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INVESTIGATING A POTENTIAL SLY1/SED5 BINDING SITE

A Major Qualifying Project

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by

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ABSTRACT

Eukaryotic vesicle targeting and fusion are conserved processes used to transport cargo between various organelles and the plasma membrane for secretion. Two components required for this trafficking are SNARE and SM proteins. The SM protein Sly1 and its cognate SNARE Sed5 are known to function in trafficking cargo between the ER and the Golgi. It is known that Sly1 has a hydrophobic pocket that binds to the N-terminal peptide of Sed5. To investigate a possible alternative binding site between Sly1 and Sed5, a truncated Sed5 mutant, Sed5 (23-324) was designed to disrupt the known binding site. This mutant construct was cloned, expressed in both *E. coli* and *S. cerevisiae*, and purified. Binding studies, including size exclusion chromatography and gel shift analyses were conducted. The results indicated that the truncated Sed5 (23-324) did not bind to Sly1, suggesting that the N-terminal binding site may be the only binding domain.

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BACKGROUND

Vesicle Trafficking

Eukaryotic vesicle targeting and fusion are conserved processes that are used to transport cargo between various organelles and the plasma membrane for secretion. These vesicle trafficking mechanisms are required for various cellular processes, including cell growth, hormone secretion, and neurotransmission, and require tight regulation to confirm specificity (Wickner and Schekman, 2008). There are many examples of vesicle trafficking, including exocytosis, endocytosis, and transcytosis. Exocytosis is the secretion of membrane bound vesicles out of the cell, which is necessary for the export of various molecules, including waste products, hormones, and various proteins. In contrast, endocytosis is the movement of extracellular material into the cell, used for the degradation and recycling of cellular materials (Salo, 2002). Transcytosis is the transport of molecules from one side of the cell to the other without disruption to the environments along the way. This process is used by endothelial cells in the gut where IgA molecules are transcytosed (Tuma and Hubbard, 2003). Although there is much variation among the different pathways of vesicle trafficking, two components required for this trafficking are SNARE proteins and SM proteins.

SNARE Proteins

Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins are crucial components for intracellular trafficking as they fuse two membranes together (Kosodo et al., 2002). SNAREs are located on opposing membranes, and can be categorized by which membrane they are on: vesicle membrane (v-SNARE also known as R-SNARE) or target

membrane (t-SNARE or Q-SNARE) (Sutton et al., 1998). Most SNAREs have a single transmembrane anchor at their C terminus (Wickner and Schekman, 2008) and a homologous ~70 amino acid repeated SNARE motif. This SNARE motif forms four-helix bundles called SNARE complexes (**Figure 1**) that force opposing membranes together (Dulubova et al., 2003). Three helices of the t-SNARE (green, orange, and yellow in the diagram) assemble with a fourth helix (blue) on the v-SNARE to form a *trans*-SNARE complex in a zippering-like mechanism (Sudhof and Rothman, 2009).

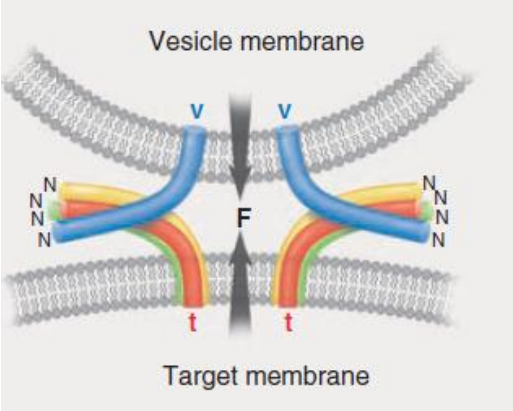

Figure-1: SNARE-Catalyzed Membrane Fusion. Shown is the model for trans-SNARE complex formation through a four helix bundle between three helices of the t-SNARE (orange, yellow, and green) and one helix of the v-SNARE (blue). (Sudhof and Rithman, 2009)

Once membrane fusion has occurred, proteins NSF and α-SNAP disassemble the *cis*-SNARE complexes. *In vitro* experiments with SNARES have shown them to be promiscuous in binding (Fasshauer et al., 1999); however, liposome fusion assays suggest the interaction among membrane-anchored SNARES is specific (McNew et al., 2000).

SM Proteins

The Sec1/Munc18 (SM) proteins are a family of cytosolic proteins that regulate SNARE complex assembly through direct interactions with their cognate SNAREs (Toonen and Verhage, 2003). The SM family of proteins is divided into seven subfamilies in higher eukaryotes, and four subfamilies in yeast (**Figure 2**). In yeast, these include Sec1 (blue in the diagram), which controls exocytosis, Vps45 (purple in the diagram), involved in endosome transport, Sly1 (red, and the subject of this MQP project) which is needed for transport between the endoplasmic reticulum (ER) and Golgi, and Vps33 (green) which is involved in vacuolar and lysosomal processes (Bracher and Weissenhorn, 2002). The structures of the mammalian Munc18 and the yeast Sly1 have been shown to be extremely similar, suggesting a conserved function among these different proteins (Peng, 2005).

