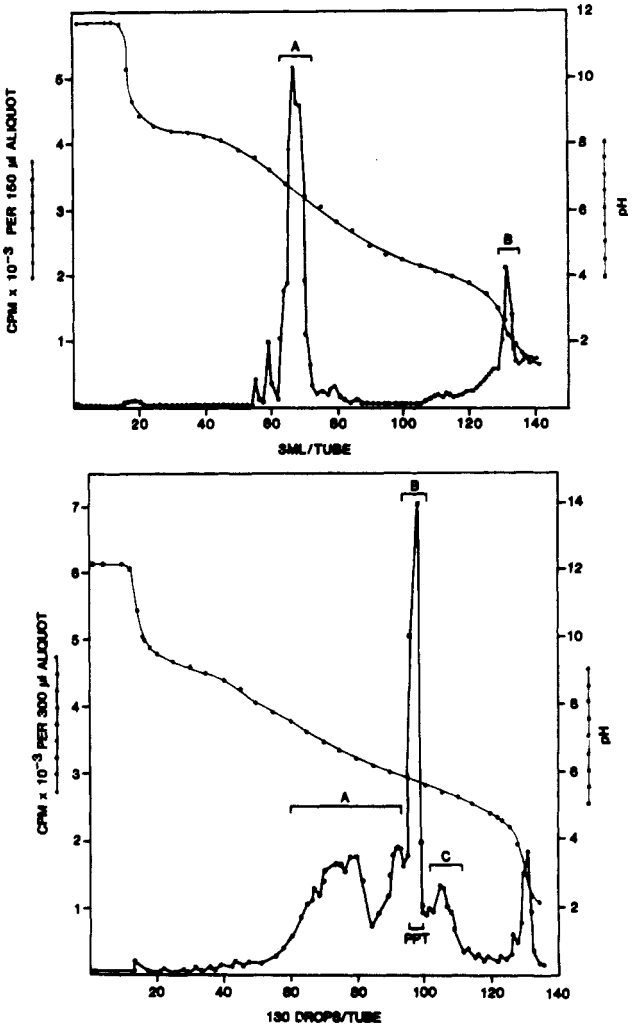

FIGURE 3: (Top) Electrofocusing pattern of the PC-binding proteins isolated from P388D$_1$ cells (0.8×10^8) which were biosynthetically radiolabeled with [^{14}C]leucine. The PC-binding proteins obtained by affinity chromatography on PC–Sepharse 4B were passed through a column (2.5 × 45 cm) of Sephadex G-100 equilibrated against 0.5% Triton X-100 made in buffer I. The gel-filtered proteins were dialyzed against 0.5% Triton X-100 and were electrofocused in the presence of 0.5% Triton X-100. (Bottom) Reelectrofocusing pattern of peak A obtained by electrofocusing of the PC-binding proteins as depicted in Figure 2A. Peak A in Figure 2A was pooled, passed through a column of Sephadex G-100 equilibrated against 6 M urea, and electrofocused in the presence of 6 M urea as in Figure 1.

materials scattered in zones A and C should constitute only a minor component which copurifies during the gel-filtration step but can be separated by electrofocusing in the presence of 6 M urea. These minor contaminants are relatively tightly associated with the PC-binding proteins, in detergent-containing solution. As seen in Figure 3 (top), the PC-binding proteins isolated from the detergent lysate of P388D$_1$ cells (0.8×10^8; biosynthetically radiolabeled with [^{14}C]leucine) focused in the presence of 0.5% Triton X-100 as a relatively broad zone between pH 6.3 and pH 6.9 (indicated as A) and as a minor acidic peak (indicated as B). When the materials designated as zone A were refocused in the presence of 6 M urea, the electrofocusing pattern shown by Figure 3 (bottom) was obtained. Thus, it appears that the purification of a major component [designated as zone B in Figure 3 (bottom)] at the isoelectric focusing step requires urea to dissociate the heterogeneous minor components by probably unfolding molecular complexes. As summarized in Table I, the yields of the PC-binding proteins after the electrofocusing step ranged from 4 to 0.7 mg per 10^9 cells in five different experiments. When

