World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 4.223

CLONING PROTEIN EXPRESSION AND CHARACTERIZATION OF HUMAN RAB39B[1] YAAMINI SUBRAMANIAN

Yaamini Subramanian*

Structural Biology Laboratory, National University of Singapore (NUS), Singapore.

***Corresponding Author: Yaamini Subramanian**
Structural Biology Laboratory, National University of Singapore (NUS), Singapore.

Article Received on 20/08/2017 **Article Revised on 10/09/2017** **Article Accepted on 01/10/2017**

ABSTRACT

The ***Rab39b*** gene encodes a member of the *Rab* family of proteins. They are small GTPases that are involved in vesicular trafficking. In *Rab* proteins, the hydrolysis of GTP to GDP is coupled with association with and dissociation from membranes and it is present in all compartments of the endomembrane system (endoplasmic reticulum, Golgi, endosomes, lysosomes), the nucleus, the plasma membrane (including cell junctions and focal adhesions), mitochondria and centrioles. Our focus is to understand the structure and interactions of *Rab39b* protein using X-ray crystallography. Specifically we can study how proteins interact with other molecules, how they undergo conformational changes, and perform catalysis in the case of enzymes. Currently very little is known about *Rab39B* in the body and how loss of *Rab39B* contributes development of neurodegenerative disorder such as Parkinson's disease, autism and Waisman syndrome. A codon optimized and the disordered regions at the C-terminal, removed *Rab39b* gene was cloned, fused with a glutathione S-transferase (GST) tag and sequenced. The cloned product was expressed in BL21 (DE3) cells resulting in a protein with molecular weight 19.8+25 kDa and verified by SDS-PAGE gel. Even though the function of *Rab39b* is less known, the protein has been implied to play important roles in synapse formation.

KEYWORDS: GTPase, Parkinson's disease, Rab39b, Rab proteins.

BACKGROUND

The rab family of proteins is a member of the ras superfamily of monomeric g proteins. They are localized to the cytoplasmic face of organelles and vesicles involved in the biosynthetic/secretory and endocytic pathways in eukaryotic cells. Twelve rab proteins have been localized to the endocytic pathway of mammalian cells; eight have been functionally characterized, and four are epithelial specific. Activated rab proteins serve as molecular scaffolds for intracellular signaling and coordinate three main membrane-trafficking steps: (1) vesicle budding (2) cytoskeletal transport (3) targeted docking and fusion (mayer and wickner, 1997).

Small GT Pases serve as molecular switches and regulate intracellular signaling. These molecules are GDP/GTP binding proteins (G-proteins) that are active when bound to GTP and inactive when bound to GDP (Fig. 1.1) (Zerial and McBride, 2001). Many small G-proteins are brought into the cytosolic form when inactive by binding to the GDP dissociation inhibitors (GDI). DENN (differentially expressed in normal and neoplastic cell) act as guanine nucleotide exchange factor (GEF) for *Rab* proteins. C9ORF72 contains the DENN domains, which dissociates GDP from *Rab* with GTP exchange and cause membrane trafficking (Hutagalung and Novick, 2011). It is involved in vesicular trafficking and plays a role in synapse formation (Progida et al., 2010). May regulate the homeostasis of SNCA/alpha-synuclein. Together with PICK1 proposed to ensure selectively GRIA2 exit from the endoplasmic reticulum to the Golgi and to regulate AMPAR compostion at the post-synapses and thus synaptic transmission. Mutations in small GTPases *Rab39b* are responsible for X-linked mental retardation associated with autism, epilepsy and macrocephaly and loss of *Rab39b* leads to dysregulation of α-synuclein and cause neurodegenerative disease such as: mental retardation, Waisman syndrome (WSSM), early onset of Parkinsons disease and intellectual disability cormobid (autism spectrum disorder and epilepsy) (Issers et al, 2010; D'Adamo et al., 1998).

METHODS AND MATERIALS

Cloning of *Rab39b* gene (514 bp)

The *Rab39b* gene was truncated by removing the disordered region from the C-terminus and was amplified from its cDNA, cloned into the pUC vector using primers that introduced the BamH1 and Xho1 restriction sites. The

amplified gene was separated on a 0.8% agarose gel and the band was extracted using a gel extraction kit. Double digestion of the amplified gene and the vector [pGEX-6-p-1, which harbors the Glutathione-s-transferase (GST) tag] was performed at 37 ºC for 1 hour and Ligation was performed for overnight at room temperature. The clones were then introduced into DH5α competent cells and grown overnight in LB agar medium containing 100µg /ml ampicillin. Colony PCR was used to identify clones that contained the inserted DNA.

Protein Expression

The confirmed plasmid were expressed in E. coli (BL21) (DE3) cells and grown in 100 ml culture with 100 µg/ml of ampicillin. Secondary culturing was performed by transferring 25 mL of the primary culture to a 2 L culture flask and incubated at 37 ºC until the optical density (OD) reaches about 0.6-0.8. 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the production of *Rab39b.* The shaker was then set to a lower speed and temperature, the culture flasks were left in the shaker for overnight .Cells were centrifuged and the bacterial pellets were washed with 1X PBS.

