

Investigating the Potential Sec6p/Sec9p Binding Site

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ABSTRACT

The interaction of the exocyst complex and SNARE proteins is essential in exocytosis, specifically to the tethering and fusion of secretory vesicles at the plasma membrane. While the specific mechanisms of vesicle trafficking are largely unknown, direct interactions have been found between the exocyst subunit Sec6p and the t-SNARE Sec9p. To investigate a possible binding site for the two proteins, a recombinant *S. cerevisiae* Sec9p mutant, designed to disrupt binding with Sec6p, was cloned, expressed in *E. coli*, and purified. Binding studies, including gel filtration and gel shift assays, were conducted. The results indicate that the mutations tested did not disrupt binding of the proteins of interest, so likely the mutagenized sites are not strongly involved in binding. Future work should include examination of other possible binding sites in Sec9p.

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BACKGROUND

Exocytosis

Exocytosis is an essential cellular function in which the cell secretes membrane-bound vesicles into the extracellular environment. Exocytosis (**Figure 1**) is necessary for the export or delivery of various molecules, including waste products, components of the extracellular matrix, various proteins (antibodies, enzymes, etc.), hormones and other lipids, and integral membrane proteins. Exocytosis is also employed to deliver and present antigens on the surface of the plasma membrane during an immune response. Products destined for exocytosis are bound by vesicles pinched off from various organelles, including the endosomes, endoplasmic reticulum, Golgi apparatus, and lysosomes. The vesicles are trafficked to sites of secretion along the cytoskeleton or microtubules of the cell to their target destinations. Interestingly, despite the necessity of this process in numerous cellular activities, the regulation and mechanisms of exocytosis remain largely unknown.

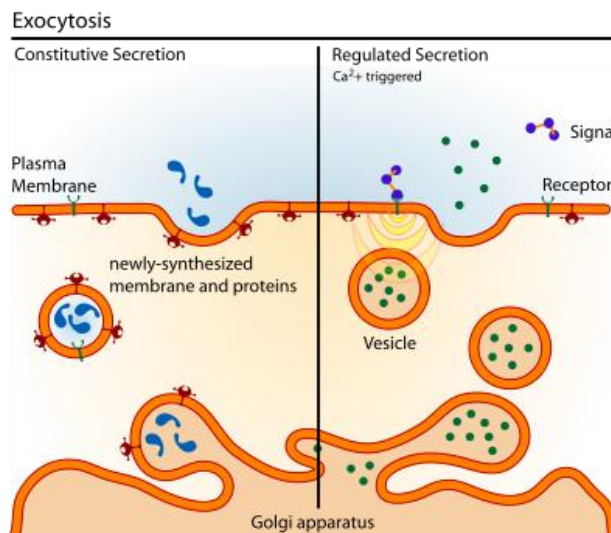


Figure 1. Diagram of Exocytosis. The left section of the diagram denotes constitutive secretion, while the right section shows secretion triggered by calcium influx.
<http://cellbiology.med.unsw.edu.au/units/science/lecture0805.htm>

Polarized Exocytosis

Polarity is a vital element of cellular functions, from embryogenesis through apoptosis. Cell division, a highly regulated and polarized process, is necessary for embryonic development, cytokinesis, chromosome segregation, membrane growth, secretion activities, and cell fate determination. Exocytosis is also highly regulated and carefully polarized, as the trafficking of cargo to specific determinate sites is essential for cell survival. Examples of polarized exocytosis abound within the human body; correct polarization is essential for the trafficking of synaptic vesicles in the nervous system (**Figure 2**), or the secretion of waste products (in the form of sweat) from epithelial cells, or the secretion of gastric enzymes in the stomach, among other processes.

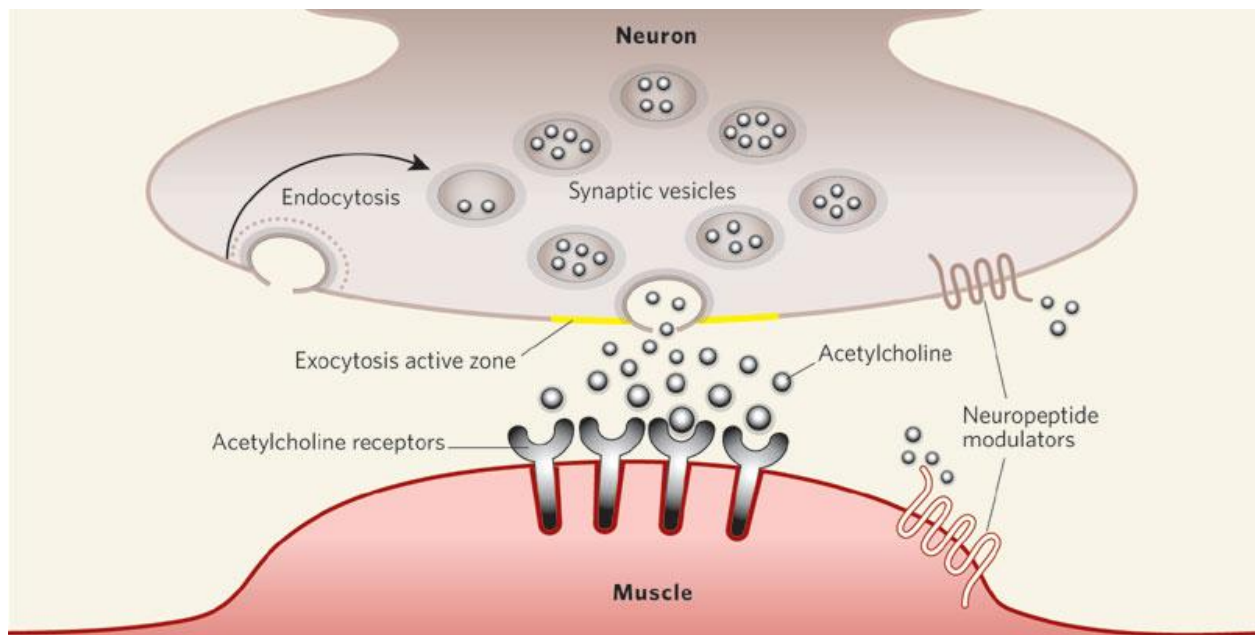


Figure 2: Vesicle Trafficking at the Neuronal Synapse. Diagram shows the exocytosis process used by neuro-muscular pre-synaptic neurons to release vesicles containing acetylcholine into the synaptic cleft. (Bargmann, 2005)

Polarized exocytosis occurs in four distinct stages: trafficking, tethering, docking, and fusion. Trafficking involves the physical, directed movement of the vesicle from the organelle of origin to the site of secretion along microtubules or the cytoskeleton. This project focuses on the trafficking of vesicles from the Golgi apparatus along the actin cytoskeleton. The budding yeast *Saccharomyces cerevisiae* is used as a model experimental system.

Yeast as a Model System

Yeast is a particularly attractive model system for the study of exocytic regulation. This organism is the simplest eukaryote, and was the first to offer a completely sequenced genome (Dujon, 1996). Analysis of this completed genome has determined that *S. cerevisiae*, also known as budding yeast, contains significantly less non-coding DNA regions than higher eukaryotes. Higher eukaryotes often possess genomes containing complex regulatory elements that render genetic manipulation and analysis difficult. Yeast offer numerous advantages to other available model systems, including rapid growth, simple culture requirements, well-dispersed cells, ease of cell cycle regulation and synchronization, low cost of maintenance, and ease of genetic manipulation.

