Abschlussbericht
Leibniz-Wettbewerb

Titel: StruViR – Structures of Viral Proteins Essential for Replication and Transcription
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Federführendes Leibniz-Institut: Bernhard-Nocht-Institut für Tropenmedizin

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Executive Summary

Negative-strand RNA viruses are responsible for a large number of infectious diseases worldwide but mainly in low and middle income countries with devastating consequences for society and economy. Several negative-strand viruses are therefore listed on the WHO R&D Blueprint for urgent development of medical countermeasures. The aim of this project is to structurally and functionally characterize L proteins of negative-strand viruses, particularly that of bunyaviruses. The L protein is a key player during virus transcription and replication harboring several enzymatic activities, all of which are potential drug targets.

Within the duration of this project we cloned a large number of L genes originating from four families of the bunyaviruses into suitable expression vectors for baculovirus-driven expression in insect cells. We optimized expression and purification procedures and produced L proteins of high purity and quality for crystallization and cryo-EM experiments. We were able to obtain L protein structures of 3 distinct bunyavirus families: *Arenaviridae*, *Phenuiviridae* and *Hantaviridae*. In addition to structures of L proteins in absence of viral RNA (apo) we also solved several structures of L proteins in key active states, such as pre-initiation, early and late replication elongation, transcription initiation and transcription elongation. These structures emphasized the stunning high flexibility of these multidomain molecular machines and allowed us to identify commonalities and differences between the bunyavirus families. The structures were published in *Nucleic Acids Research* (2020), *Nature Communications* (2021). In addition, we have one paper recently accepted at *Nucleic Acids Research* and two more publications in preparation as well as one timely review article invited by *PLOS Pathogens* accepted recently.

In addition to full-length L protein structures, we solved the structure of a functional L protein domain by X-ray crystallography and obtained solution scattering data which complemented the cryo-EM structure and are also included in one of the above mentioned publications. The array of biochemical assays we have established to characterize the different enzymatic functions of the L protein *in vitro* were used for a detailed characterization of Lassa virus L protein, which was published in the *Journal of Biological Chemistry* in 2019.

This project created a strong collaboration between three groups with complementary expertise. It allowed several young researchers to develop their skills in an interdisciplinary and international consortium qualifying them to investigate complex research problems in the future.

In summary, the structures solved within this project, the mechanistic understanding generated as well as the biochemical assays established provide a solid framework for future drug development approaches. Indeed, a project application including early drug development phases for bunyavirus L protein by one of the group members received substantial funding by a program of the German Federal Ministry of Education and Research.

Inhalt

1. Zielerreichung und Umsetzung der Meilensteine

First of all, this project was a major success for everyone involved. It resulted in many high-quality publications, advanced the field of bunyavirus structural biology and enabled strong, growing partnerships.

The reviewers of our proposal suggested to concentrate on the segmented negative-strand viruses and we decided to follow their advice. In work package (WP) 1 the tasks were to clone a large set of L proteins into expression vectors for subsequent baculovirus-driven expression in insect cells. L proteins of high quality and in high concentration should be delivered for WP2 and WP3. We successfully cloned genes of more than 35 bunyavirus L genes including arenavirus, hantavirus, phenuivirus and nairovirus sequences with different tag positions as well as mutations in functionally important elements. Expression tests resulted in soluble protein for 5 different arenavirus L proteins, 5 phenuivirus L proteins, 1 nairovirus L protein and 4 hantavirus L proteins. Only the nairovirus L protein expression yields require further optimization in the future. Purification procedures could be optimized for the L proteins of arenaviruses, phenuiviruses and hantaviruses. High quality proteins were handed both to WP2 and WP3 for structural studies using cryo-EM and X-ray crystallography. In addition to the structural studies, biochemical characterization of the L proteins was also part of WP1. We performed extensive biochemical studies for Lassa virus (LASV, arenavirus), severe fever with thrombocytopenia syndrome virus (SFTSV, phenuivirus) as well as Sin Nombre virus (SNV, hantavirus) L proteins. Analyses for LASV and SFTSV were already published in peer-reviewed high-quality journals. The biochemical characterization of SNV is currently being prepared for publication. For WP1 all planned milestones have been reached accordingly.

