STRUCTURAL CHARACTERIZATION OF THE FULL-LENGTH EXOCYST COMPLEX BY TRANSMISSION ELECTRON CRYO-MICROSCOPY

by

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ABSTRACT

The exocyst is a conserved octameric protein complex in eukaryotes that is essential for tethering and fusion of vesicles to the plasma membrane. Composed of eight subunits, it is essential for growth, secretion, and developmental processes and has been implicated in cell motility, autophagy, cell division, and exocytosis. Functional defects are linked to cancer, neurodegeneration, ciliopathies, and bacterial pathogenesis. With known biochemical data from interacting partners and four crystal structures known, architectural models have proposed extended, rod-like conformations with side-by-side packing. Isolation and physical characterization of this essential protein complex has remained elusive. Structural information is necessary to determine how this protein functions in the context of exocytosis.

Using the *Saccharomyces cerevisiae* model eukaryotic organism, this research aims to purify intact exocyst complex, characterize it structurally, and identify the spatial orientation of the carboxyl termini of Sec15 relative to the fully assembled complex. Genetic engineering of a separate subunit with a ProteinA tag allows for antibody affinity-based purification using optimized buffer conditions to retain exocyst complex stability. Subsequent proteolytic cleavage of the linker region allows for highly concentrated, purified exocyst complex and has been verified by SDS-PAGE with coomasie blue and silver staining.

Negative stain transmission electron microscopy was conducted on the FEI Tecnai G2 TF20 TEM at 200 KeV with images collected at 62,000x nominal magnification. From two-dimensional class averaging of exocyst particles, V-
shaped rod-like structures with dimensions of 10 nm x 28 nm were observed and a calculated conical volume of 2200 nm$^3$ determined. Based on known crystal structure data of Exo70 with a 150 nm$^3$ volume, and assuming other subunits have comparable widths with longer lengths (since Exo70 is the smallest subunit), estimated volume of the entire exocyst complex is approximately 1900 nm$^3$. While efforts are still being made towards localization of the GFP tag and structural characterization by cryo microscopy, we conclude these particles contain the intact exocyst complex with all eight subunits, consistent with biochemical data.
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I. INTRODUCTION

The exocyst is a conserved octameric protein complex essential in tethering vesicles to the plasma membrane for exocytosis. Originally discovered more than three decades ago, studies using various model systems have made extensive progress toward the determination of a structure and function of the complex. While direct evidence of tethering has yet to be elucidated and a molecular understanding of function not yet defined, studies argue the importance of the exocyst as a spatiotemporal regulator through numerous protein and lipid interactions of its subunits. More recent work demonstrates it is important in additional trafficking steps beyond exocytosis. With links to development and disease, further structural investigation of this protein will expand our understanding of its importance in vesicle trafficking pathways. An overview of our current understanding of the exocyst is articulated before presenting the objectives of this hypothesis-driven research project.

A. HISTORY

The exocyst is composed of eight subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84. In 1980, a famous genetic screen in budding yeast discovered six of the eight subunits by isolating temperature-sensitive secretory (sec) mutants, indicating a step required at the post-Golgi stage of the secretory pathway (1). Four other genes (sec1, 2, 4, and 9) were identified, and since then have been shown to interact with the exocyst subunits to achieve fidelity of the secretory vesicle pathway. In 1996, an epitope-tagged allele for Sec8 purified and identified seven of the eight subunits (2). The complex was called the “exocyst” and a vesicle-tethering protein was born. A later secretory screen in combination with a yeast two-hybrid assay and biochemical purification through
immunoprecipitation and velocity gradients identified Exo84 as a necessary part of exocyst (3).

B. CONSERVATION & ARCHITECTURE

The composition of the exocyst is highly conserved in eukaryotic systems, with eight single copy subunits (4). This has given the opportunity to study various model systems at a multicellular level including mice, Drosophila, and multiple plant species. While the exocyst subunits themselves share little sequence homology, partial crystal structures of four subunits display similar rod-like structures (5). Elucidated crystal structures to date include the near full-length yeast and mouse Exo70 and the C-terminal domains of Drosophila Sec15, yeast Exo84, and yeast Sec6 (6-10).

Each structure contains two or more consecutively packed helical bundles with each bundle containing three to five alpha helices. What is surprising is that- despite their less than 10% sequence identity- each structure has an identical helical-bundle topology, composed of a right-handed mixed parallel/antiparallel motif (5). Each bundle packs together in an end-on arrangement (10). Additionally, although overall helical-bundle topology is conserved, the shapes of the protein surfaces differ considerably in bundle composition (three to five helices with variable length and packing angles) and loop length (10). Moreover, surface distributions of hydrophobic and charged residues are not conserved. These observations suggest unique molecular requirements for protein-protein and in some cases protein-lipid interactions. While structures have yet to be solved for the other four subunits, modeling predictions using hidden Markov Models (11) show a similar tendency for the other subunits to adopt this topologically similar, coiled-coil structure of subunits with known crystal structures. Although it is possible that
convergent evolution brought these subunits to the same structural motif, the identical helical-bundle topology strongly suggests divergent evolution of these subunits from a common ancient ancestor.

The exocyst is a member of the complex associated with tethering containing helical rods (CATCHR) family of multisubunit protein complexes, which exhibit low sequence identity but is a conserved helical bundle structure. This family of proteins is one of two (the other is homodimeric, long coiled-coil proteins) multisubunit tethering complexes (MTCs) involved in vesicle tethering. Two other proteins, the conserved oligomeric Golgi (COG) complex and the Golgi-associated retrograde protein (GARP), are members of the CATCHR family. These proteins are involved in vesicle tethering events at other stages of membrane trafficking and share distant sequence homology (12). Four of the eight subunits of COG share modest sequence similarity to N-terminal regions of Exo84p, Sec5p, and Sec8p (13), and two of the four subunits of the GARP complex contain sequence-related domains to the exocyst and COG domains (13). These findings suggest that the exocyst, COG, and the GARP may have diverged from a common ancestor to mediate vesicle tethering at different vesicle trafficking stages.

Referred to as Sec6/8 in mammalian systems, early studies using rat brain extracts heroically purified an untagged exocyst complex. Characterization using quick-freeze/deep-etch electron microscopy showed not only putative “T/Y” shapes, but also a series of radially symmetric structures (14). These early studies were paramount in elucidating initial structures of what the exocyst may look like. However, only single particle representations were observed and depicted in the reported findings, potentially from not having enough regularity in the structure and/or not enough particles from
artifacts in the prefixation process. Additionally, advancements in electron microscopy may not have enabled the in-depth image-processing tools required to more accurately analyze the micrograph data. Despite the lack of detailed molecular information, structures have remained paramount in thinking about how the subunits assemble into a functional complex.

C. VESICLE TRAFFICKING & LOCALIZATION

Exocytosis marks the final stage of the secretory pathway, one of many trafficking pathways that membrane-enclosed spaces, called vesicles, take in eukaryotic systems. Vesicle-trafficking pathways deliver protein and lipid cargo, evident in growth, division, and organization processes (15), and are essential for membrane remodeling mechanisms in eukaryotes. Membrane trafficking can be divided into four essential steps: vesicle budding, vesicle transport along cytoskeletal tracks, tethering, and vesicle fusion to the target membrane (16). Exocytosis is accomplished in the last two steps of membrane trafficking. A set of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) catalyze the fusion of vesicles to the target membrane. Before fusion can occur, additional proteins must mediate a connection between the vesicle and the target membrane, a process termed “tethering” but also used in conjunction with “docking.” This may be defined as the initial, physical interaction at some distance and perhaps with a degree of flexibility/reversibility between a trafficking vesicle and its target membrane (13, 17). Loss of function studies of the exocyst have shown a continual delivery of secretory vesicles to the plasma membrane (18), yet SNARE complexes do not form and fusion is blocked (19). These studies imply a
function of the Exocyst in providing a physically stabilizing interaction between the vesicle and SNARE fusion proteins on the plasma membrane.