Protein Purification

The bacterial pellets were resuspended in lysis buffer containing 50 mM Tris pH 7.5, 5% glycerol, 500 µL of 150 mM, Triton X, BME and protease inhibitor tablet. The cell suspension was sonicated and the numbers of cycles were decided by visually analyzing the thickness of the cell suspension. The sonicated sample was centrifuged at 18000 g for 30 mins, and 10 µl of supernatant were collected for SDS-PAGE (Venyaminov S, 1993).

Affinity Column Purification

The sonicated sample obtained above was loaded onto a 2 ml slurry of glutathione sepharose 4B Beads, which were pre-equilibrated with the lysis buffer for 20 min at 4 ºC. The binding was performed for 4 hours at 4 ºC. The protein bound to the beads were then kept overnight at 4 ºC for cleavage with lysis buffer and of Precision protease (1.5 mg/ml). The protein was eluted (10 µL eluted sample was saved) and was further purified by size exclusion chromatography.

RESULTS AND DISCUSSION

Generation of Pcr of Insert for Cloning

Given below is agarose gel resolving the PCR product generated for cloning the gene of interest into the pGEX-6-p-1vector, containing a GST tag, and attempts with different vectors and gene lengths failed and it shows the successful amplification of the protein encoding sequences with the expected base-pair length. The product were purified and restriction enzyme digested with BamH1 and Xho1. The ligated product was transformed into DH5α and a few colonies were picked and colony PCR was performed and showed a positive result and was confirmed by restricting digest, with BamH1 and Xho 1, and amplified by insert specific primers. The plasmid miniprep was sent for sequencing and shown in the Figure 1.

Rab39b pGEX-5 Seq

NNNNNNNCAATCGGATCTGGAGTTCTGTTCCAG
GGGCCCCTGGGATCCATGGAAGCGATTTGGCTG
TACCAGTTTCGTCTGATTGTTATTGGCGATAGCA
CCGTTGGCAAGAGCTGCCTGATTCGTCGTTTCAC
CGAGGGTCGTTTCGCGCAGGTTAGCGACCCGAC
CGTGGGCGTTGATTTCTTTAGCCGTCTGGTGGAG
ATCGAACCGGGCAAGCGTATCAAACTGCAGATT
TGGGACACCGCGGGCCAAGAGCGTTTTCGTAGC
ATCACCCGTGCGTACTATCGTAACAGCGTTGGT
GGCCTGCTGCTGTTCGATATTACCAACCGTCGTA
GCTTTCAAAACGTGCACGAATGGCTGGAGGAAA
CCAAAGTGCACGTTCAGCCGTACCAAATCGTGT
TCGTTCTGGTGGGTCACAAGTGCGACCTGGATA
CCCAGCGTCAAGTTACCCGTCATGAGGCGGAAA
AGCTGGCGGCGGCGTACGGTATGAAATATATCG
AAACCAGCGCGCGTGACGCGATTAACGTTGAAA
AGGCGTTCACCGACCTGACCCGTGATATTTAACT
CGAGCGGCCGCATCGTGACTGACTGACGATCTG
CCTCGCGCGTTTCGGTGATGACGGTGAAAACCT
CTGACACATGCAGCTCCCGGAGACGGTCACAGC
TTGTCTGTAAGCGGATGCCGGGAGCAGACAAGC
CCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTG
TCGGGGCGCAGCCATGACCCAGTCACGTAGCGA
TAGCGGAGTGTATAATTCTTGAAGACGAAAGGG
CCTCGTGATACGCCTATTTTTATAGGTTAATGTC
ATGATAATAATGGTTTCTTAGACGTCAGGTGGC
ACTTTTCGGGGAAATGTGCGCGGAACCCCTATTT
GTTTATTTTTCTAAATACATTCAAATATGTATCC
GCTCATGAGACAATAACCCTGATAAATGCTTCA
ATAATATTGAAAAAGGAAGAGTATGAGTATTCA
ACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGG
CATTTTGCCTTCCTGTTTTTGCTCACCCAGAAAC
GCTGGTGAAAGTAAAAGATGCTGAAGATCAGTT
GGGTGCCCGAGTGGGTTACATCGAACTGGATCT
CAACAGCGGTAAGATCCTTGAAAGTTTTCGCCC
CGAAAAACGTTTTCCAATGATGAGCACTTTTAA
AGTTCTGCTATTGGNCCCGGTATATTCCCGG

Rab39b pGEX-3 Seq

NNNNNNNNNNANNCGCGAGGCAGATCGTCAGT
CAGTCACGATGCGGCCGCTCGAGTTAAATATCA
CGGGTCAGGTCGGTGAACGCCTTTTCAACGTTA
ATCGCGTCACGCGCGCTGGTTTCGATATATTTCA
TACCGTACGCCGCCGCCAGCTTTTCCGCCTCATG
ACGGGTAACTTGACGCTGGGTATCCAGGTCGCA
CTTGTGACCCACCAGAACGAACACGATTTGGTA
CGGCTGAACGTGCACTTTGGTTTCCTCCAGCCAT
TCGTGCACGTTTTGAAAGCTACGACGGTTGGTA
ATATCGAACAGCAGCAGGCCACCAACGCTGTTA
CGATAGTACGCACGGGTGATGCTACGAAAACGC
TCTTGGCCCGCGGTGTCCCAAATCTGCAGTTTGA
TACGCTTGCCCGGTTCGATCTCCACCAGACGGCT
AAAGAAATCAACGCCCACGGTCGGGTCGCTAAC
CTGCGCGAAACGACCCTCGGTGAAACGACGAAT
CAGGCAGCTCTTGCCAACGGTGCTATCGCCAAT
AACAATCAGACGAAACTGGTACAGCCAAATCGC

