AN ABSTRACT OF THE DISSERTATION OF <u>Murugesh Narayanappa</u> for the degree of <u>Doctor of Philosophy</u> in <u>Biochemistry</u> <u>and Biophysics</u> presented on <u>November 15, 2016.</u>

<u>Title: Characterizing the Functional Properties of Otoferlin, Essential for</u> <u>Neurotransmission in Inner Hair Cells of the Cochlea.</u>

Abstract approved:

Colin P. Johnson

Hearing loss is one of the most common defect, affecting 360 million people worldwide due to several factors including congenital, present at or soon after birth or acquired with age. Congenital hearing loss affects 32 million children in the world. The economic impact of hearing loss is estimated to cost society an average of 300,000 dollars over the lifetime of a person. It also has serious impacts on quality of life including the literacy rate in children. Although, hearing loss is clearly a major health problem its genetic basis for the pathology is poorly understood. To date, over 60 pathogenic mutations in otoferlin have been found to be associated with inherited, non-syndromic congenital hearing loss and temperature sensitive auditory neuropathy (TSAN).

of hearing depends on reliable and temporally precise The sense neurotransmitter release at the synapses of inner hair cells (IHCs) of the cochlea. Inner hair cells of the cochlea derive their name from hair bundle (stereocilia) protruding at the apical tip of the cell, that are arranged in rows of graded height. The nanometer displacement of the hair bundle opens mechanically gated ion channels that depolarize the cell. This change in membrane potential triggers calcium dependent fusion of synaptic vesicles with the plasma membrane and the release of neurotransmitter. In IHCs, Calcium-regulated exocytosis and neurotransmitter release exhibit fast kinetics in achieving exquisite temporal fidelity. To aid in fidelity and for allowing high rates of sustained synaptic neurotransmission, IHCs contain specialized structures called synaptic ribbons for tethering synaptic vesicles at release sites. The calcium triggered synaptic vesicle fusion with the plasma membrane is believed to be driven by the assembly of SNARE proteins. However, SNARE proteins are insensitive to calcium. In conventional neurotransmission in neurons, synaptotagmin 1 confers calcium sensitivity to SNARE-mediated fast synchronous neurotransmission. However, Yasunaga et al. 1999 and Beurg et al. 2010 have reported that synaptotagmin 1 was not detected in mature IHCs and it has been suggested that IHCs have evolved a unique calcium sensor, otoferlin for calcium-regulated synaptic neurotransmission. The evidence for calcium sensor hypothesis of otoferlin comes from Roux et al., 2006 who reported that mice lacking otoferlin were profoundly deaf and lack synaptic vesicle exocytosis in IHCs. Otoferlin has also

been shown to be required for calcium dependent synaptic exocytosis at immature outer hair cells (OHCs) and vestibular hair cells.

Otoferlin belongs to ferlin family of proteins and consists of six C2 domains (C2A-C2F) linked in tandem followed by a single-pass C-terminal transmembrane region. C2 domains are known to bind to calcium and lipids. The lipid and calcium binding properties of synaptic proteins are critical characteristics that define and shape the release properties of a synapse, and thus, without a quantitative characterization of these activities, an understanding of otoferlin's function in hair cells will remain elusive. This dissertation addresses the intrinsic calcium binding properties of each C2 domain of otoferlin and also the lipid binding specificity and the effects of lipids on calcium binding were also assessed using liposome sedimentation assays and laurdan fluorescence measurements. On the basis of our results, we proposed that the C2C and C2F domains of otoferlin preferentially bind PI(4,5)P2 and that PI(4,5)P2 may serve to target otoferlin to the presynapse in a calcium-independent manner. This positioning would facilitate fast calcium-dependent exocytosis at the hair cell synapse.

Many C2 domains involved in exocytosis also tilt, orient and partially insert into membranes to aid facilitating membrane fusion. To determine the orientation and structural characterization of the C2F domain at the membrane interface, we used label free sum frequency generation vibrational spectroscopy. We found that the C2F domain of the otoferlin interact with membranes DPPC:DPPS and orients 32° normal to the membrane. Our results represent the first structural view of any C2 domain of otoferlin docked at the membrane interface.

In my dissertation, I also characterized the temperature sensitive mutants of otoferlin. Deletion of glutamic acid at position 1804 in otoferlin results in a temperature-sensitive mutant in the C2F domain (C2F-TS) that causes temporary deafness in febrile patients. our results using CD-spectroscopy, tryptophan fluorescence and urea denaturation methods, we found that C2F-TS may have minor structural changes localized in the active site.

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Characterizing the Functional Properties of Otoferlin, Essential for Neurotransmission in Inner Hair Cells of the Cochlea

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Murugesh Narayanappa

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APPROVED

Major Professor, representing Biochemistry and Biophysics

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Murugesh Narayanappa, Author

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CONTRIBUTION OF AUTHORS

Nicole Hams provided Fluorescence intensity of C2F-acridone-2-ylalanine in data for chapter 2. Lee C. Speight, E. James Petersson and Ryan A. Mehl provided acridone-2-ylalanine. Thaddeus W. Golbek was involved in providing SFG spectra data and analysis and writing of chapter 3. Joe E. Baio was also involved in writing and experimental design of chapter 3. Blake Hakkila and Scott Hersberger were involved in analyzing the data for Chapter 4. Colin Johnson was involved in design, analysis and writing of all experiments and chapters.

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Chapter 1: Introduction and thesis review:

1.1. Conventional neurotransmission:

The first scientific approach to the knowledge of how the brain works started as early as the sixth century B.C, with the philosophical hypothesis by Alcmeon of Croton (a pre -Socratic philosopher) who spoke of the presence of pores and channels in the brain and sensory organs¹. However, with the advent of cellular theory in the 1830s and with enormous technical advances in the field of cytohistology, Gabriel Gustav Valentin published the first microscopic image of the nerve cell soma (cell body) in 1836¹. Following Valentin's findings, Robert Remak and Otto Karl Deiters found undivided extensions from a nerve cell that they called as "axis cylinder" (axons) and other processes that branched out extensively as "protoplasmic extensions" (Dendrites)¹. All these findings led to a theory called "the reticular theory" by Josef Von Gerlach according to which nerve cell endings would not end freely and protoplasmic prolongations form a fine nerve fibre network. This theory was defended by Camillo Golgi stating that "nerve cells lack the independence with which the rest of the organism's cells equipped". Eventually, Santiago Ramon y Cajal was the first to provide morphological evidence that nerve cells indeed terminate freely and was also able to dye the axon and cell bodies and concluding that "Each nerve cell is a totally autonomous physiological canton". In 1891, a proponent of this new theory, Heinrich Wilhelm von Waldever, coined the term "neuron" to refer to a nerve cell which is the basic and functional unit of a nervous system. Subsequently Wilhelm

His coined the term "dendrites" for protoplasmic prolongations and Von Kolliker renamed "cylinders" as axons. A neuron is the basic and functional unit of the nervous system. The neuron has three main parts, including a cell body (soma), dendrites and an axon. The cell body in a neuron contains a nucleus and other cellular organelles such as mitochondria, ribosomes and endoplasmic reticulum and carries out most basic cellular functions. Dendrites are the neuronal process that emerge from the cell body as branched tendrils to receive stimulus. Attached to the cell body is a long, thin,



Figure 1.1: Diagrammatic representation of a Neuron¹⁴⁸:

Neuron is a basic, structural and functional unit of a nervous system. It consists of three main parts including a cell body, dendrites and an axon. Cell body also known as soma contains nucleus and cellular organelles. Dendrites and the axon are the projections from the cell body required for receiving input or stimulus and propagating stimulus to another neuron respectively.

cylindrical projection called an axon, which is responsible for signal transmission within the neurons (Figure 1.1). The transmission of signals within the nerve cells occur due the membrane potential defined as the voltage difference across the cell membrane created by uneven distribution of electrically charged ions sodium (Na⁺) and (K⁺) through voltage gated Na⁺ and K⁺ channels. These channels "open" or "close" in response to changes in the membrane potential. When membrane potential exceeds a certain threshold, it depolarizes the membrane and an action potential (AP) is generated in response to the threshold which will travel along the neuron, but does not occur when there is subthreshold stimulus. Thus, the action potential is an all-or**none principle**. The action potential generated through both electrical and chemical signals in neurons ensue synaptic neurotransmission. Synaptic neurotransmission involves communication between neurons through a gap called a synaptic cleft and the area involved in connecting two neurons is called as a synapse. The neuron that sends an impulse is called as a presynaptic neuron and the neuron receiving the impulse is called as postsynaptic neuron (Figure 1.2). Synaptic neurotransmission involves following steps¹⁴⁸:

- 1. An action potential (impulse) initiated at the cell body travels along the axon and reaches the axon terminal.
- The action potential then induces opening of voltage gated Ca²⁺ channels present on the axon terminal membrane and calcium ions flow into the cell through opened channels.

- 3. The calcium signaling triggers the fusion of one or more synaptic vesicle, filled with neurotransmitter with the presynaptic plasma membrane. As membranes merge, neurotransmitters from synaptic vesicle is released into the synaptic cleft.
- 4. The neurotransmitters then diffuse through the synaptic cleft and bind to the receptors present on the postsynaptic membrane. The postsynaptic neuron then converts the chemical signal into an electrical impulse¹⁴⁸.

The relationship between the action potential and the release of neurotransmitter (synaptic vesicle fusion or exocytosis) is regulated by sophisticated molecular machinery. Though hundreds of proteins are thought to be involved in neurotransmitter release, there are several key proteins crucial for synaptic vesicle fusion whose key functions have been studied extensively. In 1967, Katz and Miledi established the link between calcium influx and membrane fusion to release neurotransmitters². Since then several approaches have been undertaken to understand the molecular mechanisms and the machinery involved in membrane fusion. In 1993, James Rothman's lab found that soluble N-ethylmaleimide sensitive fusion protein receptors (SNARE's) including syntaxin 1A, SNAP-25 and synaptobrevin -2 are essential for synaptic vesicle fusion³. SNARE proteins consists of a well conserved 70 residue sequence called the SNARE motif. SNAREs present on the synaptic vesicle are called vesicle-SNAREs (v-SNAREs) and those associated with presynaptic plasma membrane are known as target-SNAREs (t-SNAREs)^{4,5}. In neurons, the v-SNARE, synaptobrevin and t-SNAREs syntaxin-1 and SNAP-25 form a stable four helix bundle which mediates bringing the vesicular membrane and plasma membrane into close proximity to facilitate membrane fusion^{6,7}. In addition, several studies reported that SNAREs can mediate membrane fusion using reconstitution experiments. Thus, SNAREs represent the minimum protein machinery for synaptic vesicle fusion with presynaptic plasma membrane ⁴. However, membrane fusion is slow and calcium independent in vitro in contrast to in vivo synaptic membrane fusion which is rapid and calcium dependent⁸. This lead to a search for the synaptic calcium sensor that regulates SNARE mediate membrane fusion. In 1990, Perin *et al.*, found synaptotagmin, a synaptic vesicle protein referred to as P65 which has



Figure 1. 2: Synaptic Neurotransmission¹⁴⁸

When a stimulus received by dendrites exceeds certain threshold, it generates action potential (nerve impulse). The nerve impulse travels down the axon and reaches the axon terminal. 2. The action potential triggers opening of voltage gated calcium channels and calcium flows inward through opened channels. 3. The calcium signaling triggers fusion of synaptic vesicles with the presynaptic membrane to release neurotransmitters into the synaptic cleft. 4. Neurotransmitters diffuse through synaptic cleft and bind receptors on the postsynaptic membrane. 5. The receptor activation on the opens voltage gated channels across the postsynaptic membrane. 6. Activation of voltage gated channels cause postsynaptic membrane potential. 7. When postsynaptic membrane potential exceeds certain threshold, action potential is generated and the impulse travels down the axon to the axon terminal.