Table I: Yield of PC-Binding Proteins in Five Different Experiments[a]

experiment	I	II	III	IV	V
no. of cells	8×10^8	1.8×10^8	3×10^8	1.8×10^8	2.8×10^8
radiolabeled with	none	[^{3}H]Leu and -Tyr (10 μCi each)	[^{14}C]Leu (100 μCi)	[^{14}C]Leu (100 μCi)	[^{14}C]Leu (100 μCi)
protein, cpm, in lysate	64.7 mg	38.8 mg, 1.6×10^8	57.7 mg, 3.5×10^7	51.8 mg, 3×10^7	53.6 mg, 6.7×10^6
yield after IEF[c]	1.6 mg (2.4%)[b]	0.8 mg (2%), 2×10^5 (1.2%)	0.3 mg (0.52%), 1.6×10^5 (0.46%)	0.4 mg (0.77%), 2.6×10^5 (0.8%)	0.2 mg (0.37%), 3.5×10^4 (0.52%)
yield/10^9 cells	2 mg	4 mg	1 mg	2.2 mg	0.7 mg

[a] Protein concentration was determined by Lowry's method. [b] Number in parentheses represents percentages of the amounts of proteins in weight and radioactivity relative to those in the original lysate. [c] Isoelectric focusing.

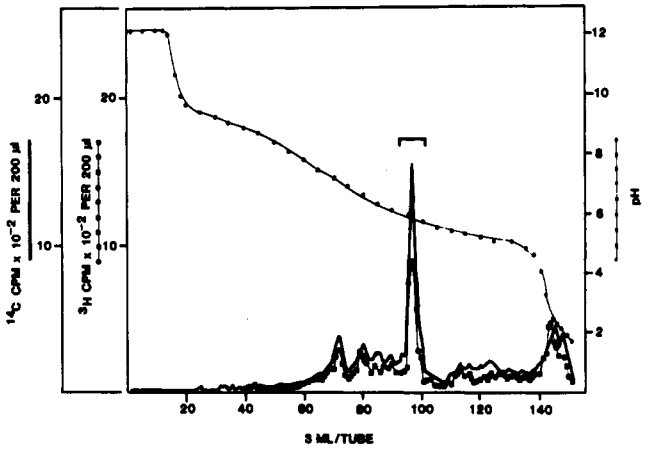

FIGURE 4: Electrofocusing pattern of the PC-binding proteins which were extracted from [^{3}H]Triton X-100 containing detergent lysate of biosynthetically radiolabeled P388D$_1$ cells as described in the text.

cells were biosynthetically radiolabeled, the yield values based on the radioactivity measurement agreed fairly well with those determined by Lowry's method.

Association of Triton X-100 with PC-Binding Proteins. Nonionic detergent such as Triton X-100 used in our studies is required to solubilize membrane proteins. Triton X-100 does not appear to induce gross denaturation of proteins, since many enzymatic activities are preserved in its presence (Soltysiak & Kaniuga, 1970; Tzagoloff & Penefsky, 1971; Suzuki et al., 1980, 1982). Also as shown above, Triton X-100 does not appear to unfold protein complexes. However, Triton X-100 has been shown to bind to hydrophobic proteins such as integral membrane proteins to varying degrees (Helenius & Simons, 1972; Makino et al., 1973). The amount of Triton X-100 that remains bound to the PC-binding proteins after the electrofocusing step was therefore estimated as follows. A total of 3×10^8 P388D$_1$ cells which include 1.1×10^8 cells previously biosynthetically radiolabeled with [^{14}C]leucine (100 μCi) were lysed in the presence of 90 mg of Triton X-100 containing a trace amount (9 μCi) of [^{3}H]Triton X-100. The amount of total protein in the lysate was 57.7 mg and the total ^{3}H and ^{14}C radioactivity were 5.2×10^6 and 3.5×10^7 cpm, respectively. The PC-binding proteins were isolated from this lysate as described (Suzuki et al., 1982). A result of the last electrofocusing step shown by Figure 4 clearly demonstrated that the PC-binding proteins (^{14}C) which focused at pH near 5.8 were also radiolabeled with [^{3}H]Triton X-100. On the basis of the radioactivity, the PC-binding proteins represented about 0.46% of the proteins in the lysate and were associated with about 0.19% of Triton X-100 present in the lysate. On the basis of Lowry's method, the yield of the PC-binding proteins in this experiment was 300 μg which represented about 0.52% of the proteins in the lysate. Thus, the approximate amount of Triton X-100 that remained bound to the purified PC-binding proteins was calculated to be 170 μg/300 μg of protein or 0.57 g of Triton X-100/g of protein.

