THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 274, No. 20, Issue of May 14, pp. 13900–13907, 1999
Printed in U.S.A.

AHNAK, a Protein That Binds and Activates Phospholipase C-γ1 in the Presence of Arachidonic Acid*

(Received for publication, January 5, 1999, and in revised form, February 25, 1999)

Fujio Sekiya‡§, Yun Soo Bae§¶, Deok Young Jhon‖, Sung Chul Hwang, and Sue Goo Rhee‡‡**

From the Laboratory of Cell Signaling, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

We have recently shown that phospholipase C-γ (PLC-γ) is activated by tau, a neuronal cell-specific microtubule-associated protein, in the presence of arachidonic acid. We now report that non-neuronal tissues also contain a protein that can activate PLC-γ in the presence of arachidonic acid. Purification of this activator from bovine lung cytosol yielded several proteins with apparent molecular sizes of 70–130 kDa. They were identified as fragments derived from an unusually large protein (~700 kDa) named AHNAK, which comprises about 30 repeated motifs each 128 amino acids in length. Two AHNAK fragments containing one and four of the repeated motifs, respectively, were expressed as glutathione *S*-transferase fusion proteins. Both recombinant proteins activated PLC-γ1 at nanomolar concentrations in the presence of arachidonic acid, suggesting that an intact AHNAK molecule contains multiple sites for PLC-γ activation. The role of arachidonic acid was to promote a physical interaction between AHNAK and PLC-γ1, and the activation by AHNAK and arachidonic acid was mainly attributable to reduction in the enzyme's apparent K_m toward the substrate phosphatidylinositol 4,5-bisphosphate. Our results suggest that arachidonic acid liberated by phospholipase A_2 can act as an additional trigger for PLC-γ activation, constituting an alternative mechanism that is independent of tyrosine phosphorylation.

Activation of phosphoinositide-specific phospholipase C (PLC)[1] is a key event in cellular signal transduction. PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), generating two second messengers, inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol. To date, a total of 10 different isozymes of PLC have been identified in mammalian cells, which can be classified into three major subfamilies, β (β1 to β4), γ (γ1 and γ2), and δ (δ1 to δ4) isozymes, based on their primary structures (1). Their structural differences correlate with varying mechanisms for their activation. Stimulation of β-isozymes by many agonists occurs through receptors coupled to heterotrimeric G-proteins and is mediated by the α-subunits of the G_q subfamily members and by βγ-subunits. In contrast, the γ-isozymes are activated when phosphorylated by various receptor-coupled protein-tyrosine kinases (1).

Several lines of evidence suggested alternative mechanisms for PLC-γ activation in the absence of tyrosine phosphorylation. Jones and Carpenter (2) reported that phosphatidic acid could activate both tyrosine-phosphorylated and -unphosphorylated forms of PLC-γ to a similar extent. Since phosphatidic acid is the immediate product of phosphatidylcholine hydrolysis by phospholipase D, activation of phospholipase D in cells may lead to subsequent activation of PLC-γ. We (3, 4) and others (5) have recently shown that the product of phosphatidylinositol 3-kinase, phosphatidylinositol 3,4,5-trisphosphate (PIP_3), is an activator of PLC-γ. A considerable portion (30–50%) of IP_3 generated in response to platelet-derived growth factor was not a consequence of tyrosine phosphorylation of PLC-γ but rather a secondary event following PIP_3 generation by platelet-derived growth factor-stimulated phosphatidylinositol 3-kinase (3, 5).

We have also shown that variously spliced forms of the microtubule-associated protein tau (6) stimulate PLC-γ activity independently of tyrosine phosphorylation in the presence of unsaturated fatty acids, such as arachidonic acid (AA). Although the concentration of AA in resting cells is quite low, a large quantity of AA can be liberated from phosphatidylcholine by the action of cytosolic phospholipase A_2 ($cPLA_2$) upon cell activation (7). Therefore, it is likely that certain stimuli that elicit $cPLA_2$ activation may indirectly cause the activation of PLC-γ if the tau proteins are present. Tau proteins are exclusively expressed in neuronal cells (8).

Here, we report that non-neuronal cells also contain a protein that can activate PLC-γ in concert with AA, and we identify it as AHNAK. Our finding further bolsters the thesis that indirect activation of PLC-γ can occur in the absence of tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Materials

Phosphatidylserine (PS) and phosphatidylethanolamine (PE) were purchased from Avanti Polar Lipids. AA and cholesterol were purchased from Calbiochem. PIP_2 was obtained from Roche Molecular Biochemicals. [*inositol*-2-^{3}H]PIP_2 and [2-^{3}H]*myo*-inositol were purchased from NEN Life Science Products. PLC isozymes (PLC-β1, -γ1, -γ2, and -δ1) were purified from HeLa cells that had been transfected with recombinant vaccinia virus containing the entire coding sequence of the respective enzyme as described (9).

* 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 1734 solely to indicate this fact.

‡ Japan Society for the Promotion of Science Fellow in Biomedical and Behavioral Research at the National Institutes of Health.

§ These authors contributed equally to this work.

¶ Present address: Dept. of Biological Sciences, College of Natural Sciences, Ewha Womans University, Seoul, Korea 120-750.

‖ Present address: Dept. of Food and Nutrition, College of Home Economics, Chonnam National University, Kwangju, Korea 500-757.

** Present address: Dept. of Pulmonary and Critical Care Medicine, Ajou University Medical School, Suwon, Korea 442-749.

‡‡ To whom correspondence should be addressed: Bldg. 3, Rm. 122, National Institutes of Health, Bethesda, MD 20892. Tel.: 301-496-9646; Fax: 301-480-0357; E-mail: sgrhee@nih.gov.

[1] The abbreviations used are: PLC, phosphoinositide-specific phospholipase C; $cPLA_2$, cytosolic phospholipase A_2; AA, arachidonic acid; GST; glutathione *S*-transferase; BSA, bovine serum albumin; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PE, phosphatidylethanolamine; PS, phosphatidylserine; IP_3, inositol 1,4,5-trisphosphate; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

Purification of PLC-γ Activator

All manipulations were performed at 4 °C unless otherwise indicated. During purification, PLC-γ1-activating activity was measured at 30 °C for 10 min in 100 μl of a reaction mixture containing 36,000 cpm of [^{3}H]PIP$_2$, 30 μM PIP$_2$, 120 μM PE, 30 μM PS, 30 μM cholesterol, 30 μM arachidonic acid, PLC-γ1 (10 ng), 3 mM $CaCl_2$, 2 mM EGTA, 0.033% (w/v) sodium deoxycholate, 50 mM Hepes-NaOH (pH 7.0), and a source of activator. To maintain the stimulated activity in the linear range of the assay, we adjusted the amount of PLC to obtain an unstimulated, basal activity in the range of a 500–1200 cpm of [^{3}H]IP$_3$ generated.

The purification procedure consisted of the following steps.

Preparation of Bovine Lung Cytosolic Extracts—Fresh bovine lungs (3.0 kg) were obtained from a local slaughterhouse and homogenized in 10 liters of a solution containing 20 mM Hepes-NaOH (pH 7.2), 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, leupeptin (1 μg/ml), and aprotinin (1 μg/ml) with a Waring blender. The homogenate was centrifuged at 1000 × *g* for 10 min, and the resulting supernatant was centrifuged further at 13,000 × *g* for 1 h.

Heat Treatment—Collected supernatant was heated at 80 °C for 10 min and centrifuged at 13,000 × *g* for 30 min. The resulting supernatant was filtered through Whatman No. 1 paper to eliminate lipids.

