Part A – Applicant

A.1 Applicant

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Part B – Scientific proposal

B.1 BASIC DETAILS

*In this proposal I used Grammarly, ChatGPT 3.5 and Word to improve spelling and grammar.

B.1.1 Title

Unravelling the Mysteries of Alternative Pathway C5 Convertase: A Combined Structural and Functional Approach.

B.1.2 Abstract

The complement system is an important component of the innate immune system, yet its mechanisms, particularly concerning the C5 convertase, remain incompletely understood. It is necessary to understand the working mechanisms of the alternative pathway C5 convertase as defects of the complement system are involved in various diseases, including autoimmune disorders, infectious diseases, inflammatory conditions, transplant rejection, and transfusion-related reactions. To advance our understanding of the alternative pathway C5 convertase, I aim to develop a liposome model capable of facilitating combined structural and functional experiments. The proposed research aims to develop and validate a liposome model to study the complement system's interaction with bacterial membranes. The liposomes will incorporate lipopolysaccharide (LPS) to mimic the bacterial outer membrane, allowing investigation into complement resistance mechanisms. Aim 1 focuses on developing and validating the model. Aim 2 aims to unravel the structure and mechanisms of alternative pathway C5 convertase using Cryo-EM. I will investigate factor P's stabilizing effect, examine LPS diversity's influence on complement activation, and assess terminal complement proteins' interaction with C5 convertase. These experiments will provide insights into bacterial resistance mechanisms and complement protein mechanism, essential for understanding host-pathogen interactions.
The insights gained from these experiments will not only deepen our understanding of the complement system but also lead the way into the development of targeted treatments for associated diseases and disorders. Moreover, the newly developed liposome model can be used to address other research questions concerning complement system functioning in the future.

B.1.3 Layman's summary

When harmful pathogens attempt to invade our bodies, our natural defences, like the skin and saliva, act as protective barriers. However, if these barriers are breached and the pathogens start causing illness, our innate immune system responds. This system targets common molecules found in many pathogens. An integral part of the innate immune system is the complement system, which comprises off three different pathways. In this research proposal, I will focus on the alternative pathway because it is involved in all complement reactions. This pathway operates continuously at a low level, attempting to attach a protein called C3b to all cells. Although our cells have mechanisms to remove C3b, when it binds to bacterial membranes, it

cannot be easily removed, leading to a process where more C3b is placed on the membrane. Eventually, when enough C3b accumulates on the membrane, it triggers the cleavage of C5 into C5a and C5b, initiating the process of creating a hole in the bacterial membrane to kill it. Some bacteria evade this process by having specific types of lipids in their membranes called lipopolysaccharides (LPS). In this study, I aim to understand how C5 is cleaved on bacterial membranes. To do this, I will utilize tiny vesicles called liposomes containing LPS to mimic bacterial membranes. These liposomes will also contain lipids where C3b can be easily attach to using a chemical reaction. By simulating an infection using only the necessary components, I can visualize these liposomes with Cryo-Electron Microscopy and specifically observe the proteins of interest, which makes it possible to identify them. If identifying the C5 convertase remains challenging, I will use an antibody to bind to C5, which will help me to identify the C5 convertase. Additionally, I will vary the type of LPS in the liposomes to observe if there is an effect on the C5 convertase. Moreover, I will introduce factor P during complement system activation, as it is believed to stabilize the C5 convertase, and visualize any differences when examining the liposomes using Cryo-EM. Furthermore, I will investigate how proteins involved in forming pores in the membrane to kill the bacterium interact with the C5 convertase. This research integrates structural studies with functional assays to enhance our understanding of the complement system and in particular the C5 convertase. Understanding the mechanisms of the C5 convertase is crucial for developing new drugs and therapies against diseases caused by defects in the complement system, including autoimmune disorders, infectious diseases, inflammatory conditions, transplant rejection, and transfusion-related reactions.

