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Engineering Factor Xa Protease Sites in the Exofacial Loop 9-10 of GLUT1

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ENGINEERING FACTOR XA PROTEASE SITES IN THE EXOFACIAL LOOP 9-10 OF GLUT1

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ABSTRACT

Cell membranes often allow small, uncharged solutes to pass freely, while larger molecular species such as nutrients and ions need the help of transporters. Glucose transporters (GLUT) are required for movement of glucose across the cell membrane. Specifically, GLUT1 catalyzes the passive facilitated diffusion of glucose across the membrane down its concentration gradient. The structure and function of GLUT1 has been studied intensively for many years but much remains unknown. Previous research identified 12 transmembrane α- helices, but a high resolution structure of GLUT1 is not available. The goal of this project was to insert a Factor Xa protease site in the exofacial loop 9-10 of GLUT1 to allow for cleavage of the protein at that loop for providing further insights into domains of the protein involved in the binding of substrates and inhibitors.

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ACKNOWLEDGEMENTS

I would like to thank Anthony Carruthers of UMass Medical School for allowing me to work in his lab and for introducing me to this project. I would also like to thank Julie De Zutter and Laura Andersh for teaching me and guiding me through this project. Lastly, I would like to thank David Adams of Worcester Polytechnic Institute for helping me get this project started and with editing this report.

BACKGROUND

Glucose Transporters

Throughout most of the body, glucose is transported by facilitative diffusion. Currently, thirteen, relatively similar, active glucose transporters have been identified in various locations, but the first four (termed Class I) are the most studied. Abbreviated GLUT for glucose transporter, GLUT1 is found predominantly in erythrocytes, GLUT2 in the liver, GLUT3 in the brain, and GLUT4 in muscle and fat (Pessin 1992). The study of glucose transporters has increased over the years in the hope that it will lead to a better understanding and treatment of glucose transport disorders, such as diabetes mellitus (Hruz 2001).

GLUT1 Structure

GLUT1 is the major glucose transporter in human cells, especially erythrocyte and endothelial cells, and has been extensively studied over the last twenty years (Zuniga 2001). Its existence in the membrane of erythrocytes was first hypothesized in 1952, and it is now known that it resides in about 10% of an rbc's cell membrane (Hruz 2001). GLUT1 is expressed highly in the endothelial cells of the blood brain barrier, but is also prevalent in tumor tissue (Pessin 1992). The GLUT1 gene resides on chromosome 1, an autosomal chromosome (Klepper 2002).

Previous work with the hydropathy of this receptor (**Figure-1**), led to the discovery that GLUT1 consists of 12 transmembrane α-helices (Hresko 1994). The

hydropathy shows that GLUT1 is exceedingly hydrophobic, with roughly 50% of the protein lying within the lipid bilayer (Pessin 1992).

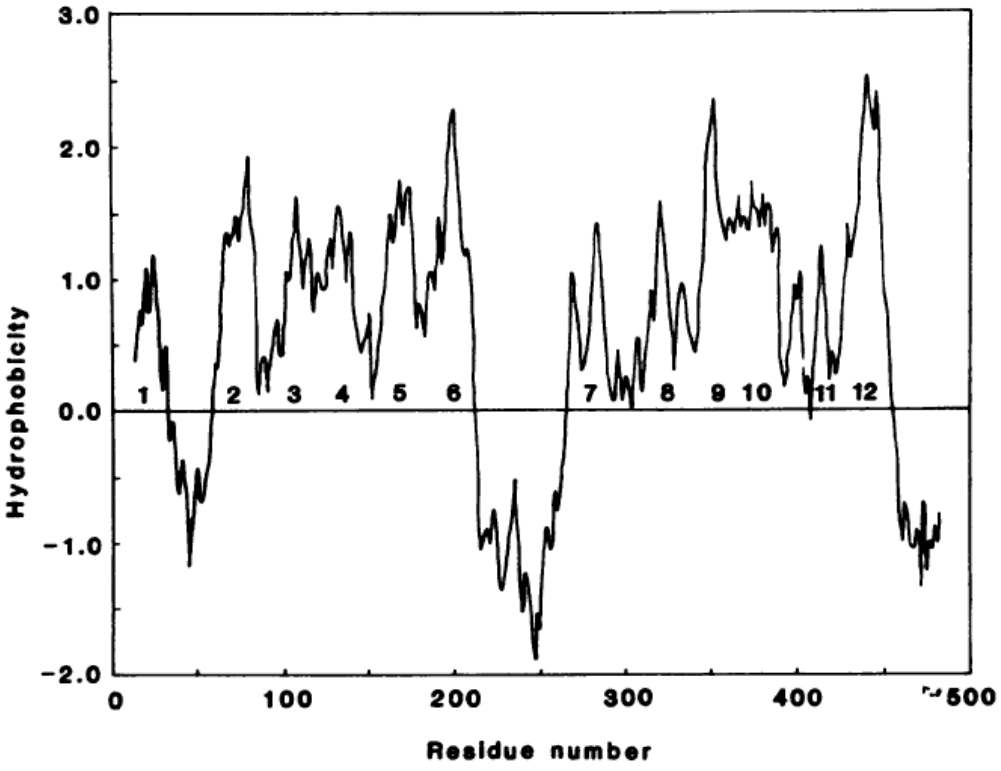


Figure-1: Hydropathy Plot For GLUT1. This diagram shows a plot of the 490 residues of human GLUT-1 (X-axis) versus the hydrophobicity of each residue (Y-axis). Note the existence of 12 hydrophobic transmembrane domains. (Mueckler 1985)

Figure 2 shows a diagram of the predicted secondary structure of GLUT1. This diagram shows the N-linked glycosylation site at ASN-45 (diagram upper left), marked in the loop between trans-membrane domain 1 and 2. The amino acid sequence of GLUT1 was partially predicted using FAB mapping (Hruz 2001). While much of the secondary structure consists of α helices, a few β-sheets and random coils have also been detected, with a distribution of 82%, 10%, and 8% respectively (Hruz 2001). It had been predicted that trans-membrane domains 3, 5, 7, 10, and 11 juxtapose to form a central aqueous channel (Hruz 2001). The other GLUT proteins are nearly identical to GLUT1 in the

trans-membrane domains, but differ significantly in the loop between segments 6 and 7, as well as the N and C terminal domains (Hruz 2001).