Heparin-Sepharose CL-4B—The heat-treated supernatant (5 g of protein) was applied to a heparin-Sepharose CL-4B column (5 × 30 cm, Amersham Pharmacia Biotech) that had been equilibrated with 20 mM Hepes-NaOH (pH 7.2), 1 mM EDTA, and 0.1 mM dithiothreitol. After washing with the equilibration buffer, bound proteins were eluted with 2 liters of a linear gradient of 0–1.0 M NaCl in the buffer at a flow rate of 10 ml/min. Fractions (20 ml) were collected and assayed for PLC-γ1-activating activity (see Fig. 1*A*). Peak fractions (fractions 59–61) were pooled and dialyzed against equilibration buffer.

HPLC on DEAE-5PW Column—The dialyzed protein (12 mg) from the previous step was applied to a TSKgel DEAE-5PW HPLC column (21.5 × 150 mm) that had been equilibrated with 20 mM Tris-HCl (pH 8.5), 1 mM EDTA, and 0.1 mM dithiothreitol. Proteins were eluted at a flow rate of 5 ml/min with equilibration buffer for 5 min followed by a linear gradient of 0–1.0 M NaCl in equilibration buffer over 40 min (see Fig. 1*B*). Fractions (5 ml) were collected and assayed for PLC-γ1-activating activity. Peak fractions (numbers 15–18) were pooled and dialyzed against equilibration buffer.

Electroelution of Proteins from SDS-Polyacrylamide Gels—Purified proteins (~700 μg) from the DEAE-5PW column were separated by preparative SDS-PAGE on 8% gel (3-mm thickness, single-well comb). The gel was stained lightly with Coomassie Brilliant Blue, and visualized protein bands were excised from the gel with a razor blade. The proteins were then eluted with an Electro-Eluter (C.B.S. Scientific, Del Mar, CA). Coomassie Brilliant Blue and SDS were extracted with isobutyl alcohol, and proteins were precipitated by cold acetone.

Tryptic Digestion and Amino Acid Sequencing

Electroeluted proteins (10 μg each) from three different bands (bands 1–3 in Fig. 1*C*) were digested with trypsin for 24 h and subjected to HPLC analysis on a Vydac C18 column (4.6 × 250 mm) equilibrated with 0.1% (w/v) trifluoroacetic acid. Peptides were eluted at a flow rate of 1 ml/min by a linear acetonitrile gradient of 0–70% (v/v) in 0.1% trifluoroacetic acid over 70 min. The peptides isolated were subjected to amino acid sequencing analysis.

PLC Assay

The mixed micellar substrate was prepared as follows. Chloroform solutions of lipids (PIP$_2$, PS, cholesterol, and PE in a molar ratio of 1:1:1:1:4 plus [^{3}H]PIP$_2$ as the tracer, together with various amounts of fatty acids where stated) were mixed and dried under N_2 stream. Dried lipids were dispersed by sonication in a buffer composed of 50 mM Hepes-NaOH (pH 7.0), 120 mM KCl, 10 mM NaCl, and 0.067% (w/v) sodium deoxycholate. The assay was started by mixing 50 μl of the micellar substrate with 50 μl of a solution containing PLC and the samples to be tested. The final assay mixture (100 μl) typically contained 10–20 ng of PLC-γ1; a 30 μM concentration each of [^{3}H]PIP$_2$ (8–10 × 10^3 cpm), PS, and cholesterol; 120 μM PE in 50 mM Hepes-NaOH (pH 7.0); 120 mM KCl; 10 mM NaCl; 0.033% deoxycholate; 1 mM $MgCl_2$; 2 mM EGTA; and 1 μM free Ca^{2+} ions, unless otherwise stated. After incubation for 10 min at 30 °C, the reactions were terminated by the addition of 200 μl of 10% (w/v) trichloroacetic acid and 100 μl of 10% (w/v) bovine serum albumin (BSA), followed by centrifugation. The amount of radioactivity in the resulting supernatant, corresponding to liberated [^{3}H]IP$_3$, was measured by a liquid scintillation counter.

In the case of the kinetic analysis, the micellar substrate was prepared with [^{3}H]PIP$_2$ without other lipids in a buffer containing 10 mM octyl glucoside instead of sodium deoxycholate.

Detection of Complex Formation of AHNAK and PLC-γ

Wells of microtiter plates (Immuno Plate MaxiSorp, Nunc) were coated with AHNAK by incubating with 100 μl of a 10 μg/ml protein solution in 50 mM Hepes-NaOH (pH 7.0) at 4 °C overnight. After further incubation with 300 μl of 1% BSA for 1 h, wells were washed twice with 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.02% (v/v) Tween 20. Incubation with PLC-γ1 was carried out in a total volume of 100 μl of a buffer containing 50 mM Hepes-NaOH (pH 7.0), 120 mM KCl, 10 mM NaCl, 1 mM dithiothreitol, 1 mM $MgCl_2$, 2 mM EGTA, 1 μM free Ca^{2+} ions, 0.1 mg/ml BSA, and 100 μM arachidonic acid plus various amounts of PLC-γ1 and proteins to be tested at ambient temperature for 1 h. Wells were then washed twice with 200 μl of the same buffer containing 0.02% Tween 20. Bound materials were eluted by incubating with 50 μl of SDS-PAGE sample buffer for 30 min, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. PLC-γ1 on the membrane was detected by using a specific monoclonal antibody (F-7–2) (10) and alkaline phosphatase-conjugated anti-mouse IgG goat antibody.

RESULTS

Purification of PLC-γ-activating Proteins from Bovine Lung—We previously noticed that the addition of crude cytosol of bovine brain or HeLa cells to purified PLC-γ markedly enhanced PIP$_2$-hydrolyzing activity measured in the presence of AA. We then identified the bovine brain activator as tau (6). Since tau is exclusively expressed in neuronal cells, we presumed that the stimulating activity seen in HeLa cell extract was due to a protein with functional similarity to tau and initiated the isolation of this presumed non-neuronal PLC-γ activator.

We screened various tissues and selected bovine lung cytosol for large scale purification of the PLC-γ activator. The increase in PLC-γ activity toward a mixed micellar substrate containing PIP$_2$ and AA was monitored throughout the purification. Treatment of bovine lung cytosol at 80 °C for 10 min resulted in a great enrichment of PLC-γ activator (data not shown). The heat-treated cytosol fraction was subjected to sequential chromatographies on heparin-Sepharose CL-4B and DEAE-5PW columns to yield a sharp activity peak that coincided with a discrete protein peak (Fig. 1, *A* and *B*). SDS-PAGE of the peak fraction showed closely spaced protein bands with apparent molecular masses ranging from 70 to 130 kDa (Fig. 1*C*, *lane T*). Further attempts at purification including gel filtration on TSKgel G3000-SW or ion exchange chromatography on Mono Q failed to separate these proteins. Thus, proteins were fractionated on a preparative SDS-polyacrylamide gel, and four major protein bands between 70 and 130 kDa were excised and electroeluted (Fig. 1*C*). After the removal of SDS, each eluted protein was tested for PLC-γ-activating activity. All of the eluted proteins activated PLC-γ1 (Fig. 1*D*).