homology to the regulatory C2 domain protein kinase C9. C2 domains are independently folded membrane binding domains of about 130-150 amino acids residues (Figure 1.3). C2 domains are known to serve in Ca²⁺ mediated cellular signaling and are also involved in membrane trafficking, vesicle fusion and signal transduction. C2 domains are β sandwich domains formed by eight antiparallel β strands and are connected by loops whose tips co-ordinate 2 to 3 Ca²⁺ ions. Most of the C2 domains bind anionic phospholipids upon Ca²⁺ binding with the exception of $(cPLA_2),$ which binds cytosolic phospholipase A_2 the neutral lipid phosphatidylcholine in the Ca²⁺ bound state ^{10,11,12,13,14}. The best known Ca²⁺ sensors are Protein Kinase C (PKC) and Synaptotagmin 1. Synaptotagmin 1 is a highly conserved, abundant synaptic vesicle transmembrane protein consisting of two cytoplasmic C2 domains C2A and C2B^{15,}. Several genetic studies using Drosophila melanogaster reported that synaptotagmin 1 is crucial for efficient synchronous neurotransmitter release ¹⁶. Similarly, eliminating synaptotagmin 1 in mice disrupted the fast synchronous phase of synaptic vesicle fusion without affecting spontaneous neurotransmitter release^{17,18}. In 1992, Reinhard Jahn's lab reported that synaptotagmin1 binds negatively charged membranes containing phosphatidylserine at physiologically relevant Ca²⁺ concentration¹⁹. Other studies confirmed the interaction between C2A and C2B domains with phosphatidylserine and have established that binding occurs with rapid kinetics on a millisecond scale ^{20,21-23}. In addition, Schiavo et al. 1996 determined that the C2B domain of synaptotagmin binds

phosphatidylinositol-4,5-bisphosphate, PI (4,5)P2 in a calcium independent manner ²⁴. PI(4,5)P2 is a plasma membrane enriched lipid, thus it was concluded that PI(4,5)P2 steers and selectively localizes synaptotagmin to the plasma membrane of neurons. This Ca²⁺ independent synaptotagmin-PI(4,5)P2 interaction would predispose synaptotagmin to penetrate into the plasma membrane in response to Ca²⁺ which serves to juxtapose the vesicle membrane and the plasma membrane together ^{25,26}. The disruption of synaptotagmin 1 and PI (4,5)P2 interaction seems to block exocytosis in vivo¹⁷. In addition to the Ca²⁺ and anionic phospholipid binding, syntaxin 1A was identified to interact with synaptotagmin by co-immunoprecipitation from rat brain extracts²⁷. Synaptotagmin has been reported to interact directly with t-SNAREs, syntaxin 1A and SNAP-25 in vitro ^{28,29,30}. Gerona et al. 2000 have reported that the alteration of C-terminus of SNAP25 resulted in diminished exocytosis ³¹. Moreover, Tucker et al. 2004 demonstrated that synaptotagmin stimulates SNARE mediated fusion between v-SNARE vesicles and t-SNARE vesicles in the presence of calcium³². Together, these studies provide compelling evidence that calcium sensor synaptotagmin, lipids and SNAREs are key components for synaptic vesicle fusion. A summary of synaptotagmin action during synaptic vesicle exocytosis is given in the figure 1.4.



Figure 1. 3: A C2 domain: an independently folded β -sandwich domain of about 130-150 amino acid residues. C2 domains mediate Ca²⁺ cellular signaling and also involved in membrane trafficking, vesicle fusion and signal transduction.



Figure 1.4: A mechanism of membrane fusion¹⁵

A minimum fusion machinery required for membrane fusion is composed of synaptotagmin 1, v-SNAREs, t-SNAREs and PI(4,5)P2. In the absence of Ca²⁺, C2B domain of synaptotagmin interacts with PI(4,5)P2 and localizes synaptotagmin to the plasma membrane. Upon calcium influx, C2 domains of synaptotagmin penetrates into the plasma membrane and drive complete assembly of trans-SNARE complex and brings the membranes into close proximity. This might result in hemifusion intermediate in which outer leaflets merge and inner leaflet forms a single bilayer. Further mixing and expansion of lipids by the fusion machinery results in the formation of pore to release neurotransmitter. Eventually, vesicle membrane collapses into the plasma membrane where it would be subsequently retrieved by endocytosis.

1.2 Neurotransmission at sensory IHC's synapse of the cochlea:

Cochlear inner sensory hair cells (IHCs) are the mammalian auditory sensory epithelial cells essential for rapid processing of sound. IHCs process and encode sound signals with high fidelity. IHCs derive their name from stereocilia known as hair bundles that protrude from the apical tip of the cell (Figure 1.5). IHCs rest on a basilar membrane that is narrower and stiffer at the base, wider and flexible at the apex of the cochlea. Sound induced vibrations in IHCs reach their maximal amplitude at specific positions along the length of basilar membrane. High frequency and low frequency sounds are encoded at the base and apex of the cochlear basilar membrane respectively. Sound waves induce nanometer displacement of stereocilia that cause opening of mechanotransducer channels, located in the tip of stereocilia. These channels allow inflow of potassium ions (K⁺) into the IHC causing cell to depolarize in proportion to the amplitude of stereocilia deflection known as graded potential. In contrast to action potentials in neurons, graded potentials die out as it spreads along the membrane. This depolarizing graded potential opens voltage gated calcium channels at the basolateral end of the cell resulting in inflow of calcium ions that triggers calcium dependent synaptic vesicle fusion as in neurons. However, the voltage gated calcium channels mediating stimulus secretion coupling in IHCs (Ltype) differ from that in neurons (N- , P/Q type Ca²⁺). In addition, unlike IHCs the active zone at neuronal synapses lack synaptic ribbons, electron dense oval shaped

structures that position vesicles proximal to the synapse ^{33,34}. Ribbon synapses are also found in other sensory cells including vestibular and lateral line hair cells as well as photoreceptors and retinal bipolar neurons³⁵. The synaptic ribbon is predominantly comprised of the protein RIBEYE and is essential for clustering calcium channels at the active zone and also acts as a conveyor belt for synaptic vesicles and tethers them at the release site^{36,37}. The Ca²⁺ inflow and a calcium sensor mediates molecular interactions between vesicle and plasma membrane proteins to facilitate membrane fusion. In neurons, as mentioned earlier, synaptotagmin 1 is thought to act as a calcium sensor of fast and synchronous neurotransmitter release at IHCs synapse. However, Safieddine *et al.* 1999 and Uthaiah *et al.* 2010 reported the absence of both classical calcium sensors, syt1 and syt2 in IHCs that are required for fast exocytosis at central nervous system (CNS) synapses^{36,38}. Beurg et al. 2011 recently confirmed that Syt 1 and Syt 2 are detected in immature IHCs but are absent in mature IHCs at the onset of hearing using immunolabeling studies. Further, knock out of Syt 1, 2 and 7 (Syt 1 -/-, Syt 2 -/- and Syt 7-/-) mice display normal exocytosis in mature IHCs³⁹. Synaptotagmin 4 (Syt 4) is the only Syt isoform that is detected both in immature and mature IHCs, however Syt 4 does not bind calcium and has no known Ca²⁺ dependent activity^{40,41}. Thus, with the absence of calcium sensors Syt 1 and Syt 2 in mature IHCs, the identification of Ca²⁺ sensor for sensory hair cells has become a major goal in the area of hearing.



Figure 1.5: IHC of the cochlea :It consists of stereocilia whose nanometer displacement causes cell depolarization resulting in evoked neurotransmitter release.

1.3. Which Ca^{2+} sensor triggers neurotransmitter release at IHCs synapse?

Otoferlin is ubiquitously expressed in brain, vestibular system and IHCs and outer hair cells of cochlea⁴². Roux *et al.* 2006 found that the ribbon synapse was found with a profound functional deficit but the morphology of the synapse between the IHCs and the afferent fibre was unaltered. Also, there was no difference between the number of docked vesicles between the normal and otoferlin defective mice. However, otoferlin knocked out mice caused profound deafness by abolishing Ca²⁺ triggered fast and synchronous exocytosis and hypothesized that otoferlin mediates the final step of exocytosis ⁴³. Roux *et al.* 2006 have also found that otoferlin is localized to the synaptic vesicles of IHCs ribbon synapse. In addition, similar to synaptotagmin 1 has C2 domains that are responsible for calcium sensing and also bind lipids, otoferlin also has six cytoplasmic C2 domains presumably for sensing calcium to act as a calcium sensor⁴³. Thus, Roux *et al.* 2006 proposed otoferlin as a putative Ca²⁺ sensor for evoked neurotransmitter release at IHCs active zone.

1.3.1. Is otoferlin the calcium sensor at IHCs synapse?

The structural and biochemical similarities between the otoferlin and a Ca^{2+} sensor synaptotagmin 1 as well as otoferlin's association with synaptic vesicles and the abolishment of Ca^{2+} dependent neurotransmitter release upon knock out lead to a hypothesis that otoferlin might act as the calcium sensor for neurotransmitter release at the IHCs synapse. Beyond IHC, otoferlin knock out mice also lose linear Ca^{2+} dependency and display slower release kinetics in both vestibular hair cells of the cochlea⁴⁴. In addition, Beurg *et al.* 2008 found that evoked neurotransmitter release is abolished in immature outer hair cells (OHCs) despite normal Ca²⁺ current⁴⁵. Thus, otoferlin appears to also contribute to neurotransmitter release in OHCs and vestibular hair cells as well. However not all studies agree, for instance immunofluorescence studies have shown that otoferlin is widely distributed beyond synaptic region throughout the cytosol of IHCs including the Golgi and early endosomes suggesting alternative or additional functional roles^{36,42,46–48}.

1.3.2. Alternative or additional functional roles of otoferlin?

Heidrych *et al.* 2009 reported that otoferlin interacts with Myosin VI and that otoferlin plays a role in targeting intracellular membranes to the active zone of IHCs. The otoferlin and myosin VI knock out mice exhibit membrane infoldings indicated disturbed transport of endocytotic membranes⁴⁹. In addition, a recent study by Duncker *et al.* 2013 found that adaptor protein complex-2 (AP2), essential for clathrin mediated endocytosis, is also an interacting partner of otoferlin by coimmunoprecipitation and mass spectrometry. Also, AP-2 co-localizes with otoferlin in mature IHCs⁵⁰. Moreover, AP-2 is also an interacting partner of Myosin VI which is involved in vesicle trafficking⁵¹. Thus, it has been speculated that otoferlin, myosin VI and AP-2 form a ternary complex to mediate clathrin mediated endocytosis.

Rab8b GTPase belongs to a Ras GTPase family that acts a regulator for vesicle delivery to the dendrites in neurons or to basolateral membrane of the sensory epithelial cell^{52,53}. Grindstaff et al. 1998 suggested that Rab8 GTPase role in transporting vesicles to the basolateral membrane of the kidney epithelial cells through trans-Golgi network and the recycling of endosomes⁵⁴. Heidrych *et al.* 2008 found that Rab GTPase is an interacting partner of otoferlin through yeast two-hybrid screen and co-immunoprecipitation using HEK cell line⁵⁵. This suggests otoferlin could be a part of protein complex involved in trans-Golgi membrane trafficking. In addition, Zak et al. 2012 found that otoferlin C2D domain directly interacted with Ergic²⁵⁶. Ergic² is a ubiquitously expressed protein and a homolog of yeast Erv41p and is presumed to play a role in vesicular transport at ERGIC (Endoplasmic reticulum-golgi intermediate compartment) station between Rough endoplasmic reticulum and Cis-Golgi^{57,58}. Revelo et al. 2014 also showed that otoferlin is colocalized with the trans-Golgi SNARE syntaxin-16 and suggested that otoferlin may play a role in membrane trafficking between the plasma membrane and trans-Golgi⁵⁹. Moreover, Redpath et al. 2016 reported that in HEK cell line, otoferlin is localized predominantly on the cytoplasmic face of the trans-Golgi network and very low levels of otoferlin was found at the plasma membrane⁶⁰.

