Biochemical Features of Anti-Amyloidogenic Activity of Gastrokine-1

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ABSTRACT

Gastrokine 1 (Gkn1) is an 18 kDa protein produced and secreted into the lumen of the stomach. It is stable and protease-resistant, which allows it to resist degradation in the gastrointestinal (GI) tract. To examine the function of Gkn1 *in vivo*, Gkn1$^{-/-}$ mice were previously generated. Gkn1$^{-/-}$ mice are healthy and have a normal lifespan. However, Gkn1$^{-/-}$ mice have markedly reduced body fat compared to wild type (WT) littermates, and they are resistant to weight gain on a high fat diet. Gkn1$^{-/-}$ mice are not diabetic, have normal appetite and physical activity, and do not malabsorb calories. We hypothesize that Gkn1 influences phenotype through regulation of the intestinal microbiota. Gkn1 prevents a fundamental amyloid function of bacteria, namely biofilm formation. This study investigates the biochemical features of Gkn1 that mediate its anti-amyloidogenic effects, in particular its BRICHOS domain, which is associated with amyloid fiber binding. For *in vitro* experimentation, a *Kluyveromyces lactis* yeast model was created for generation of large quantities of Gkn1. Site-directed mutagenesis was then used to change specific conserved amino acids in the BRICHOS domain for subsequent generation of mutant protein. To compare the abilities of Gkn1 and mutant Gkn1 to inhibit biofilm formation, biofilm assays were performed with bacterial strain LF82. Preliminary results suggest that certain amino acids in the BRICHOS domain, while conserved in evolution, are not critical for the anti-amyloidogenic activity of Gkn1. We are currently testing other mutant forms of Gkn1 to further elucidate the biochemistry of this function.

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INTRODUCTION

1.1 Obesity

The worldwide obesity epidemic has been designated as one of the greatest public health challenges of our time. Recent studies indicate that approximately one-third of the United States population is obese, which is defined by a body mass index (BMI) of greater than 30.0 (Flegal et al. 2010). This excess weight has negative consequences for the health of individuals and the economic health of the nation, since physical impairments and medical conditions associated with obesity decrease the labor supply (Renna and Thakur 2010). The cost of obesity is also reflected in healthcare expenditures. Between 0.7% and 2.8% of worldwide healthcare spending is attributable to the condition, since obesity is associated with various comorbidities such as cardiovascular disease, type 2 diabetes, stroke, and certain types of cancer (Shea et al. 2012). In 2016, the average annual medical costs of an obese person were approximately 42 percent higher than a person of normal weight (Goforth 2016). This gap will likely continue to widen, since per capita health care spending has grown faster for individuals in above-normal weight categories in recent decades (Duchovney and Baker 2010).

The obesity problem has led to research efforts in both prevention and treatment. Many doctors prescribe diet and exercise as a solution, yet studies have shown that long term, sustained weight loss is difficult to maintain (Bray 2012). In addition, behavioral interventions frequently fail to reach certain segments of the population, such as those with low incomes (Anthes 2014). Other treatment options include surgery and weight loss drugs. During bariatric surgeries, doctors shrink the

size of the stomach and reroute digestion past the early parts of the small intestine, which restricts intake and limits absorption of food (Weight-control Information Network 2011). Despite the general effectiveness of the procedures, they are costly and risky, and some patients still suffer from weight regain (Ghaferi and Varban 2018).

Prescription drugs may be a practical solution to the obesity epidemic. Researchers have found that weight loss drugs, when used in combination with lifestyle interventions, caused patients to lose 3-9% more weight than patients receiving a placebo (Yanovski and Yanovski 2014). However, medications can have serious side effects. Two popular appetite-suppressants, fenfluramine and dexfenfluramine, were pulled from the market in 1997 after being linked to the development of valvular heart disease and primary pulmonary hypertension (National Institute 2004). Few drugs are currently approved by the FDA. For years, the mainstays of treatment were phentermine, an amphetamine that suppresses appetite, and orlistat, a lipase inhibitor that interferes with the body's ability to absorb fat (Shonders et al. 2018). In 2012, two new drugs were approved: a phentermine/topiramate extended release capsule (Qsymia) and lorcaserin, which promotes satiety by activating a particular serotonin receptor in the brain (Anthes 2014). Most recently, the FDA approved naltrexone sustained release (SR)/bupropion SR and liraglutide in 2014 (Shonders et al. 2018). Each of these medications comes with a variety of adverse effects and risks. For instance, topiramate causes the development of oral clefts in fetuses of pregnant patients, while liraglutide has been linked to acute and chronic pancreatitis (The Lancet 2012;

Jensen, Saha, and Steinberg 2015). While no drug is expected to be a magic bullet, it is obvious that identification of new drug targets is vital. One promising area is the gut microbiome, which has been shown to play a critical role in host adiposity and energy metabolism (Martin et al. 2015, Mazidi et al. 2016).

1.2 Metabolism and the Gut Microbiota

Microbiota transplantation experiments have connected the composition of the gut microbiota with obesity. These studies use germ-free mice, which have no contact with bacteria and are raised in a sterile environment. When the germ-free mice are colonized with microbes from obese donors, they gain weight and fat mass without a significant increase in chow consumption (Turnbaugh et al. 2006). Germ-free mice are also protected from diet induced obesity when placed on a high-fat, high-sugar diet, indicating that alterations in the gut microbiome are an important player in the fat accumulation induced by a Western-style diet (Backhed et al. 2007). Furthermore, weight loss and metabolism changes after bariatric surgery may be partly mediated by changes in the microbiota (Tremaroli et al. 2015). Germ-free mice were transplanted with fecal microbiota of obese patients and bariatric surgery patients, and the mice receiving the bacteria from patients post bariatric surgery gained significantly less body fat (Tremaroli et al. 2015).