Exocyst subunits are localized to regions of polarized cell-growth in *Saccharomyces cerevisiae*, but appear to arrive at these sites by different mechanisms. Polarized sites are at the tip of the growing bud in daughter cells and the mother-bud neck during cytokinesis (5). One subset (Sec5, 6, 8, 10, 15, Exo84) appears to mobilize in a similar fashion as secretory vesicles to sites of polarized exocytosis by immunofluorescence and fluorescence recovery after photobleaching (FRAP) experiments (20). The other subset (Sec3 and Exo70) appears to localize independently of the cytoskeleton. These findings allude to a mechanism of tethering as dependent upon exocyst complex formation such that tethering proceeds when the first subset arrives to sites marked by the second subset. It remains to be determined whether the first set arrives already assembled and whether the disassembly of the ~850kD complex is required for fusion to occur. Since the exocyst is concentrated in subdomains of the plasma membrane that represent active vesicle fusion and many of its subunits have interactions with proteins and lipids upstream and downstream of vesicle tethering, it is strongly implicated to play an active role in spatiotemporal regulation of vesicle delivery to sites of exocytosis.

As a tethering complex, the exocyst must corroborate interactions between vesicle formation, transport, and fusion, and indeed much evidence suggests the exocyst acts within this context. The current understanding is that at the final stage of vesicle budding from the trans-Golgi, Rab GTPases Ypt31/32 along with phosphatidylinositol 4-phosphate (PI(4)P) recruit Sec2. Sec2, a guanine nucleotide exchange factor (GEF) for
Sec4, mediates a Rab GTPase cascade, recruiting Sec4 and subsequently activating Sec4 through nucleotide exchange for GTP. The unconventional myosin motor, myosin 2 or “Myo2,” is the protein that drives vesicle traffic in transport events on the cytoskeleton around the cell (21). Myo2 is recruited by Ypt31/32 and Sec4, and then at some point Ypt31/32 are released and Sec15 is recruited (21). Activated Sec4 binds to Sec15 and this is thought to facilitate post-Golgi vesicle traffic. Interestingly, the cargo-binding domain (CBD) of Myo2 is similar to subunits of the exocyst and has been shown through GST-tagged pull-downs to interact with both Sec4 and Sec15. This interaction between Myo2 and Ypt31/32 couple may vesicle formation with trafficking while the interactions between Sec4 and Sec15 with Myo2 could integrate vesicle trafficking with tethering in a targeted fashion. It is unclear how the exocyst functions in this process, however, as disruption of exocyst function and assembly by a variety of mutants does not lead to vesicle-delivery defects (1).

Secretory vesicles are targeted to the plasma membrane through the interactions of the exocyst with proteins and lipids at localized sites of membrane expansion. Interactions of Sec3 and Exo70 with Rho GTPases and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) are likely to serve as spatial landmarks for the remaining subunits and vesicles targeted to the plasma membrane. Specifically, the interaction of Rho1 and the pleckstrin homology (PH) domain on the N-terminus of Sec3 increases Sec3 concentrations locally at sites of growth (22). However, studies in which Sec3 lack this N-terminal Rho1-interaction domain showed the remaining exocyst subunits localizing normally and secretion was also normal (23). This shows the remaining exocyst components may polarize by a separate pathway that is independent of both the
N-terminal domain of Sec3 and Rho1 (22). The N-terminal domain of Sec3 also interacts with cell division cycle 42 (Cdc42) and PI(4,5)P2 (24, 25).

Exo70 is an effector for Rho3 and Cdc42, where Rho3 has been known to regulate polarized secretion and the actin cytoskeleton (26). Temperature-sensitive mutants of Rho3 and Cdc42 in yeast showed severe growth and secretion defects without affecting exocyst localization or actin polarization (27, 28). Loss of function Exo70 mutants showed a similar phenotype. Interestingly, rho3/sec3 double mutants showed no defects on growth, polarity, or localization of any exocytic machinery. However, the sec3/cdc42 double mutant was synthetically lethal, implicating functional overlap between Exo70 and Sec3 through Cdc42. Since exo70 loss-of-function mutants alone demonstrated similar phenotypes to cdc42, it is likely that Exo70 is the primary effector for this GTPase. Exo70 also has interactions with PI(4,5)P2 (29). Surprisingly, these mutants can be rescued by GTP-hydrolysis deficient versions of the Rho GTPases, implicating the Rho GTPase cycle is not required for exocyst-specific functions. Small GTPases function through GTP hydrolysis and hydrolysis-independent mechanisms. These findings suggest that Rho GTPases may act through allosteric regulatory events whereby the GTPase activates its binding partner through conformational changes as opposed to a GTP hydrolysis event. It is possible that these interactions function to activate the exocyst to polarized sites, potentially accelerating SNAREpin assembly for vesicle fusion. It is also possible that since Rho GTPase interactions are not solely responsible for exocyst localization, the phospholipid interactions of Exo70 and Sec3 with PI(4,5)P2 may provide the stabilizing interactions to anchor them at sites of marked polarization. And indeed, sequences involved in interacting with this phospholipid are
highly conserved among homologs of either subunit (24, 30), suggesting a necessary stabilizing interaction for exocyst localization. However, other interactions have been implicated to be essential to exocyst polarization, as \textit{sec6} mutants have resulted in mislocalization of all eight subunits that remain fully assembled.

Vesicle fusion happens when SNARE proteins on each membrane come together to form a twisted four-helix bundle that bridges the opposing membranes together in close enough proximity to facilitate lipid-bilayer ruffling (31). As a proposed regulator of SNAREpin assembly, Sec6 has been discovered to have interactions with SNARE proteins involved in the fusion of secretory vesicles to the plasma membrane. Sec6 binds the sec1/Munc18 (SM) cytosolic protein Sec1 (32) and the plasma membrane protein t-SNARE Sec9 on overlapping binding sites that prevent the binding of both Sec1 and Sec9 to Sec6 simultaneously. Sec1 has been known to enhance the Sso1p-Sec9p-Snc2p SNAREpin ternary complex in vivo (31). In the absence of the assembled exocyst complex, Sec6 binds to Sec9, preventing Sec9 from interacting with its syntaxin partner Sso1, thereby halting premature SNAREpin assembly at the plasma membrane. However, upon exocyst formation, Sec9 is released to bind with Sso1 and concomitant recruitment of Sec1 to Sec6 may provide the localization necessary for SNAREpin regulation and promotion by Sec1. Interestingly, Snc2p, the v-SNARE that rides the secretory vesicle to the plasma membrane for subsequent SNAREpin formation and fusion, has been shown to interact with Sec6 (33), providing a link between Sec6 and all of the SNARE machinery involved secretory vesicle fusion. It is possible that in addition to the Sec4-Sec15 interactions for vesicle recognition by the exocyst, Snc2p provides additional targeting and downstream recognition of the exocyst by interacting with Sec6. However,
it is unclear exactly how this molecular recognition cascade could occur, since Sec6 has been shown to localize concomitantly with secretory vesicles to the plasma membrane (presumably in association with Snc2p and or the partially assembled complex), preventing its association with sec9.

D. OTHER FUNCTIONS & LINKS TO DISEASE

The exocyst has been suggested by several studies to be involved in processes beyond exocytosis, where a recurring theme stresses a role in corroborating vesicle delivery to the target membrane. In mammalian cell division, exocyst localization to the midbody suggests a role in cytokinesis to direct and tethers vesicles, leading to new membrane formation to help facilitate abscission (34). Several studies also position the exocyst in areas of membrane expansion during meiosis in budding yeast, suggesting an active role for the exocyst in cell-growth and division (35, 36). In higher eukaryotes, exocyst knockdowns and mutants are linked to developmental defects and have been characterized in mouse, plant, and Drosophila model systems (37-40). It also appears important for endocytic recycling of essential surface proteins from Recycling Endosomes and Early Endosomes back to the plasma membrane (5, 41). For example, the glucose transporter Glut4 in adipocytes require the exocyst to be delivered to the plasma membrane surface when stimulated by insulin (42, 43).