These observations lead to a conflicting conclusions including if

- (a) otoferlin acts solely as a Ca²⁺ sensor in IHCs of cochlea?
- b) endocytosis?
- c) membrane trafficking at the Golgi membrane?

Also supporting the additional or alternative roles of otoferlin, other ferlin family of proteins are also implicated in vesicular and receptor trafficking through endolysosomal pathway and also trafficking at trans-Golgi network. There are two types of ferlins, Type-I and type-II based on the presence or absence of DysF domain respectively (Figure 1.6)^{61,62}. Type-I ferlins (Dysferlin and myoferlin) were readily detected at the plasma membrane and are also found to be co-localized with endosomal markers Rab5 and Rab7 and occasional co-labeling with the lysosomal maker LAMP-1 suggesting their functional role in endo-lysosomal pathway⁶⁰. Demonbreun et al. 2011 found that dysferlin null myoblasts accumulate enlarged lysosomes and transferrin-488 and showed abnormal trafficking of insulin like growth factor (IGF) receptor indicating dysferlin role in vesicular and receptor trafficking through endo-lysosomal pathway⁶³. Dysferlin is also implicated in several functions including repair of sarcolemmal damage and its association with transverse (t)-tubule membrane, it maintains Ca²⁺ homeostasis following cellular stress⁶⁴. Myoferlin has been shown to control the degradation of epidermal growth factor receptor (EGFR) via caveolae homo-oligomerization. Myoferlin is found to be overexpressed in human breast cancers and its knockdown affects the trafficking of EGF receptor through late endosome-lysosomal pathway⁶⁵. Doherty *et al.* 2008 reported the direct interaction of myoferlin with eps15 homology domain protein, EHD2 that are linked to endosomal trafficking and recycling back to the plasma membrane suggesting myoferlin role in endocytotic recycling pathway^{66–68}. Moreover, Fer1L5 is expressed in small myotubes and similar to myoferlin, it was also found to interact directly with EHD1 and EHD2 suggesting its role in endocytotic recycling for membrane trafficking events during myotubes formation⁶⁷. Fer1L6, a type-II ferlin showed profound distribution at trans-Golgi/recycling endosomal compartment and found to be co-localized with endocytosed transferrin indicating its role in trans-Golgi membrane trafficking⁶⁰. Together, these results might suggest that ferlins share multiple functions including exocytosis, membrane and receptor trafficking and endocytosis.



Figure 1. 6: Structure of ferlin family of proteins

Structure of ferlin family of proteins. Ferlins are a family of membrane anchored proteins consisting of multiple tandem cytosolic C2 domains (5-7 C2 domains). They play a pivotal role in vesicle fusion and membrane trafficking. Ferlin mutations are associated with non-syndromic deafness (otoferlin), muscular dystrophy (dysferlin), up regulation in breast cancer (myoferlin) and infertility in Caenorhabditis elegans (Fer-1). C2 domains consists of around 130 amino acids which form a beta sandwich structure. In addition, they also contain unique DysF domain and a motif specific to the ferlin family called Fer domain (60-70 conserved residues). The structure of DysF domain consists of two long antiparallel beta-strands connected by a loop region. The functions of both the domains are unknown. Ferlins are also divided into two subtypes based on the presence (Type I) or absence (Type II) of DysF domain. Abbreviations: DysF, dysferlin domain; Fer, ferlin specific motif; TM, transmembrane domain.

1.3.4. Studies to test the calcium sensor hypothesis:

The intercellular communication between mechanosensory IHCs and afferent neurons occurs on a microsecond scale and relies on rapid Ca²⁺ triggered fusion of neurotransmitter filled vesicles at the IHCs active zone. The signal transmission in IHCs should be 10 times more rapid and precise than most conventional neural synapses as IHCs must encode sound waves oscillating at thousand times per second⁶⁹. Similar to other intracellular membrane trafficking steps including those at CNS synapses, synaptic vesicle fusion requires SNARE proteins. Thus, if otoferlin is a Ca²⁺ sensor in IHCs for evoked neurotransmitter release, then otoferlin would be expected to interact with SNARE's and mediate membrane fusion. Thus, several studies have been performed to determine the interaction of otoferlin and neuronal SNARES. Johnson et al., 2010 have reported SNARE protein binding ability of individual C2 domains of otoferlin. C2A and C2B bound the neuronal SNARE heterodimer SNAP-25/ syntaxin-1A in a calcium independent manner, whereas presence of Ca²⁺ enhanced SNARE heterodimer binding of the C-terminal (C2D-C2F) C2 domains of otoferlin⁷⁰. Also, similar to Syt 1, the C2 domains of otoferlin mediate calcium dependent SNARE- mediated membrane fusion as determined using a membrane reconstitution assay. Also, Ramakrishnan et al. 2009 demonstrated a direct interaction of the C2 domains of otoferlin with the monomeric neuronal t-SNAREs, SNAP-25 and syntaxin-1A. However, the C2F domain did not bind synaptobrevin-1 ⁷¹. Both Roux et al. 2006 and Ramakrishnan et al. 2009 showed that the t-SNAREs,
SNAP-25 and syntaxin-1 co-immunoprecipitated with otoferlin and interact in a calcium dependent manner but not with v-SNARE, synaptobrevin-1⁴⁶. Ramakrishnan et al. 2009 also demonstrated that the C2D domain of otoferlin interacts with the Cav 1.3 calcium channel at its cytosolic loop II-III⁷¹. Interaction of otoferlin with SNAREs and Cav 1.3 would allow it to regulate docking of synaptic vesicles at the active zone. Cohen et al. 2003 showed how Syt 1, SNARE and Cav 2.3 channel interact to form a excitosome complex prior to Ca²⁺ activation and alter the kinetic properties of R type Ca²⁺ channel and proposed the excitosome complex is required for evoked neurotransmitter release ⁷². Also, Syt 1 is localized to the plasma membrane when Ca²⁺ channel is present, as otherwise, Syt 1 is distributed throughout the cytoplasm instead of just at synaptic active zone⁷³. The interaction of otoferlin and neuronal SNARE proteins has been established using in vitro experiments and also the presence of neuronal SNAREs in IHCs has been reported by several studies using immunostaining and western blot³⁶. However, in 2011, a study by Nouvian *et al.* cast a doubt on the physiological relevance of SNARE proteins to mediate membrane fusion in IHCs. While synaptic vesicle fusion can be blocked cleaving specific neuronal SNAREs using botulinum neurotoxins, neither botulinum neurotoxins nor genetic ablations of neuronal SNAREs in IHCs had any effect on evoked synaptic exocytosis⁷⁴. These results and observations led to a conclusion that IHCs exocytosis might operate independent of neuronal SNAREs or neuronal SNAREs are compensated by other SNAREs that are ubiquitously expressed including SNAP-23, SNAP-29 and syntaxin-4. Supporting this idea, a recent study by Codding et al. 2016

reported that dysferlin, belongs to the same family as otoferlin, interacts with nonneuronal SNAREs, SNAP-23 and syntaxin-4 and regulate SNARE mediated membrane fusion⁷⁵.

To further test the calcium sensor hypothesis of otoferlin, calcium and lipid binding properties of C2 domains of otoferlin has been studied. Johnson *et al.* 2010 reported that all the C2 domains of otoferlin interact with the Ca²⁺ and anionic lipids with an exception of C2A domain of otoferlin⁷⁶. Helfmann *et al.*, 2011 solved the crystal structure of the C2A domain of otoferlin at 1.95 A° resolution and they found calcium binding loop (CBL) is shorter compared to other C2 domains that co-ordinate calcium ions. Also, C2A of otoferlin consists of only one out of five conserved aspartate residues in its CBL 1 and CBL 3. Also, C2A domain did not bind anionic lipids in agreement with previous studies⁷⁷. Contrary to other studies, Pangrsic *et al.*, 2010 reported that the lack of calcium and lipid binding ability of the C2F domain of otoferlin. Thus, the biochemical properties of C2 domains of otoferlin is still under debate.

The Ca²⁺- sensor hypothesis has been addressed by expressing Syt 1 in IHCs of otoferlin knock out mice using viral gene transfer both in embryonic inner ear and cultured organ of corti. Despite very efficient viral transduction and localization of Syt 1 to the active zone of IHCs, Syt 1 failed to rescue synchronous neurotransmitter release³⁹. Conversely, viral transduction of otoferlin in Syt 1 knock out chromaffin

cells or hippocampal neurons, otoferlin failed to rescue exocytosis however asynchronous neurotransmitter release has been enhanced³⁹. The study suggests that though otoferlin and synaptotagmin are biochemically and structurally related proteins, they are not functionally equivalent.

The only strongest evidence for the Ca²⁺ sensor hypothesis for otoferlin came from Roux *et al.* 2006 electrophysiology work where he demonstrated that the depolarization induced evoked synchronous exocytosis was completely abolished in otoferlin knock out IHCs in mice. Typically, readily releasable pool of vesicles (RRPs) can be discharged by increasing the Ca²⁺ concentration at the release site. Thus, to test the Ca²⁺ dependency of fusion in the absence of otoferlin, Roux *et al.* 2006 used flash photolysis to increase the Ca²⁺ concentration at the active zone, but it did not elicit fast synchronous exocytosis suggesting the role of otoferlin as a Ca²⁺ sensor⁴³.

In summary, interdisciplinary studies since otoferlin has been identified in a nonsyndromic hearing loss led to the understanding of its expression pattern at different cell types; cellular and subcellular distribution; calcium and lipid binding properties; its interacting partners; its role in evoked, synchronous neurotransmitter release and endocytosis. Nevertheless, there are conflicting results on biochemical properties of otoferlin, otoferlin's interacting partners and their physiological relevance, **thus**, **extensive experimental evidence is yet required to test the Ca²⁺ -sensor hypothesis of otoferlin**.

Chapter 2

Characterization of the Lipid Binding Properties of Otoferlin Reveals Specific Interactions between PI(4,5)P2 and the C2C and C2F Domains

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2.1 Abstract

Otoferlin is a transmembrane protein consisting of six C2 domains, proposed to act as a calcium sensor for exocytosis. Although otoferlin is believed to bind calcium and lipids, the lipid specificity and identity of the calcium binding domains are controversial. Further, it is currently unclear whether the calcium binding affinity of otoferlin quantitatively matches the maximal intracellular presynaptic calcium concentrations of ~30 – 50 µM known to elicit exocytosis. To characterize the calcium and lipid binding properties of otoferlin, we used isothermal titration calorimetry (ITC), liposome sedimentation assays and fluorescence spectroscopy. Analysis of ITC data indicates that with the exception of the C2A domain, the C2 domains of otoferlin bind multiple calcium ions with moderate (K= $25 - 95 \mu$ M) and low affinities (K= 400 $-700 \,\mu\text{M}$) in solution. However, in the presence of liposomes, the calcium sensitivity of the domains increased by up to 10-fold. It was also determined that calcium enhanced liposome binding for domains C2B - C2E, whereas the C2F domain bound liposomes in a calcium-independent manner. Mutations that abrogate calcium binding in C2F do not disrupt liposome binding, supporting the conclusion that interaction of the C2F domain with phosphatidylserine is calcium-independent. Further, domains C2C and C2F, not domains C2A, C2B, C2D and C2E, bound phosphatidylinositol 4,5-bisphosphate 1,2 dioleoyl-sn-glycero-3-phospho (1' -

myoinositol-4',5'-bisphosphate) [PI(4,5)P2], which preferentially steered them toward liposomes harboring PI(4,5)P2. Remarkably, lysing mutations L478A and L480A in C2C selectively weaken the PI(4,5)P2 interaction while leaving phosphatidylserine binding unaffected. Finally, shifts in the emission spectra of an environmentally sensitive fluorescent unnatural amino acid indicate that calcium binding loops of the C2F domain directly interact with the lipid bilayer of negatively charged liposomes in a calcium-independent manner. On the basis of these results, we propose that the C2F and C2C domains of otoferlin preferentially bind PI(4,5)P2 and that PI(4,5)P2 may serve to target otoferlin to the presynapse in a calcium-independent manner. This positioning would facilitate fast calcium-dependent exocytosis at the hair cell synapse.

