Title: Discovery and Characterization of a Fic Protein from *Bordetella Bronchiseptica* with Guanylyltransferase Activity

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Keywords: *Bordetella*; guanylyltransferase; AMPylation

Fic (filamentation induced by cAMP) proteins regulate diverse cellular processes in bacteria. While Fic proteins predominantly utilize ATP to post-translationally modify target proteins, some utilize other nucleotide derivatives to alter the activity of their target. *Bordetella sp.* causes respiratory tract infections, including whooping cough in humans. A combination of waning immunity to *B. pertussis* and the emergence of human-adapted *B. bronchiseptica* strains have resulted in recent epidemics of whooping cough-like illnesses worldwide – highlighting the presence of novel *Bordetella* proteins critical for virulence and/or fitness. Such proteins would be key candidates for a more effective vaccine designed for newly circulating *Bordetella* strains. Interestingly, we discovered a Fic protein, BbFic in *Bordetella bronchiseptica*, that fits the transcriptional profile of such predicted virulence factors. Unlike most Fic proteins that preferentially bind and utilize ATP as a nucleotide source, BbFic weakly binds ATP and instead shows a 30-fold increased preferential usage for GTP. We thus report the discovery, enzymatic characterization of BbFic as a bona fide guanylyltransferases, and present structural insights into BbFic-GTP interaction. We solved the crystal structure of apo BbFic at 3.1 Å and generated an AlphaFold model of BbFic. Using molecular docking and mutagenesis, we elucidated a mechanism for GTP recognition, which implicates two arginine residues within its nucleotide-binding pocket (Flap). Furthermore, our bioinformatics analyses of the entire Fic protein superfamily (pfam 02661) to identify similarity networks using BbFic as an index protein identified a sub-cluster of proteins that also function as guanylyltransferases. The importance of our work is two-fold: 1) BbFic represents a new category of fitness genes predicted to play a role in new host-adaptations for Bordetella, and 2) BbFic frames the groundwork for understanding Fic-mediated GMPylation (addition of GMP) as a novel post-translational modification in signal transduction.

Understanding Important Lipid Interactions of SARS-CoV-2 Envelope Protein

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the pathogen responsible for COVID-19, a disease which has resulted in the death of millions worldwide over the span of a year. COVID-19 ranges in severity of symptoms which are related to the inflammatory response. This inflammation can impact cardiac health and result in damage. Although the field has progressed significantly, there is still a critical gap in understanding virus-host lipid interactions that contribute to viral assembly and budding of this lipid-enveloped virus. There are four structural proteins encoded in the SARS-CoV-2 genome: membrane, envelope (E), nucleocapsid, and spike. These proteins give shape to the bilayer lipid coat that encapsulate the genomic core necessary for virus infection and replication. Although we know that these proteins are essential for viral reproduction and how they interact with each other, little is understood on how lipid species regulate their assembly. It is well established that inflammation is common during infection. One enzyme that participates in this process is ceramide kinase (CERK), which synthesizes the pro-inflammatory lipid, ceramide-1-phosphate (C1P). CERK is a therapeutic target for a variety of inflammatory disorders. My primary goal is to define the relationship between CERK activity and SARS-CoV-2 assembly. We aim to understand the impact that CERK activity has on the localization and assembly of SARS-CoV-2 structural proteins. I am interested in how CERK regulates E protein localization and formation of virus-like particles and the process by which E-lipid interactions contributes to membrane curvature changes necessary for formation of new viral particles. Overall, I anticipate that this study will help define the relationships between a host enzyme and host membranes and the assembly of SARS-CoV-2.

BREAKING DOWN THE HAUS: MOLECULAR INSIGHTS INTO BRANCHING MICROTUBULE NUCLEATION

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Accurate segregation of chromosomes during mitosis depends on the correct assembly of the mitotic spindle, a bipolar structure composed mainly of microtubules. The augmin complex, or homologous to augmin subunits (HAUS) complex, is an eight-subunit protein complex required for building robust mitotic spindles in metazoa. Augmin increases microtubule density within the spindle by recruiting the γ-tubulin ring complex (γ-TuRC) to pre-existing microtubules and nucleating branching microtubules in a manner that maintains spindle polarity. Here, we elucidate the molecular architecture of augmin by single particle cryo-electron microscopy (cryo-EM), computational methods, and crosslinking mass spectrometry (CLMS). Augmin's highly flexible structure contains a V-shaped head and a filamentous tail, with the head existing in either extended or contracted conformational states. Our work highlights how cryo-EM, complemented by computational advances and CLMS, can elucidate the structure of a challenging protein complex. We demonstrate how this approach has provided novel insights into the function of augmin in mediating microtubule branching nucleation.