Additionally, *S. cerevisiae* exhibits polarized growth, a characteristic that makes this organism useful for the study of polarized cellular processes (including exocytosis). This particular species of yeast buds in only one direction (**Figure 3**), as cell growth and secretion are localized to the bud tip only. Further, the actin cytoskeleton of budding yeast is highly polarized, running from the mother cell to the bud tip, parallel to the mother-bud axis (Pruyne et al., 1998). The class V myosin protein Myo2p serves as the ‘motor’ that traffics vesicles along the actin filaments to sites of secretion. Myo2p, coupled with the Rab protein Sec4p, facilitates the targeted movement and delivery of secretory vesicles from the mother body to the daughter bud

tip (Pruyne et al., 1998; Karpova et al, 2000). These characteristics make *S. cerevisiae* particularly useful for *in vivo* studies of polarized exocytosis. Vesicle delivery is easily visualized using fluorescence microscopy or similar techniques. Further, yeast proteins may be recombinantly expressed in other systems (i.e. *Escherichia coli*), thereby aiding *in vitro* studies of exocytosis.

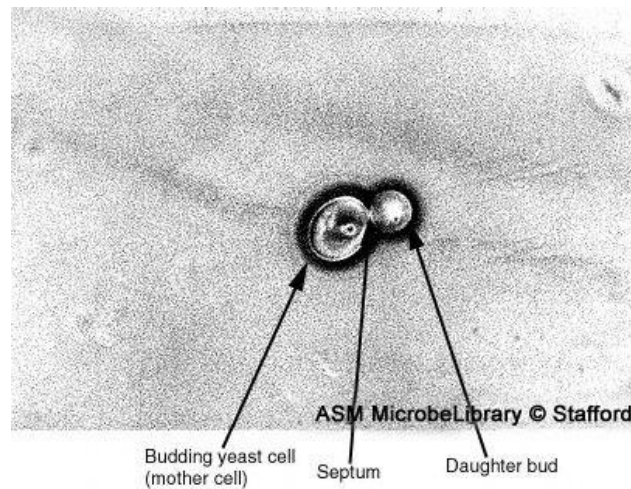


Figure 3: Photograph of the Budding of *Sacchomyces cerevisiae* as a Model of Polarized Exocytosis.
<http://pathmicro.med.sc.edu/mycology/yeast2.jpg>

The Exocyst Complex

Vesicles are trafficked to sites of secretion along the cytoskeleton , and are ‘tethered’ to the plasma membrane by the multi-subunit exocyst complex (**Figure 4**). Tethering binds the vesicle in close enough proximity to the plasma membrane for SNARE complexes to form between SNARE proteins on the vesicle and the plasma membrane. As the SNARE complexes form, the two membranes are brought together to allow membrane fusion to take place.

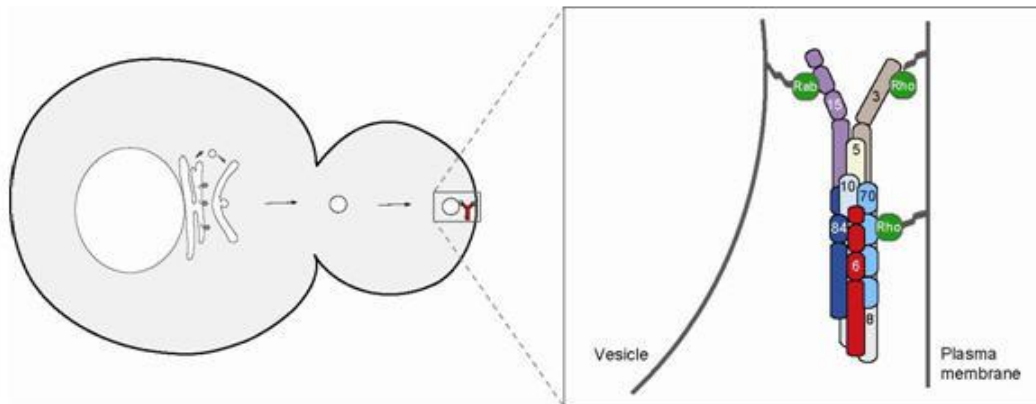


Figure 4: The Exocyst Complex in *S. cerevisiae*. The upper left diagram shows the directional budding of the yeast, with a vesicle targeted from the golgi apparatus to the cytoplasmic membrane within the bud. The upper right diagram shows the exocyst complex tethering the vesicle to the membrane. The lower diagram shows a closeup of the exocyst complex and its various proteins. Diagram courtesy of the Munson lab.

www.umassmed.edu/faculty/graphics/208/Figure1.jpg

The exocyst complex is an octameric protein complex conserved in all eukaryotes, and is essential in the tethering required for exocytosis and membrane growth. The complex is involved

in trans-Golgi network trafficking to the plasma membrane, and is composed of the subunits Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p (shown in the figure, lower panel). These subunits appear to traffic to sites of secretion via secretory vesicles; at these sites, the complex tethers vesicles to the plasma membrane in preparation for membrane fusion. Crystallographic studies of four exocytic subunits (Sec15p, Exo70p, Exo84p, Sec6p) indicate that the subunits are rod-like helical bundle structures (Sivaram et al., 2006; Munson & Novick, 2006).

SNARE Proteins

Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins are essential players in intracellular trafficking events. The SNARE proteins, found on the vesicle (v-SNAREs) and target membranes (t-SNAREs) in various pathways, these proteins assemble complexes necessary for subsequent membrane fusion. Most SNAREs are anchored to their respective membranes via C-terminal transmembrane domains (Sutton et al., 1998).

SNARE complexes are formed by the specific interactions of v-SNAREs and t-SNAREs. Each SNARE complex is a parallel four-helix bundle (Sutton et al., 1998); the specificity of bundle assembly is dictated by the SNARE motif of each protein in the complex. The SNARE motif, a homologous 60-70 amino acid region forming a coiled coil domain, is responsible for the specificity of SNARE complex assembly via cognate protein pairing (Paumet et al., 2004). In the yeast trans-Golgi network (**Figure 5**), the SNARE complex is made up of the t-SNAREs Sso1p (red) and Sec9p (blue), both localized to the plasma membrane, and the v-SNARE Snc2p (green), which is localized to the vesicle membrane.

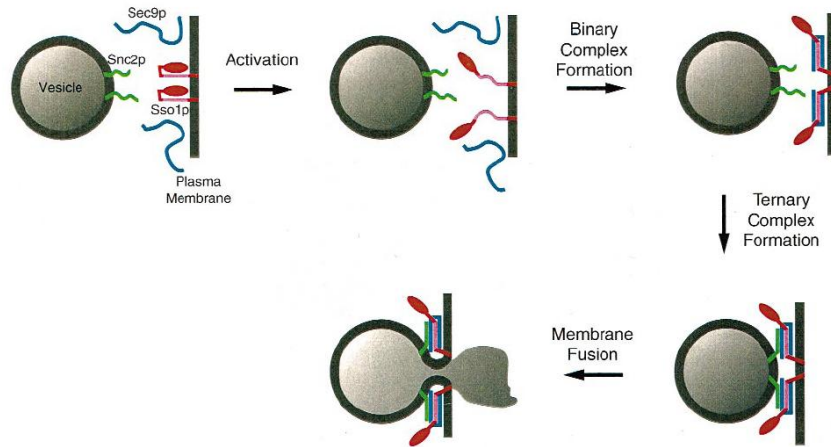


Figure 5: SNARE Complex Assembly in *S. cerevisiae*. Figure from Nicholson et al., 1998.

Sso1p and Snc2p each contribute one SNARE motif to the complex; the t-SNARE Sec9p contributes the two remaining motifs. SNARE proteins, like the exocyst, are conserved trafficking structures in eukaryotes. Sso1p, Snc2p, and Sec9p are homologous to the human SNAREs Synaptobrevin-II, Syntaxin-1A, and SNAP-25B, respectively (**Figure 6**).