2.2. Introduction

The sense of hearing depends on reliable and temporally precise neurotransmitter release at the synapses of cochlear inner hair cells (IHCs) ⁷⁸. IHCs contain sensory hair bundle structures composed of microvilli (stereocilia) arranged in rows of graded height that protrude from the apical tip of the cell. Minute displacements of the hair bundle open mechanically gated ion channels that depolarize the cell ⁷⁹. This change in membrane potential triggers an influx of calcium into the cell that in turn results in the fusion of synaptic vesicles with the plasma membrane and the release of the neurotransmitter. Calcium-regulated exocytosis and neurotransmitter release exhibit fast kinetics in achieving exquisite temporal fidelity ^{79,80}. To aid in fidelity, IHCs contain specialized structures for tethering synaptic vesicles at release sites called synaptic ribbons, allowing for high rates of sustained synaptic transmission ⁸¹.

The calcium-triggered fusion of vesicles with the plasma membrane is believed to be driven by the assembly of SNARE proteins ³⁸. However, SNARE proteins are insensitive to calcium ⁴. In neurons, the protein synaptotagmin 1 confers calcium sensitivity to SNARE-mediated fast synchronous neurotransmission ^{82,83}. However, Yasunaga et al. have reported that synaptotagmin 1 was not detected in mature IHCs, and it has been suggested that IHCs have evolved a unique calcium sensor, otoferlin, for calcium-regulated synaptic transmission ⁸⁴. However, the relationship between otoferlin and SNAREs and whether neural SNAREs are required for neurotransmitter release from hair cells have yet to be fully elucidated.

Roux et al. have reported that mice lacking otoferlin were profoundly deaf and lack synaptic vesicle exocytosis in IHCs ⁴³. Otoferlin is also required for calciumdependent synaptic exocytosis at immature outer hair cells (OHCs) and type I vestibular hair cells ^{44,45}. In addition to exocytosis, otoferlin is known to interact with the endocytotic proteins AP-2 and myosin-6, suggesting that otoferlin may also contribute to the replenishment of synaptic vesicles ^{49,50,70}.

Otoferlin belongs to the ferlin family of proteins and consists of six C2 domains (C2A – C2F) linked in tandem followed by a single-pass C-terminal transmembrane region ⁸⁵. C2 domains bind calcium and lipids and are found in proteins involved in membrane trafficking and signal transduction^{10,86}. Johnson et al. have shown that with the exception of the C2A domain, the C2 domains of otoferlin interact with calcium and bind lipids ^{55,87}. In addition, otoferlin stimulates SNARE-mediated membrane fusion in a calcium-dependent manner *in vitro*, supporting the hypothesis that otoferlin acts as a synaptotagmin-1 like calcium sensor for fusion ⁶. However, a recent study found that synaptotagmin cannot rescue the otoferlin knockout phenotype, suggesting functional differences between the proteins ⁷³. In addition, Pangrsic et al. have reported that the C2F domain lacks calcium binding and did not bind to phosphatidylserine, raising questions about which domains of otoferlin are still

controversial ⁸⁸. The lipid and calcium binding properties of synaptic proteins are critical characteristics that define and shape the release properties of a synapse, and thus, without a quantitative characterization of these activities, an understanding of otoferlin's function in hair cells will remain elusive.

In this study, the intrinsic calcium binding properties of each C2 domain were measured using isothermal titration calorimetry (ITC). The lipid binding specificity and effects of lipids on calcium binding were also assessed using liposome sedimentation assays and Laurdan fluorescence measurements. Lastly, the interaction of loop residues within the C2F domain with liposomes was tested through use of an environmentally sensitive unnatural amino acid.

2.3. Materials and Methods

2.3.1 Materials

POPS (1-palmitoyl-2-oleoyl-sn-glycero-3-phos- pho-L-serine), POPC (1-palmitoyl-2oleoyl-sn-glycero-3-phos- phocholine), PI4P [1,2-dioleoyl-sn-glycero-3-phospho(1'myoi- nositol-4'-phosphate) (ammonium salt)], and PI(4,5)P2 [phosphatidylinositol 4,5-bisphosphate 1,2-dioleoyl-sn-glycero- 3-phospho(1'-myoinositol-4',5'bisphosphate) (ammonium salt)] were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Ni2+-NTA Sepharose high-performance beads were purchased from GE-Hamersham Biosciences (Pittsburgh, PA). Other common reagents were obtained from Sigma- Aldrich (St. Louis, MO).

2.3.2 Protein Constructs

C2 domain constructs of otoferlin were reported previously ⁸⁹. For the incorporation of the noncanonical amino acid acridon-2-ylalanine, mutagenesis was conducted on the pMCSG9 vector containing the otoferlin C2F domain at phenylalanine 1833 or 1746, converting the codon to a TAG site. Primers for the mutation were designed as mismatch primers to the nucleotide site of interest. Otoferlin C2C (K478A and K480A) and C2F (D1743/1831A and D1754/1837A) domains were constructed using the Stratagene QuikChange site-directed mutagenesis kit with the pMCSG9/ 6His-MBP-otoferlin C2C and C2F plasmid templates, respectively.

2.3.3 Expression and Purification of the his-MBP-C2 domains

The pMCSG9 vectors containing the otoferlin C2 domains were transformed into BL21 Escherichia coli cells. The bacterial cultures (OD600 = 0.6) were induced for 3-4h at 37 °C with 1 mM IPTG. The C2F F1746 and F1833 acridon-2- ylalanine noncanonical amino acid constructs were expressed in autoinduction medium with 1 mM acridon-2-ylalanine using a previously reported method ^{90,91}. The cells were lysed by sonication in lysis buffer containing protease inhibitors (0.5 mM PMSF, $1-2 \mu g/mL$ aprotinin, leupeptin, and pepstatin A). The lysis buffer contained 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl. The soluble fraction of the lysate was incubated with Ni-NTA resin for 3 hrs at 4 °C, and the Ni-NTA resin was washed with lysis buffer containing Tris-HCl, 150 mM NaCl, and 20 mM imidazole before the bound protein was eluted with Tris-HCl buffer containing 500 mM imidazole. Purified proteins were extensively dialyzed in ITC buffer [20 mM Tris-HCl (pH 7.5) and 150 mM NaCl] and concentrated using an Ultrafree-10 centrifugal filter unit (Millipore Inc., Bedford, MA). The protein concentrations were determined by UV absorbance using extinction coefficients of each protein based on sequence. Figure 2.1. of the Supporting Information shows a representative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) gel illustrating the purity of the C2 domains of otoferlin.

2.3.4. Isothermal Titration Calorimetry

Isothermal titration calorimetry was conducted using a Nano ITC instrument (TA Instruments). The calcium binding experiments were con-ducted at 37 °C, and lipid binding was conducted at 30 °C. The proteins were dialyzed extensively in buffer containing 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl. Stock calcium chloride solutions were prepared in the corresponding buffers of each protein and were loaded into a 50 µL syringe. This titrant was injected with a stirring speed of 250 rpm at discrete intervals of 180 s. Calcium was added in 1 µL injections 45 times for each experiment, and the heat evolved per injection was measured. Small unilamellar vesicles (SUVs) were used to determine the binding of lipids to the C2F domain of otoferlin in the absence or presence of 1 mM calcium chloride. The lipid suspension contained the same calcium concentration as the buffer. The concentration of the C2F domain of otoferlin ranged from 40 to 400 µM, and that of the lipid suspension varied from 5 to 10 mM. The lipid suspensions were added as 1μ L injections 45–47 times with a stirring speed of 250 rpm at discrete intervals of 180 s. The heat of dilution was determined by adding the titrant to the corresponding buffer in the absence of protein and was subtracted to obtain the effective heat of binding. All ITC data were analyzed using Nano ITC analysis software.

2.3.5. Phospholipid Vesicles

The preparation of SUVs was performed according to reported methods. Briefly, chloroform solutions composed of 25% POPS and 75% POPC, 50% POPS and 50%

POPC, 95% POPC and 5% PI(4,5)P2, 95% POPC and 5% PI(4)P, or 100% POPC were mixed and dried under a stream of liquid nitrogen gas and then dried under vacuum for 3 h. The dried lipids were resuspended in buffer and extruded 20 times through a 50 nm filter (Avanti Polar Lipids, Inc.) to produce small unilamellar vesicles (SUVs).

2.3.6 Sedimentation Assay

For the binding assay, the C2 domains of otoferlin (5 µg) were mixed with SUVs (100 µg) in buffer [20 mM Tris (pH 7.5) and 100 mM NaCl] with calcium (10, 100, and 1 mM) or EGTA (1 mM). The mixture was incubated for 1 h at 37 °C and centrifuged at 85000g for 45 min in a TA-100 ultracentrifuge (Beckmann Instruments). SDS–PAGE gel data presented for calcium titration experiments consist of total protein control (total input), supernatant (soluble fraction), and pellet (lipid-bound fraction).

2.3.7 Fluorescence Spectroscopy

Fluorescence spectra were recorded on a PTI Quanta Master fluorometer with 5 nm excitation and emission slit widths. Assays were conducted at 37 °C in a quartz micro cuvette. The fluorescence intensity of oto-C2F F1833Acd and F1746Acd (2 μ M) was observed in the presence of liposomes composed of 100% POPC and 45% POPS, 50% POPC, and 5% PI(4,5)P2 in the presence of calcium or EDTA. Data were collected using FelixGX set at 1.0 nm intervals with an integration time of 0.1 s. Laurdan experiments were conducted as described previously.23 Briefly, excitation at 350 nm was used, and the generalized polarization (GP) value was calculated using emission

values at 430 and 480 nm. The reported values represent means \pm the standard deviation (SD) for three samples.

2.4. Results

2.4.1. Characterization of the Calcium Binding Properties of Otoferlin

We used ITC to determine the intrinsic calcium binding properties of the C2 domains of otoferlin in solution, adapting an approach previously described for the C2 domains of PKC and synaptotagmin I^{13,92}. ITC measures the heat exchange associated with binding by titrating the ligand to the macromolecule. It also provides a complete set of thermodynamic parameters of ligand–macromolecule interaction, including the binding affinity and changes in enthalpy and entropy. We performed the titration of isolated domains of otoferlin with calcium chloride (an overview of the constructs tested is given in Figure 2.1).

Overall, five of the six domains of otoferlin bound calcium (Figure 2.2). The C2A domain of otoferlin did not bind calcium, in agreement with previous studies ^{6,77}. The measured heats of binding for domains C2B–C2F were fit using a two-site binding model that assumes that more than one ligand can bind independently. The best-fit Kd values ranged from 25 to 95 μ M for the first site and from 400 to 750 μ M for the second (Figure 2.3). Binding of calcium to the moderate-affinity site is exothermic, whereas binding of calcium to the low-affinity site is endothermic. The fitted values are listed in Table 2.1. Typically, C2 domains interact with calcium using aspartate residues located in the loop regions of the domains, and to explicitly test whether

aspartate residues are required for otoferlin–calcium interaction, we tested two double aspartate mutants in C2F (D1743/1831A and D1754/1837A). Both mutants failed to bind calcium (Figure 2.2). We also conducted titrations with a tandem C2AB domain of otoferlin to probe for interdomain effects on calcium binding (Figure 2.2 and 2.3). The C2AB construct bound calcium with apparent K_d values of 50 and 475 μ M. This negligible deviation from the calcium affinity of the isolated C2B domain suggests that the C2A domain and linker between the domains have no effect on the calcium binding affinity of the C2B domain.

