Title
CADM1 Function in Regards to Insulin Secretion in the Beta Cell

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A Thesis submitted in partial satisfaction of the requirements of the degree of Master of Science in Biology by Shokufeh Nourollahi

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2013
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University of California, San Diego
2013
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ABSTRACT OF THE THESIS

CADM1 Function in Regards to Insulin Secretion in the Beta Cell

by

Shokufeh Nourollahi

Master of Science in Biology

University of California, San Diego 2013

Professor Steven D. Chessler, Chair

Professor Raffi V. Aroian, Co-Chair

CADM1 (Cellular ADhesion Molecule 1) is a synaptic adhesion molecule found in a multitude of tissue types. CADM1 is a very versatile protein but its main function is facilitating communication between nerve cells. Recently it has been discovered that synaptic adhesion molecules such as CADM1 are involved in insulin release and regulation in the beta cells of the pancreas. For this project, we determined which CADM isoform has the highest level of expression in the beta cell and what effect CADM1 overexpression or knockdown would have in regards to insulin secretion. Preliminary results show CADM1 has the highest level of expression in rat islets, and the overexpression and knockdown of CADM1 in rat beta cell lines in basal glucose conditions leads to an increase in insulin secretion.
Introduction:

Diabetes is an ancient metabolic disease with no definitive cause or cure, afflicting a wide variety of mammalian species without bias of age, gender, or environment. Previous data has suggested that neuronal proteins found in the pancreatic beta cell may play integral roles in the insulin secretory apparatus and its regulation. The purpose of these experiments is to explore the cellular mechanism in the beta cell in order to understand which proteins are involved in the regulation of insulin. By understanding which proteins play a significant role in insulin regulation scientists hope to find a biological cause for the diabetic phenotype.

Diabetes is a complex disease in that there are two common varieties; juvenile-onset and adult onset, also known as insulin dependent and non-insulin dependent though more commonly known as type 1 and type 2 diabetes. In type 1 diabetes the patient’s immune system targets the beta cells in an autoimmune reaction, dramatically affecting the body’s ability to produce insulin, hormone that permits glucose entry into the cell. Without insulin the patient cannot regulate blood glucose levels since insulin is a hormone normally released to decrease blood glucose levels when there is a spike in blood sugar. Glucagon hormone is released when there is a drop in blood sugar to raise blood glucose levels. These two hormones normally work in tandem to ensure blood sugar levels are maintained at constant levels. While type 1 diabetes is currently understood as an autoimmune disorder, in type 2 diabetics patients have become insulin resistant alongside progressive loss of beta cell function, leading to decreased insulin production (Cnop et al. 2007). The body becomes resistant to endogenous insulin and
blood glucose levels cannot be maintained as well as a non-diabetic patient. Because type 2 diabetes is often associated with obese patients, obesity is considered the main contributor. While these two diseases share the same name and phenotype, on a cellular level type 1 and type 2 must be considered different diseases. Type 2 diabetes progresses differently depending on treatment; often times improved diet and weight loss can lead to a decrease in symptoms. Untreated type 2 diabetics progress to insulin dependence. Currently, the only known treatment for type 1 diabetes are insulin injections, an insulin pump or a pancreas transplant, in which man-made insulin supplements endogenous production or the beta cells are replaced.

The normal human adult secretes approximately 30 units of insulin a day, with the insulin concentration in the blood stream varying (based on food consumption) between 10 µU/mL and 100µU/mL. Immediately after food is consumed, there is a spike in glucose levels in the blood; 8 to 10 minutes after food consumption there is an increase in insulin secretion that continues on for approximately 30 minutes, at which point the insulin concentration peaks and begins to decrease again. This cycle of insulin secretion functions to decrease glucose concentration levels, which decrease in inverse proportion to the increasing insulin concentration. Blood glucose levels return to basal levels approximately 120 minutes after food consumption with the help of insulin (Gardner et al 2007).

There are many clinical tests available to test for diabetes, and a common method presently used is referred to as the oral glucose tolerance test, in which individuals fast the night before, and are then give a certain portion of glucose, usually 75 grams
dissolved in water. Blood samples are taken at 0 and 120 minutes after ingestion, and if the blood glucose levels fall above 7 mmol/L in fasting state, or above 11.1 mmol/L 120 minutes after glucose ingestion, the individual is diagnosed as diabetic. Normal values are less than 5.6 mmol/L in fasting state and less than 7.8 mmol/L 120 minutes after glucose ingestion (Gardner et al 2007).

The biology behind the development of diabetes has not been entirely understood yet. Previous literature has established insulin is secreted by the beta cells of the pancreas, which is an organ located between the stomach and the small intestine, comprised of two functionally distinct regions; the endocrine and exocrine tissues. The majority of the pancreatic tissue functions as an exocrine organ and secretes digestive enzymes into the duodenum of the small intestine, but for the purpose of this study, the exocrine tissue was largely ignored to focus on the endocrine portion. The endocrine tissue makes up approximately 2% of the pancreas total mass, and is comprised of cell clusters referred to as the islets of Langerhans, which themselves are comprised of alpha, beta, delta, and F cells. Beta cells are responsible for insulin secretion and their dysfunction results in difficulties maintaining blood nutrient homeostasis (Gardner 2011). The endocrine region also produces glucagon, somatostatin, and pancreatic polypeptide hormones, all of which are responsible for regulating the metabolism of nutrients and cellular energy in an organism.

