Interactions and Phosphorylation-Dependent Roles of ARHGAP25 in Neutrophils

Ph.D. thesis

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1. Introduction

ARHGAP25, initially identified in human neutrophils, has been recognized as a Rac-specific GTPase-activating protein. Functional studies indicate that ARHGAP25 modulates key leukocyte processes: its deficiency enhances phagocytosis and superoxide production, suggesting a negative regulatory role in RAC-dependent effector functions. ARHGAP25-deficient mice exhibit attenuated inflammatory responses in the K/BxN serum-induced arthritis and allergic contact dermatitis animal models, presumably due to reduced leukocyte recruitment and altered chemokine milieu. Moreover, it has gained popularity in cancer research in recent years, demonstrating both anti- and pro-tumor properties depending on the type of cancer.

Despite its relevant role in immunology and oncology, the molecular basis of ARHGAP25 is not fully elucidated. We have demonstrated that phosphorylation of ARHGAP25 may serve as a regulatory mechanism in neutrophils, as it influences its enzymatic activity in vitro. Nonetheless, the related intracellular context is yet to be unraveled. Furthermore, existing datasets concerning ARHGAP25's interactome disregard its distinct expression profile, emphasizing the need for a cell-specific interactome to address these shortcomings and provide the opportunity to seek novel protein partners.

2. Objectives

In this current thesis, we would like to use different methodologies to find regulators or partners that partake in ARHGAP25-mediated processes to widen our understanding of small GTPase regulation in neutrophils.

The current aims are as follows:

I. Investigation of the effects of the ARHGAP25 Ser-to-Ala phospho-mutants on RAC levels and the related actin reorganization via F-actin content.

II. Creation of a neutrophil-specific interactome via GST-pulldown and co-immunoprecipitation and subsequent investigation of the proteome via bioinformatic approaches to shed light on potential protein partners and related molecular functions.

III. Investigation of the identified small GTPase partners and potential phosphorylation-related interactions of ARHGAP25

3. Methods

3.1. Creation of the plasmid constructs and corresponding cell lines

ARHGAP25 was extracted from the cDNA of human peripheral blood leukocytes and cloned into the pcDNA3.1/V5-His-TOPO vector. The LacZ gene-containing plasmid was used as the negative control. Mutations were introduced via site-directed mutagenesis using specific primers and confirmed by Sanger sequencing. The cells were transfected via electroporation with an Amaxa Nucleofector™ in accordance with the manufacturer's guidelines. The transfected PLB-985 cells were incubated in UltraMEM for 2 hours, then transferred to the geneticin-containing selective medium. PLB-985 cells were differentiated into neutrophil-like cells for 7 days using 0.5% dimethylformamide.

3.2. Measurement of active RAC content and Western blotting

Cells were lysed, and the supernatant was isolated via centrifugation. GTP-bound RAC was extracted from the supernatant utilizing the GTPase-binding domain of p21-activated kinase in a GST-tagged configuration. Equal amounts of lysates were incubated with the beads. After washing, proteins were eluted in 1 × Laemmli Sample Buffer by boiling. Cell lysates with equal protein masses were also boiled in 1 × Laemmli Sample Buffer for the measurement of the total RAC contents. Rac levels were visualized after Western blot with a RAC-specific antibody. GAPDH was used as a loading control. Measurements were normalized to the 'Vector' sample. Calculation for the total RAC content: $log_2 \frac{RAC_{total}}{GAPDH}$; calculation for the active RAC ratios: $log_2 \frac{RAC_{active}}{RAC_{total}}$. The general effect of the mutations at different positions was evaluated using three-

way ANOVA, and multiple comparisons were assessed between the 'WT' and the other groups using Dunnett's test.