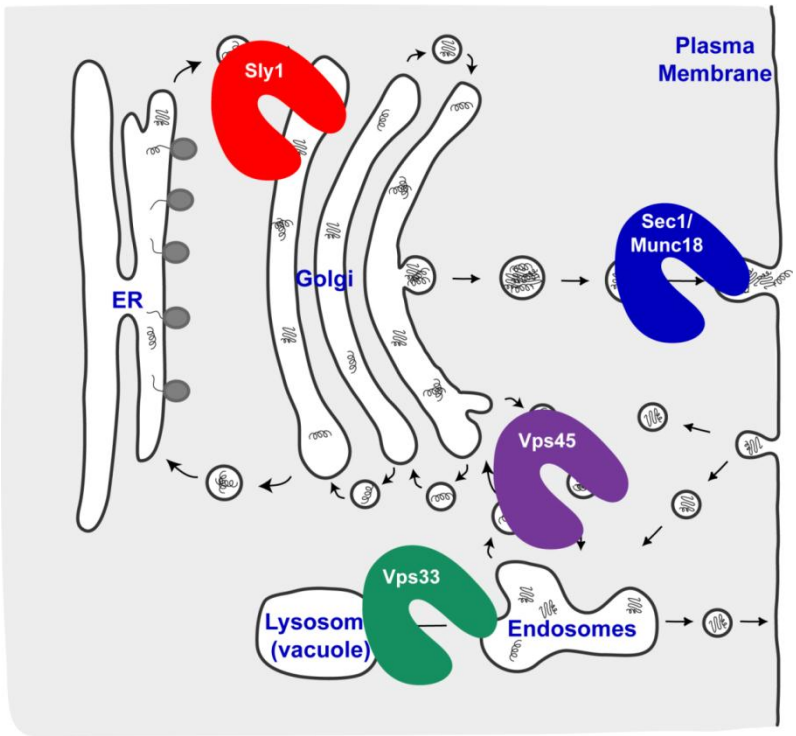

Figure-2: The Sec1/Munc18 (SM) Family of SNARE Proteins. The four families of yeast SM proteins are shown in color schematically relative to the processes they regulate. SM protein Sly1p is the subject of this project. Photo courtesy of the Munson Lab.

SM proteins associate with SNARE proteins as a clasp that binds both the t-SNAREs and v-SNARE in SNARE complexes (**Figure 3**). SM proteins contain a ~600 amino acid sequence that folds into an arch-shaped clasp (Sudhof and Rothman, 2009). There are four known binding modes between SM proteins and their cognate SNAREs. The first mode (diagram left) has been described for neuronal Munc 18a, which binds a closed conformation of syntaxin 1a (Misura et al., 2000). Mode 2 (second panel) occurs in Sly1 which has a hydrophobic pocket (shown on the lower left of SM) that binds to the N-terminal peptide of Sed5p (Bracher and Weissenhorn, 2002). Munc 18c has also been shown to bind to syntaxin 4 in this mode (Hu et al., 2011). Munc 18a can actually bind to syntaxin 1a using both Modes 1 and 2, shown as Mode 3 (Khvotech et al., 2007). Lastly, depicted as Mode 4, Sec1 mostly interacts with assembled SNARE complexes (Togneri et al., 2006).

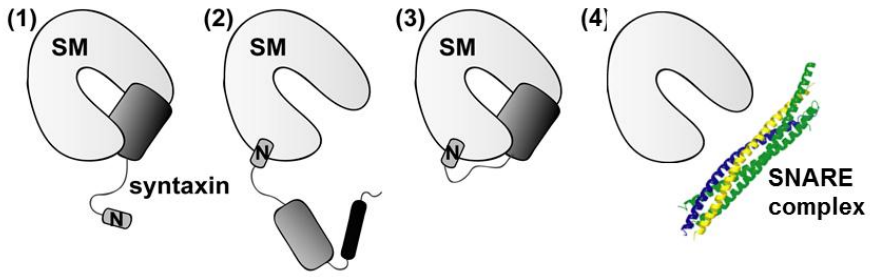

Figure-3: Diagram of the Four Known Modes of SM Protein Binding Interactions with Their Cognate SNAREs. Mode 1 is when the SM protein binds a closed conformation of the syntaxin, while Mode 2 occurs when the SM protein binds to the N-terminal domain of the syntaxin. Mode 3 combines Modes 1 and 2, and Mode 4 occurs when SM proteins interact with assembled SNARE complexes. Photo courtesy of the Munson Lab.

Despite these known binding modes, the exact role of SM protein binding to SNARES is still unclear, although the importance of SM proteins in trafficking events has clearly been shown (Toonen and Verhage, 2003). There are many models as to the role of SM proteins. The

first is that SM proteins are chaperones for syntaxins, a sub-class of Q-SNAREs. The yeast SNARE Tlg2 becomes unstable when its SM protein Vps45 is mutated, and in cell culture, syntaxin only gets delivered to the membrane in the presence of SM Munc-18. However, as shown in the various binding modes in Figure-3, not all SM proteins directly bind to syntaxins. Another model states that SM proteins negatively regulate syntaxin activity, although it has been shown that the deletion of SM proteins eliminates membrane fusion. The third model suggests that the closed conformation of syntaxins are required for the opening of it for SNARE complex formation; however, not all syntaxins have an open and closed conformation, and SM proteins can bind to both individual syntaxins and syntaxin-containing SNARE complexes (Peng, 2005).

Interactions Between Sly1 and Sed5

The SM protein Sly1 and its syntaxin Sed5 are essential for vesicular transport between the ER and Golgi (Peng and Gallwitz, 2002). Sly1 was first discovered because of a mutation that suppressed the requirement for the Rab protein Ypt1 in transport from the ER to the Golgi (Dascher at al., 1991). Sly1 was then shown to bind to the syntaxin Sed5 with nanomolar affinity (Grabowski and Gallwitz, 1997). Peng and Gallwitz (2002) found that Sly1 contributes to the specificity of SNARE complex formation *in vitro*. It was found that Nyv1-Sec22-Bet1 and Ykt6-Sec22-Bet1 could form SNARE complexes with Sed5, but this was blocked when Sly1 was bound to Sed5. Additionally, Sly1 bound to preassembled SNARE complexes, and could be precipitated with Sed5 from yeast lysates, suggesting that SNARE complexes in transport between the ER and Golgi are associated with specific SM proteins (Peng and Gallwitz, 2002).

Yamaguchi et al. (2002) characterized the binding between Sed5 and Sly1. They showed that deletion of the ~60 amino acid SNARE motif, located in the C terminus of Sed5, did not

disrupt binding to Sly1. However, a Sed5 construct containing only 40 residues of the N-terminus pulled down Sly1. Mutations of these N-terminal residues on Sed5p either weakened or abolished binding to Sly1. It was known at the time that the N-terminal domain of the syntaxins Sso1, Vam3, and syntaxin 1A had an independently folded N-terminal domain with three α-helices named the H_{abc} domain. The N-terminal domain of Sed5 was studied using NMR spectroscopy, and a well-folded domain was observed. Further tests indicated the three Sed5 α-helices were most similar to Vam3, but with a long loop in between the second and third α-helices. Sequence alignments show that the N-terminal motif that binds to Sly1 and the H_{abc} domain are very similar among Sed5 homologs in yeast, plants, and vertebrates, suggesting that H_{abc} domains are conserved within the known syntaxin families. Mammalian syntaxin 5 was then studied, and it was determined that the N-terminal 24 resides of syntaxin 5 was sufficient for binding to Sly1, and this interaction is sufficient to disrupt the steady-state structure of the Golgi complex (Yamaguchi et al., 2002).

