Spring 5-1-2012

Recombinant Expression and Purification of Human Intrinsic Factor (IF) and Mutants K159D and Q201A Designed to Interfere with Cubilin Receptor Binding

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Recombinant Expression and Purification of Human Intrinsic Factor (IF) and Mutants K159D and Q201A Designed to Interfere with Cubilin Receptor Binding

A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

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May 2012

Honors Capstone Project in Biochemistry

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Date: April 25, 201

Abstract

Although both the α- and β-domains of intrinsic factor (IF) have been previously expressed, the full crystal structure of the protein has yet to be reported. The purpose of this research is to (1) express IF in order to obtain a complete crystal structure and (2) utilize a mutant form of IF in order to orally deliver rotavirus to the ileum. The first goal of this research is to express IF in the yeast *Pichia pastoris*. The second goal is to express a K159D IF mutant protein. K159 of IF plays a role in salt bridge formation between IF and the CUB_6 domain of cubilin, a critical receptor for vitamin B_{12} enterocyte passage. The third goal is to express a Q201A IF mutant protein. Q201 of intrinsic factor plays a role in the formation of hydrogen bonding between IF and the CUB_6 domain of cubilin. We hypothesize the mutants will either prevent binding to cubilin or allow binding, but not allow for receptor-mediated endocytosis. Utilizing the vitamin B_{12} pathway with the K159D or Q201A mutated IF will allow oral delivery of antigens, such as the rotavirus vaccine, to the ileum where they can trigger an IgA immune response.

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Acknowledgements

I would like to thank Professor Doyle for giving me this topic to research and supporting me. I also would like to thank the entire Doyle group, especially Debbie Valentin. I would also like to thank my Capstone reader, Professor Korendocvych for helping to edit and proofread my Capstone.

I would also like to thank the NSF-REU program at Syracuse University for funding my project as well as the iLearn program. Finally, I would like to thank Honors for allowing me to do a Capstone project.

I. Introduction

Vitamin B_{12} Pathway: Vitamin B_{12} (B_{12}), also known as cobalamin, is a water-soluble vitamin with a molecular weight of 1355.3 required for blood cell formation, brain function, and DNA synthesis[1]. It is found in animal products such as meat, fish, and milk[1]. Because of its large size, B_{12} is not absorbed through the small intestine[1]. As a result, it requires a specific pathway to enter the blood serum[2]. The B_{12} pathway begins when the dietary source of B_{12} is broken down in the mouth (Figure 1). In the mouth it is bound by haptocorrin (HC), which brings it through the stomach and into the duodenum[2]. HC then releases B_{12} through the action by pancreatic proteases and an increase in pH from about 2 to 5.5[1]. Intrinsic factor (IF) then binds to the B_{12} and brings it into the ileum[3]. Once in the ileum, the B_{12}-IF complex binds to the cubilin receptor, which is located on the surface of the intestinal cells[4]. The –Cub-IF-B_{12} complex is then transferred across the cell through receptor-mediated endocytosis in association with the transmembrane protein amnionless[3]. Once transferred, IF is cleaved by cathepsin L and the free B_{12} is picked up by transcobalamin II (TC II) and ultimately delivered to the blood serum[1].

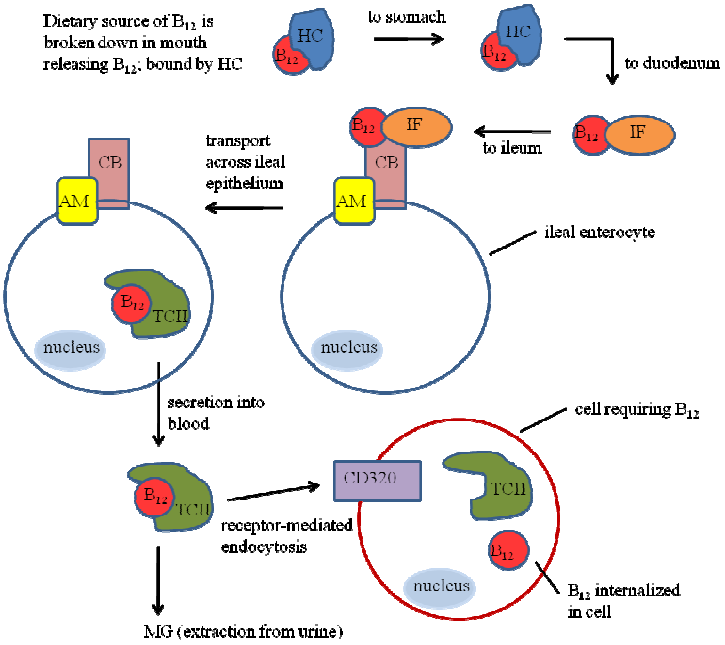


Figure 1. Dietary uptake pathway of B_{12} showing where intrinsic factor mutation will prevent B_{12} endocytosis (Red "X"). (HC: haptocorrin; B_{12}: vitamin B_{12}; IF: intrinsic factor; CB: cubilin; AM: amnionless).

Intrinsic Factor: IF is a glycoprotein in the vitamin B_{12} pathway that carries and protects B_{12} during its uptake[5]. It consists of 399 amino acids and is 43.4 kDa in humans[2]. IF is produced in the gastric mucosa and secreted in the parietal cells of the stomach[1]. It is composed of both an α- and β-domain and is about 10 to 15% glycosylated[2]. The α-domain is made up of an intertwined helical barrel while the β-domain is made up of primarily β strands[2]. The two domains are connected through a short linker segment and disulfide bridges[2]. IF has two separate binding domains; one for B_{12} and one for cubilin[1]. B_{12} is bound where the two domains meet and is mostly shielded from solute[2]. IF becomes more condensed when vitamin B_{12} binds to it, allowing it to bind to cubilin[2].

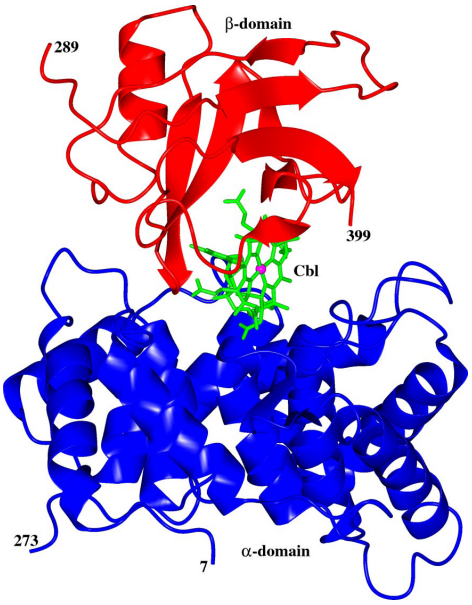


Figure 2. Crystal structure of intrinsic factor missing linker between α and β domains; Blue: α-domain, Red: β-domain, Green: Vitamin B_{12}. Image taken from reference 2 with permission (Alpers et. al.)