Gut microbes influence host energy balance by impacting energy harvest and caloric extraction from the diet (Backhed et al. 2004). Some dietary calories come from indigestible carbohydrate fibers and proteins, and bacteria in the gut are able to break down these macromolecules into smaller particles that the host can absorb. The bacteria can also affect obesity by producing metabolites. The breakdown of

dietary fiber produces short chain fatty acids (SCFAs) such as propionate and butyrate, which promote metabolic benefits in energy homeostasis by activating intestinal gluconeogenesis (De Vadder et al. 2014). Bacteria also metabolize primary bile acids into secondary bile salts, which may mitigate against weight gain and benefit host metabolic health (Sayin et al. 2013).

Recent studies have analyzed the differences in gut flora between obese and lean individuals. While the microbiota varies between individuals, the vast majority of bacteria are from the phyla *Firmicutes* and *Bacteriodetes*. A higher ratio of *Firmicutes:Bacteriodetes* is associated with obesity, while the opposite is associated with leanness, indicating that the composition of the microbiome impacts host phenotype (Turnbaugh et al. 2006). Some studies have aimed to determine which specific bacterial family, genera, or species may be linked to obesity (Bianchi et al. 2019). The genus *Bifidobacterium* has been shown to be more abundant in lean subjects compared to overweight groups and negatively correlate with visceral fat (Reyes et al. 2016; Aoki et al. 2017). Other analyses divide patients into two microbial enterotypes: the *Prevotella* P-type or *Bacteriodetes* B-type. It was shown that the *Prevotella* enterotype is predominant in individuals consuming lots of carbohydrates and fiber, while the *Bacteriodetes* enterotype was more common in individuals consuming more protein and animal fat (Christensen et al. 2018). This study, among others, provides evidence that dietary ingredients can modulate the microbiota.

Adjusting the gut flora with diet or drugs could induce healthy changes in patients with metabolic dysregulation or pathogenic intestinal conditions. A large

number of non-antibiotic human-targeted drugs have been shown to impact specific bacterial strains, indicating that some drugs could be repurposed as specific microbiome modulators (Maier et al. 2018). Furthermore, metformin, a common medication for treatment of type II diabetes, has already been observed to positively influence host metabolism through the promotion of butyrate-producing taxa (Forslund et al. 2015). Medications could also be used to target entire microbial communities and higher order bacterial structures known as biofilms.

1.3 Biofilms and Amyloid Fibers

Biofilms are communities of surface-adherent organisms that are embedded in complex extracellular matrices of self-produced polysaccharides. The colonies adhere to surfaces, and many microbes have developed systems to bind to mucin, the glycoprotein that is the major component of mucosa (de Vos 2015). The bacterial populations undergo frequent horizontal gene transfer and behave similarly to eukaryotic cells or multifaceted ecosystems; this cooperation allows the bacterial cells to grow and survive in hostile environments (Costerton et al. 1995). As a result, biofilms are inherently resistant to both antimicrobial agents and host defenses. A particular problem is their formation on therapeutic medical devices like protheses and catheters, which leads to persistent bacterial infections (Fux 2003). Furthermore, many recent studies have associated biofilm formation with disease states such as bacterial endocarditis, cystic fibrosis, and the two main types of inflammatory bowel disease (IBD): ulcerative colitis and Crohn's disease (Hall-Stoodley 2004). Studies have shown that patients with IBD have higher concentrations of adherent mucosal bacteria, indicating that the presence of

intestinal biofilms may play a role in disease pathogenesis (Swidinski et al. 2005). While the general composition of the microbiota is important, the spatial organization of the gut flora and the presence of biofilm structures may have an additional impact on microbiome function and host phenotype.

One crucial component of microbial biofilms is amyloid fibers, which are polymeric fibrils composed of folded β-sheets stacked perpendicularly. While they are frequently associated with protein misfolding and neurogenerative diseases such as Alzheimer's and Parkinson's, 'functional' amyloids are manufactured by various organisms to fulfill physiological functions (Blanco et al. 2011). These common building block structures are resistant to protease digestion and denaturation, so bacteria use them as structural materials and adhesions, as well as for protection against host defensive mechanisms (Schwartz 2013). Amyloid fibers, as the main proteinaceous component of biofilms, may also play a role in the pathogenesis of diseases. It has been suggested that leakage of microbiome amyloids through compromised gastrointestinal tract or blood-brain barriers could contribute to neuroamyloidogensis and promote the characteristic protein aggregation in Alzheimer's disease (Zhao and Lukiw 2015). On the other hand, amyloids could be therapeutic candidates. Oppong and coworkers showed that presence of curli fibers from the microbiota resulted in the production of an immunomodulatory cytokine that reduced inflammation in a mouse colitis model (2015). Finally, amyloids could serve as targets in the fight against biofilms. Several molecules that inhibit amyloid fiber formation and interfere with biofilm production or assembly are under investigation (Taglialegna et al. 2016). If these compounds have the potential to modulate

microbial biofilms and microbiota organization, they could consequently impact host phenotype.

