Signaling role of p50RhoGAP's protein domains in intracellular membrane traffic processes.

Ph.D. thesis

Dr. Gábor Sirokmány

Semmelweis University
School of PhD Studies

Supervisors: Dr. Miklós Geiszt, Dr. Erzsébet Ligeti

Opponents: Dr. Gábor Bánhegyi
Dr. Árpád Lányi

Chairman of the comprehensive exam board: Dr. Anna Faragó
Members of the comprehensive exam board: Dr. Emil Monos
Dr. András Váradi

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Own pubications

Related to the thesis:

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J Biol Chem. 2006 Mar 3;281(9):6096-105. Epub 2005 Dec 27;
2. Katalin Szászi*, **Gábor Sirokmány***, Caterina Di Ciano-Oliveira, Ori D. Rotstein, and András Kapus
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Depolarization induces Rho-Rho kinase-mediated myosin light chain phosphorylation in kidney tubular cells.
Am J Physiol Cell Physiol. 2005 Sep;289(3):C673-85. Epub 2005 Apr 27.
3. Caterina Di Ciano-Oliveira,[1] **Gábor Sirokmány**,[1] Katalin Szászi,[1] William T. Arthur,[2] András Masszi,[1] Mark Peterson,[1] Ori D. Rotstein,[1] and András Kapus[1]
Hyperosmotic stress activates Rho: differential involvement in Rho kinase-dependent MLC phosphorylation and NKCC activation.
Am J Physiol Cell Physiol. 2003 Sep;285(3):C555-66. Epub 2003 May 14.

Other publication:

1. András Masszi[1,2], Caterina Di Ciano[1], **Gábor Sirokmány[1]**, William T. Arthur[3], Ori D. Rotstein[1], Jiaxu Wang[4], Christopher A. G. McCulloch[4], László Rosivall[2], István Mucsi[2,5,6], and András Kapus[1]
Central role for Rho in TGF-beta1-induced alpha-smooth muscle actin expression during epithelial-mesenchymal transition.
Am J Physiol Renal Physiol. 2003 May;284(5):F911-24. Epub 2002 Dec 27.

Introduction

During my Ph.D. studies I worked mainly on the function of the p50 Rho GTPase activating protein (p50RhoGAP) and especially on its role in membrane traffic of eukariotic cells. To make the following chapters dealing with our objectives, results and conclusions more clear I give a short introduction about:

- GTPase cycle of RhoGTPases.
- Domain structure of p50RhoGAP protein and previous data found in the literature about this protein.
- Main steps of receptor mediated endocytosis and the role of Rab and Rho GTPases involved in these processes.

Rho GTPase cycle

Rho GTPases work like switches that bind to their effector proteins in their GTP bound form. Following the hydrolysis of GTP they release their effectors and remain inactive until they bind GTP again. Similarly to other monomer GTPase protein families Rho proteins also cooperate with numerous regulatory proteins during the GTP-GDP cycle.

The Rho specific guanin nucleotide exchange factors (GEF) promote the exchange of the nucleotide bound to the GTPase. Within the intracellular milieu it usually means that the GDP bound to the GTPase will be exchanged for GTP which is in excess in the cytosol.

The endogenous GTPase activity of monomer G-proteins is approximately only one hundredth of the activity of heterotrimeric GTPases. The Rho GTPase activating proteins (RhoGAPs) are able to enhance the GTPase activity of monomer GTPases by about five orders of magnitude. The importance of GAP activity is underlined by the fact

that in an avarage resting cell only about 5 % of a Rho GTPase is present in its active, GTP bound form and this ratio does not go above 10 % even in a cell subjected to generalized stimuli.

The third group of regulatory proteins is the group of guanin nucleotide dissociation inhibitor (GDI) proteins. They are mainly known as inhibitor proteins that are able to sequester the GTP bound small G protein and transfer the isolated protein between cellular compartments. Additionally they can solubilize a membrane bound but already inactivated small GTPase and keep it in the cytosol in a biologically inactive form.