WP2 contains structural studies of L proteins by X-ray crystallography. The postdoc situated at BNITM (Dominik Vogel) spent 6 weeks in the first two years in the lab of Stephen Cusack in Grenoble. We used 6 bunyavirus L proteins for crystallization screening testing different L protein:RNA complexes, protein concentrations and crystallization temperatures. Out of 10656 crystallization conditions tested in total we obtained 2 crystallization hits, which unfortunately turned out as being salt crystals rather than L protein crystals. However, we were successful in the crystallization of both RVFV and SFTSV cap-binding domains, which are ~11 kDa fragments of the respective L proteins containing a binding site for cap-structures which is essential for viral transcription. These domain structures were obtained in complex with a cap-analogue revealing the binding mechanism of the L protein to cap-structures. The crystallization of soluble domains was supported by the iNEXT and iNEXT-Discovery infrastructure access programmes within the Horizon 2020 programme of the European Commission. In summary, all milestones for WP2 were met.

WP3 includes structural studies of L proteins using electron microscopy (EM). Proteins provided by the BNITM (WP1) were checked for quality by negative stain EM in the Grünewald lab. After determination of optimal conditions for vitrification and to ensure sufficient density and uniform distribution of particles on cryo-EM grids initial data collection was performed. This process was informed and supported by previous studies on influenza virus polymerase complex in the Cusack lab. For LASV and SFTSV L proteins, high-resolution data collection was performed both with the apo forms of the L proteins as well as with different RNA ligands bound. The RNA ligands used in the structural studies were informed by biochemical experiments. Subsequent data processing resulted in high resolution maps suitable for *de novo* structure determination of SFTSV and LASV L proteins in the apo form, with viral RNA bound as well as in different functional states. With this strategy, we obtained snapshots of the L proteins of LASV and SFTSV in action and could follow the conformational changes associated with functional activity. Model building was done at EMBL Grenoble and BNITM. For SNV L protein we had major problems with grid preparation and data processing as the molecule adopted a strong preferred orientation on the grids. To solve this we collaborated with a team at IBS Grenoble (junior research group of Hélène Malet) and could mitigate the

orientation problem by chemical modification of the protein. In conclusion all milestones set for WP3 have been met.

2. Aktivitäten und Hindernisse

The project started in June 2018 with one postdoc, Dominik Vogel, at BNITM to work on WP1. This postdoc was supported by a PhD student, Kristina Meier, who joined in October 2018 and a technician, Carola Busch, who are both paid from institutional resources of BNITM. Sophia Reindl who was initially leading the WP1 activities left the BNITM in September 2018 and was replaced by Maria Rosenthal. Within the first months Dominik Vogel and Maria Rosenthal travelled to Grenoble to start first crystallization experiments and initiate all the contacts needed for efficient progress of the project. As the crystallization experiments with full-length L proteins were not successful and the EM facilities in Hamburg were not in regular operation, we decided to also perform cryo-EM studies in Grenoble. These cryo-EM experiments in Grenoble were supported by a postdoc of the Cusack lab, Tomas Kouba, who contributed his extensive experience with cryo-EM structural studies on the flu polymerase complex also in later stages of the project. In 2019 the PhD student in the lab of Kay Grünewald, Sigurdur Thorkelsson, started. This was a little later than initially anticipated but for technical reasons the setup of microscopes at CSSB Hamburg was delayed. However, several L protein samples were ready at that point, so that Sigurdur Thorkelsson was able to prepare and measure the first samples directly after starting. Within the first 7 months two high-resolution cryo-EM maps for the LASV and SFTSV L protein apo forms were generated. These experiments enourmously benefited from support by Tomas Kouba (EMBL Grenoble) and Emmanuelle Quemin, a postdoc in the Grünewald lab. The proximity between BNITM and CSSB as well as efficient communication also contributed to the fast progression of the project. To guarantee synchronization between WP1, WP2 and WP3 activities for the entire PhD time of Sigurdur Thorkelsson (despite the delayed starting time), Stephan Günther supported the project with institutional funding for Dominik Vogel's position for one year (June 2019-May 2020). In October 2020 Dominik Vogel left the project team and was replaced by Harry Williams, who stayed until the termination of the project. In summary, there have been changes of the initial plan (*e.g.*, operation of EM facilities, start time of PhD student, difficulties getting protein crystals) but thanks to additional financial and scientific support these changes did not reduce the scientific output of the project. The high competition in this particular research field became apparent in summer 2020, when part of our work on LASV L protein got scooped. However, we managed to generate further important insights and publish our high-quality work in a journal of high impact.
One major factor influencing the project's success has been the SARS-CoV2 pandemic leading to temporary closure of labs and facilities, general restrictions in lab occupation and limiting international travel for the second half of the project. However, we could present our data in June 2022 at two international meetings (giving 4 talks in total) and have been invited to write a review article on the recent enormous progress on bunyavirus L protein structures and mechanistic understanding, which emphasizes the visibility our work got within the scientific community despite the limited international travel in the past few years.

