Activation of Phospholipase C β4 by Heterotrimeric GTP-binding Proteins*

Huiping Jiang, Dianqing Wu, and Melvin I. Simon

From the Division of Biology, 147-75, California Institute of Technology, Pasadena, California 91125

Transient transfection assays were used to determine how the activity of phospholipase C β4, which is preferentially expressed in retina, was regulated. An expression vector carrying the full-length cDNA corresponding to phospholipase C β4 was constructed and co-transfected into COS-7 cells together with cDNA encoding the α subunits of the Gq class and various β and γ subunits corresponding to the heterotrimeric GTP-binding proteins. We found that all the α subunits of the Gq class, including Gaq, Gai1, Gai4, Gai6, and Gai8 could activate PLC β4 and that none of the Gqα subunits that we tested including Gβ1y1, Gβ1y2, Gβ1y3, or Gβ2y2 activated phospholipase C β4. In control experiments, cotransfection with cDNA encoding the α subunit of transducin or Gβ2 gave no activation of PLC β4. These results indicate that phospholipase C β4 is activated by Ga subunits that are members of the Gq class, and, like the phospholipase C β1 isofrom, it is refractory to activation in the transfection assay by many of the combinations of β and γ subunits found in the heterotrimeric G-proteins.

Heterotrimeric GTP-binding proteins mediate intracellular changes generated by ligand binding to different members of highly diversified families of cell surface receptors (1, 2). G proteins consist of three subunits, α, β, and γ. Thus far, at least 16 α subunits, 5 β subunits, and 7 γ subunits have been cloned and sequenced in mammals. The α subunits can be grouped into four classes based on their amino acid sequence identity (for details, see Ref. 3). The activated (GTP-bound) form of the α subunits as well as the free βγ subunits, which can result from interaction with ligand-bound receptors, were found to interact with a variety of effectors. These effectors, may be enzymes that generate "second messengers" or ion channels. One of the effectors is phosphoinositide-specific phospholipase C (PLC) that catalyzes the hydrolysis of phosphati-dylinositol 4,5-bisphosphate to produce two second messengers, diacylglycerol and inositol trisphosphate. Molecular cloning has revealed three classes of PLC, PLC β, PLC γ, and PLC δ, and each of these occurs in at least three isoforms (4, 5). The β isoforms of PLC, including PLC β1–3, have been shown to be activated by the α subunits of the Gq class using either the co-transfection assay (6–8) or in vitro reconstitution assays with purified or partially purified proteins (9–14). Furthermore, in co-transfection assays, the βγ subunits of G proteins were found to be able to stimulate the β2 isofrom of PLC but not the β1 isofrom (15) and to preferentially stimulate PLC β2 and β3 in reconstitution assays (16–18).

Recently, Min et al. (19) reported the purification of a protein from bovine cerebellum with a molecular weight around 100,000. This protein possesses the ability to specifically hydrolyze phosphoinositides. The partial amino acid sequence analysis of this protein suggests that it is a novel PLC β isozyme, designated PLC β4. At the same time, Pak and his colleagues (20) isolated a partial cDNA clone which appears to encode a PLC β homolog. The deduced amino acid sequence of this cDNA clone is identical with the amino acid sequences derived from the purified protein. Thus, these two groups appear to have independently identified products of the same gene. Northern analysis revealed that PLC β4 is expressed primarily in the retina, with low levels of expression also found in the cerebellum (20). Subcellular localization with specific antibodies showed that PLC β4 mainly existed in cone photoreceptor outer segments as well as in other retinal neurons such as ganglion cells. This, together with the fact that PLC β4 shares the highest homology with the Norp a protein that mediates the visual process in Drosophila (21), suggests that PLC β4 may play a role in the mammalian visual system. Therefore, it is of interest to determine how the activity of PLC β4 is regulated.