The p50RhoGAP protein

The protein is present in many different cell types and tissues. It consists of 439 amino acids that form an N-terminal Sec14 domain, a prolin rich region and a C-terminal GAP domain. Both the full length protein and the isolated GAP domain *in vitro* enhances the GTPase activity of Rac, Rho and Cdc42. In other in vitro experiments the prolin rich region of the protein was able to bind to SH3 domain containing proteins. However about the role of the Sec14 domain no previous data had been known. The cellular localization of regulatory proteins is usually not determined by their catalytic domains but rather by other additional protein domains. Therefore the role and interaction of these domains might give insight into function of the full length protein.

Rab and Rho GTPases in receptor mediated endocytosis

The membrane traffic of eukaryotic cells is a principal physiological process used for exchange of information and material between its compartments and the extracellular milieu. An important part of these processes is the receptor mediated endocytosis and recycling. (Fig.1.). The Rab and Rho GTPases play regulatory role at multiple steps in the routes diplayed on the figure. Rab5 mediates fusion between early and sorting endosomes. Rab4 and Rab11 are involved in recycling, Rab4 mainly in the rapid recycling from sorting

Results and conclusion

In our experiments we showed that p50RhoGAP colocalizes with the endogenous transferrin-receptor, with the fluorescent transferrin- and EGF-containing vesicles and Rab11 and Rab5 positive structures.

According to our experiments using Brefeldin A and nocodazole we can state that in HeLa cells p50RhoGAP very probably does not localize to the Golgi-complex and resides in a compartment distinct from the one that contains Cdc42 GTPase.

Using GFP fusion forms and single amino acid change mutants we showed that the N-terminal Sec14 domain of the molecule is responsible for the localization described above.

From BRET measurements we concluded that p50RhoGAP forms molecular complex with Rab5 and Rab11 and this interaction is mediated by the Sec14 domain of the GAP molecule. In vitro GAP activity assays showed however that Rab5 and Rab11 are not substrates for the GAP activity of p50RhoGAP.

In our quantitative transferrin uptake and recycling assays the overexpression of the Sec14 domain inhibited both the uptake and recycling of transferrin. The mislocalized L173D mutant form of Sec14 did not exhibit such inhibitory effect. Additionally both the full length wild type form and the catalitically inactive R282A form of p50RhoGAP-GFP inhibited transferrin uptake while the mislocalized L173D form did not. In our p50RhoGAP RNA interference experiments however we did not see any change in the transferrin traffic. This could be explained by the sufficient activity of the remaining 15-20 % of the endogenous protein or by the compensatory activity of p50RhoGAP homologues.

In summary the Sec14 domain of p50RhoGAP molecule plays pivotal role in the effect of the protein on membrane traffic. p50RhoGAP may provide a link between two regulators of vesicle traffic: the Rab and the Rho GTPases.

washed them once again in ice cold PBS, then cells were fixed in 1% paraformaldehyde for 15 minutes. Finally I washed them several times in PBS and kept the cells on 4 °C until the flow cytometric measurement started. GFP intensity was measured in the FL-1, Alexa647 intensity of GFP positive cells in the FL-4 channel on a Becton Dickinson FACSCalibur flow cytometer.

Bioluminescence resonance energy transfer (BRET)

Cells cotransfected with the Luciferase fusion forms of Rab GTPases and YFP fusion forms of p50RhoGAP were incubated with the cell permeable luciferase substrate coelenterazine. When interaction occurs the luminescent coelenterazine and the YFP comes into molecular proximity (<10 nm), energy transfer takes place that results in emission of 530 nm light from the excited YFP. This informs us about the interaction of the Luciferase- and YFP-tagged proteins. Measurements were carried out on a Mithras LB940 (BertoldTechnologies) luminometer.

***In vitro* GAP activity assay**

The monomer G-protein was loaded with GTP containing ^{32}P at the γ position. The radioactively labelled G-protein was put into a reaction mixture containing the protein analysed for GAP activity and incubated for 10 minutes. The reaction mixture was pipetted on a nitrocellulose membrane, the membrane was washed and then its radioactivity was measured. If the γ-^{32}P is hydrolysed by the G-protein then the radioactive phosphate will be washed off the membrane. If the γ-^{32}P is not hydrolysed then it remains bound to the protein which is bound to the nitrocellulose membrane. The radioactivity of the membrane was measured in distilled water on the basis of the Cerenkov effect in a Beckman LS 5000TD liquid scintillation spectrometer. The more GTP is hydrolyzed the less activity we measure. Therefore the GAP activity of a protein (as it enhances the GTPase activity of a G-protein) can be displayed as a decrease in membrane bound radioactivity.

endosomes while Rab11 regulates later steps of recycling starting from the perinuclear recycling endosomes.