Table II: Thin-Layer Chromatographic Analysis of Lipids Extracted from the Lysate, PC-Binding Proteins, and Pronase-Treated PC-Binding Proteins[a]

	% of ^{3}H radioactivity		
lipids	lysate	PC-binding protein	Pronase-digested PC-binding protein
origin	5.2	7.3	9.5
phosphatidylcholine	21.8	2.6	4.9
phosphatidylinositol and -serine	13.8	0	2.3
phosphatidylethanolamine	44.1	3.6	36.1
fatty acid	13.7	85.5	47.2

[a] Lipids were extracted twice with chloroform/methanol (2:1 v/v) from the Triton X-100 lysate (4 mL); the electrofocused and Pronase-treated PC-binding proteins (4 mL each) obtained from [^{3}H]arachidonate-prelabeled P388D$_1$ cells. Each extract was dried under a stream of N_2 and reconstituted in 100 μL of chloroform/methanol. An aliquot (30 μL) containing ^{3}H radioactivity of 1500 cpm from each extract was fractionated on a silica gel G-25 coated thin-layer plate with a solvent system consisting of chloroform/methanol/acetic acid/H_2O (100/60/16/1.6 v/v). Lipids were identified by comparison with the standards, scraped off, and counted. The recovery of the ^{3}H radioactivity ranged from 68% to 79%.

Association of Phospholipids with PC-Binding Proteins. Our previous studies demonstrated the association of phospholipase A_2 activity with the purified PC-binding proteins (Suzuki et al., 1982). Therefore, the PC-binding proteins may be bound with the substrates for phospholipase A_2 or their cleavage products upon isolation. It is also possible that PC-binding proteins may bind to phospholipids through hydrophobic interaction because of their integral membrane protein nature. The amount of phospholipids that remains bound to the purified PC-binding proteins was first examined by phosphorus analysis, which showed the presence of about 20 μg of phosphate/3 mg of protein (0.056 g of phospholipids/g of protein assuming the average M_r 750 for phospholipids). If all of these phosphates are part of phospholipids, such lipids must be very tightly bound to the PC-binding proteins, since the extraction with chloroform/methanol (2:1 v/v) resulted in reduction of only 14% in phosphate contents.

The association of lipids with the PC-binding proteins was further studied by radiolabeling of phospholipids of P388D$_1$ cells. To this end, a total of 1.7×10^8 cells was cultured in the presence of [^{3}H]arachidonic acid (0.2 μCi/mL) for 20 h, washed 4 times with cold phosphate-buffered saline (PBS), and cultured in the absence of [^{3}H]arachidonic acid for an additional 16 h. [^{3}H]Arachidonic acid labeled cells were then pooled with 1.1×10^8 cells which were previously biosynthetically radiolabeled with [^{14}C]leucine (0.33 μCi/mL). The cells were lysed with Triton X-100 as usual. The total protein in the lysate was estimated by Lowry's method to be 53.5 mg. The total ^{3}H and ^{14}C radioactivity was 1.9×10^7 and 0.67×10^7 cpm, respectively. Keeping with our previous results (Nitta & Suzuki, 1982), thin-layer chromatography of the chloroform/methanol (2:1 v/v) extract of the lysate showed, as summarized in Table II, that the majority of [^{3}H]arachidonic

Table III: Amino Acid Composition of the Electrofocused PC-Binding Proteins[a]

amino acid	found (μmol)	no. of residues[b]
Lys	0.023	28.8
His	0.050	6.3
Arg	0.019	23.8
Asp	0.037	46.3
Thr	0.016	20.0
Ser	0.024	30.0
Glu	0.052	65.0
Pro	0.012	15.0
Gly	0.026	32.5
Ala	0.030	37.5
$^1/_2$-Cys	0.001	1.5
Val	0.012	15.0
Met	0.007	8.8
Ile	0.015	18.8
Leu	0.033	41.3
Tyr	0.008	10.0
Phe	0.013	16.3
Glc-NH_2	0.0008	1.0
total	0.3328	416.9