A major clue that has recently been discovered is a link between diabetes and neurological diseases, such as autism (Fujita 2010) and Alzheimer’s disease (Kroner 2009). The same neuronal proteins that facilitate neuronal growth and communication
have been discovered in the pancreatic beta cell, suggesting beta cell communication may resemble neuronal communication. Recently, certain protein families have been discovered in the beta cell that link neuronal synapse machinery with beta cell insulin granule machinery, such as neurexin and neuroligin, which are known to be involved in insulin secretion and neuronal function. Another synaptic adhesion molecule, CADM, has been found in the beta cell, and therefore we suspect it is involved in a similar process (Suckow et al 2008). In this project we explore if the cellular machinery responsible for neuronal transmitter exocytosis in the synaptic cleft between neurons uses the same proteins for insulin granule release in the beta cell.
Chapter 1: Synaptic Adhesion Molecules in Diabetes

Synaptic adhesion molecules are usually located in the neurons and their function is involved with synapse formation and structure, such as neurotransmission. Recently, certain forms of autism and neurodegenerative disorders have been associated with diabetes in children (Freeman et al 2005). How or why remains a mystery, but when certain proteins such as CADM molecules, neuroligins and neurexins (referred to as a group as synaptic adhesion molecules) become mutated and/or nonfunctional there is a corresponding comorbidity of these otherwise unrelated diseases. Synaptic adhesion molecules have been studied in a variety of tissue types, but the majority of published research articles focuses on their role in neurons. Additional studies have revealed that certain families of synaptic adhesion molecules, which are known to regulate the synaptic growth and development of the central nervous system, are also located in the pancreatic islets and can control and/or influence insulin secretion, though the discovery of neuronal proteins in the beta cell is very recent.

Figure 1A shows how synaptic adhesion molecules compare in the neuron. Neurexin functions as a presynaptic adhesion molecule, neuroligin functions as a postsynaptic adhesion molecule, and CADM1 functions as both a post and pre synaptic signaling molecule, interacting with both itself and other molecules. These proteins are important in controlling synaptic function, neuronal connectivity, and recognition patterns in the developing brain, as well as the pathogenesis of epilepsy, schizophrenia, ADHD and Tourette syndrome, suggesting that some of these disorders may share common molecular pathways (Comoletti 2012). Both neurexin and neuroligin
are present in the islets of Langerhans of the pancreas, and it has been recently discovered they play a role in insulin secretory granule release, suggesting that the mechanism for insulin granule release is similar to that of synaptic neurotransmission (Suckow et al 2008). If there is a link between neurons and beta cells it might be easier to understand and manipulate the biological process of glucose regulation via insulin release.
Chapter 2: CADM Isoforms Biochemistry and Function

The CADM (Cellular ADhesion Molecule) proteins have four isoforms in most mammals; CADM1, CADM2, CADM3, and CADM4. A biochemical analysis between CADM proteins was conducted by Pietri et al in 2008 in which he compared the amino acid sequences between the CADM isoforms found in zebra fish that appeared to co-localize in neuronal growth cones and their containing tissue during embryogenesis. The results showed the sequences are highly conserved (between 80.6 and 35.8 percent alignment between isoforms), with each isoform containing three Ig-like domains, a transmembrane domain and a cytoplasmic tail with a 4.1B binding domain and a PDZ type II binding domain. Figures 2A-2D contain the mRNA sequences from rat of each CADM isoform. Below the full mRNA sequences for each isoform are the corresponding primer pairs used for PCR and qPCR.
>gi|47846863|dbj|AB114443.1| Rattus norvegicus sgigsf mRNA for spermatogenic immunoglobulin superfamily, complete cds of CADM1

1 cggcctgggct cggggacgc ccaccctgcag cagtcgtggg caggtcccgc cagatggcag  
61 tccgtctggct cccagcgcct cccagagttgc gctgtggcgc ggcgcccggc  
121 gcctcctgct gcggaccgc cccagtgtgc ggcggcagcg gctgtggcgc  
181 gcctccaggg ctccggctcc ggctcctgct gttgctcctc tcggccgccc cactgatccc  
241 cacaggtgat gggcagaatc tgtttac  
306 taag gagctgacg gtgattg  
361 aag aagaagttgc  
421 aaccatcagc tgtcaagtca ataagagtga tgactccgtg attcagctac tgaaccccaa  
481 caggcagacc atttacttca gggacttcag gccttt  
541 gaag gacagcaggt ttcagc  
601 gaatttttct agcagtgaac tcaaagtgtc actgacaaat gtctcaattt cggatgaagg  
661 gaggtacttc tgccagctct acacggacc  
721 tccgcaggag agttatacca ccatcacagt  
781 gtaaaacg caaagtcgca tccagatgac ttatctctct caaggcttca cccgggaaag  
841 ggtgctattc gatggtacg tggcgcagct cggccatggc cagcaagccg gctacgacca tcaggtggtt  
901 caaag ggaac aaggaactca aaggc aaatc agag gtggaa gagtggtcgg acatgtacac  
961 tgtgaccagt cagctgatgc tgaaggtgca caaggaggat gatggagtcc cagtgatctg  
1021 ccaggtggag caccctgcgg tcactggaaa cctgcagacc cagcggtatc tagaagtgca  
1081 gtaaaccctt tccagcttct tgcagcttct tgcagcttcc ttacggccgt ctggttagac ccacatttcc  
1141 tagtgaagtt ctgtaagagtc gagttgggct  
1201 ggagattgaa gtcaactgca cggccatggc cagcaagccg gctacgacca tcaggtggtt  
1261 caaag ggaac aaggaactca aaggc aaatc agag gtggaa gagtggtcgg acatgtacac  
1321 gtaaaacg caaagtcgca tccagatgac ttatctctct caaggcttca cccgggaaag  
1381 tagtgaagtt ctgtaagagtc gagttgggct  
1441 ggagattgaa gtcaactgca cggccatggc cagcaagccg gctacgacca tcaggtggtt  
1501 caaag ggaac aaggaactca aaggc aaatc agag gtggaa gagtggtcgg acatgtacac  
1561 atttttttgcc atattttgc cggacgacg cggcagctt gtcctctct  
1621 ggttctttcct ccttttccc cgttatttttt acatatt  
1681 atttttttgg cgttttattc cgtttatttt gccttttttc  
1741 ccttttttct cagttttttt cgttattttt acctttttttt  
1801 attttttttt ctaagtttttc cggcaggttc ggtttttttt  
1861 cattttttttta aacatacttt ttacattttttttt  
1921 atttttttttttt tttttttttttt tttttttttttttt  