All of the cloned PLC enzymes have been found to include two homologous regions designated the X and Y boxes that are thought to be associated with catalytic activity (5). The PLC β subclass can be distinguished from the others by the presence of a long stretch of sequence at the C-terminal end of the molecule following the Y box. Our previous work has identified a small segment (designated the G box) within this C-terminal region of PLC β1 that may be involved in interaction with Gαq (22). The sequences that make up the Y box in PLC β1 (23, 24), PLC β2 (25), and PLC β3 (13) are homologous, strengthening the suggestion that this region is required for activation by Gαq. Comparison of the amino acid sequence of PLC β4 (20) with that of the conserved G box reveals clear homology. Thus, we predict that PLC β4, like the other members of the PLC β class, should also be activated by Gαq and probably by other members of the α subunits of this class. To experimentally demonstrate the specificity of interaction, we first isolated the N-terminal nucleotide sequence that appears to be missing from the cDNA reported by Dr. Pak and his colleagues (20). Then we constructed an expression vector carrying the full-length cDNA of PLC β4 and tested whether PLC β4 could be activated by Ga subunits as well as Gβγ subunits using the co-transfection assay.

MATERIALS AND METHODS

Molecular Cloning of the N-terminal Nucleotide Sequence of PLC β4 by Rapid Amplification of cDNA End (5′-RACE)—The 5′-RACE protocol

* This work was supported by National Institutes of Health Grant GM34236 (to M. I. S.) and the Leukemia Society (to H. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† D. W. and H. J. contributed equally to this work.

‡ To whom correspondence and reprint requests should be addressed.

The abbreviations used are: G protein, guanine nucleotide-binding regulatory protein; PLC, phosphoinositide-specific phospholipase C; 5′-RACE, 5′ rapid amplification of cDNA end.

7593
that was used was basically the same as described in Ref. 26 with some modifications. In brief, total bovine retinal RNA (5 μg) was reverse-transcribed into single strand cDNA using the Invitrogen cDNA synthesis kit. Residual oligonucleotides were removed with 30,000 molecular weight cut-off spin filter units (Millipore). The cDNA was then tailed with dA by terminal deoxynucleotidyl transferase as described in Ref. 26 and amplified for 5 cycles with an oligonucleotide containing 16 dTs and a stretch of specific sequence (called the adaptor sequence) at low annealing temperature (40 °C). Finally, two oligonucleotides, one containing the adaptor sequence and the other derived from the known sequence of PLC β4 (20), were added, and the polymerase chain reaction was carried out at a higher annealing temperature (57 °C). The polymerase chain reaction products were separated on a 1.5% agarose gel and blotted onto a nylon membrane. The membrane was then probed with 32P-labeled PLC β4 cDNA kindly provided by Dr. Pak. The positive bands were cut out of the agarose gel and cloned into a pBluescript KS vector. The inserts were sequenced by the Sequenase Version 2 kit from U. S. Biochemical Corp. The sequences reported in this paper were confirmed by repetition with three independent clones.

Cell Culture and Transfection—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum at 37 °C with 5% CO2. For transfection, cells (1 x 10^6) were seeded into each well of a 12-well plate 1 day before transfection. The expression plasmids (1 μg) and lipofectamine (Life Technologies, Inc.) were mixed in 0.5 ml of Opti-MEM and added into one well. The medium was changed to normal growth medium 5 h after transfection. Cells were then labeled with 4 μCi of [3H]inositol the next day. The levels of inositol phosphates were determined as described (6) 1 day after the labeling.

SDS-Polyacrylamide Gel Electrophoresis and Western Analysis—Cells were lysed in SDS sample buffer and separated on an SDS polyacrylamide gel. The proteins were then electroblotted onto nitrocellulose membranes and detected with specific antibodies.

RESULTS AND DISCUSSION

In order to determine whether PLC β4 can be activated by Ga or Gβy subunits with the co-transfection assay, we must have the full-length PLC β4 cDNA. The sequence reported by Dr. Pak’s group (20) appears to be missing the N terminus based on comparisons with sequences of PLC β1, PLC β2, and PLC β3. Moreover, when the PLC β4 cDNA was subcloned into the pCMV expression vector and transfected into COS-7 cells, we could not detect any recombinant proteins using antibodies raised against a synthetic peptide derived from PLC β4 (data not shown). Thus, we concluded that the reported cDNA clone was incomplete. By using the 5′-RACE method, we have obtained a 550-base pair nucleotide sequence upstream of the reported cDNA sequence from bovine retina. This upstream sequence contains an open reading frame that specifies 165 amino acids and is in-frame with the amino acid sequence of the reported PLC β4. The alignment of this newly obtained amino acid sequence against the N-terminal sequences of other members of the PLC β family is shown in Fig. 1. The data indicate that it shares clear homology with other PLC β isoforms; the first methionine residue of this new sequence is located at a position comparable to that in the other PLC β isoforms. In addition, the nucleotide sequence upstream of the initiation codon is very similar to the Kozak consensus sequence (data not shown) (27). Therefore, the 165-amino acid sequence that we have identified is probably the N terminus of PLC β4.