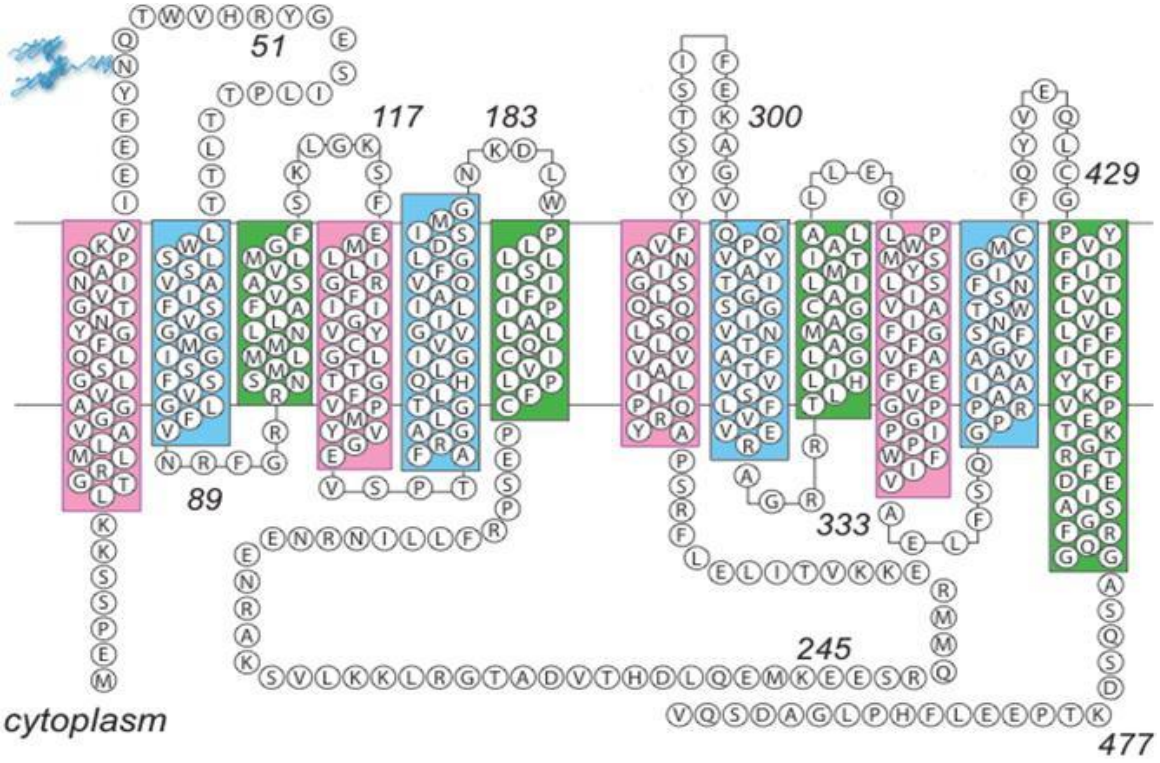


Figure-2: The Predicted Structure of GLUT1. Generated in Carruthers Laboratory from various experiments.

It is theorized that GLUT1 exists as a homotetramer of two GLUT1 dimers. Each GLUT1 dimer has two glucose binding sites, one on the internal/cytoplamic side, and the other on the external side. The homotetramer thus has two import and two export sites (Zottola 1995). Although much has been discovered about GLUT1 structure in the recent past, what is lacking is a direct experimental determination of the tertiary structure of GLUT1 (Hruz 2001). Most of the data on the structure and function of GLUT1 has been

collected by affinity labeling and site-directed mutagenesis experiments (Hruz 2001). Important regions identified in GLUT1 by affinity labeling include trans-membrane α-helix 5, 7, and 10. Helix 5 participates in external substrate binding, 7 participates in external substrate binding and transport, and 10 gives flexibility to the transporter (Hruz 2001). Another study revealed deletions of greater than 25 amino acids on the C terminus caused GLUT1 to lose function, while any deletion less than that retains wild-type functionality (Hruz 2001).

GLUT1 Function

GLUT1 is the primary glucose transporter in human fetal tissue and tissue culture cells. In human adults, it is most highly expressed in the blood brain barrier, rbc's, kidney, and colon (Pessin 1992). Low levels are expressed in muscle, liver, and adipose tissues in human adults (Olsen 1996). Studies have indicated that once sugar binds to GLUT1, an alternating conformational change takes place (Carruthers 1990). GLUT1 was previously identified as a passive uniporter and simple carrier. It is now determined to cycle between sugar import and export, and is a two site carrier (Mueckler 1994). GLUT1 has also been used to study glucose transport kinetics (Olsen 1996). According to Olsen and Pessin, the V_{max} for glucose transport in erythrocytes ranges from 15 – 366 mmol·liter of $H_2O^{-1}\cdot min^{-1}$ at 20 ºC (Olsen 1996).

Previous Research Related to this Project

In the Carruthers lab, GLUT1 has been continuously studied. Previous research has indicated an interaction between the C-terminus domain and Loop 6, both

cytoplasmic (Blodgett 2007). The results of this project can be paired with the research into the interaction between Loop 6 and the C-terminus domain by studying cleavage assays with the mutant GLUT1's that have been generated.

Inhibitors such as Cytochalasin B and Forskolin have been used in affinity labeling experiments. Work on this project will help identify CCB and Forskolin binding sites and which domains are involved. It has already been suggested that cytochalasin B binds to a site at the C-terminus of the protein (Salas-Burgos 2004). The suggested docking site of CCB is on the endofacial side of GLUT1, is positively charged, and lined with polar molecules. It is likely that the forskolin binding site is nearby so that both inhibitors interfere with glucose transport from the C-terminus end (Salas-Burgos 2004).

PROJECT PURPOSE

The goal of this project was to insert a Factor Xa binding site into exofacial loop 9-10 of GLUT1 using site directed mutagenesis. Following the engineering of this protease site, expression, glucose transport, and Factor Xa cleavage will assessed. The ultimate goal is to use the generated mutation in combination with other FXa mutants in L7-8 and L11-12 to identify regions of GLUT1 involved in substrate and inhibitor binding, as well as to assess an ATP dependent interaction between Loop 6-7 and the C-terminus.

METHODS

PCR Amplification, PCR Purification, and Ligation

These techniques were accomplished using a Qiagen Quickchange Site-directed Mutagenesis Kit. The precise protocol is shown below. Wild-type GLUT1, already purified by the lab, was used as the DNA template in the PCR. Several kinds of primers were used including Factor Xa 9-10/11-12 top and bottom and Factor Xa 9-10/11-12 scrambled top and bottom. After PCR, samples are digested with *Dpn1* and transformed in supercompetent cells. A Qiagen Herculase Enhanced DNA polymerase reaction was also used, followed by QIAquick PCR Purification or Gel Extraction to purify the amplicon. These protocols are also listed below. Wild-type GLUT1 was again used for the DNA template, while 5'HindIII/G1 tag top start, Factor Xa 9-10/11-12 Forward and Reverse, and G1 3' XhoI were the primers used in the Herculase reactions.

QuikChange Site Directed Mutagenesis

Mutant Strand Synthesis Reaction (Thermal Cycling)

1) Ensure plasmid DNA template is isolated from a dam+ E.coli strain.
2) Prepare sample reactions:
   - 5 µl of 10X reaction buffer
   - X µl (5-50ng) dsDNA template
   - 1.25 µl (125ng) of oligonucleotide control primer #1 – Fxa (9-10/11-12) Top
   - 1.25 µl (125ng) of oligonucleotide control primer #2 – Fxa (9-10/11-12) Bottom
   - 1 µl dNTP mix
   - ddH_2O to a final volume of 50 µl

   Then add 1 µl of P*fuTurbo* DNA polymerase (2.5 U/µl)
3) Cycle each reaction using the parameters outlined in Table 1. Run thermal cycling overnight.