CADM1 qPCR primers:  
LEFT PRIMER: gaaggacagcaggtttcagc  
RIGHT PRIMER: gcccttctgttcttcc  
SEQUENCE SIZE: 1954  
PRODUCT SIZE: 289

CADM1 PCR primers:  
LEFT PRIMER: taaggacgtgacggtgattg  
RIGHT PRIMER: gccttctgacgtttccgtta  
SEQUENCE SIZE: 1954  
PRODUCT SIZE: 596

Figure 2A: Full mRNA sequence of CADM1
>gi|82619335|gb|DQ272744.1| Rattus norvegicus nectin-like protein 3 mRNA, complete cds of CADM2

1 atgatttgga aacgcaacgcg ggttc tccgc ttctacagt gctg ggtcct cccattaca
61 ggcagccaag ggacgtttccc actaatccag aatgtcaggg ttgtgaaggg cggaaactgc
121 attctgacct gcagagtgta tccaaatgtg aacacctccc tccaagtgcgc gaatacgact
181 cagcagactc tgtacttccga tgacaaagaa ggtttgaggc acaatcgggt cggagctgggtt
241 cgccgcttctt gcagatggtt gacatcagt gtcaggtacc tctctcc tct cgtgagaga
301 cagctacact gcagctctatt tacaattgcct gtcaaacacct ccaagcctta tcctactgctc
361 ctggtggttcc cacagaacgc tcagattagt ggattttctt cccccatcat gggagggagac
421 tgtgagtcagc tgcacttgcaaa gacgcaggc agaaaccctg cgggtatag taaatggttc
481 aaaaatgacca aagagatcag aagatgtgaag tacaatgcct gtcaaaacct ccaaggccta
541 accttcacgg tcagcagcagc actgggatttc cggagtggacc gtagcagatga tcagcagcagc
601 cgtctctgca gtagacatca cagttccttc ctagttttcc acatgagagt gaatactgcg
661 ctaggaatag cactataacc atcaattgcct ccaactccttt ccccaacaaga
721 ggcagcagct taacagttgac ttgtagactt aagagaaac actgcgcaag cactgtttttg
781 tggagaaggg atggggccga attaacagat cctgtcagag ttggttttgg tggagggaga
841 ctcaacatcc ttctctgcaa ccaaaagccgac aatggcacttt atcagatcgc aagcaactaac
901 accagtggct aagacagcgac agaqtactct caactgttagc cctatcctgct agatcccaaa ttctctgctct
961 ggtcagatgacctg tgcagcttata ggcgcagcatg tgggttttgaatagctgaacc cactcaactcct
1021 acctgtggtct ccaatattggcattggttc cattgggagc taattaaaggg acaaattagc
1081 acaaaagaga ctgaagggcc cgaagacgcg ccaagagccct ccaagccctt tataatgcac
1141 gaagagcagc cagctaatagc cgaagagaaaa aagagtaatt tctattaag

CADM2 qPCR primers:
LEFT PRIMER ggcagccaagggcagtttccact
RIGHT PRIMER aagagcgcgaacagctcga
SEQUENCE SIZE: 1188
PRODUCT SIZE: 188

CADM2 PCR primers:
LEFT PRIMER tccgtctactagtgctgtg
RIGHT PRIMER cactgatgctcaactcatgc
SEQUENCE SIZE: 1194
PRODUCT SIZE: 246

**Figure 2B:** Full mRNA sequence of CADM2
>gi|82619333|gb|DQ272743.1| Rattus norvegicus nectin-like protein 1 mRNA, complete cds of CADM3

1 atgggggccccc tctccgcctct gcctctgtcctg cctgctcctg ggcgccgc
61 ggccgcacattttccccaggg cagacgcagc cctgctcctg ggcgccgc
121 ggtggagcgatttccccaggg cagacgcagc cctgctcctg ggcgccgc
181 tctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
241 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
301 ggctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
361 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
421 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
481 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
541 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
601 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
661 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
721 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
781 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
841 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
901 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
961 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
1021 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
1081 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct
1141 cctctcgcagy cctaggtggct cctctcgcagy cctaggtggct

CADM3 qPRC primers:
LEFT PRIMER ggaccgcagaatcctggctc
RIGHT PRIMER atttcgtctggctccctgt
SEQUENCE SIZE: 1197
PRODUCT SIZE: 184

CADM3 PCR primers:
LEFT PRIMER catgagctcagcatcagcat
RIGHT PRIMER cagtgaaggttttccccattg
SEQUENCE SIZE: 1190
PRODUCT SIZE: 298

**Figure 2C:** Full mRNA sequence of CADM3
>gi|114052914|ref|NM_001047107.1| Rattus norvegicus cell adhesion molecule 4 (Cadm4), mRNA of CADM4