[a] Results represent an average of two analysis on samples (250 μg each). [b] Number of residues was calculated based on the assumption that the number of valines is 15.

acid was incorporated into various phospholipids (44.1% in phosphatidylethanolamine, 21.8% in phosphatidylcholine, and 13.8% in the combined fraction of phosphatidylinositol and -serine). About 13.7% of the radioactivity was found as free fatty acid. The PC-binding proteins were purified from this lysate by a three-step procedure as usual. The last step of electrofocusing yielded a pattern essentially identical with that shown by Figure 4. On the basis of the radioactivity, about 0.52% of protein present in the original lysate sharply focused at pH near 5.8 and was associated with about 0.2% of [^{3}H]-arachidonic acid present in the original lysate. Since total phospholipid content in the chloroform/methanol extract of the lysate was estimated by phosphorus analysis to be 5.3 mg, and since the majority of the radioactivity in the extract was found in the phospholipid fractions, the total phospholipids associated with the electrofocused PC-binding protein were calculated to be 10 μg/200 μg of protein (0.05 g of phospholipids/g of protein). Keeping with the result of phosphorus analysis, phospholipids appear to be tightly bound to PC-binding proteins, since only 13% of the ^{3}H radioactivity associated with the PC-binding proteins could be extracted with chloroform/methanol (21: v/v). As shown by Table II, the majority (86%) of ^{3}H radioactivity extracted from the PC-binding proteins was found in the free fatty acid fraction. However, an additional 17% of the ^{3}H radioactivity associated with the PC-binding proteins could be extracted with chloroform/methanol (2:1 v/v) after the treatment with Pronase (2% of PC-binding protein by weight) for 24 h at 37 °C. Thin-layer chromatography of this extract showed that about 36% of the radioactivity was in phosphatidylethanolamine fraction, thus confirming the association of phospholipids with the PC-binding proteins.

Amino Acid Composition and Association of Carbohydrates with PC-Binding Proteins. Fcγ receptors present on J774 cells have been suggested to be glycosylated by radiolabeling of cells with NaB^3H_4 after galactose oxidase treatment (Mellman & Unkeless, 1980). Ash shown by Table III amino acid analysis of the purified PC-binding protein revealed the presence of glucosamine. Assuming a number of valine residues per mole of PC-binding protein to be 15, the total amino acid residues that comprise PC-binding proteins were calculated to be 417. The molecular weight of the peptide portion of the PC-binding protein was thus estimated to be near 38 000. The molar ratio between glucosamine and peptide was found to be 1.0. The association of glucosamine with the purified PC binding proteins was further investigated by biosynthetic labeling of protein and glucosamine. A total of 1.8×10^8 cells was first radiolabeled with [^{14}C]leucine as usual. Half of these cells were further cultured for 16 h in the presence of [^{3}H]-glucosamine (300 μCi), washed, and pooled with the rest of [^{14}C]leucine-labeled cells. The lysate obtained by the detergent treatment of these cells contained 51.8 mg of protein, and the total radioactivity was 4×10^6 and 3×10^7 cpm of ^{3}H and ^{14}C, respectively. PC-binding proteins were purified from the lysate by the three-step procedure as usual. The yield of the PC-binding proteins which focused at near 5.8 at the last step was 0.77% (400 μg) by protein determination and 0.87% by the ^{14}C radioactivity. About 1.5% of the ^{3}H radioactivity of the original lysate was found to be associated with the PC-binding proteins. Since the total glucosamine content in the lysate was found by the amino acid analysis of a 900-μg aliquot of the lysate to be 85 μg, the amount of glucosamine associated with the purified PC-binding proteins should be about 13 μg/400 μg of proteins, or about 0.7 glucosamine residue per mole of the PC-binding protein, which agrees well with the estimation previously made by the amino acid analysis.

The total carbohydrate content of the purified PC-binding proteins was estimated by the Orcinol method to be 0.25 μg/μg of protein, or 8000 g/40 000 g of protein.

