The physiological role of ARHGAP25 – a novel GTPase activating protein – in human phagocytes

PhD Theses

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INTRODUCTION

Neutrophilic granulocytes, as the professional phagocytes of innate immunity are essential in the defense against pathogenes. These pathogenes are recognized by pattern recognition receptors and opsonine receptors. Ligand binding of the receptors initiates pseudopod protrusions which allow engulfment and phagosome closure of the foreign particle. An important step of this process is the dynamic actin rearrangement which requires precise spatial and temporal regulation. Key elements of actin rearrangement signaling are the Rho family small G proteins (Rac, Rho and Cdc42).

After GTP-binding, small G proteins turn to an active state and undergo conformational changes. This allows the interaction between small G proteins and their effectors and leads to carrying forward signaling pathways. After the hydrolyzation of GTP, small G proteins go to an inactive, GDP-bound state. These molecular switches are regulated by three types of proteins. Guanine nucleotide dissociation inhibitors (GDI) keep small G proteins in inactive, GDP-bound state. Guanine nucleotide exchange factors (GEFs) facilitate the GDP dissociation, and allow binding of GTP. GTPase-activating proteins (GAPs) inactivate the small G proteins by enhancing their GTP hydrolysis. Thus, GAPs have essential role in signal downregulation or termination. However, inhibition of GAPs also could be necessary for signal initiation.

About 70 genes of the human genome are predicted to function as Rac/RhoGAPs. Their common feature is an approximately 200 amino acid long catalytic domain, which is responsible for the GTPase activating effect. There is a conserved arginine within the GAP domain, the absence of which causes significantly reduced or completely lost GAP activity. Diverse domain structure of Rac/RhoGAPs and their hitherto revealed participation in signaling pathways strongly suggest that they work in specific molecular environment.

At the beginning of my PhD studies, five Rac/RhoGAPs were known (Abr, Bcr, p50RhoGAP, p190RhoGAP, ArhGAP15) to be expressed in phagocytic cells and to be involved in the regulation of the innate immune response. Previous studies reported increased exocytosis of primary granules of neutrophils isolated from Abr-Bcr double knock-out mice. Macrophages of these mice were characterized by elongated morphology and they showed increased migration and phagocytosis.

Neutrophils from p50RhoGAP knock-out mice also showed increased migration; however, cells were not capable of directional movement. It was found that p50RhoGAP affected in this case primarily Cdc42. It was suggested that p190RhoGAP was involved in the regulation of RhoA through β_2-integrins in human neutrophils. However, a recent study demonstrated that it was not a major regulator of integrin-mediated neutrophil functions in mice. The different role of p190RhoGAP raises the question of cell-specific or species-specific regulatory roles of Rac/RhoGAPs. Investigation of ArhGAP15 knock-out mice revealed that migration, superoxide production, phagocytosis and bacterial killing of their neutrophils were increased. These experiments were carried out mostly with genetically modified mice and do not answer the question of which GAP(s) regulate(s) human neutrophilic functions.

In an *in silico* study, Katoh et al. identified the novel ARHGAP25 which has an N-terminal PH domain, a C-terminal super-helical coiled coil region and a GAP domain. According to the Expressed Sequence Tag (EST) database, ARHGAP25 shows high expression level in the lymphoid tissue and in the blood which was confirmed by the Northern blot experiments of my colleagues. However, ARHGAP25 has never been expressed as a full-length protein and its potential physiological functions in leukocytes have never been reported.

AIMS

In my PhD work I had the following objectives:

1. To investigate the tissue expression profile of ARHGAP25 and other Rac/RhoGAPs in Microarray databases.
2. To express the full-length ARHGAP25 protein and its fragments in bacteria.
3. To determine the small G protein specificity of ARHGAP25.
4. To investigate the potential role of ARHGAP25 in phagocytes – its involvement in actin cytoskeleton rearrangement, in superoxide production and in phagocytosis.

   Moreover, I had an additional objective to develop a method, which would allow investigating the changes in phagocytosis with high efficiency and high through-put.

METHODS

Analysis of microarray databases: To evaluate mRNA microarray data we used Affymetrix Human Genome U133A arrays from PubMed GEO DataSets database. Expression levels were normalized to GAPDH. Expression tables were generated by Microsoft® Office Excel® 2007 SP2 software.