B.1.4 Keywords

Complement system, liposomes, Lipopolysaccharide, alternative pathway C5 convertase, Cryo-EM

B.2 SCIENTIFIC PROPOSAL

B.2.1 Research topic (What)

When your body encounters pathogens, the innate immune system serves as an important defence mechanism[1]. It effectively combats various classes of pathogens, including bacteria, fungi, viruses, and parasites, by targeting specific classes of molecules on their surfaces[2]. One integral component of the innate immune system is the complement system, which comprises three distinct pathways: the classical, lectin, and alternative pathways[3].
The classical pathway is initiated by the formation of an antibody-antigen complex, which binds to C1, leading to its cleavage and activation. This activated C1 complex then triggers a cascade of reactions, ultimately resulting in the cleavage of C4 and C2. C4b anchors to the membrane, while C2b forms a complex with C4b, forming the classical C3 convertase C4b2b[3].
The lectin pathway, on the other hand, is activated when mannose-binding lectins (MBLs) recognize specific sugars on the surface of pathogens. Upon sugar recognition, MBLs form an active MBL complex, which can cleave C4 and C2, similar to the classical pathway to form the classical C3 convertase C4b2b[3].
In contrast, the alternative pathway works continuously at low levels, driven by the spontaneous hydrolysis of C3 into C3a and C3b. C3b binds covalently to pathogen and host cell membranes. However, certain host cell proteins inhibit or degrade alternative pathway proteins on host cell membranes, preventing further pathway activation[3]. Factor B (FB) can bind to C3b and be cleaved by factor D (FD) into Ba and Bb, forming the alternative pathway C3 convertase C3bBb. Properdin (Factor P) is thought to stabilize alternative pathway C3 convertases and help localize C3b to pathogen membranes[4,5].
The generation of C3 convertase initiates a positive feedback loop within the alternative pathway. As C3b binds FB and is subsequently cleaved by FD, new alternative pathway C3 convertases are formed. This results in the accumulation of C3b on the surface of pathogens, which leads to the formation of C5 convertase. These C5 convertases then catalyse the conversion of C5 into C5a and C5b. C5b triggers the assembly of the membrane attack complex (MAC), composed of C5b, C6, C7, C8, and 18 units of C9[6]. This MAC formation results in the creation of pores in the membranes of pathogens, leading to cellular damage and eventual cell death. Given the amplification loop within the alternative pathway and the need for high concentrations of C3b, it is probable that during infection, the predominant C5 convertase operates via the alternative pathway. This emphasises the critical role of the alternative pathway in host defence mechanisms against pathogens and the need to gain further understanding of the alternative pathway C5 convertase.
Bacteria, like all cells, have evolved immune evasion mechanisms to accomplish their evolutionary goal of reproduction. Some of these mechanisms function toward preventing the formation of MAC pores in their membrane. Staphylococcus aureus, for example, produces SpA, a membrane protein that binds to the Fc region of IgG, thereby inhibiting the classical pathway by preventing the binding of C1[7]. Additionally, it secretes SCIN, which inhibits all C3 convertases, effectively blocking the entire complement system[8]. Pseudomonas aeruginosa produces proteases that cleave C3b, preventing its binding to pathogen membranes[9]. Borrelia burgdorferi expresses a protein on its surface structurally similar to human CD59, inhibiting MAC assembly and insertion into the membrane[10]. Gram-positive bacteria, with their thick cell walls, intrinsically prevent MAC insertion into their membranes, hence my focus here on gram-negative bacteria.
In addition to proteins that inhibit the complement system, lipopolysaccharide (LPS) is suspected to play a role in preventing MAC-dependent killing. LPS consists of three parts: lipid A, core oligosaccharides, and an O-antigen. It constitutes about 70% of the outer membrane of gram-negative bacteria[11]. Lipid A anchors LPS in the membrane, while the core oligosaccharides are just above it. The O-antigen, composed of repeating units (RUs) of sugars, extends furthest from the membrane. The wide variation in O-antigens among bacteria is thought to be crucial in immune evasion. Differences in length and composition of RUs can render bacteria resistant or sensitive to complement-dependent killing. It is hypothesized that an increased length of O-antigen serves as a barrier, hindering the efficient insertion of C8 into the membrane by creating an increased distance between C8 and the membrane surface. In that way preventing the formation of MAC pores.
Currently, certain insights into the function and structure of alternative pathway C3/C5 convertases have been obtained. Berends et al. demonstrated that C3b bound to beads shows over a 100-fold increase in activity compared to the same amount of C3b in solution[12]. Moreover, they revealed that an increased concentration of C3b on the beads is necessary for the conversion of C5 to take place. Additionally, crystal structures of C3 and C3b in solution have been resolved by Janssen et al[13,14]. Furthering this, Rooijakker et al. obtained the crystal structure of C3 convertase by crystallizing C3b in complex with Bb, stabilized by the C3 convertase inhibitor SCIN used by Staphylococcus aureus to inhibit the host complement system[15]. Doorduijn et al. demonstrated that gram-negative bacteria resistant to MAC-dependent killing show heightened C5 conversion compared to MAC-sensitive counterparts[16]. This increased conversion of C5 could potentially contributes to MAC resistance. The stoichiometric ratio between C5 and C9 is 1:3 in serum, but for MAC pore formation a ratio of 1:18 between C5 and C9 is needed[6,17,18]. If a lot of C5 is converted, there may be insufficient C9 available to form MAC pores, thereby rendering the bacteria resistant to MAC-dependent killing. They also observed that MAC-resistant bacteria release more soluble MAC (sMAC)

