Localization and translocation of RhoA protein in the human gastric cancer cell line SGC-7901

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Abstract

AIM: To elucidate the localization of RhoA in gastric SGC-7901 cancer cells and its translocation by lysophosphatidic acid (LPA) and/or 8-chlorophenylthio-cAMP (CPT-cAMP).

METHODS: Immunofluorescence microscopy was used to determine the localization of RhoA. Western blotting was used to detect both endogenous and exogenous RhoA in different cellular compartments (membrane, cytosol, nucleus) and the translocation of RhoA following treatment with LPA, CPT-cAMP, or CPT-cAMP + LPA.

RESULTS: Immunofluorescence staining revealed endogenous RhoA to be localized in the membrane, the cytosol, and the nucleus, and its precise localization within the nucleus to be the nucleolus. Western blotting identified both endogenous and exogenous RhoA within different cellular compartments (membrane, cytosol, nucleus, nucleolus). After stimulation with LPA, the amount of RhoA within membrane and nuclear extracts increased, while it decreased in the cytosol fractions. After treatment with CPT-cAMP the amount of RhoA within membrane and nuclear extracts decreased, while it increased within the cytosol fraction. Treatment with a combination of both substances led to a decrease in RhoA in the membrane and the nucleus but to an increase in the cytosol.

CONCLUSION: In SGC-7901 cells RhoA was found to be localized within the membrane, the cytosol, and the nucleus. Within the nucleus its precise localization could be demonstrated to be the nucleolus. Stimulation with LPA caused a translocation of RhoA from the cytosol towards the membrane and the nucleus; treatment with CPT-cAMP caused the opposite effect. Furthermore, pre-treatment with CPT-cAMP was found to block the effect of LPA.

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Key words: RhoA; Localization; Translocation; Nucleolus; Gastric cancer cell

INTRODUCTION

RhoA, with molecular mass of 21 kDa, is the most extensively studied member of the Rho GTPase family belonging to the Ras superfamily of small G proteins. The Rho GTPase family consists of at least 11 members sharing more than 50% identity, the best known of them being Cdc42, Rac1, and RhoA. RhoA has been reported to regulate many biological activities including the formation of stress fibers and focal adhesions[1].

Like other small G proteins, RhoA is inactive in its GDP-bound and active in its GTP-bound form, these two forms can be converted by GDP/GTP exchange or GTPase reactions[2,3]. The former and latter reactions are regulated by GDP/GTP exchange protein and GTPase activating protein, respectively. Two types of GDP/GTP exchange proteins are known: one is a stimulatory type named GDS, and the other is an inhibitory type named GDI. GDS stimulates the dissociation of GDP from and the subsequent binding of GTP to each substrate small G protein, whereas GDI inhibits both reactions[6-8]. In addition to the action of GDS and GDI regulating the GDP/GTP exchange reaction, both GDP/GTP exchange proteins regulate the translocation of each substrate small G protein between the cytosol and membranes.

Lysophosphatidic acid (LPA), a commonly used RhoA activator, is a serum-born phospholipid with hormone and growth factor-like properties, which is formed by and released from activated platelets and which can be...
generated by the action of secretory phospholipase A\textsubscript{2}\textsuperscript{[16,17]}. Previous studies showed that it is able to induce the translocation of RhoA from the cytosol to the membrane accompanied with the activation of RhoA\textsuperscript{[18]}, probably \textit{via} conversion of the GDP-bound into the GTP-bound form.

\textit{cAMP} is a well-known classical secondary messenger, and a cAMP-dependent protein kinase A (PKA) phosphorylates RhoA in its C-terminal region on serine residue 188\textsuperscript{[19]}. The cAMP signal mediates down-regulation of RhoA activity\textsuperscript{[20,21]}. It was reported that cAMP could induce the translocation of RhoA from the membrane to the cytosol accompanied with the inactivation of RhoA\textsuperscript{[22]}. Moreover, translocation of RhoA was shown to be caused by stabilizing the RhoA/Rho GDIs complex by phosphorylating GTP-bound RhoA\textsuperscript{[23]}. Phosphorylation enhances translocation of RhoA to the cytosol through increased interaction of GTP-bound RhoA with Rho-GDI, possibly terminating its activity.