1 atgggccgagg cccggcgctt ccagtggccg ctgctgctac tgtgggcggc cgcggcgggg
61 cccagggacc ctttttttca aagccaccgca ggcctgaagg acgaacgatt gcaagctgga
121 ggtctcaggc tgcctctctg ccagcgtggc cctttttcaa tggcacccga gc
181 cggcagaccc ccctgaagg acgaacgatt ccagctggag
241 gagttctccc cgcgccgagt gcgcatcagg ctctcagacg cccgcctgga ggacgagggg
301 ggctctctct caaccagagg accaccaccac agatcgccaac gtcacgtgctc
ttcaggtgcct cggagaatcc ctttcttggg gaggagttgtg
gtggaactca gctgcctggt tccgcggtcg cgccccgcag cgcgtcctgc
gtggagcgcg gcgagacactg cgcgtgggct
gtcaccttc gcacggtgcc tctctgctgg
gtggaatacg cagctgctgt ggtctatgac cccggagcag tggtagaggc tcagacatcg
gttccctacg ccatcgtggg cggcatcctg gcgctactgg tgtttctgat catatgcgtg
cagttgacat ccagcggcag tgcggttccg
cgcgcagaa ccagggctgcc ctcggggcac agcaagcaga cgcagtacgt gctggatgtg
cagtatctcc ccacagcctgc cacgagctctg ctatctgctgc
tgggaatgagt ctctgccag a gagggcggag gcgttgggcg agacgctcac gctgcctggc
catgatacgg cagctgctgt ggtctatgac cccggagcag tggtagaggc tcagacatcg
cagttgacat ccagcggcag tgcggttccg
cgcgcagaa ccagggctgcc ctcggggcac agcaagcaga cgcagtacgt gctggatgtg
cagtatctcc ccacagcctgc cacgagctctg ctatctgctgc
tgggaatgagt ctctgccag a gagggcggag gcgttgggcg agacgctcac gctgcctggc
catgatacgg cagctgctgt ggtctatgac cccggagcag tggtagaggc tcagacatcg
cagttgacat ccagcggcag tgcggttccg
cgcgcagaa ccagggctgcc ctcggggcac agcaagcaga cgcagtacgt gctggatgtg
cagtatctcc ccacagcctgc cacgagctctg ctatctgctgc
tgggaatgagt ctctgccag a gagggcggag gcgttgggcg agacgctcac gctgcctggc
catgatacgg cagctgctgt ggtctatgac cccggagcag tggtagaggc tcagacatcg
cagttgacat ccagcggcag tgcggttccg
cgcgcagaa ccagggctgcc ctcggggcac agcaagcaga cgcagtacgt gctggatgtg
cagtatctcc ccacagcctgc cacgagctctg ctatctgctgc
tgggaatgagt ctctgccag a gagggcggag gcgttgggcg agacgctcac gctgcctggc
catgatacgg cagctgctgt ggtctatgac cccggagcag tggtagaggc tcagacatcg
cagttgacat ccagcggcag tgcggttccg
cgcgcagaa ccagggctgcc ctcggggcac agcaagcaga cgcagtacgt gctggatgtg
cagtatctcc ccacagcctgc cacgagctctg ctatctgctgc
tgggaatgagt ctctgccag a gagggcggag gcgttgggcg agacgctcac gctgcctggc
catgatacgg cagctgctgt ggtctatgac cccggagcag tggtagaggc tcagacatcg
cagttgacat ccagcggcag tgcggttccg
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cagtatctcc ccacagcctgc cacgagctctg ctatctgctgc
tgggaatgagt ctctgccag a gagggcggag gcgttgggcg agacgctcac gctgcctggc
catgatacgg cagctgctgt ggtctatgac cccggagcag tggtagaggc tcagacatcg
cagttgacat ccagcggcag tgcggttccg
cgcgcagaa ccagggctgcc ctcggggcac agcaagcaga cgcagtacgt gctggatgtg

CADM4 qPCR primers:
LEFT PRIMER gtcatctgtgaagcgcagaa
RIGHT PRIMER agcacatgtcagcaccagag
SEQUENCE SIZE: 1167
PRODUCT SIZE: 150

CADM4 PCR primers:
LEFT PRIMER cccctgaaggacgaagccagatt
RIGHT PRIMER gagactctttcacaaggttc
SEQUENCE SIZE: 1173
PRODUCT SIZE: 582

**Figure 2D**: Full mRNA sequence of CADM4
The CADM proteins appear to function in a similar manner, with the extracellular Ig-like domains mediating both hetero- and homophilic interactions that are Ca$^{2+}$ and Mg$^{2+}$ independent. Figure 2E shows the four CADM isoforms found in rat tissue in un-glycosylated form. The groups have been color coded based on function, and compared side-by-side in ball-and-stick model form. These images have been provided from RasMol, a free online program that generates protein structures from amino acid sequences.

Figure 2E: CADM isoforms Ball-and-Stick diagram as provided by Rasmol. Starting from upper left corner and going clockwise: CADM1, CADM2, CADM3, CADM4. Color coded according to structure.

Figure 2F shows how the CADM isoforms interact, in both cis- and trans-interactions, though each isoform also interacts with itself. It was important to understand the CADM family of isoforms before the experiment began in order to elucidate information regarding the localization and binding that might be involved with these proteins if they were found to affect insulin secretion. A particular area of interest is
which CADM isoform has the highest expression levels in the beta cell, as each isoform has its own (albeit similar) function. By determining which isoform had the highest expression level in the beta cells and focusing on that protein, there was an increased likelihood of finding the isoform with the greatest amount of involvement in insulin secretion.