Using the data described above, Bracher and Weissenhorn (2002) determined the crystal structure of Sly1/Sed5 (1-45), shown in **Figure 4**. This structure showed Sly1 to be an arch-shaped protein that has three separate domains, depicted in different colors in Figure 4. Domain I (yellow) has a Rossman fold containing a five-stranded β-sheet with α-helices on either side and domain II (blue) also has an α/β structure. Domain III (grey) is primarily α-helical with several hairpin structures. The N terminus of Sed5 (red) is in contact with the domain linker region and domain II, while the rest of the protein is in contact with domain I (Bracher & Weissenhorn, 2002).

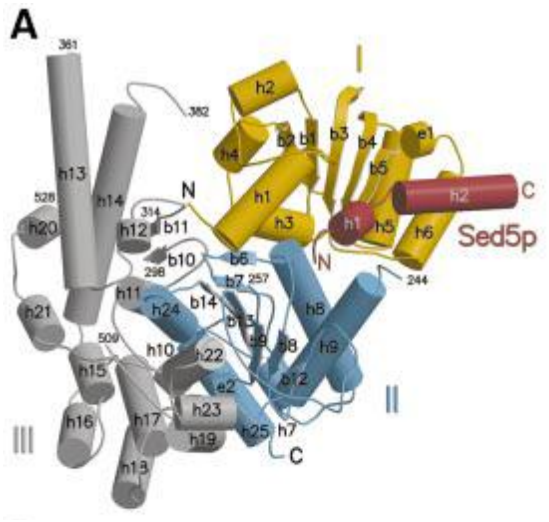

Figure-4: The Crystal Structure of the Sly1 and Sed5 (1-45) Interaction. The structure of Sly1 is shown as three domains – I (yellow), II (blue), III (grey), while the structure of Sed5 is shown in red. The N-terminus of Sed5 interacts with domain II, while the rest of the protein contacts domain I. (Bracher and Weissenhorn, 2002)

Despite this tight binding interaction, Peng and Gallwitz (2004) showed this binding is not necessary for the function of either Sly1 or Sed5. Since it has been shown that Sly1 acts after transport vesicle tethering to Golgi membranes (Cao and Barlowe, 2000), it has been proposed that Sed5, which is anchored to the cell membrane, could function to recruit Sly1 or to keep it at sites for potential *trans*-SNARE complex formation (Peng and Gallwitz, 2004).

Interactions Between Vps45 and Tlg2

Vps45, an SM protein involved in endosomal transport, and its cognate SNARE Tlg2 have also been studied to elucidate the exact role of SM proteins, and recently a new binding mode was discovered between these two proteins (Furgason et al., 2009). It had been known that Vps45 binds the N-peptide of Tlg2 and that this interaction was facilitated by the amino acid L117 in the hydrophobic pocket of Vps45. However, a mutation in L117 which causes loss of binding of Vps45 to the N-terminal domain of Tlg2 does not harm Vps45 function. Vps45 also binds endosomal SNARE complexes through binding of the N-terminal domain (Carpp et al.,

2007) and can bind the v-SNARE Snc2. Furgason et al. (2009) discovered that Vps45 interacts with Tlg2 without the N-peptide. To do so a mutant Tlg2 lacking the N-terminal domain (37-318) was purified and incubated with Vps45. Size exclusion chromatography was used to separate the complex from the individual proteins (**Figure 5**). When the two proteins were incubated together (solid line in the figure), there was a shift of elution peaks to one with a larger apparent molecular weight, demonstrating that Vps45 can interact with another binding site of Tlg2 (Furgason et al., 2009).

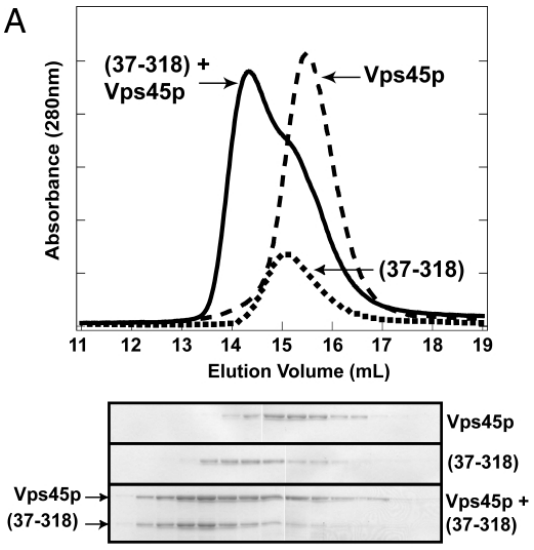

Figure-5: Vps45 Binds Tlg2 Without the N-Terminal Peptide Domain. Size exclusion chromatography was used to separate Vps45 and Tlg2 (37-318), which lacks the N-terminal domain, from the complex of these two proteins. This resulted in a shift of elution peaks (solid line) showing a complex with a larger apparent molecular weight. This complex can be also be seen in the SDS-PAGE shown below the chromatography runs. (Furgason et al., 2009)

PROJECT PURPOSE

Previous work established a direct interaction between the SM protein Sly1 and its syntaxin Sed5; the published work characterized the crystal structure of Sly1 and the N-terminal domain of Sed5 (Bracher and Weissenhorn, 2002), and showed that the deletion of the N-terminus of Sed5 abolishes binding with Sly1 (Yamaguchi et al., 2002). However, other SM proteins and their interactions with their syntaxins have been studied showing that SM proteins are not limited to solely one binding mode (Furgason et al., 2009). Studies have not yet determined if the N-terminus is completely responsible for this interaction, or if there are other binding modes between the two proteins since trafficking defects are not observed in mutants that lack the Sly1/Sed5 N-peptide interaction (Peng and Gallwitz, 2004).

The overall goal of this project was to determine whether there is an additional region of syntaxin Sed5 responsible for the protein's binding to its SM protein Sly1. A truncated Sed5 construct, Sed5 (23-324), was cloned (lacking its transmembrane domain to facilitate its solubility), and expressed in both *E. coli* and *S. cerevisiae*, and purified in order to perform *in vitro* binding studies of the two proteins. The binding of Sed5 (1-324), the cytosolic sequence of Sed5 also without the TM region, was also examined to ensure that the truncations made in Sed5p did not disrupt any other binding site. An understanding of this protein-protein interaction will provide a framework for understanding the family of SM proteins as a whole.

