PHOSPHORYLATION-DEPENDENT REGULATION OF THE RAC-SPECIFIC GTPASE ACTIVATING PROTEIN ARHGAP25

Ph.D. thesis

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I. Introduction

Rho family GTPases and their regulating proteins (including the GTPase activating proteins (GAPs)) govern the actin-cytoskeleton organization, thereby modulating a plethora of biological processes such as migration, focal adhesions, cell polarity, and diverse immunological tasks.

Our research group was the first to characterize a novel GTPase-activating protein of the Rho family, ARHGAP25. We examined its small G protein specificity and described its complex role in regulating leukocyte functions, including phagocytosis, superoxide production, and transmigration. In addition, the role of ARHGAP25 in tumor cell metastasis has also recently been recognized. However, despite its emerging immunological and oncological importance, the post-translational control of ARHGAP25 is almost entirely unknown, prompting us to explore this protein's regulation further.

To study ARHGAP25`s regulation with high yield and fidelity, we first developed a novel approach to measure its GAP activity. Current methods utilize the specific interaction between small G proteins and their downstream effectors, the radioactive or fluorescent labeling of guanine nucleotides, or

the measurement of the molecules produced in the GTPase reaction (i.e., GTP, GDP, P_i). While the radioactive filter-binding assay was considered a gold standard for a long time and fluorescent labeling of GTP or GDP works well under *in vitro* conditions, the former can be hazardous for the experimenter, and the latter is laborious and may modify the properties of the small G protein-GAP interaction. Well-based assays are admittedly easy and robust but also expensive, and since they measure an enzymatic product, they only give endpoint results. Therefore, a safe, cost-effective real-time GAP activity assay for *in vitro* measurements was in great demand.

During my Ph.D. studies, we first developed a bioluminescence resonance energy transfer (BRET)-based GAP assay that provides real-time read-outs of GAP activity. Then, we utilized this technique to characterize the regulation of ARHGAP25 by phosphorylation, one of the most common post-translational modifications, that can affect GAPs in many ways, including direct or indirect activation or inhibition of their enzymatic activity.

II. Objectives

ARHGAP25's immunological importance and its newly described role in tumor cell migration and metastasis make the investigation of its regulation all the more relevant.

During my Ph.D. studies, our first goal was to examine the possible phosphorylation of ARHGAP25 using *in silico* phosphorylation prediction analysis, *in vitro* phosphorylation assays, and mass spectrometry analysis on both the recombinant protein and ARHGAP25-expressing cell samples.

Our next goal was to develop a new method that enables the measurement of *in vitro* GAP activity in a real-time manner without the need for time-consuming and expensive techniques (e.g., radioactivity, commercial well-based assays).

Finally, utilizing our novel technique, we aimed to investigate whether phosphorylation affects ARHGAP25's RacGAP activity *in vitro* and, if yes, identify the amino acids responsible for the phosphorylation-dependent changes.

III. Methods

Full-length ARHGAP25 and its truncated fragments were cloned into the pGEX-4T-1 vector, and phosphodeletion (Ser-to-Ala) mutations were introduced via site-directed mutagenesis. The Cdc42/Rac-interactive binding motif (CRIB) specifically binding the active Rac was linked to Renilla luciferase (RLuc), Rac1 was fused with the fluorescent protein Venus and both constructs were cloned into pGEX-4T-1.

GST-tagged recombinant proteins were produced in One Shot™ BL21 Star™ *E. coli*. After lysis and sonication, the bacterial supernatant was incubated with Pierce™ Glutathione Agarose beads and eluted with a buffer containing 5 mM reduced glutathione.

Neutrophilic granulocyte cytosol was prepared from human venous blood using dextran sedimentation followed by Ficoll-Paque gradient centrifugation. Neutrophils were then lysed, and the supernatant was stored at -80ºC.

For *in vitro* phosphorylation, recombinant GST-tagged ARHGAP25 constructs were incubated with 150 µL of cytosolic extract from primary human neutrophils (intact or heat-inactivated, in which case extracts were heated for 15

minutes at 100°C) in the presence of 1 mM adenosine 5'-triphosphate (ATP) for 30 minutes on 30°C. Dephosphorylation was carried out using 400 U Lambda Protein Phosphatase in a total volume of 50 µL for 30 minutes at 30°C.

