Cloning and characterization of Rab23, a brain specific small GTPase

Chong Kooi Hoong 1 and Felicia Y.H. Teng 2

1 Department of Biochemistry, National University of Singapore, 8 Medical Drive, Singapore 117609
2 NCA Laboratory, Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609

ABSTRACT

Small GTPases of the Ras superfamily are molecular switches that control various cellular processes. The Rab-family members regulate intracellular trafficking, and are involved in vesicle targeting, docking and fusion processes. The brain-enriched Rab23 is the only Rab protein that is known to have a distinct function during central nervous system development, playing an essential role as a negative regulator of the Sonic Hedgehog (Shh) pathway via an unknown mechanism. I have cloned the mouse Rab23 cDNA, made a His6-tagged fusion protein of Rab23 and raised Rab23 specific antibodies in rabbits. I have also generated the dominant-negative and constitutively active mutants of Rab23 and expressed these in mammalian cells in comparison with the wild-type gene. Western blot analysis revealed that the Rab23 protein has a size of 27kDa. Indirect immunofluorescence showed that exogenously expressed Rab23 is cytosolic and with distinct plasma membrane staining. The latter is in line with its potential function in influencing the plasma membrane components of the Shh pathway. I also attempted to look for potential interacting membrane partners for Rab23 using the yeast-two-hybrid screen.

INTRODUCTION

Small GTPases members are highly conserved with 30% to 55% homology at the amino acid level in eukaryotes (Takai et al., 1992). More than 60 Rab proteins families had been discovered. As the largest branches of the small GTPases of Ras superfamily, Rabs regulate intracellular membrane trafficking. Rab23, a member of the Rab family of vesicle transport regulators, is predominantly expressed in the brain. Embryonic lethal mutations in mouse Rab23 results in an “open brain” phenotype (Jeong et al., 2001). Dorsalizing signals appear to activate transcription of Rab23 in order to silence the sonic hedgehog (Shh) pathway in dorsal neural cells. By acting downstream of Shh, Rab23 is postulated to participate in a process that promotes the inhibition of Smoothened by Patched1 in mice. Rab23 might also control vesicle movement by working with other proteins that contain a sterol-sensing domain. Rab23 is the only Rab protein known so far to be involved in central nervous system (CNS) development. Understanding how Rab23 works may provide further information on developmental or pathological aspects of CNS development.

MATERIALS and METHODS

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1 Student
2 Research Scientist
**Molecular cloning and DNA manipulation** – Mouse Rab23 cDNA was initially cloned from a mouse brain cDNA library (Clonetech) by PCR. Other appropriate PCR products were subsequently generated and cloned into pCIneo vector for expression studies in mammalian cells, or into pET-15b vector to produce His₆-Rab23 fusion protein. Rab23 mutants were created using nested PCR with appropriate primers and cloned into pCIneo vector for mammalian cell expression studies. The Rab23Q202L was also inserted into the pGBKT7 vector for yeast-2-hybrid interaction screen.

**His₆-Rab23 fusion protein expression in bacteria** – pET-15b constructs were transformed into BL21(DE3) cells. Cultures were induced with 0.1mM IPTG for 4 hours at 37°C. The cell pellet was sonicated, and cleared lysate was incubated with 0.5ml of nikel-nitrilotriacetic (Ni-NTA) agarose beads. The bound His₆-Rab23 was eluted with 200mM imidazole solutions in PBS.

**Animal immunization and antibody production** – The His₆-Rab23 protein was used to immunize rabbits by a regime of subcutaneous injections of a mixture with Freund’s adjuvant. Antibody from positive serum is affinity-purified using the antigen electroblotted onto nitrocellulose strips.

**In-vitro translation** – In-vitro translation was performed using the Promega TNT® Quick Coupled Transcription/Translation System and the in-vitro translated products were analyzed using Western blot with Rab23 antibody (above) or using ³⁵S-Methionine labeling followed by autoradiography.

**Mammalian cells expression** – CHO and NRK cells were cultured in RPM1-1640 media supplemented with 10% fetal calf serum FCS in a humidified incubator with 5% CO₂. Transfection was carried out with 10 to 20 µg of DNA in serum free opti-MEM medium (Invitrogen) using Lipofectamine 2000™. Cells were harvested and protein expression was analyzed though immunoblot after 24 to 36 hours.

**Immunofluorescence staining** – Cell were grown on coverslips and transfected with the Rab23 constructs. Cells are fixed with 4% paraformaldehyde, permeabilized with PBS + 0.2% Tx-100 and incubated with 5µg/ml of primary antibodies for 1 hour, followed by goat-anti-rabbit secondary antibody conjugated to FITC. Coverslips are mounted using Vectashield (Vector Labs) mounting medium. Immunofluorescence microscopy was performed with a Zeiss Axiophot microscope equipped with epifluorescence optics and a BioRad 1024 confocal system.