Structural and kinetic bases for small molecule activation and inhibition of the 3CL protease from a novel Canine coronavirus that infects humans

Abstract:

The global COVID-19 pandemic of 2019 caused by the infection of SARS-CoV-2, a *betacoronavirus*, has highlighted the importance of studying the emergence of new coronavirus strains to predict and prevent the onset of future pandemics. A novel canine coronavirus, CCoV-HuPn-2018 (CCoV), has recently been isolated from several human patients with pneumonia in Malaysia, indicating the ability of the virus to be transmitted from animals to humans. Based on sequence similarity, this novel CCoV is classified as an *alphacoronavirus*, similar to other animal-infecting coronaviruses like feline infectious peritonitis virus (FIPV) and transmissible gastroenteritis virus (TGEV), which infect cats and pigs, respectively.

Coronaviruses are single-stranded, positive-sense RNA viruses that translate their genome into two overlapping polyproteins. These polyproteins are matured through the activity of a virally encoded protease, the 3-Chymotrypsin-like protease (3CLpro). 3CLpro is highly conserved across coronavirus genera and is essential for viral replication. The protease functions as a homodimer with a catalytic dyad His41-Cys144. The absence of human analogs and its highly conserved nature makes 3CLpro an attractive antiviral drug target. Given its importance in viral replication, we sought to identify and characterize novel inhibitors of CCoV 3CLpro from existing SARS-CoV-2 3CLpro inhibitors. We also used X-ray crystallography to determine the first X-ray structures (ranging from 1.89Å to 2.6Å) of CCoV 3CLpro bound to both covalent and non-covalent inhibitors.

Upon analyzing the active site of the protease, we observed that CCoV 3CLpro has a relatively smaller S2 subsite compared to other betacoronaviruses. This is consistent with published structural data on other alphacoronaviral 3CLpro enzymes. To understand how this smaller subsite might affect inhibition, we tested small molecule inhibitors that we have previously shown to potently inhibit SARS-CoV-2 3CLpro against CCoV 3CLpro. Substantiating our observation, increasing the size of the P2 moiety on the inhibitor resulted in a greater decrease in potency against CCoV 3CLpro (>100μM to 0.26μM) which was not observed in SARS-CoV-2 3CLpro.

Unexpectedly, most 3CLpro inhibitors showed activation at lower concentrations and exhibited inhibition only at higher concentrations. A possible explanation for the observed phenomenon is weak dimerization of CCoV 3CLpro, which is induced in the presence of an inhibitor and/or substrate. Using fluorescence-based kinetic assays, the monomer-dimer equilibrium constant of CCoV 3CLpro (K_d = 6.26 ± 2.14 μM) was identified to be 25 times weaker than that of SARS-CoV-2 3CLpro (K_d = 0.25 ± 0.04 μM) . Further studies to characterize this effect can increase our understanding of the enzyme and pave the way for the creation of novel, potent inhibitors that treat this and other emerging coronavirus strains.