Cubilin-IF-B_{12} Complex: Cubilin (CUB) is the key receptor for intrinsic factor-B_{12} complexes[3]. The binding to CUB initiates receptor-mediated endocytosis[6]. The CUB_{5-8} domain of CUB interacts with IF-B_{12}[3]. The CUB_6 binding site was shown to be especially important in allowing for receptor-mediated endocytosis to occur[3]. The CUB_6 binding site is located on the α – domain of IF[3]. This binding site involves residues from helices α-7 and α-9 and the loop region between α-5 and α-6[3]. Both domains of IF recognize CUB_6 and CUB_8 through a long, positively charged side chain which forms electrostatic interactions with the glutamate and aspartate residues in the binding sites of the CUB domains (Figure 3)[3]. The K159 of IF plays a role in

salt bridge formation between the IF-B_{12} complex and cubilin[3]. The Q201 of IF plays a role in hydrogen bonding with the CUB_6 domain[3]. By mutating these regions to decrease IF-B_{12} and cubilin affinity, receptor-mediated endocytosis may be disrupted.

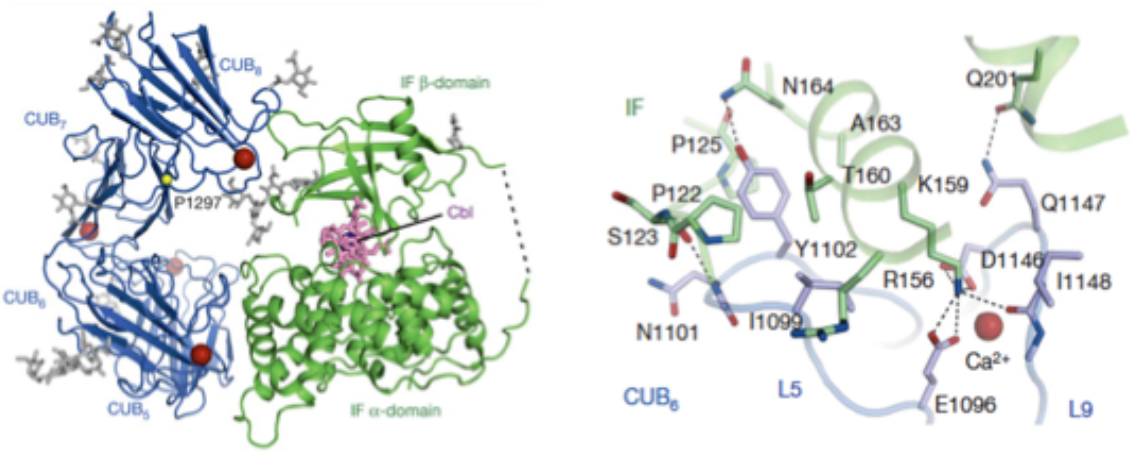


Figure 3. Left: CUB_{5-8}–IF–Cbl complex. CUB (blue), IF (green), Cbl (pink). Right: Interactions between IF and CUB_6 binding domain. Image taken from reference 3 with permission (Andersen et al.).

KM71 *Pichia pastoris* Cells: *Pichia pastoris* is a species of methylotrophic yeast, meaning it is able to reduce carbon compounds[11]. It is frequently used as an expression system for proteins because it has a high growth rate[11]. A secretion signal, *S. cerevisiae* α factor prepropeptide, was put into *P. pastoris* in order to allow the protein being expressed to be secreted into the media rather than staying within the yeast cells[12]. *P. pastoris* has two alcohol oxidase genes, AOX1 and AOX2, which allow *P. pastoris* to use methanol as an energy source as well as an inducer[12]. *P. pastoris* metabolizes methanol through the use of alcohol oxidases, which creates hydrogen peroxide as well as hydroyxmethyalene[13]. The hydroxymethyalene is sent through cycles such

as GAP and DHA[13]. In order to avoid hydrogen peroxide toxicity, peroxisomes cleave the hydroxymethyalene to produce ½ O_2 and H_2O[13].

KM71 *P. pastoris* cells were specifically chosen for the MUT^s phenotype. The MUT^s phenotype means that these colonies grow slower than the ones without the mutations, and thus can be used for selection of colonies[11]. There is a slow growth phenotype because of a mutant *aox1* locus[11].

pPIC9: The vector used for cloning was pPIC9 (Figure 4). pPIC9 is an 8000 base pair vector containing both a 5' and 3' AOX1 region[14]. The vector is ampicillin (amp) resistant and has multiple cloning sites[14]. pPIC9 is a shuttle vector, meaning it can operate in more than one organism[14]. pPIC9 can be cut by *Xho*I, *Sna*B, *Eco*RI, *Avr*II, and *Not*I[14] and also allows for secretion of the gene being expressed[14]. In order to insert at the AOX1 region, pPIC9 must be linearized with *Sac*I, which generates the MUT^s in KM71 *Pichia pastoris* cells[14].

DH5α *E. coli* Cells: DH5α *E. coli* cells are chemically competent and result in high transformation efficiency, up to $1x10^6$ transformants/μg[15]. The cells are used for efficient transformation of large plamids[15].

II. Cloning, Expression, and Purification of K159D IF Mutant

Introduction: K159 of IF plays a role in the salt bridge formation between IF and the CUB_6 domain of cubilin[3]. By mutating the lysine to an aspartic acid, we hope to disrupt the salt bridge formation, and as a result, disrupt the receptor-mediated endocytosis of IF in the B_{12} pathway. Changing the positively charged lysine to a negatively charged aspartic acid should disrupt salt bridge formation.

Site-Directed Mutagenesis: In order to create the K159D mutant, site-directed mutagenesis was conducted. Two nucleotides from AAA were changed to GAT, in order to mutate the K159 to an aspartic acid. A master mix was made consisting of 0.5 µL of *pfu* buffer, 1 µL of dNTP, 0.37 µL FWD primer, 0.47 µL REV primer, 1 µL of *pfu* turbo polymerase, 1 µL of DMSO, and 29.16 µL of dH_2O. Polymerase chain reactions (PCR) mixtures of 5 ng/µL, 10 ng/µL, 25 ng/µL, and 50 ng/µL were created using 40 µL of master mix and their respective volumes of template DNA: 1 µL, 2 µL, 5 µL, and 10 µL. Water was added to each tube to bring the final volumes to 50 µL. The primers (Figure 5) were designed in order to have a sequence that was the reverse complement of the region of DNA being mutated. They were also created to have a melting temperature between 55-65 °C.

K159D Forward:

5'CTATCGCTGTTAGATTTGCTGATACTTTGTTGGCTAACTCCTC3'

K159D Reverse:

5' GAGGAGTTAGCCAACAAAGTATCAGCAAATCTAACAGCG 3'

Figure 5. Forward and Reverse Primers for K159D Site Directed Mutagenesis

The PCR conditions can be seen in Table 1. The PCR was run for 18 cycles because two nucleotides were being mutated. The annealing temperature of 62 °C was chosen by averaging the two primer melting temperatures and subtracting 5 °C. Elongation was done at 72 °C in order for the *pfu* turbo polymerase to act most efficiently.

Segment	Cycles	Temperature	Time
1	1	95 °C	30 s
18	18	95 °C	30 s
		62 °C	1 min
		72 °C	4 min

Table 1. PCR Setup for K159D Site-Directed Mutagenesis

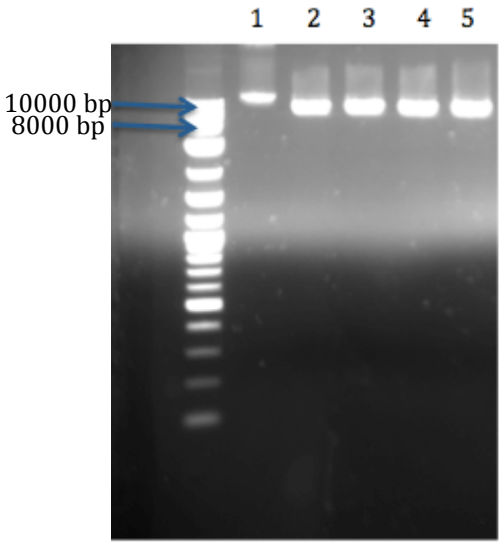
After the PCR was run, 1 µL of *Dpn*I was added to each tube in order to get rid of the methylated parent DNA. The tubes were then incubated for 1 hour at 37 °C.

Chemical Transformation into DH5α Cells: 50 µL of chemically competent DH5α *E. coli* cells were thawed on ice. 2 µL of each PCR mixture was added to a subsequent chemically competent cell tube. The tubes were left on ice for

30 minutes and then heat shocked at 42 °C in order to allow the plasmid to enter the cells. 500 µL of SOC broth was added to each tube and they were incubated for an hour at 37 °C to allow the cells to grow. Plates were streaked with each of the mixtures and then incubated overnight at 37 °C.

The next day, there were multiple colonies on all of the plates. 10 colonies were picked and grown overnight in a shaker at 350 rpm at 37 °C in 50 mL of LB with 5 µL of ampicillin (amp). The DNA was extracted from the cells using Wizard Plus SV Miniprep DNA Purification Method using the centrifugation protocol. The DNA sequences of the ten colonies were confirmed by sequencing.

Triple Digestion of pPIC9: pPIC9 was triply digested using *Eco*RI and *Not*I-hf in order to open it to insert the K159D gene. Lane 1 shows the closed vector, while Lanes 3-6 show the triply digested pPIC9 at about 8000 base pairs (Figure 6). In order to isolate the plasmid, a gel extraction was done using the QIAquick Gel Extraction Kit protocol.



Lane 1: Ladder
Lane 2: Control
Lanes 3-6: Triply Digested pPIC9

Figure 6. 1% agarose gel of triply digested pPIC9.

Double Digested pUC57.K159D: The pUC57.K159D was doubly digested using *Eco*RI and *Not*I-hf to open the vector. As can be seen in Figure 7, Lane 1 shows the pUC57.K159D vector. Lane 2 shows the doubly digested pUC57.K159D. The top band is the singly cut vector, the second band is the pUC57 vector, and the bottom band is the doubly digested K159D gene. The singly cut vector may have occurred because more vector was present than the enzymes could cut. The doubly digested gene (the bottom band) was extracted using the QIAquick Gel Extraction Kit protocol.

Lane 1: Control
Lane 2: Doubly Digested pUC57.K159D

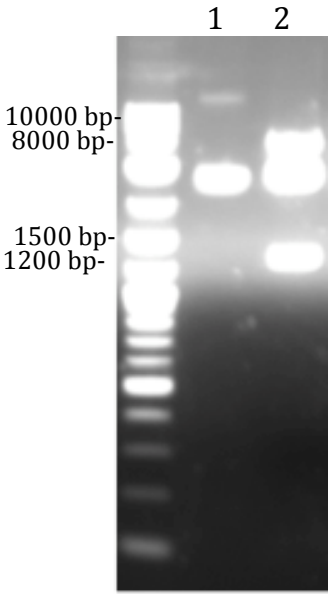


Figure 7. 1% agarose gel of double digested pUC57.K159D.

Ligation/Transformation: A molar ligation was performed at 16 °C overnight using molar ratios of 1:1, 3:1, 5:1, and 10:1. Then, a transformation was performed into chemically competent DH5α cells. Ampicillin plates were streaked and left to grow overnight at 37 °C. Eight colonies were picked for running a diagnostic agarose gel. As can be seen in Figure 8, the gel showed a

successful ligation in all colonies. Lanes 2-9 show the picked colonies. The top band is the pPIC9 plasmid while the bottom band is the K159D mutant gene. Lanes 1 and 3 were confirmed by sequencing.

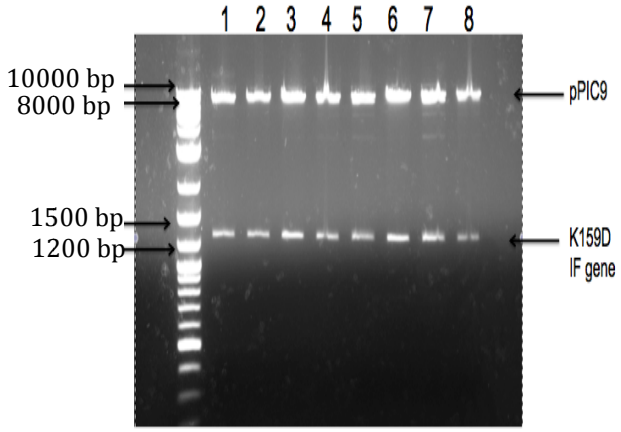


Figure 8. Diagnostic 1% agarose gel showing successful ligation of pPIC9 and K159D.

Transformation into KM71 Cells: In order to transform into *Pichia pastoris*, the plasmid must be linearized in order for a crossover to occur[12]. *Sac*I was used to linearize the DNA. As shown in Figure 9, lane 1 shows the closed pPIC9.K159D vector while lane 2 shows the linearized pPIC9.K159D vector. The closed vector is higher because closed, circular vectors move slower on gels than linearized DNA.

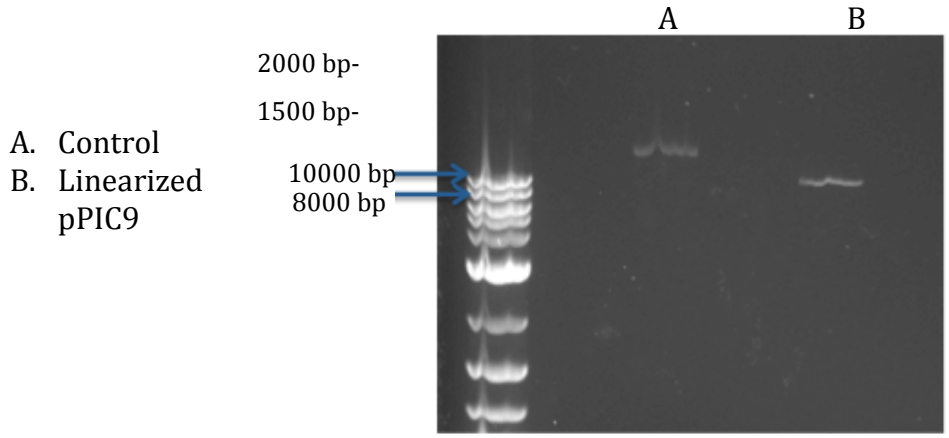


Figure 9. Linearized pPIC9.K159D cut with *Sac* I.

KM71 electrocompetent cells were thawed on ice. 10 µL of plasmid DNA was added to the cells. The mixture was electroporated at 1500 volts. 1 mL of sorbitol was added to the cuvette and incubated for 1 hour at 30 °C. The cells were streaked onto buffered media for yeast (BMY) plates and incubated at 30 °C for five days until colonies were seen.

In order to see if the transformation was successful, a colony PCR was run. A colony was picked and swirled into a tube containing a PCR mixture. It was then placed in the PCR machine and sat overnight at 16 °C. As can be seen in Figure 10, all of the colonies showed that the DNA had been successfully inserted. However, Colony D was chosen for purification because it was the brightest and did not have any other bands in the lane. The faint bands at the bottom of the gel are known as primer dimer, because it is the two primers annealing to each other rather than the DNA.

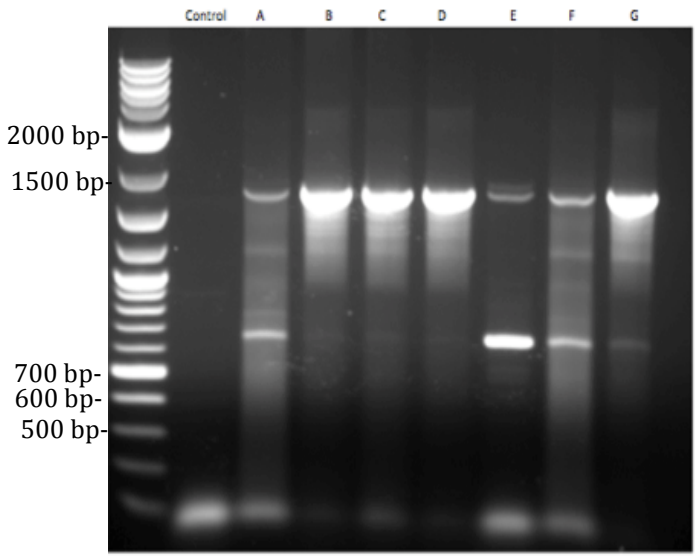


Figure 10. Colony PCR of transformed KM71 *Pichia pastoris* cells.

K159D Expression: In order to express the K159D protein, it was first induced in 10 mL of buffered media with glycerol for yeast (BMGY). After 18 hours, 1 mL of this culture was transferred into 600 mL of BMGY. After 16 hours the culture was centrifuged and resuspended in 200 mL of buffered media with methanol for yeast (BMMY). This was incubated at 30 °C for 48 hours and then centrifuged. The media was then collected and filtered for purification through the use of fast protein liquid chromatography (FPLC).

In order to find the optimum time for growth, a time course experiment was run. 200 mL of BMMY media was incubated for 96 hours. After every 24 hours, 1 mL of media was removed. After 96 hours, all of the 1 mL samples were run down an SDS-PAGE gel. As can be seen in Figure 11, 48 hours was the optimum time for growing the K159D mutant. After 72 hours (Lane 8), all of the protein bands disappeared. The bands at 48 and 72 hours

looked about the same darkness, resulting in growing the media for 48 hours for optimum expression.

The arrows on the right in Figure 11 show the three protein bands being looked for. The band at about 75 base pairs is glycosylated K159D, causing it to run higher than the 43.4 kDa of IF. The band at about 45 base pairs is unglycosylated K159D, at about the same height as unglycosylated IF. The band at about 25 base pairs is consistent with the α-domain of the K159D IF mutant

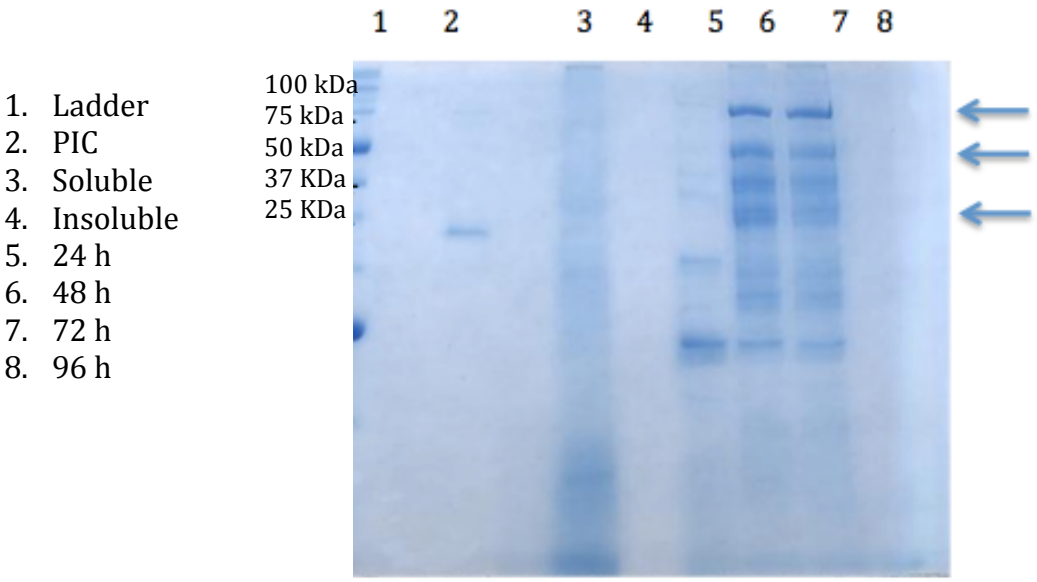


Figure 11. 12% SDS-PAGE gel of the K159D IF mutant time course over a period of 96 hours.

K159D Purification: Fast protein liquid chromatography (FPLC) was used in order to purify the K195D protein from the media. 150 mL of the media was centrifuged at 8500 rpm for 1 hour at 4 °C. The media was then filtered and injected into the FPLC. A 5 mL Histrap Ni affinity column was used with a

binding flow rate of 1 mL/min and a binding buffer containing 1x PBS (2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and 0.5M NaCl. The elution flow rate was 0.5mL/min with an elution buffer containing 1x PBS, 0.5M NaCl, and 1 M imidazole. The elution was run from 0-100% for 50 mL. The resulting data showed two peaks in the FPLC trace, as can be seen in Figure 12.