3.3. F-Actin staining of the PLB-985 cells

2×10^6 differentiated PLB-985 cells were fixed with 4% paraformaldehyde and permeabilized with PBS containing 0.1% Tween-20. Alexa fluor-488 conjugated phalloidin was used to stain F-actin. Ten thousand cells were measured using a CytoFLEX flow cytometer, and the median fluorescence intensities (MFI) were compared. We applied the same statistical approach as described in the previous section (3.2).

3.4. Isolation and cell lysis of human neutrophilic granulocytes from peripheral blood

We used buffy coats and the peripheral blood taken from healthy volunteers to isolate neutrophils, approved by the authorities (31937-7/2020/EÜIG). Dextran sedimentation procedure followed by Ficoll-Paque gradient centrifugation was utilized to separate neutrophils. Neutrophil pellets were lysed, and the supernatant was separated from the pellet via centrifugation and divided into four equal portions. Two portions remained intact, while two aliquots were subjected to pretreatment with 15 mM EDTA and either 15 mM GTPγS or 10 mM GDPβS. The reaction was stopped by $MgCl_2$ (final concentration: 60 mM).

3.5. Preparation and pulldown of GST-fused recombinant proteins

GST and full-length GST-coupled ARHGAP25 were synthesized in *E. coli*. Bacteria were pelleted and lysed with sonication in a lysis solution. Debris was eliminated by centrifugation, and the recombinant proteins were purified from the supernatant using glutathione-bound beads. GST-ARHGAP25 and GST-coated beads were incubated with the neutrophil cell lysates. After the washing steps, protein elution was performed by boiling the beads in 2× Laemmli buffer.

3.6. Co-immunoprecipitation

Magnetic beads were coated with either polyclonal ARHGAP25 antibody (created and tested by our group) or rabbit IgG (negative control) and incubated with the intact total cell lysate. After the washing steps, bound proteins were eluted by boiling the beads for 5 min in 2 × Laemmli buffer.

3.7. Sample preparation for proteomics

We performed gel-aided sample preparation as previously described. $\frac{1}{8}^{th}$ of the samples were placed in a single-use Evotip trapping mini-column. Data-dependent LC-MS/MS analysis utilized an Evosep One stainless steel emitter linked to a linear ion trap-Orbitrap mass spectrometer functioning in positive ion mode. During data acquisition, multiple charged ions were selected in a cycle time from each MS survey scan for ion-trap HCD fragmentation.

3.8. Label-free quantification

Following the conversion of raw files to .mzML format using MSConvert (v. 3.0), we employed the 'LFQ-MBR' workflow of the FragPipe program (FragPipe v. 19.1, MSFragger v. 3.7, IonQuant v. 1.8.10, Philosopher v. 4.8.1) for data analysis. The precursor and fragment mass tolerance were set to ± 5 ppm and 0.6 Da, respectively, while other database parameters remained in default settings. A human proteome database was downloaded via FragPipe and manually supplemented with the GST-tag sequence. IonQuant LFQ was selected with the 'MBR top runs' parameter configured to 25.

3.9. Data evaluation of the GST pulldown

Statistical analysis was conducted using the 'CombinedProtein.tsv' file through the FragPipe Analyst web application. The 'MaxLFQ' intensity values for each sample were subjected to $\log_2$ transformation. Samples were grouped by conditions and went through filtering and imputation. Protein-wise linear models combined with empirical Bayes statistics were employed for the differential expression analysis. A

threshold of the adjusted p-value of 0.05 (Benjamini–Hochberg method) and a that of 2 for the fold change (FC) have been chosen.

3.10. Data evaluation of the co-immunoprecipitation

We employed a score-based evaluation. Proteins were categorized into three groups based on their detection rates in the co-IP samples: 'low' (< 60%), 'good' (60% to 80%), and 'excellent' (> 80%), creating the 'confidence score' metric. Proteins were assigned an 'enrichment score' according to their fold change value relative to the median fold change ('low': FC < 0.81 and 'high': FC > 0.81). Based on these parameters, each protein was classified into four categories: 'unlikely,' 'low credibility,' 'satisfactory,' and 'high credibility.' The steps for the evaluation are available online.