Identification of the PLC-γ Activator as AHNAK—Three of the electroeluted proteins (bands 1–3 in Fig. 1*C*) were individually digested with trypsin and analyzed on a reverse phase HPLC column. The peptide maps for bands 1, 2, and 3 were very similar (data not shown), suggesting that these three proteins were related. Seven peptides including two that were common to all three electroeluted proteins were sequenced to yield 1) LKGPK, 2) VDIDVPDVDVQGPDWHL, 3) FSMPGFK, 4) VPDVNIEGPDAK, 5) VKGDVDVSLPK, 6) ADIEISGPK, and 7) GDVDVSLPK. A search of data bases revealed that the sequence of an unusually large protein named AHNAK (meaning "giant" in Hebrew; Ref. 11) carries exact coding sequences for all seven tryptic peptides; the sequences of peptides 1, 3, 4, 5, and 7 were found 29, 17, 9, 10, and 9 times, respectively, in the different regions of AHNAK, whereas the sequence of peptide 2 was found to match AHNAK residues 3923–3939 and 4387–4403, and peptide 6 corresponded only to AHNAK resi-

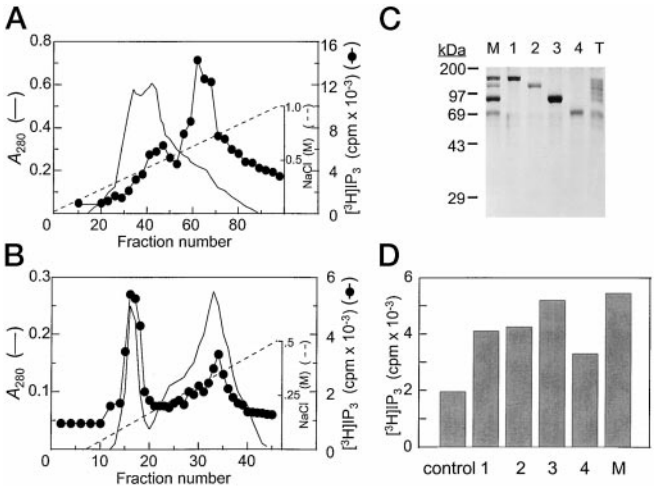

FIG. 1. **Purification of PLC-γ1-activating protein.** Heat-treated bovine lung cytosolic proteins were subjected to a heparin-Sepharose CL-4B column (*A*) and then to an HPLC DEAE-5PW (*B*) column. Fractions were assayed for PLC-γ1-activating activity (*closed circles*) as described under "Experimental Procedures." The peak fractions (700 μg) pooled from the DEAE-5PW column chromatography step were separated on a preparative SDS-polyacrylamide gel. Four protein bands (bands 1–4, beginning with the largest protein) with apparent molecular size of 130, 110, 85, and 70 kDa were excised and electroeluted from the gel. A recombined mixture of the four bands (*lane M*), band 1 (*lane 1*), band 2 (*lane 2*), band 3 (*lane 3*), band 4 (*lane 4*), and the pooled peak fraction from the DEAE-5PW column (*lane T*) were then subjected to SDS-PAGE on an 8% gel and visualized by staining with Coomassie Brilliant Blue (*C*). The PLC-γ1-activating activity of the electroeluted proteins was measured in the absence (*control bar*) and in the presence of ~200 ng of band 1 (*bar 1*), band 2 (*bar 2*), band 3 (*bar 3*), band 4 (*bar 4*), or a recombined mixture of the four bands (*bar M*) using an assay mixture containing 50 ng of PLC-γ1 (*D*). The positions of molecular size standards are shown on the *left* in *C*.

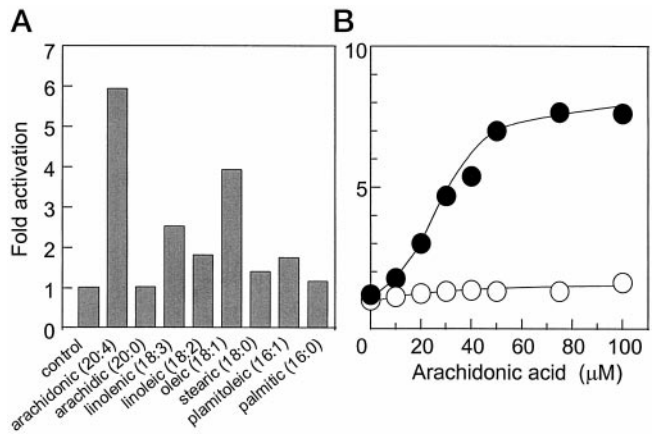

FIG. 2. **Effects of various fatty acids on PLC-γ1 activity.** *A*, the PIP$_2$-hydrolyzing activity of PLC-γ1 (20 ng/assay) was measured in the absence (*control*) or presence of the indicated fatty acid. The assay mixture contained 10 μg/ml AHNAK purified from lung and mixed micellar substrates including the indicated fatty acids at a final concentration of 30 μM. *B*, the PIP$_2$-hydrolyzing activity of PLC-γ1 was measured in the absence (*open circles*) or presence (*closed circles*) of 10 μg/ml purified activator protein with substrate vesicles containing the indicated concentrations of AA. Results are expressed as -fold activation over the activities obtained in the absence of fatty acids.

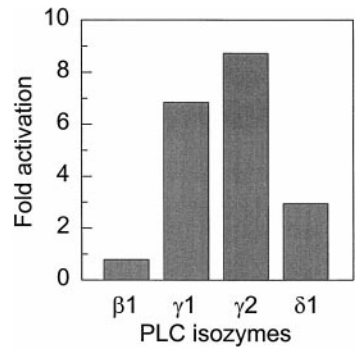

FIG. 3. **Combined effects of AA and the AHNAK fragments purified from lung on the PIP$_2$-hydrolyzing activity of various PLC isozymes.** The PIP$_2$-hydrolyzing activities of the indicated PLC isozymes (20–100 ng/assay) were measured in the absence or presence of 10 μg/ml AHNAK purified from lung with substrate vesicles containing 50 μM AA. Results are expressed as -fold activation over unstimulated activities obtained in the absence of AHNAK.

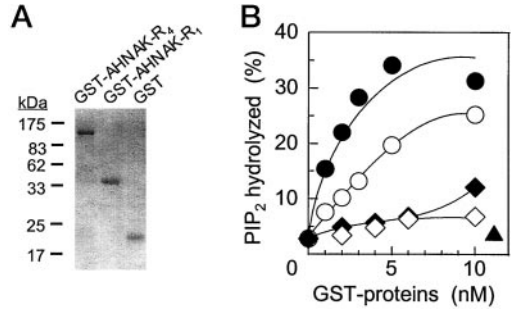

FIG. 4. **Activation of PLC-γ1 by GST-AHNAK fusion proteins.** *A*, SDS-PAGE of purified GST fusion proteins. *B*, the percentage of PIP$_2$ hydrolyzed by PLC-γ1 (20 ng/assay) was measured in the presence of indicated concentrations of GST-AHNAK-R$_4$ alone (*closed diamonds*), GST-AHNAK-R$_1$ alone (*open diamonds*), GST-AHNAK-R$_4$ plus 100 μM AA (*closed circles*), or GST-AHNAK-R$_1$ plus 100 μM AA (*open circles*) with assay mixtures containing 1 μM free Ca^{2+} ions and 1 mM Mg^{2+} ions. The percentage of PIP$_2$ hydrolyzed in the presence of 100 nM GST and 100 μM AA is indicated by the *closed triangle*.

dues 4096–4104. AHNAK is a 5643-membered protein composed of highly conserved repeated motifs (see below). Thus, the purified activator proteins are probably fragments derived from AHNAK protein.

Activation of PLC-γ by the Purified AHNAK Fragments—The ability of the purified AHNAK fragments to activate PLC-γ, measured with a mixed micellar substrate containing PIP$_2$ plus PE, PS, cholesterol, and sodium deoxycholate, was dependent on unsaturated fatty acids. As shown in Fig. 2*A*, various unsaturated fatty acids were all somewhat effective in stimulating PLC-γ in the presence of AHNAK when tested at a concentration of 30 μM, whereas these lipids alone had no effect. However, corresponding saturated fatty acids were ineffective even at higher concentrations (up to 100 μM). Among those tested, AA was the most potent stimulant of PLC-γ activity in the presence of the activator, and half-maximal stimulation was observed at 25 μM (Fig. 2*B*). AA was equally effective when added to preformed micelles as when incorporated into the substrate micelles (data not shown).

