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Evidence that 3′-phosphorylated polyphosphoinositides are generated at the nuclear surface: use of immunostaining technique with monoclonal antibodies specific for PI 3,4-P_2

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Abstract Phosphatidylinositol (PI) 3,4-P_2 is a phosphoinositide that has been shown to be important for signal transduction in growth factor stimulation. We have produced monoclonal antibodies specific for PI 3,4-P_2, which were able to detect PI 3,4-P_2 generated in 293T cells treated with H_2O_2, or in MKN45/BD110 cells expressing activated PI 3-kinase in immunostaining. Prolonged treatment with 0.05% Tween 20 resulted in detection of staining not only at the plasma membrane, but also at the nuclear surface, indicating that 3′-phosphorylated phosphoinositides can be generated and function in the nucleus.

Key words: Phosphatidylinositol 3,4-P_2; Monoclonal antibody; Immunostaining; Phosphatidylinositol 3-kinase

1. Introduction

Involvement of phosphatidylinositol (PI) 3-kinase in signal transduction is well known. PI 3-kinase can be activated by appropriate stimuli to yield PI 3,4,5-P_3 from PI 4,5-P_2 [1,2]. PI 3-kinase appears to be multifunctional. Use of selective inhibitors, dominant-negative mutants, constitutively active mutants and other reagents showed that PI 3-kinase is involved, for example, in signal transduction to the nucleus from growth factor receptors, in vesicle transport and in cytoskeletal rearrangement. There are varieties of activation mechanisms of PI 3-kinase. It can be activated by binding to autophosphorylated growth factor receptors, other proteins phosphorylated on tyrosine residues such as IRS1 or Gab1, small G proteins such as Ras, or βγ subunits of trimolecular G proteins [2,3]. These lines of evidence suggest that PI 3-kinase can act in various parts of cells which are targeted by these PI 3-kinase-binding molecules. However, it has not been clear where PI 3-kinase is activated because of the absence of appropriate probes for detecting its products. Recent work with PI 3,4,5-P_3-binding proteins has shown that PI 3-kinase is activated on the plasma membrane [4–6]. However, these signals might reflect the unique roles of each PI 3,4,5-P_3-binding protein, not showing the entire picture of the activation patterns of PI 3-kinase.

PI 3,4-P_2 can be produced by dephosphorylation of PI 3,4,5-P_3 or by phosphorylation of PI 4-P [7]. However, in many cases, elevation of the PI 3,4-P_2 level after growth factor stimulation appears to be due to activation of PI 3-kinase followed by dephosphorylation of PI 3,4,5-P_3 [8,9]. PI 3,4-P_2 has been shown to activate Akt/PKB by binding to its PH domain. Signaling of Akt/PKB has been shown to be important for the survival of cells, suggesting that PI 3,4-P_2 is an important second messenger for cell survival [10,11]. PH domains have been shown to bind to phosphoinositides. Some of them were shown to bind to PI 3,4,5-P_3 specifically, thus being able to provide probes for PI 3,4,5-P_3. However, there are no PH domains found which bind only to PI 3,4-P_2. For this reason, no attempt has been made to detect PI 3,4-P_2 in cells.

We have identified PIP_3BP as a nuclear PI 3,4,5-P_3-binding protein [12]. Because a considerable level of PI 4,5-P_2 is detected in nuclei, it is likely that PI 3,4,5-P_3 is generated in the nucleus. Indeed, we suggested the generation of PI 3,4,5-P_3 in the nuclear fraction of the cells treated with H_2O_2 or with growth factors by using biochemical methods [13]. However, further evidence should be provided to prove that PI 3,4,5-P_3 is really generated in the nucleus. Here, we demonstrate the production of PI 3,4-P_2-specific monoclonal antibodies that stain PI 3,4-P_2 generated in cells, and we show that 3′-phosphorylated phosphoinositides can be generated at the nuclear surface.

2. Materials and methods

2.1. Cell lines used in this study

Mouse SP2/0 cells were used for the production of anti-PI 3,4-P_2

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antibodies [14]. 293T and MKN45/BD110 cells were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% calf serum [15,16].

