Evidence That Phospholipase δ1 Is the Effector in the Gh (Transglutaminase II)-mediated Signaling*

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A new class of GTP-binding protein transglutaminase II (Gh) couples to a 69-kDa phospholipase C (PLC). An 8-amino acid region (Leu665–Lys672) of the α-subunit of Gh (Ghα) is involved in interaction and activation of PLC, an observation that has now been used to characterize the 69-kDa PLC further. A 20-amino acid peptide corresponding to Leu654–Leu673 of Ghα inhibited the binding of PLC-δ1 to the affinity resin-bound PLC-δ1. An antibody to the 69-kDa PLC recognized PLC-δ1 bound to the affinity resin; moreover, antibodies to PLC-δ1 recognized the 69-kDa PLC that bound to this peptide was characterized. We present, for the first time, evidence that PLC-δ1 is the effector of Gh-mediated signaling.

Phosphoinositide-specific phospholipase C (PLC)1 plays a crucial role in transmembrane signaling by producing two sec- ond messengers, diacylglycerol and inositol 1,4,5-triphosphate (1–3). A number of distinct PLCs have been purified, cloned, and classified into three types: PLC-β, PLC-γ, and PLC-δ, which are subdivided by their structural similarity (2, 3). It is now established that PLC-δ isozymes are stimulated to differ- ent extents by either the α-subunits of the Gαq family or the βγ subunits of G-proteins (4–7). Receptors involved in activation of PLC-β, especially the Gαq/PLC-β1 system, are α2-adrenergic hormone (8, 9), muscarinic acetylcholine (10), angiotensin, and bradykinin receptors (11). PLC-γ(s) are unique among the PLC isoforms in that they have two Src homology domains and are activated via tyrosine phosphorylation by growth hormone receptors, such as platelet-derived growth factor and epidermal growth factor (2, 12). The involvement of PLC-δ isozymes in transmembrane signaling is not clear, although the PLC-δ subtype is widely distributed and is more abundant than PLC-β1 (2, 13). Recently, PLC-δ1 has been implicated in thronbin-mediated transmembrane signaling using a coexpression system (14).

In the course of studying the α1B-adrenergic receptor signaling mechanism, we have identified a 69-kDa PLC that is activated by a new class of GTP-binding protein, Gαq (transglutaminase II) (9, 15). The 69-kDa PLC was initially purified by complex formation with Ghα by incubating the bovine liver membranes with α1B-agonist and GTP (15). Total reconstitution of the α1B-receptor, Ghα, and the 69-kDa PLC demonstrated that the three components effectively coupled to each other (15). This finding was further confirmed by coexpression of α1B-adrenergic receptor and Ghα in various cells (9). However, the identity of the 69-kDa PLC remains to be clarified.

Recently, we have shown that a peptide (Leu661–Lys672) derived from the COOH-terminal region of human heart Ghα inhibited coimmunoprecipitation of PLC by Ghα-antibody as well as guinea pig transglutaminase II antibody (16), indicating that the peptide binds to PLC with high affinity. Moreover, systematic deletion mutation studies with Ghα revealed that the region corresponding to this peptide of Ghα is responsible for interaction and activation of PLC. Using a peptide from this region, we sought to determine whether the 69-kDa PLC is unique or is a known PLC isozyme. A 20-amino acid peptide (Leu654–Leu673) from Ghα, which contains the PLC binding region, was used to create an affinity resin. The identity of the PLC that bound to this peptide was characterized. We present, for the first time, evidence that PLC-δ1 is structurally similar to the 69-kDa PLC and that PLC-δ1 is stimulated by Ghα.

EXPERIMENTAL PROCEDURES

Materials—Protein A-agarose, CNBr-Sepharose 4B, and chromatographic materials were obtained from Pharmacia Biotech Inc., and nucleotides were from Boehringer Mannheim. Sucrose monolaurate (SM-1200) was obtained from the Mitsubishi-Kasei Food Corp. (Tokyo, Japan), and sodium cholate was from Serva (Heidelberg, NY). [35S]GTPyS (1300–1500 Ci/mmol) and phosphatidyl[2-3H]inositol 4,5-bisphosphate (1 Ci/mmol) were from DuPont NEN. Chemiluminescence reagents were from Pierce and monoclonal PLC antibodies were obtained from Upstate Biotechnology Inc. (Lake Placid, NY).