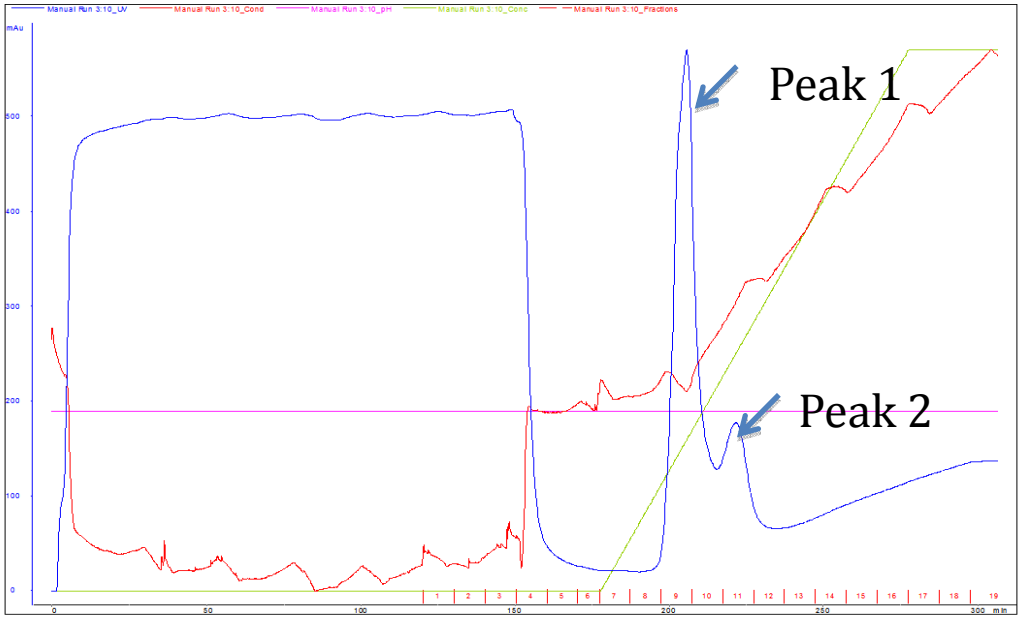


Figure 12. FPLC trace of purified K159D showing two eluted peaks.

The corresponding SDS-PAGE gel (Figure 13) showed that peak 2 contained bands that matched up with the IF control. The first band at about 55 kDa is glycosylated K159D. The second band at about 46 kDa is unglycosylated K159D. The third band at about 30 kDa may be the α-domain of the intrinsic factor mutant, suggesting that some of the K159D IF is cleaved

between the α- and β- domains during expression. This means there may be proteases native to *Pichia pastoris* that can cleave IF.

1. PIC (preinduction control)
2. FT (flowthrough)
3. Peak 1
4. Peak 2
5. IF control (2 mg/0.1 mL 10% SDS)

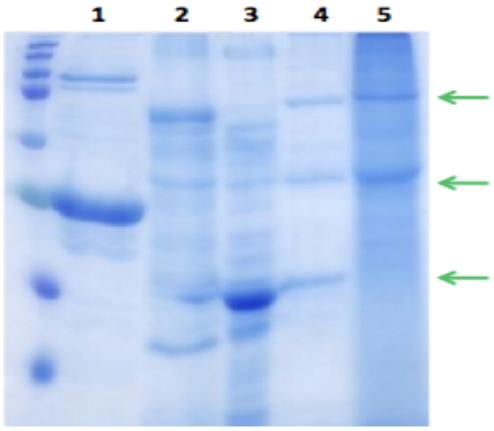


Figure 13. SDS-PAGE gel of FPLC purified K159D

Confirmation of K159D Mutant: A western blot was done in order to show that K159D mutant had been expressed and purified. 750 µL of 5 mL elution fraction samples obtained from the FPLC were trichloro acetic acid (TCA) precipitated. Then the samples were run down a 12% SDS-PAGE gel for 90 minutes at 150 V along with an IF control (2 mg dissolved in 100 µL of 10% SDS). The gel was stained overnight using commassie blue dye. The protein on the SDS-PAGE gel was transferred to a membrane using the iBlot system. The membrane was then immunoblotted using IF antibody (abcam, abcam91322 and Santacruz, sc2370) at a 1:500 dilution. A second western blot was also done using HisTag antibody (abcam, ab1187) at a 1:2000 dilution.

L: Ladder
1: crude K159D
2: eluted K159D
IF: control

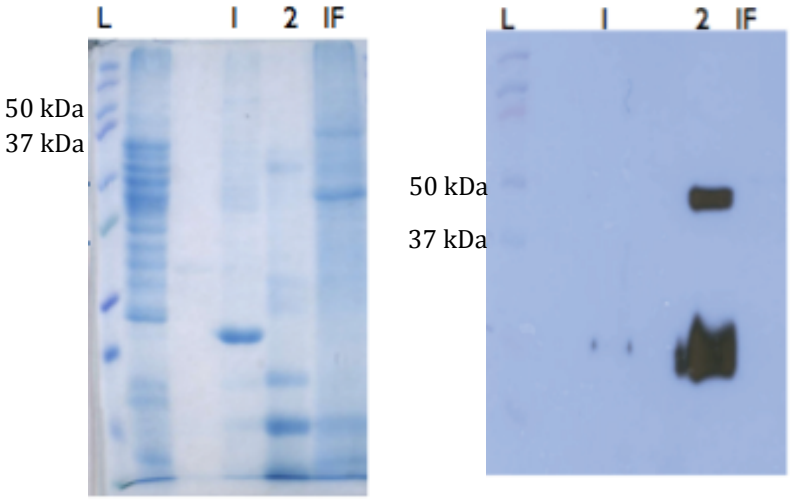


Figure 14. Left: SDS-PAGE gel of membrane used for western blots; Right: His-tag antibody western

Confocal Microscopy Uptake Studies: In order to see how the K159D mutant interacted with cubilin in vivo, confocal microscopy uptake studies were done. In order to prepare cultures for confocal uptake studies, purified protein was dialyzed in 0.1 M sodium bicarbonate buffer, pH 9.0 for 24 hours in 50 MWCO dialysis tubing. Alexa Fluor 488 was added in a 1:1 molar ration. Alexa Fluor 488 attaches to the primary amines of protein through a TFP ester moiety. The solution was rotated for 1 hr covered in aluminum foil. The protein and Alexa Fluor were then dialyzed in 1 L PBS, pH 7.4 for 24 hours three times. B_{12} was added in a 1:1 molar ratio and the samples were filtered and added to 200,000 cells/mL in confocal dishes. The cells were incubated for 45 minutes and then washed three times with PBS at pH 7.4 and three times with PBS at pH 3.

As can be seen in Figure 15, both the IF control and K159D were localized in the cytoplasm of the cells, indicating possible receptor-mediated endocytosis. The BN16 cells overexpress CUB and thus the IF would be seen

inside the cells if it is endocytosed. We had hypothesized that mutating the K159 would disrupt receptor-mediated endocytosis, but from the confocal uptake studies it could be seen that this was not the case. We were looking for the K159D mutant to bind to the cubilin, but not be taken into the cells. As can be seen n Figure 11, the K159D protein is internalized, indicating K159D is not critical for binding despite its involvement in Ca^{2+} binding.

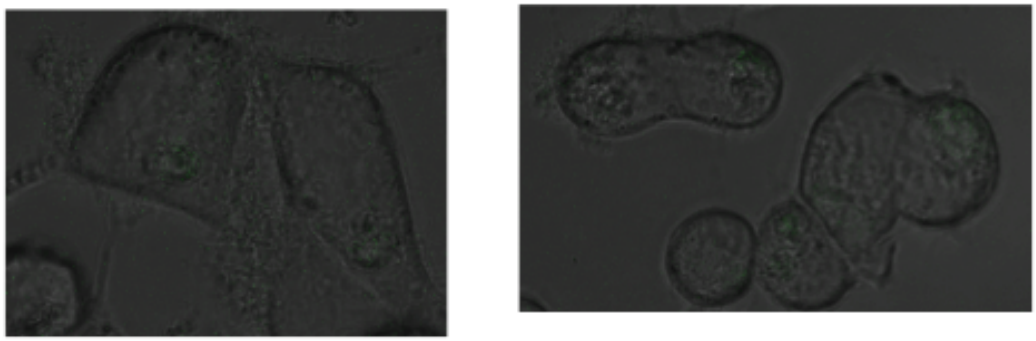


Figure 15. Right: Confocal images of IF inside BN16 cells visualized using Alexa Fluor 488. Left: Confocal images of K159D inside BN16 cells visualized using Alexa Fluor 488.