2.4.2. The C2 Domains of Otoferlin Bind Liposomes

We next sought to determine if the calcium binding affinities of the domains are modified in the presence of lipids. We therefore performed sedimentation assays on 75% PC/25% PS and 50% PC/50% PS liposomes mixed with C2 domains of otoferlin in the presence of calcium or EDTA ^{93,94}. The C2A domain of otoferlin bound liposomes poorly regardless of the presence of calcium, while the C2B domain bound liposomes in the absence of calcium, with \geq 100 µM calcium enhancing C2B– liposome interaction (Figure 2.4. A,B). By contrast, binding between liposomes and the C2C–C2E domains was sensitive to low concentrations of calcium (~5–10 µM) (Figure 2.4. A, B and Figure 2.2. of supporting information). Although the C2F domain binds calcium, binding to PS/PC liposomes was calcium-independent (Figure 2.4. A, B and Figure 2.2. of supporting information). We found that although the C2F mutants D1743/1831A and D1754/1837A failed to bind calcium, the mutants did bind to PS/PC liposomes (Figure 2.4. C). These results indicate that the calcium and lipid membrane binding activities of C2F are independent. We also conducted sedimentation assays with 50% PS and found that higher levels of PS did not enhance binding, suggesting that 25% PS is saturating (Figure 2.3. of supporting information). None of the domains bound 100% PC liposomes under any conditions, indicating the requirement for anionic lipids (Figure 2.4. D). As a negative control, PS/PC liposome sedimentation assays were also conducted with the maltose binding protein, which does not bind liposomes (Figure 2.4. E).

We next tested each C2 domain for PI(4,5)P2 binding by conducting sedimentation assays on liposomes composed of 95% PC and 5% PI(4,5)P2. Only the C2C and C2F domains of otoferlin were found to bind PI(4,5)P2, and binding was calciumindependent (Figure 2.5. A,B). Many C2 domains, including the C2B domain of synaptotagmin, interact with PI(4,5)P2 in a calcium-independent manner through a set of conserved basic lysine residues. These lysine residues (K478 and K480) appear to be conserved in the C2C domain of otoferlin. To investigate whether the lysine residues contribute to PI(4,5)P2 binding, we generated K478A, K480A, and double (K478/480A) mutants. When tested, PI(4,5)P2 binding for all mutants was attenuated relative to that of the wild type (Figure 2.5. C, D). However, lysine mutants retained the ability to bind PS/PC liposomes in a calcium-dependent manner (Figure 2.5. E). Thus, the calcium-independent PI(4,5)P2 binding activity of C2C is distinct from the calcium-dependent PS binding activity.

Previous studies have utilized the solvatochromic fluorescent membrane probe laurdan to measure the lipid binding properties of the C2 domains of otoferlin⁸⁷. These studies determined that binding of otoferlin to laurdan harboring liposomes results in a blue shift in the emission maxima and an increase in the general polarization (GP) value of the probe, and we next sought to use the changes in GP values to determine whether PI(4,5)P2 can steer otoferlin to a preferred liposome by conducting competition assays (Figure 2.6.). First, we measured the change in the laurdan GP value when each C2 domain of otoferlin (5 μ M) was mixed with liposomes composed of 69% PC, 25% PS, 5% PI(4,5)P2, and 1% laurdan in the presence or absence of calcium (Figure 2.6. black bars). Next we repeated the measurements with samples containing a mixture of 69% PC, 25% PS, 5% PI(4,5)P2, and 1% laurdan and 75% PC/25% PS liposomes lacking PI(4,5)P2 and laurdan (Figure 2.6. white bars). For domains C2C and C2F, the change in the GP values for the mixed liposome sample was equivalent to that of samples containing only PI(4,5)P2 liposomes. This suggests that C2C and C2F domains preferentially bound to PI(4,5)P2 liposomes and did not bind PC/PS liposomes appreciably. By contrast, for domains C2B, C2D, and C2E, the change in GP in the mixed liposome sample was smaller than those of samples containing only PI(4,5)P2 liposomes, suggesting that these domains were distributed between both sets of liposomes, resulting in a smaller change in GP. Thus, the C2C and C2F domains are selectively steered to PI(4,5)P2- containing lipid bilayers.

2.4.3. Characterization of the Lipid Binding Properties of the Otoferlin C2F domain

Unlike the other C2 domains of otoferlin, including C2B, the liposome binding activity of C2F appears to be completely calcium independent. However, ITC measurements indicate that the domain does bind calcium. We therefore conducted a quantitative analysis of the lipid binding activity of this domain in the presence and absence of calcium to more fully characterize any effects calcium may have on lipid binding.

ITC titrations of the C2F domain of otoferlin with PS/PC liposomes in the absence and presence of calcium are shown in Figure 2.7. After accounting for the fact that the C2F domain binds only anionic lipids on the outer leaflet of liposomes, we fit the data to a one-site binding model. In the absence of calcium, the C2F domain of otoferlin bound membranes with a Kd value of 92.1 ± 13.2 μ M. In agreement with the sedimentation results, calcium did not alter the binding affinity, with a fitted Kd value of 81.0 ± 12.1 μ M in the presence of calcium (Table 2.2). Positive Δ H and Δ S values were associated with binding ⁹⁵.

To quantify the effect of PI(4,5)P2 on the membrane binding activity the C2F domain, we titrated PC/PS/PI(4,5)P2 phospholipid membranes (Figure 2.8.). The best

fit of the data to a one-site binding model indicates that the C2F domain binds with a Kd value of $6.3 \pm 2.7 \mu$ M. Thus, the binding affinity increased by 12-fold in the presence of PI(4,5)P2 compared to that for PS/PC membranes (Table 2.3.). In agreement with the sedimentation assay results, we did not observe any effect of calcium on the binding of C2F to PC/PS/PI(4,5)P2 liposomes. Titrations with 100% POPC liposomes did not show appreciable binding (Figure 2.4 of supporting information). As a negative control, titrations of 50% PS/ 50% PC liposomes with the maltose binding protein were also conducted (Figure 2.4. of supporting information).

In addition to the presynaptic region, otoferlin also localizes to the Golgi and has been hypothesized to play a role in trans- Golgi trafficking ^{42,96}. The cytoplasmic leaflet of the Golgi membrane contains PI4P, and we therefore sought to test for the effects of calcium on C2F–PI4P binding by titrating phospholipid membranes composed of PC, PS, and PI4P in the absence and presence of calcium (Figure 2.8). In the absence of calcium, the C2F domain bound liposomes with a Kd value of 29.1 ± 4.39 μ M. In the presence of 1 mM calcium, only modest changes in affinity were found (Kd value of 32.6 ± 0.15 μ M). Thus, membrane binding is enhanced 3-fold compared to that of PS/PC membranes (Table 2.4).

The C2B domain of synaptotagmin I docks to PI(4,5)P2 in a calcium-independent manner, and elevations in intracellular calcium levels induce a reorientation of the C2 domains such that side chains found in the calcium binding loops of both C2 domains insert into the lipid bilayer. Specifically, IAEDANS fluorescence-based studies have identified loops 1 and 3 of both C2A and C2B as directly interacting with lipid bilayers ^{25,97}. If the C2F domain of otoferlin acts similarly, it would provide an explanation for the calcium binding activity of the domain. In support of this, a sequence alignment indicates that phenyl- alanine 1833 in otoferlin C2F is found at the equivalent position as the membrane penetrating phenylalanine 234 in synaptotagmin C2A. To determine if F1833 resides at the C2 domain-lipid binding interface, F1833 was replaced with acridon-2-ylalanine (Acd), a noncanonical amino acid with an environmentally sensitive fluorescence emission spectrum (Figure 2.9. A) ⁹⁰. When it was tested, the fluorescence emission profile for Acd-labeled C2F changed significantly when it was mixed with PS/PC/PI(4,5)P2 liposomes (Figure 2.9B). The observed fluorescence change was unaltered by calcium. No change in fluorescence was observed when the domain was mixed with 100% PC liposomes (Figure 2.9. B) of lipid composition or the presence of calcium. These results suggest the lipid binding region of C2F is restricted to the putative calcium binding loops of the domain and that the interaction with anionic lipids in the bilayer is calcium-independent. Results of sedimentation assays conducted with Acd C2F domain were indistinguishable from those of the wild-type C2F domain, suggesting that the Acd did not appreciably alter the properties of the domain (Figure 2.9. C). In addition, when F1746, which is located on the opposite side of the domain from F1833, was replaced with Acd, no change in the emission spectra was observed for liposomes regardless of lipid composition or the presence of calcium. These results suggest the lipid binding region of C2F is restricted to the putative calcium binding loops of the domain and that the interaction with anionic lipids in the bilayer is calcium-independent. Results of sedimentation assays conducted with Acd C2F domain were indistinguishable from those of the wild-type C2F domain, suggesting that the Acd did not appreciably alter the properties of the domain (Figure 2.9. C).

2.5. Discussion

Otoferlin is believed to be a calcium sensor required for exocytosis in inner hair cells, as well as neurotransmitter release in immature outer hair cells. Given the importance of otoferlin's calcium and lipid binding properties for neurotransmitter release, the goal of our work was to measure the intrinsic calcium binding affinity and calciummediated interaction of the of C2A, the C2 domains of otoferlin bound calcium ions with moderate (Kd = 25–95 μ M) and low affinity constants (Kd = 400–700 μ M) in solution. The moderate-affinity site is in agreement with the work of Johnson et al., who reported that the apparent dissociation constant for calcium binding ranged from 13 to 25 µM.22 Their results may represent calcium binding to the first site but not the second site. Mutations to the conserved aspartate residues of the C2F domain (D1743/ 1831A and D1754/1837A) of otoferlin abolished calcium binding. Measurements of tandem domains did not reveal any cooperative binding properties, suggesting that the domains bind calcium independently. Remarkably, despite variations in the composition of the putative calcium binding loops across the domains, all of otoferlin's C2 domains were determined to possess similar binding affinities. This contrasts with the otoferlin homologue dysferlin, which displays greater variation in calcium binding affinities, ranging from approximately 1 μ M to 1 mM ⁹⁴. In comparison to those of synaptotagmin I, otoferlin's calcium binding affinities are equivalent or slightly higher, as the C2B domain of synaptotagmin binds calcium with solution Kd values in the range of 300-600 mM 98, 99.

2.5.1. Influence of Acidic Lipids on the Calcium Binding Properties of the C2 Domains of Otoferlin.

Otoferlin bound to multiple calcium ions with apparent affinities of 20– 50 and 400–700 µM in solution. These values are low relative to the calcium concentrations believed to elicit release at hair cell synapses and thus difficult to reconcile with a role for otoferlin as the calcium sensor for neurotransmitter release. However, in the presence of PS, calcium concentrations of 10 µM resulted in significant C2–liposome interaction for C2C– C2E domains, suggesting that the presence of acidic lipids enhances the calcium binding affinity for some of the domains to physiologically relevant values. This effect was not detected for C2F and was less pronounced for C2B. Thus, otoferlin possesses domains that appear to operate using an "electrostatic switch" mechanism, as well as domains that bind regardless of calcium. Although calcium-independent membrane binding has been reported for the C2 domains of dysferlin ⁹⁴, neither C2 domain of synaptotagmin I binds appreciably to PS-containing liposomes in the absence of calcium, suggesting a difference in the mechanisms by which synaptotagmin I and otoferlin bind membranes.