2.2. Production of PI 3,4-P_2-specific antibodies

Salmonella minnesota cells washed twice with distilled water, twice with acetone and once with diethyl ether were dried in vacuo. After removal of ketodeoxyoctonates-linked oligosaccharides by heating in 1% aqueous acetic acid solution, 50 μg of the *Salmonella* were coated with 10 μg of PI 3,4-P_2 and were injected into mice via the lateral tail veins [17]. One to eight weeks after the first immunization, the mice received a booster injection, and fusion with SP2/0 was performed 3 days later [17].

Hybridoma supernatants were screened for antibodies against PI 3,4-P_2 by a liposome lysis assay.

2.3. Liposome lysis assay

A complement-dependent liposome lysis assay was performed according to the method of Miyazawa et al. with a slight modification [17]. Briefly, a dried lipid film containing 1.0 mM dimyristoyl-phosphatidylcholine (DMPC), 1.2 mM cholesterol, 2.2 mM diethylphosphate and 0.022 mM PI 3,4-P_2 or other phospholipids was dispersed in 0.25 ml of marker solution containing 7.5 mM calcein. After washing with isotonic salt solution, the resulting liposomes were reacted with monoclonal antibodies in the presence of guinea pig complement to release internally trapped calcein. Dilution of calcein by its release from liposomes results in enhanced fluorescence, which was measured for quantification of liposome lysis. Fluorescence given by bovine serum albumin instead of monoclonal antibodies was used as a 0% lysis value. For a 100% lysis value, liposomes were destroyed by freezing and thawing. In some instances, rabbit anti-mouse IgG (heavy chain+light chain) was used as a second antibody to enhance the reaction.

2.4. Dot-blot analysis

Phospholipids were dissolved in an ethanol solution containing 10 μM DMPC and spotted on a polyvinylidene difluoride (PVDF) membrane (Millipore Co., Milford, MA, USA). The membrane was incubated with 3% bovine serum albumin in HEPES-buffered saline (pH 7.4). After washing with Tris-buffered saline (TBS), the membrane was incubated with anti-PI 3,4-P_2 antibodies, and then with alkaline phosphatase-conjugated goat anti-mouse IgG3 or anti-mouse IgG and IgM. After washing with TBS, the signals were visualized with bromochloro-indoyl-phosphate (BCIP)/nitro blue tetrazolium (NBT) phosphate substrate.

2.5. Activation of PI 3-kinase

For activation of PI 3-kinase in 293T cells, the cells were treated with 10 mM H_2O_2 for 1, 3, 5 and 10 min. Under these conditions, PI 3-kinase is activated dramatically [18]. The production of PI 3,4-P_2 was analyzed as described previously [19]. Constitutively active PI 3-kinase (BD110) gene was induced in MKN45 cells carrying the PI 3-kinase gene in a silent form. The cells were infected with an adenovirus encoding the Cre recombinase AxCANCre, at a multiplicity of infection of 20, and incubated at 37°C to induce the expression of BD110 by the Cre-loxP recombination system [16].

2.6. Staining of PI 3,4-P_2 by specific antibodies

For immunofluorescence studies, cells were grown on glass coverslips coated with poly-L-lysine. They were fixed for 20 min in phosphate-buffered saline (PBS) containing 10% formaldehyde and permeabilized with 0.05% Tween 20 in PBS for 30 min or 2 h. After washing with PBS three times, a blocking reaction was carried out with DMEM containing 10% calf serum at room temperature for 30 min. The glass coverslips were incubated with culture supernatants of the hybridomas containing 50–100 μg/ml immunoglobulins for 2 h, washed in PBS three times, and then incubated with FITC-labeled anti-mouse IgG for 2 h. In some cases, the culture supernatants were preincubated with micelles containing 1 mol% of PI 3,4-P_2 or PI 4,5-P_2 in phosphatidylcholine (PC), which were produced by dispersing the dimethylsulfoxide solution of the lipids into 100 volumes of PBS. After washing with PBS three times, they were observed by fluorescence microscopy or with the CELLscan System from Scanalytics (Billerica, MA, USA) equipped with a Photometric CCD camera, a piezoelectric *Z*-axis focus device, and a computer-controlled excitation light shutter.