3.11. Reassessment of MS results using Western Blot

Equal volumes of eluates from GST-pulldown samples, along with ProSieve QuadColor Protein Marker, underwent gel electrophoresis. After the Western blot, the selected proteins (SYK, ACSL1, LDHA, RHOG, RAC2, and RAB27A) were detected using the corresponding primary antibodies against them.

3.12. Functional enrichment analysis

Functional enrichment analysis was conducted via the ShinyGO web server (v. 0.8041). The threshold was established at 0.01, utilizing the “Gene Ontology biological process” and “Reactome” data sources, filtered by term size (min: 5, max: 1000). A human neutrophil-derived background was included.

3.13. STRING analysis

The STRING platform (v. 12.043) was utilized to gather all recorded physical interactions among our prospective protein partners determined by MS. Only experimentally determined interactions and databases were included. A threshold of 0.400 was utilized.

3.14. In silico PPI prediction

The direct interaction formation of ARHGAP25 and likely partners was predicted in silico using AlphaFold optimized for multimers. Dimers were generated using ColabFold (v1.5.545) or were processed by Neurosnap Inc. (https://neurosnap.ai/), depending on the size.

3.15. **In silico prediction of 14-3-3 binding** phosphosites

We utilized an improved prediction web interface developed by Madeira *et al.*, following the authors' guidelines.

3.16. Phosphorylation of GST-coupled proteins

The phosphorylation was carried out in a solution containing 500 µL of cytosolic extract from human neutrophils (as previously described) in the presence of 30 µL kinase buffer for 30 min at 30 °C.

3.17. Statistics

The related statistical tests used for each comparison are described in the corresponding methods and results sections. The default significance threshold was set to 5% if not stated otherwise.

4. Results

4.1. Creation of PLB-985 cells expressing the ARHGAP25 Ser-to-Ala mutants

To investigate the effects of the overexpression of phospho-deficient ARHGAP25 mutants *in vivo*, we created PLB-985 cells overexpressing the Ser-to-Ala mutants of ARHGAP25 targeting four serine residues at three positions (S363, S379-380, and S488) individually and in combination, creating seven groups (S363A, S379-380A, S488A, S363A+S379-380A, S363A+S488A, S379-380A+S488A and S363A+S379-380A+S488A [referred to as: 'TM']), along with the LacZ-expressing negative control ('Vector') and the endogenous ARHGAP25-coding vector ('WT'). The presence of the V5-tagged proteins was ensured using an anti-V5 antibody after Western blot.

4.2. The effect of ARHGAP25 Ser-to-Ala mutants on the total and GTP-bound RAC levels in PLB985 cells

The total and active RAC content of the cell lysates was assessed via densitometric analysis of the Western blots as described before. Based on the three-way ANOVA, the biggest source of variation was caused by the introduction of the S379-380A mutation, but it did not reach significance ($p=0.069$). The most prominent difference was observed between the WT and the S379-380A+S488A mutant-expressing groups. However, it failed to reach the significance threshold ($p=0.081$). When comparing the active RAC levels, there was a considerable amount of variation present among the independent pulldowns. The three-way ANOVA identified the interaction of the three mutations together as the most prominent source of variation (12.8%), yet it failed to reach significance ($p=0.064$), and there was no significant difference between the 'WT' and the other groups.

4.3. The effect of ARHGAP25 Ser-to-Ala mutants on F-actin levels

We examined the quantity of filamentous actin in the differentiated PLB-985 cells. Introduction of the S379-380A mutations was significantly highlighted as the main source of variation, based on the three-way ANOVA (24.1%; p=0.0014). When compared to the 'WT' group, the single mutants S363A and S488A exhibited functional similarities. Conversely, S379-380A and the double mutants increased the F-actin amount, although only the overexpression of the combined mutant ARHGAP25, 'S363A + S379-380A', led to a significant difference compared to the 'WT' group.