**Figure 2F:** Diagram of CADM isoforms interactions. Lines indicate ability to interact. Sandau et al 2011, Kakunaga et al 2005, Nagata et al 2012, Hunter et al 2011
Chapter 3: CADM1 in the Beta Cell

The CADM1 protein has a complicated history in scientific literature. Due to the diversity of research goals regarding this protein, the nomenclature was not standardized to CADM1 by the HUGO gene nomenclature committee until recently (Koma 2008). CADM1 was repeatedly re-discovered by scientific teams specializing in different diseases (including cancer, the immune system, and CNS malfunction), unaware other research teams were studying the same protein in parallel. Due to this, CADM1 has been cross referenced under multiple names. Table 3A lists the names corresponding to the field of study the CADM1 molecule was found in.
**Table 3A:** Alternative nomenclature for the CADM1 molecule

<table>
<thead>
<tr>
<th>Name</th>
<th>Field of study</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CADM1</td>
<td>Cancer</td>
<td>Tumor suppression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mast cell survival</td>
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The CADM1 protein is found in various tissue types throughout the body, but not in the exact same form. For the purpose of this project, it was useful to focus on the previous research conducted by the CNS scientists for background information because the beta cell architecture is similar to the neuron. There are slight differences such as glycosylation based on localization and functional mechanisms, but otherwise CADM1 behaves similarly in the brain and beta cell tissue, and the information previously discovered by neuroscientists is applicable. A cell surface molecule (Figure 1A), CADM1 binds extracellularly both homophilically and heterophilically to other CADM isoforms (including itself), in both cis and trans conformations. CADM1 can be found on either the presynaptic or postsynaptic region of nerve cells, and the binding partners appear to be universal regardless is glycosolation and tissue type; namely CADM2, but also CADM3 and possibly CADM4 (Figure 2A). Intracellularly, common binding partners include CASK and similar proteins known to bind to the PDZII binding domain and the 4.1B motif (Masuda et al 2010). Figure 3A shows the schematic of the CADM1 protein structure, with the overexpression plasmid used to overexpress it beneath it. The structure of CADM1 is comprised of an extracellular region (including the N terminus), a single transmembrane pass, and a short intercellular region (including the C terminus) as seen in Figure 3A. Glycosylation takes place on the extracellular region of the molecule, both N and O linked, along the Ig-like regions. Glycosylation patterns vary between tissue types the CADM1 protein is located in, with highly glycosylated regions found in the brain and central nervous system, and regions such as the retina having less glycosylation (De Maria 2011).
**Figure 3A:** Diagram of CADM1 for overexpression (M. Masuda et al with edits) inserted at EcoR1 sites in pcDNA3.1 vector (bottom image provided by Invitrogen)
Glycosylation can be visualized via western blot of differing tissue types, with highly glycosylated proteins appearing at higher molecular weights. In the rat model, without glycosylation the 445 amino acid protein (Figure 3B) is estimated to be 36kDa, but in the beta cell CADM1 appears at a distinct 90-100 kDa band (See figure 3C). In the brain, a distinct banding pattern can be seen around 75kDa. As can be seen in Figure 3D, the blotting pattern in the brain tissue of rats in a western is very different then the binding pattern in a rat based beta cell line. Also, there is less protein available in the beta cell line, since the antibody doesn’t have a high enough affinity to strongly visualize naturally occurring CADM1.

MASPVLPSGS QCAAAAAVAA AAAPGLRLR LLLLLSAAA LIPTGDQONL FTKDVTVIEG EVATISCQVN KSDKSVQQQL NPNRQTIYFRE FFRPLKDSRF QLLNFSSSEL KVSHTNVSIS DEGRYFCQLY TDPQGQESTT ITVLVPPRNL MIDIQKDTAV EGEEIEVNT AMASKPATTI RWFKGNKELK GKSEVEESWD MVTQTVQQLM KVHDGGQVQVQI VGQVEHPSAV TGNLQQTQRLQ EVQYKQVSQOQI QMTPLQQQTQI REGDAELTC EATGKQPVQV MVTWVRVDEDM PQHVLGSPN LFIVNQLKD TDYRCEASN TVRQALVSEK YVYDPPCTT PTPPTTTTT TTTTTTTTTL TIIITTTATT EAPVHGLTQL PNSAEELDSE DLSDSRGEE GAIGAVDHAV IGGVAVVVVF AMLCLIIILG RYFARHGTY FTHEAKGADD AADADTAIIN AEGGQNNSEE KKEYFI

**Figure 3B:** CADM1 full protein sequence in *Rattus norvegicus*, with 476 amino acids

**Figure 3C (left):** Western blot of INS1E protein lysate probing for CADM1. Left lane contains overexpression plasmid, right lane contains negative pcDNA3.1 empty control

**Figure 3D (right):** Western blot of rat brain protein lysate probing for CADM1
The extracellular region of CADM1 is comprised of three Ig domains, with the outmost region referenced as Ig1, the middle Ig2, and the third (closest to the transmembrane domain) Ig3, as can be seen in Figure 3A, with the entire extracellular region comprised of 373 amino acids. Included in that region is an area between amino acids 343-356, referred to as a “shedding domain” and is comprised of an excess of T amino acids. This is where the protein’s extracellular domain can be spliced off (Nagara et al 2011). This shedding process includes a series of events in which the extracellular region of the protein is cleaved by an enzyme complex to modulate function. This may explain why CADM1 is found in different banding patterns in different tissues; there is post-translational modification in order to regulate cellular function. After the extracellular region is the transmembrane region of CADM1, comprising of 23 amino acids, and lastly an intracellular region, of only 46 amino acids. The intracellular region of CADM1 is comprised of a PDZ domain, and a 4.1 protein motif (Fogel 2011). Figure 3A also shows the site where a FLAG motif was added to the overexpression protein, on amino acid 363 (Figure 3B).