3. Results

3.1. Specificity of anti-PI 3,4-P_2 antibodies

A series of monoclonal antibodies reactive with PI 3,4-P_2 was established (Table 1). To determine the specificity of the antibodies, we tested liposomes containing various phospholipids. As shown in Table 1 and Fig. 1A, the monoclonal antibodies were reactive with PIP_3 or PI 4,5-P_2 except for one; however, their affinity for PI 3,4-P_2 was much higher than that for these lipids. Phospholipids other than PI 3,4-P_2 required too much concentration of antibodies to deduce EC_{50}.

The liposome lysis assay is applicable only to lipids. To test water-soluble compounds such as Ins 1,3,4-P_3, which has a structure similar to the head group of PI 3,4-P_2, we established a liposome competition assay. The antibodies were incubated with various concentrations of compounds for testing prior to the liposome lysis assay of PI 3,4-P_2. As shown in Fig. 1B, preincubation with PI 3,4-P_2 abolished the liposome lysis reaction, suggesting that this assay was successfully done. Ins 1,3,4-P_3, Ins 1,4,5-P_3 or Ins 1,3,4,5-P_4 was not effective in blocking the liposome lysis, suggesting that these compounds did not react with the antibodies. To confirm this further, we performed dot-blot analysis with phospholipids. The phospholipids were mixed with PC, which did not react with the antibodies, and were blotted onto a PVDF membrane and tested for their reactivity with the antibodies. As shown in Fig. 1C, these antibodies showed a much higher reactivity with PI 3,4-P_2 than with the other lipids in this assay.

3.2. Staining of PI 3,4-P_2 in 293T cells

To visualize the formation of PI 3,4-P_2, we used 293T cells

Table 1
Summary of anti-PI 3,4-P_2 monoclonal antibodies used in this study

Mab	Isotype	Liposome lysis assay using various lipids[a] (Mab concentration required for 50% liposome lysis (μM))									Inhibition of liposome lysis by various competitors[b] (competitor concentration required for 50% inhibition of liposome lysis (μM))				
		PC	PE	PS	PA	PI	PI 4-P	PI 4,5-P_2	PI 3,4-P_2	PIP_3	PI 3,4-P_2	I 1,3,4-P_3	I 1,4,5-P_3	I 1,3,4,5-P_4	IP_6
8C2	IgG3	–	–	–	–	–	–	–	6	–	16	–	–	–	–
#10	IgG2a	–	–	–	–	–	–	1 600	1	32 000	63	–	–	–	–
#14	IgG3	–	–	–	–	–	–	3 200	6	3 200	250	–	–	–	–
#18	IgG3	–	–	–	–	–	–	160	6	1 600	10	–	–	–	–

[a]The liposome lysis assay was done with PC used as a carrier. Mab concentrations required for 50% lysis of liposomes carrying various lipids (1 M%) are shown. '–' indicates no effect with 10 mg/ml of the Mabs. 'PC' means that no other lipids were added to the liposome.
[b]Various competitors were added to the lysis reaction of the liposomes carrying PI 3,4-P_2. Competitor concentrations required for 50% inhibition of the liposome lysis are shown. '–' indicates no effect with 10 mM of the competitors.