Other membrane expansion processes such as ciliogenesis, tubulogenesis, and cell-migration in mammalian systems require functional exocyst (44-46). Polarized functions in neurons such as neurite-branching and synaptogenesis, but not vesicle delivery to synapses, utilize the exocyst (38). Lateral membrane delivery in epithelial cells (47) and membrane delivery to the base of ciliary bodies appear to utilize the
Three subunits, Sec3, Sec5, and Sec8, have also been implicated in ER inheritance (48). Additional evidence suggests a role for the exocyst in motile processes (49) and autophagosome biogenesis (50). There is no atomic-level, mechanistic understanding yet for the exocyst in these processes but the interactions suggest important clues for roles in vesicle transport up to SNARE complex assembly.

As an active protein in cell growth and cell migration, links have been made to diseased states such as cancer-cell metastasis, neurodegeneration, ciliopathies, and bacterial pathogenesis. In one example, an isoform switching of Exo70 (51) suggest a pathway for epithelial cancers to become motile by variable isoform expression to mesenchymal phenotypes. *Salmonella* highjack the vesicle transport pathway via exocyst machinery for membrane ruffling and macropinocytosis (52). Additionally, the cholera toxin and autosomal-dominant polycystic kidney disease (ADPKD) seems to corroborate malfunctions of the exocyst (53).

### E. TETHERING COMPLEX DIFFICULTY

Vesicle trafficking is an essential mechanism required for secretion, growth, and developmental processes. The exocyst has remained an elusive challenge in the field because of its tendency to fall apart, presumably because of the dynamic role in exocyst assembly and disassembly in order to tether vesicles to the plasma membrane. Other factors such as protease degradation and solubility have also contributed to the difficulty in isolation of the intact complex.

Many of the subunits are readily degraded in the cell, especially the largest subunit Sec3 (2, 54). Degradation is an important cellular process that prevents unnecessary aggregation of proteins, while maintaining their controlled function as
required for cell vitality. It was not until Dr. Munson (our collaborator) and her lab delved into the purification based on cryo-grinding and 11ynabeads utilization that Sec3 was effectively pulled out in an un-proteolyzed state (unpublished data). Other genetically engineered tags have been used, including GST, MBP, and monoclonal antibodies, but have not always been very clean or efficient.

Solubility is also an issue that has prevented isolation of the complex and individual subunits. Insolubility happens for several reasons. The protein may not fold correctly when overexpressed, they may not have the correct post-translational modifications, or they may be insoluble in the absence of cofactors or binding partners (11). Known crystal structures of exocyst subunits show surface distributions of charged and hydrophobic residues, presenting unique molecular surfaces (5). As a result, the absence of specific binding partners can invoke insolubility from the tendency to drive hydrophobic features away from the aqueous cytosolic environment (10). As such, no stable isolation of all eight subunits has been easily accomplished.

F. FRAMING THE PROJECT

This research utilizes the most commonly studied and simplest eukaryotic model organism: *Saccharomyces cerevisiae*. As a multisubunit complex, isolation and purification has proven a challenge in the field. When adequately isolated and purified, common structural methods are a challenge because of the large molecular weight and requirement of large amounts of purified product. Transmission electron microscopy tackles both of these challenges. It does not require large amounts of protein and has a track record suitable for characterization of macromolecular complexes. The current understanding of the exocyst raises several questions regarding the molecular specifics of
its function. The exocyst has a proposed function in vesicle tethering and spatiotemporal regulation of exocytosis, but how is the molecular structure of the exocyst involved? A universal theme is how function fits form. There has been no atomic or molecular level characterization of the fully assembled complex. What does the molecular structure look like? Where are the binding sites for the G-proteins, for lipids on the plasma membrane and vesicles, and for Sec1/Sec9? What are the orientations of the subunits? Answering these fundamental questions all pivot on resolving a high-resolution, atomic structure of the exocyst. I hypothesize that, in the fully assembled complex, Sec15 and Exo70 subunits are structured in the same orientation with their carboxyl termini located at the flexible arms of the implicated “V” shape while Sec3 is in an opposite orientation. Additionally, negatively stained 2-D class averages in this collaborative effort have shown what appear to be either doublets or poorly resolved “V’s.” While it is possible that by mere statistical chance, these may be exocyst doublets, I hypothesize that these poorly resolved 2-D averages are open, “V”-shaped complexes of the single particle exocyst. In order to test these two hypotheses, I propose to spatially locate the carboxyl and amino termini of these subunits relative to the fully assembled complex by negative stain electron microscopy and/or electron cryo-microscopy.

To spatially locate the amino and carboxyl termini of all three subunits, six genetically engineered strains will need to be constructed, each with a GFP or alternate Maltose Binding Protein (MBP) on the terminus in question. A requirement is that the strain is not compromised (i.e. dies) from the potential loss of essential function in the genetic construct. As a negative control, negative stain and/or cryo-electron microscopy of the native, untagged exocyst complex will be undertaken as well. Deletion mapping of
the termini in question may be used additionally as negative controls. If the fusion construct proves fatal for the strain, an alternative approach in amino/carboxyl truncations may be pursued to juxtapose against deletion mapping and the native structural determination. Localization of the Sec15-GFP tag will most readily test the second hypothesis (and part of the first) in the time-constrained endeavor of this undergraduate research project. From truncations, deletions, and fusion constructs, delineating the boundaries of Sec3, Sec15, and Exo70 may be achieved.

G. FUTURE RESEARCH DESIGNS

In an effort to sculpt future endeavors utilizing the isolated exocyst complex, a brief discussion is entertained on other possible in vitro experiments involving physical interactions between the exocyst, known binding partners such as lipids and GTPases, and lipid membranes.

The exocyst is believed to be the initial physical interaction for vesicles to the plasma membrane. If the exocyst is actually a tethering complex as all of the evidence points to, how does the structure of the exocyst tether vesicles to the plasma membrane? Evidence points toward the amino terminal PH domain of Sec3 in binding PI(4,5)P₂ (25) while the carboxyl terminus of Exo70 is implicated to bind to PI(4,5)P₂ (29), yet a molecular understanding remains to be determined for the fully assembled complex in the proposed interactions. How does the exocyst physically engage the plasma membrane and its membrane-associated partners? Are lipids alone sufficient for exocyst localization to the plasma membrane? What lipids do the fully assembled exocyst bind to? And with what affinity does the fully assembled exocyst bind to these lipids? With the exceptional track record of Adam Frost in elucidating membrane dynamics, lipid-based interactions
are an attractive avenue to pursue in answering the last two questions. I hypothesize that the fully assembled exocyst preferentially binds negatively charged phospholipids such as PI(4,5)P₂, PI(3,4,5)P₃, phosphotyidylinositolserine(PS), and phosphotidylglycerol. To test this hypothesis, I propose to purify the intact exocyst for *in vitro* qualitative lipid-binding assays and quantitative lipid-binding assays using model membranes.

To test what lipids the fully assembled exocyst binds to, a PIP-strip protein-lipid overlay assay may be utilized. This assay is generally comprised of a hydrophobic membrane that has been spotted with a specified amount (typically in the pmol range) of biologically relevant lipids, each in a separate well. These include all eight phosphoinositides as well as positively and negatively charged biologically relevant lipids. A specified amount of exocyst may be loaded into each well, incubated for a specific amount of time under appropriate buffer conditions, subsequently washed, then detected through an antibody against the exocyst or alternatively by an affinity tag. As a negative control, a well with no lipids is used while a positive control, a known lipid binding protein such as synaptotagmin 1 for PI(4,5)P₂, is used (56). The advantage of this assay is the rapidity of assessment and range of lipids tapped as potential binding partners for the exocyst. The disadvantage, however, is that false positives may be observed. Repetition of the experiment may help repress the possibility of false positives. This assay will give a qualitative assessment for the lipid types that the fully assembled exocyst interacts with. From this data, candidate lipids may be selected for subsequent quantitative binding experiments.