3. Ergebnisse und Erfolge

The work on this project resulted in five major peer-reviewed publications in recognized journals, one manuscript currently under review and two more manuscripts in preparation. In addition, as mentioned, we recently submitted a review article on the recent progress in L protein structures and published a review about the bunyavirus cap-snatching mechanism in 2020. Two additional papers related to this project have also been published. We published the biochemical characerization of LASV L protein in the *Journal of Biological Chemistry* in 2019. In 2020 the apo-structure of SFTSV L protein was published in *Nucleic Acids Research*. A publication on the LASV L protein structure including functional states was published in *Nature* Communications in 2021. A matters arising article was published in Nature

Microbiology correcting a flawed L protein structure of another group. We submitted a study on SFTSV L protein genome replication structures to *Nucleic Acids Research*, where it is currently under review. An invited review article was recently submitted to *PLOS Pathogens*. Manuscripts on SNV L protein structures and SFTSV transcription-relevant structures are currently being prepared.
What has been particularly successful is the transfer of knowledge between the partners. Cryo-EM experiments at CSSB have benefited from experience of the Cusack group. Model building for these large L proteins at BNITM was supported by the long-standing experience of Stephen Cusack with viral polymerase structures. Basic know-how about model building was also transfered from BNITM to the Grünewald group. The Grünewald group as well as Tomas Kouba taught BNITM scientists basic knowledge about cryo-EM experiments and data processing. The recent connection to the junior research group of Hélène Malet at IBS Grenoble further strengthens the scientific network created with this grant.
In context of annual project meetings in 2019 and 2020 we organized public scientific talks of Stephen Cusack and Tomas Kouba in Hamburg to foster scientific exchange. However, due to the SARS-CoV2 pandemic, all following meetings were held in a virtual formate. We presented our work in June 2022 at two international meetings (4 talks in total) and also several meetings of the institutes. Sigurdur Thorkelsson got awards for best speed talk at LCI summer school in 2022 and for the best presentation at the LIV scientific retreat in 2022.
Another important aspect of success is the qualification of the young scientists within this project. Kristina Meier was accepted for an EMBO course on high-throughput protein production and crystallization held in Oxford in 2019. Dominik Vogel participated in an Instruct-funded course on computational approaches in integration of structural biology techniques in Vestec near Prague in 2019. Maria Rosenthal was also accepted for an EMBO course on integrated structural and cellular biology held in Paris in 2019. Sigurdur Thorkelsson successfully applied for a Gordon research conference on three-dimensional electron microscopy in 2020, which was postponed due to the SARS-CoV-2 pandemic. He also obtained a travel grant from the Leibniz Institute for Virology (LIV) to gain expertise in data processing of flexible macromolecular complexes and model building at the MRC-LMB Cambridge, UK. Sigurdur Thorkelsson has now already moved on to do a postdoc at MRC-LMB in Cambridge, UK. Emmanuelle Quemin got a permanent position at the CNRS in December 2021 and started her own research group at I2BC in France with funding from ATIP-Avenir. Tomas Kouba moved on to become a cryo-EM facility head at IOCB in Prague.
Third-party funding acquired to support the work includes annual funding for beamtime by EMBL Hamburg to perform both crystallography and solution scattering experiments. Furthermore, we got support from the European Commisson Horizon 2020 programme (iNEXT and iNEXT-Discovery) for infrastructure access. This funding allowed us to screen a large number of crystallization conditions for bunyavirus cap-binding domains resulting in crystallization of SFTSV cap-binding domain, which is included in the publication in *Nucleic Acids Research* in 2020. While most of screening and data collection was done at the multiuser cryoEM facility at CSSB, headed by Kay Grünewald and supported by the Universität Hamburg and the DFG (grants INST 152/772-1, 774-1, 775-1 and 776-1), we also received measurement time on a cryo-EM microscope at ESRF Grenoble for this project. Related to the project and the herein developed functional assays the DFG also granted funding for a project on virus-host protein-protein interactions to Maria Rosenthal in 2019 and the BMBF provided funding for her junior research group to investigate the L protein polymerase, RNA binding sites and cap-binding function as potential inhibitor targets. This work is also basis for a grant application of Harry Williams to be submitted soon. Overall, the project has been successful in many different ways and fostered scientific collaboration as intended.