Characterization of the β-barrel Assembly Machinery in *Fusobacterium*

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Fusobacterium nucleatum is a Gram-negative, anaerobic human oral microbiome constituent. Though a steady state commensal, this pathobiont plays a role in periodontal disease, endodontic infections, pre-term births, and colorectal cancer. *F. nucleatum* effects its pathogenicity through a class of outer membrane proteins (OMPs) called adhesins. Several key adhesins present as β-barrel OMPs in the form of Type VA secretion system autotransporters or porins. The β-Barrel Assembly Machinery (BAM) complex plays a vital role in the biogenesis of β-barrel OMPs in Gram-negative bacteria. The *E. coli* BAM complex is composed of five proteins, OMP BamA and accessory lipoproteins BamB-E. Though there is diversity in the number of BAM complex components among Gram-negative bacteria, BamA and BamD are essential for viability across multiple organisms. However, preliminary searches of the *F. nucleatum* genome have revealed only the presence of BamA, the integral membrane protein of the complex, to the exclusion of any apparent periplasmic auxiliary proteins. Though our lab has previously solved BAM components from various organisms, the structure of BamA in *F. nucleatum* remains unresolved. Thus, the objective of this study is to determine both the structure of BamA and to identify the accessory proteins with which it binds. We employ techniques including X-ray crystallography and cryo-electron microscopy to accomplish the structural aims of this proposal. Pull-down assays coupled with proteomics elucidate the identity and nature of proteins accessory to BamA. This research is innovative in its exploration of membrane protein biology in *F. nucleatum* through examining BAM, a difficult system unstudied in this challenging anaerobic organism. We expect to advance the mitigation of *F. nucleatum* pathogenesis in the oral microbiome and beyond by detecting the binding partners and solving the structure of BamA in *Fusobacterium nucleatum*.

Characterizing the structure of HCV envelope proteins using pseudoparticles

Ryan Peters, Shishir Poudyal, Richard Kuhn

Hepatitis C virus (HCV) has chronically infected an estimated 58 million people around the globe, and it infects another 1.5 million every year. The viral envelope proteins E1 and E2 are the major targets for the immune system. However, rational design of an E1E2-based vaccine has been hindered by a lack of structural information. Here we outline the production of lentiviral particles pseudotyped to express full-length HCV E1E2. These HCV pseudoparticles (HCVpp) were produced by transfecting a combination of packaging (HIV-GAG) and HCV E1E2 expressing plasmids in Expi293F cells. The resulting supernatants were clarified, concentrated, and vitrified in liquid ethane. Cryo-electron microscopy of the samples revealed the presence of heterogenous particles (HCVpp). We propose that a better production and purification scheme of HCVpp which is being optimized will enable high-resolution cryo-electron microscopic and tomographic studies of native HCV E1E2 proteins. These studies will also include characterizing human antibodies in complex with HCVpp. This would provide insight into the conformation in which these proteins are presented to the immune system which is crucial for the design of an HCV vaccine.

Title: Bridging the Gap: reconstitution of engineered T cell activation with single-cell, single-molecule resolution

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Chimeric antigen receptor (CAR) binding to tumor associated antigen leads to signaling events that initiate anti-tumor CAR T cell responses. This engineered receptor likely couples to native T cell signaling machinery yet has been shown to inefficiently signal, requiring an estimated 2 or 3 orders of magnitude more binding events for activation. Additionally, average molecular binding dwell times of CARs are up to two orders of magnitude longer than native TCR off rates. Surprisingly, this long mean dwell time far exceeds the typical timescale of lytic degranulation of about 4-8 minutes. Taken together, the molecular mechanism of CAR T cell activation remains paradoxical. We apply *in vitro* reconstitution using supported lipid bilayers functionalized with adhesion and tumor-antigens as a tumor mimetic and measure the minimal, threshold criteria for CAR T cell activation. Reconstitution enables control over the density of tumor markers, spanning orders of magnitude, that reflect the normal-to-disease spectrum as well as expression level changes that occur during disease progression. Our current work focuses on a second-generation, anti-FITC CAR that targets tumor cells via a small molecule ligand (bridge). This bridging molecule consists of a folic acid (tumor ligand) moiety linked to FITC. Binding is captured using high spatiotemporal resolution imaging in a Total Internal Reflection Fluorescence (TIRF) configuration and mapped to cytotoxic degranulation. A mechanistic understanding of CAR activation may improve therapeutic implementation by tuning bridge and T cell dosing to yield a precision treatment based on tumor stage and type.