and cause significantly more bystander cell damage. Besides that their study suggested that MAC resistance in certain bacterial strains could be due to differences in the O-antigen. Menny et al. demonstrated how the terminal complement pathway can insert into liposomes and elucidated the mechanisms underlying membrane disruptions using Cryo-EM[19].
In this proposal, **I aim to deepen our understanding of the structural and functional aspects of the alternative pathway C5 convertase**. To achieve this, I will use a model system utilizing liposomes on which I will activate the complement system. Unlike the study performed by Berends et al. using beads, liposomes provide a more biologically relevant platform, also MAC pores can insert into liposomes but not into beads. Furthermore, the structures obtained here will be more biologically relevant as they will be situated on a membrane, unlike the current elucidated crystal structures. I will incorporating LPS into the liposome model to enhance its physiological relevance. Additionally, liposomes possess an optimal thickness for cryo-EM, unlike bacteria, which are currently too thick for effective imaging with cryo-EM. Next to that, I will extend the study conducted by Menny et al. by investigating the interaction between terminal complement pathway proteins and the alternative pathway C5 convertase.
The proposed research will start with the development and validation of the liposome model containing alternative pathway convertases. Subsequently, three distinct sublines will utilize this model to unravel the structure and working mechanisms of alternative pathway C5 convertase: first, to unravel the structure of alternative pathway C5 convertase and the stabilizing effect of factor P; second, to investigate the impact of lipopolysaccharides diversity on complement system activation; and third, to investigate the interaction of terminal pathway complement proteins with alternative pathway C5 convertase

B.2.2 Approach (How)

Aim 1: Development and validation of the liposome model
The outer membrane of gram-negative bacteria serves as a target for the complement system. However, the complexity of both bacteria and serum requires simplification to be able to study specific mechanisms of the complement system. In this regard, I will use purified complement components instead of serum and utilize liposomes as a simplified model of the bacterial outer membrane. Liposomes, being small vesicles with adjustable lipid compositions, offer a suitable model for mimicking bacterial membranes. To improve the similarity to bacterial outer membranes, I will incorporate LPS into the liposomes.
Building upon the methodologies of Kraus et al. and Erridge et al., I will replicate their approach to incorporate LPS into liposomes[20,21]. Specifically, I will utilize three types of LPS from Klebsiella pneumoniae: one lacking an O-antigen and two with different O-antigens, one complement-sensitive (O2) and the other complement-resistant (O1)[22]. This selection allows for subsequent investigation into the role of LPS in complement resistance.
Initially, I will employ LPS lacking an O-antigen to ensure complement sensitivity, which will facilitate the validation of the liposome model. To enable the attachment of C3b to the liposomes, I will adopt a method similar to that of Boero et al[23]. This involves the chemical modification of C3b at its thioester domain with a maleimide linker containing a DBCO group, followed by click chemistry with a low percentage of azide-containing lipids incorporated into the liposomes. Subsequently, the amplification of C3b deposition will mimic the physiological process during infection by adding all the purified complement components necessary for C3b amplification. The presence of C3b on the liposomes will then be confirmed via western blot analysis.
When I am using LPS containing an O-antigen, it remains uncertain whether C3b can effectively penetrate through the glycans of the O-antigen to attach to the azide group on certain lipids. Moreover, I aim to explore potential variations in the distance of C3b deposition from the liposomal membrane surface. To address this, it is essential to validate the activation of the complement system initially with C5-depleted serum, followed by the amplification of C3b using purified components. This approach allows me to determine if different LPS variants influence the distance of C3b deposition to the liposomal membrane. To confirm the existence of functional C3 and C5 convertases on the liposomes, I will measure C3a and C5a in the supernatant using ELISA. Cryo-Electron Microscopy will be employed to visualize the liposomes, followed by Cryo-Electron Tomography to examine protein complexes from different angles[24]. Subtomogram averaging will then allow for the extraction of high-resolution data on the studied protein complexes, laying the groundwork for subsequent investigations under Aim 2.