TTCCATGGATCCCAGGGGCCCCTGGAACAGAAC
TTCCAGATCCGATTTTGGAGGATGGTCGCCACC
ACCAAACGTGGCTTGCCAGCCCTGCAAAGGCCA
TGCTATATACTTGCTGGATTTCAAGTACTTATCA
ATTTGTGGGATAGCTTCAATACGTTTTTTAAAAC
AAACTAATTTTGGGAACGCATCCAGGCACATTG
GGTCCATGTATAAAACAACATCAAGAGCGTCAT
ACAACATGAAGTCAGGATGGGTTACATGATCAC
CATTTAAATATGTTTTATGACATAAACGATCTTC
GAACATTTTCAGCATTTCAGGTAGCTTGCTAAGA
AAATCAACTTTGAGAGTTTCAAAGTCTTTACTAT
ATGCAATTCTCGAAACACCGTATCTAATATCCA
AAACCGCTCCTTCAAGCATTGAAATCTCTGCAC
GCTCTTTTGGACAACCACCCAACATGTTGTGCTT
GTCAGCTATATAACGTATGATGGCCATAGACTG
TGTTAATTTAACATCACCCTCCAATATAATAANG
GAAGATTGGGAAACTCCAAACCCAATTCAAACT
TTTTGTTTCCCCATTTATCACCTTCATCGGGCTCA
TACAAATGGCTCTTCATATTTTTCTTCAAAATAT
TCCAAAAAAAGTCCNNNGGGGTTGGCCAAAGGC
CCTTAATTTTCCAATAACNTNNTNAAGGGGACA
TGNAAANNGGTTCCCGGGGGAAAATTGTTTCCC
NCCCCAATTCCCACCTTTT

Figure 1: The sequencing results of Human *Rab39b.*

Expression and Purification of *Rab39B*

The expression clones were transformed into chemically competent BL21 (DE3). Colonies were grown against Ampicillin. Once the OD of the cultures reached 0.6, expression was induced by adding 0.5 mM IPTG. Cells were grown overnight at 16 °C. The un-induced and induced aliquots were loaded onto an SDS-PAGE for comparison. Fig. 4.4 shows that in the uninduced lane, our protein with the fusion tag is not expressed. In the induced lane, the *Rab39b* protein is overexpressed as a prominent band at 45.8 kDa (including GST).

From the Figure 2 it is clearly seen tthe protein solubility is low as depicted by the high protein content in the pellet (Burgess, 2009). The presence of insignificant protein in the flow through and wash shows that the purification process was efficient where the GST tag was cleaved using Precision protease and the cleaved protein was eluted. In the "elute lane" the desired protein of molecular weight 19.8 kDa is seen with other non-specific proteins. The protein was purified by GST affinity purification and most of the protein came in the elution, along with several impurities, and will require further purification steps.

By optimizing IPTG concentration and temperatures, so that high concentration yield can be obtained with purity so that crystals can be set where the structure can be solved and understand how loss of *Rab39B* contributes development of.

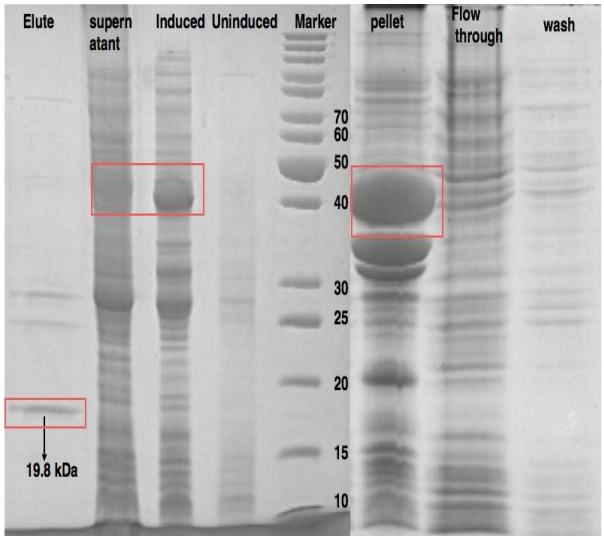

Figure 2: Expression of *Rab39b* clone at 0.5 mM IPTG and the profile of GST affinity column purification. The lanes are: elute, supernatant, induced, uninduced, marker, pellet, flow through and wash. The red box indicates the position of the band corresponding to the size of *Rab39b* +GST.

Neurodegenerative disorder and further studies will focus on the expression of *Rab39B* protein and the role of human *Rab39B.*

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