Design, Synthesis, and Antibacterial Evaluation of 4-Hydroxy-benzylidenebenzofuran-3-ones as c-di-AMP Synthase Inhibitors

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Abstract: The rise of antibacterial resistance in recent decades has led to the demand of novel pharmaceuticals that will defend against drug-resistant pathogenic bacteria. Cyclic-di-adenosine monophosphate (c-di-AMP) is implicated in several essential bacterial processes including cell wall homeostasis, biofilm formation, virulence, and others. To this end, the Sintim Lab has been targeting bacterial enzymes responsible for the synthesis of the secondary messenger c-di-AMP. A high throughput screen was performed to identify potential c-di-AMP synthase and c-di-AMP phosphodiesterase inhibitors. We herein describe the efforts made towards identification of hit compounds and their derivatization to improve solubility, potency, and drug-likeness.

Drug Discovery for *Neisseria Gonorrhoeae* Carbonic Anhydrase

Molly Youse

Neisseria gonorrhoeae is a bacterial pathogen that causes the sexually transmitted disease gonorrhea. In recent years, gonorrhea cases in the United States have been on a steady rise. More than 675,000 new cases of gonorrhea were reported to the CDC in the United States in 2020, an increase of 45% from 2016. Additionally, *N. gonorrhoeae* strains are becoming increasingly resistant to the antibiotics typically used to treat the disease. To address the need for new therapeutics, we have developed *N. gonorrhoeae* carbonic anhydrase (NgCA) inhibitors. Carbonic anhydrases are metalloenzymes that play a role in maintaining pH homeostasis by catalyzing the hydration of carbon dioxide. Our structure-based approach has focused on repurposing the scaffolds of acetazolamide (AZM) and ethoxzolamide (EZM), two FDA-approved carbonic anhydrase inhibitors (CAIs). To assess potency and selectivity, compounds have been tested against NgCA and human carbonic anhydrases hCAI and hCAII in a CO_2 hydration assay. A subset of these molecules was also found to have potent antimicrobial activity according to their MIC_{50} data, validating their potential to be effective drugs. Interestingly, there also exist compounds that exhibit potent NgCA inhibition but poor antimicrobial activity, indicating other factors play a role in CAI efficacy. Thus, an assay has been developed to assess the accumulation of our analogs in *N. gonorrhoeae*, as it is our hypothesis that that there are certain properties a molecule must have to pass through the outer membrane of the bacteria. Quantifying the amount of compound that enters *N. gonorrhoeae* will allow us to incorporate design features in our analogs based on permeability. Additionally, surface plasmon resonance (SPR) studies have begun to elucidate protein-ligand binding kinetics of our molecules, with the hopes of understanding how to selectively inhibit NgCA over the human enzymes. Using the accumulation assay and SPR experiments on a large scale will further inform drug design as we continue to develop molecules that have the potential to treat gonococcal infections.

Title:

Bacterial Ubiquitin De-ADP-Ribosylating Metaeffector Expands the Macrodomain Landscape

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Abstract:

ADP-ribosylation is a reversible post-translational modification in which ADP-ribose (ADPR) from nicotinamide adenine dinucleotide (NAD^+) is added onto target proteins. Removal of ADP-ribosylation requires (ADP-ribosyl)hydrolases, with macrodomain enzymes being a major family in this category. The bacterial pathogen *Legionella pneumophila* mediates atypical ubiquitination of host targets using the SidE effector family in a process that involves ubiquitin ADP-ribosylation on arginine 42 as an obligatory step. Using LC-MS/MS, I performed a proteome-wide search of ubiquitin-interacting effectors in *L. pneumophila* and found that the *Legionella* macrodomain effector MavL regulates this SidE-mediated pathway by reversing the arginine ADP-ribosylation, probably to minimize potential detrimental effects caused by modified ubiquitin. I determined the crystal structures of ADP-ribose and ubiquitin-bound MavL, providing structural insights into substrate recognition and catalytic mechanism. Further bioinformatical analyses reveal that MavL and its homologs define a new class of macrodomain enzymes uniquely selective for mono-ADP-ribosylated arginine residue. Such enzymes are also present in eukaryotes, as exemplified by two previously uncharacterized MavL-type hydrolases in *Drosophila melanogaster*. Crystal structures of several proteins in this class provide insights into arginine specificity and a shared mode of ADP-ribose interaction distinct from previously characterized macrodomains. Collectively, this study reveals a new regulatory layer of SidE-catalyzed ubiquitination and expands the current understanding of macrodomain enzymes.