We here describe RhoA to be localized in the nucleus of SGC-7901 cells and a cross talk between LPA/RhoA-mediated and cAMP/PKA mediated signal transduction pathways\textsuperscript{[22]}. Whether there is interaction between LPA induced and cAMP induced RhoA distributions in different cellular compartments deserves further investigation. Our experiments were designed to explore the localization of RhoA and to investigate the effects of a treatment with LPA, cAMP, and cAMP + LPA.

**MATERIALS AND METHODS**

**Cell lines**

The human gastric cancer cell line SGC-7901 and human cervix cancer cell line HeLa were provided by the Institute of Cell Biology (Shanghai, China).

**Reagents**

Dulbecco’s Modified Eagle Media (DMEM) was purchased from Gibco (Grand Island, NY), new-born calf serum (NBCS) from Minhai Bio-engineering C (Lanzhou, China), the antibody against RhoA from Santa Cruz Biotechnology (Santa Cruz, CA), the antibody against GAPDH (glyceraldehyde phosphate dehydrogenase) from Kangcheng (China), the horseradish peroxidase (HRP)-conjugated secondary antibody from Jackson ImmunoResearch Laboratories (West Grove, PA), the cell transfection reagent Lipofectamin 2000, TRizol reagent, and reverse transcription kit from Invitrogen (Carlsbad, CA), the cellular permeable cAMP analog 8-chlorophenylthio-cAMP (CPT-cAMP) from Calbiochem, LIPA and nuclear fluorochrome Hoechst 33342 from Sigma (St. Louis, Missouri), electrochemiluminescence (ECL) reagents from Amersham Biosciences (Buckinghamshire, England), and pGEmol/L vector from Promega (USA). Plasmid DNA constructs of RhoA pcDNAEE-wt (wild type of RhoA), pcDNAEE-63L (a constitutive active form of RhoA), and pcDNAEE-N19 (an inactive mutant of RhoA) were kind gifts from Dr. Renate Pilz, University of California, San Diego.

**Cell culture and transfection**

SGC-7901 cells were cultured in DMEM supplied with 10% NBCS. The medium was changed every two days and the cells were subcultured at confluence. For transfection, the cells were subcultured the day before the process. The seeding amount of cells was adjusted to attain a density of 80%-90% confluence on the day of transfection. Transfection was performed according to the instruction of the manufacturer.

**Immunofluorescence microscopy**

The cells grown on cover slips were incubated to reveal nuclei with 0.2 \textmu mol/L Hoescht 33 342 for 10 min before fixed with freshly prepared 40 g/L paraformaldehyde in PBS at 4\textdegree C overnight (o/n). After being penetrated with 30 mL/L Triton X-100 and blocked with 30 g/L BSA, the cells were incubated with primary antibodies at 4\textdegree C o/n, and then with FITC- or TRITC-conjugated secondary antibodies for 1 h at room temperature (RT), with three washes after each incubation. The morphologic changes of the cells were analyzed by fluorescence microscopy.

**Membrane, nucleus, and cytosol preparation**

According to the method from Jeffrey C. Chen \textit{et al}\textsuperscript{[23]}, four 100 mm plates of cells were prepared, one of them considered as control, and the others treated with LPA (1 \textmu mol/L, 15 min), CPT-cAMP (250 \textmu mol/L, 30 min), or CPT-cAMP (250 \textmu mol/L, 30 min) followed by an incubation with LPA (1 \textmu mol/L, 15 min). Cells were extracted by Dounce homogenization in 10 mmol/L Hepes pH 7.5, 2 mmol/L EDTA, 1 mmol/L MgCl\textsubscript{2} (HEM buffer). The homogenate was centrifuged at 500 g at 4\textdegree C for 5 min to obtain nuclear proteins, and the supernatant was centrifuged at 37 000 g at 4\textdegree C for 30 min. The supernatant and pellet from the second centrifugation are referred as cytosol and membranes, respectively, with the membrane preparation washed twice in HEM buffer to remove contaminating cytosol. Protein concentrations were determined and equal amounts of protein from each preparation (30 \textmu g) were subjected to SDS-PAGE/ Western blotting using mouse monoclonal anti-RhoA (1:200) and GAPDH (1:5000) antibodies.