1.4 The BRICHOS domain and Gastrokine-1

Certain protein domains are also known to have anti-amyloidogenic properties. One such domain is the BRICHOS domain, a domain of ~ 100 amino acids that was first described in 2002 (Sánchez-Pulido, Devos, and Valencia 2002). Its name comes from three proteins: Bri2, chondromodulin-I, and prosurfactant protein C (proSP-C), which are members of the diverse superfamily of BRICHOS-containing proteins including the gastrokines, tenomodulins, arencins, and the group C proteins (Knight et al. 2013). The proteins in this superfamily share a common overall architecture and secondary structure (figure 1), despite the fact that there is low amino acid sequence conservation within the BRICHOS domain (Willander et al. 2011). However, two of the three amino acids conserved across the entire family are cysteines that form a disulfide bridge present in all proteins (Willander et al. 2012). After identification, it was proposed that the BRICHOS domain may have a chaperone-like function which prevents β-sheet aggregation and amyloid fibril formation. Consequently, the domain has been linked to a variety of amyloid diseases such as familial dementia and respiratory distress syndrome (Knight et al. 2013).

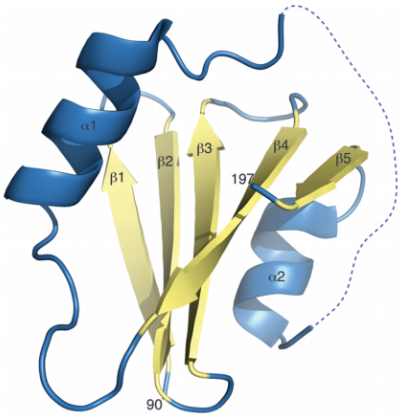

Figure 1. Ribbon diagram illustrating the fold of the proSP-C BRICHOS domain. α-helices and β-sheets are labeled (Knight et al. 2015).

Like other members of the family, Gastrokine-1 (Gkn1), has been shown to have anti-amyloidogenic properties that prevent the aggregation of amyloid fibrils and therefore biofilm formation (Altieri et al. 2014). Gkn-1 was initially identified in 2003 and originally named antral mucosal protein (AMP)-18, since it is expressed in the stomach antrum and is 18 kilodaltons in size (T.E. Martin et al. 2003). It is highly conserved across mammals, including human and mouse, and is abundantly expressed by the mucus secreting cells of the stomach antrum. Nearly 1-5% of stomach RNA is thought to be Gkn1 (T.E. Martin et al. 2003; Oien et al. 2004). Two main functions of Gkn1 have been identified. First, studies have shown that Gkn1 is downregulated in gastric carcinoma, and it may play a role in gastric cancer suppression through the promotion of apoptosis of cancerous cells (Oien et al. 2004; Mao et al. 2012). Gkn1 also functions to protect the colonic mucosal barrier from injury and promote intestinal epithelial cell growth. In one study, administration of exogenous Gkn1 limited the extent of colonic mucosal injury in mice by increasing the accumulation of tight junction proteins (Walsh-Reitz et al. 2005). While the

protective functions of Gkn1 suggest its importance for pathologies like inflammatory bowel disease, the protein likely also plays a role in obesity.

To advance understanding of Gkn1, Dr. David Boone and coworkers use Gkn1 knockout mice ($Gkn1^{-/-}$ mice) for *in vivo* experiments. $Gkn1^{-/-}$ mice are healthy and have a normal lifespan, but they have markedly reduced body fat compared to wild type (WT) mice and are resistant to weight gain on a high fat diet. It was found that they are not diabetic, have normal appetite and physical activity, and do not malabsorb calories. Instead, altered fat metabolism or storage may cause the difference in adipose accumulation. Notably, WT mice given chicken anti-Gkn1 antibodies gained less fat and weight than control mice on a high fat diet, indicating that blocking of Gkn1 activity can mediate against weight gain (Bakke 2016). As a result, modulation of the activity of this protein could serve as a potential treatment against obesity. Further understanding of Gkn1 is crucial for development of these potential treatments. In this thesis, I investigate the mechanism of action of Gkn1. We hypothesize that Gkn1 exerts its function via its anti-amyloidogenic properties, which allow it to modulate the gut microbiome and influence adiposity. Two aims were defined: 1) Successful production of Gkn1 for use in assays. 2) Investigation of the impact of Gkn1 on biofilm formation *in vitro*.

1.5 Site Directed Mutagenesis

A third aim was also defined: 3) Use of site-directed mutagenesis to identify critical amino acids and elucidate the importance of the BRICHOS domain on Gkn1 function. BRICHOS-domain containing proteins like Gkn1 have a similar pattern. They all contain a short cytosolic region, a hydrophobic domain, a linker region, the

BRICHOS domain, and a C-terminal region (Knight et al. 2015). Previous research on the tumor suppressive mechanism of Gkn1 utilized deletion and point mutant forms of the protein in order to examine which regions were responsible for the protein's effects (Yoon et al. 2013). In the present study, point mutants are generated using site-directed mutagenesis. Amino acids were chosen based on their conservation among all BRICHOS-containing proteins. In this study, the amino acids in Gkn1 (C11P_Mouse) were the cysteines (C) at position 81 and 140, and the aspartates (D) at positions 65 and 128 (figure 2).