METHODS

Cloning of Sed5 and Sly1 Constructs in *E. coli*

Two Sed5 mutants were created for this project, one lacking the N-terminal domain and transmembrane region, and the other lacking just the transmembrane domain. Data from prior experiments showed that the N-terminal peptide of twenty amino acids is sufficient for Sly1 interaction (Bracher & Weissenhorn, 2002; Yamaguchi et al., 2002). This data was used to determine the truncation of the Sed5 mutant, Sed5 (23-324), in which the first 22 amino acids were removed hoping it would allow the mutant to bind to Sly1 using a different domain, and this mutant also lacked the transmembrane domain to make it more soluble for purification. In addition to this mutant, another Sed5 construct was made that contained the wild type Sed5 sequence minus the transmembrane domain, Sed5 (1-324). These constructs were cloned into plasmid pET15 using the NdeI and BamHI restriction sites. Since both constructs do not have the transmembrane region, the same reverse primer was used for PCR:

Forward Primer Sed5 (23-324): N-Sed5-Δ1-22
5’ GGAATTCCATATGAACTTTAGAGAACAGCAGAGGGAACG 3’

Forward Primer Sed5 (1-324): N-Sed5-1-324
5’ GGAATTCCATATGAACATAAAGGATAGAACTTCAGAATTTC 3’

Reverse Primer: Sed5-B
5’ CGGGATCCTTACTTTGCGGCTAACCATCTATTACTC 3’

The complete Sly1 sequence was also cloned into the pETDuet vector in the NcoI and HindIII sites. Since the NcoI site is upstream of the polyhistidine tag, the Met-$(His)_6$ was encoded into the primer. Two forward primers were used and the PCR was done in two steps:

Forward Primer 1: N-Sly1-1
5’ CATCACCACAGCCAGGACCCTGCTGTGGAGGAAATTGCGTCC 3’

Forward Primer 2: N-Sly1-2
5’ GGCCATGGGCAGCAGCCATCACCATCATCACCACAGCCAGGACCC 3’

Reverse Primer: Sly1-H
5’ CCCAAGCTTTTATGCATCGTTGTTGCTGCTATTAC 3’

The cloning for these three constructs had already been performed by Caroline Duffy and James Ritch at the start of this project.

Purification of Sed5 and Sly1 Proteins Produced in *E. coli*

To obtain purified Sed5 (23-324) and Sed5 (1-324) proteins, the following protocol was used. A volume of 1 μL of plasmid DNA was transformed into BL21 (DE3) competent cells, and allowed to incubate on ice for one hour. Cells were plated on LB plus 1X carbenicillin and grown overnight at 37°C. Cells were scraped into a 100 mL starter culture of LB medium plus 1X ampicillin, and grown at 37°C shaking at 200 rpm to an OD_{600} of 1.0. Four liters of LB medium plus 1X ampicillin were inoculated in equal volumes with the starter culture, and allowed to grow to an OD_{600} of 0.8. The cultures were induced for expression off the plasmid at a final concentration of 0.1 mM IPTG, and grown at 37°C at 200 rpm for another three hours. Cells were harvested by spinning in an Evolution centrifuge at 4°C at 5,000 rpm for 10 minutes, then the cell pellets were scraped into conical tubes, and stored at -80°C.

Cells were resuspended in 48 mL of cold lysis buffer (50 mM Tris-HCl, 200 mM KCl, 10 mM imidazole, 10% glycerol; pH 7.5. Fresh 5 mM (final) β-mercaptoethanol, 1 mM (final) PMSF, 1 Protease Inhibitor tablet, and DNase was added to the buffer prior to lysis). The cells were lysed in a cell disrupter at 80 psi, and spun for 10 minutes at 13,000 rpm at 4°C in an Evolution centrifuge to pellet cell debris. The supernatant was added to 4 mL of Ni-NTA agarose bead slurry pre-equilibrated in lysis buffer, and was rocked on a Nutator at 4°C for one

hour to allow the expressed his-tagged protein to bind to the beads. The beads were then poured into a column to create a column bed. The bed volume of beads was washed with 70 mL of wash buffer (50 mM Tris-HCl, 200 mM KCl, 20 mM imidazole, 10% glycerol; pH 7.5. Fresh 5mM (final) β-mercaptoethanol was added to the buffer just prior to washing) and then eluted with 30 mL elution buffer (50 mM Tris-HCl, 200 mM KCl, 250 mM imidazole, 10% glycerol; pH 7.5. Fresh 5 mM (final) β-mercaptoethanol was added to the buffer just prior to elution in 2 mL fractions. Each fraction was spotted onto Whatman paper, which was stained with Coomassie Blue to determine which fractions contained protein. The fractions containing protein were pooled, diluted to 50 mM NaCl, and filtered to remove particulates. This entire sample was loaded onto a MonoQ 5/5 column pre-equilibrated in 5% Buffer B (10 mM Tris pH 8.0, 1 M NaCl; 1 mM DTT) diluted with Buffer A (10 mM Tris pH 8.0; 1 mM DTT). The column was eluted over a 5%-50% gradient of Buffer B over 20 column volumes with 0.5 mL fractions collected throughout. Fractions corresponding to chromatograph peaks indicated the elution of various proteins, and aliquots of these fractions were run on 12% SDS-PAGE gels and stained with Coomassie Blue. Those fractions corresponding to the purest Sed5 samples were pooled. Using Milipore Amicon Ultra 0.5 mL 3K Centrifugal Filters, these samples were concentrated to 1/5 their volume by spinning at 13,200 rpm at 4°C in an Evolution centrifuge for 10 minutes. The protein was then divided into 50 μL aliquots, flash frozen in liquid nitrogen, and stored at -80°C until use.

In order to determine the final concentration of the purified proteins, a ninhydrin assay was used. A series of dilutions (5 μL, 7.5 μL, 10 μL, 12.5 μL, 20 μL) of a 10 mM leucine standard and protein sample were set up. A sample of 5 μl of water was used for the blank sample. These samples were added to polypropylene tubes containing 0.15 mL 13 N NaOH.