2.5.2. PI (4,5)P2 Binding Properties of the C2 Domains of Otoferlin

PI(4,5)P2 is a major signaling molecule at the presynapse, and thus, we sought to determine if the C2 domains of otoferlin have a preference for phosphoinositide lipids. Our studies indicate that only the C2C and C2F domains of otoferlin bind

PI(4,5)P2 and that binding is calcium- independent. This result differs from that of a recent study of the C2F domain that reported the calcium sensitivity of the C2F–PI(4,5)P2 interaction ⁴⁶. However, this study used free PI(4,5)P2 lipids instead of mixed composition liposomes, and thus, a direct comparison cannot be made. However, we found that aspartate mutant forms of C2F that do not bind calcium did bind PI(4,5)P2 liposomes, supporting the conclusion that PIP2 binding is calciumindependent. Many C2 domains contain a polybasic region with cationic and aromatic residues (YXK/Q Xn1 KXK) that is known to interact with the phosphate moieties of the inositol ring ¹⁰⁰, ¹¹. This polybasic region appears to be conserved (YVQ VFFAGQ KGK) in the C2C domain of otoferlin but does not appear to be conserved in C2F. Mutation of these lysines in C2C (K478A and K480A) weakened PI(4,5)P2 binding. However, the lysine mutants bound POPS/POPC liposomes like the wild-type C2C domain, suggesting that different residues mediate phosphatidylserine and phosphatidylinositol binding and that that these binding activities can be selectively abrogated. ITC measurements indicate that PI(4,5)P2 enhanced liposome binding for the C2F domain by 12-fold but by only 3-fold for PI4P relative to PS/ PC liposomes, indicating a specificity for the bisphosphate. The C2B domain of synaptotagmin interacts with PI(4,5)P2 in a calcium-independent manner, and this interaction is believed to target the protein to the presynaptic membrane ^{92,25}. The C2C and C2F domains of otoferlin may act in a similar manner, targeting otoferlin to the presynaptic region of the cell and positioning it for calcium-induced neurotransmitter release (Figure 2.10. B). Indeed, our laurdan measurements suggest C2C and C2F preferentially target PI(4,5)P2-containing liposomes.

Given that C2F–lipid membrane interaction is calcium- independent, the exact reason for the calcium binding activity of this domain is unclear. Studies of the domains of synaptotagmin have demonstrated a calcium-triggered reorientation of the C2 domains as well as penetration of hydrophobic side chains into the lipid bilayer, as demonstrated using the fluorescent probe AEDANS ²⁵, ¹⁰¹. Our study using a fluorescent unnatural amino acid in one of the putative calcium binding loops indicates that the side chain interacts with liposomes in an anionic lipid-dependent manner. However, no evidence of calcium-induced changes was detected, suggesting that although the loop does interact with lipids, the mechanism may be different from that of the C2 domains of synaptotagmin. Rather than lipid interaction, calcium binding to C2F may facilitate other actions, including vesicle priming and fusion. In support of this, several studies have demonstrated calcium sensitive protein binding and membrane fusion activity in vitro.

2.6. Abbreviations

POPS, 1-palmitoyl-2-oloeyl-sn-glycero-3-phospho-L-serine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PI4P, 1,2-dioleoyl-sn-glycero-3-phospho(1'-myoinositol-4'-phosphate) (ammonium salt); PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate 1,2-dioleoyl-sn-glycero-3-phospho(1'-myoinositol-4',5'-bisphosphate) (ammonium salt); PS, phosphatidylserine; SUVs, small unilamellar vesicles; MBP, Maltose binding protein; Acd, Acridon-2-ylalanine.



Figure 2. 1: Schematic diagram of full length otoferlin and the otoferlin constructs used in this study.

Otoferlin is composed of six C2 domains and a transmembrane segment (TM). Recombinant proteins composed of C2 domains were generated according to the amino acid designations listed at the right.



Figure 2. 2: Representative thermograms for the interaction of CaCl₂ with the C2 domains of otoferlin

Titrations were performed at 37°C in 20 mM Tris buffer (pH 7.5) and 150 mM NaCl. The following concentrations of C2 domains were used: 350 μ M C2A, 420 μ M C2B, 400 μ M C2C, 390 μ M C2D, 450 μ M C2E, 390 μ M C2F, 380 μ M C2AB, 320 μ M C2F D1743/1831A, and 340 μ M C2F D1754/1837A. The syringe contained 20-30 mM CaCl₂.



Figure 2.3: ITC data for the interaction of CaCl2 with the C2 domains of otoferlin.

The panels show the integrated heat as a function of the $Ca^{2+}/protein$ ratio after substraction of the heat of dilution. The solid line in the panel corresponds to the best fit to a two-site model. The values for the fitted parameters are listed in Table 2.1.



Figure 2.4: Association of C2 domains of otoferlin with phospholipid membranes in the presence of increasing calcium concentrations.

(A) Interactions of the C2 domains of otoferlin with liposomes composed of 25% POPS and 75% POPC (B) Quantitation of the results of the liposome binding assay from panel A. (C) Interaction of calcium binding mutants of the C2F domain (D1743/1831A and D1754/1837A) of otoferlin with liposomes composed of 25% POPS and 75% POPC (D) Sedimentation assays conducted with 100% POPC liposomes and the C2 domains of otoferlin.
(E) Sedimentation assays conducted with MBP and liposomes composed of 25% POPS and 75% POPS and 75% POPC (± standard deviation; N=3). S denotes supernatant and P the pellet.



Figure 2. 5: Association of C2 domains of otoferlin with PI(4,5)P2 in the presence or absence of calcium.

(A) Interaction of the C2 domains of otoferlin with liposomes composed of 95% of POPC and 5% PI(4,5)P2. (B) Quantification of results of the liposome binding assay from panel A (± standard deviation; N=3). (C) Interaction of C2C WT, K478A, K480A and K478/480A domains of otoferlin with liposomes composed of 95% POPC and 5% PI(4,5)P2 in the presence or absence of calcium. (D) Quantification of the results of the liposome binding assay from panel C (± standard deviation; N=3). (E) Association of the K478A, K480A and K478/480A mutants with liposomes composed of 25% POPS and 75% POPC. (F) Interaction of calcium binding mutants of the C2F domain (D1743/1831A and D1754/1837A) of otoferlin with liposomes composed of 95% POPC and 5% PI(4,5)P2 in the presence or absence of calcium (± standard deviation; N=3). S denotes the supernatant and P the pellet.



Figure 2. 6: PI(4,5)P2 steers C2C and C2F domains

(A) Schematic illustrating the changes in laurdan emission spectra upon C2 domain binding. Arrows indicate the decrease in long wavelength emission and the rise in shorter wavelength intensity accompanying C2 domain binding. (B) Mean change in GP values (\pm standard deviation) in samples containing 5 μ M C2 domain and either POPC/POPS/PI(4,5)P2/laurdan liposomes (black bars) or a mixture of POPC/POPS and POPC/POPS/PI(4,5)P2/laurdan liposomes (white bars) in either 100 μ M EDTA or 500 μ M calcium (N=3; * P< 0.05).



Figure 2. 7: Representative thermograms for the interaction of C2F with 50% PC/50% PS liposomes in the presence or absence of calcium.

(A) Representative thermograms and integrated heat changes after subtracting the heat of dilution for the titration of 360 μ M C2F with 10 mM lipid vesicles in the presence of 1 mM CaCl2. (B) Thermograms and integrated heats of binding after subtracting the heat of dilution for the titration of 360 μ M C2F with 10 mM lipid vesicles in the absence of calcium. The binding isotherms were fit using a one-site model. The values of the fitted parameters are summarized in Table 2.



Figure 2. 8: Representative thermogram of the interaction between C2F and liposomes composed of 45% POPC, 50% POPS, and 5% PI(4,5)P2 or 45% POPC, 50% POPS, and 5% PI4P.

(A) Thermogram and integrated heat changes after subtracting the heat of dilution for the titration of 360 μ M C2F with 10 mM POPC/POPS/PI(4,5)P2 liposomes in the presence of 1 mM CaCl2. (B) Thermogram and the integrated heats of binding after subtracting the heat of dilution for the titration of 360 μ M C2F with 10 mM POPC/POPS/PI(4,5)P2 liposomes in the absence of calcium. (C) Thermogram and integrated heat changes after subtracting the heat of dilution for the titration of 80 μ M C2F with 10 mM POPC/POPS/PI4P liposomes in the presence of 1 mM CaCl2. (D) Thermogram and the integrated heats of binding after subtracting the heat of dilution for the titration for the titration of 80 μ M C2F with 10 mM POPC/POPS/PI4P liposomes in the presence of 1 mM CaCl2. (D) Thermogram and the integrated heats of binding after subtracting the heat of dilution for the titration of 80 μ M C2F with 10 mM POPC/POPS/PI4P liposomes in the presence of calcium. The values of the fitted parameters are summarized in Tables 3 and 4.



Figure 2. 9: Fluorescence intensity of C2F-acridon-2-ylalanine (Acd) in the presence of varying liposome and calcium concentrations.

(A) Schematic illustrating the two acridone-2-ylalanine-labeled C2F domains and their interaction with liposomes. (B) C2F-Acd fluorescence intensity measured in the presence or absence of calcium and liposomes. The fluorescence intensity of C2F F1833Acd and F1746Acd did not increase in the presence of 100% POPC liposomes. A marked increase in fluorescence was observed for F1833Acd but not F1746Acd in the presence of 45% POPS/50% POPC/5% PI(4,5)P2 liposomes. The addition of calcium did not affect the fluorescence for any of the samples. Error bars represent the standard deviation (N = 3). (C) Interaction of the F1833Acd and wild-type C2F with liposomes composed of 100% POPC, 25% POPS and 75% POPC, or 95% POPC and 5% PI(4,5)P2. S denotes the supernatant and P the pellet.


Figure 2. 10: Summary and possible mechanisms of action.

(A) Diagram of otoferlin with calcium phosphatidylserine (PS) and PI(4,5)P2 binding properties denoted for each domain. (B) Possible models of membrane binding. In the absence of calcium, synaptic vesicle-bound otofelrin contacts the presynaptic membrane through the interaction of PI(4,5)P2 with the C2C and C2F domains. Increased intracellular calcium concentrations would drive additional C2 domain–lipid interaction with either the synaptic vesicle membrane or presynaptic membrane.

Chapter 1: Supporting Information



Figure 2.1. SDS-PAGE gel-illustrating purity of MBP-C2 domains used in the studies.



Figure 2.2. (S= supernatant, P= pellet). (A) Interaction of the C2 domains of otoferlin with liposomes composed of 25%POPS: 75% POPC (B) Quantitation of the results of liposome binding assay from panel A \pm STD.



Figure 2.3. Interaction of C2 domains of otoferlin with liposomes composed of 50% POPS:50%POPS, (S= supernatant, P=pellet).