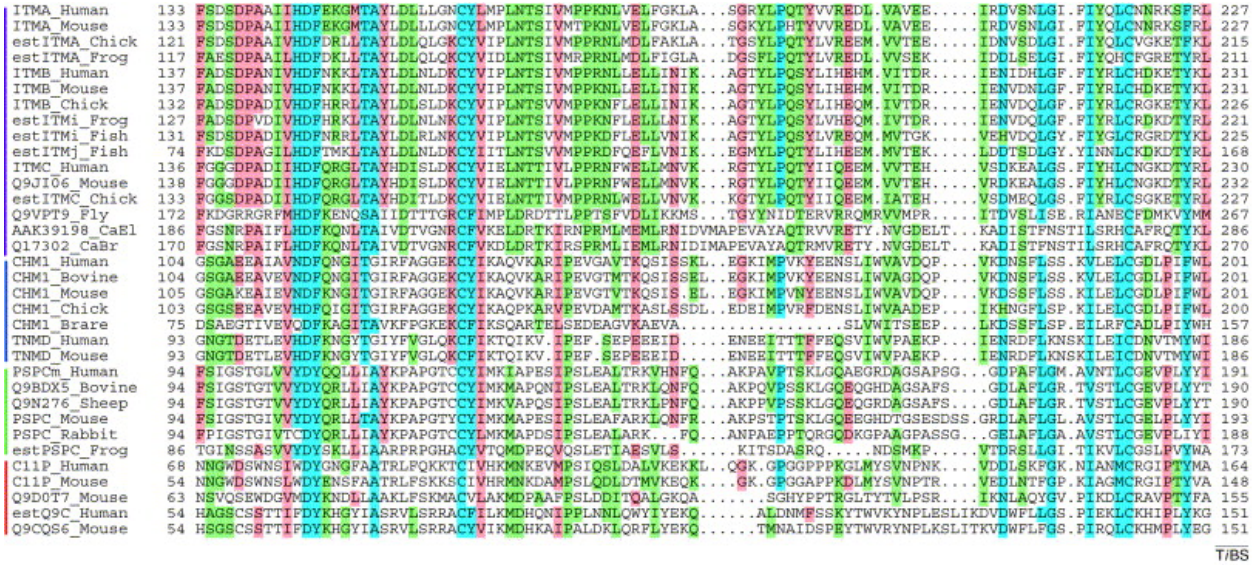

Figure 2. Multiple alignment of the BRICHOS domain (Sánchez-Pulido, Devos, and Valencia 2002). In the figure, mGkn1 is C11p_Mouse. Some amino acids are highly conserved.

We hypothesized that mutation of these amino acids would negatively impact the ability of Gkn1 to modulate *in vitro* biofilm formation. These experiments allowed for investigation of the biochemical features of the anti-amyloidogenic activity of Gkn1.

MATERIALS AND METHODS

2.1 Site directed mutagenesis

Primers were designed to change conserved amino acids in the BRICHOS domain and are shown in table 1. Amino acids chosen were the cysteines (C) at positions 81 and 140, and the aspartates (D) at position 65 and 128. Serine and alanine were selected to be the new amino acids because they are stereochemically different than cysteine and aspartate, respectively and because only one nucleotide change was required for mutation to the new amino acid. pKLAC2 plasmids containing the mouse Gastrokine-1 (mGkn1) gene were used for mutagenesis, which was performed using the QuikChange II Site-Directed Mutagenesis kit (Agilent).

mGkn1 mutant name	Amino Acid Change	Nucleotide Change	Forward Primer	Reverse Primer
Mutant 242	C 81 to serine	g242c	5'-ctcttctccaagaagtcatcca ttgtgcacagaatgaac-3'	5'-gttcattctgtgcacaatgg atgacttcttggagaagag-3’
Mutant 419	C 140 to serine	g419c	5'-gattgctggcatgtccagg ggcatcccta-3'	5'-tagggatgcccctggac atgccagcaatc-3'
Mutant 194	D 65 to alanine	a194c	5'ctggaatagcctctgggcc tatgaaaacagtttcg-3’	5'cgaaactgttttcatagg cccagaggctattccag-3’
Mutant 383	D 128 to alanine	a383c	5'-cctaccagagtggagg ccctgaatacattcgga-3’	5'tccgaatgtattcagggc ctccactctggtagg-3’

2.2 Generation of Yeast Expression Model

PKLAC2 plasmids containing the mouse Gkn1 (mGkn1) gene or mutated mGkn1 gene were used to transform One Shot TOP10 chemically competent *E. coli* cells according to manufacturer’s instructions. Successfully transformed colonies

were cultured in LB broth (Bertani et al. 1951). and miniprepped using the PureLink™ HIPure Plasmid Miniprep Kit (Invitrogen) for sequencing. 3 μg pKLAC2 plasmid DNA was linearized using 30 units SacII in 50 μL 1X CutSmart Buffer at 37°C for 2 hours (NEB). Before transformation, restriction digests were purified through the addition of an equal volume of phenol:chloroform (1:1, v/v) and isolation of the top (aqueous) phase. This step was repeated with only chloroform before addition of 10 μg glycogen and 1/10 volume sodium acetate. After mixing, an equal volume of 100% isopropanol was added, and the mixture was incubated for 10min. Tubes were microcentrifuged for 15min at 12,000 *x g*, then pellets were rinsed once with 70% ethanol before resuspension in 25 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Nanodrop was used to measure DNA concentration. *K. lactis* GG799 competent cells were then transformed according to the *K. lactis* Protein Expression Kit (NEB). Colonies were maintained on YCB Agar Medium plates containing 5 mM acetamide per kit protocol.