Segment	Cycles	Temperature	Time
1	1	95^{0}C	30 Seconds
2	30	95^{0}C	30 seconds
		55^{0}C	1 minute
		68^{0}C	2 minute/kb of plasmid length

Dpn I Digestion of the Amplification Products

1) Add 1 µl of the *Dpn* I restriction enzyme (10 U/µl) directly to each amplification reaction using a small pointed pipet tip.
2) Gently and thoroughly mix each reaction mixture by pipetting up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate at 37^{0}C for 1 hour to digest the parental supercoiled dsDNA.

Transformation of XL1-Blue Supercompetent Cells

1) Thaw XL1-Blue supercompetent cells on ice. For each control and sample reaction, aliquot 50 µl of the supercompetent cells to prechilled and labeled 14 ml BD Falcon polypropylene round-bottom tubes.
2) Transfer 1 µl of the *Dpn* I treated DNA from each control and sample reaction to their respectively labeled tubes.
3) Swirl reactions to mix and incubate on ice for 30 minutes.
4) Heat pulse transformation reactions for 45 seconds at 42^{0}C and then place reaction on ice for 2 minutes.
5) Add 0.5 ml NZY+ broth preheated to 42^{0}C and incubate reactions at 37^{0}C for 1 hour shaking at 225-250 rpm.
6) Plate 250 µl control reaction on carb+ agar plate, and 250 µl of sample reactions on carb+ agar plates, two plates for each sample reaction.
7) Incubate transformation plates at 37^{0}C for >16 hrs (overnight).

Expected Results

- Control reaction should have between 50-800 colonies with greater than 80% appearing blue.
- Mutagenesis efficiency: ME = # blue colonies/total # colonies X 100%
- Expected colony number for sample reactions is between 10 and 1000 colonies depending on base composition and length of DNA template used.

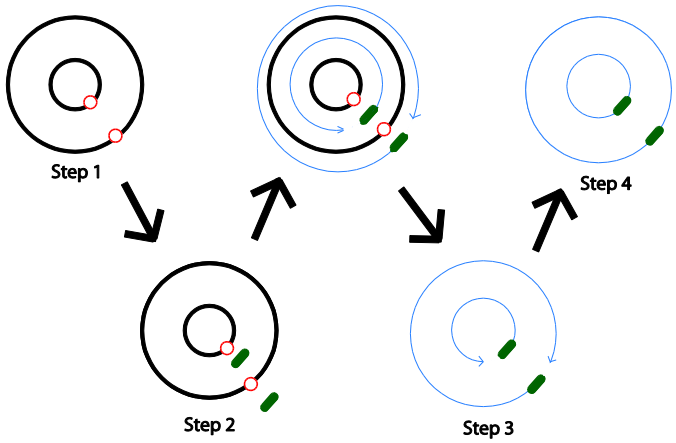


Figure-3: Schematic of Quickchange PCR.

Herculase Enhanced DNA Polymerase

1) Prepare sample reactions:
   5 µl 10X Herculase Reaction Buffer
   0.4 µl dNTP mix (25 mM of each dNTP)
   X µl (15-50 ng) DNA Template
   1 µl (100ng) Primer # 1 – 5' HindIII
   1 µl (100ng) Primer # 2 – 3' XhoI
   0.5 µl Herculase Polymerase (5 U/µl)
   X µl ddH_2O to final volume of 50 µl

2) Aliquot mixtures into sterile thin-walled PCR tubes.
3) PCR using single block temperature cycler
4) Samples may be purified directly or run on 0.7% agarose gel.

QIAquick PCR Purification

1) Add 5 volumes of Buffer PBI to 1 volume of the PCR reaction and mix.
2) Place a QIAquick column in a provided 2 ml collection tube or into a vacuum manifold.
3) To bind DNA, apply the sample to the QIAquick column and centrifuge for 30 – 60 seconds and discard flow through, or apply vacuum to manifold until all samples have passed through the column.
4) To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30 – 60 seconds and discard flow through, or apply vacuum.
5) Centrifuge the column in a 2 ml collection tube provided for 1 minute.
6) Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7) To elute DNA, add 50 µl dH_2O to the center of the QIAquick membrane and centrifuge the column for 1 minute.

8) If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

QIAquick Gel Extraction

1) Excise DNA fragment from gel with scalpel
2) Weigh gel slice in colorless tube
3) Add 3 volumes Buffer QG to 1 volume gel (100mg = ~100µl)
4) Incubate at 50°C for 10 minutes, vortex every 2-3 minutes
5) After gel is dissolved, make sure color appears yellow
6) Add 1 volume gel volume of isoproponal to sample and mix
7) Place QIAquick spin column in 2 ml collection tube
8) Apply sample to column, centrifuge for 1 minute
9) Discard flow-through, place column back in collection tube
10) Add 0.5 ml Buffer QG to column, centrifuge for 1 minute
11) Add 0.75 ml Buffer PE to column, centrifuge for 1 minute
12) Discard flow-through, centrifuge again for 1 minute
13) Place column in clean 1.5 ml microcentrifuge tube
14) Elute DNA with 50 µl distilled water, add to center of QIAquick membrane and centrifuge for 1 minute

Transformation

Transformations of DH5α cells followed the protocol shown below. These were used in cases when plasmids with low yields needed to be transformed again.

1) Thaw supercompetent cells on ice
2) Pre-chill DNA to transform on ice
3) Mix 1µL of DNA and 50 µL of competent cells together with pipette tip
4) Incubate on ice for 30 minutes
5) Heat shock cells for 20 seconds at 42°C
6) Place cells back on ice for 2 minutes
7) Add 950 µL pre-warmed media to cells and place the tubes in a 37°C water bath with shaking platform for 1 hour
8) Plate 200 µL of the transformed cells onto LB carbanicillin resistant plates Incubate overnight at 37°C

DNA Isolation and Quantification

DNA isolation and quantification was accomplished using Qiagen MaxiPreps or MidiPreps. The protocols are listed below. After transformation, colonies were cultured in LB + carbanicillin broth. After ~16 hrs of growth, the DNA was isolated using the Qiagen kits and quantified with a spectrometer. A concentration of 300 µg/ml or greater was ideal.

Midi Prep

1) Pick a single colony from a freshly streaked selective plate and transfer it to 3mL day culture containing LB nutrient broth.
2) Transfer the culture into a 500mL Erlenmyer flask containing 200 mL LB nutrient broth typically containing 100µL/mL carbenicillin (if stock is 100mg/mL add 200µL of carbanicillin)

Note: Antibiotic chosen based on plasmid

3) Incubate for approximately 12-16hr. at 37°C with vigorous shaking.
4) Harvest the bacterial cells by centrifugation at 5000RPM for 15 minutes at 4°C.
5) Carefully remove supernantant while leaving behind the pellet.
6) Resuspend the bacterial pellet in 6mL of Buffer P1 (refrigerated). Ensure that the pellet is uniformly resuspended. Make sure that the RNase and the blue lysis indicator has been added to Buffer P1.
7) Add 6mL of Buffer P2 and mix thoroughly by vigorously. Incubate at room temperature for 5 minutes. Upon addition of the P2 buffer the solution will turn blue, indicating that the cells have all been lysed.
8) Add 6mL of Buffer P3 (refrigerated) and mix thoroughly. Upon addition of the buffer the beige color of the solution will reappear ensuring that the cells are completely saturated and SDS, the denaturant in Buffer P2, is effectively precipitated.
9) In order to get rid of the precipitate, pour lysate into the QIAfilter Cartridge (a syringe type apparatus) with cap placed on to the end of the cartridge. Incubate at room temperature for 10 minutes or until all the precipitate floats to the top of the lysate.
10) While waiting, equilibrate QIAGEN HiSpeed Midi Tip by applying 4mL Buffer QBT and allow the column to empty by gravity flow. Collect the QBT buffer in a 50mL conicle.
11) Using the plunger, dislodge the fluid in the QIAfilter Cartridge (making sure to take off the cap first) into the equilibrated column and gravity filter the lysate into the 50mL conicle.
12) Wash QIAGEN-tip (column) with 20mL Buffer QC.
13) Elute DNA with 5mL Buffer QF in a new sterile 50mL conicle.