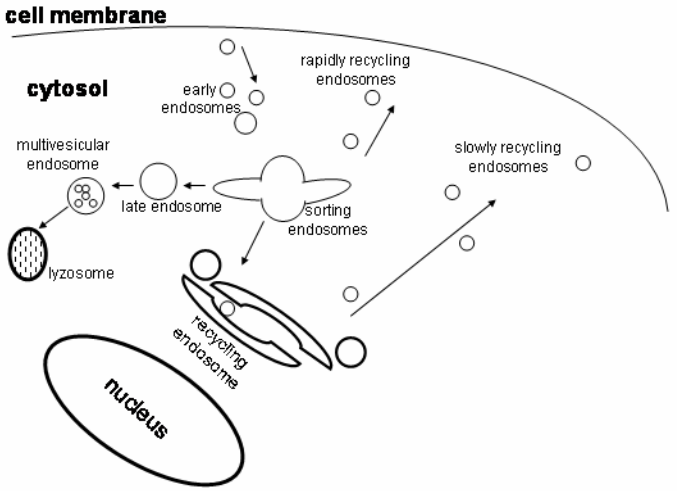

Fig.1. Main routes of receptor mediated endocytosis.

Rho GTPases possess regulatory role at many points of endocytosis. Interestingly the constitutively active, GTP bound mutant forms of Rho GTPases exert inhibitory effects at these points of endocytosis. It indicates that a G-protein „frozen" in its GTP – and effector protein - bound form is far not enough to fulfil its physiological role, but it rather requires cyclic hydrolysis and binding of GTP. RhoGAPs are indispensable for this cycling.

Main Objectives

Apart from the *in vitro* results described above there were no data available about the *in vivo* role and function of p50RhoGAP. Therefore our major objectives were the followings:

1. To determine the intracellular localization of p50RhoGAP and to define also the protein domain(s) responsible for the localization.

2. Functional study of p50RhoGAP in intracellular processes that seem to be relevant regarding our results from step 1.

3. To look for and possibly identify molecular partners in the above processes.

Methods

Immunofluorescent experiments

The localization of the endogenous p50RhoGAP protein in paraformaldehyde fixed HeLa cells was determined using an affinity purified polyclonal rabbit anti-p50RhoGAP antibody (made at our Department) and fluorescent anti-rabbit secondary antibody The cells were imaged using a Zeiss LSM510 confocal laser microscope. The specificity of p50RhoGAP antibody was verified by p50RhoGAP specific siRNA.

DNA constructs, RNA intereference

We prepared the GFP and YFP fusion forms of p50RhoGAP and of Sec14 domain. Beyond the wild type forms we also prepared the R282A mutant form of p50RhoGAP that is catalitically inactive. Using Qiagen's Quickchange site directed mutagenesis kit we introduced the L173D mutation into the Sec14 domain and also into the p50RhoGAP protein which according to our observations resulted in the loss of endosomal localization of these proteins.

Ambion's Silencer siRNA kit was used to synthetize p50RhoGAP specific siRNAs. Sequences starting at nt 127 and 875 from start codon were effective. Scrambled siRNAs were used as controls. We also used vector based RNAi by devising a vector coding the hairpin form of the „875" target sequence behind an H1 promoter and GFP behind a CMW promoter.

Quantitative measurement of transferrin uptake

HeLa cells grown on tissue culture plates were transfected with Sec14-GFP or p50-GFP constructs 24-30 hours before the assay. 10 µg/ml Alexa647-transferrin was added for the indicated times. Cells were then washed in ice cold medium, then treated for two minutes in trypsin-EDTA at 37 °C. After cells became detached from surface I immediately resuspended them in ice cold PBS,