Cells and cell lines: In our experiments we used human polymorphonuclear cells, mixed population of monocytes and lymphocytes (PBMC), T and B cells from human tonsils, primary human macrophages differentiated from PBMC with M-CSF, PLB-985 myelomonoblast cell line differentiated to neutrophils, COS7 cells and COSphoxFcγR cells. COSphoxFcγR are COS7 cells transfected with the components of phagocyte NADPH oxidase and FcγRIIa receptor. Transfection of PLB-985 cells for ARHGAP25-silencing was carried out with siSTRIKE U6 hairpin cloning system. Primary macrophages were transfected with ARHGAP25-specific or control siRNA using Amaxa Human Macrophage Nucleofector Kit. COS7 and COSphoxFcγR cells were transfected with Fugene HD reagent.

Plasmids and proteins: GST-tagged ARHGAP25 and its fragments were amplified from human leukocyte cDNA with PCR reaction. PCR products were cloned into pGEX4T-1 or pCFP-C1 vector. Conserved arginine in the catalytic domain of ARHGAP25 was changed to alanine using QuickChange site-directed mutagenesis kit.

Western blot: We used polyclonal anti-ARHGAP25 and anti-p50RhoGAP antibodies that were prepared by our team, and commercial anti-GST or anti-β-actin monoclonal antibodies.

Measurement of GTPase activity in cell-free conditions: GTPase activity of recombinant proteins was measured with radiolabelled GTP using nitrocellulose filter-binding assay. Radioactivity was counted with Beckman LS 5000TD liquid-scintillation spectrometer.

Investigation of membrane ruffling: We used COS7 cells transfected with CFP-ARHGAP25, mutant CFP-ARHGAP25R192A, CFP-p50RhoGAP, or control CFP vector. Cells were stimulated with EGF for 20 min and filamentary actin was labeled with Alexa-568-phalloidin. The results were evaluated by confocal microscopy.

Measurement of superoxide production: Phagosomal superoxide production of ARHGAP25-silenced and control PLB-985 was determined by lucigenin-based chemiluminescence. Cells were stimulated with zymosan opsonized with pooled human serum or heat-inactivated pooled human serum. Extracellular superoxide production induced by phorbol ester (PMA) was measured with cytochrome c.

Labeling of filamentary actin in PLB-985 cells: Filamentary actin was labeled with Alexa488-phalloidin. The results were evaluated with flow cytometry.

Investigation of yeast-uptake by flow cytometry: Heat-inactivated *Saccharomyces cerevisiae* yeast cells were labeled with Cell Tracker Green fluorescent dye and opsonized with normal or heat-inactivated pooled human serum. Yeast cells and phagocyte cells were co-incubated for 10 min. The results were evaluated with flow cytometry.

Measurement of phagocytosis with confocal microscopy: We used COSphoxFcγR cells transfected with CFP-ARHGAP25, CFP-ARHGAP25R192A, CFP-p50RhoGAP or control CFP vector. Cells were co-incubated for 1 hour with pooled serum opsonized yeast labeled with Cell Tracker Red fluorescent dye. Phagocytosis was evaluated with confocal microscopy.

Statistical analysis: Data were evaluated by one-sample t-test or one way or two ways factorial analysis of variance (ANOVA). Tukey honest significant difference test was used for post-hoc comparisons. Level of significance was set at $p<0.05$. For statistical analysis, STATISTICA 7.0 (Statsoft Inc., Tulsa, USA) software was used.

RESULTS

The first part of my PhD work was to investigate the tissue distribution of Rac/RhoGAPs in public databases. First we identified 75 human Rac/RhoGAPs which makes database and literature search easier, because the nomenclature of GAPs is quite difficult. Next we evaluated the mRNA microarray expression profile of the identified Rac/RhoGAPs and we generated a color-coded expression table. The observations were concordant with the results from Expressed Sequence Tag (EST) database and from our Northern blot experiment: ARHGAP25 showed high mRNA level in the spleen and in the peripheral blood leukocytes. The expression profile of the other GAPs can give initial information for further experiments.

The next part of my PhD work was the investigation of the role of ARHGAP25 in human phagocytes. We expressed the full-length ARHGAP25 and its fragments as recombinant proteins. We generated a specific polyclonal antibody against the coiled coil fragment of the protein. Using this antibody we could detect the ARHGAP25 protein in the major human leukocyte types and also in human neutrophil. Next we investigated the small G protein specificity of ARHGAP25 in cell-free experiments using recombinant proteins and radiolabeled GTP. We found, that ARHGAP25 can regulate only Rac and it has no effect on Rho or Cdc42. These findings were confirmed in living cells: overexpressed ARHGAP25 inhibited Rac-mediated ruffling in COS7 cells whereas there was no sign of modification of Rho-dependent function. We generated a mutant variant of the protein with changing the conserved arginine of the GAP domain to alanine. This mutant ARHGAP25R192A lost its RacGAP activity so we could use it as loss-of-function mutant in further experiments.