14) Add 3.5mL of isopropanol in order to precipitate the DNA. Mix and incubate at room temperature for 5 minutes.
15) Remove the plunger from a 25mL syringe and attach the QIAprecipitator Midi Module onto the outlet nozzle.
16) Over a 50mL conicle place the QIAprecipitator and pour the eluate in to the 25mL syringe. Using constant pressure filter the eluate through the precipitator.
17) Remove the QIAprecipitator from the syringe and pull out the plunger. Over the 50mL conicle place the QIAprecipitator and add 2.0mL of room temperature 70% ethanol in to the 25mL syringe. Using constant pressure filter the ethanol through the precipitator to wash the DNA.
18) Remove the QIAprecipitator from the syringe and pull out the plunger. Using constant pressure dry the QIAprecipitator by passing through air about 3 times. Detach the 25mL syringe from the QIAprecipitator.
19) Remove the plunger from a new ds5mL syringe and attach the QIAprecipitator onto the outlet nozzle. Hold the outlet of the QIAprecipitator over a 1.5 microcentrifuge tube and add 500μL of water to the 5mL syringe. Insert the plunger and elute the DNA into the collection tube using constant pressure.
20) Quantitate DNA using spectrophotometer.

Maxi Prep

1) Pick a single colony from a freshly streaked selective plate and transfer it to 3mL day culture containing LB nutrient broth.
2) Transfer the culture into a 500mL Erlenmyer flask containing 200 mL LB nutrient broth typically containing 100μL/mL carbenicillin (if stock is 100mg/mL add 200μL of carbanicillin)

Note: Antibiotic chosen based on plasmid

3) Incubate for approximately 12-16hr. at 37°C with vigorous shaking.
4) Harvest the bacterial cells by centrifugation at 5000RPM for 15 minutes at 4°C.
5) Carefully remove supernantant while leaving behind the pellet.
6) Resuspend the bacterial pellet in 10mL of Buffer P1 (refrigerated). Ensure that the pellet is uniformly resuspended. Make sure that the RNase and the blue lysis indicator has been added to Buffer P1.
7) Add 10mL of Buffer P2 and mix thoroughly by vigorously. Incubate at room temperature for 5 minutes. Upon addition of the P2 buffer the solution will turn blue, indicating that the cells have all been lysed.
8) Add 10mL of Buffer P3 (refrigerated) and mix thoroughly. Upon addition of the buffer the beige color of the solution will reappear ensuring that the cells are completely saturated and SDS, the denaturant in Buffer P2, is effectively precipitated.
9) In order to get rid of the precipitate, pour lysate into the QIAfilter Cartridge (a syringe type apparatus) with cap placed on to the end of the cartridge. Incubate at room temperature for 10 minutes or until all the precipitate floats to the top of the lysate.

10) While waiting, equilibrate QIAGEN HiSpeed Maxi Tip by applying 10mL Buffer QBT and allow the column to empty by gravity flow. Collect the QBT buffer in a 50mL conicle.
11) Using the plunger, dislodge the fluid in the QIAfilter Cartridge (making sure to take off the cap first) into the equilibrated column and gravity filter the lysate into the 50mL conicle.
12) Wash QIAGEN-tip (column) with 60mL Buffer QC.
13) Elute DNA with 15mL Buffer QF in a new sterile 50mL conicle.
14) Add 10.5mL of isopropanol in order to precipitate the DNA. Mix and incubate at room temperature for 5 minutes.
15) Remove the plunger from a 30mL syringe and attach the QIAprecipitator Maxi Module onto the outlet nozzle.
16) Over a 50mL conicle place the QIAprecipitator and pour the eluate in to the 30mL syringe. Using constant pressure filter the eluate through the precipitator.
17) Remove the QIAprecipitator from the syringe and pull out the plunger. Over the 50mL conicle place the QIAprecipitator and add 2.0mL of room temperature 70% ethanol in to the 25mL syringe. Using constant pressure filter the ethanol through the precipitator to wash the DNA.
18) Remove the QIAprecipitator from the syringe and pull out the plunger. Using constant pressure dry the QIAprecipitator by passing through air about 3 times. Detach the 25mL syringe from the QIAprecipitator.
19) Remove the plunger from a new ds5mL syringe and attach the QIAprecipitator onto the outlet nozzle. Hold the outlet of the QIAprecipitator over a 1.5 microcentrifuge tube and add 500µL of water to the 5mL syringe. Insert the plunger and elute the DNA into the collection tube using constant pressure.
20) Quantitate DNA using spectrophotometer.

Sequencing

Sequencing was accomplished through the use of Davis Sequencing in California. DNA samples must be purified and quantified before sending for sequencing. Samples were sent at 300 ng/µl. Primers used were 750-start, 575-1200, and siteIII, all at 3 pmol/µl. Results were viewable using the MacVector program and Sequencher programs.

Transfection

Transfection follows the concept of transformation using tissue cultures. Passaging of tissue cultures is listed below followed by the transfection protocol from Invitrogen. Other transfection protocols may be used depending on the kit purchased. The Carruthers lab utilizes human embryonic kidney (HEK) cells for cultures.

Passaging HEK Cells

1) Warm DMEM media and trypsin in 37°C water bath
2) Aspirate media from plated HEK cells
3) Add 3 ml warmed trypsin to plated HEK cells
4) Allow to incubate for a couple minutes until holes develop and cells begin to slide around. This can be viewed under inverted microscope
5) Aspirate trypsin
6) Re-suspend cells in 10 mls of warmed media
7) For a 1:6 dilution, add 10 mls warmed media to 2 new plates
8) Add 2 mls of re-suspended cells to each plate, so that each plate contains a total of 12 mls
9) Place freshly passaged HEK cells in incubator at 37°C
10) Passage again in 3-4 days

Transfection

1) The day before transfection, trypsinize and count cells. Plate 0.5-1.25X10^5 cells per well in 0.5 ml of complete growth medium. Cell density should be 50-80% confluent on the day of transfection.
2) The day of transfection, remove growth medium from cells and replace with 0.5 ml of complete growth medium.
3) For each well of cells to be transfected, dilute 0.5 µg of DNA in 100 µl Opti-MEM I Reduced Serum Media without serum.
4) For each well of cells, add 0.75-1.75 µl of Lipofectamine LTX Reagent into the above diluted Opti-MEM:DNA solution, mix gently and incubate for 30 minutes at room temperature to form DNA-Lipofectamine LTX Reagent complexes.
5) After 30 minutes incubation, add 100 µl of the DNA-Lipofectamine LTX Reagent complexes directly to each well containing cells and mix by gently rocking the plate back and forth.
6) Complexes do not have to be removed following transfection. Incubate the cells at 37°C in a CO_2 incubator for 18-24 hours post-transfection before assaying for transgene expression.