**Purification of nucleoli from SGC-7901**

Nucleoli were purified using a procedure adapted from previously published protocols\textsuperscript{[24]}. For a typical experiment, $1 \times 10^8$ SGC-7901 cells were plated onto 245 mm Petri dishes in DMEM containing 10% NBCS. Cells were incubated in a humidified atmosphere containing 5% CO\textsubscript{2} at 37\textdegree C. At 80% confluence, cells were washed with cold PBS, and scraped off on ice. Cells were collected by centrifugation at 500 g at 4\textdegree C for 5 min. Cells were resuspended in 5 mL hypotonic buffer (10 mmol/L Tris-HCl pH 7.4, 10 mmol/L NaCl, and 1 mmol/L MgCl\textsubscript{2}) and incubated on ice for 30 min. Cell lysis was performed by addition of a final concentration of 0.3% Nonidet P-40 (Roche Applied Science, Mannheim, Germany) and homogenization was performed using a 0.4 mm clearance Dounce homogenizer (Kimble/Kontes, Owens, IL) for 15 times on ice. Nuclei were collected by centrifugation at 228 g at 4\textdegree C for 5 min and resuspended in 3 mL 0.25 mol/L sucrose-containing 10 mmol/L MgCl\textsubscript{2}. Nuclei were centrifuged at 1430 g at 4\textdegree C.
for 5 min through a 0.35 mol/L sucrose cushion containing 0.5 mmol/L MgCl\(_2\) and resuspended in 3 mL 0.35 mol/L sucrose-containing 0.5 mmol/L MgCl\(_2\) and then sonicated on ice for ten bursts of 10 s with 1 min intervals between them. Nuclei were then purified by centrifugation at 2800 g at 4°C for 10 min through a 0.88 mol/L sucrose cushion containing 0.5 mmol/L MgCl\(_2\). Nuclei were washed with 500 μL 0.35 mol/L sucrose containing 0.05 mmol/L MgCl\(_2\) twice, and centrifuged at 2800 g at 4°C for 10 min. Purified nuclei were resuspended in 50 μL 0.35 mol/L sucrose containing 0.05 mmol/L MgCl\(_2\) for further analyses.

**Protein extraction from Hela cells in interphase and mitosis**

Hela cells were seeded on four 100 mm plates and treated with 5 μg/mL Taxol for 12 h. Cells in mitosis phase were collected by “shake off”, and the cells left on plates considered in interphase were scraped. Cells were extracted with 500 μL RIPA (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L PMSF, 1 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), incubated on ice for 30 min, and then centrifuged at 14,000 g at 4°C for 15 min to obtain total protein extracts. Protein concentrations were determined and equal amounts of protein from each preparation (30 μg) were subjected to SDS-PAGE/Western blotting using mouse monoclonal anti-Rhoa (1:200) and GAPDH (1:5000) antibodies.

**Western blotting**

SDS-PAGE gels of different concentrations were cast according to the molecular size of target proteins. Sample proteins were run on 10% SDS-polyacrylamide gels, and were blotted onto polyvinyl difluoride (PVDF) membrane. The PVDF membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in TBS-T for 1 h at room temperature (RT). Membranes were incubated with the primary antibody at 4°C o/n, and with the secondary antibody for 1 h, with three washes after each incubation. Electrochemiluminescence reagents were used to show the positive bands on the membrane. Briefly, same volumes of solution A and solution B were mixed and added to the protein side of the membrane. The incubation was 5 min at RT. The exposure time of the first film was 15 s. The exposure time of the second film was adjusted according to the intensity of the signal on the first film. The bands on film were analyzed with GeneSnap/ Gene Tool software from Syngene (Cambridge, UK).