To test the affinity by which the fully assembled exocyst binds to candidate lipids, cosedimentation or coflotation experiments may be conducted. These commonly use
large unilamellar vesicles (LUVs), synthetically produced with a specified lipid composition and concentration. For the purposes of this experiment, one type of candidate binding lipid is incorporated into a general framework of phospholipids. Both assays involve mixing a known concentration of the exocyst in buffer with LUVs. Cosedimentation assays use high-speed centrifugation to sediment the protein with the lipid while coflotation assays use density-gradient centrifugation to sediment the protein with the lipid. The amount of unbound exocyst and bound exocyst-lipid may be measured and plotted for varied lipid percentages to obtain an apparent $K_d$. If the exocyst has the tendency to oligomerize or aggregate (though no previous indication in the literature suggests such), then coflotation assays may be more advantageous. A drawback from coflotation assays is that a larger quantity of protein is required and is less versatile in certain buffer conditions. Another possible drawback for both assays is that fragmentation or vesicle fission may occur readily with lipid-binding proteins, though no evidence suggests the tendency for the exocyst alone to induce such processes. As a measure of lipid membrane integrity, a small amount of labeled (BODIPY) lipids may be incorporated to make them visible to the naked eye.

As a positive control for cosedimentation/coflotation assays, known lipid binding proteins may be utilized (56). As a negative control, a model membrane without any negatively charged candidate lipids may be used. As a biological control, LUVs with a representative composition of plasma membrane lipids may be used, about 5% PI(4,5)P$_2$ and 20% PS. The experiment can be repeated for varied concentrations of either lipid or protein. Lipid variation is often more advantageous because, in general, it is in such a large excess that it gives more information in determining the $K_d$. Then the concentration
of “bound” protein-lipid must be computed and plotted against free lipid. A hyperbola depicting the degree of interaction can be fitted from multiple data sets (presuming the interaction is not cooperative), where the concentration of the free lipid required to convert half of free protein to the protein-lipid bound state is equal to the $K_d$. Caution should be advised with determining the lipid-protein dissociation constant, as proteins often interact with lipids in a multivalent manner (i.e.- Exo70 and Sec3 can each bind to lipids), and thus only an apparent $K_d$ value may be obtained from the interaction. This will give useful information for the degree to which the fully assembled exocyst preferentially binds one lipid over another.

Studies have shown in vivo that the GTPases Rho1, Rho3, and Cdc42 are important for exocyst localization to the plasma membrane and interact with subunits Sec3 (Rho1, Cdc42) and Exo70 (Rho3, Cdc42). However studies have indicated these localization patterns may be independent of the GTPase hydrolysis function in these proteins (36). Therefore, it would be interesting to examine the binding capabilities of the exocyst complex relative to these GTPases utilizing model membrane systems. In other words, is the exocyst capable of independently targeting to regions of higher GTPase concentrations in their GTP or GDP bound state? Is there a preference for the fully assembled exocyst to bind to one GTPase protein in their GTP or GDP bound state over the other? What is the affinity of fully assembled exocyst to the GTPases? I hypothesize that the assembled exocyst complex binds stronger to Cdc42 in its GTP-bound state over the other GTPases (Rho1, Rho3, Sec4) in either state. In order to test this hypothesis, I will isolate the intact exocyst complex for protein-binding experiments using chemical equilibrium assays with the GTPases in their GTP/GDP bound states (57).
One experiment that can measure the $K_d$ between the fully assembled exocyst and a GTPase, Cdc42 for example, is to use a chemical equilibrium assay that separates the bound complex from the unbound complex without disturbing the equilibrium. While both the exocyst and Cdc42 are known to associate with the plasma membrane, they are readily soluble in solution. Therefore, utilizing a strategy to change the sedimentation coefficient of one of them will allow for an easy separation between bound and unbound states. For example, the exocyst has been tagged with PrA. Incubation with a small amount of Cdc42 and dynabeads followed by pelleting (or using a magnet) allows for the separation between all Cdc42 that’s attached to the exocyst and free Cdc42. Similarly to the PrA construct, a GST tag may be used in similar design with a bead that is nonmagnetic. Measuring the concentration of Cdc42 in the supernatant allows for a calculation by difference to obtain the concentration of exocyst-Cdc42 in the pellet. Free Cdc42 may be measured by SDS-PAGE with coomassie blue staining, quantitative immunoblot, or ELISA if the assays are available. An important negative control is to use the PrA and dynabeads without the exocyst construct. Another important control experiment is for every supposed GTPase, a non-hydrolysable analogue and a hydrolysable form must be used in order to juxtapose the binding affinity between the GTPase in its unhydrolyzable and hydrolyzable forms. As a positive control, free exocyst in a competition experiment must be used to ensure no additional binding is associated with tagged construct. The tagging strategy can also work in reverse to ensure reproducibility of the computed $K_d$ value. The $K_d$ value is computed in similar fashion to lipid-binding experiments, where several trials using different concentrations of one component (Cdc42 for example) is used to generate a hyperbola graph from the data set.
An alternative experiment to measure the $K_d$ value can take advantage of fluorescence anisotropy, where one subunit is tagged with a fluorescent dye such as rhodamine. Fluorescence anisotropy measures the rotational diffusion of a molecule. As the two proteins bind, the larger complex has a lower rotational diffusion coefficient and therefore a larger fluorescence anisotropy. To a low concentration of the tagged protein (Cdc42 for example) is titrated in a range of concentrations of the untagged protein (exocyst). A plot of anisotropy to the free, untagged protein gives a hyperbola similar to the previously proposed experiment. The $K_d$ can be determined from a subtly different set of calculations but is the same correlational strategy in principle. Controls of hydrolyzable and non-hydrolyzable analogues must be used to again juxtapose the two forms of the GTPase in question. Another essential control is to include a competition experiment where free Cdc42 exists in equal concentration to its tagged form, thereby allowing for the determination of affinity measurements of the untagged molecule for its partner.

As a vesicle-tethering complex composed of eight subunits, many studies reveal localization of only one or of a specific few subunits to the plasma membrane. Additionally, difficulty with a stable, isolatable, fully assembled exocyst indicates it is not always fully assembled in the cell. While vesicle tethering remains to be viewed in real time at a molecular/pseudo-atomic scale, the question arises as to when and under what conditions do assembly and disassembly occur? Disassembly is thought to be pertinent to proceeding with vesicle fusion by SNAREs, yet no evidence exists to support this notion. Purification of the fully assembled complex gives an opportunity to work backwards and investigate under what lipid membrane conditions disassembly occurs. Is
it the relative lipid membrane composition (such as the ratio of PI(4,5)P₂ to PI(4)P)? Is it simply the driving force of the membrane fusion or some consequence of interacting with SNAREs or GTPases that tell the exocyst to disassemble? Is it the curvature of the membrane? Is there a physical/shape characteristic of plasma membranes that destabilizes the fully assembled complex? I hypothesize that disassembly of the exocyst occurs readily above a specific curvature of the membrane. In order to test this hypothesis, a way of detecting disassembly will be instigated and used as an indicator for varying membrane curvatures.