4.4. Evaluation of the neutrophil-specific pull-down with GST-fused ARHGAP25

GST-tagged ARHGAP25 was incubated with neutrophil cell lysates, and the samples were analyzed using tandem mass spectrometry. In six instances, the cell lysate was supplemented with nucleotide derivatives (GTPγS or GDPβS) to evaluate their impact on the detected protein pool. Recombinant GST served as a control bait to eliminate false-positive targets. The label-free quantification intensity (LFQ) values were utilized to create a Principal Component Analysis (PCA) plot and a Pearson correlation matrix that served as quality control checks.

Distinct clusters were delineated on the PCA plot: the control samples, where the experiment was conducted with GST alone, were separate from the GST-ARHGAP25 pulldowns, as well as the pulldowns with the modified protein pools. Similarly, pulldowns from identical protein pools (intact cell lysates, GDPβS-, and GTPγS-loaded cell lysates) exhibited a strong correlation with one another. No clear indications of batch effect were observed. The total amount of unique peptides and proteins identified in each sample before data imputation was assessed. GST-ARHGAP25 pulldowns consistently exhibited higher counts than GST control samples, indicating effective

enrichment. Among the 775 identified proteins, 9.16% (71) were exclusively detected in GST, 60.77% (471) solely in GST-ARHGAP25 eluates, while 30.07% (233) were common to both. These findings imply that our recombinant GST-ARHGAP25 protein established direct or indirect interactions with multiple proteins.

4.5. Assessment of label-free quantitative proteomics to identify the ARHGAP25 proteome

For the creation of the initial interactome pool, we compared the GST-ARHGAP25 pulldown samples (eluted from intact neutrophil lysates) with the control GST pulldowns (eluted from the same lysates). 90 proteins were identified as significantly enriched in GST-ARHGAP25 eluates.

The reliability of the MS data was assessed using six proteins chosen from the pulldown measurements. ACSL1, RAB27A, RAC2, RHOG, and SYK were picked from the significant candidates, whereas LDHA was chosen from the proteins exhibiting low fold change and high adjusted p-value. All the chosen proteins were consistently identified by antibodies and exhibited substantial enrichment, except for LDHA, which was not detected.

4.6. Refinement of the interactome by co-immunoprecipitation

In parallel, co-immunoprecipitation was performed, and the relative abundances of the previously identified 90 proteins were quantified via mass spectrometry. 13 candidates were eliminated because of insufficient detection rates, whereas the results validated the enrichment of 76 proteins (excluding ARHGAP25). In addition to the anticipated presence of cytoskeletal and GTPase-related proteins, several kinases, phosphatases, and chaperones were found. Members of the 14-3-3 family were also present, along with proteins associated with membrane trafficking, protein synthesis, and degradation.

To explore the known interactions among these candidates, we employed the STRING platform to compile all experimentally validated interactions. This yielded a total of 49 connections involving 45 interactors, suggesting the potential presence of eluted multimers in the samples.

4.7. Investigation of biological functions of the revealed proteome

Functional enrichment analysis was performed utilizing the ShinyGO web server. Compared to the neutrophil proteome, our results indicated over-represented gene sets that predominantly grouped into 10 principal themes. First, we identified a cluster of five gene sets connected with the Fc receptor signaling pathway and phagocytosis. The corresponding proteins were also represented in interleukin-mediated signaling and phosphorylation. Several terms that involve cell projection and cytoskeletal organization emerged, with some tied to GTPase-mediated signaling. Additionally, gene sets associated with mast cell and platelet activation, hemostasis, and organelle maintenance were over-represented as well.