**RNA isolation, reverse transcription, and real-time quantitative PCR**

To detect the difference in expression of Rhoa gene between interphase and mitosis phase in HeLa cells, total RNA of interphase and mitosis cells was isolated using TRIzol reagent. cDNA was synthesized in a 20 μL reaction containing 4 μg of total RNA and SuperScript™II RT according to the manufacturer's instructions. Primers designed for amplification of the human Rhoa gene and GAPDH gene were forward 5'-GGCTGCTACATCGGAA GAAA-3’ and reverse 5’-CACAAGAAGAACACCC AGA-3’ and forward 5’-GGATTGTGCTGATTTGG G-3’, and reverse 5’-GGAAGATGTGATGGGAT T-3’, respectively. The 25 μL reaction contained 0.5 μL cDNA, 10 pmol/L of each primer, 2 mmol/L MgCl\(_2\), 2.5 μL of 200 mmol/L dNTP, and 1 unit of Taq DNA polymerase. The thermal cycle profile for PCR was 94°C for 5 min followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature (63.8°C for Rhoa and 55°C for GAPDH), and 30 s at 72°C with a final 10 min incubation at 72°C. The amplified fragments of the target genes were cloned into pGEMmOl/L vector and DNA sequencing was performed by Gene Co. Ltd. (Shanghai, China).

For real-time quantitative PCR, standard curves were prepared by serial dilution of pGEM-Rhoa and pGEM-GAPDH vector. Reactions of 25 μL included 1 μL of 1:1500 SYBR Green 1, 0.5 μL cDNA, 10 pmol of each primer, 2 mmol/L MgCl\(_2\), 2.5 μL 200 mmol/L dNTP, and 1 unit of Taq DNA polymerase. To compensate for variations of input RNA amounts and efficiency of reverse transcription, an endogenous 'housekeeping' gene (GAPDH) was also quantified and used to normalize the results. The thermal cycle profile for PCR was 94°C for 2 min followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature (63.8°C for Rhoa and 55°C for GAPDH) and 30 s at 72°C. After the cycling of PCR assay was completed, the samples were subjected to a temperature ramp (from 45°C-95°C at 0.5°C/2 s) with continuous fluorescence monitoring for melting curve analysis. Analyses were done in triplicates and all reactions were repeated six times independently to ensure the reproducibility of the results. Data were analyzed by Rotor-Gene real-time analysis software (Rotor-Gene 2000, CR, Australia). For each sample the amplification plot and the corresponding dissociation curves were examined. To obtain standardized quantitative results, external controls consisting of cDNA plasmid standards were constructed.

**Statistical analysis**

Data were expressed as mean ± SE. Statistic significance was examined with Wilcoxon signed-rank test and Student's t test, respectively. Probability below 0.05 (P < 0.05) was considered as significant.

**RESULTS**

The subcellular localization of endogenous and exogenous Rhoa in SGC-7901 cells

With immunofluorescence staining (Figure 1) and Western blotting (Figure 2) endogenous Rhoa was detected in the membrane, the cytosol, and the nucleus of SGC-7901 cells. As shown by Western blotting (Figure 3) exogenous Rhoa was also localized in the membrane, the cytosol, and the nucleus of SGC-7901 cells.

The existence of Rhoa in nucleoli of SGC-7901 cells

Both immunofluorescence staining (Figure 4) and Western blotting (Figure 5) indicated the nucleoli as the precise localization of Rhoa protein in interphase SGC-7901 cells.

The change of Rhoa expression during cell cycle

In mitotic active Hela cells, Western blotting indicated that the expression of Rhoa protein in interphase (Figure 6A) was higher than that in mitosis phase (Figure 6B).
Consistent with this finding, the amounts of RhoA transcripts were also found to be higher in interphase than in mitosis phase (Figure 7B).

**LPA-induced membrane and nucleus translocation of RhoA in SGC-7901 cells**

As shown by Western blotting, a stimulation with 1 μmol/L LPA at 37°C for 15 min causes an increase in the amount of RhoA in the membrane (Figure 8A) and the nuclear (Figure 8B), but a decrease in the cytosol fraction (Figure 8C).

**CPT-cAMP-induced cytosol translocation of RhoA in SGC-7901 cells**

An incubation with 100 μmol/L cAMP at 37°C for 30 min led to a decrease in the amounts of RhoA protein in membrane (Figure 9A) and nuclear (Figure 9B) fractions, but to an increase in cytosol preparations (Figure 9C).

**CPT-cAMP could block LPA-induced RhoA translocation in SGC-7901 cells**

Stimulation with 100 μmol/L cAMP at 37°C for 30 min.
our laboratory reported the existence of RhoA in the and cytosol, and mainly in the latter one, the subcellular localization of RhoA was in the membrane and tumor progression.