The glycosylation patterns are not completely understood yet, but based on the addition or deletions of the sugar chains, the CADM1 protein is either enabled or disabled from interacting with certain extracellular proteins on neighboring cells, and thus cell-to-cell interactions and signaling can be controlled. In the pancreas, the islets are richly innervated allowing a large surface area interaction between the CADM1 proteins and their binding partners, but there appears to only be one CADM1 glycosylation pattern (Figure 3C).
The beta cell is the only cell of the body that contains insulin granules. In response to glucose stimulation, it will release these granules via exocytosis and insulin is released into the blood stream. This is the first phase, in which all the docked insulin granules which have been waiting for glucose stimulation are exocytosed in an immediate and almost instantaneous fashion. If the glucose stimulation continues, the second phase of insulin is released, though slower because new insulin must be synthesized in the beta cell, packaged into granules, and then exocytosed. While the finer details of how this process is carried out are not entirely understood at the present time, we believe that learning how the beta cells functions on a cellular level will lead to new insights as to how insulin is released, and perhaps give researchers new insight as to the pathogenesis of diabetes. Previous studies by Ito et al 2012 have confirmed in a mouse model that CADM1 knockdown affects glucagon secretion, but it tells us little of what CADM1 is actually doing, or more importantly, what the presence or absence of CADM1 does to affect insulin levels; if it has a direct effect on the mechanism of granule release, or is a signaling molecule in the pathway somewhere in that process.

We chose to work with cell lines INS1 and INS1-E, specialized rat beta cell lines, with INS1 cells being a polyclonal cell line and INS1-E a monoclonal version. There are minor differences between them in terms of morphology, but both produce reasonable insulin responses to glucose stimulation, allowing us to observe the beta cells alone, in an isolated environment. Since there are so few islets in pancreatic tissue, and by virtue of this even fewer beta cells, by using these cell lines we can perform multiple experiments
in an effective fashion and control for extraneous factors that might contribute to insulin release.
Chapter 5: The Process of Insulin Synthesis and Secretion via Glucose Stimulation

Insulin is synthesized within the beta cell. The precursor molecule is preproinsulin, which is cleaved into proinsulin in the ER immediately after synthesis. Proinsulin is transported to the Golgi apparatus and packaged into clathrin-coated secretory granules. These granules mature by loss of clathrin coating. Proinsulin is converted into insulin and C-peptide by proteolytic cleavage at two sites in the peptide chain (Gardner et al. 2007). This storage pool of insulin granules is transported by various proteins including microtubules and microfilaments, ATP, MAP-2, kinesin, RAB3A, kinase and phosphatase to the plasma membrane of the beta cell, where the granule is docked and primed through a complex series of events involving multiple proteins and ATP, with these primed granules referred to as the readily releasable pool. Upon receiving appropriate stimuli, the primed granules release their contents into the blood stream to decrease blood glucose levels. (Gardner et al 2007) Figure 5A illustration this process.

Basal insulin secretion occurs in the absence of exogenous stimuli, and is the insulin secreted while in a fasting state. Stimulated insulin secretion occurs in response to exogenous stimuli, usually a response to increased glucose concentrations. A sudden glucose spike in the blood stream causes a short-lived burst of insulin release, referred to as first phase insulin secretion (see figure 5A); if the glucose concentration is maintained, insulin release gradually falls off and then begins to rise again to a steady level, referred to as second phase. At this point, insulin secretory granules are transported from the storage pool directly to the plasma membrane for exocytosis, skipping the docking step.
Sustained levels of high glucose stimulation result in reversible desensitization of the beta cell in response to glucose. (Gardner et al 2007)

**Figure 5A**: Schematic representation of the putative steps in the mechanism of insulin exocytosis and beta-granule trafficking in pancreatic beta cells
Chapter 6: Insulin Granule Docking and Secretion

There are several proteins involved in the docking steps, notably the SNARE complex, in which v-SNAREs and t-SNAREs form complexes to allow vesicle fusion to take place. This entails the pairing at the target membrane of a vesicle-associated membrane protein (VAMP), a v-SNARE, with a binary cognate receptor complex that comprises SNAP25 or SNAP23 and a syntaxin protein (both of which are t-SNAREs) (Wang et al 2009, Izumi et al 2007). This phenomenon has been well studied in neurons, in which the SNARE complex is responsible for the release of neurotransmitters between the synaptic cleft. The same phenomenon has been observed in beta cells with regard to release of insulin granules, further suggesting that the beta cells must maintain contact and communication in order to properly release insulin granules.

Glucose stimulated insulin secretion takes place on a cellular level, where glucose enters the beta cells through GLUT2, a transmembrane protein in the plasma membrane that functions as a passive glucose transporter into the cell. The glucose molecule enters glycolysis and the respiratory cycle within the beta cell, leading to ATP formation via oxidation, leading to a rise in the ATP:ADP ratio within the cell. This increase in the ATP:ADP ratio closes ATP-sensitive potassium channels, leading to a buildup of potassium ions, causing depolarisation of the cell surface membrane, leading to voltage-gated calcium ion channels to open and allow calcium ions to enter the cell. This increase in intracellular calcium ion concentration causes the activation of phospholipase C, which cleaves the membrane phospholipid phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate (IP3) binds
to receptor proteins in the plasma membrane of the endoplasmic reticulum, allowing the release of calcium ions from the ER via IP3-gated channels, and further raising the intracellular concentration of calcium ions (Cawston et al 2010).

Significantly increased amounts of calcium ions in the cell causes the release of previously synthesized insulin, which has been stored in secretory vesicles. This is the primary mechanism for release of insulin. Other substances known to stimulate insulin release include the amino acids arginine and leucine, parasympathetic release of acetylcholine (via phospholipase C), sulfonylurea, cholecystokinin (CCK, via phospholipase C), and the gastrointestinal-derived incretins glucagon-like peptide-1 (GLP-1) and glucose-dependent insulino-tropic peptide (GIP). (Cawston et al 2010).