An experimental design for testing this hypothesis will have to meet certain criteria defined for “(fully) assembled” or “disassembled.” As experimental design revises, it may be more advantageous to broaden the definitions to a range of assembly/disassembly. A fluorescent quenching strategy between two subunits may be used as an indication of whether they are in a bound, fully assembled state or whether they are disassembled. The warrant is that these two subunits are representative of whether the exocyst is completely assembled or disassembled. Binding predictions can articulate which two subunits may be the weakest or strongest binding partners. A weak binding partner may have the most surface area to the cytosol, being more easily stripped from the complex by solution conditions, or it may be a landmark protein such as Sec15, Sec3, or Exo70. A strong binding protein may take the physical core of the exocyst, buried away from the cytosol. Choosing strong binding pairs may give more information about complete dissolution all subunit/subcomplex interactions while weak-binding pairs will give preliminary insights into the first steps of disassembly. A drawback from this strategy is that the labeling may disrupt exocyst stability. Therefore, for the purposes of
this experiment, labeling must occur on sites near each other and not within the core of the complex. Structural investigation will be advantageous to elucidate candidates more suitable to these requirements. Using a range of synthetic vesicle size or supported lipid bilayers, each of its own specific curvature, fluorescent quenching may be observed in perspective to the physical curvature of the membrane. A simple way to think about this is using three curvatures, one at each extreme (really curved vs. flat/curved opposite). Genetic engineering of a fluorophore on one subunit and a quencher on the other subunit will therefore give more fluorescence upon disassembly of the complex. As a positive control, a construct with no quencher may be used while a negative control may be the absence of the quenching construct. A positive control for membrane curvature binding may utilize a protein of known, specified, higher affinities for specifically curved membranes such as the Bar family of proteins (58). From this experiment, disassembly may be viewed in the light of physical properties of the lipid bilayer.

Through these proposed experiments objectives of analyzing protein/lipid binding interactions and disassembly tendency, anticipated results will either validate or falsify the proposed hypotheses. Efforts to address the first two hypotheses are undertaken in this project while the remaining hypotheses are a direction for future research.

H. TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy has been used as a tool to visualize the substructure of cells and tissues, but improvements in microscopes, specimen-preparation techniques, image-recording procedures, and digital image processing in the last two decades have allowed for advanced pseudo-atomic studies of macromolecular complexes.
While X-ray crystallographic and nuclear magnetic resonance (NMR) spectroscopic methods predominant as the staples of determining atomic-scale, high-resolution structures, they have drawbacks when it comes to larger, asymmetric complexes.

Electron microscopy is more suitable for macromolecular complexes, cellular scaffolds that are essential for a variety of cellular processes such as signal transduction, DNA replication, RNA splicing, and membrane trafficking.

Negative stain microscopy involves the application of a chemical cross-linker to coat the macromolecule of interest on a continuous, copper-based carbon grid. The shape is deduced from stain distribution, which scatters most of the electrons to enhance the normally low-intrinsic contrast of the macromolecule. The process of staining involves the dehydration of the sample by air-drying and may disrupt native conformations. Additionally, high concentrations of heavy-metal salts and pH are also a concern. The methodology has been useful for a range of specimens and is quick, serving as a prelude to cryo-microscopy. Additionally, only surface features are depicted, yielding little information about internal structure.

Cryogenic transmission electron microscopy, commonly referred to as electron cryo-microscopy, preserves the protein in an unstained grid, a technique suitable for high-resolution structure determination. The sample is blotted onto a thin, porous carbon-based grid and submerged in liquid ethane (-180 degrees Celsius) such that the solution freezes in a thin film of ice, preventing physical disruption by ice-crystal formation. The water molecules form an amorphous state, preserving the sample when freezing is fast enough and the grid maintained at liquid nitrogen temperatures (59).
II. EXPERIMENTAL METHODS

This research utilized the most commonly studied and simplest eukaryotic model organism *Saccharomyces cerevisiae*. We have formed an ongoing collaboration with Dr. Mary Munson from UMASS to provide the desired *Saccharomyces cerevisiae* strains and biochemical support for structural investigation experiments. The principally provided strain consisted of two genetically engineered modifications, one Protein-A tag on the carboxyl terminus of one subunit (Sec6) and a green-fluorescent protein (GFP) tag on the carboxyl terminus of another subunit (Sec15). The yeast strains were grown to log phase, harvested, and flash frozen (cryogrinded) in liquid nitrogen using a Planetary Ball Mill (Retsch GmbH, Germany). These were then shipped to the University of Utah along with homemade IgG-conjugated dynabeads (Invitrogen). This project followed a general strategy: isolate the complex and confirm by SDS-PAGE, visualize low-resolution structures by negative stain, then move to cryogenic grid preparation and screening. Once optimized cryogenic grid-preparation can be achieved, a number of grids can be prepared and sent to an off-site facility (more powerful microscope) to collect data for image analysis. Data processing reconstructs a pseudo-atomic structure, where known crystal structures and biochemical data from interacting partners can localize the GFP-tagged subunit within the context of the fully assembled complex.
Figure 1: Strategy for isolation & purification of intact exocyst

A. PURIFICATION

The lysate strain was taken directly from the -80 degrees Celsius freezer and placed in liquid nitrogen to prevent rewarming of the lysate. Both the spatula and eppendorph tube were pre-chilled in liquid nitrogen for the same justification. To the pre-chilled eppendorph tube was weighed 150-600mg of lysate powder depending on desired product quantity. The eppendorph tube was placed immediately over ice after weighing and 15-60\(\mu\)L of 50x protease inhibitor cocktail (PIC, a roche product) added to prevent protein degradation from proteases present in the lysate. The tube was subsequently suspended in 600-900\(\mu\)L of lysis buffer (undisclosed conditions). The tube was then vortexed on high for approximately 30 seconds before centrifuging for 10 minutes at 13.2rpm. The supernatant was drawn into a fresh tube and centrifuged for another 10 minutes at 13.2rpm. This step was repeated. The supernatant from the third spin-down was then added to the prepped dynabead resin.

Dynabead resin was prepped by diluting 5\(\mu\)L of home-made (from Dr. Munson’s Lab) IgG-conjugated Dynabeads in 1mL TBST, pH = 7.68. This mixture was placed onto a magnet to separate the resin from supernatant phases. The supernatant was taken off
and discarded. Two additional washes with 1mL TBST were done in similar fashion, with one last wash in 100μL of TBST. The supernatant from the lysate was added and allowed to incubate at 4 degrees Celsius for one hour. Following incubation, the mixture was placed onto the magnet and the supernatant drawn off. Three wash steps followed with the first two washes consisting of 500μL lysis buffer. The buffer was added to the tube, mixed with the pipet and placed back onto the magnet to allow dynabead/supernatant separation before extracting off and discarding the supernatant. The last wash was done with 250μL lysis buffer with transfer of the mixture to a fresh eppendorph tube before extracting the final wash. The resin was then resuspended in 10-40μL of lysis buffer depending on the prep scale.

To test the expected mass shift of the ProteinA tag before and after proteolytic cleavage, two separate paths were taken from this step. To visualize by SDS-PAGE the protein bands with the ProteinA tag still in tact with the Sec6 subunit (the negative-control for PPX cleavage), 1μL of DTT and an equal volume of 2x SDS loading dye was added to the eppendorph tube, vortexed on medium for a 30 seconds to one minute, spun down at 14k rpm for 30 seconds, and allowed to incubate at 72 degrees Celsius for 10 minutes. The other route involved the addition of 0.4μL-1.6μL PPX for proteolytic cleavage of the ProteinA linker. Incubation on the rocker/spinner was allowed to occur for two hours at 4 degrees Celsius for ProteinA cleavage. A magnet was then used to separate phases and draw off the supernatant into a fresh tube. An equal volume of 2x SDS was added and followed the same vortex, centrifuge, and incubation protocol as the first route. These samples were then loaded onto a Bio-rad gradient or non-gradient (4-12, 4-15, 4-20, 10, and 7% all used) gel with a 3.5-5μL protein ladder loaded into lane
one for reference. The gel ran on a Biorad SDS-PAGE assay at 170V, 30mA, and 5W for 40-80 minutes. The gel was then stained for with coomassie blue stain, warmed in the microwave for 10-15 seconds and agitated for 15-60 minutes before being destained for a similar amount of time. The gel was allowed to resolve on water overnight. Visualization of the gel was done on the GelDoc-IT transilluminator imager and processed using Vision Works software.