Figure 2.4. (A) Representative thermogram of MBP (320 μ M) titrated with 50% POPS: 50% POPC. (B) Representative thermogram of C2F domain of otoferlin titrated with 100% POPC liposomes.

construct	$K_{\rm D}$ (μ M)	ΔH (kJ/mol)	$\Delta S (J \text{ mol}^{-1} \text{ K}^{-1})$	no. of bound ligand molecules (n)
C2B	$K_1 = 95.4 \pm 6.4$	$\Delta H_1 = -0.5 \pm 0.1$	$\Delta S_1 = 75.3 \pm 0.6$	$n_1 = 0.9 \pm 0.1$
	$K_2 = 485.1 \pm 12.5$	$\Delta H_2 = 2.9 \pm 0.0$	$\Delta S_2 = 72.8 \pm 0.3$	$n_2 = 2.0 \pm 0.1$
C2C	$K_1 = 26.1 \pm 3.0$	$\Delta H_1 = -0.6 \pm 0.1$	$\Delta S_1 = 87.9 \pm 1.0$	$n_1 = 1.2 \pm 0.1$
	$K_2 = 379.4 \pm 3.7$	$\Delta H_2 = 1.9 \pm 0.1$	$\Delta S_2 = 71.6 \pm 0.2$	$n_2 = 3.0 \pm 0.0$
C2D	$K_1 = 51.6 \pm 2.5$	$\Delta H_1 = -0.3 \pm 0.1$	$\Delta S_1 = 78.6 \pm 4.6$	$n_1 = 0.9 \pm 0.0$
	$K_2 = 591.6 \pm 19.4$	$\Delta H_2 = 1.9 \pm 0.1$	$\Delta S_2 = 67.8 \pm 0.9$	$n_2 = 1.2 \pm 0.2$
C2E	$K_1 = 34.3 \pm 1.4$	$\Delta H_1 = -0.14 \pm 0.1$	$\Delta S_1 = 86.0 \pm 0.1$	$n_1 = 1.1 \pm 0.1$
	$K_2 = 771.0 \pm 76.7$	$\Delta H_2 = 1.3 \pm 0.1$	$\Delta S_2 = 63.9 \pm 0.7$	$n_2 = 2 \pm 0.0$
C2F	$K_2 = 25.3 \pm 7.2$	$\Delta H_2 = -0.2 \pm 0.0$	$\Delta S_1 = 88.9 \pm 2.4$	$n_1 = 0.9 \pm 0.1$
	$K_1 = 568.3 \pm 25.5$	$\Delta H_1 = 2.3 \pm 0.0$	$\Delta S_2 = 69.3 \pm 0.3$	$n_2 = 1.0 \pm 0.0$
C2AB	$K_1 = 50.4 \pm 4.6$	$\Delta H_1 = -0.3 \pm 00$	$\Delta S_1 = 92.2 \pm 2.2$	$n_1 = 1.1 \pm 0.1$
	$K_2 = 475.9 \pm 12.6$	$\Delta H_2 = 2.8 \pm 0.0$	$\Delta S_2 = 72.1 \pm 0.3$	$n_2 = 2.1 \pm 0.1$

Table 2. 1: Thermodynamic parameters of binding of calcium to different otoferlin constructsmeasured by ITC

Table 2. 2: Thermodynamic Parameters of POPC/POPS (50:50) SUVs Binding to the Otoferlin C2F Domain in the Presence and Absence of Ca2+.

Ca ²⁺	$K_{\rm D}$ (μ M)	ΔH (kJ/mol)	$\Delta S (J \text{ mol}^{-1} \text{ K}^{-1})$
without	$K_1 = 92.1 \pm 13.2$	$\Delta H_1 = 1.8 \pm 0.1$	$\Delta S_1 = 75.9 \pm 0.4$
with	$K_1 = 81.0 \pm 12.1$	$\Delta H_1 = 1.8 \pm 0.1$	$\Delta S_1 = 76.9 \pm 0.4$

Table 2. 3: Thermodynamic Parameters of POPC/POPS/PIP2 (45:50:5) SUVs Binding to theOtoferlin C2F Domain in the Presence and Absence of Ca2+.

Ca ²⁺	$K_{\rm D}$ (μ M)	ΔH (kJ/mol)	$\Delta S (J \text{ mol}^{-1} \text{ K}^{-1})$
without	$K_1 = 8.7 \pm 1.3$	$\Delta H_1 = -0.4 \pm 0.0$	$\Delta S_1 = 76.2 \pm 5.4$
with	$K_1 = 6.3 \pm 2.7$	$\Delta H_1 = -0.5 \pm 0.0$	$\Delta S_1 = 86.2 \pm 1.4$

Table 2. 4: Thermodynamic Parameters of POPC/POPS/PI4P (45:50:5) SUVs Binding to the
Otoferlin C2F Domain in the Presence and Absence of Ca2+.

Ca ²⁺	K_{D}	ΔH (kJ/mol)	$\Delta S (J \text{ mol}^{-1} \text{ K}^{-1})$
without	$K_1 = 29.1 \pm 4.4$	$\Delta H_1 = -3.7 \pm 0.1$	$\Delta S_1 = 65.1 \pm 0.3$
with	$K_1 = 32.6 \pm 0.2$	$\Delta H_1 = -2.8 \pm 0.0$	$\Delta S_1 = 71.2 \pm 0.0$

Chapter 3

Direct Observation of Otoferlin C2 Domain Bound to Lipids

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3.1. Abstract

C2 domains are widely used beta-sandwich motifs that target proteins to subcellular membranes in response to rises in intracellular calcium. Many C2 domains involved in exocytosis also tilt and partially insert into membranes to aid in facilitating membrane fusion. While the importance of the C2-membrane interface and the angle of the domain has been recognized, there is currently no method for monitoring these characteristics without using perturbing interfacial labels. Here we report the development of a label free method using sum frequency generation vibrational spectroscopy to structurally characterize the interaction of a C2 domain of the exocytotic protein otoferlin with a lipid bilayer. We found the C2F domain to interact with membranes in a 1,2-Dipalmitoyl-sn-glycero-3-phospho-L-serine (DPPS) dependent, calcium insensitive manner. In addition, when coupled with theoretical calculated SFG spectrum, we found the C2F domain of otoferlin orients 32° normal to the membrane. Our results represent the first structural view of any C2 domain of otoferlin docked at the membrane interface, and provides a new, generally applicable methodology for structurally characterizing the membrane-protein interface without the need for perturbing labels.

3.2. Introduction

The protein-lipid membrane interface represents an important but understudied area of biological chemistry. Characterization of this interface has been a major challenge due to the considerable difficulties in applying traditional structural biology-based techniques to probe protein structure at lipid surfaces. For example, crystallography efforts on lipid-protein samples have had relatively limited success, and crystal structures cannot capture the dynamics of the interaction. Efforts to obtain this information through site-directed labeling with fluorescent or spin probes have been made, but require labeling of the protein at the interfacial region, resulting in an alteration of the chemical properties of the binding site. While Attenuated Total Internal Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR) has the ability to provide some structural information¹⁰², the technique falls short in the characterization of properties like the protein-membrane orientation and the distribution.

An example for the need to characterize protein-membrane interfaces is exemplified in the activity of the sound encoding protein otoferlin. Found in sensory hair cells, otoferlin is a 240kDa protein with six C2 domains that couples rise in intracellular calcium to membrane exocytosis and neurotransmitter release⁸⁵. Typically composed of an eight stranded beta-barrel fold, the C2 domain represents one of the largest families of membrane binding motifs, and is found in numerous

protein families, including the synaptotagmins, protein kinases, DOC2, and ferlins^{15,76,103,104}. Mechanistically, it is believed that C2 domains target the protein to a particular subcellular location based upon membrane lipid composition, with the loops at one edge of the domain making electrostatic and hydrophobic interactions with the lipid headgroup¹². In addition, hydrophobic residues within the loops of some C2 domains insert into the bilayer, resulting in a change in the spontaneous curvature of the membrane, which may facilitate membrane fusion and exocytosis ^{101,105}. For otoferlin, the C2F domain located on the C-terminus of the protein has garnered considerable interest due to deafness-causing mutations that abrogate membrane binding having been mapped to this domain⁷⁶. Liposome cosedimentation and noncanonical amino acid studies have established that this domain binds anionic lipids including phosphatidylserine via loops on one side of the domain which contact the bilayer¹⁰⁶. Like other studies on membrane binding domains, measurements on the C2F domain have relied on site direct mutagenesis of the loops that are thought to interact with the membrane, which can alter the binding affinity, specificity, and depth of insertion. The docking angle between the domain and membrane is thought to be a critical parameter that may throttle protein activity, however the aforementioned mutagenesis studies were incapable of determining this angle.

A method that can both characterize the protein and lipid bilayer without chemical perturbation of the interface would be ideal in the characterization of membranebinding proteins. Recently, nonlinear surface spectroscopy techniques including sum frequency generation (SFG) vibrational spectroscopy have emerged as a methodology for directly probing protein-membrane interactions in a label-free manner.^{107,108,109,110} SFG is a second-order nonlinear optical technique, which is capable of detecting biomolecule adsorption and orientation in the submicromolar concentrations at physiological pH.¹¹¹ The technique involves a fixed wavelength visible laser that is pulsed in temporal and spatial synchronicity with a tunable IR laser, and the product is a vibrational spectrum encoding information on the structure and orientation of the system under study.¹¹¹ SFG can provide molecular-level structural information and track changes in the sample over time with small peptides that are simplistic in structure and larger proteins that have multiple structures such as protein G B1 (8) kDa) and Cytochrome C (12 kDa).^{112,113,114,115,116,117} Here we report the use of experimental SFG spectra coupled with calculated theoretical SFG spectra to characterize the interaction and docking angle between a membrane binding protein domain (15 kDa) and a lipid bilayer surface by characterizing the structural orientation of the C2F domain of otoferlin when in contact with a model lipid bilayer. To obtain structural information on the C2F domain-lipid layer system, we collected SFG spectra corresponding to the amide I region of the domain and observed vibrations assigned to the C2F domain of otoferlin with a peak near 1621cm⁻¹ and a dip near 1672 cm⁻¹ consistent with an β -barrel secondary structure.^{117,118} Simulations of theoretical SFG spectra were then applied to interpret our experimental SFG results and indicate that the C2F domain of otoferlin orients with a tilt angle of 32° with respect to the surface normal of the lipid layer with the loops on one side of the

domain interdigitated with the membrane. Our results provide the first structural view of any C2 domain of otoferlin docked at a lipid membrane.

3.3. Materials and Methods

3.3.1 Materials

DPPS (1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (sodium salt), DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids, Inc. Glutathione Sepharose beads were purchased from GE-Hamersham Biosciences (Pittsburgh, PA). Other common reagents were obtained from Sigma Aldrich (St. Louis, MO).

3.3.2. Protein Construct and Purification

The C2F domain of otoferlin was cloned as a GST-fusion construct into PGEX-6P3 vector. The subcloned C2F domain was verified by DNA sequencing. The PGEX-6P3 vector containing the C2F domain was transformed into BL21 *Escherichia coli* cells. The bacterial culture ($OD_{600} = 0.6$) was induced for 12 hrs at 18°C with 1mM IPTG. Cells were pelleted at 4,000 rpm and re-suspended in lysis buffer containing 40mM HEPES (pH = 7.5), 200mM NaCl and 1mM DTT. The cells were lysed by sonication in lysis buffer containing protease inhibitors (1mM PMSF, 1-2 µg/ml aprotinin, leupeptin and pepstatin A). The soluble fraction of lysate was incubated with Glutathione resin for 2 hours at 4°C and The resin was washed with lysis buffer before the bound protein was eluted with elution buffer containing HEPES (pH = 7.5) and 200mM NaCl and 200mM reduced glutathione. Purified protein was extensively dialyzed in buffer

containing 40mM HEPES (pH 7.5), 200mM NaCl, 1mM DTT and concentrated using an Ultrafree-10 centrifugal filter unit (Pall Corporation, MI, USA). The protein was cleaved from the GST-tag with precission protease enzyme overnight at 4°C and the cut GST tag was removed by incubating with Glutathione Sepharose resin. The pure oto-C2F domain concentration was determined by UV absorbance at 280 nm.

3.3.3. Phospholipid Vesicles

The preparation of SUVs was performed according to reported methods ⁹³. Powders of DPPS and DPPC were dissolved in Chloroform, methanol and Water at the ratio (65:35:8) and in Chloroform respectively. 50% DPPC and 50% DPPS were mixed and dried under a stream of liquid nitrogen and then dried under vacuum overnight. The dried lipids were re-suspended in buffer containing HEPES (pH 7.5), 200mM NaCl and 1mM DTT and extruded 20 times through 50 nm filter (Avanti Polar Lipids Inc.) to produce small unilamellar vesicles (SUVs).