Aim 2: Unravelling the structure and working mechanisms of alternative pathway C5 convertase

Upon validation of the liposome model in Aim 1, I will proceed with a series of experiments aimed at elucidating novel structural and functional insights into C5 convertase. Each experiment is detailed below:

<u>2A Unravelling the structure of alternative pathway C5 convertase and the stabilizing effect of factor P</u>
Utilizing the liposome model established in Aim 1, which incorporates C3b on its surface through click chemistry followed by amplification using purified complement components, I will determine C3 and C5 conversion activity by measuring C3a and

C5a levels in the supernatant using ELISA. Once conversion activity is confirmed, I will replace factor B with an in house produced mutant variant that exhibits stronger binding to C3b and lacks cleavage activity for either C3 or C5.
Following the exchange, I will confirm the absence of C3 and C5 conversion activity via ELISA and verify the presence of C5 still attached to the liposomes through western blotting. Subsequently, monoclonal antibodies targeting C5 will be employed to identify C5 convertase complexes on the liposomes. Cryo-Electron Tomography coupled with subtomogram averaging will then be utilized to visualize the structure of C5 convertase with trapped C5. In the case that subtomogram averaging fails to achieve the desired resolution, I will explore single-particle analysis as an alternative approach to obtain higher resolution of the C5 convertase complex.
Additionally, I will investigate the stabilizing effect of factor P on C3 and C5 convertases at the molecular level, as was demonstrated by Medicus et al. that factor P stabilizes both convertases[4]. To this end, I will use the liposome model described under aim 1 and use two groups of similar liposomes with the addition of factor P to one group of liposomes at each step. Monitoring convertase activity over time will reveal if factor P indeed stabilizes the complexes.
Furthermore, visualization of liposomes with and without factor P will elucidate the interaction between factor P and C3/C5 convertases. Comparison with the crystal structure of factor P in complex with C3b will provide insights into the potential formation of a meshwork of factor P between C3b molecules on the liposome surface[5].

2B. Investigating the impact of lipopolysaccharides diversity on complement system activation

LPS is a vital component of the gram-negative bacterial outer membrane, with its sugar groups serving as protective moieties for the bacterium. A study by Doorduijn et al. proposed that MAC resistance may be due to specific types of LPS in the outer membrane and Grossman et al. demonstrated that variations in LPS sizes and distributions can lead to MAC resistance[16,25]. Moreover, Doorduijn et al. observed that MAC-resistant bacteria show increased C5 conversion compared to MAC-sensitive strains, suggesting a potential correlation between LPS and C5 conversion.
To elucidate the extent to which LPS contributes to MAC resistance and affects C3 and C5 convertase activity, three distinct batches of liposomes will be prepared, each containing different types of LPS: LPS lacking an O-antigen, LPS with Klebsiella LPS O1-antigen, and LPS with Klebsiella LPS O2-antigen. Prior to experimentation, the quantity of LPS in each liposome group will be quantified and adjusted if necessary, following the methodology outlined by Kraus et al[20].
The complement system will be activated using C5-depleted serum with a brief incubation period to generate initial amounts of C3 convertases on the liposomes. Subsequently, extensive washing will be conducted, followed by the addition of purified alternative pathway components to amplify the amount of C3b on the surface of the liposomes. Initial attachment of C3b to liposomes will occur through serum activation to mimic a biologically relevant scenario. This approach will also facilitate the investigation of whether LPS influences the distance between where C3b is anchored on the liposome and the liposomal membrane, as outlined later in the proposal.
Following complement system activation, C3 and C5 convertase activity among the different liposome groups will be determined by measuring C3a and C5a in the supernatant using ELISA. Terminal pathway induction will be achieved by supplementing the system with purified components C5, C6, C7, C8, and C9 to observe if specific LPS types can cause MAC resistance to liposomes. Liposome integrity will be assessed by incorporating a fluorescent molecule during liposome production and measure leakage of the fluorescent molecule in the supernatant.
Furthermore, Cryo-Electron Microscopy will be used to visualize the liposomes and analyse potential differences in convertase localization on the liposomal membrane. A key focus will be on identifying variations in the distance between the convertase and the membrane, particularly examining the potential impact of O-antigens. These variations may prohibit the efficient insertion of C8 into the membrane, crucial for MAC pore formation and rendering the bacteria MAC resistant. Initially, a comparative analysis will be conducted to determine any general differences in the proximity of the complement proteins to the membrane among the different groups of liposomes. Subsequently, a more detailed investigation will be performed by replicating the experiment proposed under aim 2A to generate C5 convertases with trapped C5 inside. These convertases will be tagged with antibodies to precisely determine the distance differences between the various liposome groups. This approach will provide insights into how specific lipid compositions, particularly the presence of O-antigens, influence the localization of C5 convertases on the liposomal membrane, thereby contributing to our understanding of MAC resistance mechanisms.