Association of RNA with the Purified PC Binding. Nucleic acid (such as DNA or RNA) can bind covalently through their 5′ end to hydroxyl groups of serine or threonine of proteins (Revie et al., 1976; Lee et al., 1977). The apparent tight association of a mole of small RNA (7*S*, 300 nucleotides long) with a mole of highly purified signal recognition protein was recently reported (Walter & Blobel, 1982). Since such 7S RNA has been shown to be relatively abundant in cytoplasmic fractions in association with polysomes or microsomal membrane (Gunning et al., 1981), a possible association of such RNA with the PC-binding proteins was investigated. To this end, 1.7×10^8 cells which were previously biosynthetically radiolabeled with [^{14}C]leucine were pulse labeled for 30 min with 200 μCi of [^{3}H]uridine in serum-free RPMI 1640 medium. Cells were washed 4 times with cold PBS, pooled with [^{14}C]leucine-labeled cells (5.1×10^8), and lysed with 0.5% Triton X-100. This lysate contained 72.6 mg of protein and had a ^{3}H radioactivity of 3.9×10^7 and 3.5×10^7 cpm, respectively. The PC-binding proteins which focused at pH near 5.8 at the final step of purification comprised 0.11% of the ^{14}C radioactivity and 0.17% (130 μg) of the proteins present in the lysate. ^{3}H radioactivity associated with the purified PC-binding proteins was 0.03% of the original lysate. Since the total RNA content in the lysate determined by the spectrophotometric method was 6.5 mg, uridine associated with the PC-binding protein should be about 0.5 μg/120 μg of protein, or 160 g/40 000 g of protein.

Reaction of 2.4G2 Antibody with PC- and IgG-Binding Proteins. Our previous studies demonstrated that the phospholipase A_2 active PC-binding proteins isolated from the detergent lysate of $P388D_1$ cells inhibited the rosette formation between $P388D_1$ cells and EA_{2b} (Suzuki et al., 1982). Since this inhibition could be reversed by preincubating the PC-binding proteins with IgG_{2b} but not with IgG_{2a}, it appears that the PC-binding proteins contain $Fc\gamma_{2b}R$ originated from the cell surface. Our previous data also suggested that the phospholipase A_2 inactive IgG-binding proteins should contain $Fc\gamma_{2a}R$ originated from the cell surface. It follows that mo-

Table IV: Binding of ^{125}I-Labeled 2.4G2 F(ab')$_2$ Fragments to [^{14}C]Leucine-Labeled PC- and IgG-Binding Proteins[a]

	^{125}I-labeled 2.4G2 F(ab')$_2$ (cpm)	goat anti-rat F(ab')$_2$	cpm in ^{14}C sup	cpm in ^{14}C ppt	cpm in ^{125}I sup	cpm in ^{125}I ppt	% ^{14}C precipitated found	% ^{14}C precipitated net
PC-binding proteins (μg)								
0.7	900	+	128	194	129	744	60	47
1.0	900	+	178	238	168	687	57	44
1.5	900	+	245	307	212	634	56	43
1.5	0	+	467	68	0	0	13	
0	900	+	0	0	107	685	0	
IgG-binding proteins (μg)								
1.5	900	+	282	70	159	645	20	6
1.5	0	+	290	47	0	0	14	

[a] Different amounts of [^{14}C]leucine-labeled PC-binding and IgG-binding materials in TBS–0.5% Triton X-100 were mixed in microfuge tubes with a constant amount (900 cpm, 1 μg) of iodinated F(ab')$_2$ fragment of 2.4G2 monoclonal antibody and (150 μL) was then added to each tube. After the additional incubation period (18 h), the immunoprecipitates were separated by centrifugation (10000 rpm/5 min) and washed once in TBS–0.5% Triton X-100. Both supernatants (sup) and precipitates (ppt) were finally processed for γ and then scintillation counting.