The combined effects of the purified lung AHNAK and AA on PIP$_2$ hydrolysis by PLC-β1, -γ1, -γ2, and -δ1 were compared in the presence of an excess amount of AHNAK (Fig. 3). Activation by AHNAK and AA were apparent with both PLC-γ1 and PLC-γ2 (7–9-fold). Stimulation of the activity of PLC-δ1 was less apparent, and PLC-β1 activity was not affected at all.

Activation of PLC-γ1 by Glutathione S-Transferase (GST)-AHNAK Fusion Protein—AHNAK contains approximately 30 repeats of a highly conserved motif, most of which are 128 amino acids in length (see Fig. 13). We investigated if one or several units of this repeated motif are capable of stimulating PLC-γ1. We prepared two GST-AHNAK fusion proteins (Fig. 4*A*), one containing only one repeat (GST-AHNAK-R$_1$) corresponding to AHNAK residues 3470–3882 and the other containing three full repeats and two split one-half repeats (GST-AHNAK-R$_4$) corresponding to residues 3817–4412 (see Fig.

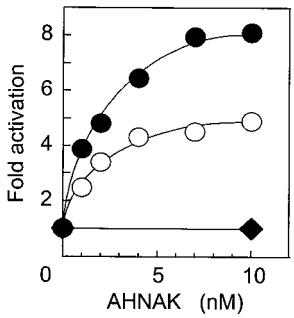

FIG. 5. **Effects of various concentrations of recombinant AHNAK protein on the PIP$_2$-hydrolyzing activity of PLC isozymes.** The PIP$_2$-hydrolyzing activities of PLC-β1 (*closed diamonds*), PLC-γ1 (*closed circles*), and PLC-δ1 (*open circles*) were measured in the presence of 100 μM AA plus the indicated concentrations of GST-AHNAK-R$_4$. Other conditions were as described in the legend to Fig. 4. Results are expressed as -fold activation over unstimulated activities obtained in the absence of AHNAK.

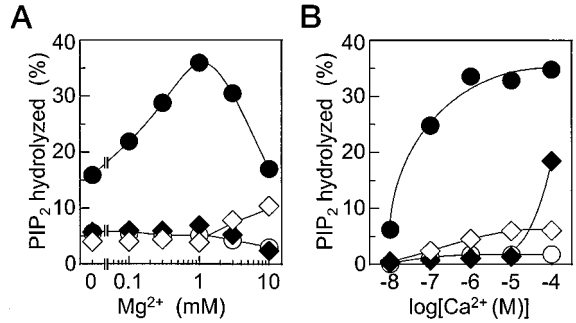

FIG. 6. **Effect of Mg^{2+} and Ca^{2+} ions on the AHNAK- and AA-dependent PLC-γ1 activity.** *A*, the percentage of PIP$_2$ hydrolyzed by PLC-γ1 (20 ng/assay) was measured at the indicated Mg^{2+} concentrations in the absence (*open circles*) or in the presence of 10 nM GST-AHNAK-R$_4$ alone (*closed diamonds*), 100 μM AA alone (*open diamonds*), or 10 nM GST-AHNAK-R$_4$ plus 100 μM AA (*closed circles*). The assay mixture contained 1 μM free Ca^{2+} ions. *B*, the effect of Ca^{2+} ions on the percentage of PIP$_2$ hydrolyzed by PLC-γ1 was assessed using assay mixtures containing 1 mM Mg^{2+} ions and indicated concentrations of free Ca^{2+} ions. The *symbols* used to represent the different combinations of 100 μM AA and 10 nM GST-AHNAK-R$_4$ are the same as in *A*.

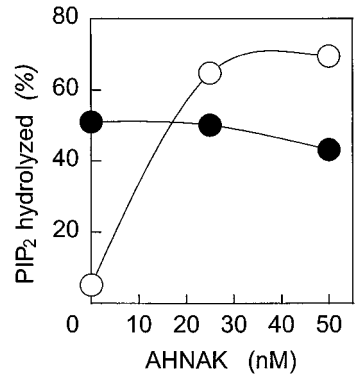

FIG. 7. **Effect of tau on the PLC-γ1 activation by AHNAK.** The percentage of PIP$_2$ hydrolyzed by PLC-γ1 was measured in the absence (*open circles*) or the presence of 20 μg/ml tau (*closed circles*) using assay mixtures containing 100 μM AA and the indicated concentrations of GST-AHNAK-R$_4$.

13*B*). The two GST-AHNAK fusion proteins from *E. coli* could stimulate the PIP$_2$-hydrolyzing activity of PLC-γ1 in a dose-dependent manner in the presence of AA, whereas GST alone in concentrations up to 100 nM had little effect on the activity (Fig. 4*B*). GST-AHNAK-R$_1$ was somewhat less potent than the longer form, but both stimulated apparent PLC-γ1 activity more than 10-fold at concentrations as low as 10 nM. Half-maximal stimulation by the longer form was observed at 1.5 nM in the presence of 100 μM AA. In the absence of AA, both forms were ineffective in stimulating PLC-γ1, consistent with the behavior of the AHNAK fragments purified from bovine lung. We also reevaluated the sensitivity of PLC-β1, -γ1, and -δ1 isozymes using the GST-AHNAK fusion protein (Fig. 5). Activation of PLC-γ1 was again most efficient. PLC-δ1 was also activated by the combination of AA and GST-AHNAK-R$_4$ but to a lesser extent compared with PLC-γ1, and the activity of PLC-β1 was not affected at all, thus confirming the result with AHNAK fragments purified from bovine lung (Fig. 3).

In addition, the stimulation of PLC-γ1 activity was partially dependent on Mg^{2+} ions. At a free Ca^{2+} concentration of 10^{-6} M, the stimulation was maximum at an Mg^{2+} concentration of 1 mM, which is close to physiological concentrations, and declined at higher concentrations (Fig. 6*A*). The mechanism by which Mg^{2+} ions potentiated AHNAK-stimulated activity was not clear, but the basal activity of PLC-γ1 in the absence of AHNAK and AA was not significantly affected by the addition of Mg^{2+} ions. Stimulation of PLC-γ1 activity by GST-AHNAK-R$_4$ plus AA was also investigated at different concentrations of free Ca^{2+} ions (Fig. 6*B*). Activities of all PLC isozymes are dependent on Ca^{2+} ions (12), and the activity of PLC-γ1 increases rapidly from 10^{-8} M of Ca^{2+} to reach a plateau at 10^{-6} M of Ca^{2+} (13). The PLC-γ1 activity stimulated by GST-AHNAK-R$_4$ plus AA showed a similar Ca^{2+} dependence. AA alone had some stimulative action at 10^{-4} M of Ca^{2+} (Fig. 6*B*).

Activation of PLC-γ1 by AHNAK in the Presence of Tau—Because the tau protein also stimulates PLC-γ1 and PLC-γ2 rather specifically among various PLC isozymes in the presence of AA (6), we asked whether tau and AHNAK share a common activation mechanism. As shown in Fig. 7, AHNAK was no longer effective at enhancing the PLC-γ1 activity in the presence of a saturating concentration of tau. This result indicates that the tau interaction site on PLC-γ overlaps with that of AHNAK.

Kinetic Analysis of the Activation by AHNAK—The effect of AHNAK and AA on PLC-γ activity at various concentrations of PIP$_2$ was evaluated using a micellar substrate system composed of PIP$_2$ and octyl glucoside. In the presence of 2–20 mM octyl glucoside, the PIP$_2$-hydrolyzing activity of PLC-γ was comparable with that measured with the mixed micellar substrate containing PIP$_2$ plus PE, PS, cholesterol, and sodium deoxycholate (data not shown). With this simplified substrate system, AHNAK and AA activated PLC-γ activity only 3-fold, which compares with the 7–9-fold activation observed in Figs. 2–4 with the substrate consisting of PIP$_2$, PE, PS, cholesterol, and sodium deoxycholate. However, the effective concentrations of AHNAK and AA were unchanged. The initial rate of PIP$_2$ hydrolysis was measured with varying concentration of PIP$_2$ in the presence and absence of GST-AHNAK-R$_4$ and AA. A plot of these data according to the Eaddie-Hofstee equation gave fairly straight lines (Fig. 8). It appeared that the apparent V_{max} was not affected by the presence of saturating concentrations of GST-AHNAK-R$_4$ and AA, whereas the apparent K_m for PIP$_2$ was considerably reduced from 24 ± 4 μM (mean ± S.E., n = 4) to 8 ± 1 μM (n = 4).