Protein Expression and Analysis

Proteins were analyzed using Western blots. Preparation of the 15% SDS-PAGE gels in listed below. The primary antibody, rabbit anti-GLUT1 C-terminus, and secondary antibody, goat anti-rabbit were generated by New England Peptide at a dilution of 1:10K in PBS + 0.1% Tween. The protocol for use of the gel and remaining components of the Western blot is listed below. Protein was quantitated by the BCA Assay also listed below.

Preparation of 15% SDS-PAGE Gel

	Separating	**Stacking**
ProtoGel Acrylamide	7.5 ml	1.3 ml
Distilled Water	3.75 ml	6.1 ml
Separating Buffer	3.75 ml	0 ml
Stacking Buffer	0 ml	2.5 ml
APS	75 µl	50 µl
TEMED	15 µl	10 µl

1) Prepare the separating and stacking gels in separate conical tubes without adding APS or TEMED.
2) Add APS and TEMED into separating tube.
3) Add separating gel layer to 2 cassettes three quarters of the way to the top.
4) Immediately add a layer of butanol to the top, approximately 100 – 200 µl.
5) Allow gel to set.
6) Rinse butanol off with distilled water and dry cassette completely.
7) Add APS and TEMED to stacking tube.
8) Fill cassettes to the top with stacking layer and insert comb.
9) Allow gel to set.

Western Blot

15% SDS-PAGE Electrophoresis

1) Insert 15% SDS-PAGE gel to apparatus. Make sure blank cassette is in back of apparatus. Tighten to seal.
2) Cover with 1X Tank Solution.
3) Insert 10 µl ladder into first well. Insert protein samples mixed with buffer into remaining wells until out of samples. If empty wells remain, fill will buffer to ensure samples run straight.
4) Run at 75 Volts, infinite Current until samples reach separating gel.

5) Run at 125 Volts, infinite Current until finished. Purple band should be in middle of gel.

Transfer

1) Cut PVDF membrane to the size of the gel
2) Wet in methanol, then in 1X transfer buffer
3) Wet whatman paper in 1X transfer buffer
4) In case, black side down, layer: sponge, 1 sheet of whatman, 15% SDS-PAGE gel, PVDF membrane, 1 sheet of whatman, sponge. Close case with white side on top.
5) Place case in apparatus with black side to the back, matching to the black wire.
6) Run at 250 Current, infinite Voltage for 1 hour 15 minutes.

Block

1) Block with 10% BSA in PBS-Tween (can be left overnight)
2) Wash 2 times, 10 seconds each, with PBS-Tween
3) Add 1 µl 1° antibody diluted in 10 ml 3% BSA in PBS-Tween
4) Incubate for 1 hour at room temperature
5) Wash 3 times, 5 minutes each, with PBS-Tween
6) Add 2 µl 2° antibody diluted in 10 ml 3% PBS-Tween
7) Incubate for 45 minutes at room temperature
8) Wash 6 times, 5 minutes each, with PBS-Tween
9) Develop with Lumiglo Chemiluminescence (5:5) for 1 minute
10) Wrap in saran wrap and x-ray or computer process

BCA Assay

1) Using a 96 well plate, fill the first two columns in duplicate with 10 µl of the standards 0, 200, 400, 600, 800, and 1000 µg/ml concentrations.
2) Fill 10 µl of unknowns in duplicate in remaining wells
3) Using repeater pipetter, fill 200 µl of BCA solution in each well (BCA solution = 5 ml BCA Protein Assay Reagent A mixed with 100 µl Reagent B)
4) Incubate for 30 minutes at 37°C
5) Read with BioRad Microplate Reader
6) View Standard Curve Report and Unknown Concentration Report

RESULTS

Factor Xa Protease Sites

Originally, a Factor Xa protease cleavage site was inserted into loops 7-8, 9-10, and 11-12 by previous members of the Carruthers Lab. The sites of insertion are shown in **Figure 4**, and a schematic showing the expected cleavage results is shown in **Figure 5**.

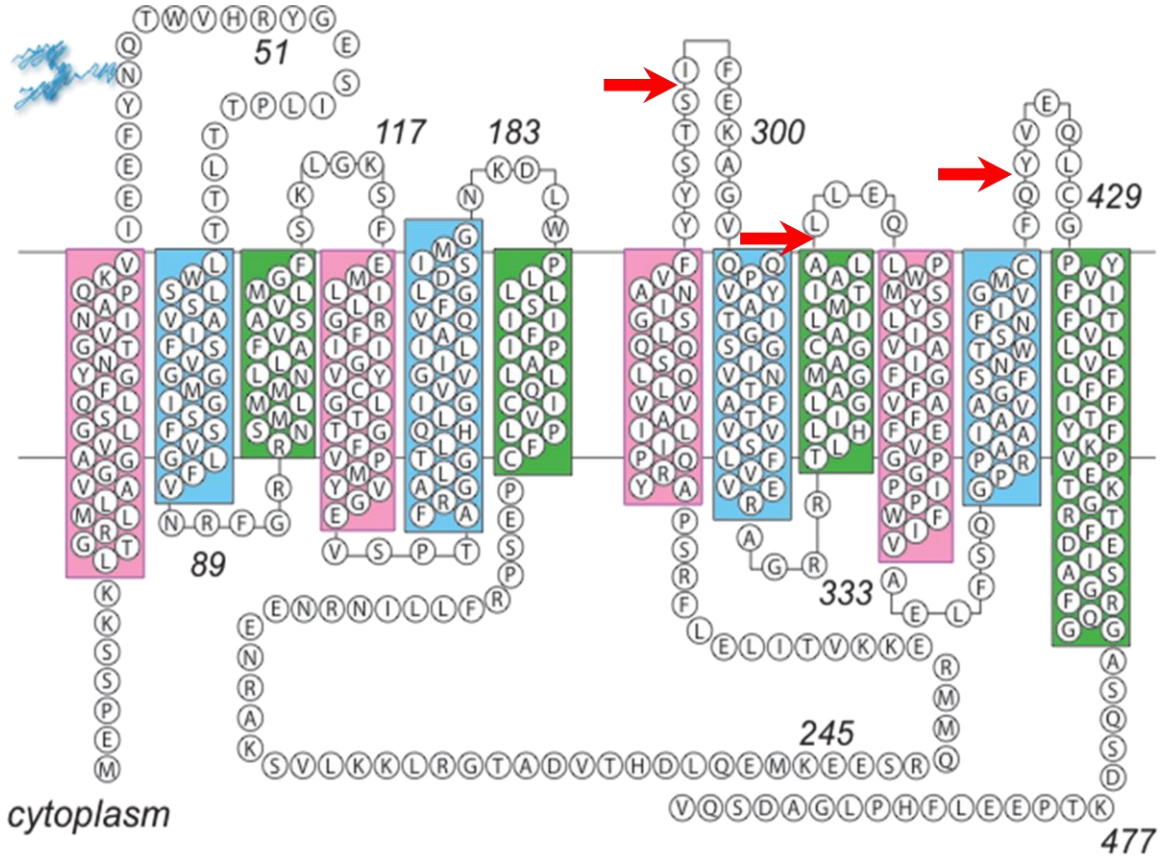
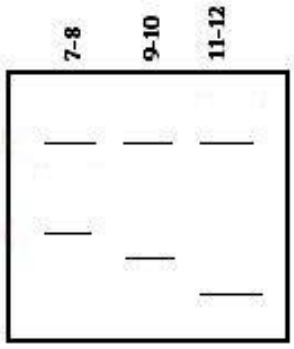