Publications resulting from this project:

1. Sigurdur Thorkelsson. Structural analysis of bunyavirus L protein during replication and transcription. PhD thesis. Submitted to the University of Hamburg 12/2022
2. Kristina Meier. Structure and function of bunyavirus L protein. PhD thesis. Submitted to the University of Bremen 09/2022

3. Hélène Malet, Harry M. Williams, Stephen Cusack, Maria Rosenthal. The mechanism of genome replication and transcription in bunyaviruses. PLoS Pathog. Accepted 12/2022
4. Williams HM[#], Thorkelsson SR[#], Vogel D, Milewski M, Busch C, Cusack S, Grünewald K, Quemin ERJ, Rosenthal M. Structural insights into viral genome replication by the Severe fever with thrombocytopenia syndrome virus L protein. *BioRxiv* 10.1101/2022.08.25.505333, *Nucleic Acids Res.* Accepted 12/2022
5. Kouba T[#], Vogel D[#], Thorkelsson SR[#], Quemin E, Williams HM, Milewski M, Busch C, Günther S, Grünewald K, Rosenthal M*, Cusack S*. Conformational changes in Lassa virus L protein associated with promoter binding and RNA synthesis activity. *Nat. Comms* 2021
6. Meier K[#], Thorkelsson SR[#], Quemin E*, Rosenthal M*. Hantavirus Replication Cycle-An Updated Structural Virology Perspective. Viruses 2021
7. Cusack S, Rosenthal M. Matters arising: Errors in the deposited SFTSV L protein structure (PDB:6L42). *Nat. Microbiol*. 2021
8. Vogel D[#], Thorkelsson SR[#], Quemin E, Meier K, Kouba T, Gogrefe N, Busch C, Reindl S, Günther S, Cusack S, Grünewald K, Rosenthal M. Structural and functional characterization of the Severe fever with thrombocytopenia syndrome virus L protein. *Nucleic Acids Res*. 2020 Apr 20. doi: 10.1093/nar/gkaa253.
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4. Chancengleichheit und Internationalisierung

This project included the Cusack lab from EMBL Grenoble as an international partner. The PhD student positions that had to be filled have been internationally advertised and students of different nationality were hired. Relatively frequent travel of the postdoc from BNITM to EMBL Grenoble as well as regular video conferences and annual in-person project meetings until 2020 with all involved scientists ensured a close collaboration and efficient communication between the partners. We had people of different gender working within all levels of the project. Similarly, scientists and technicians with different levels of expertise were contributing to the success of the project. In summary, within this project we have provided equal opportunities for scientists and technicians of different nationality, gender, and career stage.