Next we investigated the role of overexpressed ARHGAP25 in phagocytosis. We used COSphoxFcγR cells previously transfected with the components of the phagocyte oxidase and the FcγRIIa receptor. We observed that overexpressed ARHGAP25 inhibited the uptake of pooled human serum opsonized yeast in these cells. The loss-of-function mutant ARHGAP25R192A had no effect on phagocytosis which suggests, that ARHGAP25 operates as a RacGAP in the regulation of phagocytosis. The control protein p50RhoGAP could also inactivate Rac, but it had no effect on yeast engulfment which suggests the specific role of ARHGAP25 in this process. The mutant

ARHGAP25R192A localized mostly in the cytosol, however, in phagocytosing cells it was enriched around the phagosome. Although, phagocytosis was inhibited by overexpressed ARHGAP25, in ~10% of the cases the wild-type GAP also translocated to the phagosome.

We developed a flow cytometric method to investigate phagocytosis of fluorescent particles with high efficiency and high through-put. In flow cytometry, particle size is characterized by forward scatter (FSC) and granularity is characterized by side scatter (SSC). Phagocytosing cells and free yeast particles are well separated by these two parameters in a dot-plot diagram. In order to make sure that we can distinguish between the engulfed and the non-phagocytosed yeast cells, we labeled yeast with fluorescent dye. After gating of phagocytosing cells in the dot-plot diagram we generated a histogram which showed the fluorescence intensity of the engulfed yeast particles. Using this histogram we can calculate the rate of phagocytosing and non-phagocytosing cells.

Using this method we investigated the phagocytosis of ARHGAP25-silenced PLB-985 cells differentiated to neutrophils. Pooled serum opsonized yeast uptake into silenced cells showed significant increase as compared to control cells. We confirmed this result in primary human macrophages transfected with ARHGAP25-specific siRNA. Heat-inactivation of the pooled serum before opsonization causes the inactivation of complement components and switch–off the complement-mediated phagocytosis, thus we can investigate the FcR-mediated yeast uptake. Engulfment of heat-inactivated serum opsonized yeast by PLB-985 cells was decreased compare to normal pooled serum opsonized yeast. The reason of the decreased phagocytosis may be the low expression level of Fc receptors on PLB-985 cells. However, silenced cells phagocytosed more particles compared to control, but the difference was statistically not significant.

Actin-rearrangement, which is regulated by Rac, is an essential step of phagocytosis. Thus, we investigated the role of ARHGAP25 in filamentary actin reorganization. We observed increasing tendency in f-actin level of ARHGAP25-silenced PLB-985 cells compared to control cells.

To measure the phagosomal superoxide production of PLB-985 cells we used opsonized zymosan stimulation. Silencing of ARHGAP25 caused an approx. threefold

increase in the rate of superoxide production upon stimulation with normal pooled serum opsonized zymosan. Opsonization of zymosan with heat-inactivated serum (HOPZ) abolished the difference. HOPZ stimulus caused a relatively slow superoxide production both in knock-down and control cells that started to increase after a 5-min lag phase. Extracellular superoxide production of PLB-985 cells was stimulated with PMA. We observed that PMA stimulation caused no significant difference between ARHGAP25-silenced and control cells. This suggests that ARHGAP25 is not a direct regulator of superoxide production.

CONCLUSION

The results of our study lead us to the following conclusions:

1. According to database search and our Northern blot experiment ARHGAP25 is expressed in leukocytes.
2. We were able to express the full-length ARHGAP25 and its fragments as recombinant protein.
3. ARHGAP25 regulates Rac small G protein, but it has no effect on Rho or Cdc42.
4. Silencing of endogenous ARHGAP25 results in an increase of phagocytosis whereas overexpression of the protein clearly inhibits phagocytosis.
5. ARHGAP25 localizes mostly in the cytosol, however, it translocates to the phagosomal membrane during phagocytosis.
6. Silencing of ARHGAP25 causes increased tendency in the amount of filamentary actin.
7. Silencing of ARHGAP25 causes an increase in phagocytosis-related superoxide-production.

PUBLICATIONS

Publications relevant to the dissertation

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