Figure-4: Initial Location of Three Factor Xa Cleavage Sites in GLUT1. The red arrows (diagram upper right) denote the location of protease sites inserted into extracellular loops 7-8, 9-10, and 11-12. The Factor Xa protease site consists of the amino acids IEGR with cleavage occurring after the R.



Full Length GLUT1 50 kDa

Expected
ProteolyticFragments

Figure-5: Expected Results of Factor Xa Cleavage.

The GLUT1 western blot in **Figure 6** shows the actual results of proteolytic cleavage of this initial mutant. The western blot was conducted using Rabbit anti-GLUT1 C-terminus as the primary antibody diluted 1:10000, and Goat anti-rabbit HRP as the secondary antibody diluted 1:5000. The cleavage at loops 7-8 and 11-12 were as expected, as proteolytic fragments were detected around 20 kDa for loop 7-8 (figure left side), and 10 kDa for loop 11-12 (figure middle). However, the cleavage of loop 9-10 failed to occur as expected, at approximately 15 kDa (figure right side). Possible reasons for the Fxa failure to cleave are that the loop was too short and that it was inaccessible to the protease. The failure of this previously constructed mutant to cleave at the loop 9-10 site is why that loop needed to be extended while also adding a new Factor Xa site in this MQP. The hypothesis is that extension of the loop would allow easier access of the protease to the site.

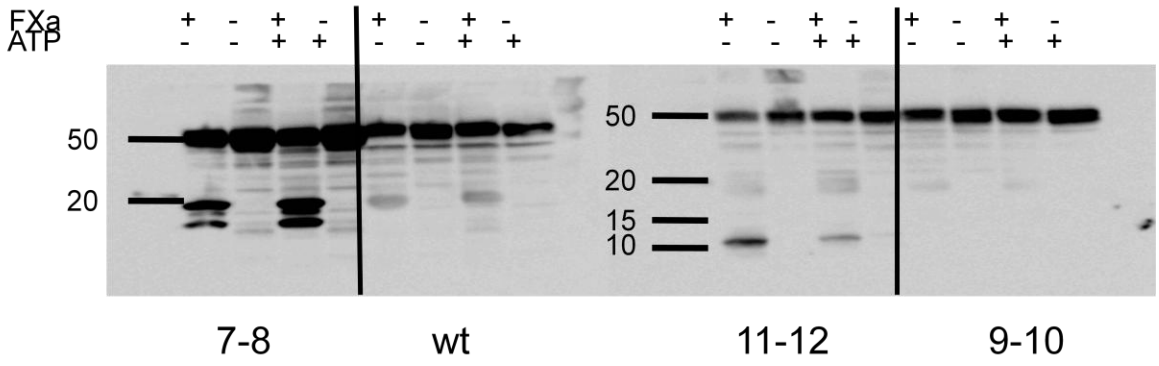


Figure-6: Western Blot of Factor Xa Proteolytic Cleavage of GLUT1 Mutagenized at Extracellular Loops 7-8, 9-10, and 11-12.

Mutagenization of GLUT1 by QuickChange PCR

To extend loop 9-10, the loop 11-12 was used as an example since that loop cut successfully in the previous experiment. The first sets of Quickchange PCR experiments were conducted using the sequence identical to loop 11-12 in the primers. These primers were already designed by the lab. After several failed attempts to create the mutant GLUT1, it was determined that because of the primers homology to loop 11-12, the annealing process was not occurring correctly. Some problems that were encountered included neither being able to generate the correct sequence nor being able to isolate proper colonies.

At this point, new primers were designed. The nucleotide sequence of loop 11-12 was scrambled in order to prevent the improper annealing. The exact sequence constructed for primer 1 (9-10 scrambled top) was 5' – ACC ATC GCG CTA GCA CTG CTG GCA GAA CAC CAA ATA GAA GGA CGA TCA GCA GAA GTA GAG CAG CTA CCC TGG ATG TCC TAT – 3'. The sequence for primer 2 (9-10 scrambled bottom) was 5' – ATA GGA CAT CCA GGG TAG CTG CTC TAC TTC TGC TGA TCG TCC TTC TAT TTG GTG TTC TGC CAG CAG TGC TAG CGC GAT GGT – 3'.

The new sequence, supplemented with the amino acids IEGR (nucleotides ATA GAA GGA CGA in primer 1) for Factor Xa, was inserted as shown in **Figure 7**. The site of cleavage occurs after the arginine residue R, as noted by the red arrow.

