Identification of ribosomal protein S3a as a candidate for a novel PI 3-kinase target in the nucleus

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Abstract

Phosphatidylinositol 3,4,5-trisphosphate (PIP₃) is an important lipid second messenger that mediates various cell responses. We have searched for the nuclear PIP₃ binding proteins using PIP₃ analogue beads. A 33 kD protein was detected in this method, which was identified as ribosomal protein S3a by the mass spectrometric analysis. The recombinant S3a protein bound specifically to PIP₃. S3a localized not only in the cytosol but also in the nucleus. Interestingly, not cytosolic but nuclear S3a bound to PIP₃, suggesting different roles of S3a in the cytosol and the nucleus.

Abbreviations: PI 3-kinase, Phosphatidylinositol 3-kinase; PIP₃, Phosphatidylinositol 3,4,5-trisphosphate; PI 4,5-P₂, Phosphatidylinositol 4,5-bisphosphate; PI 3,4-P₂, Phosphatidylinositol 3,4-bisphosphate.

Introduction

Phosphoinositides, components of biomembrane, are important signaling molecules for signal transduction of gene expression or cytoskeletal rearrangement although their levels are relatively low among various phospholipids. Phosphatidylinositol (PI) 3-kinase is one of the key enzymes that regulate various cell responses (Vavhaesebroeck et al., 2001). PI 3-kinase phosphorylates 3' position of PI 4,5-bisphosphate (PI 4,5-P₂) to produce PIP₃, which is an important second messenger generated on the membrane. PIP₃ may recruit its binding proteins to the membrane. This relocalization can provide a chance to the proteins to meet their partners on the membrane, which may start the signaling reaction. Or sometimes binding of PIP₃ directly regulates the activities of the binding proteins to transduce signals to the downstream molecules (Toker and Cantley, 1997; Fukui et al., 1998; Vavhaesebroeck et al., 2001).

Phosphoinositides are found in the nucleus as well. Cocco et al. first proposed a evidence that inositol lipid metabolism also exists within the nucleus (Cocco et al., 1987), now that the role and regulation of the nuclear PI turnover is understood to some extent (D'Santos et al., 1998; Neri et al., 1999; Divecha et al., 2000; Cocco et al., 2001; Martelli et al., 2001). Localization and activity of PI 3-kinase in the nucleus were observed in several cell types (Neri et al., 1999; Tanaka et al., 1999; Yokogawa et al., 2000). For example, translocation of the enzyme is studied in NGF-stimulated PC12 cells (Neri et al., 1994). Consequently, PKC ζ translocates to the nucleus in a PI 3-kinase dependent manner in these cells (Neri et al., 1999). Since PKC ζ is known to be a PI 3-kinase target, it is possible that this molecule functions as a PI 3-kinase effector also in the nucleus. *In vitro* labeling of the nuclei prepared from rat liver revealed the production of PIP₃ from PI 4,5-P₂, suggesting the PI 3-kinase activity in the nucleus (Lu et al., 1998).

Translocation of PI 3-kinase into the nucleus was also detected in IL-1 stimulated Saos-2 cells (Bavelloni et al., 1999). A regulatory subunit, p85 α , was also detected in the nuclear membrane and nuclear matrix by the immunofluorescence with anti p85 α antibody (Zini et al., 1996). The requirement of nuclear PI 3-kinase in the cell responses has been shown only in a limited cells. HL60 is an example, in which translocation of PI 3-kinase to the nucleus and its binding to Vav after treatment with ATRA to introduce differentiation of the cells to granulocytes has been reported (Bertagnolo et al., 1998, 1999).

Although nuclear target of PI 3-kinase is not yet clearly determined, there are some candidates to date. PKC ζ , as described above, and Akt/PKB are the possible targets, since translocation of Akt/PKB after growth factor stimulation has been observed (Borgatti et al., 2000). PIP₃BP, which is homologous to centaurin α , is a nuclear PIP₃ binding protein (Tanaka et al., 1999). It contains two PH domains responsible for the PIP₃ binding and a region homologous to Arf-GAP (GTPase activating protein) although its GAP activity has not been detected. This protein moves to the cytosol after activation of PI 3-kinase, suggesting the role of PI 3-kinase in the nucleus (Tanaka et al., 1999).

In this study, we intended to identify PI 3-kinase targets in the nucleus by a similar method which was used for identification of PIP₃BP (Tanaka et al., 1999). We found that ribosomal protein S3a is a candidate for a novel PI 3-kinase target in the nucleus.

Materials and methods

Materials

PIP₃ and PIP₃-APB (PIP₃ analogue) were chemically synthesized as described before (Shirai et al., 1998). PI 3,4-P₂ was synthesized by a similar method. Synthetic PI 4,5-P₂ was purchased from Echelon Research Laboratories (Salt Lake City, U.S.A.).

Plasmids and antibodies

A cDNA fragment encoding human ribosomal protein S3a was PCR-amplified using following primers (5'-CAGATCTCATGGCGTTGGCAAGAAC-3', 5'-CTGAACCTTAAACAGATTCTTGGAC-3') and cloned into pT7blue vector (Novagen). The cDNA was subcloned into pGEX4T-3 (Amersham Pharmacia) for bacterial expression of GST-fusion S3a, pEF-BOS-HA for mammalian expression of HA-tagged

S3a, pEGFP-C1, or pEGFP-N1 (Clontech) for mammalian expression of GFP-fusion S3a (Mizushima and Nagata, 1990). pEF-BOS-HA-S3a produces a product with a molecular weight of about 39 kD due to the long linker sequences derived from pT7blue between the sequences for HA tag and S3a. Anti-S3a mouse polyclonal antibody was generated by the following procedure. GST-S3a was expressed in *E. coli* and affinity-purified through Glutathione Sepharose 4B column (Pharmacia), and the resulting protein was mixed with Freund's complete adjuvant. Balb/c mice were injected with the antigen to induce ascite fluid, which was used as an anti-S3a antibody.

Synthesis of PIP₃-APB beads and the binding assay

Synthesis of PIP₃-APB beads was done as described before (Shirai et al., 1998). Briefly, Affigel-10 (100 μ l, Bio-Rad) was incubated with PIP₃-APB (0.84 mg) in the buffer (100 mM Hepes-NaOH pH 7.4, 80 mM CaCl₂) at room temperature for 48 h. After washing with distilled water for 3 times, the PIP₃-APB beads were used in the following study.

Proteins were preincubated with water-soluble PIP₃, PI 3,4-P₂, or PI 4,5-P₂ at 4 °C for 1 h, or not, followed by incubation with PIP₃-APB beads at 4 °C for 3 h to over night. After washing beads 3 times with buffer A (20 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF), the proteins binding to beads were eluted by SDS sample buffer, separated by SDS-PAGE, and visualized by silver staining (Wako) or immunoblotting with appropriate antibodies.

Cell culture and transfection

293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS). Transfections were performed by calcium phosphate method.

Fractionation of the cells

All the following procedures were carried out at 0–4 °C. Cells were harvested and homogenized with a Dounce homogenizer in buffer B (20 mM Tris-HCl pH 7.0, 10 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF). The resulting samples were centrifuged at 800 $\times$ g for 10 min. After taking the supernatant, the pellet was resuspended with buffer B and collected by centrifugation at 800 $\times$ g for 10 min. The two supernatants were combined and used as cytosolic fraction.

Figure 1. Detection of 33 kD nuclear PIP₃ binding proteins. (a) Identification of 33 kD proteins as nuclear PIP₃ binding proteins. Cytosolic (lane 1) or nuclear (lane 2) fraction of 293T cells was mixed with PIP₃-APB beads and the proteins bound to the beads were analyzed by SDS-polyacrylamide gel electrophoresis. The bands were visualized by silver staining. (b) Specific binding of the 33 kD proteins to PIP₃. The nuclear fraction of 293T cells was mixed with 1.6, 8, or 16 μ M PIP₃ (lanes 2, 4, and 6) or 1.6, 8, or 16 μ M PI 4,5-P₂ (lanes 3, 5, and 7) and then with PIP₃-APB beads. The proteins bound to the beads were analyzed by SDS-polyacrylamide gel electrophoresis. The bands were visualized by silver staining. Lane 1, control experiment with no preincubation with phospholipids. PIP₃BP, the position of PIP₃BP. Arrowheads indicate 33 kD proteins detected for the PIP₃ binding proteins. The positions of molecular weight markers are shown on the left.

The pellet was suspended with buffer C (20 mM Tris-HCl pH 7.0, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF) and incubated for 15 min to extract the nuclear proteins and centrifuged at $125,000 \times g$ for 1 h. The supernatant was used as a nuclear fraction in the following study. Protein concentrations of both cytosolic and nuclear fractions were quantified by the assay described by Bradford (Bradford, 1976).

Analysis of the 33 kD protein by peptide mass mapping

Twenty mg protein of the nuclear extract from 293T cells was incubated with 300 μ l PIP₃-APB beads, followed by washing with buffer A and eluting with SDS sample buffer. The sample was electrophoresed on a 12% polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Problot, Applied Biosystems), followed by CBB staining to visualize the protein. The immobilized protein were reduced, S-carboxymethylated, and digested *in situ* with *Achromobacter* protease I (a Lys-C)

(Iwamatsu and Yoshida-Kubomura, 1996). Molecular mass analyses of Lys-C fragments were performed by Matrix-assisted Laser Desorption/Ionization time-of-flight (MALDI-TOF) mass spectrometry using a PerSeptive Biosystem Voyager-DE/RP. Identification of the protein was carried out by comparison between the molecular weights determined by MALDI-TOF/MS and theoretical peptide masses from the proteins registered in NCBI (5.14.2001).

Results

Identification of ribosomal protein S3a as a PIP₃ binding protein from nucleus of 293T cells

293T cells were fractionated into cytoplasm and nucleus. Both fractions were tested for PIP₃ binding. As shown in Figure 1a, different patterns of the proteins bound to the beads were obtained in cytosolic and nuclear fractions. A few bands including two bands with molecular weight of about 33 kD were detected only

a

1 MAVGKNKRLT KGGKKGAKKK VVDPFSKKDW
 31 YDVKAPAMFN IRNIGKTLVT RTQGTAKIAD
 61 GLKGRVFEVS LADLQNDIVA FRKFKLITED
 91 VQGKNCITNF HGMDLTRDKM CSMVKKWQTM
 121 IEAHVDVKIT DGYLLRLFCV GETKKRNNQI
 151 RKTSYAQHQQ VRQIRKKMME IMTREVQTN
 181 LKEVVNKLIP DSIKGDIEKA QSIYPLHDV
 211 FVRKVKMLKK PKFELGKLM LHGEGSSSGK
 241 ATGDETGAKE ERADGYEPPV QESV

b

Figure 2. Identification of ribosomal protein S3a as a PIP₃ binding protein. (a) Lys-C peptidase fragments identified in the mass spectrometric analysis. The amino acid sequence of ribosomal protein S3a is shown. The Lys-C peptidase fragments identified in the mass spectrometric analysis are underlined. (b) Specific binding of ribosomal protein S3a to PIP₃. Nuclear fraction of 293T cells transfected with an expression vector for HA-S3a was prepared and mixed with 4 μM PIP₃ (lane 3), 4 μM PI 3,4-P₂ (lane 4), or 4 μM PI 4,5-P₂ (lane 5). The samples were then mixed with PIP₃-APB beads and the proteins bound to the beads were analyzed by western blotting with anti-HA antibody (12 CA5). Lane 1, Affigel-10 was used instead of PIP₃-APB beads. Lane 2, without preincubation with phospholipids.

in the nuclear fraction. The two bands were examined further. Pre-incubation of the nuclear extracts with free PIP₃ resulted in disappearance of the bands. Since PI 4,5-P₂ and PI 3,4-P₂ did not block the binding of the proteins to the beads, the binding of the proteins to PIP₃ was suggested to be specific (Figure 1b and data not shown).

These two bands were purified from 20 mg protein of the nuclear extract. The proteins were blotted onto a PVDF membrane and analyzed by mass spectrometry after digestion with *a* Lys-C. These two bands were identified as ribosomal protein S3a (Figure 2a) and histon H1B, respectively. A careful study revealed that most of the members of the histon family can bind to PIP₃ through their abundant basic amino acid residues and that histon H1B may have been separated from other members of the family by the mild condition of the nuclear extraction. We therefore chose ribosomal protein S3a and explored further.

The cDNA of ribosomal protein S3a was cloned by a PCR method and an expression vector for S3a tagged with the epitope of the monoclonal antibody for hemagglutinine (HA-S3a) was constructed. After transfection of 293T cells with the expression vector, the expressed protein was tested for the PIP₃ binding. As shown in Figure 2b, HA-S3a specifically bound to the PIP₃-APB beads but not to affigel-10, empty beads. The binding was inhibited by preincubation with 4 μM PIP₃. Slight inhibition was observed with 4 μM PI 3,4-P₂ and none with 4 μM PI 4,5-P₂. These results suggest that ribosomal protein S3a is the 33 kD protein detected in Figure 1.

Only the nuclear ribosomal protein S3a binds to PIP₃

Since ribosome is present in the cytosol, ribosomal protein S3a is expected to be present in the cytosol as well. We therefore fractionated the cells into cytosol and nucleus and analysed for the binding. Since there are a number of PIP₃ binding proteins in the cytosol, it was hard to distinguish ribosomal protein S3a from the other proteins in the cytosolic fraction. To accomplish this, we raised an antibody against bacterially expressed ribosomal protein S3a. This antibody detected endogenous ribosomal protein S3a as well as HA-S3a (Figure 3a). Using the antibody, we analyzed localization of ribosomal protein S3a. As shown in Figure 3b, ribosomal protein S3a was mainly detected in the cytosolic fraction and only a small population was present in the nucleus. However, the binding was largely detected only in the nucleus. This binding was inhibited by preincubation with PIP₃ (data not shown). Only a slight binding was observed in the cytosolic fraction.

In contrast, HA-S3a overexpressed in the cells bound to the beads even from the cytosolic fraction (Figure 3c). It is likely that ribosomal protein S3a in the cytosol may be in a different status from that in the nucleus to be unable to bind to PIP₃ by the unknown regulation. Overexpression of it will provide S3a free from the inhibitory situation to bind to PIP₃.

Localization of ribosomal protein S3a

Ribosomal protein S3a was expressed in 293T cells as GFP-fusion proteins. As shown in Figure 4, ribosomal protein S3a fused with GFP at the amino terminus (GFP-S3a) was detected mainly in the nucleus. Distribution with GFP at the carboxyl terminus (S3a-GFP) was also abundant in the nucleus but more fluorescence was seen in the cytosol compared with

Figure 3. Not cytosolic but nuclear S3a can bind PIP₃-APB beads. Binding of ribosomal protein S3a to PIP₃ was analyzed. (a) Detection of S3a in 293T cells using anti-S3a antibody. 293T cells were transfected with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) an expression vector for HA-S3a. Cells were fractionated into cytosol (lanes 1, 2, 5, and 6) and nucleus (lanes 3, 4, 7, and 8) and HA-tagged S3a and endogenous S3a were detected by western blotting with anti HA antibody (lanes 1 to 4) or anti-S3a antibody (lanes 5 to 8). (b) Binding of endogenous S3a with PIP₃-APB beads. The cytosolic or nuclear fraction (300 μ g) of the untransfected 293T cells was used for the binding to PIP₃-APB beads. S3a was detected by western blotting with anti-S3a antibody. Lane 1, cytosolic fraction, lane 2, nuclear fraction, lane 3, S3a bound to PIP₃-APB beads from cytosolic fraction, lane 4, S3a bound to PIP₃-APB beads from nuclear fraction. (c) Binding of HA-S3a with PIP₃-APB beads. The cytosolic or nuclear fraction (300 μ g) of 293T cells transfected with an expression vector for HA-S3a was used for the binding to PIP₃-APB beads. S3a was detected by western blotting with anti-HA antibody. Lane 1, cytosolic fraction, lane 2, nuclear fraction, lane 3, HA-S3a bound to PIP₃-APB beads from cytosolic fraction, lane 4, HA-S3a bound to PIP₃-APB beads from nuclear fraction.

GFP-S3a. The two proteins were detected intact in the western blotting with anti-GFP antibody (data not shown). GFP alone distributed equally in the cytosol and the nucleus. These results suggest that ribosomal protein S3a can distribute to the nucleus, supporting the results in this article.

Discussion

It is well known that PIP₃, a product of PI 3-kinase, transduce signals by binding to the downstream molecules to trigger cell growth, cell survival, vesicle transport, and cytoskeletal rearrangement. However, the image of function of PI 3-kinase in the nucleus has not been clear to date.

Besides several known candidates for nuclear target of PI 3-kinase, we identified ribosomal protein S3a in this report. One may argue that detection of ribosomal protein S3a is due to binding to histon H1B which binds to PIP₃-APB beads by its basic amino acid residues. However, cytosolic ribosomal protein S3a overexpressed in 293T cells bound to the PIP₃-APB beads in the absence of histon H1B (Figure 3c). This may argue against the criticism. Ribosomal protein S3a is a component of the 40S small subunit (Lutsch et al., 1990) and binds to 3' OH end of 18S rRNA (Svobada and McConkey, 1978). S3a is known to be involved in interaction of 40S subunit with initiation factors (Westermann et al., 1979; Nygard et al., 1981; Tolan and Traut, 1981; Westermann and Nygard, 1983), initiator-tRNA (Westermann et al.,

Figure 4. Distribution of ribosomal protein S3a fused with GFP. 293T cells were transfected with expression vectors for GFP-S3a, S3a-GFP, and GFP. After 24 h, cells were observed under the fluorescence microscope.

1981), or mRNA (Stahl and Kobets, 1981), and also in the interaction of the 80S subunit with EF-2 (Nygard et al., 1987; Nagahisa et al., 1996), which implies its role in regulation of translation. In addition to this, recent studies revealed that ribosomal protein S3a can be involved in cell growth, transformation, and apoptosis (Kho and Zarbl, 1992; Kho et al., 1996; Naora et al., 1998; Russell et al., 2000). For instance, Fte (v-fos transformation effector), a factor that restores the transforming phenotypes to the flat revertant of the v-fos transformed cells, has been shown to be identical to ribosomal protein S3a, suggesting the possible role of ribosomal protein S3a in cell transformation (Kho et al., 1996). High expression of ribosomal protein S3a followed by rapid decline of it was shown to induce apoptosis (Naora et al., 1998). This is supported by the findings that ribosomal protein S3a binds CHOP or PARP, apoptosis inducers, or Bcl-2, an apoptosis suppressor (Cui et al., 2000; Song et al., 2002). Ribosomal protein S3a can inhibit the activity of PARP cooperatively with Bcl-2 *in vitro* (Song et al., 2002). Given these information, it is likely that ribosomal protein S3a may be multi functional.

We found that only the nuclear ribosomal protein S3a binds to PIP₃ in this report. This result suggests that functions of nuclear and cytosolic ribosomal protein S3a may be different. Cytosolic ribosomal protein S3a may be incorporated in ribosome, which can interfere access of PIP₃. However, our preliminary results suggest that ribosomal protein S3a free from ribosome presents in the cytosol in addition to the ones incorporated in ribosome (data not shown). This observation

suggests that there may be some specific modification on ribosomal protein S3a in the cytosol, interfering the activity to bind PIP₃. Since overexpressed cytosolic ribosomal protein S3a binds to PIP₃ (Figure 3c), the modification may be given to the limited proteins. It has been reported that ribosomal protein S3a binds to CHOP free from ribosome (Cui et al., 2000). It is possible that nuclear ribosomal protein S3a free from cytosolic modification complexes with CHOP and contributes to cell survival in cooperation with PI 3-kinase/Akt system (Datta et al., 1997; Eves et al., 1998; Zhou et al., 2000).

The biological importance of PIP₃ binding activity of ribosomal protein S3a is yet to be known. We are currently working on the possibility of involvement of PI 3-kinase in the signaling of apoptosis through ribosomal protein S3a.

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