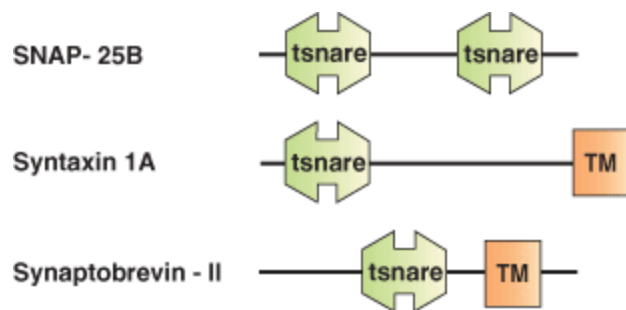


Figure 6: Domains of Human SNARE Proteins.
<http://www.cellsignal.com/reference/domain/snare.html>

Interactions Between Sec6p and Sec9p

The exocyst subunit Sec6p is a predominantly helical, 88kD protein. Various mutant strains of this protein in *S. cerevisiae* exhibit the accumulation of secretory vesicles at the bud tip

at the restrictive temperature (37°C), indicating that the subunit is essential for the targeting of vesicles to sites of secretion (St. Pierre et al., 1994). Recent studies (Songer & Munson, 2009) implicate Sec6 in the polarization of the other exocyst subunits to sites of secretion. This more recent work (**Figure 7**) found that patch mutants exhibited temperature sensitive growth. Interestingly, vesicles were still correctly trafficked to the plasma membrane; however, the *intact* exocyst complex was mislocalized.

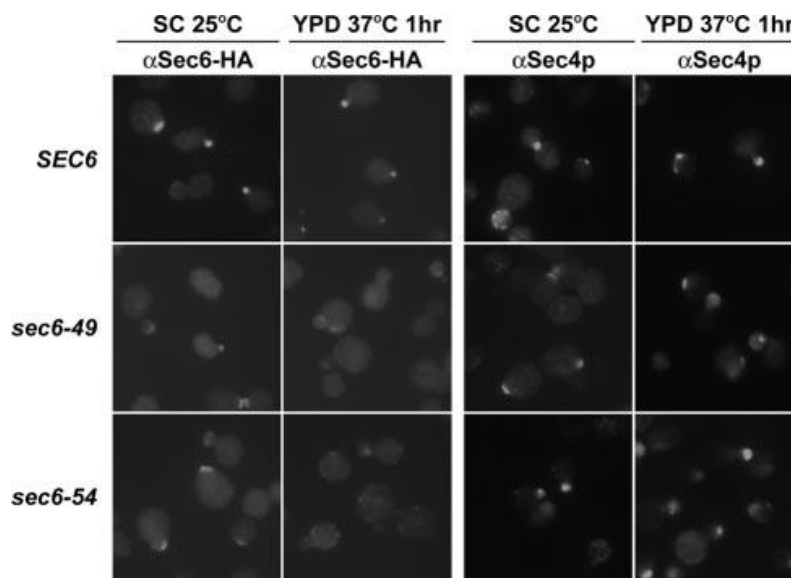


Figure 7. Patch mutations in Sec6 result in proper vesicle trafficking, but mislocalization of the exocyst complex, at 37°C. Songer & Munson, 2008

The t-SNARE Sec9p, unlike other t-SNAREs, is not localized to sites of secretion (Brennwald et al., 1994). The C-terminal region of Sec9p (residues 416-651), homologous to human SNAP-25, has been used for Sec9p binding analysis in previous work (Sivaram et al., 2005), and has been shown to have fully functional characteristics *in vivo* (Brennwald et al,

1994). This C-terminal construct, called Sec9CT, was used in this study to examine the potential Sec6p binding site in Sec9p.

Previous work (Sivaram et al., 2005; Sivaram et al., 2006) established a direct interaction between the exocyst subunit Sec6p and the t-SNARE Sec9p. This published work characterized the structure of Sec6p, and established that the protein's interaction with Sec9p inhibits the ternary complex assembly of the t-SNARE protein and its partner t-SNARE Sso1p. However, studies have not yet determined the Sec9p region responsible for Sec6p binding.

PROJECT PURPOSE

The main goal of this project was to elucidate the region of the *S. cerevisiae* t-SNARE Sec9p responsible for the protein's binding to the exocyst subunit Sec6p. A mutant Sec9 construct (9KAKR) was cloned, expressed in *E. coli*, and purified in order to perform *in vitro* binding studies of the two proteins. The binding of Sec9p (and the 9KAKR mutant) to its partner t-SNARE, Sso1p, was also examined to ensure that the mutations made in Sec9p did not disrupt any other binding site.

METHODS

Cloning

To clone the Sec9 mutant necessary for this investigation, a truncated construct of Sec9p containing a C-terminal fragment (known as Sec9CT) was used. Prior data gathered from Sec6/Sec9 cross-linking experiments (personal communication) suggested that the peptide QRKNVLEKAKRYQ in Sec9CT contained residues possibly essential for the binding to Sec6. A 'patch' of mutations in the KAKR peptide was determined to be a starting point for this project's investigation. The residues in this 4-amino acid peptide (lysine, alanine, lysine, and arginine) were to be mutated to four alanine residues, as alanine is an uncharged amino acid, rendering the mutations far less likely to disturb the native structure of the Sec9CT protein.

To create the mutant Sec9KAKR-AAAA (herein referenced as 9KAKR), two cloning primers were created:

Forward primer: 9KAKR-AAAA-F

5' GGAAGAATGTTCTAGAAGCGGCAGCGGCATATCAGTTTGAG 3'

Reverse primer: 9KAKR-AAAA-R

5' CTCAAACCTGATATGCCGCTGCCGCTTCTAGAACATTC 3'

Plasmid pLM-1 was selected to be the cloning vector for the 9KAKR mutant. The mutant was cloned into the vector in the EcoRI and BamHI restriction sites, using the following primers:

5' (EcoRI) primer: E-sec9ct

5' CGGAATTCTAAGGAGGATATTAATGG 3'

3' (BamHI) primer: sec9ct-B

5' CGGGATCCCTATCTGATACCTGCC 3'

To ensure that proper cloning occurred, mutant DNA samples were sent for sequencing using the commercially available SP6 and T7 primers:

SP6 sequencing primer
5' ATTTAGGTGACACTATAG 3'

T7 sequencing primer
5' TAATACGACTCACTATAGGG 3'

Expression Trials

Expression trials were necessary to determine if the 9KAKR mutant could be expressed in sufficient levels for protein purification. A volume of 1 μ L of the 9KAKR DNA was transformed into 100 μ L of BL21 (DE3) Codon Plus competent *E. coli* cells, and incubated on ice for 1 hour. Cells were plated on LB (plus 1X carbenicillin and 1X chloramphenicol) plates, and grown overnight at 37°C. After overnight incubation, the cells were scraped from the plates into two 50mL starter cultures of LB media containing 1X ampicillin and 1X chloramphenicol. The starter cultures were grown at 37°C, shaking at 200 rpm, until they reached an OD₆₀₀ of 0.6. At this density, 50 μ L samples were taken for later gel analysis; the samples were spun down in a cold room centrifuge (13,200 rpm at 4°C), the pellets resuspended in 30 μ L of 1X loading dye, and then boiled at 95°C for 5 minutes. The samples were stored at -20°C until gel analysis was performed. The starter cultures were inoculated with 0.1 mM and 0.3 mM (respectively) final concentrations of isopropyl β -D-1-thiogalactopyranoside (IPTG) to induce protein expression, and grown for 3 hours at 37°C. Post-induction samples were taken and prepared similarly to the pre-induction samples; 7 μ L samples from both trial cultures were run on 15% SDS-PAGE gels. The gels were then stained with Coomassie Blue to visualize the protein in each sample to determine if the 9KAKR mutant expressed in levels similar to Sec9CT.

Growth and Purification of 9KAKR Protein

To obtain purified 9KAKR protein, 1 μ L of DNA was transformed into BL21 (DE3) Codon Plus competent cells, and allowed to incubate on ice for 1 hour. Cells were plated on LB (plus 1X carbenicillin and 1X chloramphenicol) plates, and grown overnight at 37°C. Cells were scraped into a 200 mL starter culture of LB media (plus 1X carbenicillin and 1X chloramphenicol), and grown at 37°C shaking at 200 rpm to an OD₆₀₀ of 1.0. Six liters of LB media (plus 1X carbenicillin) were inoculated, in equal volumes, with the starter culture, and allowed to grow to an OD₆₀₀ of ~1. Cultures were induced at a final concentration of 0.1 mM IPTG, and grown another 3 hours at 37°C. Cells were harvested by spinning at 4°C, 5000 rpm in an Evolution centrifuge, scraping into appropriately sized conical tubes, and stored at -80°C.

Cells were resuspended in cold Tris lysis buffer (pH 8), lysed in a cell disupter at 80 psi, and spun for 15 minute minutes at 5000 rpm in an Evolution centrifuge to pellet cell debris. The supernatant was removed, and placed into a glass beaker surrounded by ice. The supernatant was stirred slowly while approximately 5 mL of a 10% polyethyleneimine (PEI) solution was added; the PEI served to precipitate the nucleic acids remaining in the supernatant. An additional 100 μ L PMSF was added to the supernatant after the precipitation of nucleic acids was complete; the supernatant was spun as above to pellet the precipitated nucleic acids. After spinning, an additional 100 μ L PMSF was again added. The supernatant (containing proteins) was filtered to remove any remaining particulates.

The supernatant was loaded onto an 85 mL column volume Q Sepharose column pre-equilibrated in a 10 mM Tris, pH 8, 50 mM NaCl buffer. A secondary buffer (Buffer B: 10 mM Tris, pH 8; 1M NaCl) was also used to create a linear elution gradient. The protein was eluted from the column in 9 mL fractions over a 5-50% buffer B gradient, following a 2 column volume

(~170 mL) column wash. Samples from the chromatograph peaks were run on 15% polyacrylamide SDS-PAGE gels and stained with Coomassie Blue; those samples containing protein of the approximate correct size were pooled.

To continue the purification process, ammonium sulfate was added to an 800 mM final concentration to the pooled solution. The solution was then loaded onto a pre-equilibrated 35 mL phenyl sepharose column; the column was pre-equilibrated in a 43% buffer of 50 mM sodium phosphate, pH 7 ('Buffer B'), and 53% Buffer A (50 mM sodium phosphate, pH 7; 1.4M ammonium phosphate). The column was eluted in 5 mL fractions over a 10 column volume (~350 mL) gradient from 43-100% Buffer B, following a 2 column volume (~70 mL) wash at 43% Buffer B. Samples from the chromatograph peaks were run on 15% polyacrylamide SDS-PAGE gels and stained with Coomassie Blue; those samples containing protein of the approximate correct size were pooled.

The volume of pooled fractions totaled approximately 55 mL; this was concentrated to 8 mL using a nitrogen spin concentrator, then brought to a total final volume of 50 mL with a 10 mM HEPES buffer (pH 7.5). The volume of sample was then loaded onto a MonoQ 10/10 column pre-equilibrated in 10 mM HEPES buffer (pH 7.5). A secondary buffer ('Buffer B') was also used (10 mM HEPES, pH 7.5; 1M NaCl) to create a linear elution gradient. The column was washed with 2 column volumes of buffer at 5% Buffer B, then eluted over a 20 column volume gradient from 5-50% Buffer B. Fractions were collected throughout (9 mL each during wash, and 2 mL each during elution). Samples from the chromatograph peaks were run on 15% polyacrylamide SDS-PAGE gels and stained with Coomassie Blue; those samples containing protein of the approximate correct size were pooled.

The pooled sample volume (approximately 39 mL) were concentrated with a nitrogen spin concentrator, and exchanged to potassium phosphate buffer (10 mM K_2PO_4 , 10 mM KH_2PO_4 , 140 mM KCl; pH 7.4) to a final volume of ~2.5 mL. The protein was then divided into 150 μ L aliquots and flash frozen in liquid nitrogen. The protein aliquots were stored at $-80^\circ C$ until use.

Growth and Purification of Sec6N Protein

BL21 (DE3) competent cells were transformed with 1 μ L each of Sec6N DNA, incubated on ice for ~20 minutes, and plated on LB (plus 1X carbenicillin) plates. The plates were incubated at $37^\circ C$ overnight. After overnight incubation, colonies were scraped into a starter culture of LB media (plus 1X ampicillin), and placed in an incubator shaking at 200 rpm for 1 hour at $37^\circ C$. At an OD_{600} of ~1, the starter culture was evenly divided into six 1-liter bottles of LB media (plus 1X ampicillin), and incubated at $37^\circ C$, shaking at 200 rpm, until the OD_{600} reached 0.4. At this point, flasks were moved to $20^\circ C$ and shaken at 200 rpm until the OD_{600} reached 0.8. The cultures were then induced with 0.1 mM IPTG (final concentration), and incubated at the same conditions for 3 hours. The cultures were spun down in an RC-3C centrifuge ($4^\circ C$, 5000 rpm for 10 minutes); the supernatant was poured off, and the cells were scraped and frozen at $-80^\circ C$ until purification was performed.

The cells were resuspended in approximately 300 mL of cold His lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole; pH 8. Fresh leupeptin, pepstatin, 5 mM (final) B-mercaptoethanol, 1 mM (final) PMSF were added to the buffer just prior to cell lysis.). Cells were lysed using a cell disrupter at 80 psi, and spun for 30 minutes at 13000 rpm, $4^\circ C$, in an Evolution centrifuge. The supernatant was added to 6 mL of an Ni-NTA agarose bead slurry pre-

equilibrated in His lysis buffer; beads were rocked on a Nutator at 4°C for 1 hour to allow binding of the His-tagged Sec6 protein to the beads. Beads were then spun at 800 rpm at 4°C for 5 minutes, resuspended in a small volume of HIS lysis buffer, and poured into a BIO-RAD column to create a column bed. The bed volume of beads was then washed three times with a total of 18 mL of His wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole; pH 8. A fresh 5 mM final concentration of β-mercaptoethanol was added to the buffer just prior to washing). The beads were eluted with ten 3mL aliquots of His elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole; pH 8. Fresh β-mercaptoethanol was again added just prior to elution) at 4°C. Each aliquot was collected as a separate fraction, and 5 μL of each was spotted onto Whatman paper; the Whatman paper was then stained with Coomassie Blue to determine which fractions contained protein.

The fractions containing protein were pooled, filtered for aggregates/particulate, and diluted to 50 mM NaCl. This entire sample was loaded onto a MonoQ 10/10 column preequilibrated in 20% Buffer B (10 mM Tris, pH 8; 1M NaCl; 1 mM DTT). The column was eluted over a 20-60% gradient of Buffer B (where Buffer A was: 10 mM Tris, pH 8; 1 mM DTT) over 20 column volumes, with 0.5 mL fractions collected throughout. Fractions corresponding to chromatograph peaks indicated the elution of various proteins; samples from those fractions were run on 12% SDS-PAGE gels, and stained with Coomassie Blue.

Those fractions containing protein were pooled, and loaded onto a Superose 200 16/60 preparation-grade column (GE Healthcare) that was pre-equilibrated in potassium phosphate buffer (10 mM K₂PO₄, 10 mM KH₂PO₄, 140 mM KCl; pH 7.4. 1 mM DTT was added fresh to the buffer before column equilibration.). The column was eluted in 1mL fractions with potassium phosphate buffer. Samples from the fractions corresponding to chromatograph peaks were again

run on 12% SDS-PAGE gels. Those fractions corresponding to the purest Sec6 samples were pooled, yielding approximately 14.5 mL of sample. Using a spin nitrogen concentrator pre-equilibrated with potassium phosphate buffer, this sample was concentrated down to approximately 0.85 mL in volume. Glycerol was added to the concentrated protein to a 10% final volume, along with a 1 mM final concentration of DTT; the protein was then divided into 50 μ L and 100 μ L aliquots, and flash frozen in liquid nitrogen. The protein aliquots were stored at -80°C until use.

Determination of Protein Concentration: Ninhydrin Assay

A ninhydrin assay was used to quantitatively determine the final concentration of the proteins used for this project. A 10 mM leucine standard was prepared, and an aliquot of the protein sample to be tested was thawed out and kept cold on ice. A series of dilutions (0 μ L, 5 μ L, 7.5 μ L, 10 μ L, 12.5 μ L, 20 μ L) of the leucine standard was set up to create a standard curve for comparison; the same series of dilutions was used for the protein sample. A sample of 5 μ L of filtered potassium phosphate buffer (10 mM K_2PO_4 , 10 mM KH_2PO_4 , 140 mM KCl; pH 7.4) was used for the 0 μ L samples. These samples were added to polypropylene tubes containing 0.15 mL 13N NaOH; the tubes were then covered with aluminum foil and autoclaved on the liquid cycle to hydrolyze the protein to amino acids. After autoclaving, the tubes were allowed to cool to room temperature before the addition of 0.25 mL glacial acetic acid, which was used to neutralize the hydrolysis reaction.

A 0.4 mL volume of CN-ninhydrin solution was added to each reaction; the tubes were then loosely capped and placed in a water bath, where they were allowed to boil for exactly 15 minutes. Immediately after boiling, 2mL of 50% isopropanol was added to each reaction. Each

tube was then tightly capped and shaken, then allowed to again cool to room temperature. The A_{570} of each sample was taken using a UV spectrophotometer, and a standard curve was created for each sample. The curve for the 10 mM leucine sample served as the standard/reference for the protein sample curve; the concentration of the protein sample in question was determined using the following formulas:

$$\frac{(10\text{mM})(\text{slope of sample curve})}{(\text{slope of leucine standard curve})} = \text{mM of amino acids}$$

$$\frac{\text{mM of amino acids}}{\# \text{ amino acids in protein sample}} = \text{mM of protein in sample}$$

Binding Assay I: Gel Filtration

In order to quantitatively determine the Sec6/Sec9 interactions for this project, gel filtration runs of the proteins and their complexes were run using a Superose 200 10/30 analytical column (GE Healthcare). 150 μL samples were prepared for each protein, each at a 10 μM concentration per protein in the sample, in potassium phosphate buffer (10 mM K_2PO_4 , 10 mM KH_2PO_4 , 140 mM KCl ; pH 7.4). Complex samples were incubated at 18°C for the time period desired for binding examination, and the column pre-equilibrated in potassium phosphate buffer. Samples were spun at 13,200 rpm in a cold room centrifuge (4°C) to pellet any precipitation or contaminants, then injected using a sterile 1mL syringe into a 100 μL loading loop. Samples were loaded onto the gel filtration column using a 100 μL injection of potassium phosphate buffer through the loading loop; the column was then eluted in 0.5 mL fractions using potassium phosphate buffer. Absorbance at 280nm was used to determine protein elution from the column.

Binding Assay II: Gel Shift

In order to validate the results determined by gel filtration assay, native gels were used to examine the binding of Sec6/Sec9 in their native structures. 10 μ L samples were prepared, with each protein at 10 μ M concentration, in potassium phosphate buffer (10 mM K_2PO_4 , 10 mM KH_2PO_4 , 140 mM KCl; pH 7.4). Complex samples were incubated for 24 hours at 18°C; control samples were prepared fresh just prior to gel loading.

Native gels (6% polyacrylamide) were pre-equilibrated at 4°C by running in 1X native gel buffer (10.75 mM imidazole, 8.75 mM HEPES; pH 7.4) at 30 mA for 15 minutes. A 2 μ L volume of 6X native gel loading buffer was added to each sample prior to gel loading; 12 μ L of each sample was loaded into gel wells (at the bottom, to prevent curved bands). Gels were run at 4°C for 45 minutes at 30 mA, then stained with Coomassie Blue to visualize the proteins.

RESULTS

Purification of Sec9 9KAKR Mutant

The 9KAKR mutant was grown in *E. coli*, and the lysates purified over three preparatory columns using FPLC: an 85mL Q Sepharose column, a 35mL phenyl sepharose column, and a MonoQ 10/10 preparatory column. Several peaks were eluted from the Q Sepharose column (**Figure 8**); fractions corresponding to peak #5 (**Figure 8**, blue box) were pooled and prepared for loading onto the phenyl sepharose column as described in Methods.

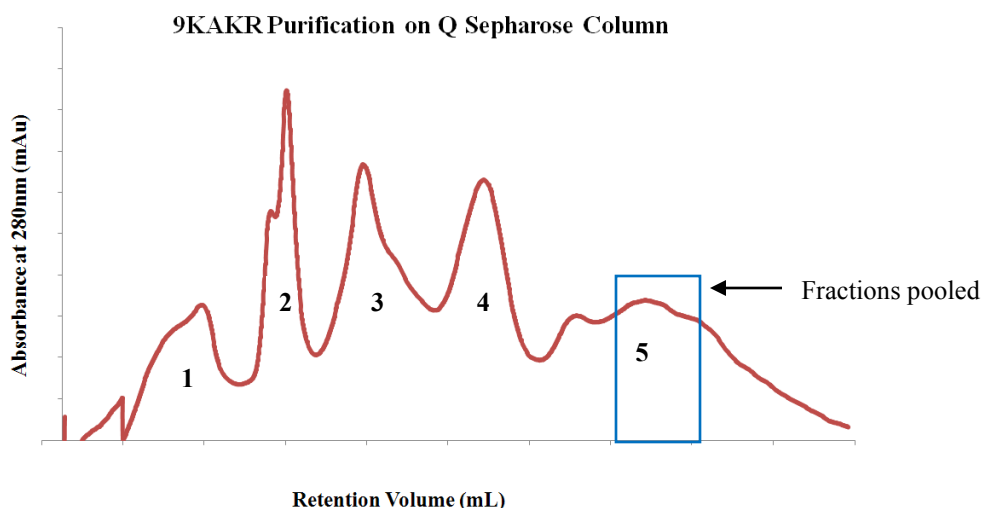


Figure 8. Purification of 9KAKR, Column #1. This figure illustrates the chromatograph obtained from eluting the 85mL Q Sepharose column. Fractions corresponding to peak #5 (blue box) were pooled and prepared for loading onto the phenyl sepharose column.

Three main protein peaks were eluted from the phenyl sepharose column (**Figure 9**), and fractions corresponding peak #2 (blue box) were pooled and prepared for loading onto the MonoQ 10/10 as described in Methods.

9KAKR Purification on Phenyl Sepharose Column

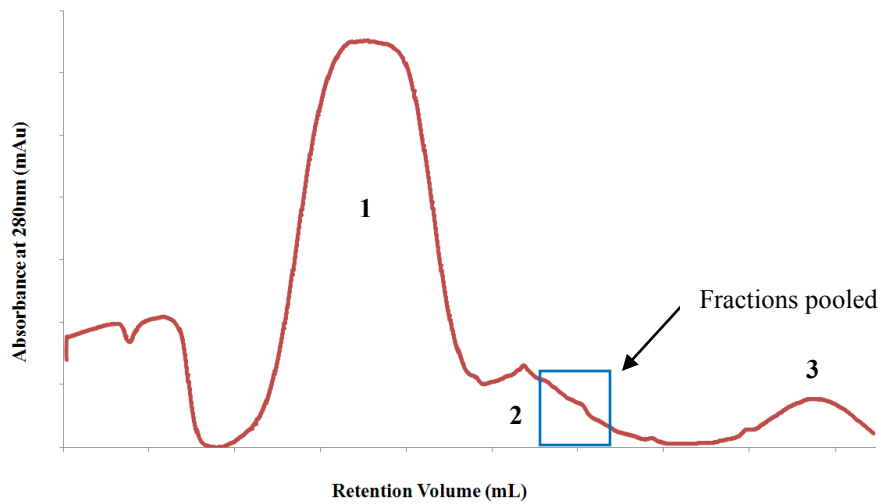


Figure 9. Purification of 9KAKR, Column #2. This figure illustrates the chromatograph obtained from eluting the 35mL phenyl sepharose column. Fractions corresponding to the second peak (blue box) were pooled and prepared for loading onto the MonoQ 10/10 column.

Two peaks eluted from the MonoQ 10/10 column; fractions containing >95% pure protein from peak #3 (**Figure 10**, blue box) were pooled and concentrated.

9KAKR Purification on MonoQ 10/10 Column

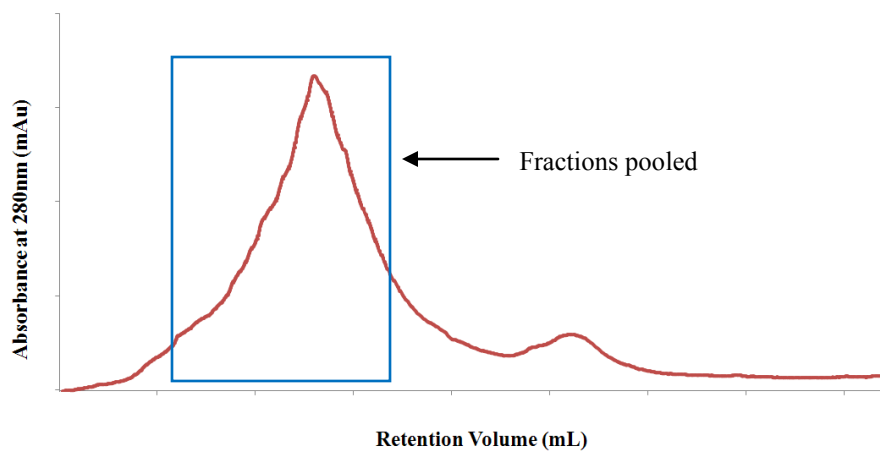


Figure 10. Purification of 9KAKR, Column #3. This figure illustrates the chromatograph obtained from eluting the MonoQ 10/10 column. Fractions containing >95% pure protein (blue box) were pooled and concentrated.

Purification of Sec6N

Sec6N was purified over two preparatory columns using FPLC: a MonoQ 10/10 column, followed by a Superose 200 16/60 column. Two main peaks eluted from the MonoQ 10/10 (**Figure 11**); fractions corresponding to peak #2 (red box) were pooled and prepared for loading onto the Superose 200 16/60, as described in Methods.

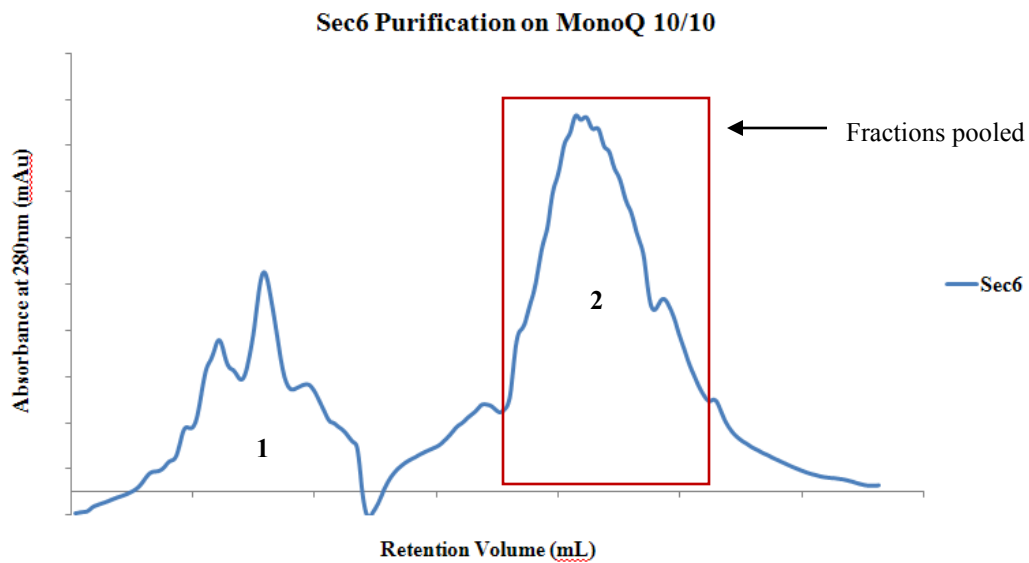


Figure 11. Purification of Sec6N, MonoQ Column #1. This figure illustrates the chromatograph obtained from eluting the MonoQ 10/10 column. The fractions corresponding to the second peak (red box) were pooled and prepared for elution over the Superose 200 16/60 column.

One main peak eluted from the Superose 200 16/60 column (**Figure 12**). Fractions containing >95% pure protein (red box) were pooled and prepared for concentration, as described in Methods.

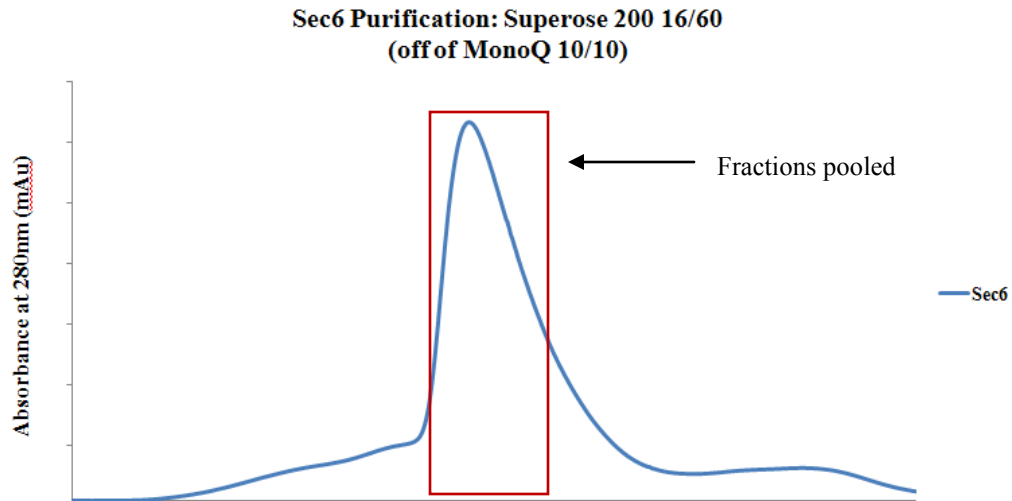


Figure 12. Purification of Sec6N, Column #2. This figure illustrates the chromatograph obtained from eluting the Superose 200 10/60 column. Fractions with >95% pure protein (red box) were pooled and concentrated.

Gel Filtration is Sufficient for Separation of the Complexed Proteins of Interest

A Superose 200 10/30 gel filtration column was used to analyze interactions of purified Sec6p, Sec9p, Sso1p, and the 9KAKR mutant. 100 μ L samples of each protein or complex were prepared at 10 μ M concentrations; then complexes were incubated at 18°C for 24 hours. The samples were loaded onto the column in separate runs, and eluted from the column with potassium phosphate buffer, as described in Methods. Analysis of the gel filtration runs for each individual protein indicated that the Superose 200 10/30 column was sufficient to differentiate the proteins of interest (**Figure 13**). Thus, the resolution of this separation technique was sufficient to proceed with the mixing experiments. Note that Sec9CT (red) and 9KAKR (green) absorb weakly due to their lack of phenylalanine residues.

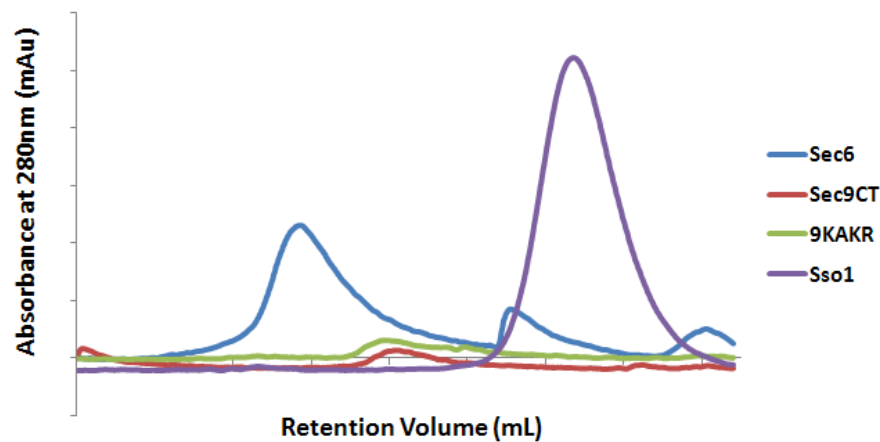


Figure 13. Gel Filtration is Sufficient for Separation of Individual Proteins. This figure shows the difference in retention volume per protein tested.

Gel Filtration Assay: 9KAKR Mutant Binds to Sso1

100 μ L samples of each purified protein or complex were prepared at 10 μ M concentrations. Samples of Sso1p/Sec9CT and Sso1/9KAKR were prepared to determine the ability of the 9KAKR mutant to bind to Sso1p in a manner similar to Sec9CT. The complex samples were incubated at 18°C for 24 hours prior to gel filtration. Samples were loaded and eluted individually. Analysis of the gel filtration runs indicated that the 9KAKR mutant (turquoise) and Sso1p (green) individual proteins when mixed together (red) form a complex (left peak eluting with low retention volume) whose position is larger than either of the individual proteins alone. The height of the 9KAKR/Sso1p complex is similar in size and mass to the complex formed by Sec9CT and Sso1p (dark blue, left peak) (**Figure 14**).

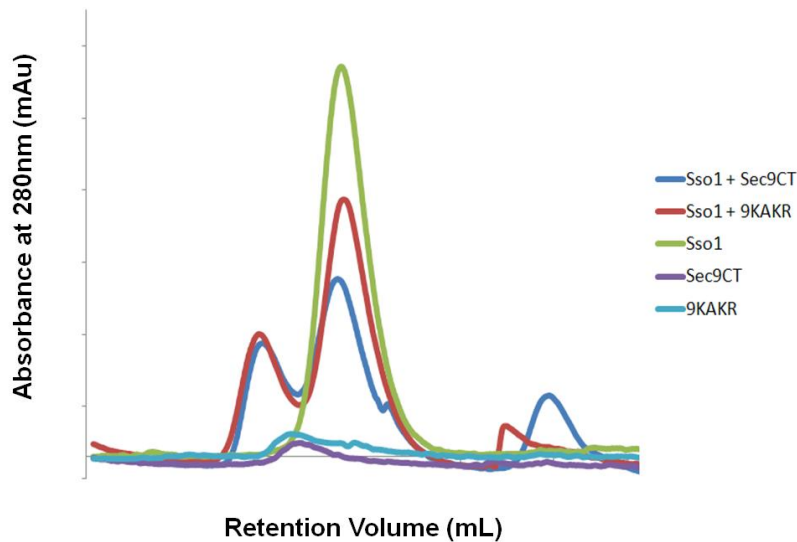


Figure 14. The 9KAKR Mutant Does Not Disrupt Binding to Partner t-SNARE Sso1p. This figure demonstrates negligible differences in retention volume and peak absorbance for the Sso1/Sec9CT (dark blue) and Sso1/9KAKR (red) complexes.

Gel Filtration Assay: 9KAKR Mutant Binds to Sec6p

100 μ L samples of each protein or complex were prepared at 10 μ M concentrations. Samples of Sec6/Sec9CT and Sec6/9KAKR were prepared in order to determine the ability of the 9KAKR mutant to bind to Sec6p in a manner similar to Sec9CT. The complex samples were incubated at 18°C for 24 hours prior to gel filtration. Samples were loaded and eluted individually. Analysis of the gel filtration runs indicated that the 9KAKR mutant and Sec6p form a complex (purple, upper left curve) in a similar amount as Sec9CT and Sec6p (light blue, upper left curve) (**Figure 15**).

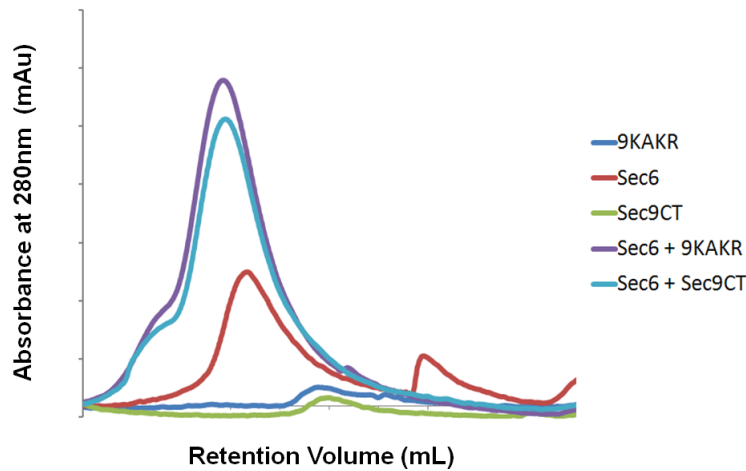


Figure 15. The 9KAKR Mutant Does Not Disrupt Binding to Exocyst Subunit Sec6p. This figure demonstrates negligible differences in retention volume and peak absorbance for the Sec6/Sec9CT (light blue) and Sec6/9KAKR (purple) complexes.

Gel Shift Assay: 9KAKR Mutant Binds to Sec6

10 μ L samples of proteins and samples were prepared at 10 μ M concentrations; complex samples were incubated for 24 hours at 18°C. Samples were loaded onto a 6% polyacrylamide native gel, which was run at 4°C to separate the proteins and complexes. Analysis of the results by gel shift assay indicated that the 9KAKR mutant binds to Sec6p (center red circle) and Sso1p (right red circle) in a manner similar to Sec9CT, as indicated by the similar mobilities of the complex pairs in the native gel (**Figure 16**). However, 9KAKR exhibits different gel mobility than Sec9CT (left red circle) (**Figure 16**).

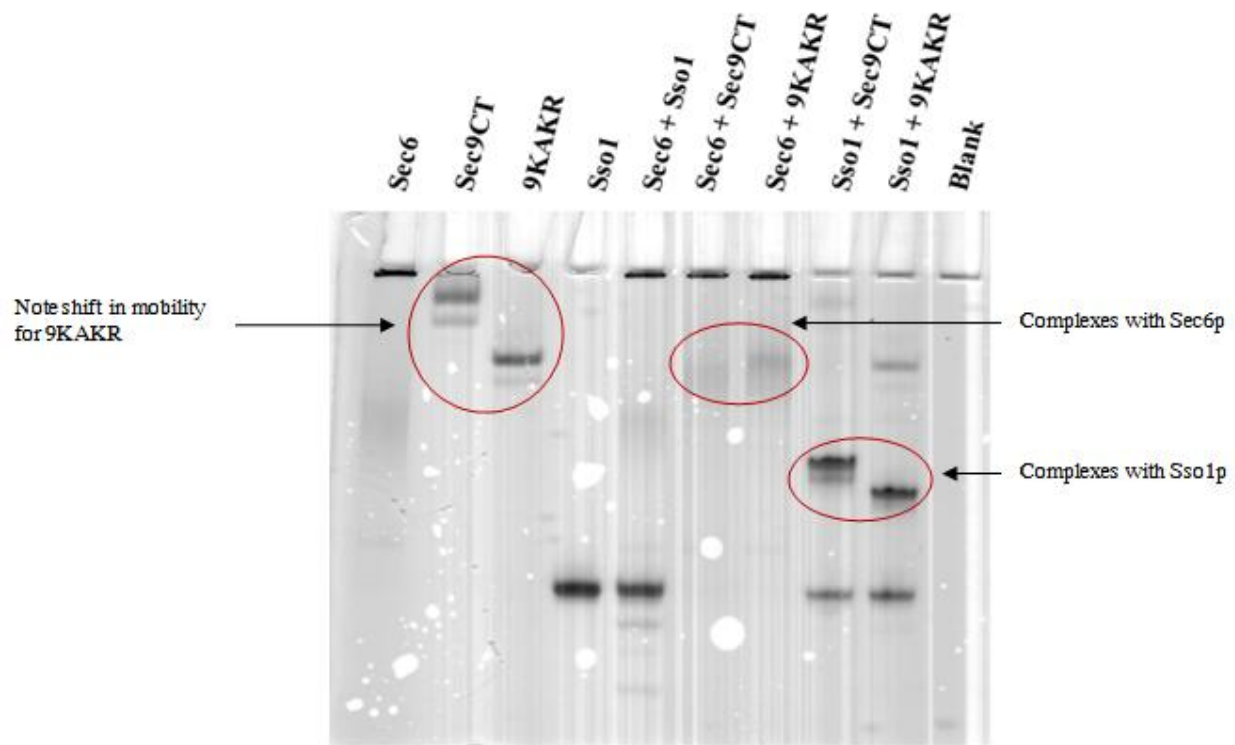


Figure 16. Gel Mobility Assay. 9KAKR exhibits shift in mobility, but does not disturb binding to Sec6p or Sso1p.

DISCUSSION

This study aimed to examine the *in vitro* effects of a Sec9p ‘patch’ mutant, 9KAKR, on the binding of the Sec9 t-SNARE to the exocyst subunit Sec6p. Previous work in the Munson lab suggested a role of Sec6p in the regulation of SNARE complex assembly at the plasma membrane (Sivaram et al., 2005; Sivaram et al., 2006), however, the binding site for Sec6p was not determined in Sec9p. The 9KAKR mutant was designed to disturb residues in a peptide region (Appendix B) suspected to play a role in Sec6/Sec9 binding, as suggested by cross-linking experiments and mass spectrometry (Munson, personal communication). The residues KAKR were mutated to a patch of four alanine residues in order to create an uncharged ‘pocket’ that would potentially disrupt binding, but not disrupt the proper folding of the protein. The mutant was cloned, recombinantly expressed in *E.coli*, and purified using FPLC, and its interactions with Sec6 tested *in vitro* using gel filtration and gel shift assays.

All protein complex samples were incubated at 18°C for 24 hours prior to analysis by each method. These parameters had been used in previous examinations of Sec6/Sec9 binding (Sivaram et al., 2005), and were kept the same for the sake of continuity. Further, the t-SNARE Sso1 exhibits a closed conformation that requires opening time *in vitro* for the binding to Sec9 to occur (Munson et al., 2000). Complete complex formation requires approximately 48 hours *in vitro* for these two proteins; the 24 hour incubation time was selected in order to visualize each uncomplexed individual protein in the complex in addition to complex assembly (**Figure 16**).

Gel filtration separates proteins according to their relative sizes; smaller proteins diffuse into the porous beads of gel filtration columns and take greater time to elute from the column

than larger proteins, which do not diffuse as easily. The 9KAKR mutant was not a truncation or expanded construct of Sec9CT, and eluted from the gel filtration column similarly to Sec9CT (**Figure 10**). The low absorbance of the Sec9CT and 9KAKR constructs can be attributed to the residues in each protein; the analysis of eluted proteins using FPLC was taken at 280nm, and since the Sec9CT and 9KAKR constructs do not contain phenylalanine residues, they do not absorb well at that particular wavelength. However, the results indicated that the gel filtration column provided sufficient resolution to separate the complex and individual proteins of interest for this project (**Figure 13**).

Gel filtration analysis of 9KAKR binding to Sec6p and Sso1p indicated that the mutations made did not disrupt binding to the partner t-SNARE or to the exocyst subunit (**Figures 14 and 15**). In each case, the results of the complex samples for the 9KAKR construct were compared to the complex samples of Sec9CT. Complex formation is indicated by a shift in elution volume; since complexes are larger in size, they elute from the gel filtration column more quickly than the proteins within them. The results demonstrate that the 9KAKR mutant was not sufficient to disrupt binding to Sec6p or Sso1p; further, the mutant binds to Sec6p and Sso1 in a manner that forms the same amount of complex as Sec9CT.

Gel shift assays were used to determine the *in vitro* binding of 9KAKR while demonstrating if any structural changes occurred due to the mutations made. The data suggests that the 9KAKR mutant has a difference in structure, pH, or another characteristic in comparison to Sec9CT, as the two proteins exhibit different mobilities within the native gel (**Figure 16**).

Interestingly, there is also a shift in the formation of complex with Sso1p, again suggesting a change in some physical characteristic of the 9KAKR mutant.

Recommendations for Future Work

The data collected during this project suggest that the peptide sequence of interest (**Appendices A & B**) may not be the Sec6p binding region in Sec9p. However, this conclusion should be considered carefully due to the significant shift in mobility of the 9KAKR mutant as compared to Sec9CT in the gel shift assays performed. Should interested parties wish to pursue this mutant further, structural analysis (ex. circular dichroism) of the 9KAKR construct should be performed to analyze its native structure. This analysis would shed light on the construct's conformation and enable the determination of whether the mutations made disrupted proper folding. Other possible binding sites in Sec9p should also be pursued, as it is possible that other residues in the peptide region are more influential in binding to the exocyst. It may also be possible that the peptide region itself is wrong – examination of other regions in Sec9p should be undertaken as well.

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Appendix I. Amino Acid Sequence of Sec9p

Note that the highlighted peptide region (QRKNVLEKAKRY) was the region of interest for the Sec6/Sec9 binding site.

MGLKKFFKIKPPEEATPEQNKDTLMELGISVKNPSKKRKE KFAAYGKFAN
DKAEDKVVYAPPGYEYARPDLELDLNASPLDANANEATAGSNRGSSGTQ
DLGNGAESNSMQDPYAIENDDYRYDDDPYARFQANKSNGRGVSNAAPYGD
YGGGYNGTSLNSYNNDGPYSNQNTSNSWVNANGRNSLNHSNSTLNVGPSR
QTRQPPVSTSTNSLSLDQRSPLANPMQEKRNPYADMNSYGGAYDSNTNRS
SGTRQGSSKNANPYASMANDSYSNGNLNRSANPYSSRSVRQPQSQQAPMT
YTPSFASDEAARNSEVDLNEEPRTGEFDFEEVYADKSAENRAALDEPDL
NAVMTNEDSIDLNASEVDHSSRQQQQQQWFMDEQQQQQQHFNATNNQYGD
QRGYKTFEEIQKEEEARQQQEEDEAVDEIKQEIKFTKQSSVASTRNTLKM
AQDAERAGMNTLGMLGHQSEQLNNVEGNLDMKVQNKVADEKVAELKKLN
RSILAVHVSNPFNSKRRRREREEQLKNRKEEKL MREQTSQQLSQSTQRI
EGAMNANNISEVRERYQRKNVLEKAKRYQFENDEEDDEMELEIDRNLDQ
IQQVSNRLKKMALTTGKELDSQQKRLN NIEESTDDLDINLHMNTNRLAGI R

Appendix II. Amino Acid Sequence of Sec9CT

Note that the highlighted peptide region (QRKNVLEKAKRY) was the region of interest for the Sec6/Sec9 binding site.

MARQQQEEDA VDEIKQEIKFTKQSSVASTRNTLKMAQDAERAGMNTLGMLG
HQSEQLNNVEGNLDMKVQNKVADEKVAELKKNRSILAVHVS NPFNSKRRR
REREEQLKNRKIEEKL MREQTSQQLSQSTQRIEGAMNANN NISEVRERY QRKN
VLEKAKRY QFENDEEDDEMELEIDRNLDQIQQVSNRLK KMALTTGKELDSQQ
KRLNNIEESTDDL DINLHMNTNRLAGIR