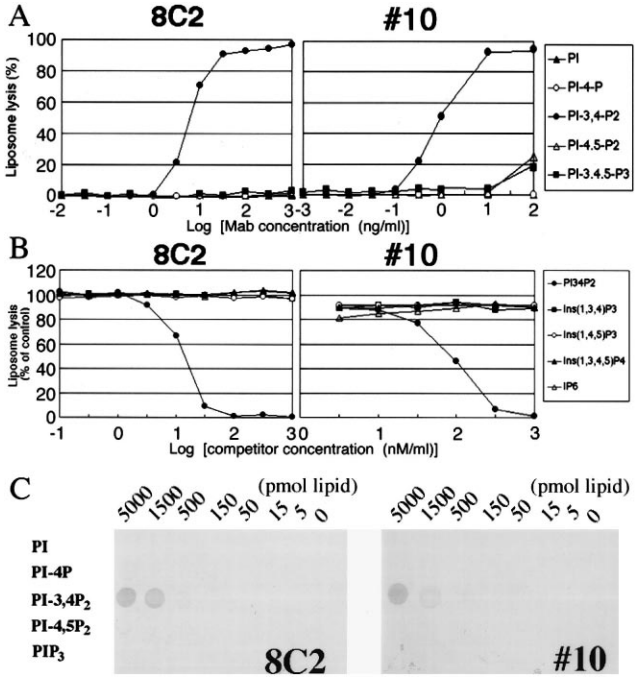

Fig. 1. A: Liposome lysis assay of the monoclonal antibodies used. Lysis of liposomes carrying various phosphoinositides shown on the right side with different concentrations of monoclonal antibodies (final concentration) is shown. This figure shows concentration of monoclonal antibodies required for the liposome lysis. A second antibody was used for the assay of monoclonal antibody #10. B: Various inositol phosphates shown on the right side were added as competitors to the liposome lysis assay of PI 3,4-P_2 in A. The competitor concentration is shown as a final concentration. C: Various lipids were blotted on a PVDF membrane with PC as a carrier. The membrane was probed with the monoclonal antibodies, and the signals were visualized with anti-mouse IgG conjugated with alkaline phosphatase and a BCIP/NBT phosphate substrate system. The amounts of lipids are shown in mol quantities.

treated with H_2O_2. After superactivation of PI 3-kinase, a great accumulation of PI 3,4-P_2 was observed, probably due to the absence of activation of PI 3,4-P_2-metabolizing enzyme in these cells [13,18] (Fig. 2A). Elevation of the PI 3,4,5-P_3 level was observed prior to that of PI 3,4-P_2. After fixation with formaldehyde, the membrane structure of these cells was permeabilized with Tween 20. PI 3,4-P_2 was stained with the monoclonal antibody 8C2 and a second antibody conjugated with FITC. Strong staining of PI 3,4-P_2 was observed at the plasma membrane 3 or 10 min after H_2O_2 treatment. The time course of PI 3,4-P_2 staining was similar to the PI 3,4-P_2 levels detected by high performance liquid chromatography analysis (Fig. 2B). Another antibody, #10, gave similar results.

To rule out the possibility that this staining was due to a non-specific interaction of the antibody with substances other than PI 3,4-P_2, we performed a competition assay with phospholipids. As shown in Fig. 2C, the presence of micelles containing PI 3,4-P_2 abolished this staining, whereas that containing PI 4,5-P_2 or control micelles showed no effects on the staining, suggesting that the staining was specific for PI 3,4-P_2. Further evidence was provided by an experiment using wortmannin, a PI 3-kinase inhibitor (Fig. 2B). The staining was completely inhibited by treatment with wortmannin. These lines of evidence indicate that the staining by the antibodies indeed reflects the positions of PI 3,4-P_2.

3.3. PI 3,4-P_2 is generated at the nuclear membrane as well as in the plasma membrane

We previously showed using a biochemical method that PI 3,4,5-P_3 is generated in the nucleus [13]. Because the staining of the plasma membrane may be strong, it was possible that the staining at the nucleus would not be detectable in the presence of the high background of the fluorescence of the membrane. We, therefore, used a CELLscan system to analyze the cells.