Direct Binding of AHNAK to PLC-γ1 in the Presence of AA—Because AHNAK could activate PLC-γ1 efficiently even at concentrations as low as 1 nM (Fig. 4*B*), we presumed that AHNAK might form a tight complex with PLC-γ1. To prove this, GST-AHNAK-R$_4$ was immobilized onto the wells of microtiter plates, and PLC-γ1 was incubated in the coated wells containing AA. Bound PLC-γ1 was eluted from the wells with a SDS-containing buffer and detected by immunoblot analysis. Binding of PLC-γ1 to immobilized AHNAK was clearly detected,

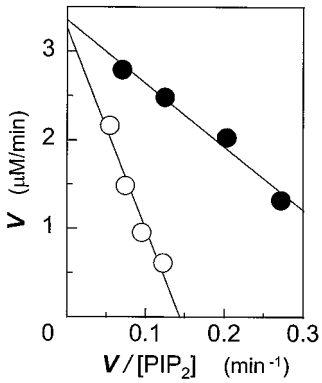

FIG. 8. **Eddie-Hofstee plot of PLC-γ1 reaction measured in the absence and presence of GST-AHNAK-R$_4$ plus AA.** The hydrolysis of substrate PIP$_2$ by PLC-γ1 (20 ng/assay) was followed in the absence (*open circle*) and presence of 20 nM GST-AHNAK-R$_4$ plus 100 μM AA (*closed circle*) using micellar substrates containing 5 mM octyl glucoside. The initial velocities (V) monitored over 5 min were plotted against V/[PIP$_2$] according to the Eddie-Hofstee equation ($V = -K_m \times V/[PIP_2] + V_{max}$). The V_{max} values that correspond to the intercept at the y axis were nearly identical in the absence and presence of the activators. The K_m values, represented by the slope, were 24 ± 4 and 8 ± 1 μM, respectively, in the absence and presence of the activators. Data are expressed as means of four independent determinations.

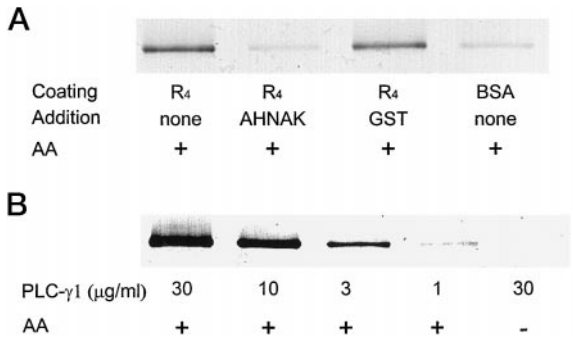

FIG. 9. **Direct interaction of AHNAK with PLC-γ1.** *A*, wells of microtiter plates were coated with GST-AHNAK-R$_4$ (R_4) or BSA. After blocking unoccupied surface with BSA, a solution of 10 μg/ml PLC-γ1 was added and incubated in the presence of 100 μM AA with 100 nM GST-AHNAK-R$_4$ or 100 nM GST or without additional proteins. After washing, proteins bound to the wells were eluted and subjected to immunoblot analysis with a monoclonal antibody to PLC-γ1. *B*, PLC-γ1 at the indicated concentrations was incubated in the absence or presence of 100 μM AA in the wells coated with GST-AHNAK-R$_4$, and the amount of bound PLC-γ was measured by immunoblot analysis as in *A*.

while no binding was seen with a control well that had been coated with BSA (Fig. 9*A*). The binding to GST-AHNAK-R$_4$ was completely abolished by the addition of GST-AHNAK-R$_4$, but not by the addition of GST, to the well containing PLC-γ (Fig. 9*A*). Moreover, we found that the binding was dependent on AA. As is shown in Fig. 9*B*, in the presence of AA the binding was detectable at PLC-γ1 concentrations as low as 1 μg/ml and increased in a PLC-γ1 concentration-dependent manner. However, in the absence of AA, the binding was hardly seen even at 30 μg/ml PLC-γ1. These results indicate that PLC-γ1 directly interacts with AHNAK, and the role of AA is to potentiate the interaction.

We could also precipitate the complex of GST-AHNAK-R$_4$ and PLC-γ1 in the presence of AA using glutathione-conjugated agarose beads (data not shown). However, this experiment suffered from high background, probably due to nonspecific binding of PLC-γ1 to the beads.

Stimulation of PLC in Crude Cell Extract by AA—Increasing amounts of cytosolic fractions of HeLa cells, which are rich in AHNAK and PLC-γ1, were incubated with a mixed micellar

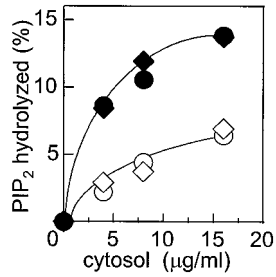

FIG. 10. **Effect of AA and AHNAK on PLC activity in crude cell lysate.** A confluent culture of HeLa cells grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum was harvested; washed with phosphate-buffered saline; suspended in 50 mM Hepes-NaOH (pH 7.0), 1 mM EDTA, 1 mM EGTA; and then lysed by sonication. The lysate was centrifuged at 100,000 × g for 15 min. PIP$_2$ hydrolyzed by indicated amounts of the resulting supernatant was measured by incubating with mixed micellar substrates for 5 min at 30 °C in the absence (*open circles*) or presence of 100 μM stearic acid (*open diamonds*), 100 μM AA (*closed diamonds*), or 100 μM AA plus 20 nM GST-AHNAK-R$_4$ (*closed circles*).

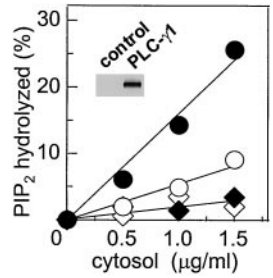

FIG. 11. **Identification of PLC-γ as the major target of activation by AHNAK and AA in fibroblast extracts.** PLC-γ1 null TV-1 cells, which were established from embryonic fibroblasts of *Plcg1* (−/−) mouse (14), were infected with vaccinia virus harboring PLC-γ1 gene or with empty virus and cultured for 2 days. Cytosolic fractions were obtained from these cells as described in the legend to Fig. 10. PIP$_2$ hydrolyzed by the indicated amounts of the cytosolic fractions from PLC-γ1-expressing cells (*circles*) and control cells (*diamonds*) were measured by incubating with the mixed micellar substrates for 10 min at 30 °C in the absence (*open symbols*) or in the presence of 100 μM AA (*closed symbols*). *Inset,* the presence of PLC-γ1 in the cytosolic fractions of control and PLC-γ1-expressing cells was detected by immunoblot analysis with a monoclonal antibody to PLC-γ1.

substrate containing [^{3}H]PIP$_2$ and octyl glucoside. Hydrolysis of [^{3}H]PIP$_2$ was stimulated by the addition of AA, but a saturated fatty acid (stearic acid) was ineffective (Fig. 10). The addition of recombinant AHNAK to the reaction mixture did not further the effect of AA. Thus, it appeared that these cells contained a saturating amount of AA-dependent activator for PLC and also that no other components in the soluble fraction of HeLa cells significantly inhibited this activation process. Background activity in the absence of AA was probably due to PLC-γ1 as well as other PLC isozymes like PLC-β1, PLC-β3, and PLC-δ1 that are known to exist in HeLa cells (HeLa cells do not contain PLC-γ2 isozyme).[2]

Since AHNAK and AA stimulated PLC-δ1 isozyme to a smaller but significant extent (Figs. 3 and 5), we evaluated the contribution of PLC-γ1 to the AHNAK/AA-stimulated activity by employing TV-1 embryonic fibroblasts derived from the PLC-γ1 null mouse (14). The TV-1 cells expressed no detectable amount of PLC-γ1 and very low levels of PLC-γ2 (14, 15). PLC-γ1-expressing cells were obtained by infecting TV-1 cells with vaccinia virus harboring the PLC-γ1 gene. Expression of PLC-γ1 by the viral vector was detected by immunoblot analysis (Fig. 11, *inset*). Cytosolic fractions were obtained from either control TV-1 cells infected with empty virus or the PLC-

[2] S. B. Lee and S. G. Rhee, unpublished observation.