Glucose is not the sole stimulate for insulin release. The “incretin effect” has been observed in studies, in which orally administered glucose leads to greater insulin secretion than the same glucose administered intravenously. This effect is mediated by the incretin hormones glucose-dependent insulino-tropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), secreted from endocrine cells located in the small intestine. Insulin secretion in response to these hormones is strictly glucose-dependent and in healthy individuals accounts for up to 70% of the insulin response to a meal (Russell 2012). Because the studies for this project were conducted strictly in beta cell lines, the incretin effect could not be accounted for.
Methods

Overexpression:

A kind gift of pCAGGS synCAM1(363)FLAG expression vector was received from Dr. Thomas Biederer of Yale University, a proven overexpression plasmid of CADM1 used in neurons. It comprised of the full CADM1 protein construct with the addition of FLAG at amino acid number 363 (Biederer 2005). pCAGGS was found to be an inefficient overexpression vector in the beta cells, so the CADM1 fragment was spliced out via restriction enzymes Eco-R1 and replaced in a pcDNA3.1 vector (Figure 3A). Sigma 3X FLAG CMV-7-BAP Positive control plasmid was used as a negative control for a FLAG plasmid.

Overexpression was successful in both INS1 and INS1E cell lines, using Lipofectamine2000 transfection reagent with the CADM1-pcDNA3.1 plasmid over a 48 hour time course, with 80% confluent cells at time of transfection, confirmed via western blot. Polyclonal antibody used to probe for CADM1 was purchased from Novus Biologicals Antibody #NB300-186. The affinity of the CADM1 antibody is somewhat low to detect endogenous CADM1 in the rat beta cell, so it was confirmed using rat brain protein lysate instead (Figure 3D).

Knockdown:

ON-TARGETplus SMARTpool siRNA was purchased from ThermoScientific Cat#L-101011-01-0005 Rat Igsf4a (363058) comprising of target sequences GGGUGAGAGAGUCAUGACGA, GUGGAAGAGUGGUCGGACA, GGGAAAGCUCAUUCGGACU, and CGUAACUUGAUGAUCGAUA. Cat#D-
001810-10-05 ON-TARGETplus Non-targeting Pool was purchased as a negative control. Lipofectamine2000 was found to be an ineffective transfection reagent for knockdown, so electroporation was performed instead. Lonza VACA-1003 Cell Line Nucleofector Kit V serial number T-01968 was successful using program code T-020 with an efficiency of 70-96% over a 48 hour time course, using 3 x 10^6 INS1 cells per condition. Dr. George Sen of the UCSD Dermatology lab kindly allowed use of his Lonza transfection machine. This experiment was performed in INS1 cells healthy and confluent in a 24 well plate over a 48 hour knockdown time period.

CADM1 knockdown was tested via qPCR using CADM primers GAAGGACAGCAGGTTTCAGC and GCCTTTGAGTTTCTTGTTCC and control 18s primers CGCCGCTAGAGGTGAAATTC and TTGGCAAATGCTTTCGCTC, purchased from Valuegene Inc. qPCR reagent SyberGreen was purchased from Quanta. Absolute qPCR primers were also purchased from Valuegene, as seen in Figures 2A-2D.

**Absolute qPCR**

PCR products of the four CADM isoforms were made using the corresponding primers listed in Figures 2A-2D with INS1E cDNA, reverse transcribed using RNA extracted from confluent INS1E cells using the GenElute Mammalian Total RNA MiniPrep Kit purchased from Sigma. The products were run on an electrophoresis gel (not show) and the DNA fragments extracted, measured for concentration, and diluted to a standard curve of copy numbers, from 10^9 to 10^2. The standard curve (of each isoform) was run on a qPCR plate alongside normal INS1E cDNA. The results were back-
calculated using the Ct values of the standard curve in order to determine exact quantities of each CADM isoform found in INS1E cDNA.

**Glucose stimulated insulin secretion**

Prepared cells were grown in a 24 well transfection plate and incubated in low glucose Krebs media for one hour. The medium was aspirated and then replaced with either high or low glucose Krebs media for an hour. This medium was then collected and diluted 1:50 in assay buffer. The beta cells were lysed using RIPA buffer with a protease inhibitor cocktail and PMSF and diluted 3:12500 in assay buffer. Both the medium and cell lysate were then analyzed for insulin content using a RIA kit with $^{125}$I labeled insulin. GSIS results were calculated by normalizing the insulin in the medium by the insulin in the cell lysate of each condition. Statistical significance was tested using the student’s t test. Error bars show standard error.
Results:

Isoform amounts

Figure 7 shows the most abundant CADM isoform found in INS1E is CADM2, but Figure 8 shows in normal rat islet tissue CADM1 is the most abundant isoform. It is possible this is due to INS1E being an insulinoma cell line with mutations. Therefore, islet cDNA has a higher likelihood of showing true physiological conditions. Because we were interested in determining the natural state of CADM isoforms in normal tissue, we decided to pursue the CADM1 molecule for further studies in the beta cell.

![Absolute qPCR of CADM isoforms](image)

**Figure 7:** Absolute qPCR of CADM isoforms in INS1E cDNA results
Figure 8: PCR products of CADM isoforms in varying tissue types. From lanes left to right: INS1E cDNA, islet cDNA, negative control (water). From top image down PCR primers for CADM1, CADM2, CADM3, CADM4
**CADM1 knockdown**

Knockdown was achieved at levels between 70% and 96%, confirmed via qPCR normalized to 18s (figures not show) using electroporation. Knockdown results show that when CADM1 was successfully knocked down, CADM2 and CADM3 levels were also decreased by 20-30% (data not shown). Figure 9 shows a statistically significant increase in insulin secretion in INS1 cells with knockdown levels CADM1 in low glucose conditions. High glucose conditions show no significant change.