To visualize the phosphorylation, ARHGAP25 constructs were run in SDS-PAGE gels, fixed, and stained with Pro-Q™ Diamond Phosphoprotein Gel Stain, and later with SYPRO™ Ruby Protein Gel Stain for the total protein amount, following the manufacturer's protocol.

For phosphor screen autoradiography, recombinant ARHGAP25 was incubated with 200 µL neutrophilic granulocyte cytosol extract in the presence of 10 µCi ^{32}P-ATP and 20 µM ATP for one hour at 37°C. After running the samples in SDS-PAGE gel, the autoradiograph was taken on a phosphor screen with an exposure time of 48 hours and developed with a Bio-Rad GS-525 Molecular Imager®.

The newly developed *in vitro* bioluminescence resonance energy transfer (BRET) GAP assay was carried out in 96-well plates in 100 µl/well total volume. First, 5 µg GST-Venus-Rac1 was preincubated ('loaded') with 30 µM GTP (or 300 µM GTPγS or GDPβS for positive and negative controls, respectively) for 10 minutes at room temperature, and the GTP-binding was stabilized by adding 25 mM $MgCl_2$. Each well

contained 2 µg GST-CRIB-RLuc and 5 µM coelenterazine h complemented with 1 mM GDP. The reaction was started with the addition of 15 µg GST-ARHGAP25. Emissions were measured every 30 seconds for 15 minutes using 535BP/30 nm (Venus) and 475BP/30 nm (RLuc) filters at 25°C with a CLARIOStar luminometer (BMG Labtech, Ortenberg, Germany).

For the GTPase-Glo™ Assay, 2 µg GST-Rac was loaded with 1 µM GTP, and 0.5 µg, 1 µg, 5 µg, or 10 µg recombinant GST-ARHGAP25 was added in a final reaction volume of 25 µl on 96-well plates. Reactions were incubated for 30 minutes at room temperature, and luminescence was recorded using a CLARIOStar luminometer (BMG Labtech, Ortenberg, Germany).

In the filter binding assay, 150 ng GST-Rac1 was loaded with [γ-^{32}P]GTP (more than 5000 Ci/mM) for 10 min at room temperature, and the GTP-binding was stabilized by adding 20 mM $MgCl_2$. After incubating it with 375 ng recombinant ARHGAP25, samples were filtered through nitrocellulose membranes (0.45-µm pore size), and radioactivity of the filters was measured using a Beckman LS 5000TD liquid-scintillation spectrometer (Beckman Coulter, Brea, CA, USA).

All data were analyzed and plotted using GraphPad Prism 8.0.1 software. Comparison of experimental groups was carried out with paired t-test, one-way ANOVA, or two-way ANOVA followed by Tukey's *post hoc* test as appropriate, and p values <0.05 were considered statistically significant.

IV. Results

1. ARHGAP25 is phosphorylated under *in vitro* conditions

GST-ARHGAP25 and its truncated fragments were incubated with neutrophilic granulocyte cytosolic extract as a kinase source in the presence of radiolabeled ^{32}P-ATP. Samples were then eluted and ran in SDS-PAGE, and radioactivity was measured directly in gel with phosphor screen autoradiography. After intact cytosol incubation, we detected a clear phosphorylation signal, which was the strongest in ARHGAP25's 'interdomain' region, referring to a ca. 200 amino acid long section between the GAP and coiled coil domains. This result was corroborated by staining ARHGAP25 in gel with a non-radioactive phosphorylation-specific dye, the Pro-Q™ Diamond. Using heat-inactivated cytosol and lambda protein phosphatase as negative controls, we also confirmed that the signal, in fact, reflects phosphorylation.

2. *In silico* evaluation of potential phosphorylation sites within ARHGAP25

In order to identify potential phosphorylation sites in the ARHGAP25 sequence, we conducted *in silico* data analysis using four online servers: NetPhos 3.1, Group-based Prediction System 5.0, MusiteDeep, and KinasePhos 3.0. We found 29 amino acids that were present in all predictions.