noclonal antibody, 2.4G2, directed against Fcγ_{2b}R (Unkeless, 1979) is expected to bind to PC-binding proteins but not to IgG-binding proteins. In order to test this possibility, PC- and IgG-binding proteins were incubated in TBS containing 0.5% Triton X-100 at 4 °C for 16 h with a constant amount (900 cpm in 100 μL) of the radioiodinated F(ab')$_2$ fragment of 2.4G2 antibody. At the end of the incubation period, F(ab')$_2$ fragment of goat anti-rat IgG antibody was added to the mixtures. The mixtures were incubated at 4 °C for an additional 16 h and then centrifuged at 10000g in a Beckman microfuge. The radioactivity in supernatants and pellets was separately measured by γ and liquid scintillation counters. Results summarized in Table IV clearly show that 2.4G2 antibody coprecipitated with about 43–47% of ^{14}C counts associated with PC-binding proteins, suggesting the presence of molecules reacting with 2.4G2 antibody in these preparations, whereas 2.4G2 antibody coprecipitated with 6% of ^{14}C radioactivity associated with IgG-binding proteins.

Further evidence for the binding of 2.4G2 antibody to PC-binding proteins was sought by gel filtration. As shown by Figure 5, when the mixture of ^{125}I-labeled F(ab')$_2$ fragments of 2.4G2 antibody and ^{14}C-labeled PC-binding proteins was passed through a column (1 × 60 cm) of Sephadex G-150 gel equilibrated against the buffer I, the two proteins clearly co-migrated during the gel filtration. The two proteins were completely separated when passed through a column of Sephadex G-150 gel equilibrated against 6 M urea (data not shown).

The above results suggest that 2.4G2 antibody reacts with the purified PC- but not IgG-binding proteins. Since 2.4G2 antibody has been shown to react with Fcγ_{2b}R on the cell surface (Unkeless, 1979) and since phospholipase A_2 active site associated with the PC-binding proteins should be embedded within the lipid bilayer, 2.4G2 antibody-bound material could be extracted by PC–Sepharose from the detergent lysate of P388D$_1$ cells, if the Fcγ_{2b}-binding site and phospholipase A_2 active site resides on a same molecule. In order to examine this possibility, P388D$_1$ cells (1.9×10^7) which were washed 3 times with cold HBSS were first incubated with ^{125}I-labeled 2.4G2 F(ab')$_2$ fragments (8300 cpm) for 1 h at 4 °C in the presence of cytochalasin D (10 μg/mL). At the end of the incubation period, cells were collected, washed with PBS, and counted for ^{125}I radioactivity. About 20% (1700 cpm) of the ^{125}I radioactivity was found to be associated with the washed cells. After detergent lysis of 2.4G2 antibody-binding cells with Triton X-100, the PC-binding proteins were extracted from the lysate by affinity chromatography on PC–Sepharose and were found to be associated with about 17% (1,400 cpm) of the original ^{125}I radioactivity. Thus, about 82% of P388D$_1$ cell-bound ^{125}I radioactivity was found in association with the PC-binding protein. In the second experiment, ^{125}I-labeled 2.4G2 F(ab')$_2$ fragments were added to the detergent lysate of 2.1×10^7 P388D$_1$ cells. Affinity chromatography on PC–Sepharose of this lysate gave the PC-binding proteins which were associated with about 37% (3300 cpm) of the original ^{125}I radioactivity.

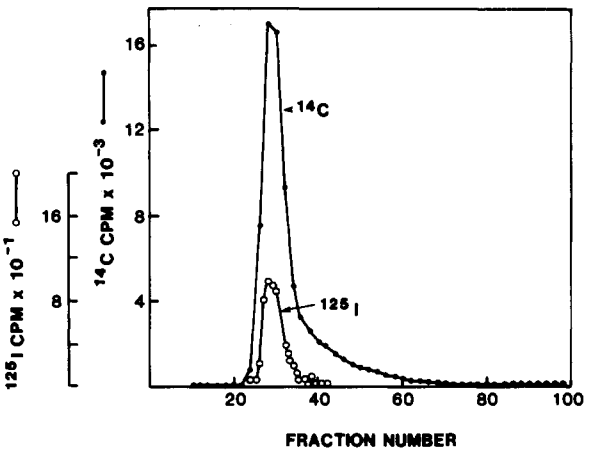

FIGURE 5: Sephadex G-150 gel filtration of the mixture of ^{125}I-labeled F(ab')$_2$ fragment of 2.4G2 antibody and the ^{14}C-labeled PC-binding proteins.

Discussion

The data presented in this paper show that the biosynthetically radiolabeled, therefore, viable cell origin PC-binding proteins copurify with heterogeneous minor cellular components throughout the affinity chromatography and gel filtration steps of purification. The separation of the contaminants from the major PC-binding proteins could be achieved by electrofocusing in the presence of 6 M urea but not in the presence of Triton X-100 (0.5%), suggesting the necessity of unfolding of heterogeneous complex for further purification of the PC-binding proteins. The biosynthetically radiolabeled PC-binding proteins focus as a single peak, based on the absorbancy at 280 nm, at pH near 5.8 where the surface-radioiodinated PC-binding proteins usually focus (Suzuki et al., 1982). The yields of this type of PC-binding proteins, based on the Lowry's method, ranged from 4 to 0.7 mg per 10^9 cells in five different experiments and constituted about 2–0.4% of the total proteins estimated to be present in the lysate. The relatively large variations in the yields are due to the differences in the recovery of the focused proteins which isoelectrically precipitate and adhere to the glass surface of an Ampholine column. Neither pretreatment of a column with silicon or bovine serum albumin

nor change of the materials to form gradient from sucrose to polyethylene glycol or glycerol could prevent the problem of adherence of focused material to the glass surface.

The purified PC-binding proteins appear to consist of 417 amino acid residues (giving a minimum peptide molecular weight of 38 000) and to be glycosylated with an oligosaccharide chain. Triton X-100 and phospholipids are tightly bound to this protein in a weight ratio of 0.57 and 0.05 g/g of protein, respectively. We have previously reported the yield of the PC binding from $P388D_1$ cell lysate as 7 mg per 10^9 cells based on their dry weights (Suzuki et al., 1982). The maximum yield obtained in this study was 4 mg per 10^9 cells based on protein determination by Lowry's method. The yield based on the dry weight in this case should be about 7.3 mg, when we count the contribution of tightly bound Triton X-100 and oligosaccharide chain to the weight.

On the basis of the binding assay, the number of FcγR on macrophage has been estimated to be $(1-10) \times 10^5$ per cell (Unkeless, 1977; Unkeless & Eisen, 1975; Segal & Hurwitz, 1977). FcγR should then represent 0.002–0.02% of total protein of a cell, if we assume the molecular weight of FcγR to be 50 000 and total protein per cell to be 300 pg. Our data based on the Lowry's method as well as on the radioactivity show that about 2–0.4% of the total proteins in the lysate can be obtained as the purified PC-binding proteins. The similar magnitude of the yield was also reported by two other laboratories who utilized the same $P388D_1$ cell line in their studies. Loube et al. (1978) thus reported that IgG_{2a}-binding proteins obtained from the detergent lysate of the surface-radioiodinated cells represent about 0.74% of the proteins in the lysate. D'Urso-Coward & Cone (1978) reported that 62% of the ^{125}I radioactivity in the lysate bound to aggregated IgG–Sepharose and that 0.76% of aggregated IgG-binding proteins could be eluted with acetate buffer. Using 2.4G2 Fab–Sepharose, Mellman & Unkeless (1980) reported, however, that Fcγ_{2b}R of the J774 cell line represents about 0.011% by protein and 0.041% by the radioactivity of the proteins present in the lysate. The exact reasons for such large differences between the yield reported by Mellman and Unkeless and those by three other laboratories are unknown at present.

The binding assay could potentially result in the underestimation of the number of receptors per cell, if FcγR aggregates on the cell surface and/or binding of ligands is sterically hindered. The fact that the affinity-purified PC-binding proteins are excluded from Sephadex G-100 even in the presence of 6 M urea suggests a strong tendency of PC-binding proteins to aggregate (Suzuki et al., 1982). FcγR materials purified by 2.4G2 antibody-affinity chromatography are also shown to have a strong tendency to aggregate to form a large molecular complex consisting of more than 10–20 subunits, since they were shown to be excluded from Sephadex G-150 in the presence of 0.5 M NaCl and to sediment during sucrose density gradient centrifugation as a heterogeneous broad zone ranging from 7 S to well over 19 S (Mellman & Unkeless, 1980). However, whether or not FcγR form high molecular weight complexes on the cell surface needs to be examined. Steric hindrance is another possible cause for the underestimation of the number of FcγRs on a cell. Scanning electron micrograph shows that macrophage surface is not smooth but is uneven with numerous blebs, blisters, and projections. The surface area of a cell should be much greater than that calculated as a simple sphere. In addition, macrophage surface carries not only FcγR but also other receptors and antigens. The binding of such a large ligand as IgG of Fab fragment of monoclonal antibody can be sterically hindered by lingand itself or cell surface proteins other that FcγR. Either aggregation of FcγR or steric hindrance would not interfere for an apparent saturation binding. Additional potential problem is the binding affinity, which is reported to be 7.5×10^6 for Fcγ_{2b}R (Unkeless & Eisen, 1975) and $(2.3–130) \times 10^6$ L/M for Fcγ_{2a}R (Unkeless, 1977; Segal & Hurwitz, 1977). Some of of the bound ligands may have been released during washing. To avoid this problem, Unkeless & Eisen (1975) measured the amount of IgG bound to $P388D_1$ cells which had been allowed to adhere to the plastic surface, thus allowing a rapid wash. What is unclear is how much surface area of $P388D_1$ cells became unaccessible to the ligand by plastic adherence. The oil centrifugation method employed by Segal & Hurwitz (1977) allowed them to eliminate the washing process and to measure binding constants as low as 5×10^6 L/M. In both techniques, however, a possible presence of FcγR with binding affinity $<5 \times 10^6$ on $P388D_1$ cells cannot be excluded. Thus, the estimation of the number of FcγR molecules per cell by binding assay can only be regarded as minimal.

On the other hand, the affinity procedures employed by us and by others can give rise to an erroneously high yield if FcγR molecules copurify with a large amount of nonreceptor proteins or other cellular components or if methodology to quantitiate the yield is inappropriate. The fact that three different methods (dry weight, biosynthetic radiolabeling, and surface radioiodination) employed by three different laboratories gave a similar yield of about 1% suggests the validity of the methods. The tight association of Triton X-100 certainly increased the yield of dry weights as much as 57%, but not to the extent of 100–1000-fold. Indeed, if the estimation by binding assay is correct, the purified PC-binding proteins which could react with 2.4G2 antibody must be 99–999-fold impure. Isoelectric focusing used at the final step of purification should, with its superb resolution power, separate some of the impurities particularly under unfolding condition. In fact, the PC-binding proteins focus reproducibly as a sharp single peak at pH near 5.8–6.0, suggesting their charge homogeneity. However, since a possibility that FcγR could be linked covalently to a large amount of homogeneous nonreceptor proteins remains, the PC-binding proteins after the gel filtration step were also electrofocused in the presence of 1 mM dithiothreitol and 6 M urea. Such experiments gave no indication of the heterogeneity, since the PC-binding proteins focused again as a single peak at more acidic pH (near 4.5).

The data shown in Table IV and Figure 5 show that the purified PC-binding proteins do react in Triton X-100 containing buffer with the $F(ab')_2$ fragment of 2.4G2 antibody which is presumably directed against cell surface Fcγ_{2b}R (Unkeless, 1979), whereas IgG-binding proteins, which we have shown previously to bind specifically to IgG_{2a} (Suzuki et al., 1982), did not. Probable reasons why not all of PC-binding proteins coprecipitated with 2.4G2 antibody in these experiments include (1) an incomplete renaturation of PC-binding proteins upon isolation, (2) a decrease in binding affinity of 2.4G2 antibody due to pepsin digestion, and (3) the presence of impurities in the isolated PC-binding proteins.

Thus, our present studies show that the material isolated as PC-binding protein shares a common antigenic determinant with Fcγ_{2b}R originated from the cell surface. Furthermore, the data showing that PC-binding proteins could be extracted from the lysate of 2.4G2 antibody-bound $P388D_1$ cells suggest a possible presence of two biologically distinct Fcγ_{2b}-binding and phospholipase A_2 active sites on a single molecule. This possibility requires, however, further extensive biochemical

studies of the purified PC-binding proteins.

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