2C. Investigating the interaction of terminal pathway complement proteins with alternative pathway C5 convertase

The potential regulatory role of proteins from the terminal complement pathway on C5 convertase remains elusive. To address this, I aim to elucidate how the addition of terminal pathway complement components influences C3 and C5 convertase activity. Liposomes containing either MAC-resistant or MAC-sensitive LPS will be initially complement-activated using C5-depleted serum followed by the addition of purified components to increase the amount of C3b on the surface of the liposomes. Subsequently, the addition of C3/C5 will be accompanied by the simultaneous addition of other terminal

complement pathway proteins C6, C7, C8, and C9, both individually and in combinations. The conversion of C3 and C5 will be determined by measuring C3a and C5a in the supernatant using ELISA.
Furthermore, I want to investigate the attachment of C5b and downstream MAC proteins to the C5 convertase. While previous experiments could have identified the density of C5b on the C5 convertase, I here aim to explore whether downstream MAC proteins also remain associated, particularly considering potential differences between liposomes containing MAC-resistant and MAC-sensitive LPS. Following sequential addition of MAC proteins (C6 to C9), the presence of each protein on the liposomes will be determined using western blot analysis. Subsequently, Cryo-Electron Microscopy will be employed to visualize the protein complexes that have been confirmed by western blot and examine interactions between different proteins.
Additionally, I aim to assess the specificity of MAC action, particularly focusing on bystander cell damage and the role of C5 convertase presence on the membrane. Liposomes containing MAC-sensitive LPS, labelled with a fluorescent dye, will be initially complement-activated using C5-depleted serum followed by the addition of purified components to increase the amount of C3b on the surface of the liposomes. They will be mixed with liposomes that have the same lipid composition but contain a different fluorescent dye and are not complement activated. The subsequent addition of purified MAC components will be followed by measurement of leakage to determine the presence of MAC pores. This experiment will be conducted with MAC-resistant LPS and combinations thereof to determine the requirement of C3/C5 convertase presence for MAC killing and identify whether certain types of LPS promote or prevent bystander liposome damage. These experiments provide valuable insights into the role of LPS in bystander cell damage within a simplified environment, shedding light on the dynamics of complement protein interactions and MAC specificity.

B.2.3 Feasibility / Risk assessment

Combination of techniques
While individual techniques used in the proposal have been successfully performed, there is a risk associated with combining them. Specifically, generating liposomes with LPS and azide-labeled lipids may pose a challenge. However, an alternative approach using serum to initiate C3b convertase formation followed by amplification with purified complement components has been demonstrated in literature and serves as a feasible backup plan. Additionally, an alternative method, as developed by Boero et al., could be employed to obtain lipids with azide groups specifically attached to a sugar moiety[23]. This alternative method provides a valuable contingency plan, ensuring flexibility and adaptability in the experimental design.

Imaging challenges
Cryo-EM is a well-established technique, but there may be challenges in distinguishing C5 convertases on the membrane from other attached proteins. That is why I use a monoclonal antibody specific to C5. In case that doesn't work we could utilize super-resolution CLEM with GFP-tagged C5. This approach uses fluorescence to locate the C5 convertase with high precision before employing Cryo-EM to visualize its structure, ensuring accurate characterization.
Additionally, it may not be necessary to use the in-house produced factor B mutant, as natural conditions may sufficiently retain C5 attached to the C5 convertase in the absence of further terminal pathway complement components. This alternative strategy offers a simplified approach while maintaining experimental integrity and reliability.

Resolution limitations
Achieving sufficient contrast and resolution in Cryo-EM imaging to obtain desired structural details may be challenging. However, using a phase-plate in the microscope, as demonstrated by Sharp et al., offers a promising solution to enhance contrast[26]. Additionally, transitioning from tomography to single-particle analysis (SPA) can further improve resolution, potentially reaching atomic levels of detail. In this approach, the lower-resolution density obtained from tomography can serve as references for SPA, aiding in refining the resolution and enhancing the accuracy of structural reconstructions.

Complex identification
Investigating terminal pathway complexes on liposome surfaces may be complicated due to the presence of abundant C3b and LPS. Using monoclonal antibodies to tag terminal pathway proteins can be used to identify the complexes accurately.

B.2.4 Scientific (a) and societal (b) impact

The complement system is an important part of the innate immune system, playing a central role in defending against a wide range of pathogens and maintaining human well-being. Dysregulation of the complement system has been shown to be involved in various diseases, including autoimmune disorders, infectious diseases, inflammatory conditions, transplant

rejection, and transfusion-related reactions[27,28]. Understanding the fundamental processes of C5 convertases is crucial for elucidating its mechanisms and exploring therapeutic interventions for conditions where it is not working properly.
The model system proposed in this research holds immense potential for advancing our understanding of the complement system. By providing a platform to study both structural and functional aspects, this model enables researchers to unravel complex details of complement activation and regulation. Furthermore, the established workflows can serve as a model for integrating structural and functional data, facilitating thorough investigations into the complement system's complexities.
This research holds significant promise for societal impact through the potential development of novel therapeutics. By gaining a deeper understanding of the mechanisms of C5 convertase activity, particularly in the context of MAC-resistant bacteria, new approaches for targeted interventions can be explored. For instance, novel drugs could be designed to inhibit C5 conversion activity, as MAC-resistant bacteria likely employ this mechanism to prevent the formation of MAC pores by increasing the abundance of C5a and C5b, leading to a stoichiometric deficiency of C9 in serum for MAC pore formation. Additionally, excessive conversion of C5 results in the generation of more C5a, a potent anaphylatoxin that can trigger autoimmune diseases. Understanding the complex working mechanisms of C5 convertase opens doors to innovative drug design approaches aimed at modulating its activity. By doing so, these drugs could render resistant bacteria susceptible to MAC-dependent killing. This research not only has the potential to address antibiotic resistance but could also offers insight to help tackling autoimmune diseases by targeting C5 convertase activity. Given the increasing threat of antibiotic resistance, alternative treatments targeting the complement system represent a promising approach to fight against infectious diseases[29].
Furthermore, this research can be used as starting point to further understand the mechanism of action of existing therapeutics, such as the C5 inhibitor Eculizumab. Despite its efficacy, the precise mechanism of action of Eculizumab remains elusive. The model proposed in this research can be used to elucidate the binding of Eculizumab to C5 and its interference with C5 convertase. Such insights could lead the way to optimize the drug's efficacy and affordability, potentially reduce its high cost and the requirement for long-term administration. This has significant implications for improving access to effective treatment options and enhancing patient outcomes, particularly in the context of chronic conditions requiring prolonged therapeutic intervention.
Overall, this research holds significant scientific and societal implications, offering insights into fundamental biological processes and potential approaches for therapeutic intervention in diseases influenced by complement dysregulation and in particular C5 convertase. Through its multifaceted approach, this research contributes to the broader scientific understanding of the complement system and its role in health and disease.

B.2.5 Ethical considerations

Ethical considerations have been thoroughly examined, and to the best of my knowledge, no ethical issues arise from the proposed experiments. It will be ensured that the C5-depleted serum needed for the research is obtained through ethically correct practices. Ethical standards will be upheld throughout the study, with a commitment to conducting research in a responsible and respectful manner.

B.2.6 Literature/references

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