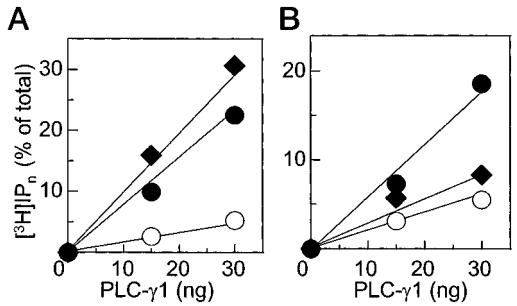

FIG. 12. **Isolated cell membrane as the substrate of PLC-γ1 activated by AHNAK and AA.** *A*, HeLa cells were labeled with [^{3}H]*myo*-inositol (10 μCi/10-cm dish) for 24 h in inositol-free Dulbecco's modified Eagle's medium; suspended in 50 mM Hepes-NaOH (pH 7.0), 3 mM EDTA, 3 mM EGTA; and lysed by sonication. Membranes were isolated by centrifugation (100,000 × *g*, 15 min); washed once with the sonication buffer and then with the sonication buffer containing 3 M KCl; and suspended in 50 mM Hepes-NaOH (pH 7.0), 120 mM KCl, 10 mM NaCl, and 0.067% deoxycholate. Labeled membranes (4000 cpm/assay) thus prepared were incubated with the indicated amounts of PLC-γ1 at 30 °C for 10 min, and liberation of [^{3}H]inositol phosphates ([^{3}H]IP$_n$) was measured in the absence of both AA and GST-AHNAK-R$_4$ (*open circles*) or in the presence of 100 μM AA alone (*closed diamonds*) or 20 nM GST-AHNAK-R$_4$ plus AA (*closed circles*). *B*, one volume of the membrane suspension from *A* was extracted with 6 volumes of a solution containing chloroform, methanol, and concentrated HCl (50:50:0.3, v/v/v) and then centrifuged. To the resulting supernatant, 2 volumes of 1 M HCl were added, and the organic phase was separated. The extracted lipids in the organic layer were dried under an N_2 stream and suspended in 50 mM Hepes-NaOH (pH 7.0), 120 mM KCl, 10 mM NaCl, 0.067% sodium deoxycholate. Using this extracted lipid preparation, PLC-γ1 reactions were carried out as in *A*.

γ1-expressing TV-1 cells, and the PIP$_2$-hydrolyzing activity of the cytosolic fractions was measured in the presence and absence of AA (Fig. 11). The PLC activity in the control cells was low and not affected by the addition of AA. In contrast, the PLC activity in the cytosol from PLC-γ1-expressing cells was higher than that from control cells and substantially enhanced by the addition of AA. These results indicate that the γ-isozyme is the main target of AA stimulation.

[^{3}H]Inositol-labeled Membrane as the Substrate of PLC-γ1 Activated by AHNAK and AA—Membranes were isolated from HeLa cells that had been metabolically labeled with [^{3}H]*myo*-inositol for 24 h, washed, and used as substrate for exogenously added PLC-γ1. AA again augmented the release of inositol phosphates by PLC-γ1, but the addition of recombinant AHNAK did not cause further augmentation (Fig. 12*A*). In a separate experiment, the labeled membranes were extracted with organic solvents to remove proteins and then used as the substrate. This time, AA alone did not cause much stimulation of PLC-γ activity, whereas the addition of AHNAK rendered AA capable of stimulating PLC-γ activity. (Fig. 12*B*). These results suggest that the buffer-washed membrane preparation contained enough AHNAK to activate PLC-γ1 in the presence of AA and that AHNAK and AA were capable of stimulating the activity of PLC-γ1 toward cell membrane phosphoinositides.

DISCUSSION

In the present study, we find that, like tau, AHNAK activates PLC-γ in the presence of AA. AHNAK was initially isolated as a 680-kDa protein uniquely localized to the desmosomes of bovine muzzle epidermal cells and termed desmoyokin (16). The same protein was also independently identified by Bishop and colleagues as the product of a gene whose expression is repressed in human neuroblastomas and several other types of tumor cell lines and named AHNAK to convey its exceptional size (11). Bishop's group showed that AHNAK was translated from a 17.5-kb mRNA and isolated two cDNA clones of 5.5 kb (GenBank™ accession number M80902) and 4.0 kb

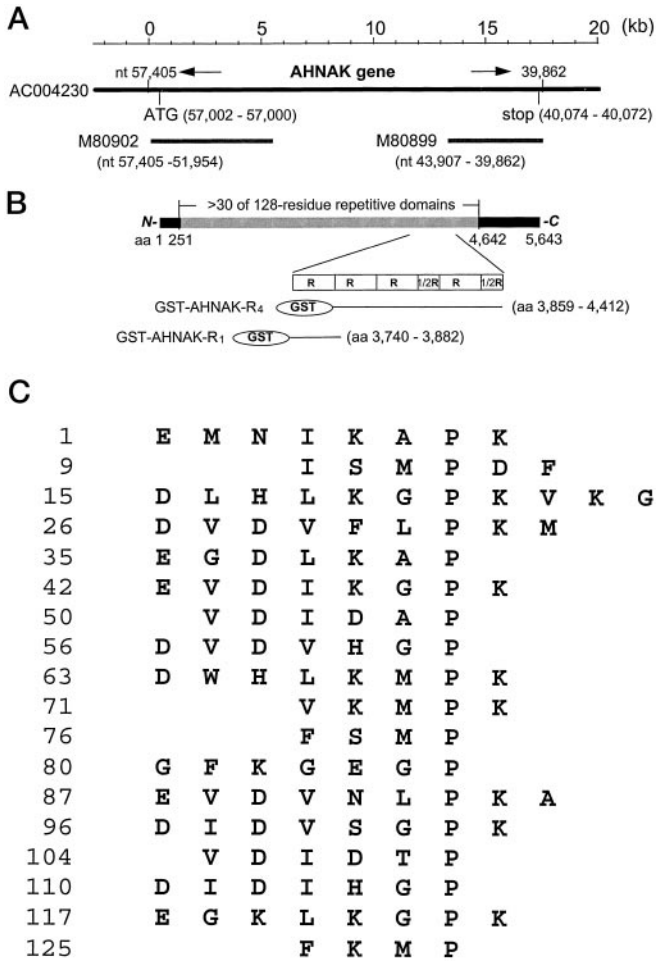

FIG. 13. **AHNAK sequence.** *A*, AHNAK gene sequence derived from the sequence of chromosome band 11q12 (GenBank™ accession number AC004230) includes the sequences of two cDNAs (accession numbers M80902 and M80899) identified in the laboratory of Bishop (11). The nucleotide residue numbers derived from AC004230 are indicated. *B*, amino acid (*aa*) sequence deduced from the sequence of AC004230 indicates that AHNAK protein can be divided into three structural regions, the amino-terminal 251 amino acids (*black line*), a large central region of 4391 amino acids with multiple repeated motifs (*gray line*), and the carboxyl-terminal 1002 amino acids (*black line*). The regions included in the GST fusion proteins, GST-AHNAK-R$_4$ and GST-AHNAK-R$_1$ are indicated. *R* and ½*R* represent 1 and ½ unit, respectively, of the 128 amino acids motif. *C*, repetitive units within the 128-amino acid motif of amino acid residues 3746–3873 are shown.