![CADM1 knockdown in INS1 cells](image)

**Figure 9**: Graph of glucose stimulated insulin secretion in CADM1 knockdown conditions in INS1 normalized to cellular insulin. Compilation of experiments, n = 18.
CADM1 overexpression

CADM1 had been successfully overexpressed in both INS1 and INS1E cell lines, as confirmed by western blot (Figure 3C). There is only one band shown at approximately 90 kilodaltons, suggesting the glycosylation pattern yields a single band and thus is uniformed. This situation may mask any glycosylation changes that occur in the plasmid overexpression form of CADM1 compared to the naturally occurring CADM1, which may also affect function of the molecule. GSIS analysis of CADM1 overexpression results showed that with increased CADM1 protein, there was an increase in insulin secretion after both high and low glucose stimulation compared to control, in both INS1E (Figure 10) and INS1 (Figure 11) cell lines, though only statistically significant at low glucose in INS1.
**Figure 10:** Graph of glucose stimulated insulin secretion in CADM1 overexpression conditions in INS1E normalized to cellular insulin. Compilation of experiments, n = 15.
CADM1 overexpression in INS1 cells

**Figure 11**: Graph of glucose stimulated insulin secretion in CADM1 overexpression conditions in INS1 normalized to cellular insulin. Compilation of experiments, n=10.
Discussion & Conclusion

Recent diabetes-related literature has suggested that neuronal proteins play a significant role in insulin release, though this role is still largely unclear, and the cellular mechanisms that are being employed in this capacity is still unknown. Papers by Koma et al 2008 suggest that CADM1 mediates nerve-islet cell interactions though CADM1’s role in insulin secretion is unclear, and papers by Suckow et al 2010 suggest that other neuronal proteins such as neuroligin and neurexin play a role in insulin secretion. Poy et al 2009 has shown that gene mutations that lead to an increase in CADM1 regulate alpha and beta cell mass. CADM1 malfunction also appears to have a role in neuronal diseases such as autism (Fujita et al 2010). Preliminary lab results suggested that the CADM family of molecules might yield interesting information in this field, as they function as both a pre- and post-synaptic adhesion molecule in neuronal cells and are present in neuronal and pancreatic tissue in vertebrates.

In this project, the initial question asked was which CADM isoform had the highest expression level in the beta cell. Due to the nature of primer binding efficiency and low levels of CADM molecules in beta cell tissue, an absolute qPCR was performed on the four isoforms found in INS1E cDNA. Ins1E was chosen because the RNA is readily available for isolation and it has been established for diabetes research as a monoclonal rat beta cell line. Absolute qPCR results showed significantly more CADM2 mRNA compared to the other four CADM molecules (Figure 2F). A PCR reaction was performed to confirm those results, comparing INS1E cDNA to normal islet cDNA
extracted from rat pancreatic tissue. CADM isoforms are known to be involved in cancer metastasis, so it is possible that the expression level in a cell line is not an accurate representation of beta cell protein levels. The results in Figure 2G show that CADM1 has the highest level of endogenously expressed mRNA in islet cDNA. While the PCR results conflict with the absolute qPCR results, the project moved to focus on the CADM1 molecule because islet cDNA reflects more physiologically correct conditions.

Glucose stimulated insulin secretion (or GSIS) was the main assay that was used in our experiments to test beta cell insulin response to changing levels of glucose stimulation. By setting up plates of beta cells with CADM1 either overexpressed or knocked down, it was possible to compare insulin secretion rates across cellular conditions. This was very useful in determining if this protein has an effect on insulin release, and to what extent. The insulin secretion rates were normalized by dividing insulin in the media (insulin that had been exocytosed) to insulin in the cell lysate (insulin that had been produced but not yet secreted in the beta cells). These result showed that the INS1 polyclonal cell line had a smaller standard error then the INS1E monoclonal cell line, and also tended to be healthier in knockdown and overexpression conditions (images not shown).

Knockdown of CADM1 was the next step in this project, but it became difficult to perform based on the nature of the molecule; it is endogenously expressed at low levels and is not well documented in beta cells. We wished to perform these assays in a beta cell line, such as INS1, in order to observe the beta cell in isolation and transfection worked best using electroporation, since it kept the cells healthy and confluent. GSIS results
show that a knockdown of CADM1 in INS1 cells results in a statistically significant increase in insulin secretion in low glucose conditions (Figure 3F). This is the first time CADM1 has been shown to influence insulin secretion, and while knockdown increases insulin secretion at basal glucose levels, it does not affect insulin secretion at high glucose levels.

The next step in this experiment was to test overexpression. Overexpression by definition is a non-physiological event, but CADM1 overexpression was performed in both INS1 and INS1E rat beta cell lines, with the same results. As seen in Figure3D and E, overexpression shows a trend of increased insulin secretion, both in high glucose and low glucose conditions.

Under both overexpression and knockdown conditions, insulin secretion is shown to increase, notably in low (basal) glucose conditions. These results suggest that altered CADM1 levels in beta cells can affect insulin secretion, but why or how remains unknown. Perhaps changing levels of CADM1 causes the beta cells to alter levels of insulin granules, and different results would be seen if alpha cells were also present. It is also possible that CADM1 plays an inhibitory role in first phase insulin secretion, leading to increased insulin secretion when it is knocked down, and an excitatory role in second phase insulin secretion, leading to an increase in insulin secretion in both high and low glucose stimulatory conditions when it is overexpressed. This hypothesis can be further tested in future experiments such as visualizing insulin docking under overexpression and knockdown conditions and observing granule distribution.
Papers Cited:


