CLONING, EXPRESSION, AND CHARACTERIZATION OF RECOMBINANT BACE ISOFORMS

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Abstract

In Alzheimer's disease (AD), β-site amyloid precursor protein cleaving enzyme 1 (BACE1) proteolysis is the rate limiting step that leads to amyloid beta (Aβ) formation, which is thought to be the main cause of Alzheimer's disease. The process begins with BACE1 cleaving the amyloid precursor protein (APP), an integral Type I membrane protein, with subsequent cleavage by γ-secretase creating the 40 or 42 amino acid Aβ peptides. BACE1 and BACE2 form a small family of Type I membrane aspartyl proteases with 75% homology, which are both expressed in the brain and able to cleave APP at the β-secretase cleavage site. In addition, both BACE1 and BACE2 have alternatively spliced isoforms. Published studies indicated that the shorter BACE1 isoforms did not contribute to Aβ production; however, the purpose of these isoforms remains unclear. No information was available on the BACE2 isoforms until a recently published report indicated BACE2C protein concentration and activity were associated with the progression of neurodegenerative disease. Since alternative splicing is a common event that typically results in changes of activity and function, we hypothesize that this is also the case for the BACE isoforms. BACE1 is a prime therapeutic target for the treatment of AD but its inhibition could yield unexpected results if the shorter BACE1 or BACE2 isoforms are also inhibited, especially if they are active and have specific physiological functions. Our goal was to express and purify recombinant BACE1 and BACE2 isoforms and characterize their activity utilizing in vitro FRET assays. Recombinant BACE2 isoforms were synthesized and cloned into the pSec insect expression vector and subsequently expressed in *Drosophila* S*2* cells along with

previously synthesized deglycosylated BACE1 isoforms. Isoforms were partially purified using ion exchange chromatography and characterized using two FRET peptide substrate assays. All isoforms showed activity and K_m values for most isoforms were determined. Using a BACE1 inhibitor, IC_{50} values were also determined for the BACE isoforms. These data show that the isoforms are active and have distinct binding affinities, suggesting that the BACE isoforms likely have alternative functions when compared to the full length proteins. New BACE clones have also been constructed to aid in the purification of the isoforms which will allow for better kinetic analyses.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Alzheimer's disease (AD)

Anterior pharynx defective 1 (APH-1)

Amyloid beta (Aβ)

Amyloid precursor protein (APP)

APP Intracellular domain (AICD)

Apolipoprotein E (APOE)

Basepair (bp)

Beta-galactoside alpha 2/6 sialyltransferase (ST6Gal)

Beta-site APP Cleaving Enzyme (BACE)

Calcitonin-gene-related protein (CGRP)

Central nervous system (CNS)

Down syndrome (DS)

Endoplasmic reticulum-associated degradation (ERAD)

Familial Alzheimer's disease (FAD)

Interleukin-1 receptor (IL-1R2)

Intracellular domain (ICD)

Lipoprotein receptor-related protein (LRP)

Long-term potentiation (LTP)

Neuregulin 1 (NRG1)

Nicastrin (NCT)

Peripheral nervous system (PNS)

Presenilin 1 (PSEN1)

Presenilin 2 (PSEN2)

Presenilin enhancer 2 (PEN-2)

P-selectin glycoprotein ligand 1 (PSGL-1)

Reactive oxygen species (ROS)

Sporadic Alzheimer's disease (SAD)

Trans-Golgi network (TGN)

Very low density lipoprotein (VLDL)

Voltage gated sodium channel (VGSC)

Introduction

"Complete helplessness." Those words were used by Alois Alzheimer to describe the state of a 51 year old woman back in 1907 in the first description of what would later be diagnosed as Alzheimer's disease (AD).[1] He was the first to report the now well characterized plaques and tangles that are hallmarks of the disease. Today as many as 5.4 million Americans are living with AD, which is the sixth leading cause of death in the United States.[2] Billions of dollars each year are spent on care and research, yet there is no cure and few agents that slow the progression of AD. An official diagnosis of AD can still not be definitively made until post-mortem examination. Once diagnosed with the illness, the average patient lives for another four to eight years. On average 1 in 8 people over the age of 65 have AD.[2] While the incidence of AD may be on the rise, progress is being made to help those with this debilitating disease.

Aβ Genesis

The first hallmarks of AD that Alois Alzheimer described are now known to be aggregates of the amyloid beta (Aβ) peptide and neurofibrillary tangles (NFT). Though these are the common characteristics of the disease, they constitute only a small part of the AD pathology. The most widely accepted hypothesis for AD is the Aβ cascade, which postulates that the production of Aβ peptides is the first domino to fall, setting off a series of other events such as the disruption of membrane transporters, synaptic plasticity, ion concentrations, and the formation of NFTs, all of which ultimately lead to neuronal loss.[3] The Aβ peptide originates from amyloid precursor protein (APP), a Type

I transmembrane protein found in the brain, and is usually 40 (Aβ40) or 42 (Aβ42) amino acids in length with Aβ42 being more prone to aggregate and more toxic to cells.[4]

As previously mentioned, the Aβ peptide originates from APP and arises from two sequential cleavage events. The first cleavage of APP is catalyzed by the β-secretase enzyme. The major β-secretase enzyme was identified as β-site APP cleaving enzyme 1 (BACE1), which was discovered in 1999.[5–9] This cleavage releases a soluble N-terminal fragment $APP_{s\beta}$. The remaining C-terminal fragment of APP retained in the membrane then undergoes the second cleavage event by the γ-secretase complex. This second cleavage by γ-secretase releases an APP intracellular domain fragment (AICD) and the amyloid-beta (Aβ) peptide. The γ-secretase complex is comprised of nicastrin (NCT), anterior pharynx defective 1 (APH-1), presenilin 1 (PSEN1) or presenilin 2 (PSEN2), and presenilin enhancer 2 (PEN-2). Mutations within the PSEN genes have been found to play a major role in familial AD (FAD) or early onset AD.[10] This discovery was not so surprising when multiple groups showed that the PSEN subunit of the γ-secretase complex contained the catalytic site aspartate residues.[11–13] An alternate APP processing pathway does exist in which APP is cut first by an α-secretase enzyme that results in a larger N-terminal fragment ($APP_{s\alpha}$). The remaining C-terminal fragment is still cleaved by γ-secretase with concomitant release of the AICD fragment, but the secondary fragment (P3) is shorter and does not aggregate like those produced by β-secretase. The processing by either β-secretase or α-secretase of APP is shown in Figure 1.

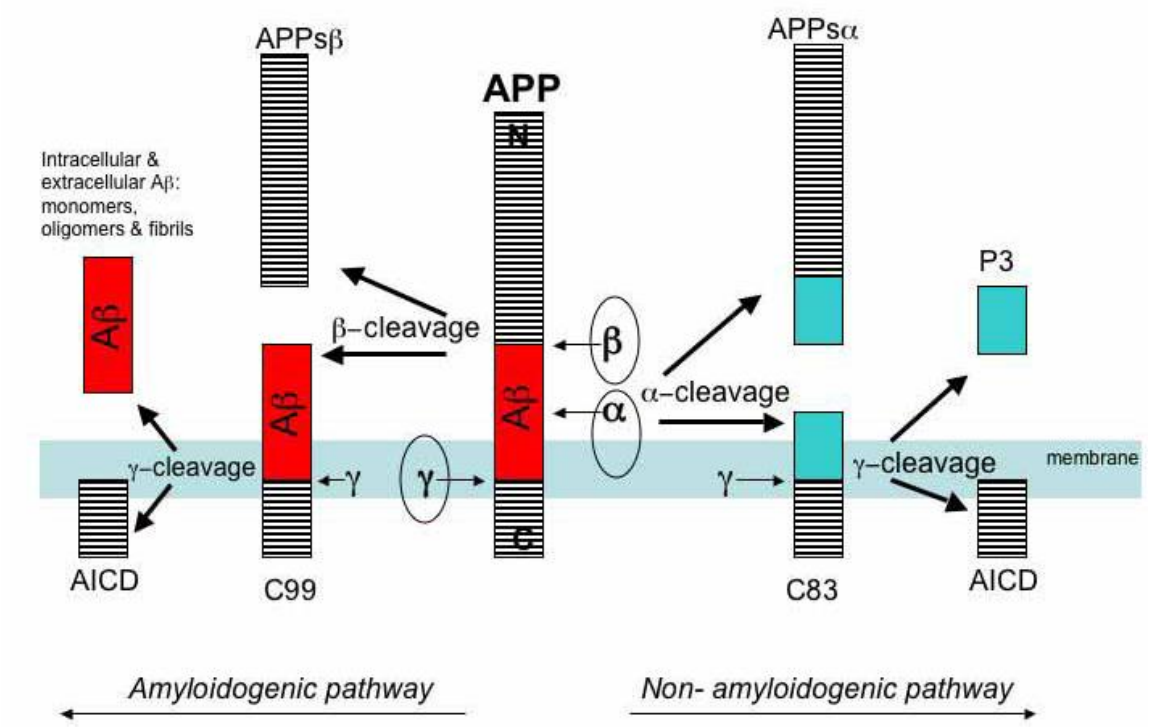

Figure 1: Processing of amyloid precursor protein (APP) occurs by one of two distinct pathways. The amyloidogenic pathway to the left highlights an initial cleavage by β-secretase and formation of Aβ peptides. Cleavage by β-secretase generates the N-terminal APPsβ fragment and the reaming C-terminal fragment C99. Subsequent cleavage by γ-secretase liberates the Aβ peptide and the AICD fragment. The pathway to the right is the non-amyloidogenic pathway in which α-secretase initiates the cleavage of APP to generate the soluble APPsα and the remaining C83 fragments. Further cleavage by γ-secretase releases the AICD fragment as in the β-secretase pathway, but also the shorter P3 fragment. Figure modified from refence.[14]

Familial and Sporadic AD

There are two distinct classifications of AD depending on whether the onset of AD is early or later in life. Mutations in certain genes lead to increased generation of the longer Aβ peptide, which in turn leads to the early onset of AD. These cases of AD are referred to as familial AD (FAD). Genetic mutations in the APP and presenilin genes have been linked to FAD. The first described case was discovered in a Swedish family that had a mutation in the APP gene that resulted in APP being a more preferred substrate for β-secretase.[15,16] This enhancement led to the generation of more Aβ and ultimately

the early onset of AD. Mutations in the presenilin active site of the γ-secretase complex cause the preferential cleavage of APP at the site that generates Aβ42 instead of the less toxic Aβ40. People with Down syndrome (DS), who have an extra copy of APP (located on chromosome 21), also develop FAD.[17] It is believed that the extra copy of the APP gene results in an increased concentration of available APP and amount of Aβ peptides.

Patients who develop early onset AD only represent a small portion of people who get AD; the remaining people are said to have late onset AD or sporadic AD (SAD). Certain genes have been identified that are associated with SAD, but the role that they play in the development of AD or increased formation of Aβ peptides is not clearly understood. One gene that has been implicated as a risk factor in the development of SAD is apolipoprotein E (APOE). APOE is a component of very low density lipoproteins (VLDLs) which are crucial for transporting cholesterol in the blood stream. Three different isoforms of APOE exist, and a genetic study showed that individuals homozygous for isoform ε4 had increased risk of developing AD by eight times.[18] A recent genome wide association study has further provided susceptible gene loci associated with AD. In the study they identified ABCA7, MS4A6A/MS4A4E, CD33, CD2AP, and EPHA1 as potential genes associated with an increased risk of AD.[19,20] The function of these gene products range from being involved in the immune system (ABCA7, CD33, and EPHA1) to cell membrane processes (CD33 and CD2AP) to endocytosis (ABCA7). Again, the role these proteins play in AD remains unclear.

AD Pathology

Significant efforts have been directed towards understanding how Aβ peptides lead to AD. Early studies reported the toxicity of Aβ peptides towards cells.[21] While Aβ plaques are the hallmark of AD, they may not play the casual role in the AD cascade. Many groups have discounted the role of Aβ plaques due to the lack of correlation between the number of plaques in an AD brain and the severity of the symptoms. However, recent studies have found that it may actually be the smaller, soluble Aβ aggregates that play a more important role. The level of expression of soluble Aβ has a much greater correlation with the symptoms where there is a progressive increase in the amount of soluble Aβ in non-dementia patients to those with AD.[22–25] It has also been shown that synaptic transmission is decreased due to diffuse Aβ peptides in vivo.[26] The exact mechanism by which this happens is not yet clear, but long-term potentiation (LTP) between neurons has also been found to be affected by Aβ in rats.[27] LTP is a type of transmission between neurons that is thought to be a major component of learning and memory formation. This group further characterized the form of Aβ that was causing the decreased synaptic plasticity and found that trimer formation of Aβ lead to complete inhibition of LTP. (For an in depth review of soluble Aβ and its effects see reference [28].)

Oxidative stress is a common pathology seen in postmortem AD brains that arises from overproduction of reactive oxygen species (ROS). This is hypothesized to occur when Aβ interacts with redox active metals such as Fe^{2+} and Cu^{+} which then generate ROS.[29–31] Aβ plaques can generate hydrogen peroxide in an oxygen-dependent fashion, and this generation is increased in the presence of Fe^{2+} and Cu^{+}.[29,30] These ROSs can in turn affect membrane ion and glutamate transporters which disrupt ion concentrations

and mitochondrial function. ROS could also be responsible for the alteration of the protein Tau, a microtubule associated protein that is key in cytoskeletal support. Under disease conditions Tau becomes hyperphosphorylated and forms NFTs, another hallmark of AD.

Calcium ion concentrations are greatly affected by the AD cascade. During synaptic transmission, calcium plays a crucial role. When a neuron is going to send a signal to another cell, there is an electronic transmission or action potential that results in an influx of calcium ions. This increase in calcium ion concentration on the interior of the cell activates the release of neurotransmitters that propagate the signal. As a result of Aβ damage to ion transporters due to oxidative stress, calcium homeostasis is disrupted. Without the proper calcium concentrations inside and outside the cell, signaling adversely affected. Studies have shown that Aβ can disrupt the cell membrane or activate different cell receptors that enhance an influx of calcium into cells.[32,33] Many NFTs have been found to contain a high concentration of calcium as well.[34] Much more work is needed to completely understand the Aβ cascade in order to discover ways to stop the progression or alleviate symptoms of AD.

BACE1

Since its discovery in 1999, BACE1 has garnered significant interest as a promising therapeutic target for the treatment of AD due to the fact that it catalyzes the rate limiting step in the generation of Aβ. BACE1 is a 501 amino acid, Type I transmembrane aspartic protease and a member of the pepsin family. It contains the expected aspartyl protease active site motifs, DTGS (residues 93-96) and DSGT (residues

289-292), with the active site located on the luminal side of the membrane. Each of these aspartate residues of BACE1 are essential for activity; mutating either residue renders the enzyme inactive.[5,35] Other features include a N-terminal signal sequence (residues 1-23), a pro-domain (residues 24-48), and a C-terminal transmembrane domain (residues 455-478). BACE1 undergoes post-translational modification at four N-linked glycosylation sites.[36] The glycosylation of BACE1 may be important for enzymatic activity. One study found that mutating two of the four glycosylation sites reduced BACE1 activity between 30 and 40%.[37]

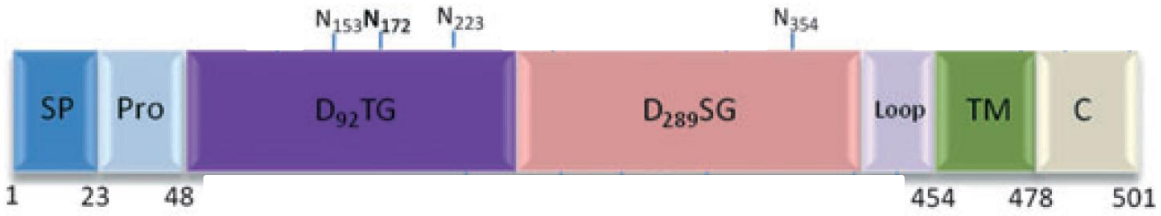

Figure 2: Amino acid sequence diagram for BACE1. Shown is the signal peptide sequence (SP), pro-domain (Pro), active site motifs, four N-linked glycosylation sites, transmembrane domain (TM), and C-terminal tail (C). Figure modified from reference.[38]

Prior to the discovery of BACE1, the majority of β-secretase activity was localized to mainly neuronal cells compared to peripheral astrocytes.[39] Consistent with this finding was the fact that BACE1 mRNA expression was determined to be highest in neuronal tissues.[7] This study also showed that BACE1 was expressed in other tissues as well, such as the pancreas which had comparable expression levels to those seen in the brain. Further characterization of BACE1 showed that it had optimal activity at a low pH (~ 4.5) and was found in the secretory pathway, specifically in the trans-Golgi network (TGN) and early endosomes, both of which have a low pH.[7] These characteristics coincided well with early studies demonstrating the increased β-secretase activity in an

acidic environment and its localization to the compartments of the secretory pathway.[40] BACE1 is initially expressed in the ER before it is quickly modified from immature BACE1, or pro-BACE1, by removal of its prodomain. The prodomain is predominantly removed in the Golgi by furin, a proprotein convertase.[35,41] Unlike many proproteins that are inactive, pro-BACE1 still shows β-secretase activity in vivo, ruling out the possibility of targeting BACE1 maturation as an effective therapeutic strategy.[35,41]

BACE1 Substrates

A number of substrates in addition to APP are cleaved by BACE1. While most research has centered on the role BACE1 plays in AD pathology, BACE1 likely has other physiological functions and surely does not exist to cause AD. BACE1 is considered a prime therapeutic target for the treatment of AD. Identification of the other native BACE1 substrates may help to predict the potential side effects arising from chronic BACE1 inhibition. Identification of other BACE1 substrates is crucial to understanding not only its role in AD, but also the physiological significance of the enzyme.

The most obvious and well-studied substrate of BACE1 is APP. BACE1 cleaves APP at two sites, the preferred β-secretase cleavage site which forms the Aβ peptide and an additional site within the Aβ sequence.[7] Changing the sequence of APP can have a dramatic effect on the activity of BACE1 as evidenced by the enhanced proteolysis of the Swedish mutation of APP, in which Lys-Met is replaced with Asn-Leu. This mutation increases BACE1 cleavage of APP by almost tenfold.[16] Subsequent cleavage by γ-secretase liberates the AICD fragment that is thought to play a role in regulating gene

transcription.[42] This suggests at least one physiological function for BACE1, even though the AICD peptide is generated in the alternative α-secretase pathway as well.

Not surprisingly, BACE1 has other substrates besides APP. To date, all identified BACE1 substrates are single transmembrane domain containing proteins. Two substrates that have been identified are homologues of APP, namely the amyloid precursor-like proteins APLP1 and APLP2.[43,44] Like APP, these proteins are cleaved by γ-secretase, though they do not share the same Aβ cleavage sequence with APP. These fragments, like the AICD fragment of APP, may also function as transcriptional activators.[45,46] Specifically the AICD domains may be responsible for the regulation of neprilysin, a protease that can cleave Aβ.[45] Unlike APP, these proteins are not cleaved by α-secretase.[43]

One of the best documented functions of BACE1 is its role in myelination. Multiple studies have shown that BACE1 regulates myelination through its cleavage of neuregulin 1 (NRG1).[47,48] NRG1 is an initiator of myelination in the peripheral nervous system (PNS) and controls myelin sheath thickness in the central nervous system (CNS). Myelination is a process that primarily occurs early in development where the axons of neurons are insulated with myelin. This myelin sheath serves to increase increasing the speed of electronic impulse or action potential transmission down the axon. The cleavage of NRG1 by BACE1 was confirmed in BACE1 knockout mice. Decreased levels of cleaved NRG1 and hypomyelination was observed in $BACE1^{-/-}$ mice.[47,48] This discovery may be important since BACE1 is a potential therapeutic target, and its inhibition could impact myelination.

Another well-studied BACE1 substrate is the voltage gated sodium channel (VGSC) β2 subunit.[49,50] These channels consist of multiple subunits, one α subunit and at least three β subunits. The VGSC is involved in neuron action potentials and is partly responsible for propagating the signal. Again this is another protein that is cleaved by both BACE1 and γ-secretase where the intracellular domain (ICD) of the β subunit is involved in transcriptional regulation.[49,50] The β2-ICD fragment enhanced the mRNA expression of the VGSC α-subunits resulting in increased α-subunit concentration. The subunits ultimately aggregate or pool within the cell and actually decrease the number of functional channels.[49] This reduction in functional sodium channels reduces neuronal action potentials and impairs synaptic transmission.

Another candidate substrate of BACE1 is the lipoprotein receptor-related protein (LRP). LRP functions as a receptor that interacts with APOE, which brings cholesterol into the neurons. APOE, as previously discussed, is also implicated as susceptible gene loci in SAD. Increased cholesterol levels have been implicated in the regulation of Aβ production and the hyperphosphorylation of tau.[51] In vitro studies suggest that LRP is a BACE1 substrate and also a γ-secretase substrate.[52]

Other substrates that have been identified are various inflammatory proteins. Neuroinflammation is another common characteristic of AD and BACE1 cleavage of these proteins could play a role in this pathological feature. The inflammatory proteins identified include p-selectin glycoprotein ligand 1 (PSGL-1), beta-galactoside alpha 2/6-sialyltransferase (ST6Gal1), and interleukin-1 receptor II (IL-1R2).[53–56] All three substrates have been tested in vivo, though the data for Il-1R2 did not show a significant reduction in expression levels in BACE1 deficient mice. The resulting hypothesis was

that α-secretase could also cleave at the BACE1 site and compensate for the lack of BACE1.[54]

More recently a quantitative proteomic study was performed with BACE1 which discovered 68 potential substrates.[57] They found that the majority of these proteins were Type I transmembrane proteins. A few of the identified substrates were tested and confirmed as substrates in cells. This confirmed the validity of their methods in identifying potential BACE1 substrates. Among the many types of proteins identified were a significant number that are involved in axon guidance. Recently, BACE1 was shown to be required for the proper formation of glomeruli in olfactory bulbs.[58] Glomeruli are comprised of axons and neuron receptors and are an important part of odor signaling. Regeneration of olfactory sensory neurons is a continuous process throughout a person's life, and understanding the role of BACE1 in the regulation of various axon guidance proteins may be extremely important. This new information identifies novel putative physiological functions for BACE1 if it is indeed confirmed to cleave these identified substrates and co-localizes with them in cells. Understanding the enzymatic properties and physiological functions of BACE1 will likely be crucial for the advancement of AD therapeutics targeting BACE1.

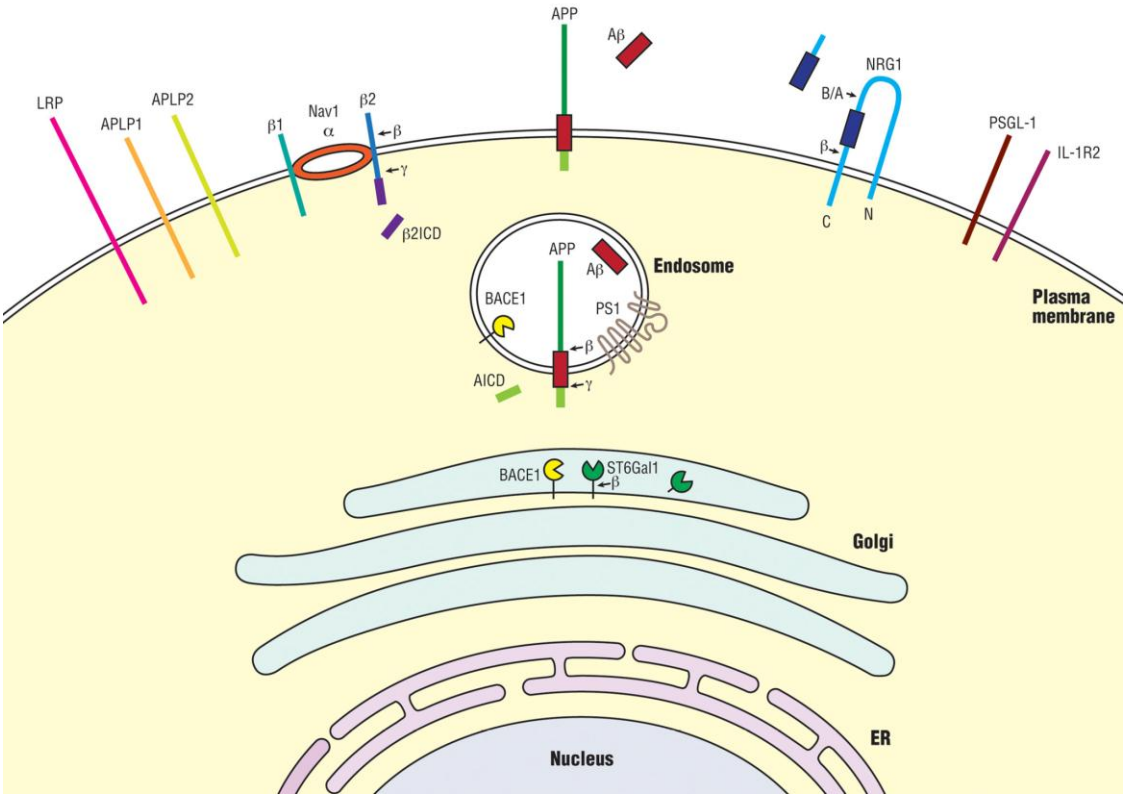

Figure 3: Location and cleavage products of well-studied BACE1 substrates. BACE1 cleavage of these proteins has been shown to regulate myelination (NRG1), sodium channels (VGSCβ2), inflammatory responses (ST6Gal1, PSGL-1, and Il-1R2), and gene regulation or signaling (APP, APLP1, and APLP2). Figure modified from reference.[59]

BACE1 Deficiency

Since BACE1 is viewed as a prime therapeutic target for the treatment of AD, understanding the physiological side effects of its inhibition is crucial. One way to shed light on these potential side effects is to knock out the BACE1 gene in mice. Initial studies of BACE1 knockout mice indicated that there were no discernible differences or significant phenotypes when compared to wild type mice.[60,61] There were no reported differences in embryonic or adult stage mice. This finding was encouraging at first since BACE1 is a prime target for AD treatment. γ-Secretase has also been investigated as a

potential target for treatment, though the finding that the Notch protein was a substrate of γ-secretase posed potential problems.[62] The Notch signaling pathway is important in development and crucial for neuronal differentiation. Indeed knocking out PSEN1 in mice proved to be embryonic lethal.[63] However, selective inhibition has been found to have potential and current studies targeting the inhibition of γ-secretase while sparing the Notch signaling pathway are under investigation.[64–66]

BACE1 knockout studies identified BACE1 as the major contributor to Aβ production. One study showed that in transgenic mice expressing the Swedish form of APP (APPsw), only the $BACE1^{-/-}$ mice lacked Aβ.[60] When compared to $BACE1^{+/-}$ and $BACE1^{+/+}$, there was a drastic decrease in the amount of isolated Aβ. Similar results were seen by another group using different transgenic mice. No amyloid plaques were observed in the BACE1 deficient mice even after thirteen months, whereas the control APPsw expressing mice developed plaques as early as nine months.[67]

Follow-up studies of the BACE1 knockout mice studies revealed that the mice do indeed show subtle abnormalities that were not appreciated in the original studies. The mice were shown to suffer from cognitive and emotional impairment, hypomyelination, and spatial and reference memory deficits.[68–70] The underlying cause of these observed effects was not immediately obvious, though it is possible that it is due to the fact that BACE1 has other substrates besides APP as discussed above. A lack of BACE1 would not only reduce Aβ production but also eliminate the ability to process its native substrates as well.

One interesting discovery suggesting that BACE1 is still a viable therapeutic target comes from the research looking at heterozygous BACE1 knockout mice

($BACE^{+/-}$). Production of Aβ was reduced by 60 to 70% in heterozygous BACE1 knockout mice compared to transgenic mice expressing BACE1 and APPsw.[68] The heterozygous mice also displayed normal spatial memory and cognitive functions early in development, while older mice eventually had a buildup of Aβ and behavioral deficits. In contrast to this study, another group found basically the exact opposite, observing that the heterozygous mice showed minimal reduction in Aβ early in development, while the Aβ plaque burden in older mice was drastically reduced.[71] These differences necessitate further investigation into the consequences of BACE1 activity.

Multiple hurdles exist in the designing of potential BACE1 inhibitors. Perhaps the greatest hurdle is penetrating the blood brain barrier. BACE1 has a large active site which makes it difficult to design small, selective inhibitors that can cross the blood brain barrier. Early peptidomimetic inhibitors mimicked the APP sequence with a statine moiety incorporated as a transition state analog. While the first reported inhibitor achieved an IC_{50} of 30 nM, the length (14 amino acids) and hydrophobic character of the inhibitor limited its viability as a true candidate to inhibit BACE1 in vivo.[72] Even the design of a six amino acid inhibitor based on the Swedish sequence of APP proved to be too large, though it showed great efficacy with a K_i of 1.6 nM.[73] More recently a non-peptidic inhibitor was developed that was ready for phase II clinical trials when the drug was discovered to cause retinal damage in rat subjects.[74] Since then the clinical trial has been discontinued, but it is clear progress is being made in the design and delivery of BACE1 inhibitors.

BACE1 Isoforms

Alternative mRNA splicing is common for many human genes. BACE1 alternative splicing yields five different isoforms. Alternative splicing occurs when the transcribed mRNA has exons or parts of exons removed in addition to the introns, ultimately leading to distinct proteins. This allows tissue specific or developmental stage specific expression of the different protein isoforms without requiring multiple genes. Calcitonin is a well-studied example of alternative splicing. In the thyroid, the inclusion of exon 4 encodes for the mature calcitonin protein that regulates calcium and phosphorus metabolism, whereas the exclusion of exon 4 in neuronal cells encodes a protein calcitonin-gene-related protein (CGRP) that acts as a vasodilator.[75] Since BACE1 is a promising drug target for treating AD, it is important to understand the function of the full length protein in addition to its isoforms, which may have altered specificity and function.

As mentioned previously, BACE1 has five alternatively spliced variants. The full length isoform contains 501 amino acids and is referred to as BACE1A. The other known BACE1 isoforms result from different splicing events at or within exons three and four. Figure 4 shows that the splicing events that give rise to the shorter isoforms lacking 25 (BACE1B), 44 (BACE1C), 69 (BACE1D) or 46 amino acids (BACE455). Each isoform retains its active site aspartates; however, the latter three isoforms are missing two of the four N-linked glycosylation sites. In vivo studies have shown that the shorter isoforms of BACE1 do not make significant contributions to the production of Aβ and activity data suggests that the isoforms are considerably less active than the full length

isoform.[76–78] This differential activity towards APP suggests that the isoforms resulting from alternative splicing have altered activities.

Analysis of BACE1 mRNA distribution showed that the isoforms were expressed in the brain as well as the pancreas like that of BACE1A.[76,78,79] BACE1D had the lowest expression levels and BACE455 showed greater expression in the pancreas than in any other tissue.[76] Overall, the mRNA levels of BACE1A were highest followed by BACE1B. One study comparing the expression levels of the different isoforms in human and rat brain found that BACE1C was not expressed in the human cerebellum and that BACE1D was found in the frontal cortex of only the rat brain.[80] Another report looked at the differential expression of BACE1 isoform mRNA between mice at four months and one year. They found that there was no significant change in the mRNA expression levels for any of the isoforms except for BACE1B, which showed an increase in expression in the one year old mice compared to the four month old mice suggesting that BACE1B may have a unique physiological function later in life.[81]

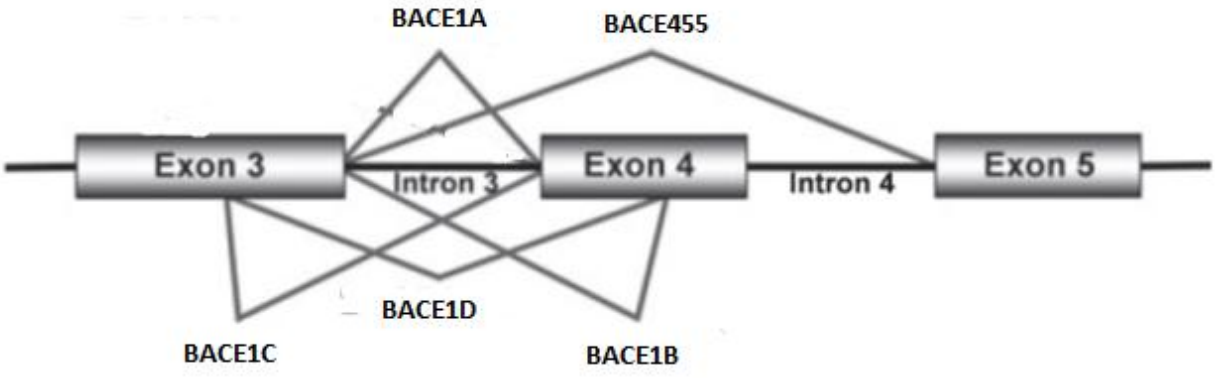

Figure 4: Alternative splicing of BACE1 involves exons 3 and 4. Shown above are the splicing events that give rise to BACE1A, BACE1B, BACE1C, BACE1D and BACE455. BACE1A is formed by the removal of Intron 3. BACE1B has the same 5' splice site, but an alternate 3' splice site within Exon 4. BACE1C is the opposite, having the same 3' site, but a different 5' splice site within Exon 3. BACE1D is the combination of 1C and 1B using both alternate 5' and 3' sites. BACE455 skips exon four altogether. Figure modified from reference.[82]

BACE2

BACE2 is an aspartyl protease and is the closest homolog to BACE1. BACE2 shares 50% sequence identity with BACE1 and has the conserved active site residues. BACE2 is processed in the ER where the protein is glycosylated at two N-linked glycosylation sites. In the mature form, after the autocatalytic removal of its prodomain, BACE2 is approximately 60 kDa.[83] Alternative splicing of BACE2 can generate three unique isoforms. The full length isoform is referred to as BACE2A (518 aa) and the variants are BACE2B (396 aa) and BACE2C (468 aa). Interestingly BACE2B is a naturally soluble protein because alternate splicing leads to a change in reading frame resulting in a stop codon before its transmembrane domain. Both BACE2B and BACE2C retain the active site aspartate residues, and therefore may be active.

BACE2 has been implicated in AD not only due to its similarity to BACE1, but also because it is located in the 'Down critical region' on chromosome 21.[84,85] Since APP and BACE2 are located on chromosome 21, the extra copy of each gene has been hypothesized to play a role in the early development of AD in people with DS. However, the significance, if any, of this has yet to be shown. Like BACE1, BACE2 can also cleave at the β-secretase site and even shows an increased activity towards the Swedish mutation of APP.[86–88] However, one study showed that BACE2 preferred to cleave within the Aβ sequence at a site similar to the Flemish sequence of APP. The Flemish APP sequence is a mutated form of APP that was discovered in a Dutch family. The mutation is located at a single site within the Aβ sequence closer to the α-secretase site.[89] Further research found that BACE2 in fact had increased activity towards the Flemish

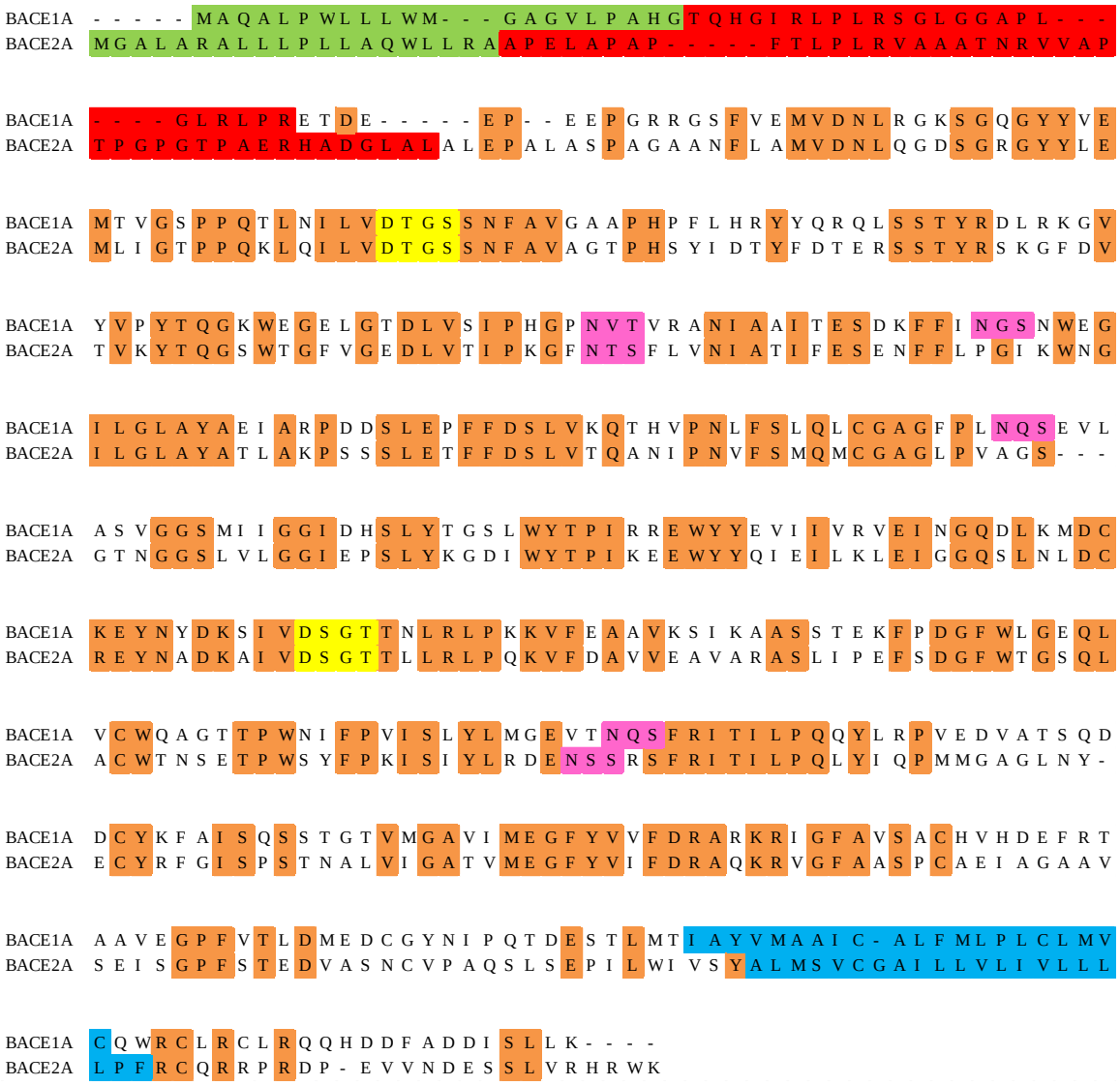

Figure 5: Pairwise sequence alignment of BACE1 and BACE2 with highlighted similarities. They share 50% sequence identity. The conserved residues are highlighted in orange, the conserved active site motifs in yellow, the glycosylation sites in pink, the transmembrane domain in blue, the signal sequences in green, and in red is the prodomains for BACE1 and BACE2.

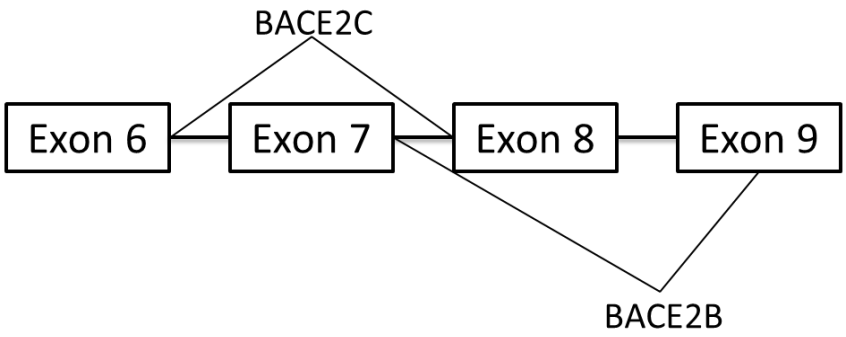

Figure 6: Alternative splicing of BACE2. The full length BACE2A retains all nine exons only removing the introns, which are represented as lines connecting each exon. The splicing for BACE2B removes exon 8 and part of exon 9. This event causes a reading frame shift and results in a novel C-terminal tail and no transmembrane domain. Alternative splicing of BACE2C only removes exon 7, but does not alter the reading frame.

APP sequence compared to the wild type APP sequence.[88] Unlike BACE1, however, BACE2 overexpression actually reduced the amount of Aβ peptides isolated from cells.[86] This indicated that BACE2 may cleave preferentially at an alternate site within APP. The alternate site has been termed the θ-secretase site and is located within the Aβ sequence just downstream of the α-secretase site (Figure 7).[90] So while BACE2 may be able to cleave APP to generate Aβ, most results indicate that Aβ would only be a minor side product.

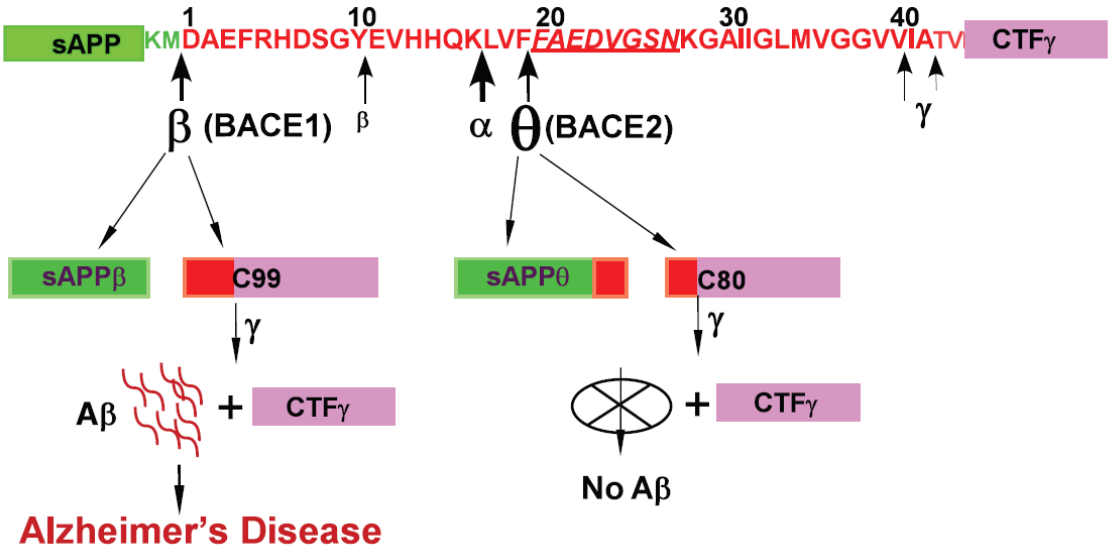

Figure 7: Represented is the APP sequence with the location of BACE1, BACE2 and the α-secretase cleavage sites. The pathway on the left highlights the formation of Aβ following the cleavage by BACE1; while on the right cleavage by BACE2 within the Aβ sequence at the θ site does not form plaques and lead to AD. Figure modified from reference.[90]

Early studies showed that BACE2 mRNA expression was distinct from BACE1. BACE2 was not highly expressed in the brain, but rather was constitutively expressed in other peripheral tissues.[91] This added to the already mounting evidence that BACE2 was not involved in the AD pathway due to its not being expressed in neurons where APP is localized and Aβ plaques are found. Other expression data showed that BACE2 was expressed in muscle tissue along with BACE1, where they may play a role in muscular disorders, suggesting a physiological function for both BACE1 and BACE2.[92] One piece of evidence pointing to BACE2 having some involvement in AD came from the study of BACE knockout mice. When BACE1 was knocked out, Aβ expression in neurons was almost zero, whereas the expression of Aβ in glia was still measurable.[93] It was only in BACE1/BACE2 double knockout mice that the expression of Aβ in glia became

undetectable.[93] These data suggest that BACE2 does in fact contribute to the pool of Aβ in glia, which is significant because glia outnumber neurons almost ten to one.

More recent investigations of BACE2 expression contradict previous work. The previous data suggested that BACE2 mRNA was expressed at much lower levels than that of BACE1, but now it appears that BACE2 is found at comparable levels to BACE1 in the brain.[94,95] BACE1 and BACE2 protein concentrations were comparable and the activity and protein concentration for both increased in neurodegeneration.[94] There was not, however, a corresponding increase in mRNA levels, nor was there a correlation between Down syndrome and an increase in BACE1 or BACE2 activity.[94] Perhaps the most interesting discovery from the study was that the BACE2 mRNA detected was actually that of BACE2C.[94] Upon further investigation, BACE2C mRNA was found to increase along with a corresponding increase in protein expression and activity with the progression of neurodegenerative disease.[94] Not only does this research change the way BACE2 and its role in AD is viewed, but it also provides evidence that the splice variant BACE2C is in fact biologically significant. More work is needed to clarify the role of the BACE2 isoforms not only in AD, but also their physiological role.

Here we report the cloning of BACE2 isoforms into an insect expression vector and the expression of recombinant BACE1 and BACE2 isoforms. Michaelis-Menten kinetic studies were performed with partially purified enzyme, and K_m and IC_{50} values were determined for the isoforms. We show that the isoforms are indeed active and have distinct binding affinity and activity for two different peptide substrates. These data show that the BACE1 and BACE2 isoforms are likely to be active in vivo and could

cleave different substrates. More work is needed with purified enzyme to further characterize the isoforms which may help elucidate their physiological function.

Materials and Methods

Cloning of BACE2 Isoforms

BACE2B and BACE2C isoform genes were generated from a pcDNA3 BACE2A plasmid template kindly provided by Dr. Suzana de la Luna (Medical and Molecular Genetics Center, IRO, Barcelona, Spain). Primers were designed to remove exon 8 and part of exon 9 for BACE2B, while the primers for BACE2C removed exon 7. For BACE2B the forward primer (5'-TTATCGTCTCTGAAATTGCAGGTGCTGCAGTGT-3') sat at the 5' end of exon 9 and the reverse primer (5'-TATTCGTCTCTTTTCTGAGG CAGGATTGTGATACGGA) annealed at the 3' end of exon 7. The forward primer (5'-TTATCGTCTCACTTTACATTCAGCCCATGATGGGG) for BACE2C annealed at the 5' end of exon 8 and the reverse primer (5'-TATTCGTCTCACAGAGATGCGCGGGC) sat at the 3' end of exon 6. These primers were used to PCR amplify the entire plasmid, minus the removed exons. Both sets of primers had a built in Bsm BI recognition sequence (underlined in primer) that allowed for "sticky" end ligations after digestion with Bsm BI. All PCR products were digested with Dpn I to remove PCR template and then PCR purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Inc.) prior to ligation. The linear PCR product was then ligated shut with T4 ligase (Promega, Inc.) and used to transform JM109 *E. coli* cells. Agarose gels were stained with SYBR Safe gel stain (Invitrogen) and imaged using a Bio-Rad ChemiDoc XRS.

Once the isoforms were generated, the genes were sub-cloned into the pSec *Drosophila* S2 expression vector that also included a C-terminal thrombin-V5-6XHis tag. Primers were designed to amplify only the gene of interest to create a linear insert. The amplified gene was then inserted into a linear pSec vector. Ligations of the BACE2

genes were performed using In-Fusion HD (Clontech) according to manufacturer's protocol. Linear pSec vector was generated by digesting with Eco RV and Sac II restriction enzymes and gel purified using the Promega kit listed above. The same forward primer (5'–GGTACCCAGCTTGATATCATGGGCGCACTGGCC) was used for all clones. Since the transmembrane domain was being removed in order to generate soluble recombinant protein, two different reverse primers were used in making the BACE2A and BACE2C clones. Two primers were used to be sure that the location of truncation had no effect on the activity of the protein. The notation of the primers refers to the last five amino acids of the sequence with SEPIL being four amino acids shorter than the LWIVS construct.

SEPIL:

5'-GGGCTTGCCGCTGCCGCGGGCACCAGGGGCCCCAAAATGGGCTCGCTCAAAGACTGA

LWIVS:

5'-GGGCTTGCCGCTGCCGCGGGGCACCAGGGGCCCGGACACAATCCACAAAATGGGCTC.

For BACE2B, only the SEPIL reverse primer was used due to the fact that it naturally lacks a transmembrane domain, so it was not necessary to have two forms of the protein. Ligated constructs for all isoforms were confirmed through DNA sequencing. The underlined portion of the primers corresponds to the sequence that anneals to the linear pSec vector.

Expression and Purification of BACE Isoforms

Drosophila S2 cells were transfected with BACE2 and previously generated BACE1 clones using FuGENE HD (Promega, Inc.) transfection reagent according to manufacturer's protocol. Insect cells are advantageous because BACE is a glycosylated

protein and *E. coli* cells would only produce deglycosylated protein that would also need to be refolded. Gycosylated-Thrombin-V5-6XHis constructs of BACE2 and Deglycosylated-Thrombin-V5-6XHis constructs of BACE1 were transfected. Deglycosylated isoforms were used for BACE1 because it was found that glycosylated BACE1C was recognized as a misfolded protein and directed to the endoplasmic reticulum-associated degradation (ERAD) pathway.[96] The deglycosylated isoforms were created by mutating the asparagine in the glycosylation motifs to glutamine. Previously purified glycosylated BACE1A was also tested for comparison with the deglycosylated isoform. Transfections were performed in 12-well plates and included positive and negative controls. Transfection with a pSec vector containing the enzyme acetylcholinesterase served as the positive control since it has a very sensitive colorimetric assay able to confirm successful transfection as early as one day post transfection. A total of 2 µg of DNA at a 10:1 ratio of pSecBACE DNA to pSecHph (plasmid containing hygromycin resistance gene) was used to generate stable cell lines. Cells were grown in Express Five serum free media (Invitrogen) supplemented with penicillin (50 units/ml final from Gibco) streptomycin (50 µg/mL final from Gibco) and gentamicin sulfate (10 µg/mL final from Cellgro) at 25°C and passaged every ten days. Cells were placed under hygromycin selection three days post-transfection at a final concentration of 150 µg/mL. Expression of isoforms was confirmed through Western blot detection with a V5-HRP conjugated antibody (Abcam). Positope (Invitrogen), a control peptide containing the V5 epitope, was used to confirm antibody recognition and activity. Cell media was harvested from confluent cells for protein purification by centrifugation of insect cells for 30 minutes at 7700 x g. The collected cell media was

dialyzed against 20 mM sodium phosphate pH 7.4 buffer. Dialyzed media was then filtered through a 0.2 μm filter and loaded onto a column with Q sepharose fast flow ion exchange resin (GE Healthcare). The column was washed with a 20 mM sodium phosphate pH 7.4 and 100 mM sodium chloride buffer, then eluted with a 20 mM sodium phosphate pH 7.4 and 350 mM sodium chloride buffer.

Activity Assays

Enzyme activity assays were performed using one of two FRET peptide substrates synthesized by Genscript, Inc. One substrate (Abz-VNLDAE-DNP) was based on the APP sequence with the Swedish mutation and will be termed the High K_m substrate because it has a lower affinity. The other substrate (Abz-YIWDEIDLMVLD-DNP) was based on the peptide described by Jordan Tang's group in identifying subsite specificity of BACE1A and shown to be a tighter binding substrate (and therefore a lower K_m) when compared to the Swedish APP sequence.[97] The tighter binding substrate we have termed the Low K_m substrate. Stock substrate solutions were prepared in DMSO and the final DMSO concentration for all assays was kept at 5%. All assays were performed in 50 mM sodium acetate buffer pH 4.5 with substrate concentrations ranging from 35 μM to 0.39 μM. FRET hydrolysis was measured in half-volume 96-well plates with the Molecular Devices M5 Multifunction Platereader with excitation and emission wavelengths of 320 nm and 420 nm, respectively. The resulting plots were fit using the Michaelis-Menten equation with constant percent error weighting in SigmaPlot.

$$v_0 = \frac{[\mathrm{S}] \cdot V_{\max}}{K_m + [\mathrm{S}]} \quad \text{Eq 1}$$

BACE2 isoforms were activated (by removing prodomain) by incubation in sodium acetate pH 4.5 buffer at 37°C for up to 16 hours as previously described.[98] Assays were performed at various times over the 16 hour period with the Low K_m substrate at a concentration of 25 μM. BACE2B and BACE2C were additionally activated using a minute amount of stock BACE2A (<1% total activity) and incubated as previously stated.

Inhibition assays were performed using the Low K_m substrate at a concentration of 25 μM. Dose-response curves were generated using a BACE1 inhibitor (STA-200, MP Biomedicals, LLC) by varying inhibitor concentration between 100 μM and 0.49 nM. Enzyme and inhibitor were incubated in buffer for thirty minutes at room temperature prior to addition of substrate. The inhibition dose-response curves were fit using Eq 2:

$$\frac{v_i}{v_0} = 1 - \frac{1}{1 + \frac{IC_{50}}{[I]}} \quad \text{Eq 2}$$

Where v_i refers to the initial velocity in the presence of inhibitor and v_o refers to the uninhibited velocity. IC_{50} values were determined from the plot and used to solve for the K_i corresponding to competitive inhibition using Eq 3:

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}} \quad \text{Eq 3}$$

Cloning of Thrombin-6XHis and 6XHis only Constructs

The V5 sequence or thrombin and V5 sequences were removed from both BACE1 and BACE2 clones due to the possibility of BACE recognizing either sequence as a

substrate and cleaving the 6XHis tag off. Our goal was to use PCR to remove these sequences from the C-terminal tail in order to express protein that retained the 6XHis tag. Removal of four Bsm BI recognition sites within the pSec vector was performed in pSec BACE1A in order to utilize primers with the "sticky" ends formation of PCR products as previously described without digesting the vector. Four sets of primers were designed with a single basepair change, each specific for one of the four Bsm BI sites, while not changing the amino acid coding sequence. PCR with these primers generated circular constructs that were digested with Dpn I and transformed as described above. The methods used were analogous to the QuikChange mutagenesis protocol (Agilent Technologies). Successful mutations were confirmed by digesting with Bsm BI to ensure each restriction site was successfully removed.

Table 1: Listed below are the forward and reverse primers for each of the four Bsm BI restriction sites that were mutated. Bsm BI recognition sequence is underlined with the mutated basepair highlighted.

Site 1	Forward 5'-ACAAGCTGTGACCCTCTCCGGGAGCTG Reverse 5'-CAGCTCCCGGGAGAGGGTCACAGCTTGT
Site 2	Forward 5'-GAAACGCGCGAGAGGAAAGGGCCTCGT Reverse 5'-ACGAGGCCCTTTCCTCTCGCGCGTTTC
Site 3	Forward 5'-GCTTGTGTGTGAGGCCTCTCTCTCTTCGTCT Reverse 5'-AGACGAAGAGAGAGAGGCCTCACACACAAGC
Site 4	Forward 5'-GGCCTCTCTCTCTTCCTCTCTGTTGCGCAAA Reverse 5'-TTTGCGCAACAGAGAGGAAGAGAGAGAGGCC

Primers were designed that remove V5 or thrombin and V5 with the Bsm BI recognition sequence in the primer tail. The forward primer (5'-ATTATCGTCTCAGGC CACCACCACCACC) for both anneals just after the V5 sequence and the reverse primers sat either just before the V5 sequence (5'-ATTATCGTCTCAGCTGCCGCGGG

GCAC) or the thrombin sequence (5'-ATTATCGTCTCAGGCTATGGTCATGAGGGT TGACTC). The reverse primer sits just before the thrombin sequence anneals within the BACE gene and is specific for BACE1 or BACE2. The one listed above was the one used to for BACE1. The BACE2 LWIVS reverse primer was: 5'-ATTATCGTCTCAGG ACACAATCCACAAAATGGGCTC. PCR was performed to generate a linear PCR product without the V5 sequence or the thrombin and V5 sequences. Linear products were treated as described above for making the BACE2 isoforms.

A mutated pSec BACE1A template minus V5 or thrombin and V5 was used to create the shorter BACE isoforms. Previously designed primers were used to generate BACE1B, 1C, 1D and 455. Linear PCR products were treated in the same manner as described above in creating the BACE2 isoforms prior to ligation. Ligated products were confirmed through DNA sequencing. Both glycosylated and deglycosylated BACE1 isoforms were created.

BACE2 isoforms were made by doing In-Fusion ligations with the same primers and methods listed above, but the linear vector used was a previously mutated pSec vector without V5. The thrombin sequence was removed from BACE2 clones using the same forward primer listed above along with a reverse primer designed for BACE2A and BACE2C-LWIVS (5'-ATTATCGTCTCAGGACACAATCCACAAAATGGGCTC) or SEPIL (5'-ATTATCGTCTCACAAAATGGGCTCGCTCAAAGACTGA). A unique reverse primer (5'-ATTATCGTCTCACATCCTCTGTTGAGAAAGGCCCG) was required for BACE2B due to the change in reading frame due to alternative splicing.

Results and Discussion

Cloning of BACE2 Isoforms

In order to express the BACE2 isoforms, the shorter isoforms (BACE2B and BACE2C) needed to be generated and then sub-cloned into the pSec insect expression vector. The BACE2A gene started in the pcDNA3 vector. The full length BACE2A isoform was used as the template to remove the correct exons for 2B and 2C. The designed primers amplified the entire vector minus the alternatively spliced exon. The PCR was designed to remove 165 bp (2B) or 150 bp (2C) from the approximately 7,200 bp plasmid. PCR was performed for both 2B and 2C to generate a linear PCR product. Shown in Figure 8 is the BACE2C PCR product at the expected length of 7,000 basepairs. A similar PCR was performed for BACE2B and Figure 8 is representative of the gel for the BACE2B PCR product. Also seen in the figure is a contaminating band just over 2,000 bp that is the result of mispriming. Although optimization of the PCR reaction did not produce the single desired band, we moved forward with this product knowing there was a possibility of getting an undesired ligation product. The linear PCR product for both BACE2B and 2C were ligated and subsequently transformed into *E. coli*. All *E. coli* colonies that grew after ligation were screened for the correct size indicating a successful ligation. The DNA purified from colonies that appeared to correct, missing 165 bp for BACE2B or 150 bp for BACE2C, were sequenced before moving forward to subsequent steps.

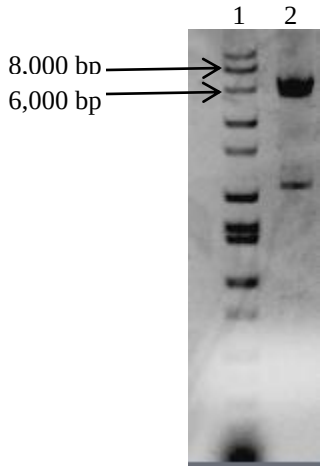

Figure 8: DNA agarose gel of the PCR product generated in the creation of BACE2C. The 6,000 basepair (bp) and 8,000 bp bands of the ladder in lane 1 are indicated. The lane 2 on the right is the PCR product generated using primers designed to remove exon 7. The expected length of the BACE2C PCR product is approximately 7,000 bp. Similar results were seen for 2B.

After creating the shorter BACE2 isoforms, the genes needed to be sub-cloned into the pSec insect expression vector. S2 insect cells were used for expression in order to express glycosylated, folded protein, which *E. coli* cells cannot do. Because we were creating two forms of both 2A and 2C, there was a total of five constructs that were cloned into the pSec vector. The generated isoforms were: pSecBACE2A-SEPIL, 2A-LWIVS, 2B, 2C-SEPIL and 2C-LWIVS. Previous undergraduate students had tried the more typical ligation method to create these constructs by PCR amplifying the gene of interest and ligating that into a linear vector with T4 ligase. This approach was unsuccessful, so we used the alternate In-Fusion method.

The In-Fusion ligation process is similar in that you still generate a PCR insert and linear vector, but the primers are engineered with 15 homologous bp to each end of the linear vector. The In-Fusion enzyme recognizes the homology between the ends and performs a homologous recombination to form the ligation product as opposed to T4 ligase connecting the ends of the insert and vector. The linear vector was created by cutting out BACE1 from the pSec vector with Eco RV and Sac II restriction enzymes.

This digest created two bands, one band corresponded to the BACE1 gene, and the other was the desired linear vector. The linear vector was gel purified to isolate it away from the BACE1 gene. The purified product can be seen in lane 3 of Figure 9. This purified linear vector was used to create all constructs. The PCR product for the isoform genes ranged from 1,235 bp to 1,400 bp depending on the isoform. The product for 2A-SEPIL is shown in lane 2 of Figure 9. Similar results were seen for the other BACE2 isoforms.

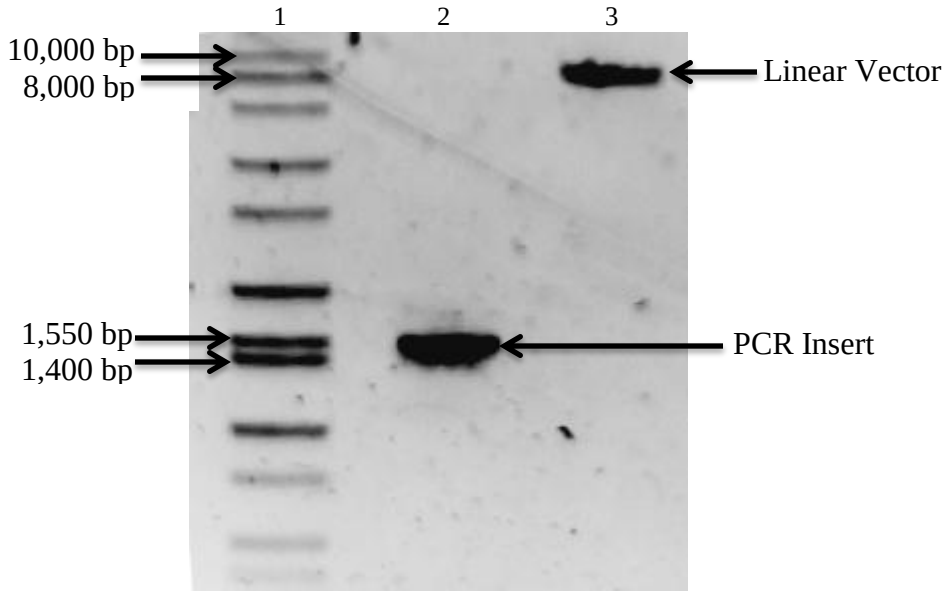

Figure 9: DNA agarose gel of insert and vector precursors used for In-Fusion ligation. Lane 1 is the DNA ladder with bp lengths indicated for selected bands. Lane 2 shows the PCR product for BACE2A-SEPIL at the expected value of approximately 1,400 bp. Lane 3 shows the linear pSec vector generated from the restriction digest with Eco RV and Sac II. The expected length is approximately 7,800 bp.

The successful In-Fusion ligation reactions when run on a gel should show a newly formed third band. The bands seen correspond to the PCR insert, the linear vector, and also the ligated product. Figure 10 clearly shows that for the 2A-SEPIL ligation, the third band appears just above the linear vector in lane 2. The insert and vector are the same as those shown in Figure 9. When combined, the length of the ligated product should be approximately 9,200 bp. However, the ligated product was circular, so it did

not appear at 9,200 bp because the supercoiling of circular DNA affects its migration. Visualization of this third band was not always indicative of a successful ligation. For some of the isoforms this band was not seen after ligation but correct colonies were still isolated, and on one occasion the third band was present but no colonies grew after transformation. Successful In-Fusion ligations were confirmed by DNA sequencing.

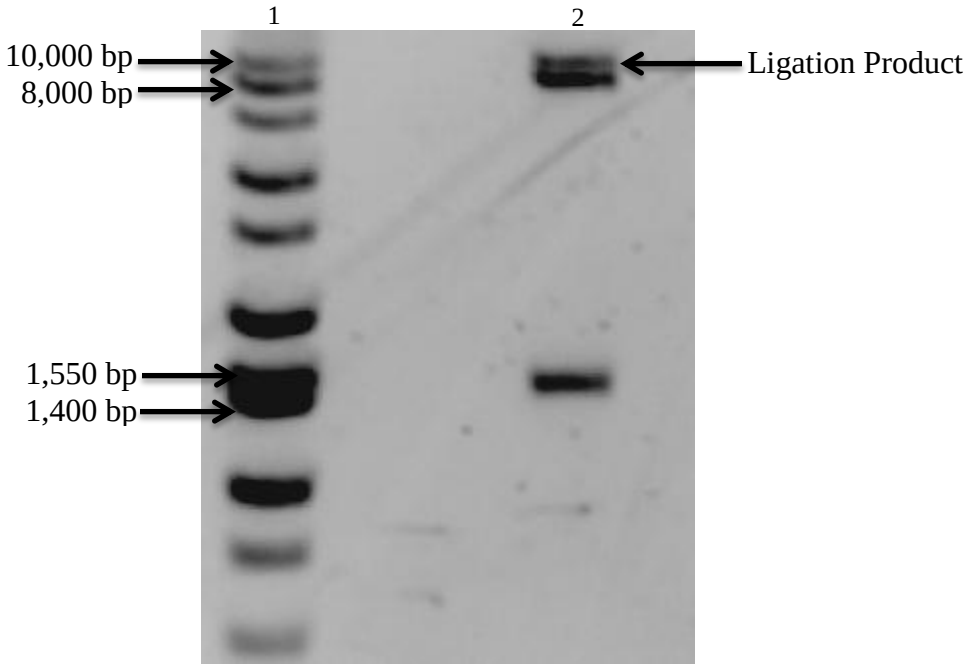

Figure 10: DNA agarose gel after In-Fusion ligation. The gel shows the ligation product of pSec BACE2A-SEPIL. Seen in lane 2 at approximately 1,400 bp is the gene insert, at approximately 7,800 bp is the linear vector and above that band is the newly formed ligation product.

Expression and Purification of BACE Isoforms

In order to characterize the activity of the BACE isoforms, recombinant protein needed to be expressed and purified from insect cells. The BACE2 isoform constructs that were created as described above were used to transfect S2 cells along with previously generated deglycosylated BACE1 isoforms. Expression of the isoforms was confirmed through anti-V5 Western blot detection approximately three days post-transfection.

BACE1A was detected using an antibody directed towards the V5 epitope in the C-terminal tail as seen in Figure 11; however, detection of 1A was not seen over time indicating the loss of the V5 sequence. Using a bioinformatics algorithm that predicts BACE1A cleavage sites, we found that the V5 sequence was a predicted substrate for 1A (Johnson et al.). It was possible that our initial detection of 1A by Western blot was due to testing cell media early after transfection and before the C-terminal tail was removed. We also saw detection of 2A, but no other isoforms. Not detecting the other isoforms could have been due to lower protein concentrations or because the C-terminal tail had already removed by the time Western blot was performed.

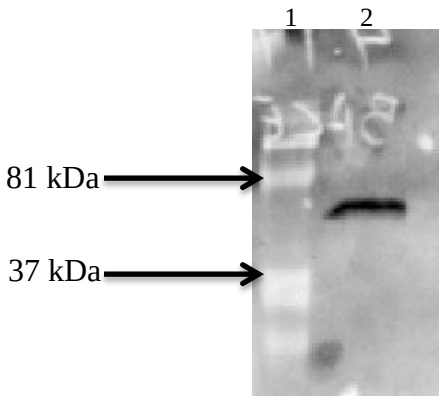

Figure 11: Western blot of deglycosylated BACE1A with a V5 antibody. Ladder is shown in lane 1. The dark band in lane 2 shows positive confirmation of the BACE1A isoform. The same media a week later had no detectable bands by western blot, indicating the loss of the V5 tag.

Protein was purified using ion exchange chromatography. The 6XHis tag at the end of the constructs was incorporated to facilitate further purification using immobilized metal affinity chromatography. The attempt to purify the isoforms using affinity chromatography was unsuccessful. Protein loaded over the column did not adhere and instead passed through further indicating the probable loss of the C-terminal tail. With this knowledge and the fact that Western blot did initially show expression, we

hypothesize that the isoforms are in fact cleaving within the V5 sequence which also removes the 6XHis tag. This only allowed for partial purification of the isoforms through ion exchange chromatography. The purified isoforms were subsequently characterized with FRET assays in order to determine activity and binding affinity of the BACE1 and BACE2 isoforms. The characterized isoforms are highlighted in Figure 19.

Kinetic Analysis of BACE1 Isoforms

Initial activity studies of BACE1 isoforms were performed using the High K_m substrate, a hexapeptide that corresponds to the Swedish mutation sequence of APP. FRET assays were performed using this substrate over a concentration range of 0.39 μM to 35 μM. All sets of assays were performed in at least triplicate with the data being fit with the Michaelis-Menten equation (Eq 1) with constant percent error weighting. Assays could not be performed at higher substrate concentrations due to observed inner filter effects above 40 μM.[99] Inner filter effects are seen at high substrate concentrations when the emitted photons of the fluorophore are reabsorbed by nearby quencher molecules. In our case the aminobenzoyl (Abz) fluorescence emission is reabsorbed by the dinitrophenyl (DNP) moiety on the FRET peptide. This effect greatly reduced our ability to lock in on meaningful values for K_m due to the inability to saturate the enzyme; hence, we do not report any K_m values for the BACE1 isoforms because any value would not be an accurate assessment of the true K_m. However, results in Figure 12 indicate that there are significant differences between the BACE1 isoforms. We also observed a difference between the deglycosylated and glycosylated forms of 1A. The K_m value for glycosylated 1A appears to be larger than the apparent K_m of deglycosylated 1A. This

same difference in binding affinity has been reported by another group comparing the activity of glycosylated and deglycosylated BACE1A. They found that deglycosylated BACE1A expressed in *E. coli* had a lower K_m as compared to glycosylated 1A expressed from human or insect cells.[100] We also show that all the isoforms are indeed active, but without meaningful K_m values, we cannot draw significant conclusions beyond the appearance that they have different binding affinities.

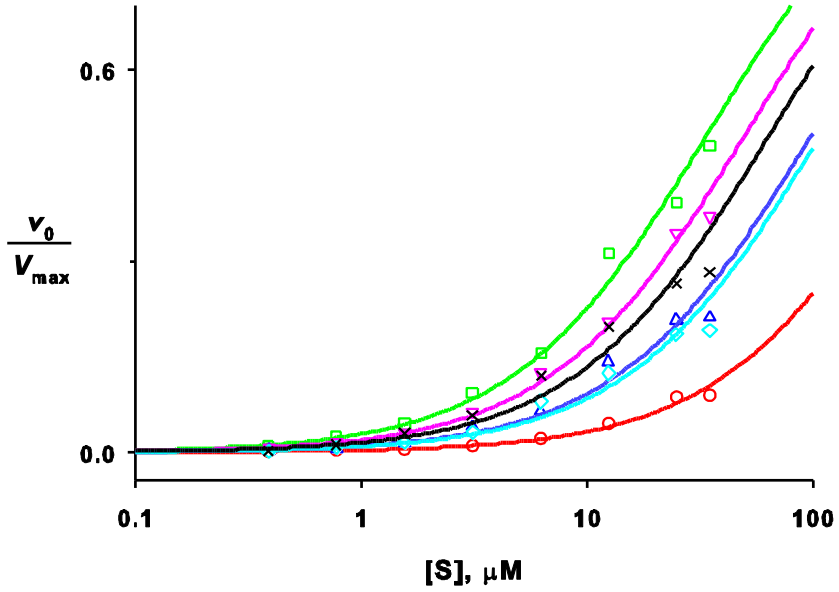

Figure 12: Michaelis-Menten plots with the High K_m substrate for BACE1 isoforms. Data was normalized to V_{max} and fit using the equation 1. Deglycosylated isoforms 1A (green squares, solid line), 1B (blue triangles, solid line), 1C (pink triangles, solid line), 1D (cyan diamonds, solid line), 455 (black x, solid line) and glycosylated 1A (red circles, solid line) are shown.

While the High K_m substrate assays were able to confirm that the isoforms were active, we were unable to assay at substrate concentrations high enough to saturate the enzyme and determine accurate K_m values. In order to saturate our enzymes, we needed a substrate that had tighter binding (or lower K_m) than the High K_m peptide substrate that we used. A previous study described a substrate that showed tighter binding for BACE1.[97] This peptide in fact showed tighter binding and allowed us to obtain more

reliable K_m values for some of the isoforms. Glycosylated 1A had a K_m value of 16.6 ± 3.58 μM. Deglycosylated 1A, 1B, 1C, 1D and 455 had values of 4.34 ± 0.45 μM, 85 ± 15 μM, 39 ± 21 μM, 21.7 ± 6.8 μM, and 51 ± 35 μM, respectively. The large error for 1B, 1C and 455 is reflective of the inability to achieve the higher substrate concentrations necessary to calculate a meaningful K_m. Again we see tighter binding from the deglycosylated 1A isoform as compared to glycosylated 1A, which is consistent with previously reported data.[100] Even without accurate K_m values for some of the isoforms, it is clear from Figure 13 that there are differences between the BACE1 isoforms. Surprisingly 1D, which lacks 69 amino acids, more closely resembled 1A when compared to the other isoforms. It was hypothesized that 1B, only 25 amino acids shorter than 1A, would have the most similar activity to 1A. However, 1B appears to have the highest K_m of the isoforms for the Low K_m substrate.

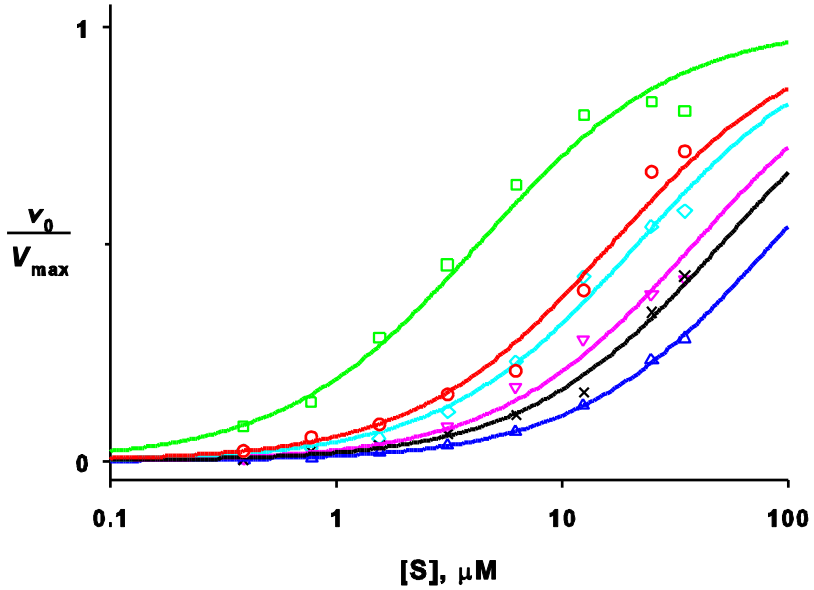

Figure 13: Michaelis-Menten plots for BACE1 isoforms with the Low K_m substrate. Data was normalized to V_{max} and fit using the equation 1. Deglycosylated isoforms 1A (green squares, solid line), 1B (blue triangles, solid line), 1C (pink triangles, solid line), 1D (cyan diamonds, solid line), 455 (black x, solid line) and glycosylated 1A (red circles, solid line) are shown.

Further characterization of the BACE1 isoforms utilized inhibition assays with a known BACE1 inhibitor (STA-200). Assays were performed using the Low K_m substrate and dose response curves were fit using Eq 2. Enzyme concentration was held constant while inhibitor concentration was varied between 0 µM and 10 µM. The dose response curves allowed for the determination of an IC_{50} for all BACE1 isoforms. All isoforms displayed competitive inhibition and K_i was determined with Eq 3 for only the isoforms which had a meaningful K_m below 35 µM. Therefore a K_i value is not reported for 1B, 1C and 455 in Table 2. As is seen in Figure 14, there is a clear difference in the affinity of the inhibitor between isoforms. BACE1D and 455 had the lowest IC_{50}, whereas the rest of the isoforms, including glycosylated 1A, were more closely related.

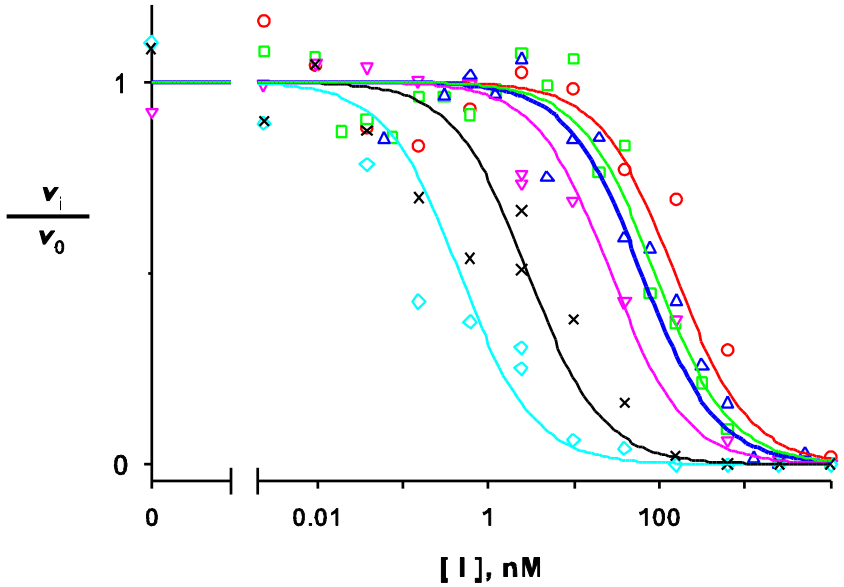

Figure 14: Inhibition assays for BACE1 isoforms using STA-200. Data was fit using equation 2. Deglycosylated isoforms 1A (green squares, solid line), 1B (blue triangles, solid line), 1C (pink triangles, solid line), 1D (cyan diamonds, solid line), 455 (black x, solid line) and glycosylated 1A (red circles, solid line) are shown.

Table 2: Kinetic Values for BACE1 isoforms as determined using the Low K_m substrate and Eqs 2 and 3.

Isoform	K_m	**[S]**	K_i	IC_{50}
Glycosylated BACE1A	16.6 μM (fixed)	25.0 μM (fixed)	102 ± 25 nM	149 ± 29 nM
Deglycosylated BACE1A	4.34 μM (fixed)	25.0 μM (fixed)	13.9 ± 2.3 nM	62 ± 5 nM
Deglycosylated BACE1B	ND	ND	ND	88 ± 16 nM
Deglycosylated BACE1C	ND	ND	ND	27 ± 7 nM
Deglycosylated BACE1D	21.7 μM (fixed)	25.0 μM (fixed)	1.1 ± 0.3 nM	0.46 ± 0.16 nM
Deglycosylated BACE1 455	ND	ND	ND	2.4 ± 0.7 nM

Kinetic Analysis of BACE2 Isoforms

BACE2 isoforms were initially characterized using the High K_m substrate as described for the BACE1 assays to determine if the isoforms were active. Previously reported activity and substrate specificity data for BACE2 suggested this peptide would be hydrolyzed by BACE2 as well and would be a suitable substrate to detect activity.[101] Figure 15 shows the data fit with the Michaelis-Menten equation with constant percent error weighting. As seen for the BACE1 isoforms with the High K_m substrate, inner filter effects only allowed for rough approximations of K_m. We were unable to assay at substrate concentrations above K_m. Even though we are unable to report meaningful K_m values, the data does show that the BACE2 isoforms are indeed active and we report the first activity data for the shorter isoforms with a FRET substrate. No significant

difference in activity was seen between the SEPIL and LWIVS forms of 2A, so only the SEPIL isoforms were used in the determination of kinetic values. From here on any reference to 2A or 2C will refer to the SEPIL isoforms.

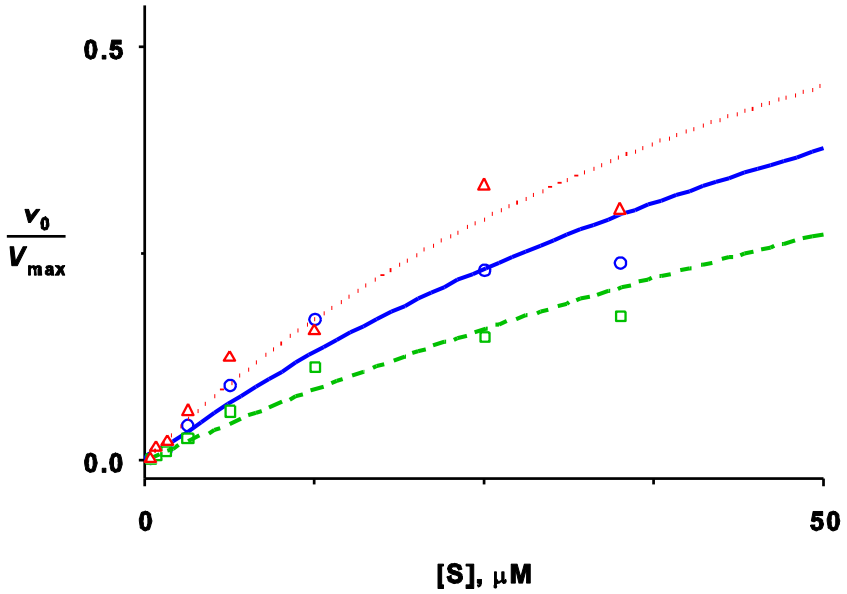

Figure 15: Michaelis-Menten data for BACE2 isoforms with the High K_m substrate normalized to V_{max}. Fits shown are for BACE2A-SEPIL (blue circles, solid line), BACE2B (green squares, dashed line), and BACE2C-SEPIL (red triangles, dotted line). All assays were performed in at least triplicate.

Assays with the Low K_m substrate were then performed with the BACE2 isoforms in the hopes of observing tighter binding and allowing for the determination of K_m values. Again we saw higher affinity binding of the substrate by the BACE2 isoforms. As with the High K_m substrate, the same conditions and parameters were used to determine K_m values with the Low K_m substrate. The tighter binding with this substrate allowed for more accurate values to be determined, and we were able to get meaningful values for K_m. The values for BACE2A, 2B, and 2C were 8.2 ± 0.9 μM, 35.6 ± 10.9 μM, and 10.1 ± 3.1 μM, respectively. Only 2B shows a large error which is due to the fact that we could not assay above the apparent K_m of 35 μM. Figure 16 clearly shows that the isoforms are being saturated with substrate and approaching a maximum velocity which

was not seen with the High K_m substrate. The data further show that the isoforms of BACE2 yield active enzymes that have distinct activity compared to the full length isoform. BACE2B is unique because not only because is it shorter, but it also has a unique C-terminal sequence. With this in mind, the fact that it had the greatest difference in binding compared to 2A was not surprising. Interestingly, BACE2C does not show a drastic change in binding affinity even though the alternate splicing results in the removal of 40 amino acids. A recent report showed that 2C activity and protein concentration increased with neurodegenerative disease progression.[94] This report along with our finding that 2C has similar activity to 2A demonstrates the need to understand the activity of the BACE2 isoforms.

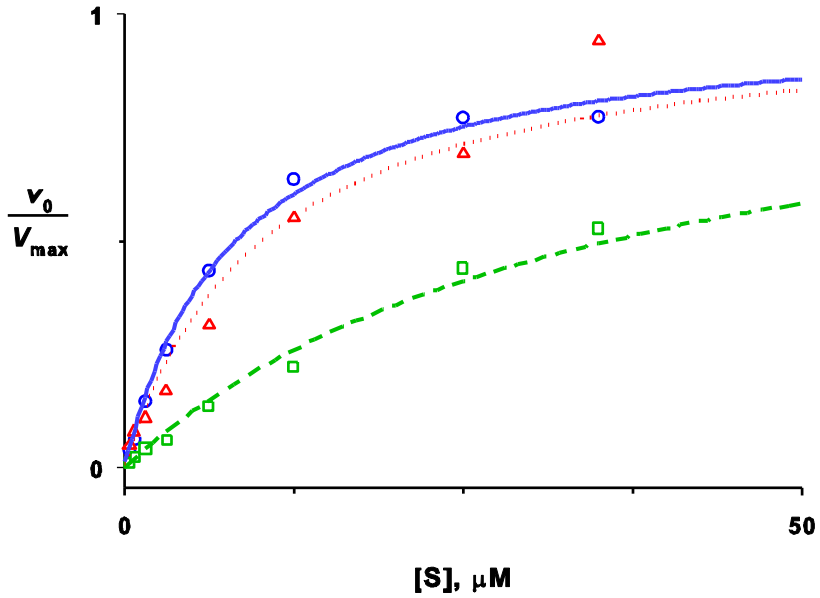

Figure 16: Michaelis-Menten data for BACE2 isoforms with the Low K_m substrate normalized to Vmax. Fits shown are for BACE2A-SEPIL (blue circles, solid line), BACE2B (green squares, dashed line), and BACE2C-SEPIL (red triangles, dotted line).

Inhibition assays for the BACE2 isoforms were also performed with the STA-200 transition state analog inhibitor to further characterize the differences in activity between the isoforms. The dose response curves were generated using the Low K_m substrate and

varying the inhibitor concentrations from 0 µM to 100 µM. Eq 2 was used to create the fit lines and determine IC_{50} values for the isoforms. All isoforms displayed competitive inhibition, and the values for K_i shown in Table 2 were determined using Eq 3 along with the K_m values found using the Low K_m substrate. Similar trends can be seen in the IC_{50} and K_i values that were observed in the K_m values for the BACE2 isoforms. BACE2A had the lowest IC_{50} and K_i values with 2C being most similar to 2A.

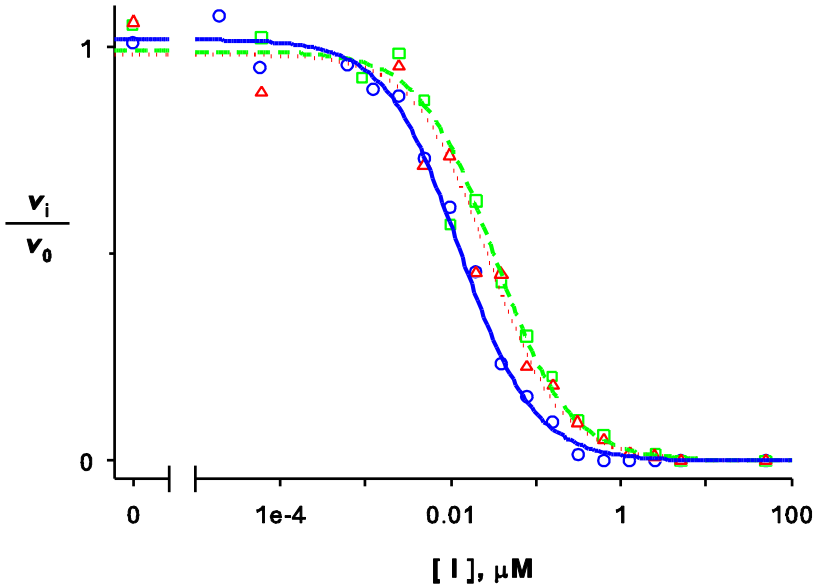

Figure 17: Inhibition of BACE2 isoforms with STA-200 BACE inhibitor and the Low K_m substrate at a fixed concentration of 25µM. Initial velocity with inhibitor (v_i) is normalized with the initial velocity in absence of inhibitor (v_0). Fits shown are for BACE2A-SEPIL (blue circles, solid line), BACE2B (green squares, dashed line), and BACE2C-SEPIL (red triangles, dotted line). Plots fit using Eq 2.

Table 3: Kinetic values for BACE2 inhibition with STA-200 inhibitor. K_m values are those from Low K_m Michaelis-Menten fits.

Isoform	K_m	**[S]**	K_i	IC_{50}
BACE2A	8.2 μM (fixed)	25.0 μM (fixed)	3.6 ± 0.2 nM	14.5 ± 0.9 nM
BACE2B	35.6 μM (fixed)	25.0 μM (fixed)	16.6 ± 1.9 nM	28.3 ± 3.2 nM
BACE2C	10.1 μM (fixed)	25.0 μM (fixed)	6.7 ± 0.8 nM	23.1 ± 2.8 nM

Lastly for BACE2 we wanted to determine if the prodomain was removed upon expression in S2 cells. As previously mentioned BACE2 prodomain removal is autocatalytic under acidic conditions. To test if the prodomain was present, we incubated each BACE2 isoform in pH 4.5 buffer at 37°C for up to 6 hours and measured the initial velocity using the Low K_m FRET peptide. BACE2A activity increased nearly tenfold and had a $t_{1/2}$ of 2.3 hours, which matches previously reported data on autoactivation of BACE2A.[98] Conversely 2B and 2C initial velocity decreased over the same period of time. The $t_{1/2}$ of deactivation of 2B and 2C was 0.76 hours and 0.54 hours respectively. Overnight incubations of 2B and 2C resulted in complete loss of activity. Control measurements were performed in parallel for 2B and 2C in which they were incubated in 20 mM sodium phosphate buffer pH 7.4 at 37°C. They only showed a decrease in activity of approximately 10%. It is unclear whether the removal of the prodomain of 2B and 2C forms a less stable enzyme or if the temperature causes the reduction of activity. Rapid removal of the prodomain was achieved using BACE2A (<1% total activity) to activate 2B and 2C. This showed an increase in the rate of deactivation. Activation of

2A had no effect on binding parameters as determined through inhibition assays previously described.

Removal of Thrombin and V5

While we were able to determine K_m values for most of the BACE isoforms and can clearly see distinct differences in binding between them, more work is needed with purified enzyme. In order to accurately determine the kinetic values for BACE such as V_{max} or $k_{cat,}$, pure enzyme was needed. These values require knowing an accurate enzyme concentration and the ion exchange purified enzymes are not pure enough to obtain accurate enzyme concentrations. While the BACE constructs had a 6XHis tag engineered into the vector to allow for further purification, we believe the His tag was being removed during expression via cleavage within the V5 sequence. To circumvent this problem, we have designed new gene constructs that have the V5 sequence removed or both thrombin and V5 removed.

To remove these sequences we designed primers with Bsm BI recognition sites that could be used to amplify our plasmid minus the desired sequence. We engineered the Bsm BI recognition sites into the primers so that "sticky" ends would be formed after digesting the linear PCR product with Bsm BI. The created "sticky" ends allow for easier ligations and circularization of the linear vector. In order to utilize this feature, we first had to mutate four Bsm BI recognition sites that were naturally in the pSec vector. Four sets of primers were designed, one for each restriction site, where one bp was mutated while not changing the amino acid coding sequence. With the single point mutation, Bsm BI would no longer recognize the sequence as a restriction site. Then when we digested

PCR products with Bsm BI the vector was not digested, only the primers were. One site was mutated at a time and once all four sites were mutated the, final mutated product was confirmed by digestion with Bsm BI. If all four sites were successfully mutated, the digested product would look like undigested plasmid. Mutations were successful and the mutated templates included pSec BACE1A glycosylated thrombin-V5-6XHis, 1A deglycosylated thrombin-V5-6XHis and 1A glycosylated 6XHis only.

With the Bsm BI sites mutated these templates could be used to remove V5 or both thrombin and V5 via PCR. Primers were designed to amplify the pSec vector minus the sequence we wanted to remove. The linear PCR product was digested with Bsm BI and ligated shut with T4 ligase. Colonies were screened by restriction digest and compared to the template to confirm the successful removal of the desired region. Successful cloning of the constructs was confirmed through DNA sequencing.

Lastly, the mutated 1A templates without V5 or thrombin and V5 were used to create the other BACE isoforms. To create the BACE1 isoforms, previously designed primers with Bsm BI restriction sites were used to amplify the entire pSec vector except the specific region missing for each isoform. Figure 18 shows the PCR products for the BACE1 deglycosylated thrombin-6XHis isoforms. The desired band for all isoforms is approximately 9,000 bp which is the upper most band in all the lanes. It is clear that this is not the major band from the PCR reaction. Attempts to optimize the reaction by modification of the PCR conditions were not successful; however, ligations with these products were successful. The same process was repeated to create the BACE1 isoforms in the pSec vector as glycosylated 6XHis only, glycosylated thrombin-6XHis, and deglycosylated 6XHis only. Again all constructs were confirmed through sequencing.

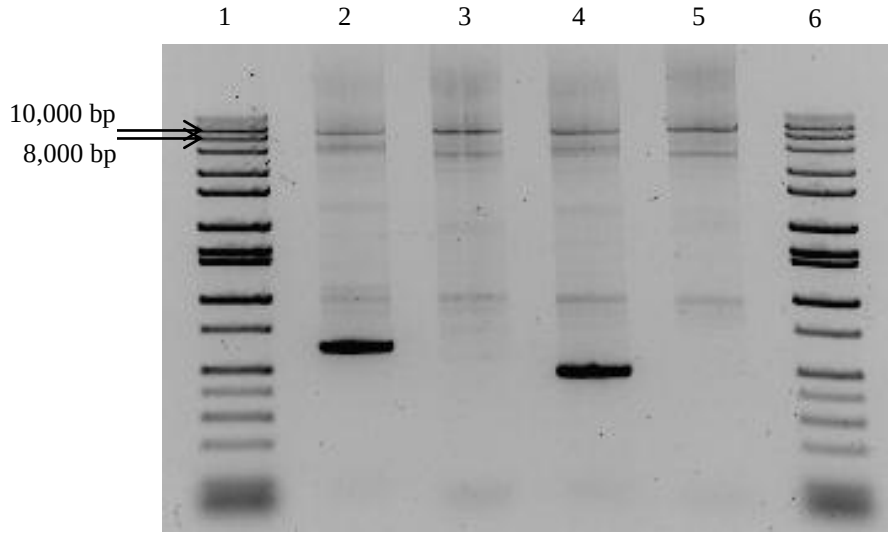

Figure 18: DNA agarose gel showing the PCR product removing specific regions to create each isoform. The template for all PCR reactions was pSec BACE1A deglycosylated thrombin-6XHis. The order from lane 2 to lane 5 is 1B, 1C, 1D and 455 respectively with ladder in lanes 1 and 6. The top band in all lanes is the desired band at approximately 9,000 bp. This gel is representative of the creation of all other isoform variations.

To create the alternate BACE2 isoforms, we performed In-Fusion ligations similar to those previously described. The same primers were used to generate the gene insert, and the only difference was that the plasmid used to isolate the linear vector for ligation was pSec BACE1A mutated vector lacking V5. Successful BACE2 clones were then used as a template to remove the thrombin site as described above for BACE1A. So far only the LWIVS form for 2A and 2C have been created along with 2B. Figure 19 highlights all the BACE1 and BACE2 constructs created.

	Glycosylated			Deglycosylated		
	TVH	TH	H	TVH	TH	H
BACE1A	X	X	O	O	X	X
BACE1B		X	X	O	X	X
BACE1C		X	X	O	X	X
BACE1D		X	X	O	X	X
BACE455		X	X	O	X	X
	Glycosylated					
	TVH	TH	H			
BACE2A-SEPIL	O			Already Existed		
BACE2A-LWIVS	X	X				
BACE2B	O	X	X	Isoforms Created		
BACE2C-SEPIL	O					
BACE2C-LWIVS	X	X	X			

Figure 19: The table highlights all the plasmid constructs created in the lab. Glycosylated and deglycosylated isoforms for BACE1 were created. Three possible C-terminal tails were engineered with either Thrombin-V5-6XHis (TVH), Thrombin-6XHis (TH), or 6XHis only (H). Highlighted in orange are the constructs that were either created by previous students or BACE1A glycosylated 6XHis only was the original template for all BACE1 constructs. All isoforms in green are newly created isoforms overall during my time and have been sequenced. An "O" designates any isoform expressed, purified and characterized by FRET assays. This figure does not capture the Bsm BI mutants that were necessary in order to create some of the isoforms.

Conclusions

Alternative splicing is a common event that often leads to proteins with unique activity and physiological functions.[75] To this point, not much was known about the BACE isoforms. It had been shown that the BACE1 isoforms do not contribute to Aβ production, but the relevance of their existence remained unexplained.[76–78] More recently BACE2C was found to be active in neurons and its increasing activity was correlated with the progression of neurodegenerative disease.[94] The fact that the BACE isoforms are seen in other species and that they still retain their active site residues suggests that the isoforms are active and serve a physiological role. Our goal was to characterize BACE1 and BACE2 isoforms through kinetic analysis using a FRET substrate to determine if they were active and characterize the differences in their binding properties. A further understanding of the BACE isoforms will help elucidate their role in AD as well their physiological function.

Previous students had cloned deglycosylated BACE1 isoforms for expression in S2 cells. Deglycosylated isoforms were chosen because it was found that glycosylated BACE1C was recognized as a misfolded protein and degraded via the ERAD pathway in human cells.[96] BACE2 isoforms had yet to be cloned and only the full length 2A isoform existed, though it was in a non-insect expression vector. The isoforms of BACE2 were generated via PCR amplification and ligation in the pcDNA3 vector. Successful clones of BACE2 were then sub-cloned into the pSec insect expression vector by the use of In-Fusion ligations. Five clones were successfully created, SEPIL and LWIVS isoforms for 2A and 2C, plus 2B.

Drosophila S2 cells were transfected with recombinant BACE1 and BACE2 isoform constructs. Expressed protein was secreted into the cell media and separated from cells for purification. The isoforms were purified using ion exchange chromatography. Affinity chromatography was also attempted but the 6XHis tag appeared to be cleaved off during expression, which only allowed for partial purification. We were able to confirm expression of BACE isoforms through anti V5 Western blot detection, but saw the signal decrease over time. We hypothesize that the V5 sequence is a cleavable substrate by BACE and recognition of this sequence by the BACE isoforms cleaves the 6XHis tag off.

Kinetic analysis of the isoforms was carried out with two distinct FRET peptide substrates. The High K_m peptide corresponded to the Swedish APP sequence and had been shown to be a suitable substrate for both BACE1 and BACE2.[101] Activity for all isoforms was confirmed using this peptide; however, inner filter effect limited the upper limit of substrate concentrations we could assay. This did not allow us to reach substrate concentrations high enough to get an accurate value for K_m. Knowing that the isoforms were indeed active led us to try another FRET peptide. The Low K_m substrate was predicted to be a tighter binding substrate for BACE1.[97] Through our assays we found this was the case for BACE1 and also for BACE2. The tighter binding of this substrate allowed us to accurately determine K_m values for most of the BACE isoforms.

While we were not able to get meaningful K_m values for all the BACE1 isoforms, we did see clear differences between the binding affinities through the use of the Low K_m substrate and inhibition using the STA-200 BACE inhibitor. The most notable differences in K_m values were seen between the deglycosylated and glycosylated isoforms

of 1A. We found that the deglycosylated isoform showed tighter binding than its glycosylated counterpart. This difference in binding affinity has also been seen by another group expressing deglycosylated BACE1A in *E. coli* and comparing to glycosylated BACE1A expressed from human and insect cells.[100] Perhaps most surprising from the BACE1 data was the fact we were able to get a meaningful K_m value for BACE1D, which is the shortest of the BACE1 isoforms. We expected that BACE1D would behave least like the full length BACE1A, whereas BACE1B (only lacking 25 amino acids) was predicted to be more similar to BACE1A.

We were able to get a meaningful value for K_m for all BACE2 isoforms. Distinct differences were seen between the isoforms, where BACE2A had the lowest K_m with BACE2C having activity more similar to BACE2A than 2B. This was not surprising because 2B is the shortest isoform and the alternative splicing event shifts the reading frame resulting in a unique C-terminus. The fact that BACE2C had the closest binding affinity to 2A was an interesting discovery since BACE2C protein expression and activity increases with neurodegenerative disease progression. The role that BACE2C plays in neurodegenerative disease is worth investigating now that we know it has comparable activity to BACE2A. We also found that we could activate 2A by removal of its prodomain. Though activity did increase almost tenfold for 2A, this did not have an effect on its binding affinity. This same test resulted in inactivation of BACE2B and 2C. It is unclear whether removal of the prodomain or the buffer conditions was responsible for the loss of activity. Overall, we have found that all BACE isoforms are in fact active, and report the first detailed kinetic analysis of the BACE2 isoforms using a FRET assays.

More work is needed to better understand the activity and binding properties of the BACE isoforms. One approach that should help achieve this goal is to generate more pure enzyme. To do this we need to retain the 6XHis tag in order to purify the isoforms by affinity chromatography. Since it is our belief that the tag was being removed from cleavage within the V5 sequence, we have begun to genetically remove that sequence along with the thrombin sequence just in case BACE could cleave within that sequence as well. So far deglycosylated and glycosylated BACE1 isoforms have been successfully created that include a thrombin-6XHis tag or the 6XHis tag only. The same is true for the BACE2 isoforms; however, only the LWIVS version of 2A and 2C have been created so far. These new constructs will help with future purification and characterization of the BACE isoforms. Our data has shown that the isoforms are active and have distinct binding affinities. Future analysis of the isoforms will further help elucidate their functional properties and physiological function.

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