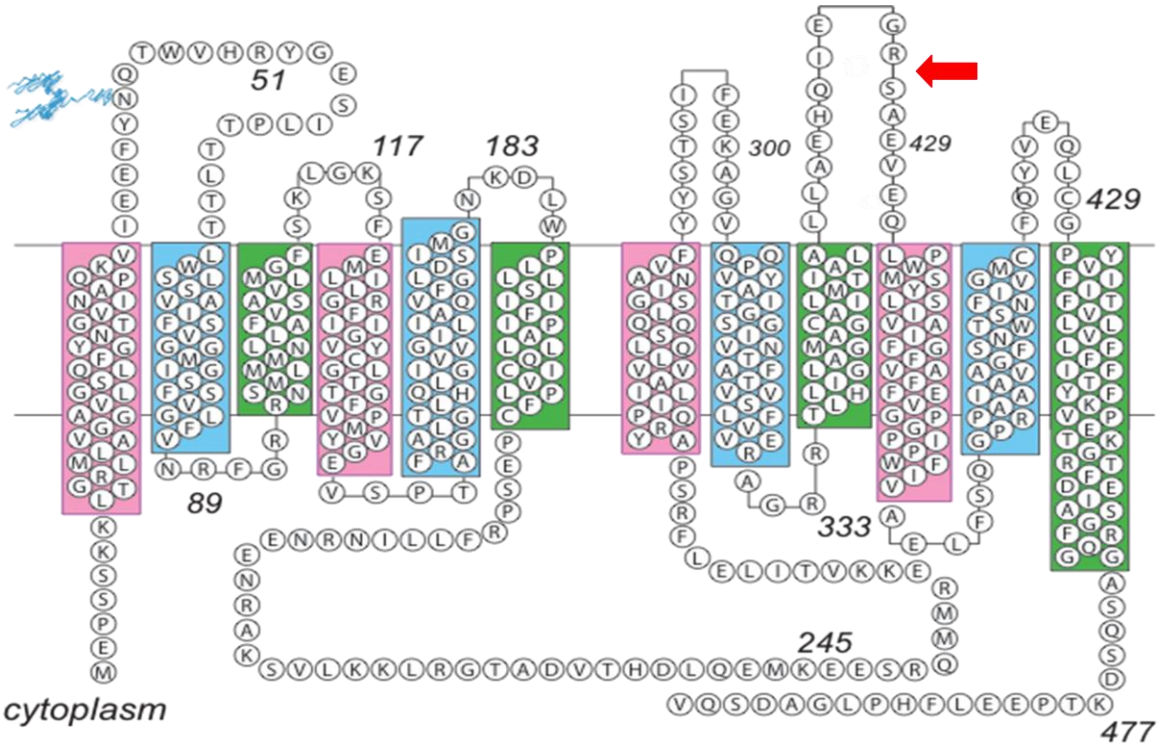


Figure-7: Structure of the New Mutant GLUT1 Expressed in this Project. Extracellular loop 9-10 was modified to extend its structure to allow easier access to the protease Xa site to allow a more efficienct cleavage.

After performing the Quickchange PCR, the PCR samples were transformed into *E. coli* XL-1 supercompetant cells. Colonies were cultured in LB + carbanicillin broth and purified using Qiagen midipreps. In total, 30 samples were sent out for sequencing. When sent to Davis Sequencing, the samples were sent at a concentration of 300 ng/µl. The primers used to compare wild-type GLUT1 were 750-start, 575-1200, and siteIII. These were sent at a concentration of 3 pmol/µl. Initial problems included no insertion of

the Fxa site, occasional double insertion of the Fxa at the 9-10 loop, and some random insertions throughout the protein. The most recent sequencing data indicates the correct plasmid was successfully cloned. Subsequent experiments to be performed include expression, *in vitro* glucose transport, and Fxa cleavage.

Mutagenization of GLUT1 by Herculase PCR

A second GLUT1 mutant was simultaneously developed using a nested Herculase PCR. This process involves 3 total PCR reactions. The first two PCR reactions, labeled 1A and 1B, involved a forward and reverse amplification of the plasmid in opposing directions. Reaction 1A used primers 5' HindIII/G1 Tag Top Start and Factor Xa 9-10/11-12 Reverse, while Reaction 1B used primers Factor Xa 9-10/11-12 Forward and 3' XhoI (schematic shown in **Figure-8**). The amplified samples were run on a 0.7% agarose gel, resulting in bands at approximately 1000 bp for 1A, and 400 bp for 1B (**Figure-9**).

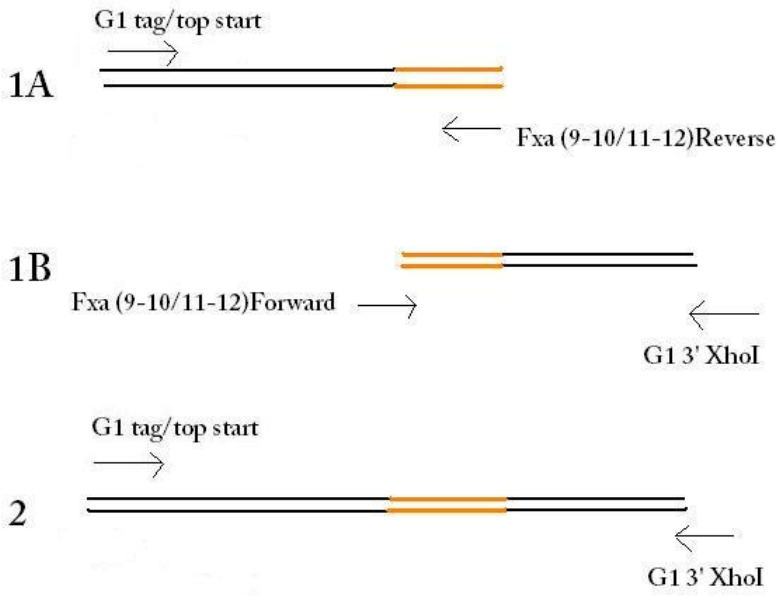


Figure-8: Schematic of Nested Herculase PCR Reactions.

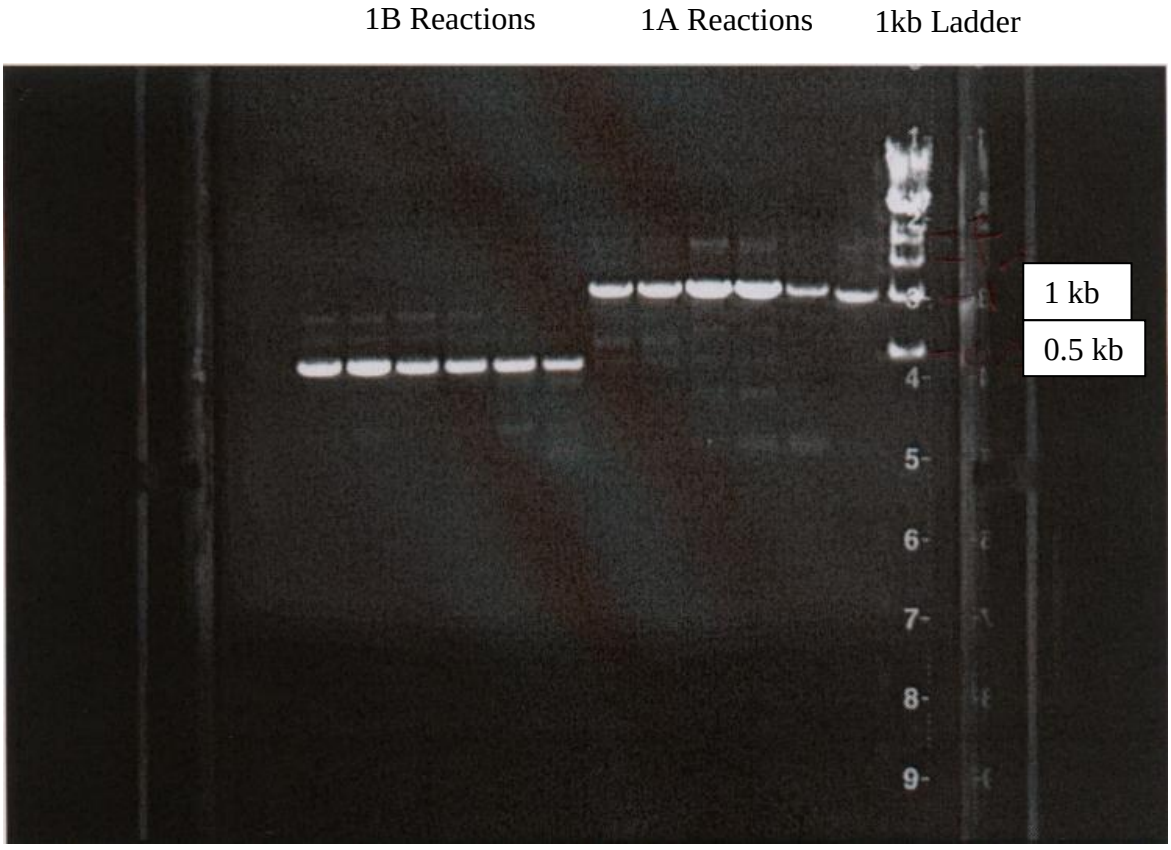


Figure-9: Gel Analysis of Herculase PCR Reactions 1A and 1B for Construction of the Second GLUT1 Mutant.

The exact expected fragment lengths are calculated to be 1074 bp from the start to loop 9-10 in 1A, and 402 bp from loop 9-10 to the stop in 1B. The samples were purified using the Qiagen gel extraction kit.

The third PCR involved combining the results from 1A and 1B. Four reaction samples were constructed with different template concentrations. Reaction 2A contained 50 ng of each 1A and 1B, reaction 2B contained 100 ng of each, reaction 2C contained 250 ng of each, and reaction 2D contained 500 ng of each. The samples were run on a 0.7% agarose gel after PCR, along with samples of 1A and 1B. The resulting gel is

shown in **Figure-10**. Two samples of 1kb DNA ladder were run alongside the first set of PCR samples and the second set of PCR samples.

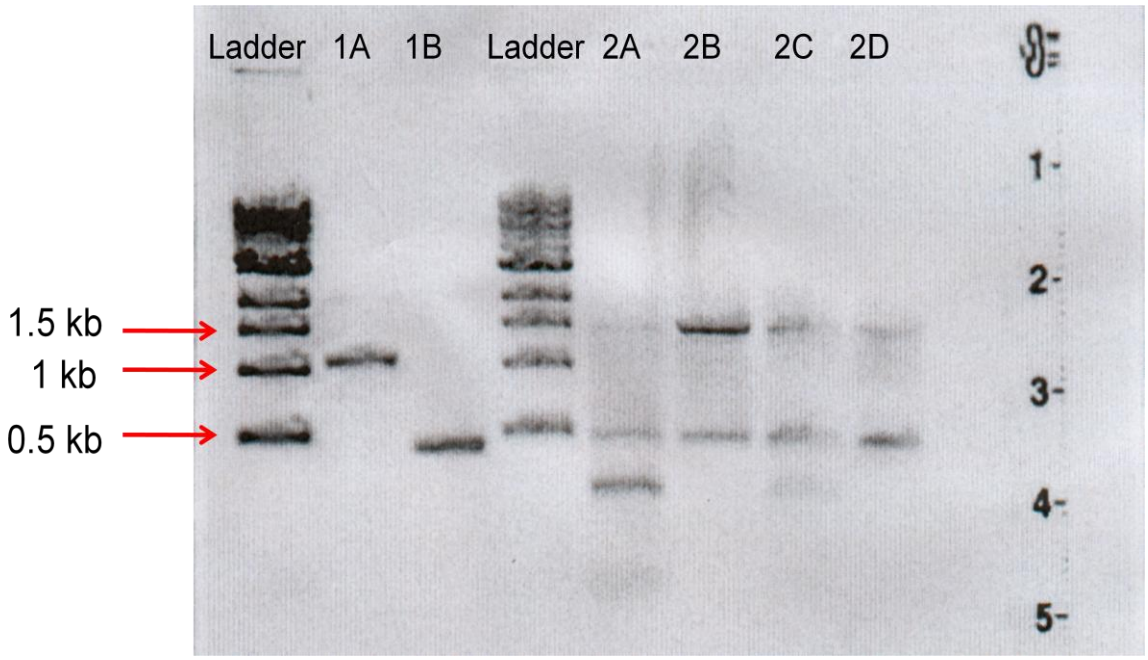


Figure-10: Gel of PCR Reactions From the Herculase PCR Approach.

The reactions 2A through 2D were purified using the Qiagen gel extraction kit. It appears from the figure that sample 2B, containing 100 ng each of template 1A and 1B, worked most efficiently to create a full length plasmid of 1.5 kb. Thus the data from the Herculase PCR approach shows the apparent production of the correct GLUT1 mutant. However, the plasmid has not been sequenced yet nor expressed in *E. coli* to produce mutant GLUT1 for protease testing. Subsequent steps would be to purify the GLUT1 fragment digest w/ HindII/XhoI, clone into pcDNA3.1+ expression vector and cut with same enzymes followed by checking expression, glucose transport, and Fxa cleavage.

Future Experiments

The mutant GLUT1 generated by the Quickchange PCR, and the other mutant generated by Herculase PCR now needs to be transfected into human embryonic kidney (HEK) cells that have been cultured. Western blotting will be used to analyze GLUT1 expression. If the mutant is successfully expressed in the HEK cells, then its testing by Factor Xa cleavage should indicate correct cutting of loop 9-10 as expected in Figure 5.

Following the assessment of expression, this Fxa (9-10/11-12) mutant will be used in Cytochalasin B cross-linking studies to determine domains of GLUT1 involved in radioactive CCB binding (**Figure-11**). The figure below shows what is likely to appear from the radioactive labeled CCB binding experiments with Fxa Cleavage. For example, if the CCB binding site is near loop 9-10, using this mutant after binding radioactive CCB will allow cleavage near the binding site. The main idea is that using the Fxa mutants for cleavage allows one to narrow down the area when looking for the domains in which substrates and inhibitors bind. Lastly, this mutant will be used in cross-linking studies coupled with proteolysis to help identify a ATP-dependent interaction between loop 6-7 and the C-terminus of GLUT1.

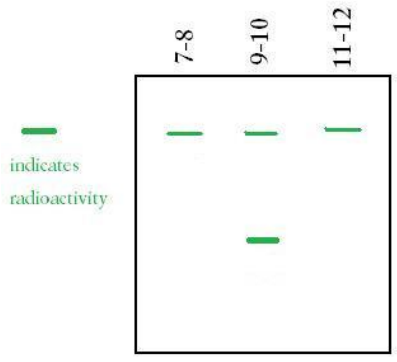


Figure-11: Plausible Results of CCB Binding Gel.

DISCUSSION

As explained in the Results, there were many failed attempts to make the GLUT1 9-10 mutant using the Quickchange PCR. Some plasmids were sequenced with no insertion of the Factor Xa site, some had double insertion of the Factor Xa site in the correct location, while still others had random insertions at areas other than the 9-10 loop. It was suspected that the original 9-10/11-12 primers were not annealing in the proper location and were annealing with the homologous sequence in loop 11-12. Scrambling of the nucleotides in the primers of the Quickchange PCR eventually led to a confirmed mutant, sequenced as in Figure 6. The mutant can now be used to transfect HEK cells to express the GLUT1.

Use of the Herculase enhanced PCR yielded a mutagenic GLUT1 faster than the trial and error method used with the Quickchange PCR. Though it has yet to be sequenced, the results of the gel shown in Figure 8 show what looks to be the correct mutant plasmid because of its length. The Quickchange experiments lasted several weeks before the correct GLUT1 mutant was identified, while the Herculase experiment lasted only 1 week. Once checked for sequencing, the GLUT1 mutant from the Herculase PCR can also be used to transfect HEK cells for expression.

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