The tubes were covered with aluminum foil, and were then autoclaved on the liquid cycle to hydrolyze the proteins to amino acids. After autoclaving, the tubes were allowed to cool to room temperature and then 0.25 mL glacial acetic acid was added to neutralize the hydrolysis reaction. To each tube, 0.4 mL CN-ninhydrin solution was added. The tubes were then loosely capped and spun for a minute in a tabletop centrifuge in order to get all of the condensation to the bottom of the tube. The tubes were then placed in a water bath where they were allowed to boil for 15 minutes. Immediately after boiling, 2 mL of 50% isopropanol was added to each tube. Each tube was tightly capped and shaken, then allowed to cool to room temperature. The A_{570} of each sample was taken using a UV spectrophotometer, and a standard curve was created for comparison to each protein sample. The curve of the 10 mM leucine sample served as the reference for the protein curves. The concentration of the protein sample was determined using the following equations.

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

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

The Sly1 construct was purified using this same protocol, except the protein sample was loaded onto a MonoQ 10/10 column pre-equilibrated in 5% Buffer B (10 mM Tris pH 8.0, 1 M NaCl; 1 mM DTT). The column was eluted over a 0%-100% gradient of Buffer B over 20 column volumes, with 0.5mL fractions collected throughout. Fractions corresponding to chromatograph peaks indicated the elution of various proteins, and aliquots of the fractions were run on 12% SDS-PAGE gels and stained with Coomassie Blue. Those fractions corresponding to the purest Sly1 samples were pooled. To concentrate the protein, Milipore Amicon Ultra 30K

Centrifugal Filters were used. These samples were concentrated to 1/5th their volume by spinning at 13,200 rpm at 4°C in a centrifuge for 3.5 minutes. The protein was then divided into 50 μL aliquots, flash frozen in liquid nitrogen, and stored at -80°C until use. The concentration of the protein was determined using the same ninhydrin assay described above.

Assay of Binding Interactions: Gel Filtration of *E. coli* Produced Sed5 and Sly1 Proteins

In order to quantitatively determine the Sed5/Sly1 interactions, gel filtration runs of the purified proteins and their complexes were run using a GE Healthcare Superose 200 10/30 analytical column. Stock samples were prepared for each protein, at either 5 μM (Sed5 (1-324)) or 10 μM (Sed5 (23-324), Sly1) concentration of protein, in potassium phosphate buffer (10 mM K_2PO_4, 10 mM KH_2PO_4, 140 mM KCL, pH 7.4). The complex samples were prepared at either 5 μM (Sed5 (1-324)/Sly1) or 20 μM (Sed5 (23-324)/Sly1), depending on the concentration of the purified protein, and were incubated at 4°C for one hour. The column was pre-equilibrated in potassium phosphate buffer. Prior to sample injection, each protein sample was spun at 13,200 rpm at 4°C in a centrifuge to pellet any contaminants. The samples were then injected into the column using a sterile 1 mL 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 eluted in 0.25 mL fractions using potassium phosphate buffer. An absorbance at 280nm was used to determine where the protein eluted from the column.

Gel Shift Assay of *E. coli* Produced Sed5 and Sly1 Proteins

In order to confirm the results of the gel filtration assay, native gels (non-denaturing) were used to examine the binding of the two protein constructs: Sed5 (23-324) and Sly1. For each individual sample, 15 μl was prepared at a concentration of 10 μM in potassium phosphate

buffer (10 mM K_2PO_4, 10 mM KH_2PO_4, 140 mM KCL, pH 7.4). Complex samples were prepared at increasing concentrations (5, 7, 10, 12, 15 μM) and incubated for 1 hour at 4°C, while the single protein samples were prepared just prior to loading.

Native gels (6% polyacrylamide) were pre-equilibrated at 4°C by pre-electrophoresing 1X native gel buffer (10.75 mM imidazole, 8.75 mM Hepes; pH 7.4) at 30 mA for 15 minutes. A 2 μl volume of 6X native gel loading dye was added to each sample, and 17 μl of each sample was loaded into the gel well. Gels were run for 90 minutes at 30 mA at 4°C. The gels were stained with Coomassie Blue to visualize the proteins.

Cloning of Sed5 and Sly1 Constructs in *S. cerevisiae*

To clone the Sed5 and Sly1 constructs in yeast, the DNA from the plasmid constructs cloned into *E. coli* were cloned into plasmid pPP450 (pRS315-GALp) using the XmaI and HindIII restriction sites. The Met-$(His)_6$ was encoded into the 5' primer. All three constructs used the same forward primer, but a different reverse primer was used between the Sed5 and Sly1 constructs:

Forward primer Sed5 (23-324), Sed5 (1-324), Sly1: Xma-His6
5' CCCTCCCGGGATGGGCAGCAGCCATCACCATCATC 3'

Reverse primer Sed5 (23-324), Sed5 (1-324): Sed5-H
5' CCCAAGCTTACTTTGCGGCTAACCATCTATTACTC 3'

Reverse primer Sly1: Sly1-H
5' CCCAAGCTTTTATGCATCGTTGTTGCTGCTATTAC 3'

Induction Trials of Sed5 and Sly1 Constructs in *S. cerevisiae*

Induction trials were necessary to determine if the three proteins, Sed5 (23-324), Sed5 (1-324), and Sly1, could be expressed by the galactose promoter to sufficient levels for protein

purification. First, the DNA was transformed into yeast. The strain INV was used, which is a diploid strain with the genotype MATa, his3DI, leu2, trp1-289, ura3-52. A 5 mL culture of INV in YPD medium was grown overnight on a room temperature rollerdrum. For each of the three constructs, 500 μL of the culture was spun down for five seconds at 13,200 rpm in an Evolution centrifuge. Each tube was decanted to leave a small amount of media in the bottom of the tube, and then the cell pellet was resuspended by pipetting up and down. To each transformation tube, 2 μL of 5 mg/mL prepared salmon sperm DNA, 1 μL of the corresponding construct DNA, and 250 μL of PLATE mixture (45% poly-ethyleneglycol, 1M lithium acetate, 1M Tris-HCl, 0.5 M EDTA; pH 7.5) was added. Each sample was inverted and vortexed for a few seconds on the fourth speed. To each sample, 10 μL of 1M DTT was added, and again the tubes were inverted and vortexed for a few seconds on the fourth speed. The samples were then incubated at room temperature for one hour, then were subsequently heat shocked at 42°C for 8 minutes. The samples were then spun for 30 minutes at 13,200 rpm in a centrifuge, and the supernatant was aspirated off. The cell pellet was washed in 1 mL of sterile water, and the spin was repeated. Each sample was decanted to leave some water in the tube. The pellets were resuspended in the remaining water and plated on SC-Leu plates grown at room temperature. After incubation for four days, each plate was restreaked onto new SC-Leu plates.

After a 4-day incubation at room temperature, the cells from the plates (Sed5 (23-324), Sed5 (1-324), and Sly1) were scraped from the plates into two 150 mL cultures of SC-Leu medium containing 2% glucose. The cultures were grown at 30°C shaking at 200 rpm until they reached an OD_{600} of 0.6. The samples were spun down in a centrifuge (13,000 rpm at 4°C) and the cell pellets were resuspended in 150 mL cultures of SC-Leu medium containing 2% galactose to induce protein expression. The samples were induced for 16 hours. Post-induction samples

were taken; 1 mL samples were spun down in a cold room centrifuge (13,200 rpm at 4°C) and the cell pellets were resuspended in 1x loading dye. The amount of loading dye was calculated by multiplying the absorbance by 50 µl. For each sample, 15 µl were run on 12% SDS-PAGE gel, and visualized using Coomassie Blue staining.

Purification of Sed5 and Sly1 Proteins in *S. cerevisiae*

To purify the Sed5 (23-324) protein expressed in *S. cerevisiae*, a similar protocol to that of the constructs in *E. coli* was used since all the proteins contain a polyhistidine tag. The yeast transformations were re-streaked for single colonies on SC-Leu plates. Cells were scraped into a 100 mL starter culture of SC-Leu medium containing 2% glucose, and were grown at 30°C shaking at 200 rpm to an OD_{600} of 1.0. Equal volumes of the starter culture were added to 1L SC-Leu medium containing 2% glucose. These cultures were grown at 30°C shaking at 200 rpm to an OD_{600} of 0.8. The cultures were spun down at 5,000 at 4°C in an Evolution centrifuge. The cell pellets were resuspended in 1L cultures of SC-Leu medium containing 2% galactose to induce protein expression. The samples were induced for 16 hours, and then cells were harvested by spinning in an Evolution centrifuge at 4°C 5,000 rpm for 10 minutes. The cell pellets were scraped into conical tubes, and stored at -80°C.

Cell pellets were resuspended in 20 mL of cold Buffer A (50 mM Hepes, 150 mM KCl, 15 mM imidazole, 10% glycerol; pH 7.4; fresh 5 mM (final) β-mercaptoethanol, 1mM (final) PMSF, 1 Protease Inhibitor tablet, and DNase was added to the buffer prior to lysis), lysed in a cell disrupter three times at 100 psi, and spun for 300 minutes at 13,000 rpm at 4°C in an Evolution centrifuge to pellet cell debris. The supernatant was added to 6 mL of Ni-NTA agarose bead slurry pre-equilibrated in lysis buffer, and rocked on a Nutator at 4°C for one hour to allow the his-tagged protein to bind to the beads. The beads were then poured into a column

to create a column bed. The bed volume of beads was washed with 30 mL of a 5% Buffer B solution (50 mM Hepes, 150 mM KCl, 500 mM imidazole, 10% glycerol; pH 7.4. Fresh 5 mM (final) β-mercaptoethanol and 1 mM (final) PMSF was added to the buffer just prior to washing) and then eluted with 20 mL of a 40% Buffer B solution in 2 mL fractions. Each fraction was spotted onto Whatman paper, which was stained with Coomassie Blue to determine which fractions contained protein. The fractions containing protein were pooled, filtered to remove particulates, and diluted to 50 mM NaCl. This entire sample was loaded onto a MonoQ 5/5 column pre-equilibrated in 5% Buffer B (10 mM Tris pH 8.0, 1 M NaCl; 1 mM DTT). The column was eluted over a 5%-50% gradient of Buffer B diluted with Buffer A (10 mM Tris pH 8.0; 1 mM DTT), over 20 column volumes, with 0.5mL fractions collected throughout. Fractions corresponding to chromatograph peaks indicated the elution of various proteins, and these were run on 12% SDS-PAGE gels and stained with Coomassie Blue. Those fractions corresponding to the purest samples were pooled. To concentrate the protein, Milipore Amicon Ultra 3K Centrifugal Filters were used. These samples were concentrated to 1/5 their volume by spinning at 13,200 rpm at 4°C in a centrifuge for 10 minutes. The protein was then divided into 50 µL aliquots, flash frozen in liquid nitrogen, and stored at -80°C until use. The concentration of the protein was determined using the ninhydrin assay as described above.

The purification of the Sly1 protein from yeast proceeded in the same manner as with the Sed5 constructs, except for the use of the MonoQ 5/5 column. The column was eluted over a 0%-100% gradient of Buffer B diluted with Buffer A (10 mM Tris pH 8.0; 1 mM DTT) over 20 column volumes with 0.5mL fractions collected throughout. As described above, fractions corresponding to chromatograph peaks indicated the elution of various proteins, and these were run on 12% SDS-PAGE gels and stained with Coomassie Blue. Those fractions corresponding

to the purest samples were pooled. To concentrate the protein, Milipore Amicon Ultra 30K Centrifugal Filters were used. These samples were concentrated by spinning at 13,200 rpm at 4°C in a centrifuge for 3.5 minutes. The protein was then divided into 50 μL aliquots, flash frozen in liquid nitrogen, and stored at -80°C until use. The concentration of the protein was determined using the same method as described above.

RESULTS

The goal of this project was to determine whether there is an additional binding domain between the SM protein Sly1 and its cognate SNARE Sed5 aside from the known N-terminal binding domain. To achieve this, plasmid DNAs encoding three proteins were constructed: Sed5 (23-324), lacking the N-terminal domain known to bind Sly1, and also lacking the transmembrane domain to facilitate its dissolution, Sed5 (1-324), containing the cytosolic Sed5 sequence and lacking the transmembrane domain, and Sly1. The proteins were expressed in both *E. coli* and *S. cerevisiae* as his-tagged proteins to facilitate purification for testing by *in vitro* binding experiments.

Purification of the Sed5 and Sly1 Proteins from *E. coli*

The two Sed5 constructs, Sed5 (23-324) and Sed5 (1-324), and the Sly1 construct were initially grown in *E. coli*, and the lysates were purified over a MonoQ 5/5 column. **Figure 6** shows a 10% SDS-PAGE gel of the purified proteins.

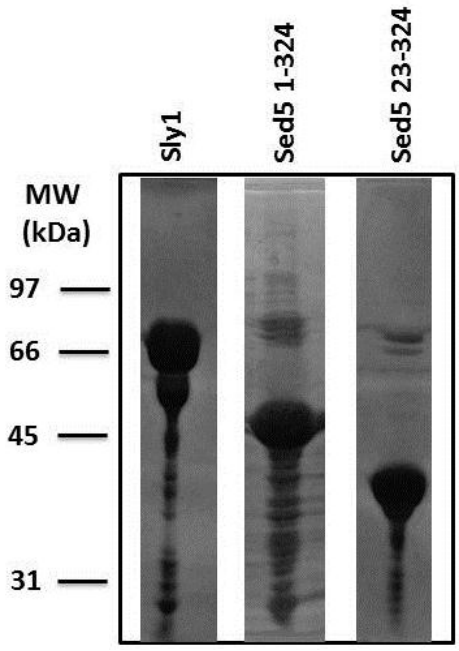

Figure 6: 10% SDS-PAGE of Purified Sly1, Sed5 (1-324), Sed5 (23-324) Proteins Isolated From *E. coli*. Each construct was cloned, grown, and purified as described in the Methods.

Gel Filtration Analysis: Sed5 (23-324) Does Not Bind Sly1

A Superose 200 10/30 gel filtration column was used to analyze the interactions between the purified Sed5 and Sly1 constructs. The samples were loaded onto the column in separate runs and eluted using potassium phosphate buffer as described in the Methods section. Samples of the individual proteins were prepared at either 5 μM (Sed5 (1-324)) or 10 μM (Sed5 (23-324), Sly1). The complex samples were prepared at either 5 μM (Sed5 (1-324)/Sly1) or 20 μM (Sed5 (23-324)/Sly1). **Figure 7** displays the chromatographs of the individual proteins, Sed5 (23-324) and Sly1, alongside the complex Sly1/Sed5 (23-324), as well as 10% SDS-PAGE gels of the corresponding fractions. The data for Sed5 (1-324) is not shown.

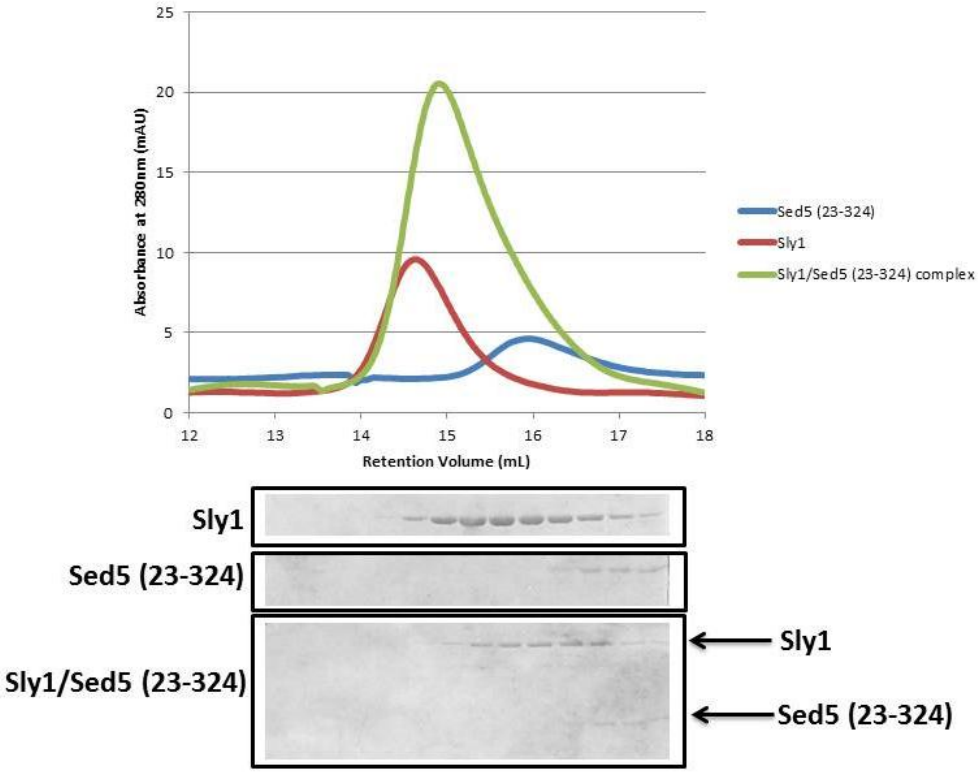

Figure 7: Gel Filtration Analysis of the Sly1/Sed5 (23-324) Complex. A Superose 200 10/30 column was used to separate the proteins Sly1, Sed5 (23-324), and the Sly1/Sed5 (23-324) complex. The individual proteins were run at 10 μM and the complex at 20 μM. 0.25 mL fractions were collected and then run on a 10% SDS-PAGE gel and stained by Coomassie. The corresponding fractions are shown below the chromatograph.

Gel Shift Analysis: Sed5 (23-324) Does Not Bind Sly1

Samples of Sly1 and Sed5 (23-324) at 10 μM, as well as the Sly1/Sed5 (23-324) complex at increasing concentrations (5-15 μM) were prepared as described in the Methods and loaded onto 6% acrylamide native gels. **Figure 8** displays this native gel.

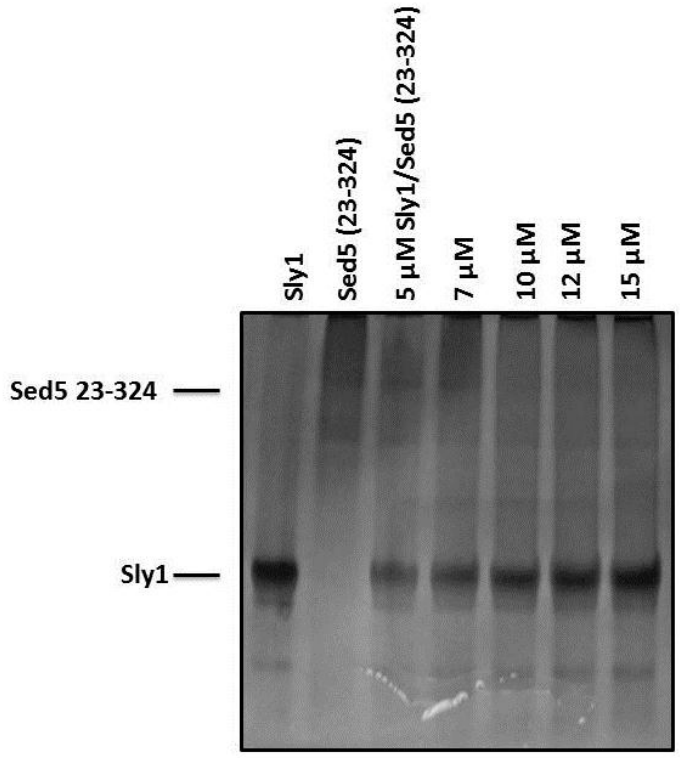

Figure 8: Native Gel of the Sly1, Sed5 (23-324), and Sly1/Sed5 (23-324) Complex. The individual proteins (at 10 µM) and the complex (5-15 µM) were run on a non-denaturing 6% polyacrylamide gel and then stained using Coomassie.

Preliminary Purification of the Sed5 and Sly1 Proteins from *S. cerevisiae*

Two constructs, Sed5 (23-324) and Sly1, were grown in *S. cerevisiae* and the lysates were purified over a MonoQ 5/5 column. **Figure 9** shows a Western blot of the two proteins.

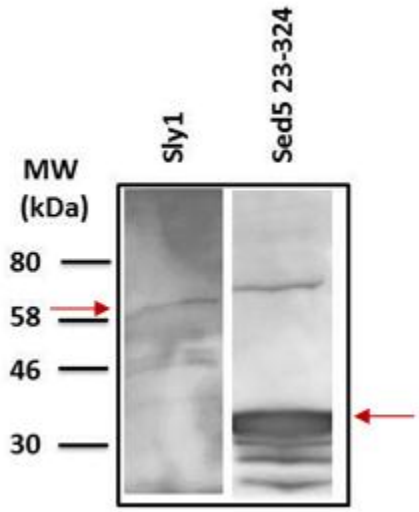

Figure 9: Western Blot of Preliminary Purification Sly1 and Sed5 (23-324).
The two proteins were cloned, grown and purified according to the Methods. The red arrows correspond to the bands of interest.

DISCUSSION

This work aimed to investigate the binding sites of the SM protein Sly1 and its cognate SNARE Sed5. Previous work in the Munson lab showed that another SM protein Vps45, involved in endosomal trafficking, can bind to its cognate SNARE Tlg2 through a different binding site when the N-terminal 36 residues of Tlg2 are deleted (Furgason et al., 2009). The Sed5 (23-324) truncation, lacking the N-terminal domain, was designed to test this hypothesis to see whether Sly1 can bind to Sed5 in an alternative manner. This truncation was cloned, expressed in both *E. coli* and *S. cerevisiae*, and purified using FPLC. The interactions with Sly1 were then tested *in vitro* using gel filtration and gel shift assays.

Gel filtration separates proteins according to their relative sizes. Larger proteins cannot diffuse as easily into the porous beads of the column so are excluded from the bead and take less time to elute compared to smaller proteins that can diffuse more easily. Gel filtration analysis of Sly1 and Sed5 (23-324) indicated that the truncation did not bind to Sly1 (**Figure 7**). Had a binding interaction been observed, there would have been a shift in the chromatograph of the Sly1/Sed5 (23-324) complex line. A complex of the two proteins would have had a higher molecular weight than the individual proteins and would have eluted earlier. This can also be confirmed through the SDS-PAGE. The corresponding fractions did not shift in location and appear in the same location on the gel as the runs with the individual proteins alone. The gel filtration was also run using the Sed5 (1-324) construct as a control. However, this construct is prone to degradation (**Figure 6**) and could only be concentrated to at most 5 μM. There is a ten-fold dilution of protein concentration down the gel filtration column, and for these reasons protein could not be detected in the fractions corresponding to the peaks on the chromatograph. Therefore, this construct could not be used as a control to show that a complex could be detected

using gel filtration. However, because previous work in the Munson Lab showed complex formation of other SM and SNARE proteins using the same gel filtration columns (Furgason et al., 2009), the work was continued.

To allow for higher protein concentrations to be tested without dilution, a gel shift analysis was performed. The results showed that even at a concentration of 15 μM of both Sly1 and Sed5 (23-324), a complex does not form between the two proteins (**Figure 8**). Since both the Sly1 and Sed5 (23-324) constructs were more successfully concentrated, only these two proteins were used in this analysis.

The data collected during this project suggests that the N-terminal domain of Sed5 is the only binding domain to Sly1. However, this conclusion should be carefully considered. It is possible that at the concentrations tested, the gel filtration and gel shift assays were not sensitive enough to detect a second binding site. Although Sly1 and Sed5 bind with nanomolar affinity (Grabowski and Gallwitz, 1997) at the primary binding site, a secondary binding site could have a much weaker interaction. To continue this project, the purification protocols in *E. coli* should be optimized for higher protein concentrations. The purification of the constructs in *S. cerevisiae* should also be improved. It is also possible that production of these proteins in *E. coli* gives rise to proteins that are not folded properly. The same binding experiments, gel filtration and gel shift assays, should be repeated with the *S. cerevisiae* constructs and the results compared to those from *E. coli*. Structural analysis (i.e. circular dichroism) could be performed on the purified constructs to ensure they are properly folded. An additional epitope tag could also be added to Sly1, for example a V5 tag, for additional *in vitro* pull-down experiments. This information would be useful in understanding the family of SM proteins as a whole and their role

in binding to SNAREs. If it is found that all SM proteins bind to SNAREs in a similar manner, it is possible that they also have similar functions.

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