III. Cloning, Expression, and Purification of Q201A Mutant IF

Introduction: Q201 of IF plays a role in the formation of hydrogen bonds between IF and the CUB_6 domain of cubilin[3]. By mutating a different part of the binding site, we hope to disrupt the receptor-mediated endocytosis of IF in the vitamin B_{12} pathway.

Site-Directed Mutagenesis: In order to create the Q201A mutant, site-directed mutagenesis was done. Two nucleotides were changed in order to mutate the Q201 to an alanine. A master mix was made consisting of 0.05 µL of *pfu* buffer, 1 µL of dNTP, 0.37 µL FWD primer, 0.47 µL REV primer, 1 µL of *pfu* turbo polymerase, 1 µL of DMSO, and 29.16 µL of dH_2O. PCR mixtures of 5 ng/µL, 10 ng/µL, 25 ng/µL, and 50 ng/µL were created using 40 µL of master mix and their respective volumes of template DNA: 1 µL, 2 µL, 5 µL, and 10 µL. Water was added to each tube to bring the final volumes to 50 µL. The primers (Figure 16) were designed in order to have a sequence that was the reverse complement of the region of DNA being mutated. The primers were also created to have a melting temperature between 55-65 °C.

FWD Primer:
5'-GATACAGATCATTGTTCGGAGCAGTTTTGAAGGACATCGTTGA-3'
REV Primer:
5'-GTCCTTCAAAACTGCTCCGAACAATGATCTGTATCCCTC-3'

Figure 16. Forward and reverse primers for Q201A mutagenesis.

The PCR conditions can be seen in Table 2. The PCR was run for 18 cycles because two nucleotides were being mutated. The annealing temperature of 62 °C was chosen by averaging the two primer melting temperatures and

subtracting 5 °C. The final stage was done at 72 °C in order for the *pfu* turbo polymerase to act most efficiently.

Segment	Cycles	Temperature	Time
1	1	95 °C	30 s
18	18	95 °C 62 °C 72 °C	30 s 1 min 4 min

Table 2. PCR conditions for Q201A site-directed mutagenesis.

After the PCR was run, 1 µL of *Dpn*I was added to each tube in order to get rid of the methylated parent DNA. The tubes were then incubated for 1 hour at 37 °C.

Chemical Transformation into DH5α Cells: 50 µL of chemically competent DH5α *E. coli* cells were thawed on ice. 2 µL of each PCR mixture was added to a subsequent chemically competent cell tube. The tubes were left on ice for 30 minutes and then heat shocked at 42 °C in order to allow the plasmid to enter the cells. 500 µL of SOC broth was added to each tube and they were incubated for an hour at 37 °C to allow the cells to grow. Plates were streaked with each of the mixtures and then incubated overnight at 37 °C.

The next day, there were multiple colonies on all of the plates. Six colonies were picked and grown overnight at 350 rpm at 37 °C in 50 mL of LB with 5 µL of amp. The DNA was extracted from the cells using Wizard Plus SV Miniprep DNA Purification Method using the centrifugation protocol. The DNA sequences of the six colonies were confirmed by sequencing.

Triple Digestion of pPIC9: pPIC 9 was triply digested using *Eco*RI and *Not*I-hf in order to open it to insert the Q201A gene. Lane 1 shows the closed vector, while Lanes 3-6 show the triply digested pPIC9 at about 8000 base pairs (Figure 17). In order to isolate the plasmid, a gel extraction was done using the QIAquick Gel Extraction Kit protocol.

Double Digested pUC57.Q201A: The pUC57.Q201A was doubly digested using *Eco*RI and *Not*I-hf to open the vector. As can be seen in Figure 17, Lane 7 shows the pUC57.Q201A vector. Lanes 8-9 show the doubly digested pUC57.Q201A. The first band is the pUC57 vector, and the bottom band is the doubly digested Q201A gene. The doubly digested gene (the bottom band) was extracted using the QIAquick Gel Extraction Kit protocol.

1. Ladder
2. pPIC9 control
3-6. Triple digested pPIC9
7. Q201A control
8-9. Double digested Q201A

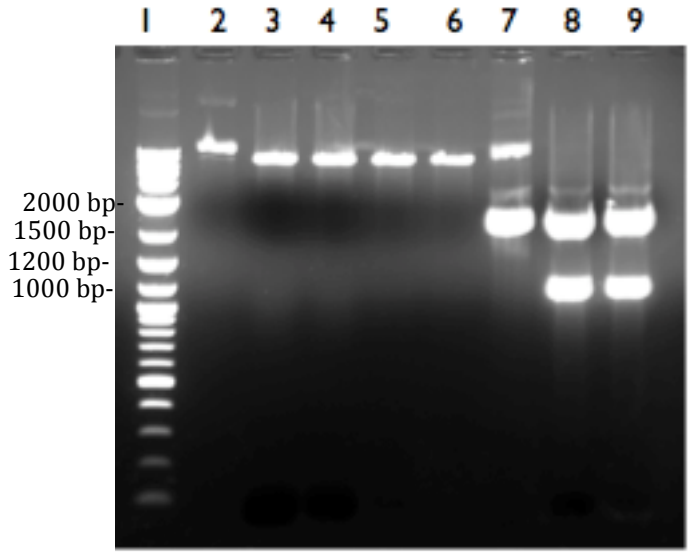


Figure 17. 1% agarose gel of triply digested pPIC9 and double digested pUC57.Q201A.

Ligation/Transformation: A molar ligation was performed at 16 °C overnight using molar ratios of 1:1, 3:1, 5:1, and 10:1. Then, a transformation

was performed into chemically competent DH5 α cells. Ampicillin plates were streaked and left to grow overnight at 37 °C overnight. Eight colonies were picked for running a diagnostic agarose gel. As can be seen in Figure 18, the gel showed a successful ligation in all colonies except for colony 6. Lanes 2-9 show the picked colonies. Colony 6 showed the pPIC9 vector at a much lower height than it should have been. The top band is the pPIC9 plasmid while the bottom band is the K159D mutant gene. Lanes 2 and 3 were confirmed by sequencing.

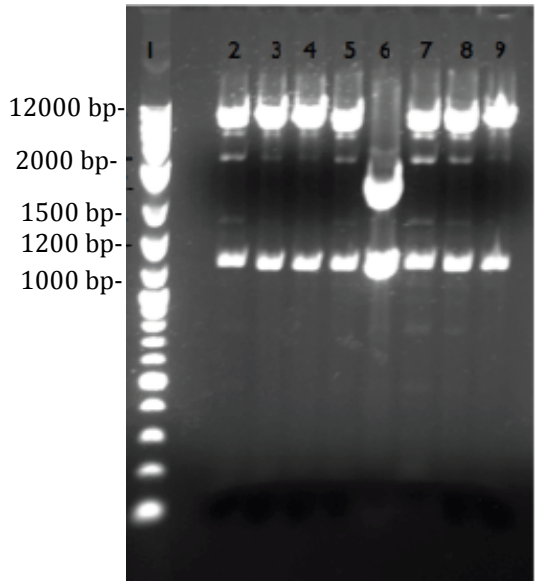


Figure 18. Diagnostic gel showing successful ligation of pPIC9 plasmid and Q201A gene in lanes D and E.

Transformation into KM71 *P. pastoris* Cells: In order to transform into *P. pastoris*, the plasmid must be linearized in order for a crossover to occur[12]. *Sac*I was used to linearize the DNA.

KM71 electrocompetent cells were thawed on ice. 10 µL of plasmid DNA was added to the cells. The mixture was electroporated at 1500 volts. 1 mL of sorbitol was added to the cuvette and incubated for 1 hour at 30 °C.

The cells were streaked onto BMY plates and incubated at 30 °C for five days until colonies were seen.

In order to see if the transformation was successful, a colony PCR was run. A colony was picked and swirled into a tube containing a PCR mixture. It was then placed in the PCR machine and sat overnight at 16 °C. As can be seen in Figure 19, only colonies D and E suggested successful transformations. Lane 1 was the control.

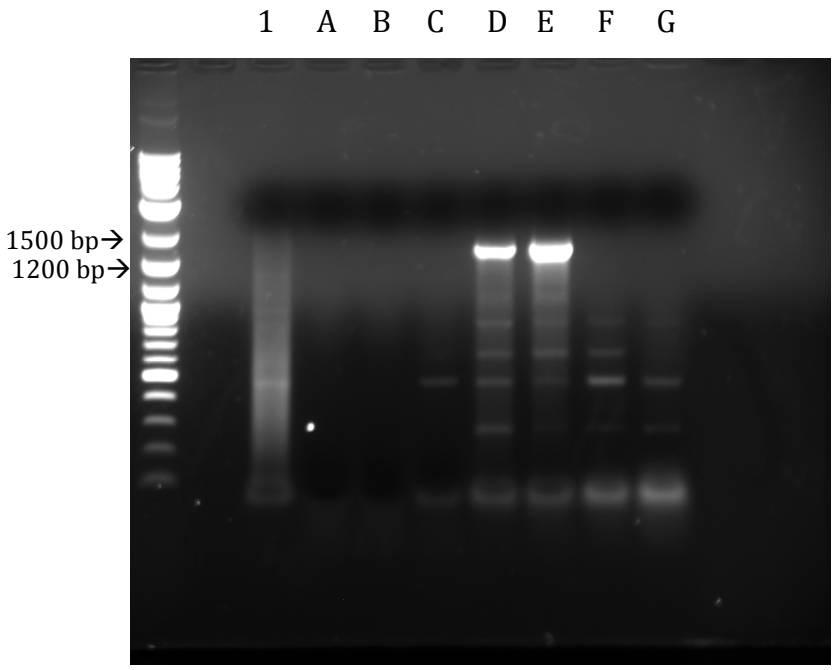


Figure 19. Colony PCR of transformed KM71 *Pichia pastoris* cells.

Q201A Expression: In order to express the Q201A protein, it was first induced in 10 mL of BMGY. After 18 hours, 1 mL of this culture was transferred into 600 mL of BMGY. After 16 hours the culture was centrifuged and resuspended in 200 mL of BMMY. The media was incubated at 30 °C for

48 hours and then centrifuged. The media was then collected and filtered for purification.

In order to find the optimum time for growth, a time course experiment was run. 200 mL of BMMY media was incubated for 96 hours. After every 24 hours, 1 mL of media was removed. After 96 hours, all of the 1 mL samples were run down an SDS-PAGE gel. 48 hours was the optimum time for growing the Q201A mutant. The bands at 48 and 72 hours looked about the same darkness, resulting in growing the media for 48 hours for optimum expression. The bands at 96 hours look a little darker, but not enough to increase the expression period by 48 hours.

Lane 1: Ladder
Lane 2: PIC
Lane 3: soluble
Lane 4: insoluble
Lane 5: 24h
Lane 6: 48h
Lane 7: 72h
Lane 8: 96h

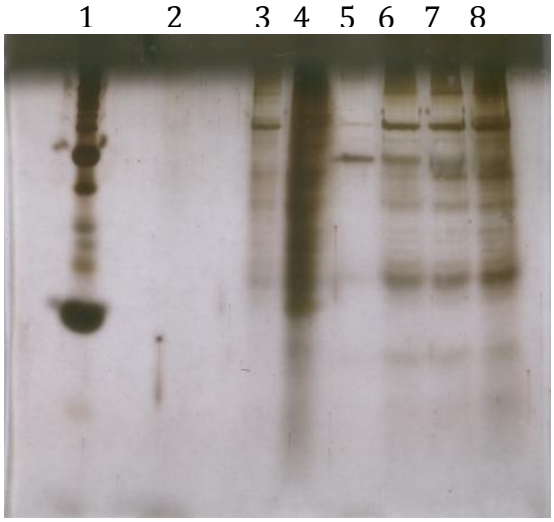


Figure 20. SDS-PAGE gel of the Q201A IF mutant time course over a period of 96 hours.

Q201A Purification: A fast protein liquid chromatography (FPLC) was used in order to purify the Q201A protein from the media. 150 mL of the media was centrifuged at 8500 rpm for 1 hour at 4 °C. The media was then filtered and run down the FPLC. A 5 mL Histrap Ni affinity column was used with a

binding flow rate of 1 mL/min and a binding buffer containing 1x PBS (2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and 0.5M NaCl. The elution flow rate was 0.5mL/min with an elution buffer containing 1x PBS, 0.5M NaCl, and 1 M imidazole. A stepwise elution was done with three different percentages of elution buffer: 4, 10, and 25 percent. The resulting data showed three peaks as can be seen in Figure 21.

The first peak was eluted with 4% elution buffer. In the corresponding SDS-PAGE gel, see Figure 22, there were many bands, showing that the first peak was other stuff that had stuck to the column. The second peak was eluted with 10% elution buffer, and had bands showing the correct height for the IF mutant. The third peak was eluted with 25% elution buffer and did not show any bands on the SDS-PAGE with commassie staining.

Figure 21. FPLC trace of Q201A stepwise elution.

Confirmation of Q201A Mutant: A western blot was conducted in order to show that the Q201A mutant had been expressed and purified. 750 µL of 5 mL elution fraction samples obtained from the FPLC were TCA precipitated.

Then the samples were run down a 12% SDS-PAGE gel for 90 minutes at 150 V along with an IF control (2 mg dissolved in 100 µL of 10% SDS). The gel was stained overnight using commassie blue dye. Western blots were also performed on the samples obtained from the FPLC. The protein on the SDS-PAGE gel was transferred to a membrane using the iBlot. The membrane was then immunoblotted using HisTag antibody (abcam, ab1187) at a 1:2000 dilution.

As can be seen in Figure 22, bands at about 46 kDa appeared on the western blot in both the 4% elution and 10% elution peaks. In the 10% (lanes 8-10), the α fragment can also be seen at about 25 kDa.

Lane 1: PIC
Lane 2: FT
Lane 3: Ladder
Lane 4-6:4%
Lane 7-10: 10%
Lane 11-12: 25%

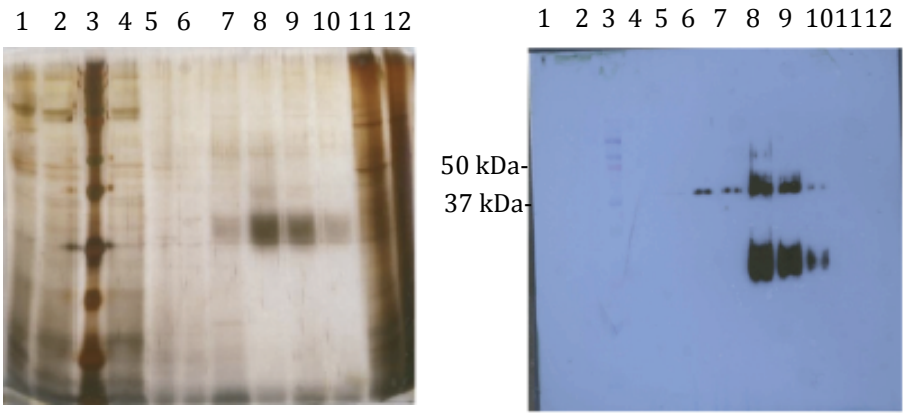


Figure 22. Left: SDS-PAGE gel of FPLC peaks of Q201A Right: His-tag western blot of Q201A

IV. Conclusion

In the future, the K159D confocal micrscopy has to be redone. Also, the Q201A confocal micrscopy has to be done. The IF, K159D, and Q201A proteins are being tested for specific cubilin binding in collaboration with Professor Mette Madsen, University of Aarhus, Denmark. Professor Madsens's lab will look at the specific binding affinities between each of the mutants and cubilin, allowing us to compare them quantitatively rather than just through images on the confocal.

Research on the K159D and Q201A mutants will help to shed light on important intrinsic factor binding sites. By binding rotavirus to the mutants, a safer alternative to the current vaccine may be created, leading to better third-world access to the drug. Finally, utilizing the B_{12} uptake pathway in this way, it could become a delivery system for other vaccines, offering a new platform for drug delivery.

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Capstone Summary

The vitamin B12 pathway is an important delivery mechanism for the body to absorb B12[5]. Food containing B12, such as meat, is broken down in the mouth where the B12 is then picked up by the transport protein haptocorrin (HC)[5]. The HC-B12 complex goes through the stomach and HC is broken down by a change in pH and proteases[5]. B12 is picked up by IF and brought into the small intestine. The IF-B12 complex is then bound to cubilin (CUB) and transported into the epithelial cells of the small intestine[3]. The B12 is eventually delivered into the blood serum[3].

In order to use this pathway for drug delivery, we had to create a mutant form of IF that would bind to cubilin but not enter the cell. This way, the drug would stay localized in the small intestine to trigger an immune response. It has been shown that the CUB_{5-8} binding domains are important for transporting the IF-B12 into the cell, especially the CUB_6 and CUB_8 domains[3]. We examined the binding between the CUB_6 domain of cubilin and IF and distinguished two amino acids that could be changed to disrupt binding. The first mutation was a K159D mutant, which changed the lysine of IF to an aspartic acid. This would disrupt salt bridge formation between cubilin and IF. The other mutation was a Q201A mutant, which changed the glutamine to an alanine. This would disrupt hydrogen bonding between cubilin and IF.

A site-directed mutagenesis was done in order to create both the K159D and Q201A mutants. Site-directed mutagenesis is a procedure in

which a specific area of a DNA molecule is mutated. The IF DNA molecule was mixed with primers containing the mutations we were trying to create in the IF protein. A polymerase chain reaction (PCR) was run in order for the primers to bind to the IF DNA molecule and make many copies of it that contained the mutations. The original non-mutated IF DNA was removed through the use of the enzyme *Dpn*I, which gets rid of methylated DNA. We confirmed the correct mutations were made and that no other mutations had occurred during the process by sending off the samples for sequencing.

Next, we needed to express both the K159D and Q201A mutants so that we could get enough of each for experiments. This was done through the use of *Pichia pastoris*, a yeast. The K159D and Q201A mutants were placed inside the yeast cells through the use of a vector. As the yeast grew, it also secreted the mutant proteins into the media.

The IF mutants were expressed in *Pichia pastoris* cells, which are yeast cells. The DNA was inserted into the cells and grown up in media, while also secreting the IF mutants into the media. However, the protein is mixed in with all of the nutrients and other things the yeast is secreting while growing. As a result, we had to purify the media in order to collect only the protein. This was done through the use of fast protein liquid chromatography (FPLC). The media is injected into the FPLC and run through a nickel affinity column. Both of the IF mutants are tagged with six histidine residues at the end of their amino acid chains, allowing them to bind to the nickel column while everything else is flushed away. In order to collect the protein, the

column is washed with a buffer containing imidazole. Imidazole has a similar structure to histidine; allowing it to bind to the nickel column and in the process, knock off the IF mutants.

In order to ensure we were expressing and purifying the correct proteins, we ran two experiments to examine the samples. The first method we used was running an SDS-PAGE gel. The SDS-PAGE gel separates proteins based on their size. By running our samples on the gel along with an IF control, we were able to compare the bands of our protein to the band of the IF protein. IF is about 45 kDa and the mutants should appear at the same height, as one amino acid switch will not affect its size by very much. Both proteins showed up at the correct heights. However, there was also a higher band at about 55 kDa and a lower band at about 30 kDa. The 55 kDa band is glycosylated IF, meaning it has had sugars added to it, while the lower band is the α domain of IF.

The other test that we performed on the purified samples was a western blot. Another SDS-PAGE gel was run and the protein bands were transferred to a membrane. Antibodies specific to IF are washed over the membrane and bind to anything with the IF binding site for the antibody. A second antibody is washed over the membrane that will bind to the first antibody to enhance the signal. These sites are then detected by spraying a luminescent solution over the membrane and then transferring it to a film. The film is developed and the resulting bands can be seen on it. As with the SDS-PAGE gel, an IF control was run with the samples so that we could

compare bands. As with the SDS-PAGE gel, the bands matched and also ensured that the IF mutants were being created, as the antibodies are specific only to IF.

Confocal studies allowed us to see how the mutants interacted with cubilin. The K159D protein was dyed green so that we could track their interactions with BN16 cells, which overexpress cubilin. Preliminary experiments showed that the protein entered the cell, which is not what we had attempted to do. As previously mentioned, we had wanted to stop the proteins from entering the cells.

However, future work entails redoing this experiment with both mutants as well as sending off samples to a lab in Denmark. They will look at the specific binding affinities between each of the mutants and cubilin, allowing us to compare them quantitatively rather than just through images on the confocal.

Research on the K159D, Q201A, and K159D Q201A mutants will help to shed light on important intrinsic factor binding sites. By binding rotavirus to the mutants, a safer alternative to the current vaccine may be created, leading to better third-world access to the drug. Finally, utilizing the B_{12} uptake pathway in this way, it could become a delivery system for other vaccines, offering a new platform for drug delivery.