3. Identification of the phosphorylation sites with mass spectrometry

In collaboration with the Biological Research Centre in Szeged, Hungary, we performed mass spectrometry experiments to pinpoint phosphorylated amino acid residues in ARHGAP25. Phosphosites were analyzed in four different sample types: recombinant ARHGAP25, COS-7 cells transfected with ARHGAP25, differentiated PLB-985 cells, and human neutrophilic granulocytes, the latter two expressing endogenous ARHGAP25. Altogether, 12 amino acid residues were found to be phosphorylated, from which 11 showed an overlap with the result of the computational, *in silico* analysis.

4. Development of a bioluminescence resonance energy transfer (BRET)-based GAP assay

In our design, we used the basic principle of intermolecular energy transfer: the luciferase-tagged CRIB domain of p21-activated kinase (PAK) exclusively binds active Rac and excites its linked Venus protein so that we can continuously infer the active Rac amount from the BRET ratio (emission of Venus (530 nm) divided by the emission of RLuc (480 nm)). The addition of ARHGAP25 and the subsequent enhancement of the

GTPase reaction is reflected by a decrease in the BRET ratio. We used a single turnover approach, meaning that all reactions started with a fully GTP-loaded Rac, and after hydrolysis and inactivation, the binding of another GTP was prevented by using high GDP concentration. During assay development, we optimized the quantity and ratio between the interacting partners, the negative and positive controls, and the GTP concentration. Following that, we verified that the BRET-based method has comparable sensitivity to other established GAP assays (GTPase-Glo™ assay, radioactive filter-binding assay) and still provides low variability.

5. Measuring the effect of phosphorylation on ARHGAP25's GAP activity

Recombinant GST-tagged ARHGAP25 was phosphorylated with neutrophil cytosol extract and 1 mM ATP for 30 minutes, and the GAP activity of the phosphorylated and non-phosphorylated (treated with heat-inactivated cytosol) constructs was measured using the newly developed BRET-based GAP assay. Non-phosphorylated wild type ARHGAP25 greatly reduced the active Rac amount by its relatively high GAP activity. In contrast, ARHGAP25, which was phosphorylated by neutrophilic granulocyte cytosol, proved to be significantly less active as a GAP. This inhibitory effect

could be reversed with lambda phosphatase treatment and was not observed in enzymatically compromised R200A ARHGAP25 mutant, implying that the phosphorylation acts (directly or indirectly) on ARHGAP25's enzymatic activity. The inhibitory effect of phosphorylation was also confirmed with the filter-binding radioisotope assay.

6. Identifying the amino acids involved in the phosphorylation-dependent regulation of ARHGAP25's GAP activity

Out of the 11 identified phosphorylation sites of ARHGAP25, three proved to be also functionally relevant. In a collaborative study, we found that phosphorylation on serine residues S363, S379-380, and S488 has the most significant effect on hematopoietic stem cell and progenitor cell (HPSC) mobilization from murine bone marrow. Thus, we first mutated S363, S379-380, and S488 individually or in pairs to alanine (S-A mutants). The GAP activity of these mutants was then measured under control or phosphorylated (*i.e.*, treated with neutrophil cytosol) conditions and compared to that of the wild type ARHGAP25. We revealed that the RacGAP activity of ARHGAP25 is regulated directly by the phosphorylation of serine residues at positions 363 and/or 488 but not at 379-380 *in vitro.*

V. Conclusions

- ARHGAP25, a Rac-specific GTPase activating protein essential to neutrophilic granulocyte functions, can be phosphorylated by neutrophilic granulocyte cytosolic kinases.
- Based on mass spectrometry data and *in silico* analysis, we identified eleven potential phosphorylation sites in ARHGAP25.
- We developed a novel real-time bioluminescence resonance energy transfer (BRET) assay to monitor the GAP activity of ARHGAP25 *in vitro*.
- Using both the radioactive filter-binding assay and our newly developed GTPase assay, we showed that ARHGAP25's phosphorylation weakens its enzymatic GAP activity under *in vitro* conditions.
- Using phosphodeletion mutants, we revealed that phosphorylation of S363 and S488, but not that of S379-380, controls ARHGAP25's RacGAP activity.

VI. Bibliography of the candidate's publications

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