The entire coding sequence of PLC β4 was obtained by combining the 550-base pair N-terminal sequence with the sequence reported by Dr. Pak’s group at the XbaI site and subcloned into a pCMV expression vector (28). When this plasmid was transfected into COS-7 cells, an ~120-130-kDa protein could be detected by antibodies specific for PLC β4 (Fig. 2). The size of this recombinant PLC β4 is clearly larger than the one purified by Min et al. (19), which is around 100 kDa. It is possible that the purified PLC β4 protein from bovine cerebellum results from alternative splicing or proteolytic degradation.

We have previously found that when COS-7 cells were cotransfected with cDNAs that express different α subunits of the Gq class together with cDNA constructs that express PLC β1 or PLC β2 there was a marked increase in inositol phosphates that could be clearly distinguished from levels found in cells transfected with either cDNA alone (6-8). These results provided evidence that the recombinant Ga subunits of the Gq class, some of which presumably exist in the GTP-bound form or are being activated by endogenous receptors, can activate PLC β1 and PLC β2. In this report, a similar co-transfection assay was used to test whether PLC β4 can be activated by any Ga or Gβy subunits. Our results indicate that the α subunits of the Gq class can activate PLC β4 (Fig. 3A). The expression level of each Ga protein was estimated by Western analysis using an antibody raised against a common sequence shared by all five Ga proteins. As shown in Fig. 2, all of the recombinant proteins were clearly expressed at detectable levels. Since it is very difficult to control the expression level of each transfected component in this assay, we cannot make precise quantitative comparisons among the Ga subunits with regard to their ability to activate PLC. However, it appears that the activation of PLC β4 by Go16 is rather poor (Fig. 3A). This is not the result of differences in protein expression. Go16 apparently activates PLC β1 and PLC β2 quite well, and, in these experiments, the expression level of Go16 is similar to or higher than that of Ga14 (Fig. 3B). We do not understand the basis for the rela-
A, or PLC 134 were co-transfected with cDNA encoding 13-galactosidase.

Differences in this region may underlie the different activation efficiencies of these Ga subunits.

The By subunits released from activated G proteins were shown to be able to activate PLC, which may account for the pertussis toxin-sensitive activation of PLC by many of the receptors such as IL-8 (32), C5a, etc. There appears to be some specificity in activation of PLC By isoforms by GBy subunits. Only PLC p2 but not PLC p1 or PLC p4 can be activated by GBy in co-transfection assays (15), whereas purified PLC p2 and PLC p3 as well as PLC p1 to a much less extent can be activated by GBy in reconstitution assays (16–18, 33). PLC p3 has not been tested in the co-transfection assay because the full-length cDNA is not available. We do not understand the discrepancy regarding activation of PLC p1 by GBy in the two different assay systems. However, these two assay systems are rather consistent in showing that PLC p4 could not be activated by GBy (34). It is very interesting to understand the molecular basis for the specificity in activation of PLC isoforms by GBy subunits. In a previous report, we determined that the region of PLC p2 required for activation by GBy includes the 400-amino acid sequence that ranges from the beginning of the X box to the end of Y box (8). Due to the large size of this region, we were not able to obtain any useful information about the molecular mechanism involved in activation. The inability of PLC p4 to be activated by retinal-specific GBy subunits or transducin suggests that PLC p4 is not activated directly in the phototransduction cascade and that it is most probably regulated by receptors other than rhodopsin. Nevertheless, the highly restrictive expression pattern and extensive homology to Norp A protein suggest that PLC p4 may play another role in maintaining the visual response in mammalian systems.

Acknowledgment—We would like to thank Drs. W. Pak for the partial PLC p4 cDNA and C.-H. Lee for the PLC p4 antibodies.

REFERENCES


3 D. Wu and M. I. Simon, unpublished results.
Activation of PLC β4