We utilized this system on MKN45/BD110 cells. These cells express an activated PI 3-kinase, BD110, upon introduction of Cre recombinase by an adenovirus vector. They lose cell–cell contact and show enhanced secretion of mucinous compounds after induction of PI 3-kinase. We used these cells for detection of PI 3,4-P_2. As shown in Fig. 3A, the cells became rounded after infection with the AxCANCre virus. These round cells were stained well with the antibody, whereas those which did not show a round morphology, escaping from infection by the virus, were not stained, suggesting that the staining was due to the expression of BD110 (Fig. 3A). Careful examination of these cells revealed that staining was observed not only in the plasma membrane, but also on the nuclear surface (Fig. 3B). This staining was not seen in control cells (data not shown). Treatment of the cells with wort-

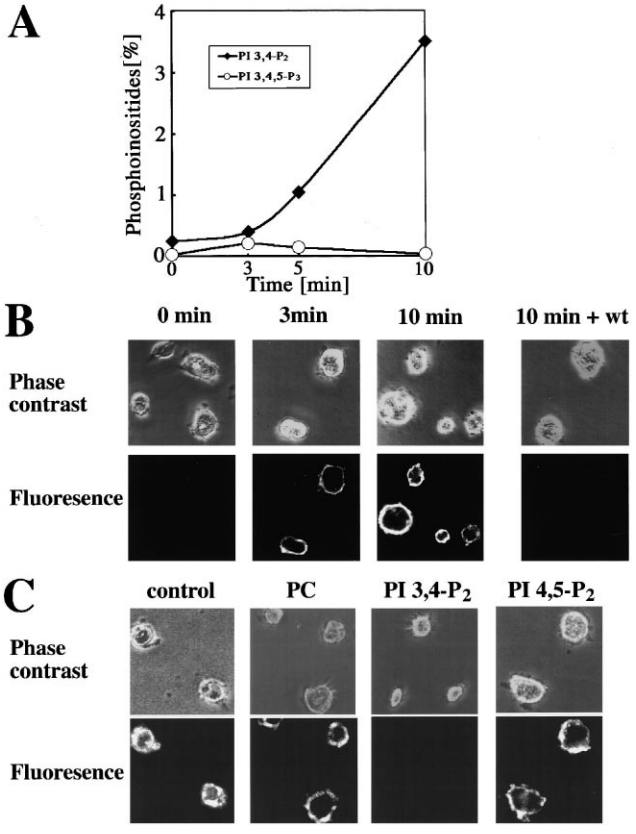

Fig. 2. A: 293T cells labeled with ^{32}P-orthophosphate were stimulated with 10 mM H_2O_2, and the production of PI 3,4-P_2 was analyzed. The levels of PI 3,4-P_2 are shown as percentage of the total lipids. B: 293T cells stimulated with H_2O_2 were fixed and stained with monoclonal antibody 8C2. In one experiment, 100 nM wortmannin was added 30 min prior to stimulation of the cells (right panel). C: Monoclonal antibody 8C2 was preincubated with the phospholipids (50 μM) indicated in the figure prior to immunostaining. Then 293T cells treated with H_2O_2 for 5 min were stained with the antibody.

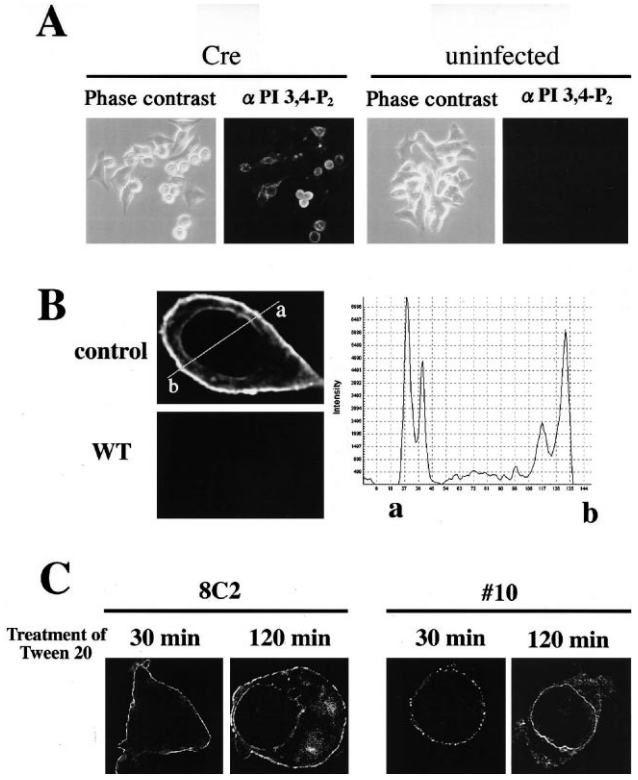

Fig. 3. A: MKN45/BD110 cells were infected with Cre. After 1 day, cells were fixed and stained for PI 3,4-P_2. Arrows indicate adenovirus-infected round cells, which are well stained with the antibody. B: MKN45/BD110 cells infected with Cre treated with (bottom panel) or without (top panel) 100 nM wortmannin were stained for PI 3,4-P_2 with monoclonal antibody 8C2 and analyzed by the CELLsan system. The right panel shows the strength of the signal between the line of a to b. C: The cells treated with 10 mM H_2O_2 for 5 min were fixed and permeabilized for 30 min or 120 min with 0.05% Tween 20. Then PI 3,4-P_2 was stained with monoclonal antibody 8C2 or #10.

mannin blocked the staining, confirming that the antigen is produced by PI 3-kinase (Fig. 3B).

293T cells were also used for detection of nuclear PI 3,4-P_2. In this case, 30 min treatment with Tween 20 did not allow the detection of nuclear PI 3,4-P_2; however, prolongation of the treatment time enabled us to detect nuclear PI 3,4-P_2, although the staining of the plasma membrane was reduced somewhat (Fig. 3C). Wortmannin treatment abolished the staining, confirming that the signal depends on activation of PI 3-kinase (data not shown). This indicates that stronger detergent treatment was required for the antibodies to reach the nucleus in these cells. Treatment with 10 mM H_2O_2 is an artificial condition; however, these observations may still reflect cell responses after a physiologic activation of PI 3-kinase to some extent. Taken together, these results suggest that 3′-phosphorylated phosphoinositides are generated at the nuclear surface.

4. Discussion

In this paper, we describe the production of monoclonal antibodies specific for PI 3,4-P_2, which can be used for immunostaining. The staining was shown to be specific because it was abolished by a PI 3-kinase inhibitor, wortmannin, and by the presence of PI 3,4-P_2 as a competitor. Staining was also detected in cells expressing activated PI 3-kinase. Although the systems used in this study were artificially inducing superactivation of PI 3-kinase, the results shown here open the new possibility that signaling lipid molecules can be visualized by staining with antibodies. Unfortunately, our system was not sensitive enough to detect PI 3,4-P_2 under physiologic conditions; however, this can be improved by the use of antibodies with greater affinity for PI 3,4-P_2 or by amplifying the signals by use of appropriate second antibodies.

We previously reported that PIP_3BP, a PIP_3-binding protein, is located in the nucleus and that it can be exported from the nucleus after expression of activated PI 3-kinase [13]. Consistent with this, generation of PIP_3 was detected in the nucleus by biochemical methods. However, the nuclear fraction can include endoplasmic reticulum attached to the nuclear membrane, suggesting that confirmation by other methods is needed. Our staining results reported in this paper clearly show that PI 3,4-P_2, which may arise from PI 3,4,5-P_3, is present in the nuclear membrane, supporting the idea that PI 3,4 5-P_3 is generated in the nucleus and that PI 3,4,5-P_3-binding proteins such as PIP_3BP can function as PIP_3-binding proteins in the nucleus.

PI 3-kinase has been suggested to play a role in vesicle transport. According to this hypothesis, PI 3,4-P_2 should be detected in other membranes such as the endoplasmic reticulum or Golgi apparatus. However, we failed to detect such staining by our method. This can be due to the method we used for preparation of samples. Treatment with Tween 20 is required for detection of the staining. Other detergents were not effective for staining. This suggests that a delicate condition is required for staining of membrane structures other than the nuclear membrane and plasma membrane, and that they are destroyed by the Tween 20 treatment in this study. Although staining of the nuclear structure was observed in this study, it is not clear whether PI 3,4-P_2 is present inside the nucleus. Strong detergent treatment is required to permeabilize the nuclear membrane, which would destroy all other membrane structures. Further studies on the conditions of fixation may be required for the detection of the whole picture of PI 3,4-P_2.

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