**Yeast-two-hybrid screening** – Rab23Q202L(pGBKT7) is transformed into AH109 and expression was confirmed by immunoblotting. Pretransformed Matchmaker Libraries (Clontech) were mated with Rab23Q202L-AH109. Plasmids from the yeast were transformed into DH5α bacteria cells and the inserts were sequenced and identified with the BLAST programs at the NCBI website [http://www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/).

**RESULTS and DISCUSSIONS**

**Making His₆-Rab23 Fusion Protein** – His₆-Rab23 protein was produced in large scales by processing 4 liter cultures. The size of the His₆-Rab23 obtained was about 36kDa (fig.1), which is larger than its predicted size. Protein sequencing using Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MALDI) revealed that the 36kDa band contains several peptide sequences corresponding to that of Rab23.
Fig.1. The His<sub>6</sub>-Rab23 proteins are inducible and soluble. 5 fractions (1ml each) were collected from large-scale production and 10µl aliquot was resolved by SDS-PAGE. 50µl of the uninduced and induced cultures were centrifuged, the pellets solubilized in sample buffer and loaded. For determination of His<sub>6</sub>-Rab23 solubility, equivalent amount of the supernatant and pellet was loaded after sonication. The proteins were visualized by Coomassie blue staining. Fractions 1 to 4 were combined and dialysed.

**Raising Rab23 Antibody in rabbits** – The affinity-purified antibody from each bleed was subjected to analysis and characterization by Western blotting of rat brain lysate. The antibody picks up a band at around 27kDa. The titer and specificity of the Rab23 antibody improved with time (Fig.2).

**In-vitro translation of Rab23 cDNA** – Translation of two separate clones (5 and 7) yielded a band of 27kDa in size (fig.3).

**In-vivo expression of Rab23** – Rab23(pCIneo) was transfected into human kidney 293T mammalian cells and western blot analysis with Rab23 antibody revealed a distinct band at 27kDa (fig.4), in agreement with that obtained by in-vitro translation.

**Fig.2. Western Blot analysis of Rab23 antibody efficiency.** 1.25µg of Rab23-His<sub>6</sub> protein was used as the positive control and different indicated amounts of rat brain Tx-100 lysate was loaded. The blot was incubated with 2ng Rab23 antibody followed by Goat-anti-rabbit antibody conjugated with HRP. Chemiluminescence substrate was diluted 8x with water and the blot was exposed for 10 seconds. Comparing the Western blots performed on November 2001(B), and December 2002 (A) showed distinct improvement of the titer and specificity of Rab23Ab.

**Fig.3. (A) Western Blot of “cold” in-vitro translated products.** The blot is first incubated with His<sub>6</sub>-Rab23 antibody followed by Goat-anti-rabbit antibody conjugated to HRP. Chemiluminescence substrate was diluted 8x with water and exposed for 5 seconds. (B) Phospho-imaging visualization of the “hot” in-vitro translated product. T7 Luciferase (monomeric protein with size 61kDa) was used as a positive control for the translation reaction. The exposure times were 3 days.

**Fig.4. Western Blotting of Rab23(pCIneo) in 293T cells.** The blot is incubated with His<sub>6</sub>-Rab23 antibody followed by Goat-anti-rabbit antibody conjugated with HRP. 8x diluted Chemiluminescence substrate was added and was exposed for 5 seconds. Empty pCIneo vector was transfected as a negative control.
Subcellular localization of Rab23 by indirect immunofluorescence microscopy – Rab23 wild type and mutants were transiently transfected into CHO and NRK cells. Indirect immunofluorescence analysis revealed that exogenously Rab23 are primarily cytosolic (fig.5). There also appear to be clear staining of the plasma membrane by the antibody (especially Rab23 wild type and Rab23Q202L), as illustrated by the labeling of cell edges and extensions (fig.5C).

Fig.5. Immunofluorescence analysis of Rab23(pClneo), Rab23Q202L(pClneo) and Rab23S67N(pClneo). Cells were fixed with 4% paraformaldehyde and incubated with Rab23 antibody, following by fluorescein isothiocyanate-conjugated Goat anti-rabbit IgG. (C) is an magnified view of a cell in (B), the labeling of plasma membrane edges is marked by arrows. Legend: Bars = 10 µm

Rab23Q202L(pGBKT7) expression in AH109 yeast strain – Analysis of two separate clones (1 and 2) revealed an immunoreactive band at 51kDa. This band is not present in empty pGBKT7 vector transfected AH109 cells.

Identification of Rab23 interacting partners – 287 clones were obtained in this yeast-two-hybrid screen. However, most of the clones represent DNA fragments of unknown identity. Amongst those with an apparent identity, most do not correspond to the coding region of the known gene, or are fused with Gal4-AD in a wrong reading frame. The only potential interacting candidate is a new gene recently deposited into database, koyt binding protein (KBP) (Li et al., 2003).

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REFERENCES