2.3 Polymerase Chain Reaction

Transformants were tested using polymerase chain reaction (PCR) to verify integration of the pKLAC expression fragment. From YCB Agar Medium Plates containing 5 mM acetamide, cells were harvested from an area approximately 1 mm^2 and incubated in 25μL of 1 M sorbitol with 2mg/mL lyticase for 60min at 30°C and 10min at 98°C. 50 μL of master mix (2.5μL DNTP, 5μL 10X DreamTaq Green Buffer, 32μL DI H_20, 0.5μL DreamTaq polymerase, 5μL 1X Integration primer 1, 5μL 1X Integration primer 2 from *K. lactis* Protein Expression Kit) was then added to each tube and samples were thermocycled at 94°C for 30sec, 50°C for 30sec, and 72°C

for 2min for 30 rounds before final extension at 72°C for 10min. Amplification reactions were run on 1% agarose gels before visualization using Gel Logic 1500 Imaging System and KODAK MI software.

2.4 Gastrokine-1 production and purification

Cells containing the integrated Gkn1 gene were harvested and resuspended in YPGal medium in a sterile culture tube, then incubated at 30°C with 250 rpm shaking. After centrifugation, supernatant was filter sterilized. Anti-hemagglutinin (Anti-HA) immunoprecipitation was performed for purification.

2.5 Western blot

Supernatant samples were boiled in Laemmli buffer for 5 minutes and loaded into 12% polyacrylamide gels to run at 200 V for 50min. Gels were then rinsed and placed in a transfer apparatus to transfer proteins onto polyvinylidene difluoride (PVDF) membrane. Transfer was performed on ice for 60min at 30 V. The membrane was rinsed briefly in water and blocked with 5% non-fat dry milk (NFDM) for 60min, then incubated overnight in 1:200 anti Gkn-1 rabbit antibody in 5% NFDM (Proteintech). After overnight incubation, membrane was washed three times for 15min by rocking in PBS-T. Membrane was then incubated in 5% NFDM with 1:2000 dilution of horse radish peroxidase-tagged anti-rabbit secondary antibody. After washing three more times in PBS-T, the membrane was incubated in WesternSure Chemiluminescent reagent and imaged using a c-digit blot scanner (Li-Cor).

2.6 Ammonium sulfate protein precipitation and Coomassie stain

Ammonium sulfate was added to supernatant samples while vortexing at low speed to reach 50% saturation. Samples were incubated on ice for 30min before

centrifugation at 16,000 *x g* to pellet insoluble material. Supernatant was poured off and pellets were resuspended in 10 μL Laemmli buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% DTT, 0.004% bromphenol blue) for each mL of initial supernatant. After running, gels were rinsed and stained with SimplyBlue SafeStain (Thermo Fisher) per manufacturer's protocol.

2.7 Biofilm Assay

Biofilm Assays were performed according to the protocol of Chassaing and Darfeuille-Michaud (2013) with modifications. mGkn1 protein concentration was first determined via comparison with proteinase K standards after running on 12% Bis-tris gel and staining with SimplyBlue Safestain. mGkn1, mutant mGkn1, and control HA peptide were UV sterilized using a GS GeneLinker UV Chamber (Bio-Rad) prior to use in assays. Plates were incubated for 8 hours before washing, then wells were entirely filled with 0.1% crystal violet in milliQ water. After crystal violet staining, wells were dried overnight and solubilized with 260 μL of absolute ethanol before reading using a SpectraMax Pro 384 (Molecular Devices).

RESULTS

3.1 Confirmation of successful site-directed mutagenesis

After site directed mutagenesis and successful transformation of One Shot TOP10 chemically competent *E. coli* cells, plasmid DNA was sequenced to confirm successful mutation. Sequencing results were translated using ExPASy and amino acid sequences were compared to the known sequence of mGkn1 (figure 3).

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Native mGkn1          62 SLWDYENSFAATRLFSKKSCIVHRMNKDAMPSLQDLDTMVKEQKGKGPGGAPPKDLMYSVNPTRVEDLNTFGPIAGMCRGIPT 145
mGkn1 mutant 242      62 SLWDYENSFAATRLFSKKSSIVHRMNKDAMPSLQDLDTMVKEQKGKGPGGAPPKDLMYSVNPTRVEDLNTFGPIAGMCRGIPT 145
mGkn1 mutant 419      62 SLWDYENSFAATRLFSKKSCIVHRMNKDAMPSLQDLDTMVKEQKGKGPGGAPPKDLMYSVNPTRVEDLNTFGPIAGMSRGIPT 145
mGkn1 mutant 194      62 SLWAYENSFAATRLFSKKSCIVHRMNKDAMPSLQDLDTMVKEQKGKGPGGAPPKDLMYSVNPTRVEDLNTFGPIAGMCRGIPT 145
mGkn1 mutant 383      62 SLWDYENSFAATRLFSKKSCIVHRMNKDAMPSLQDLDTMVKEQKGKGPGGAPPKDLMYSVNPTRVEALNTFGPIAGMCRGIPT 145
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Figure 3. Sequencing results showing successful mutation of cysteine and aspartate residues of the mGkn1 gene in pKLAC2 plasmids. The cysteines at positions 81 and 140 were mutated to serine, while the aspartates at positions 65 and 128 were mutated to alanine. Numbers refer to the position of the amino acids within the mGkn1 gene.