Expression of PLC-δ1 in Escherichia coli and Purification—The rat cDNA clone for PLC-δ1 in a pBl21 vector was used to transform a DH-5α strain of E. coli. The transformants were grown at 37°C overnight in 50 ml of Luria-Bertani (LB) medium containing ampicillin (100 µg/ml). The cells were further incubated in the presence of 0.1 mM isoproterenol-β-thiogalactopyranose in LB medium (500 ml) at 20°C for 20–24 h (17) and then collected by centrifugation at 4°C for 2,000 × g for 10 min. The pellets were suspended in HDGD (20 mM Hepes, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT) buffer containing 1% sodium cholate and lysed by incubating with lysozyme (100 µg/ml) at 4°C for 20 min. The pellets were collected by centrifugation at 40,000 × g at 4°C for 30 min and solubilized with 1% sodium cholate and 200 mM NaCl in HDGD buffer containing protease inhibitors (10 µg/ml phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM and 1 µM aprotinin) and lyophilized for 2 days. The resulting material was dissolved in 20 mM Hepes at 4°C.

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§ The abbreviations used are: PLC, phospholipase C; G-protein, GTP-binding regulatory protein; Ghα, holo enzyme consisting of α74-kDa GTP-binding α-subunit and 50-kDa β-subunit; Ghαδ, 74-kDa guanine nucleotide-binding regulatory protein and a tissue type transglutaminase; Ghα, antibody; a polyclonal antibody raised against 78-kDa Ghαδ and previously designated as Ghαδ-antibody; GTPyS, guanosine 5'-O-3(thiotriphosphate); PIP2, phosphatidylinositol 4,5-bisphosphate; DTT, 1,4-dithiothreitol.
sulfonyl fluoride, 2 μg/ml bacitracin, 100 μg/ml benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml soybean trypsin inhibitor, 20 μg/ml antipain). Purification of PLC-δ1 was achieved by chromatography using heparin-agarose and Q-Sepharose according to the method of Ginger and Parker (18), except sodium cholate (1%) used for the solubilization of PLC was exchanged for 0.05% sucrose monolaurate (SM). The peptides (P5 or P6) were then washed with HSD buffer containing 0.01% SM. The bound proteins were eluted in three portions (300–500 μl) or ethanolamine cross-linked to Sepharose 4B (Resin), that had been equilibrated with HSD buffer (100 mM NaCl). The peptide-Sepharose was synthesized using CNBr-Sepharose 4B with a slight modification of the method recommended by Pharmacia. The peptides (P5 or P6, shown in Fig. 1) were dissolved in minimum amounts of dimethyl sulfoxide (Me2SO) and diluted with equal amounts of water. After washing with 20 mM HCl, the activated resin (10 ml, wet volume) was incubated with 100 mM NaHCO3/Mε2SO (1:1, v/v) solution (40 ml), and the residue was washed with 20 mM Tris-HCl (pH 7.4) containing 100 mM NaCl. The peptide-Sepharose was stored in the same buffer containing 0.02% azide at 4°C.

Binding of PLCs to Peptide-Sepharose—For the binding of PLCs to peptide-Sepharose, PLC preparations (pure, 20 ng + 1 μg of bovine serum albumin; crude, 50 μg) were incubated with the resins (30 μl of gel/tube) at 4°C for 1 h with gentle rotation. The final volume was 100 μl in HSD buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, and 0.5 mM DTT) containing 0.01% SM. After centrifugation at 1,900 × g for 5 min, supernatants and pellet fractions were collected, and the pellets were washed three times with 1 ml HSD buffer containing 0.05% SM. The bound proteins were eluted in a small volume (300–500 μl) with a 9 M urea, 1 M NaCl solution.