Some of the early experiments indicated that the exocyst components were bordering the detection limit of coomassie blue as a staining agent. Initially this was overcome by loading more product per well to achieve detectable measurements. As experiments proceeded, it seemed advantageous to utilize silver staining as a means of reducing product waste and maximizing visualization of subunit bands by SDS-PAGE. Procedure followed fast-protocol for Qiagen’s silver-stain test kit. The gels were again visualized using the GelDoc-IT transilluminator imager and processed using Vision Works software. Silver staining achieves greater sensitivity than coomassie blue, a 50-fold difference on an average basis. However, for cryogenic grid preparation, a coomassie blue detection threshold was desired. This was reasoned because cryogenic microscopy typically has requirements an order of magnitude greater than for negative stain visualization.

B. NEGATIVE STAIN MICROSCOPY

Negative stain grid preparation followed confirmation of the expected mass shift of around 30kDa indicated by SDS-PAGE analysis. Carbon-backed, copper-based negative stain grids (Electron Microscopy Sciences) were glow-discharged on a Pelco easiGlow glow discharger with a 25 second charge preceded by a 10 second wait at a
pressure of 0.39mBar. To a prepped (glow-discharged), continuous carbon-coated grid was added 5µL of product sample (following PrA cleavage step) and allowed to sit for 2-3 minutes. Filter paper was used to wick off remaining liquid from the grid, and the grid was placed over a droplet of water for two-three seconds before being wicked with filter paper once more. To the grid was then added 3.5µL of uranyl acetate and allowed to sit for 20-30 seconds before the remaining liquid was wicked away with filter paper. The grid was allowed to finish air-drying for a few minutes before being placed in a grid box for later visualization on the electron microscope.

Negative stain electron microscopy was performed on one of three electron microscopes based on availability, feasibility, and desired image quality. These microscopes were the TF20 (Tecnai), T12 (Tecnai), and the Jeol 1400 (JEM). Negative stain microscopy followed standard protocols of operation to ensure a well-behaved beam was present and alignments correctly obtained. Visualization using the fluorescent screen ranged from 8,000x mag to 35,000x nominal magnification while visualization using the charge-coupled diode (CCD) camera was conducted at 12,000x up to 62,000x nominal magnification. These images were saved in format appropriate to be processed on electron micrograph program software (ImageJ for raw EM data and RELION and E2boxer for advanced single-particle analysis).

C. CRYO-MICROSCOPY

Upon adequate negative stain visualization, moving to cryo-microscopy was the next step. Grid preparation for cryogenic samples was performed on the Vitrobot (Tecnai). Standard grid-preparation procedures entailed cooling the cryogenic container with liquid nitrogen, making liquid ethane via condensation in the center compartment,
replacing the blotting filter pads, preparing cryogenic grids, insertion of the tweezers (with grid in place) onto the holder, and operation of the Vitrobot computer software. Grid preparation on “holy carbon,” copper-based grids (Electron Microscopy Sciences) followed either glow-discharging in the first attempt or a hydrophobic prep (via chloroform) in the second attempt, presenting different types of physical properties to investigate whether one method was preferred to the other in attempting to localize the exocyst into regions of well-formed, vitreous ice. Once the settings were in order and the specimen loaded onto the grid, an automated blotting and submersion of the grid into the liquid ethane was conducted. Careful handling from then on was maintained to ensure the grid was not compromised. Quick transfer of the grid from liquid ethane to the liquid nitrogen was done before placing the grid in a submersed, four-sample cryogenic grid holder. A plastic top was fastened and tightened over the grid-holder to hold it in place. The grid holder was then quickly transferred with a precooled instrument into the transport vessel (filled again with liquid nitrogen) and placed in a 50mL falcon tube precooled in the transport vessel. These grids were preserved by maintaining them at liquid nitrogen temperatures at all times and transferred down to the USTAR building for subsequent storage and/or taken directly to the TF20 or T12 for visualization.

The grids were kept under liquid nitrogen during all transfers in the loading process. A specialized cryogenic specimen holder for the Tecnai microscopes allowed safe storage of the fragile cryo grids. A pre-bake cycle was run on the cryo-holder to prevent any contamination before being placed in a loading station. The loading station was prepared by adding liquid nitrogen and allowing the apparatus to cool. The grid holder in the 50mL falcon tube was taken from the transport vessel and poured directly
into the loading station. The fastener on the holder was unscrewed and turned to expose the grid. Pre-cooled tweezers were then used to place the grid onto the cryo-holder and a specialized instrument used to fasten a metal ring (equal in circumference to the grid) over the grid before the stage shutter was closed over the specimen (preventing sudden rises in temperature). The cryo-holder was placed into the microscope after appropriate settings and pre-pump vacuum cycles were performed on the microscope.

For electron cryo-microscopy, preserving the grid from radiation damage must be maintained at all possible costs. This was done through a three-step process. In “search” mode, a lower magnification was set to prevent the grid from receiving too much radiation per square angstrom, thereby preserving the vitreous ice from heating to crystalline ice. This mode was used to find areas of interest, regions of well-formed ice on the grid. A “focus” mode permitted an increased magnification on a location away from the area of interest, allowing for proper alignments, astigmatism corrections, and a defocus value to be obtained. An “exposure” mode was then used to capture images in areas of interest- set by a specified degrees and distance from the targeted region- where electron dosage per square Angstrom could be adjusted to capture higher quality EM data. Between each mode the electron beam was blanked (covered by a physical object above the stage of the grid) so as to prevent unnecessary radiation damage from the electron beam.

D. CROSS-LINKING

Initial screening of cryogenic grids indicated no particles of proportional size to the exocyst complex in regions of well-formed, vitreous ice or on the carbon-backbone of the grids from either the glow-discharged or hydrophobic grid-prep methods. These
findings indicated the exocyst may have been liable, or fell apart, when subjected to the vitrification process. From these results, cross-linking experiments were performed as a way to stabilize the octameric complex via glutaraldehyde. This cross-linker forms amide-bonds with amines (namely, lysine) between molecules in close spatial proximity to each other. This served to covalently link the eight subunits together to improve stability for the vitrification process. A standard pull-down of the Sec6PrA/Sec15-GFP strain was performed. At the end of the purification, separate tubes were each tested for cross-linking efficiency based on different concentrations of glutaraldehyde stock, where 1μL was added each tube and allowed to cross-link for ten minutes at room temperature before being quenched with 1μL of 20mM Tris-Cl. As a negative control, a lane for un-cross-linked protein was prepared as well. A gel was performed with cross-linked samples to evaluate the efficiency of exocyst subunit cross-linking.
III. RESULTS & DISCUSSION

A. PURIFICATION

The isolation of the exocyst complex has remained an elusive challenge in biochemistry for decades. For many, the presumption was that it was unstable and disassembled readily. This project utilized combined efforts between the labs of Dr. Mary Munson and Dr. Adam Frost. The key to stable isolation of the complex appears to depend on three main factors: cryogrinding of yeast strains to inhibit subunit proteolysis, use of home-made magnetic IgG Dynabeads (at least an order of magnitude more efficient than commercially available beads) to effectively pull-down all eight subunits, and the optimization of lysis buffer conditions to retain the structural integrity of the complex. These conditions must be met before characterization experiments can be conducted.

Results from the project highlighted the difficulty in exocyst purification, both early on and during cross-linking experiments. While negative stain visualization adequately visualized structures of proper dimensions to be the exocyst, cryo-microscopy did not reproduce the anticipated results. Conditions for cryo and negative stain sample preparation differ considerably, and an effort to stabilize the octameric protein via covalent cross-linking was pursued. Stable isolation of the product again proved difficult, and a compromise between prep procedure and 2-D averaging by negative stain was undertaken. This was in an effort to resolve a lower resolution structure (relative to cryo) of the exocyst in a time-constrained effort to localize the GFP marker relative to the proposed “V”-shape. While data collection for the negatively stained grids was not done in time to include, this paper represents the effort taken up until this point in the project.
The first initial purifications performed at the onset of this project related to the Sec8-PrA strain. Standard pull-down protocol showed no initial results as determined from the lack of protein bands on the coomassie blue stained SDS-PAGE gels (not shown). These results were disappointing but were postulated to be from the lack of pull-down efficiency such as pipetting errors in properly mixing solutions or transferring samples. Additionally, not prepping the dynabeads or keeping the samples over ice may have also likely contributed to the lack of results. The pull-down protocol was revised with two more washes of the initial lysate before incubation to ensure the lysate pellet may not have inadvertently contributed to subunit degradation. With consideration to temperature, dynabead preparation and lysate washes were performed on ice. Careful pipetting was also ensured. This pull-down was performed with the Sec8-PrA strain and the recently received Sec6-PrA/Sec15-GFP strain. Results showed faint bands on the gel (figure 2), indicating that while the pull-down may not have been as good a yield or as pure as desired, it was starting to work.
**Figure 2**: first pull-down with observable protein bands

The second co-immunoprecipitation (referring to the second pull-down of both Sec8-PrA and Sec6-PrA/Sec15-GFP) yielded no results as determined from the lack of protein bands on the coomassie blue stained SDS-PAGE gel (not shown). This was again disappointing but was postulated to be from a lack of efficiently reproducing staining/destaining protocol with use of the coomassie blue stain. Specifically, destaining was performed overnight and quite possibly destained the bands right off the gel. Restaining was performed in hopes of making the bands more detectable, but to no avail. While re-staining is not always as effective as the first round, potentially being a factor, a
lack of efficient yield or simply not enough protein product to visualize may have also contributed to the unsuccessful purification.

Successful pull-down protocol was achieved in the third attempt of pulling down both the Sec8-PrA and Sec6-PrA/Sec15-GFP strains (figure 3). Up until this point, proof of pull-down efficiency was the objective in isolating the exocyst complex. The protein-A linker region was not cleaved since it was unnecessary in the initial proof of concept experiments.

**Figure 3:** First successful purification

As can be seen from the coomassie-blue stained gel, no additional proteins above 50kD were observed while all eight subunits were qualitatively observed in stoichiometric proportion. The smears below 50kD in each lane may have represented non-specific, sub-stoichiometric amounts of proteins, but were not in appreciable enough yield to distinguish the relative molecular weights.
B. NEGATIVE STAIN MICROSCOPY

The next objective was to move to negative stain imaging to ensure the complex was retaining structural integrity. Earlier work from this collaborative effort between labs with a Sec15-PrA strain revealed rod-like structures by negative stain imaging. Particles from the negative stain electron micrographs were taken and class-averaged, generating 2-dimensional pictures of what these particles looked like. To accomplish this, they were sorted into similar classes, where each image within a class set was aligned transnationally (x, y) and rotationally (about the axis perpendicular to the image). Then, each aligned set could be averaged. These class-averages showed the purified exocyst complex as having a “V”-shaped, rod-like structure with dimensions of 10 nm x 28nm. This was used as a baseline for reference when investigating negatively stained grids of the Sec6/Sec15-GFP complex.

![Figure 4: 2-D class averages from the Sec15-PrA strain](image)

Two pull-downs were performed with the Sec6-PrA/Sec15-GFP strain in the first couple of attempts for exocyst complex purification for negative stain grid preparation and visualization. The first attempt failed simply because the product was stored for a week before PPX could be located in time to perform the cleavage (protein stability rapidly degrades over time). The second attempt was performed and from that three grids
were prepared and visualized, to a disappointing result of not being able to see any significant structures under the microscope. It is important to note that these grids were prepared with the purified sample at room-temperature and not washed with water between sample loading and application of uranyl acetate. Additionally, not enough of the product was pulled-down to ensure the integrity of the purification was maintained by SDS-PAGE analysis for that specific purification. Therefore, it could be presumed that either this specific pull-down may have possibly not worked or that, since this grid preparation did not include a wash step, negative stain grid preparation was low quality. This was indicated from large, aggregated uranyl acetate crystals.

In the third attempt for negative stain, a pull-down was performed with enough product to run a SDS-PAGE and to prepare three more negatively stained grids. The SDS-PAGE showed a downward band-shift (less molecular weight) in Sec6 by ~25kD, indicating that the PPX cleavage of the linker region between Sec6 and PrA worked (figure 5). The gel was run such that the 25kD bands were eluted off, running the PPX off the gel. The intention was to separate the bands between Sec8, Sec5, Sec10, Sec6, and Exo84 since their molecular weights are relatively close to each other. Upon negative stain visualization of the grids from this pull-down, many rod-shaped structures were observed in very similar shapes and dimensions to the images previously obtained from the Sec15-PrA strain. These results were promising and indicated the GFP-tagged Sec15 did not appear to compromise the structure of the complex (figure 6), a condition that must be met if this strain is to be a candidate for cryogenic-electron microscopy.
**Figure 5:** Purification with negative stain grid prep

**Figure 6:** Negative stain electron micrograph
C. CRYO-MICROSCOPY

Cryogenic electron microscopy was the next avenue to pursue. A pull-down was done with the Sec6-PrA/Sec15-GFP strain with enough product to run a SDS-PAGE (figure 7) and to prepare six cryo grids. Results yielded no particles of predicted size and shape to exocyst. A second attempt utilizing a different grid-preparation technique (hydrophobic grids) was undertaken as well (figure 8). Again, results yielded no particles of predicted size and shape to the exocyst. These results were disappointing but could have come from several factors. The vitrification process can be highly disruptive to the point that multisubunit complexes may be physically separated from one another. Another possibility is that particles may prefer the carbon-grid to the vitreous ice. However, observations of the cryo-EM data, where the grid bars were within the electron micrograph image taken, showed not indication of this being the case. Additionally, a requirement for cryo-EM typically pertains to an order of magnitude difference in concentration to that for negative stain grid preparation. Therefore, it may be noted that the difficulties in coomassie blue detection of these subunits reflect the challenge for obtaining optimal concentrations. The requirement that product yield be detectable by coomassie blue staining became a concern in subsequent experiments conducted. It may have also simply been poor statistical representation of the grids, a chance of events where there may have been exocyst particles present, but not in the areas chosen of well-formed, vitreous ice.
Figure 7: Silver-stain for first attempt at cryo-microscopy

Figure 8: Second attempt at cryo-microscopy
D. CROSS-LINKING, PURIFICATION, & THE FUTURE

Due to the absence of particles in cryo-microscopy grid screening, a strategy of covalently linking the exocyst complex was pursued. This strategy has been previously used in the Frost lab under a different project, where a year of hard work was wasted to solve a naked, 60s ribosome complex in hopes of elucidating proteins associated in the quality control of translation. In short, all associated proteins that were attempting to be solved dissociated from the complex when subjected to cryo-microscopy characterization. While cryogenic grid preparation can be a step-wise process utilizing different grid properties, buffer conditions, and salt concentrations to optimize variables, a great deal of the process is still largely empirical based on how the protein of interest behaves under the varying conditions. As such, protein complexes can dissociate or fall apart when subjected to the vitrification process. The exocyst, already a difficult protein to purify, was presumed to act in this manner from the lack of any particles in the two screens with cryogenic samples. Cross-linking with glutaraldehyde was pursued as a means of stabilizing the complex for the vitrification process. The strategy of cross-linking uses just enough of a concentration not to cross-link nonspecific proteins but still cross-link the protein in question. Once cross-linking conditions were optimized, verifying by negative stain microscopy ensured the lower-resolution structure, seen previously for the Sec6/Sec15-PrA, was still being maintained. Four experiments were conducted in an effort to optimize these conditions. In the first attempt, a concentration range from 0.0025% to 0.02% was chosen in doubling concentrations (figure 9). While the negative control could be discerned only faintly by coomassie blue staining, protein band intensity could not be discerned for the cross-linked lanes. This could have been
from a lack of pull-down efficiency from those specific pull-down samples and/or from a
detection limit in the stain where a marginal removal of subunits by cross-linking could
have easily removed the amount sufficient to be visualized.