Plasmids with mutations were isolated, linearized, purified, and transformed into *K. lactis* GG799 competent cells. PCR was then utilized to confirm successful integration of pKLAC2 plasmid DNA into the *K. lactis* genome (figure 4).

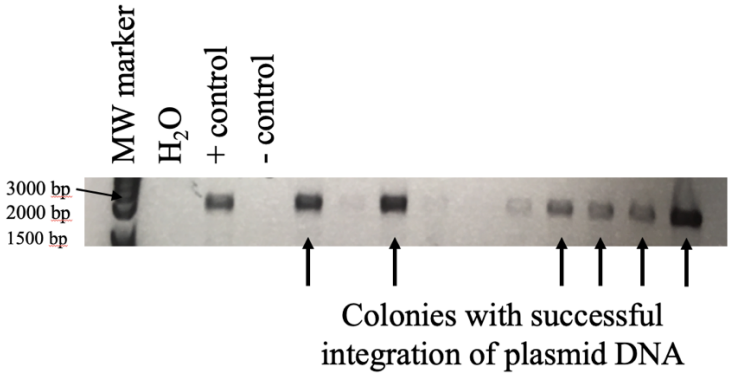

Figure 4. PCR amplification of pKLAC2 DNA after successful integration into *K. lactis* genome. Amplification products were approximately 2.4 kB in size.

3.2 Confirmation of mGkn1 generation

Successful generation of mGkn1 was confirmed using various methods. While mGkn1 and mGkn1 mutants 383 and 194 were successfully purified from yeast supernatant, mGkn1 mutants 242 and 419 were unable to be generated. For higher purity preparation, protein was purified from yeast supernatant using HA immunoprecipitation and visualized via Coomassie blue staining or Western blot (figure 5). Ammonium sulfate precipitation with Coomassie blue staining was used for crude preparation and more rapid visualization (figure 6).

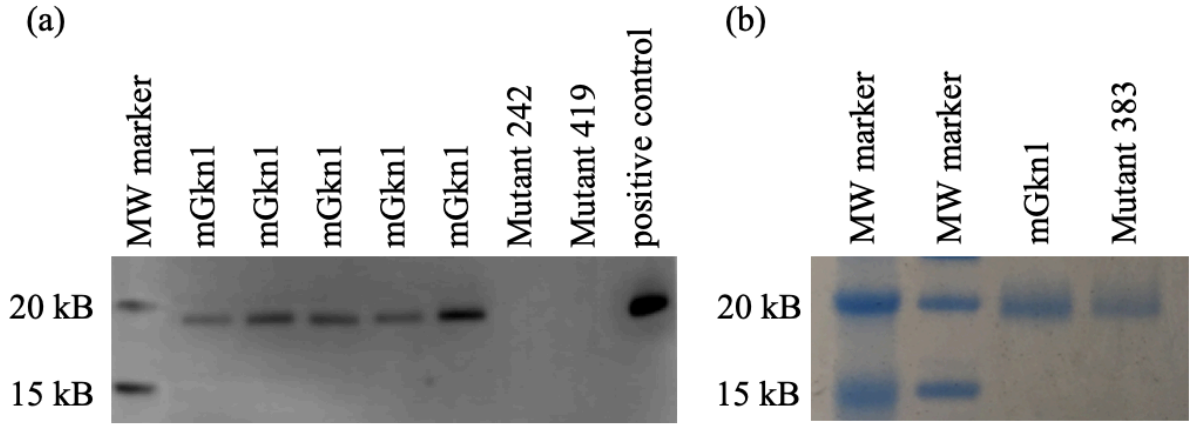

Figure 5. Examples of Western blot and Coomassie blue stain of mGkn1 and mGkn1 mutants after HA immunoprecipitation. (a) Western blot results demonstrate that mGkn1 mutants 242 and 419 were unable to be generated, unlike mGkn1 (b) mGkn1 mutants 383 and 194 (not shown) were successfully isolated from yeast supernatant, as demonstrated via Coomassie blue staining results.

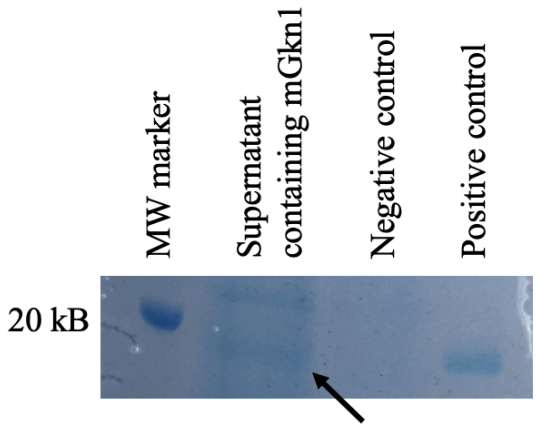

Figure 6. Coomassie blue stain of 12% polyacrylamide gel after protein precipitation using ammonium sulfate. The band at approximately 18 kD indicates the presence of mGkn1 in the supernatant of cultures of transformed *K. lactis*.