Reconstitution of Peptides and Gα with PLC-δ1—The purified PLC-δ1 was reconstituted with peptides in detergent solution and with Gα, in phospholipid vesicles (15). PLC-δ1 (7 ng/tube) in HSD buffer containing 0.02% SM was incubated with various concentrations of peptides (0–220 μM). The PLC-δ1 activity was measured in the presence of 3.74 mM of free Ca2+ at 30°C for 8 min. The free Ca2+ concentration was obtained in the presence of 0.8 mM EGTA and 75 μM CaCl2 in HSD buffer. Reconstitution of Gα with PLC-δ1 was achieved by the dilution method (20). Gαi (6 pmol) was preincubated with GTPγS, GDP, or buffer only in the presence of 5 mM MgCl2 at 30°C for 30 min. Ligand-pretreated Gαi was mixed with PLC-δ1 (200 ng) and a phospholipid mixture (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine; 3:1:1) in an ice bath. The PLC activity was measured with various concentrations of CaCl2 (0–16 μM) at 30°C for 8 min (15, 20).
The PIP$_2$ concentration was 100 μM (1200 cpm/nmol) in a 100-μl final volume.

Western Blotting—Polyclonal antibody against 69-kDa PLC was generated in New Zealand White rabbits according to the method of Baek et al. (21). 69-kDa PLC (40–60 μg in 200 μl/rabbit) was used to raise the antibody. Rabbit antisera were characterized by immunoblots with purified 69-kDa PLC (21, 22), except antibody cross-reactivity was determined using chemiluminescence. Anti-rabbit Ig horseradish peroxidase (1:1000 dilution) was used for the secondary antibody. Immuno blotting with monoclonal antibody against bovine brain PLC-δ1, PLC-γ1 and PLC-γ2 was performed using the same protocol (21, 22). Anti-mouse Ig horseradish peroxidase was used for the secondary antibody.

Coimmunoprecipitation—For the coimmunoprecipitation studies, G$_h$, (monoclonal antibody raised against guinea pig liver transglutaminase and G$_{h15}$) antibodies and preimmune sera were cross-linked with protein A-agarose (15, 23). Reconstituted samples of G$_h$ with PLC-δ1 in HSD buffer containing 0.02% SM were incubated with or without 2 μM GTPyS in the presence of 2 mM MgCl$_2$. After incubation at room temperature for 30 min, the samples were further incubated with the antibody-agarose (30 μl suspension/tube in HSD buffer) at 4°C for 2 h with gentle rotation. The supernatant and pellets were collected by centrifugation at 1,900 × g for 5 min. The pellets were washed three times with 1 ml HSD buffer containing 0.05% SM and 2 mM MgCl$_2$. PLC activity was measured in both supernatant and pellets at 30°C for 10 min (15).

Other Assays—G$_h$ concentration was measured using 1 μM [35S]GTPyS (specific activity, 1000–1500 cpm/pmol) (19). The protein concentration was determined by the method of Bradford (24) using a Bio-Rad protein determination kit and bovine serum albumin as a standard.

RESULTS AND DISCUSSION

We have shown previously that a stretch of 12 amino acids (P4, Leu$^{661}$–Lys$^{672}$) near the COOH terminus of G$_{h15}$ contained the high affinity PLC binding site (16) (Fig. 1). This observation was further applied to identify the PLC isozyme activated by G$_{h15}$. We synthesized four peptides (P3–P6) (Fig. 1) and cross-linked P5 and P6 to CNBr-Sepharose 4B for use as peptide-affinity resins. A partially purified PLC preparation from rat liver membranes was incubated with the peptide affinity resins and their ability to bind PLC was determined (Fig. 2A). The P6 affinity resin bound 3–6 times more PLC activity than the P5 affinity resin or resin alone did, consistent with P6 containing the PLC binding site. The partially purified PLC preparation was then loaded onto a P6-Sepharose column, and the bound proteins were eluted and subjected to immunoblot analysis with antibodies to the 69-kDa PLC (Fig. 2B). The antibodies recognized two proteins with molecular sizes of ~69 and 85 kDa (lane 2) as well as the purified 69-kDa PLC (lane 3). The antibodies did not recognize any proteins in the eluate from the P5 affinity resin (lane 1). Furthermore, nonimmune serum did not recognize the ~69- and 85-kDa proteins in the eluate from the P6 affinity column (data not shown). These results suggested that the 85-kDa protein, which has approximately the same molecular size as PLC-δ1, is structurally similar to the 69-kDa PLC. PLC activity measurements revealed that both purified 69-kDa PLC and PLC-δ1 bound to the P6 affinity resin (Fig. 3A). Furthermore, both purified proteins were recognized by a monoclonal antibody to PLC-δ1 (Fig. 3B), suggesting that the 69-kDa PLC is structurally similar to PLC-δ1 and may be a proteolytic fragment of this enzyme. The polyclonal antibodies to the 69-kDa PLC cross-reacted with PLC-δ1 (data not shown). The 69- and 85-kDa proteins present in the partially purified preparations that bound to the P6 affinity resin were recognized by the monoclonal antibody to PLC-δ1 but not by monoclonal antibodies to PLC-β1 or PLC-γ1.