(accession number M80899) that encode the N-terminal 1683 amino acids and the C-terminal 1277 amino acids, respectively (Fig. 13*A*). From the amino acid sequence derived from the two cDNA clones, which represented about 50% of the entire AHNAK coding sequence, it was suggested that the AHNAK protein can be divided into three structural regions: the amino-terminal 251 amino acids, a large central region of about 4300 amino acids with multiple repeated motifs, and the carboxyl-terminal 1002 amino acids (Fig. 13*B*). Subsequently, the AHNAK gene was localized to human chromosome band 11q12 (17). The region 11q12 that includes the sequences of clones M80902 and M80899 has recently been established (accession number AC004230). The genomic sequence predicts a length of 5643 amino acids for AHNAK. As suggested before, the central region of AHNAK is composed of approximately 30 repeated motifs. The repeated unit is 128 amino acids in length, but some of the units are less than 128 residues. The 128-residue motifs are on average approximately 80% identical to each other with respect to amino acid sequence. Examination of the

amino acid sequence within the repeated unit reveals a number of recurrences of heptasequence (or octasequence) (D/E)ΦΩΦK(A/G)P(K), where Φ represents a hydrophobic residue, Ω represents a hydrophilic residue, and other letters correspond to conventional one-letter representation of amino acid residues (Fig. 13*C*).

The AHNAK genomic locus has no intron, suggesting that the 70–130-kDa activator proteins purified from bovine lung are probably proteolytic fragments of AHNAK rather than splice variants. To study this possibility, immunoblot analyses of HeLa cell extracts were carried out using rabbit serum prepared against the purified 110-kDa activator protein. A major protein band with an approximate size of 700 kDa was seen together with several minor bands of smaller size when extracts were freshly prepared in the presence of a mixture of protease inhibitors. However, when the extracts were prepared in the absence of protease inhibitors or the extracts were prepared with protease inhibitors but aged for several hours prior to immunoblot analysis, the 700-kDa band was not detectable, and instead the intensity of lower molecular bands was increased. This result suggests that AHNAK is readily susceptible to proteolysis.

AHNAK appears to be an abundant and ubiquitous protein found in various cellular compartments. AHNAK was located primarily (but not exclusively) in the nucleus of HeLa cells and phosphorylated on serine and threonine (18). In contrast, desmoyokin (AHNAK) was found mainly in the plasma membrane and to a lesser extent in the cytoplasm in squamous cell carcinomas, and it showed strong cytoplasm staining in melanomas (19). It was also claimed that different antibodies could yield different staining patterns in a given cell. In keratinocytes, AHNAK was mainly in the cytoplasm when cells were kept in low Ca^{2+} medium but translocated to the plasma membranes with a concomitant increase in the degree of phosphorylation upon an increase in extracellular Ca^{2+} concentrations or treatment with phorbol ester (20). The level of expression as well as the state of phosphorylation of the protein in several transformed cells was altered during growth and differentiation (18).

Despite these extensive studies on structure and cellular localization of AHNAK, no information has been available that might hint at the cellular function of AHNAK. Our current report is the first such insight.

Activation of PLC-γ isozymes by AHNAK was indistinguishable in many aspects from activation by tau (6), although there is no similarity in their primary structures except that both proteins are replete with proline residues. These two activators competed against each other in activating PLC-γ, indicating that they may share a common mechanism of activation. Recent reports have revealed that tau can interact with all three of the activation reaction components, PLC-γ (21), PIP_2 (22), and AA (23). Tau was shown to co-immunoprecipitate with PLC-γ, but not with β- or δ-isozymes of PLC, from lysate of neuroblastoma cells (21). Tau was also shown to interact with PIP_2, since divalent cation-induced aggregation of PIP_2 vesicles was modified by tau in a manner similar to that caused by the known PIP_2-binding protein, gelsolin (22). Modulation of tau's function by fatty acids was also evident from the fact that filament formation by tau was stimulated by free fatty acids, with AA as the most effective and saturated fatty acids much less potent (23).

We have shown here that AHNAK can directly interact with PLC-γ1 in the presence of AA. It seems that AHNAK and tau serve as the receptors for unsaturated fatty acids, whereas PLC-γ1 is not affected by fatty acids, since the activity of the enzyme was unchanged by fatty acids in the absence of the activator proteins. As a consequence of interaction with the AA-bound AHNAK, the apparent K_m for PIP_2 decreased. It should be noted that enzymes that catalyze reactions on the water-lipid interface usually do not obey Michaelis-Menten kinetics but rather obey "surface dilution kinetics" (24). This is also the case for PLC-γ1 (13). The "apparent K_m" values we obtained here actually contain two parameters, the affinity for micellar surface and the affinity for PIP_2 molecule *per se*. More experiments are needed to dissect the apparent K_m value into these two parameters. It is nevertheless apparent that the effect of AHNAK plus AA results in more efficient recognition of the micellar substrate, which can be caused by increase in the affinity for hydrophobic surface, for PIP_2, or for both. In this connection, it is interesting to note that the activation of PLC-γ via tyrosine phosphorylation is also attributable to an increase in the affinity for substrate but not to an increase in the turnover number (V_{max}) (13). It is also important to note that although the extent of activation somewhat varied, the AHNAK- and AA-dependent activation was observed with vesicular substrates prepared without detergents as well as with micellar substrates in the presence of deoxycholate, octyl glucoside, or Tween 20.

Given that both tau and AHNAK are known to be phosphoproteins, phosphorylation may play a role in regulation of their function as PLC-γ activators. Tau is a substrate of various protein kinases, including cAMP-dependent protein kinase, Ca^{2+}/calmodulin-dependent protein kinase, and Cdc2-like protein kinase (reviewed in Ref. 25), and can be heavily phosphorylated. AHNAK is also shown to be phosphorylated *in vivo* (18, 20).

In a classical scheme, binding of a variety of agonists to their cognate receptors causes the activation of PLC-γ through tyrosine phosphorylation. However, accumulating evidence shows that the activation mechanism for PLC-γ is not as simple as once thought. Activation of phospholipase D may also lead to activation of PLC-γ1 through accumulation of phosphatidic acid (2). We and others have recently shown that PIP_3 activates purified PLC-γ and that receptors coupled to phosphatidylinositol 3-kinase are capable of activating of PLC-γ indirectly in cells through the generation of PIP_3 (3–5, 26). Our previous (6) and present studies suggest that intracellular accumulation of AA can be another trigger for PLC-γ activation in the presence of tau or AHNAK proteins. AA is mainly released from phosphatidylcholine by the action of $cPLA_2$ (7) and serves as the precursor of various eicosanoids. The resulting eicosanoids in turn activate cells by autocrine and/or paracrine mechanisms. AA is also known to modulate several biological processes without conversion to eicosanoids. It can activate protein kinase C (27), guanylate cyclase (28), and neutral sphingomyelinase (29, 30), while it inhibits Ca^{2+}/calmodulin-dependent protein kinase (31) and GTP-binding to Gzα (32). Now PLC-γ is added to the list of AA effectors.

Activation of $cPLA_2$ requires intracellular Ca^{2+} mobilization (7), and it can thus be a secondary event following PLC activation, either PLC-β activation by G-protein-coupled receptors or PLC-γ activation by growth factor receptors. $cPLA_2$ activation in turn may activate PLC-γ if AHNAK or tau are present, constituting a positive feedback loop in the hydrolysis of PIP_2. On the other hand, PIP_2 is a potent activator of $cPLA_2$ (33). Therefore, hydrolysis of PIP_2 by PLC will attenuate the activity of $cPLA_2$, constituting a negative feedback loop in terms of AA mobilization.

In vitro results presented here all suggest that intracellular accumulation of AA can induce PLC-γ activation, and we have also shown that HeLa cells possess the necessary machinery for AA-induced activation. Several *in vivo* studies have suggested that endogenously released AA stimulates PLC activity inde-

pendently of its conversion to eicosanoids. Incubation of human trophoblasts with AA stimulates PLC activity, and neither cyclooxygenase nor lipoxygenase inhibitors blocked this response (34). They also found that activation of PLA_2 was involved in the stimulation of phosphoinositide metabolism and placental lactogen release in these cells (35). AA stimulated phosphoinositide metabolism in and catecholamine release from bovine adrenal chromaffin cells, and eicosanoid inhibitors were without effect (36). The fatty acid was also shown to increase phosphoinositide breakdown and glutamate release in rat hippocampal tissue (37) and to increase intracellular Ca^{2+} ions by mobilizing an IP_3-sensitive Ca^{2+} pool in isolated rat pancreatic islets (38) and in a human leukemic T cell line (39). The AA-induced Ca^{2+} release was shown to be independent of its metabolites (38). We believe that most, if not all, of these earlier observations now can be explained by the participation of AHNAK and tau.

REFERENCES

1. Rhee, S. G, and Bae, Y. S. (1997) *J. Biol. Chem.* **272,** 15045–15048
2. Jones, G. A., and Carpenter, G. (1993) *J. Biol. Chem.* **268,** 20845–20850
3. Bae, Y. S., Cantley, L. G., Chen, C. S., Kim, S. R., Kwon, K. S., and Rhee S. G. (1998) *J. Biol. Chem.* **273,** 4465–4469
4. Rameh, L. E., Rhee, S. G., Spokes, K., Kazlauskas, A., Cantley, L. C., and Cantley, L. G. (1998) *J. Biol. Chem.* **273,** 23750–23757
5. Falasca, M., Logan, S. K., Lehto, V. P., Baccante, G., Lemmon, M. A., and Schlessinger, J. (1998) *EMBO J.* **17,** 414–422
6. Hwang, S. C., Jhon, D. Y., Bae, Y. S., Kim, J. H., and Rhee S. G. (1996) *J. Biol. Chem.* **271,** 18342–18349
7. Leslie, C. C. (1997) *J. Biol. Chem.* **272,** 16709–16712
8. Lee, G. (1990) *Cell Motil. Cytoskeleton* **15,** 199–203
9. Park, D., Jhon, D. Y., Kriz, R., Knopf, J., and Rhee, S. G. (1992) *J. Biol. Chem.* **267,** 16048–16055
10. Suh, P. G., Ryu, S. H., Choi, W. C., Lee, K. Y., and Rhee, S. G. (1988) *J. Biol. Chem.* **263,** 14497–14504
11. Shtivelman, E., Cohen, F. E., and Bishop, J. M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 5472–5476
12. Rhee, S. G., Suh, P. G., Ryu, S. H., and Lee, S. Y. (1989) *Science* **244,** 546–550
13. Wahl, M. I., Jones, G. A., Nishibe, S., Rhee, S. G., and Carpenter, G. (1992) *J. Biol. Chem.* **267,** 10447–10456
14. Ji, Q. S., Winnier, G. E., Niswender, K. D., Horstman, D., Wisdom, R., Magnuson, M. A., and Carpenter, G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2999–3003
15. Ji, Q. S., Ermini, S., Baulida, J., Sun, F. L., and Carpenter, G. (1998) *Mol. Biol. Cell* **9,** 749–757
16. Hieda, Y., Tsukita, S., and Tsukita, S. (1989) *J. Cell Biol.* **109,** 1511–1518
17. Kudoh, J., Wang, Y., Minoshima, S., Hashimoto, T., Amagai, M., Nishikawa, T., Shtivelman, E., Bishop, J. M., and Shimizu, N. (1995) *Cytogenet. Cell. Genet.* **70,** 218–220
18. Shtivelman, E., and Bishop, J. M. (1993) *J. Cell Biol.* **120,** 625–630
19. Hashimoto, T., Amagai, M., Parry, D. A., Dixon, T. W., Tsukita, S., Tsukita, S., Miki, K., Sakai, K., Inokuchi, Y., Kudoh, J., Shimizu, N., and Nishikawa, T. (1993) *J. Cell Sci.* **105,** 275–286
20. Hashimoto, T., Gamou, S., Shimizu, N., Kitajima, Y., and Nishikawa, T. (1995) *Exp. Cell Res.* **217,** 258–266
21. Jenkins, S. M., and Johnson, G. V., (1998) *Neuroreport* **9,** 67–71
22. Flanagan, L. A., Cunningham, C. C., Chen, J., Prestwich, G. D., Kosik, K. S., and Janmey, P. A. (1997) *Biophys. J.* **73,** 1440–1447
23. Wilson, D. M., and Binder, L. I. (1997) *Am. J. Pathol.* **150,** 2181–2195
24. Carman, G. M., Deems, R. A., and Dennis, E. A. (1995) *J. Biol. Chem.* **270,** 18711–18714
25. Billingsley, M. L., and Kincaid, R. L. (1997) *Biochem. J.* **323,** 577–591
26. Gratacap, M. P., Payrastre, B., Viala, C., Mauco, G., Plantavid, M., and Chap, H. (1998) *J. Biol. Chem.* **273,** 24314–24321
27. Nishizuka, Y. (1992) *Science* **258,** 607–614
28. Gerzer, R., Brash, A. R., and Hardman, J. G. (1986) *Biochim. Biophys. Acta* **886,** 383–389
29. Tomiuk, S., Hofmann, K., Nix, M., Zumbansen, M., and Stoffel, W. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 3638–3643
30. Jayadev, S., Linardic, C. M., and Hannun, Y. A. (1994) *J. Biol. Chem.* **269,** 5757–5763
31. Piomelli, D., Wang, J. K. T., Sihra, T. S., Nairn, A. C., Czernik, A. J., and Greengard, P. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 8550–8554
32. Glick, J., Santoyo, G., and Casey, P. J. (1996) *J. Biol. Chem.* **271,** 2949–2955
33. Mosior, M., Six, D. A., and Dennis, E. A. (1998) *J. Biol. Chem.* **273,** 2184–2191
34. Zeitler, P., and Handwerger, S. (1985) *Mol. Pharmacol.* **28,** 549–554
35. Zeitler, P., Wu, Y. Q., and Handwerger, S. (1991) *Life Sci.* **48,** 2089–2095
36. Negishi, M., Ito, S., and Hayashi, O. (1990) *Biochem. Biophys. Res. Commun.* **169,** 773–779
37. Lynch, M. A., and Voss, K. L. (1990) *J. Neurochem.* **55,** 215–221
38. Wolf, B. A., Turk, J., Sherman, W. R., and McDaniel, M. (1986) *J. Biol. Chem.* **261,** 3501–3511
39. Chow, S. C., and Jondal, M. (1990) *J. Biol. Chem.* **265,** 902–907

AHNAK, a Protein That Binds and Activates Phospholipase C-γ1 in the Presence of Arachidonic Acid

Fujio Sekiya, Yun Soo Bae, Deok Young Jhon, Sung Chul Hwang and Sue Goo Rhee

J. Biol. Chem. 1999, 274:13900-13907.
doi: 10.1074/jbc.274.20.13900