3.3 Gastrokine-1 inhibits biofilm formation

Biofilm assays were performed with bacterial strain LF82, which is associated with ileal Crohn's disease (Chassaing and Darfeuille-Michaud 2013). LF82 was grown in M83 media, and equivalent volumes of either TBS, HA peptide, or mGkn1 were added before incubation. The level of biofilm formation by LF82 was measured using optical density (OD) readings. OD 620 nm was first recorded to measure the level of general LF82 growth over the 8-hour incubation. The wells were then washed, and biofilms were stained with crystal violet. OD 570 nm, a wavelength in the yellow range of the visible light spectrum, was subsequently measured to detect the intensity of crystal violet stain and thus the amount of biofilm. The OD 570 nm / OD 620 nm ratio therefore indicates the level of biofilm formation in relation to bacterial growth. Results show that LF82 formed biofilms when grown in M63 media with TBS or HA added, as indicated by the increased OD 570 nm / OD 620 nm compared to M63 media alone (figure 7). This formation was suppressed by the

addition of increased amounts of mGkn1 to cultures of LF82, as indicated by the reduced OD 570 nm / OD 620 nm compared to HA peptide alone.

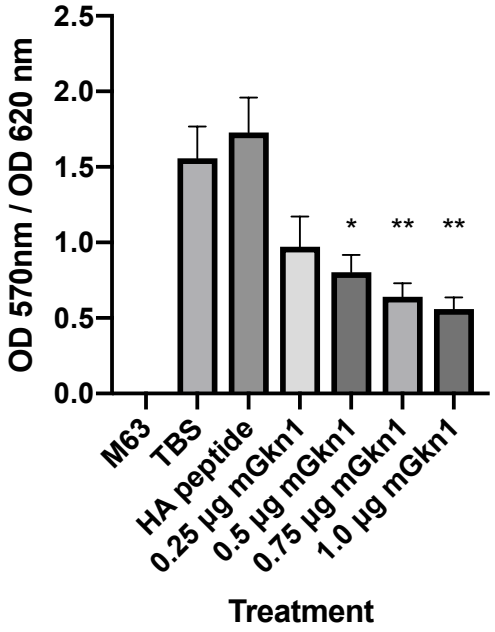

Figure 7. The presence of Gkn1 inhibits biofilm formation of bacterial strain LF82. Increased concentrations of mGkn1 resulted in further inhibition when compared to HA peptide control ($p = 0.0233$, $p = 0.0119$, $p = 0.0088$).

3.4 mGKn1 mutant 383 shows similar level of biofilm inhibition as mGkn1

Biofilm assays were also used for comparison of mGkn1 and mGkn1 mutants. Like unmutated mGkn1, mutant 383 demonstrated biofilm inhibition that increased with increasing levels of protein concentration. However, a significant difference in the amount of biofilm inhibition was not found between mGkn1 and mutant 383 (figure 8).

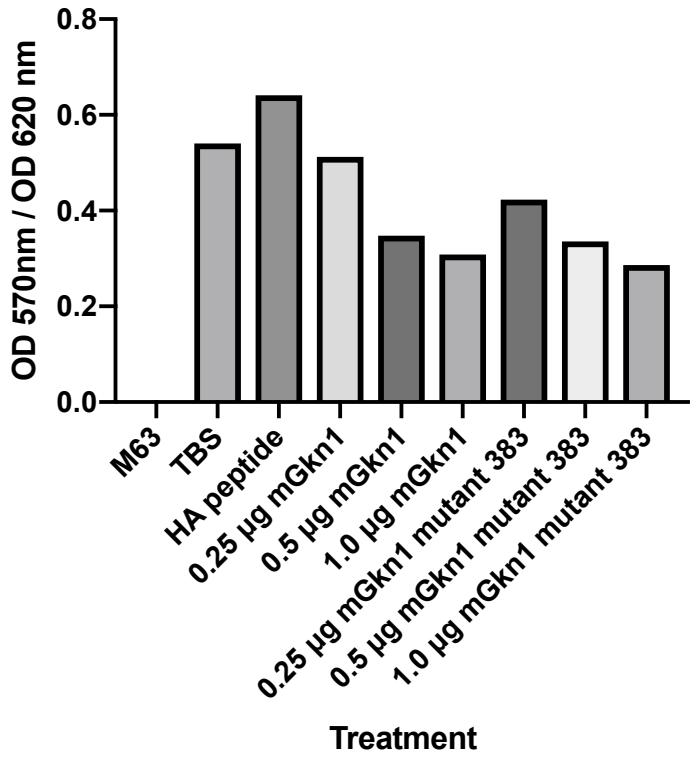

Figure 8. Biofilm assay results comparing mGkn1 and mGkn1 mutant 383. While increasing concentrations of either mGkn1 protein led to greater biofilm inhibition, no significant difference was found when comparing treatments of mGkn1 and mGkn1 mutant 383.

DISCUSSION AND FUTURE DIRECTIONS

While some mutant versions of mGkn1 were successfully generated, other versions were unable to be isolated from yeast supernatant. These results suggest that the transformed *K. lactis* was either unable to produce that version or unable to secrete the final folded protein. Notably, the common feature of each failure was the site directed mutagenesis of a cysteine residue. All BRICHOS domain containing proteins contain a conserved pair of cysteine residues that are predicted to form a disulfide bridge. Previous research on proSP-C, another protein in the BRICHOS superfamily, has suggested that these residues are vital for the trafficking and processing of the proprotein (Mulugeta et al. 2005). While mutant versions of proSP-C with point mutations of conserved cysteines are expressed, they are misfolded and mistargeted, leading to retainment and aggregation in aggresomes (Kabore et al. 2001). As such, it is unsurprising that our similarly mutated versions of mGkn1 were unable to be produced and secreted. Further experimentation would involve the analysis of cell lysates of *K. lactis* producing mGkn1 cysteine mutants, to identify possible misfolding, mistrafficking, or degradation of cysteine mutant Gkn1.