The specificity of PLC-δ1 binding to the P6 affinity resin was then evaluated. PLC-δ1 was incubated with P5 or P6 affinity resins in the presence or absence of P4 (Fig. 4A). PLC-δ1 did bind to the P6 affinity resin but not to the P5 affinity resin, and the peptide P4 blocked the PLC-δ1 binding to the P6 resin. In addition, peptide P3 did not inhibit the binding of PLC-δ1 to the P6 affinity resin, showing that the P4 region contributes to the binding of PLC to G$_{h15}$ (data not shown). Since this P4 region of G$_{h15}$ interacts with and activates PLC (16), we investigated whether peptide P4 activates PLC-δ1. Indeed, the peptide P4 did activate PLC-δ1 in a concentration-dependent manner with an EC$_{50}$ of ~50 μM, and a maximal activation was reached at ~150 μM (Fig. 4B). Peptide P3 did not stimulate PLC-δ1 (Fig. 4B), indicating that stimulation of PLC-δ1 by P4 is specific.

Finally, coupling between PLC-δ1 and G$_h$, reconstituted in phospholipid vesicles was investigated. We have previously reported that EGTA inhibits the coupling of G$_h$ with 69-kDa PLC (15). Similarly, coupling between G$_h$ and PLC-δ1 was not observed in the presence of chelator. Therefore, coupling was assessed in the absence of chelator under the conditions de-
scribed previously (15, 20). Reconstitution of PLC-δ1 with G_{i} reduced basal PLC activity by two-thirds (data not shown). Activated (GTPγS-bound) G_{i} increased PLC-δ1 activity in a Ca^{2+} concentration-dependent manner (Fig. 5A); the activity was maximal at \( \leq 12 \mu M \) Ca^{2+}, similar to the concentration required for maximal activation of the 69-kDa PLC by G_{i} (15). Enzyme activity was less sensitive to Ca^{2+} in the presence of GDP or buffer alone; under these conditions, PLC-δ1 was not fully activated at \( \geq 16 \mu M \) Ca^{2+}. Thus, activation of PLC-δ1 by G_{i} resulted in an increase in the affinity of the enzyme for Ca^{2+}, as observed when G_{i} and the 69-kDa PLC were coupled (15). We also showed that antibodies to G_{i} coimmunoprecipitated PLC-δ1 when the enzyme was reconstituted with GTPγS-bound G_{i} (Fig. 5B); PLC-δ1 was not coimmunoprecipitated in the absence of GTPγS. Antibodies from nonimmune serum coupled to protein A-agarose did not coprecipitate PLC-δ1 in the presence or absence of GTPγS (data not shown).

It has been suggested that low molecular mass PLCs might be proteolytic fragments of PLC-ζ, γ, and δ isoforms (2, 25). Previous studies have indicated that the 69-kDa PLC is probably a proteolytic fragment of PLC-δ1. Thus, the elution profile of 69-kDa PLC is similar to PLC-δ1 on ion exchange chromatographic resins, particularly on heparin-agarose (15). Digestion of PLC-δ1 by proteases yields fragments of 52–68 kDa (25); in the presence of PIP_{2}, trypsin generates a 68-kDa fragment from PLC-δ1 (26). Indeed, the original purification of the 69-kDa PLC-G_{i} complex may have contributed to an increased sensitivity of PLC-δ1 to proteases, because the complex was induced by \( \alpha_{2} \)-adrenoreceptor activation in the presence of GTP \((15)\), which increases the binding of PIP_{2} to PLC-δ1. On the other hand, we previously stated that the purified 69-kDa PLC did not cross-react with antibodies to PLC-ζ1 and that G_{i} did not stimulate PLC-ζ1 activity (15). The reason for the failure to stimulate PLC-ζ1 or to detect the 69-kDa PLC by PLC-ζ1 antibody is not clearly understood. It is probably due to the vitality of the purified enzymes and affinity of the antibody for the 69-kDa PLC, since we previously used a polyclonal antibody to PLC-ζ1.

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