4.8. Investigation of the effects of GTPγS or GDBβS loading of small GTPases on the proteome

Among the 76 proteins, four small GTPases were identified in the GST-ARHGAP25 eluates (RAC2, RHOG, ARF4, RAB27A). We performed GTPγS and GDPβS loading of the cell lysate prior to incubation with the beads to examine the influence of the GTP/GDP cycle on the interactome. We observed notable changes between GTPγS and GDPβS loading for RAC2 ($\log_2FC = 0.785$) and RHOG ($\log_2FC = 0.589$), but not for ARF4 and RAB27A ($\log_2FC < 0.2$). The data may indicate ARHGAP25's affinity for the active (GTP-bound) form of RAC2 and RHOG, consistent with the GAP domain's recognized specificity for the Rho family. Nonetheless, the binding appears to be independent of the GDP/GTP-bound state concerning ARF4 and RAB27A.

4.9. In silico investigation of the RHOG-ARHGAP25 dimer

We utilized AlphaFold Multimer prediction to ascertain reliable direct binary interactions between ARHGAP25 and the candidates. For each dimer, ten predictions were executed, and the predictions were compared based on the estimates established by the creators of Alphafold: the TM-score (pTM) and intracellular TM-score (ipTM). In accordance with the directives of *O'Reilly et al.*, a threshold of ipTM = 0.85 was chosen to identify the most reliable predictions. The software generated three dimers involving ARHGAP25 with RAC2, RHOG, and RAB27A above the initial cut-off value. Given that only the predictions for RHOG and RAC2 had the highest credibility, we only focused on their predicted conformation in this study. Both small GTPases were situated next to the GAP domain of ARHGAP25, exhibiting the lowest predicted alignment errors. Based on the simulations, the GAP domain predominantly participates in the interaction, whereas the PH domain and the coiled-coil region contribute to a lesser extent.

4.10. Demonstration of the phosphorylation-dependent interaction between 14-3-3 proteins and ARHGAP25

Given that three members of the 14-3-3 proteins were identified as potential interaction partners, we aimed to evaluate the likelihood of their binding to ARHGAP25 by using the 14-3-3-Pred web server, developed by Madeira et al. T102, S395, S500, and T502 achieved high scores by a single approach, while S25, T343, T442, and S487 received high scores across all three methods. To see whether phosphorylation facilitates the interaction in vitro, we performed pulldowns with and without prior phosphorylation on ARHGAP25. The amount of the eluted 14-3-3 proteins was visualized by an anti-pan 14-3-3 antibody. Only pre-phosphorylated GST-ARHGAP25 associated with a detectable amount of 14-3-3 proteins.

5. Conclusions

In this thesis, I sought to further our understanding of ARHGAP25 at a molecular level.

The following goals were achieved:

I. We quantified and compared the total and activated RAC content in PLB-985 cell cultures overexpressing the Ser-to-Ala ARHGAP25 mutants. Even though the introduction of the mutations did not have a clear effect on RAC-GTP levels, the presence of the S379-380A mutation influenced ARHGAP25's effect on the filamentous actin content, dampening the F-actin-lowering effect of ARHGAP25 overexpression.

II. We established a neutrophil-specific interactome combining GST-pulldown and co-immunoprecipitation techniques, resulting in 76 identified partners that complement ARHGAP25's known profile, as well as highlighting new directions for ARHGAP25-related research.

III. Apart from the most anticipated RAC2-ARHGAP25 interaction, we identified three other small GTPases (RHOG, ARF4, RAB27A) as potential interaction partners. We showed via *in silico* simulations that ARHGAP25's associations with RHOG and RAC2 were GAP-domain related, and *in vitro* manipulation of the small GTPases' activity influenced the interactions in both cases. We identified kinases and phosphatases, mainly related to the Fc-receptor and interleukin-mediated signaling, and we confirmed a novel phosphorylation-dependent interaction between ARHGAP25 and 14-3-3 protein family members, serving as promising new directions for the research related to ARHGAP25 regulation.

6. Bibliography of the candidate's publications

Publications related to the thesis:

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∑IF: 28.6