Figure 9: First attempt at cross-linking

The second trial utilized the ease of silver staining in an experiment that showed a
gradual increase in cross-linking efficiency from 0.005% to 0.02% in steps of doubling
concentration (figure 10). Results of cross-linking were observed in the 0.01% and 0.02%
lanes where a gradual disappearance of subunit bands was correlated with an increase in
band intensity just below the loading well. While faint subunits bands could still observed
in all three lanes, it was determined that doing the experiment again would be optimal.
That way, SDS-PAGE with silver staining could observe an upper limit of cross-linking.
Thus, a broader range of cross-linking concentrations was chosen for the third trial.
Figure 10: Second attempt at cross-linking

The third cross-linking experiment was done in a range from 0.01% to 0.04% in steps of 0.01%. Enough product was prepared in order to make three negatively stained grids with a sufficiently cross-linked sample and run a SDS-PAGE (figure 11). Sufficient cross-linking was perceived from the accumulation of a high molecular weight (far above 250kD) smear that did not move down the gel into the wells. Due to the strategy of utilizing just enough glutaraldehyde to cross-link the exocyst subunits to each other, 0.02% was chosen as the concentration of choice. An interesting result from this experiment was that what appeared to be the Sec3 subunit did not cross-link, an observation previously not observed. However, negative stain microscopy showed particles of anticipated dimensions representative of the complex (not shown).
Figure 11: Third attempt at cross-linking.

While the micrographs from the 0.02% grids appeared promising, the indication that Sec3 did not cross-link was a peculiar result. In an effort to verify if the uncross-linked subunit was in fact Sec3, redoing the pull-down procedure was advised. By obtaining a coomassie blue stained gel instead of a silver-stained gel, submission for mass spectrometry may discriminate whether or not the subunit band was actually Sec3. However, Sec3 did not appear to be uncross-linked in the next cross-linking attempt (figures 12 and 13). Additionally, the 0.01% appeared cross-linked while the previous two attempts for 0.01% (figures 10 and 11) were observed as uncross-linked.

The concern with the pull-downs at this point was that the fourth attempt doubled the amount of product per well (relative to the first attempt) while yielding similarly undetectable results of cross-linking lanes by coomassie blue staining. The question of
pull-down efficiency was revisited, and a silver stain was run on the same gel as well as running the dynabeads on a separate gel. Dynabeads showed that around 50% of the complex was still being stuck or conjugated to IgG and the dynabeads (figure 14). The dynabeads had never been run on the gel after the PPX-cleaved elution was taken off, presenting a result not previously observed.

**Figure 12:** Fourth attempt at cross-linking
Figure 13: Fourth attempt at cross-linking, silver-stained.

Figure 14: dynabeads from the fourth cross-linking attempt
To address the concern of pull-down efficiency, the lysate with the Sec15-PrA construct was used as a tester to not unnecessarily waste the GFP-construct. Potential factors that could have contributed to the decrease in yield could’ve stemmed from the denaturation of the IgG-dynabeads or from the PPX. While the pull-down was working to an extent of silver-stain detectability, any subtle denaturation in the IgG could potentially expose a hydrophobic pocket, providing the driving force to make the IgG “sticky,” trapping any nearby molecules such as other IgG antibodies or cleaved/uncleaved exocyst particles. This could have contributed to seclusion of the cleavage sight for PPX. The protease PPX could have also been denaturing to the point of proteolytic inefficiency. To test these possibilities, a pull-down was performed to compare different PPXs (figures 17 and 18) and a different protein construct with the same methodology of PrA and PPX cleavage (figure 20). Additionally, a step-wise digestion was run on a sample to investigate the possibility of cleavage efficiency over time (figures 15 and 16). From these results it can be seen that the non-digested sample appeared by coomassie while the others did not, an observation supporting the notion that while the dynabeads may still be working, the digestion elution was not as efficient as before. Upon silver staining, a gradual decrease in subunit cleavage was observed for the stepwise digestion while the dynabeads were still retaining what appeared to be roughly half of the subunits. This supported the notion that while digestion still worked with subsequent cleavage, the dynabeads appeared “sticky” from still retaining a significant amount of subunits. These observations seemed to support both the possibility of an inaccessible cleavage site or of the exocyst complex being “stuck” to the beads after digestion.
To test further whether the PPX was degrading, a side-by-side pull-down with the PPX and a different protease was used. The other protease, Precision Protease (PP), recognizes and cleaves the same amino acid linker region between the exocyst subunit
and PrA. This experiment tested whether a different protease would cleave with a higher efficiency relative to the hypothesized PPX degradation. A negative control was again used with an undigested sample. Results from coomassie blue indicated observable bands from each of the dynabeads while the elution steps appeared unobservable (figure 17), indicating again an inefficient cleavage from either protease. However, upon silver-stain preparation (figure 18), subunit bands were easily observed in the elution lanes in similar band intensity to that of the dynabeads. From the comparison to band intensity between the two methods, cleavage efficiency indicated 60:40 or 50:50 between subunit retention on the dynabeads and elution of product. PPX appeared to be marginally more efficient than PP, indicating that protease degradation was not occurring at an appreciable rate.

**Figure 17:** PPX vs PP digestion efficiency
Figure 18: PPX vs PP digestion efficiency, silver-stained

In an effort to utilize what time was left for the research endeavor undertaken, the Sec6-PrA/Sec15-GFP was again revisited. This time however, multiple negatively stained grids were prepared for data collection and image analysis. A silver-stained SDS-PAGE was run as well to ensure the pull-down worked (figure 19). Since pull-down optimization was not fully remedied to the extent of reproducing coomassie blue detectable gels, electron cryo-microscopy with cross-linked (or uncross-linked) exocyst particles was left as an avenue to revisit in post-bachelor studies.
Figure 19: silver-stained gel for negative stain grid prep and data collection

Figure 20: silver-stained gel for negative stain grid prep and data collection. Also assessed relative cleavage efficiency of exocyst-PrA construct relative to another construct.
In addition, a second pull-down was performed to prepare six more negatively stained grids to obtain a larger pool of potential “candidate” grids. This was undertaken because of slightly less ideal grid preparation techniques from the previous attempt, specifically relating to accidentally letting some grids fall on the counter (causing potential contamination), bending the grids, and mishandling that may have led to the sample being washed off. Along with the silver-stained SDS-PAGE, a sample from another lab member using the same dynabeads-PrA pull-down strategy loaded their respective sample (figure 20, shown on the right-hand side, lanes 7 and 8). This result showed a more efficient cleavage using with the same exact pull-down methodology and reagents. The construct for the exocyst-PrA tag was cleaved less efficiently (50:50) while the other construct was cleaved far more efficiently (90:10 or 80:20). This result indicated that the Sec6-PrA cleavage site was less accessible than the cleavage site for the other construct. It may have been a result from hydrophobic residues or binding pockets on the exocyst (such as the PH on Sec3) that prevented accessibility for the PPX to cleave its recognition sequence. Or it may have been just the relative bulkiness of the ~850kD exocyst on the cleavage site. From all attempts at optimizing and trouble-shooting the purification, the exocyst pull-down appeared “half” efficient at best.
CONCLUDING REMARKS

The exocyst has been structurally mischievous. While the evidence is consistent with the exocyst in vesicle tethering and spatiotemporal role in exocytosis, a molecular understanding of how the tethering takes place remains to be shown. This research effort focused on the difficulties of structural characterization that have before prevented the study of the exocyst at a high-resolution, atomic scale. Purification of the intact complex was overcome and a “V”-shaped, rod-like structures were observed by negative stain microscopy. While initial efforts with cryo-microscopy were unpromising, more attempts will be required to confirm the exocyst is indeed labile under vitrification conditions, whether it is cross-linked or not. With continued efforts utilizing electron microscopy and \textit{in vitro} lipid and protein binding assays, structural characterization and tethering dynamics of the exocyst will allow a more comprehensive understanding of its essential role as a vesicle tethering complex.

\textbf{Figure 21}: Negative stain micrograph of grid chosen for data collection.
IV. REFERENCES


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