5. Strukturen und Kooperationen

Collaboration between the partners LIV(HPI)/CSSB Hamburg, EMBL Grenoble and BNITM was coordinated by Maria Rosenthal at the BNITM. Regular telephone or video conferences and yearly personal project meetings (until 2020, virtual meetings only afterwards) ensured efficient communication and quick progression of the project. The BNITM postdoc traveled to Grenoble three times to perform experiments in the Cusack laboratory. The PhD student Kristina Meier visited IBS Grenoble twice in 2022 to collaborate with Helene Malet's group.

The proximity between BNITM and CSSB was particularly beneficial for the scientific exchange between BNITM and LIV(HPI)/CSSB. Both the BNITM postdoc and the BNITM PhD student were able to support and learn about EM experiments and data processing. The PhD student of the Grünewald group spent time at BNITM to learn some basics about model building. Cryo-EM experiments at CSSB were supported remotely by the postdoc from the Cusack lab and twice by direct visit for grid preparation and data collection. The decision to perform additional cryo-EM experiments in the Cusack lab was accompanied by an agreement about main responsibilities of each partner and re-arrangements concerning the use of funds for the EMBL partner site. Overall, the interaction and cooperation within this project was characterized by a strong team spirit as well as clear and straightforward communication promoting the scientific and methodological exchange between the partners.

6. Qualitätssicherung

All data generated within the project are shared and discussed with the partners ensuring checks of data integrity and plausibility. Students, postdocs and technicians are closely supervised by the respective WP leaders. All experimental data are recorded in detail in lab books, which are regularly checked by the WP leaders for plausibility and completeness. The project group recognizes the contributions of everyone involved, of all previous publications and acknowledges all third-party funding providers in the publications. Data were published in peer-reviewed open-access journals. To accelerate research, we made several of the initial manuscript versions publicly available on a preprint server (BioRxiv) prior to peer review. All experimental data for structure determination and modelling as well as the final models are deposited in public databases, such as the protein databank PDB, the EM databank EMDB and the databank for solution-scattering data SASBDB.

7. Zusätzliche eigene Ressourcen

This specific field of research is extremely competitive at the moment with several new structures published in the last years. Therefore, the partner institutions have been financially supporting the project with different measures:
(1) Scientific and technical support by postdocs, PIs, PhD students and technicians from the respective labs not included in the budget (Stephan Günther/BNITM, Kay Grünewald/LIV(HPI), Stephen Cusack/EMBL, Maria Rosenthal/BNITM, Emmanuelle Quemin/LIV(HPI) now I2BC, Tomas Kouba/EMBL now IOCB Prague, Carolin Seuring/LIV(HPI), Kristina Meier/BNITM, Carola Busch/BNITM, Nadja Gogrefe/BNITM).
(2) For synchronization Dominik Vogel, a postdoc included in the project budget for 3 years, was paid from institutional funding for 12 months to compensate the delayed start of the LIV(HPI) PhD student Sigurdur Thorkelsson due to technical reasons.
(3) LIV(HPI) support allowed for more EM experiments than initially planned, paying for microscope time for data collection and access to the cluster for data processing and archiving.
(4) Costs for open access publications were higher than initially estimated and have been supported by the Leibniz community through open access funding provided to BNITM, by EMBL and by LIV(HPI).
(5) Due to the additional people working on the project the costs for consumables at BNITM were considerably higher than initially planned. This was also compensated by institutional funding.

8. Ausblick

The huge success of this project and the momentum in bunyavirus structural biology is being harnessed to prepare further funding applications. These will help to preserve and further strengthen the strong and supportive scientific partnerships developed.