Furthermore, the BRICHOS domain mutants of proSP-C are linked to proliferative and interstitial lung diseases, since the misfolded proteins cause cell injury and death through the deposition of toxic aggregates and induction of ER stress (Mulugeta et al. 2005). Most proteins in the BRICHOS superfamily are associated with degenerative diseases, and Gkn1 may play a role in the development of inflammatory bowel disease (IBD). Perhaps patients suffering from Crohn's disease or ulcerative colitis have mutations in Gkn1 that prevent the protein

from being secreted, leaving the mucosa more susceptible to injury. Furthermore, it has been shown that proSP-C and mutant proSP-C undergo heteromeric assembly, and the formation of these multimers produces a dominant-negative effect, exacerbating the effect of the mutations (Wang et al. 2002). To examine the impact of Gkn1 mutations on mucosal health, samples from patients suffering from IBD and control patients could be collected, and the amino acid sequence would be analyzed to potentially pinpoint a Gkn1 mutation. Currently, most information on genetic susceptibility to IBD is limited to genome-wide association studies which identify genetic regions that contain risk modifying alleles. These studies have not identified the Gkn1 region as a risk modifying region for IBD. However, rare variants that cause disease remain to be fully explored, as there have been only limited published studies on whole genome sequencing or exome sequencing in IBD. Although genetic mutations in Gkn1 associated with IBD or other diseases have not been found, it remains possible that such mutations could exist in rare cases. In addition, since Gkn1 is resistant to degradation in the GI tract, there is potential for oral administration of Gkn1 as a treatment method for IBD. Purified Gkn1 protein could be fed to mice to assess its effect on promoting or restoring mucosal health.

We hypothesize that Gkn1 exerts its protective and obesogenic functions via its anti-amyloidogenic properties and ability to modulate the microbiota. Biofilm assay results show an inverse relationship between the concentration of Gkn1 and the level of biofilm formation; these results support earlier research on the anti-amyloidogenic effects of Gkn1 (Altieri et al. 2014). Further research on these effects will involve *in vitro* protein assays. In these assays, the formation of amyloid fibers

can be traced in real time. These assays will offer more substantial evidence that inhibition of amyloids fibrils leads to the loss of biofilm formation in our biofilm assays. The comparison between mGkn1 and mGkn1 mutants could also be performed using future *in vitro* protein assays.

Surprisingly, the mGkn1 mutant 383 displayed a similar level of inhibition compared to WT mGkn1, which indicates that this amino acid, while conserved in evolution, is not critical for the anti-biofilm activity of Gkn1 and therefore not likely critical for the anti-amyloidogenic activity of Gkn1. The creation of different mutants is underway in the hopes of pinpointing specific crucial amino acids and elucidating the biochemical interaction between the protein and the amyloid fibers. This information could allow for the development of anti-amyloid drugs that mimic the function of Gkn1. As mentioned previously, oral administration of Gkn1 could be a potential treatment option for digestive diseases. Synthesis of drugs that mimic its properties would be more efficient and cost-effective to produce when compared to synthesis of the whole protein.

On the other hand, loss of Gkn1 results in resistance to adiposity, as seen in the Gkn1$^{-/-}$ mice. Selectively inhibiting or fully inhibiting this protein in the GI tract could be a potential treatment for obesity. Since mice models fed anti-Gkn1 antibody gained less fat and weight than control mice (Bakke 2016), drugs that inhibit Gkn1 could also be developed. However, since Gkn1 has multiple functions, selective inhibition may be required for successful obesity treatment without compromising the health of the GI tract (Bakke 2016). More information is still needed about the mechanism, or mechanisms, of action of Gkn1. It is possible that different parts of

the protein, or different parts of the BRICHOS domain, have varying importance with regards to carrying out each function. Knowledge about specific biochemical features could allow for the development of drugs with higher specificity that target, for example, the obesogenic activity of Gkn1, but not the IBD suppressing activity of Gkn1.

It is also possible that Gkn1 exerts multiple functions because the microbes or metabolites that promote epithelial cell health are different than those promoting weight gain. In a broad sense, the specifics of the modulation of the gut microbiome by Gkn1 are still unknown. While prevention of biofilm formation may alter the organization or structure of microbial communities, we lack information on compositional changes of the microbiota in the presence or absence of Gkn1. Many previous research studies have shown that the microbial composition of the GI tract plays a critical role in the phenotype of the host. To investigate the impact of Gkn1 on microbiome composition, a comparative analysis of 16S rRNA from multiple different sections of the GI tract of WT and Gkn1$^{-/-}$ mice should be performed. This information may show large differences in the microbiome, and particular bacterial species could be associated with the weight gain caused by Gkn1. Knowledge of the biochemistry of the interactions between Gkn1 and particular bacteria may permit the development of drugs that target specific bacterial species and health conditions.

Overall, our data show that Gkn1 exerts anti-amyloidogenic activity but that not all conserved amino acids in the BRICHOS domain are crucial for this function. Further experimentation on the biochemistry of the mechanism of action of Gkn1 is

still required. Ultimately, this information may be used to create specific drugs that can target and treat obesity and its related health problems.

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