RhoA acts as a molecular switch in the cells, regulates intracellular target molecules, and is involved in a variety signal transduction from cell surface receptors to RhoA protein in the membrane (Figure 10A) and the nuclear (Figure 10B), but an increase in the cytosol (Figure 10C) RhoA protein followed by an incubation with 1 μmol/L LPA at 37°C for 15 min was found to cause a decrease in the amounts of RhoA gene was detected by real-time quantitative PCR. Each bar represents mean ± SE obtained from five independent experiments. *P < 0.05.

DISCUSSION

RhoA acts as a molecular switch in the cells, regulates signal transduction from cell surface receptors to intracellular target molecules, and is involved in a variety of biological processes, including cell morphology[25,26], motility[27,28], cytokinesis[29,30], smooth muscle contraction[31,32], and tumor progression[33,34]. It was widely accepted that the subcellular localization of RhoA was in the membrane and cytosol, and mainly in the latter one[35,36]. Recently, our laboratory reported the existence of RhoA in the nuclei of SGC-7901 cells[37]. In this study, we not only confirm the nuclear localization of RhoA; we were able to localize RhoA proteins more precisely, namely within the nucleolus.

Protein import into the cell nucleus is a typical transport process between the cytosol and the nucleus. It occurs through nuclear pore complexes (NPCs)[38,39]. These elaborate proteinaceous structures act not only as molecular sieves, allowing free diffusion of ions and small molecules, but also mediate the active transport of proteins and ribonucleoprotein particles. In order to enter the nucleus, proteins larger than about 60 kDa generally require a specific nuclear localization signal (NLS). Protein molecules can bind with transporting protein such as...
importin via NLS and get into the nucleus through ATP-dependent process. However, the molecular mass of RhoA is 21 kDa and there is no NLS with its protein structure. Our primary experiment did not detect the combination of RhoA with nuclear transporting proteins such as importin α and importin β (data not shown). So, it was reasonable for us to presume that the molecular mechanism of RhoA protein transportation to the nucleus was not through the classic transportation pathway, but diffusion.

The nucleolus of eukaryotic cells was first described in the early 19th century and was discovered in the 1960s to be the site of ribosome synthesis. It owned a variety of roles of cell activity, rRNA transcription, rRNA processing, and ribosome assembly have been clearly established as major functions of the nucleolus. As research progresses, many other functions of the nucleolus have been recognized, including gene expression, processing of nuclear export of certain mRNAs, processing of U6 RNA, one of the spliceosomal small nuclear RNAs, and processing of tRNA precursors. Recently, the plurifunctional nucleolus concept has been widely accepted.

According to the concept of plurifunctional nucleolus, it is reasonable to speculate that RhoA will share, at least, part of the function of the nucleolus. A previous study suggested a role for major nucleolar proteins in the nucleocytoplasmic transport of ribosomal components, and transient exposure of shuttling proteins to the cytoplasm may provide a mechanism for cytoplasmic regulation of nuclear activities. The expression of some nucleolar proteins, such as B23, changes during cell cycle. In this experiment, the results showed that the amount of RhoA protein also changed during cell cycle, exhibiting high amount in S phase, and the change of RhoA mRNA expression was the same as that of the protein. This suggested that RhoA probably played a role in regulation of cell mitogenesis. In this way, our results obviously provide a new insight into investigating some unknown functions of RhoA according to its new distribution.

In our previous study, with immunofluorescent microscopy, we did not detect significant translocation of RhoA in SGC-790 treated with RhoA activator LPA or RhoA activation inhibiting reagent cAMP. Since some other studies reported RhoA translocation according to its activity with Western blot assay, in this experiment, we applied different methods to separate cellular components and western blotting to investigate RhoA translocation, especially in nucleus. With these more concise and sensitive methods, we showed that after the treatments with LPA, cAMP, and cAMP + LPA, nuclear translocation of RhoA was the same as its membrane translocation. Furthermore, since it was reported that RhoA in membrane represented its active form and our results showed that RhoA moved into the nucleus and onto the membrane during the stimulation of LPA, we supposed that RhoA in nucleus is also in the active status.

In conclusion, our results indicated that the nucleolus provided a new compartment for RhoA in SGC-7901 cells and that the activation of RhoA had an obvious effect on its translocation, stimulating the distribution of this protein not only in membrane but also in nucleus.

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