3.3.4. Sedimentation assay

For the binding assay, the C2F domain of otoferlin (5µg) was mixed with SUVs (100µg) in buffer (40mM HEPES (pH 7.5), 200mM NaCl, and 1mM DTT) with calcium (5, 10, 100, 500 and 1mM) or EGTA (1mM). The mixture was incubated for 1h at room temperature and centrifuged at 75,000g for 45 min in a TA-100 ultracentrifuge (Beckmann Instruments). SDS-PAGE gel data presented for calcium titration experiments consists of total protein (total input), supernatant (soluble fraction), and pellet (lipid-bound fraction).

3.3.5. Lipid Bilayer Setup

A flow cell made from Teflon was used for all experiments. A Lipid bilayer prepared from a 50:50 molar ratio of phospholipids DPPC (1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine, Avanti Polar Lipids Inc.) and DPPS (1,2-Dipalmitoyl-*sn*-glycero-3-phospho-L-serine, Avanti Polar Lipids Inc.) was deposited on equilateral CaF₂ prisms (vendor). DPPS/DPPC vesicles were prepared by rapid solvent exchange by first dissolving the lipids in chloroform, vacuum drying for 1.5 hours, then finally adding D₂O (Carl Roth GmbH) for a final concentration of 1 mg/ml. The vesicles were allowed to fuse forming a bilayer for 2 hours before PBS (0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl, pH 7.4, Sigma Aldrich) made with D₂O was flowed in to wash away the remaining vesicles. Otoferlin C2F domain was flowed in (concentration) and allowed to interact for (time).

3.3.6. Molecular Dynamic (MD) Simulation

The SFG spectra calculation were performed using the formalism described by Roeters et al. [J. Phys. Chem. A, 2013, 117, 6311–6322.]. Briefly, the amide-I excitonic Hamiltonian for the amide backbone modes that generate the experimentally observed SFG response was calculated from the protein conformation. The amide I couplings of nearest neighbors was obtained using an *ab initio* 6-31G +(d) B3LYP-calculated map that produces the inter-bond coupling for the specific dihedral angles between the amide moieties [Gorbunov, R. D., Kosov, D. S. & Stock, G. Ab initio-based exciton model of amide I vibrations in peptides: Definition, conformational

dependence, and transferability. J Chem Phys 122, - (2005).]. Couplings, between nonnearest neighbor amide-I vibrations were estimated using the transition dipole coupling model [Hamm, P., Lim, M. & Hochstrasser, R. M. Structure of the Amide I Band of Peptides Measured by Femtosecond Nonlinear-Infrared Spectroscopy. J Phys Chem B 102, 6123-6138 (1998).]. The frequency shifts induced by hydrogen bonding were calculated using an empirical model [J. Phys. Chem. A, 2013, 117, 6311-6322.]. The resulting Hamiltonian was then diagonalized to obtain the amide normal modes of the system. We determined the SFG spectra by taking the tensor product of the calculated infrared and Raman spectra. The spectra were then transformed into the lab frame by averaging the Euler transformation between the two reference frames over the orientation distribution of the protein, which was set to $\pm 15^{\circ}$. The structure of the related dysferlin C2A domain (PDB: 4IQH) were used for the calculation. The domain was oriented as shown in figure 2B using the VMD software package [Humphrey, W., Dalke, A. and Schulten, K., "VMD - Visual Molecular Dynamics", J. Molec. Graphics, 1996, vol. 14, pp. 33-38.].

3.3.7. Sum Frequency Generation (SFG) Vibrational Spectroscopy A femtosecond Ti: Sapphire laser oscillator coupled with a Nd: YLF laser pumped regenerative amplifier (Spitfire Ace, Spectra Physics) was used to generate a visible beam (35 fs pulse duration and 4.65 mJ) centered at 791.8 nm. The amplified visible beam was split into two parts. The first was used as the visible pulse for sum frequency generation and was passed through a Fabry-Perot etalon to spectrally narrow the pulse to ~15 cm⁻¹. The second part was used to pump the optical parametric amplifier (OPA) system (Light Conversion, TOPAS). The generated tunable infrared pulse (3.1-6.1 μ m) was polarized by a half-wave plate before use as the sum frequency generation IR pulse. The IR and visible pulses were overlapped temporally and spatially at the liquid-air interface generating an SFG signal. SFG spectra of the samples were collected in the polarization combinations *ppp and spp* (*s*-polarized SFG, *p*-polarized visible, and *p*-polarized IR) Amide I region (1500 cm⁻¹ – 1800 cm⁻¹). The resulting SFG signal was focused onto a spectrograph (Action, Princeton Instruments), dispersed by grating, and re-focused on an electron multiplying charge coupled device (CCD) camera (Newton, Andor). SFG spectra were normalized by division of a clean gold reference. The SFG spectrum were fit with the following equation:¹⁰²

$$\chi_{eff}^{(2)}(\omega) = \chi_{NR}^{(2)} + \sum_{q} \frac{A_q}{\omega - \omega_q + i\Gamma_q}$$
(1)

where Γ_q , A_q , and ω_q are the full width half max (FWHM), amplitude, and resonant frequency of the qth vibrational mode, respectively, and $\chi_{NR}^{(2)}$ and $\chi_{eff}^{(2)}$ are the nonresonant background and effective seconded nonlinear susceptibility tensor, respectively.

3.4. Results

3.4.1. The C2F Domain of otoferlin binding to liposomes made up of DPPS and DPPC.

As a first step in characterizing C2F-membrane interaction, we sought to verify the binding of the C2F domain to liposomes composed of a 1:1 mole ratio DPPS: DPPC. We therefore performed sedimentation assays on a 1:1 mole ratio DPPS: DPPC liposomes mixed with the C2F domain of otoferlin in the presence of calcium or EDTA (Figure 3.1A and Figure 3.1B). We found that regardless of calcium, the C2F domain of otoferlin bound liposomes, in agreement with previous studies.

3.4.2. Experimental sum frequency generation (SFG) vibrational spectroscopy and simulated spectra

We next sought to apply SFG to structurally characterize the C2F-membrane interaction. For our experimental setup, a flow cell made from Teflon was used for all experiments (Figure 3.2). A lipid bilayer prepared from a 1:1 molar ratio of phospholipids DPPC (1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine) and DPPS (1,2-Dipalmitoyl-*sn*-glycero-3-phospho-L-serine) was deposited on equilateral CaF₂ prisms. DPPS/DPPC vesicles were prepared by rapid solvent exchange by first dissolving the lipids in chloroform, vacuum drying for 1.5 hours, then finally adding D₂O for a final concentration of 1 mg/ml. The vesicles were allowed to fuse forming a bilayer for 2 hours before PBS made with D₂O was flowed in to wash away the remaining vesicles. Otoferlin C2F domain was flowed in at 1 μ M and allowed to interact for 1 hr before SFG vibrational spectra were taken.

Our experimental SFG vibrational spectra in the Amide I region (1500 cm⁻¹ -1800 cm⁻¹) were collected to monitor and observe the secondary structure of otoferlin C2F domain interacting with a model DPPS/DPPC lipid bilayer at the CaF₂-water interface (Figure 3.2 A). Vibrations related to the secondary structure of proteins have a vibrational mode in the Amide I region between 1600 cm⁻¹ and 1700 cm⁻¹, water does have a vibrational mode in this region but using deuterium oxide (D₂O) shifts the vibrational mode from this region. Analysis of vibrational spectra in PPP (P-SFG, Pvisible, P-IR) polarization combination revealed a peak near 1621 cm⁻¹ and a dip near 1672 cm⁻¹ suggesting a mostly beta-barrel secondary structure (Figure 3.2 C). To further interpret the collected experimental SFG vibrational spectrum we conducted sum frequency generation vibrational spectroscopy simulations to generate theoretical spectra representing a C2-bilayer system. The experimental PPP polarized SFG vibrational spectrum can only be reproduced with the C2F domain orientated with a tilt angle of $32^{\circ} \pm 2^{\circ}$ with respect to the bilayer surface normal (Figure 3.2 B, C). The small tilt angle to the surface normal suggests that there is a region of the otoferlin C2F domain that can bind to lipids. This part is known as the binding loop and is comprised of amino acids that have the ability to interact with both the bilayer and the calcium present near the membrane (Figure 3.2 D).

3.5. Discussion

In the present study, we demonstrated a new label free method for structurally characterizing protein-membrane interfaces. Applied to the C2F domain of otoferlin, we found that the domain binds to DPPC/DPPS membranes with a tilt angle 32° to the surface normal. This tilt angle suggest that the C2F domain is docked in a nearly perpendicular orientation relative to the membrane with the loops on one side of the domain in contact with the membrane (Figure 3.2 B, C). Similar to the C2F domain of otoferlin, synaptotagmin 1 C2A domain (Syt 1C2A) also orients at the membrane interface at a tilt angle of 37.1± 9.5° ^{119, 120}. However, the tilt angles of the C2 domains of synaptotagmin have also been reported to adopt other angles, in an apparently calcium sensitive manner. It is hypothesized this calcium induced change in the tilt angle allows the C2 domains to insert hydrophobic residues into the bilayer which aids in facilitating membrane fusion ¹²¹. While our study, as well as other have found the membrane binding properties of C2F to be largely calcium independent, we speculate that adjacent C2 domains in the otoferlin protein may confer calcium sensitivity. In addition to phosphatidylserine, the C2F domain also binds phosphatidylinositol 4,5-bisphophate, and it would also be of interest to determine if this lipid alter the contact angle of the domain.

In summary, this work introduces a new means to study the membrane docking of C2 domains and other domains that contain β - sandwich fold by SFG vibrational spectroscopy. Since the C2F domain is one of 6 domains in the otoferlin

protein, it would be of interest to study the effects of linked domains on the tilt angle of a given C2 domain. However, if two domains are present in tandem interacting with the lipid bilayer, experimental SFG tilt angle calculations will become increasingly difficult as new parameters will have to be introduced to solve the nonlinear equations associated with peptide orientation in laboratory coordinates. Proteins that have a bent secondary structure or multiple domains may become more difficult to characterize with experimental SFG data alone and the use of simulated SFG spectrum will help in meeting this challenge. Finally, several studies have noted that certain peripheral membrane binding proteins alter the acyl chain ordering and distort the membrane, and SFG has the capability to probe change in acyl chain ordering. Thus future studies should focus on developing SFG methods to aid in characterizing the effects of protein binding on bilayer structure.

3.6. Abbreviations

POPS, 1-palmitoyl-2-oloeyl-sn-glycero-3-phospho-L-serine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; DPPC, (1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine); DPPS, (1,2-Dipalmitoyl-*sn*-glycero-3-phospho-L-serine); PS, phosphatidylserine; PC, phosptotidylcholine; SFG, sum frequency generation spectroscopy, SUVs, small unilamellar vesicles; ATR-FTIR, Attenuated Total Internal Reflection Fourier Transform Infrared Spectroscopy.



Figure 3.1. (A) Interaction of the C2F domain of otoferlin with phospholipid membranes in the presence of increasing calcium concentrations. (B) Quantification of the results of the liposome binding assay.



Figure 3.2. The SFG experimental setup is depicted such that the incident beams are polarized perpendicular (s-polarized) or parallel (p-polarization) to the plane of incidence (A) Experimental PPP polarization SFG Amide I region spectra of otoferlin C2F domain interacting with a lipid bilayer is compared to MD simulation calculated SFG spectra. (B) Oriented otoferlin C2F domain with respect to the surface normal of a POPS/POPC lipid bilayer is depicted with a theoretical calculated tilt angle. (C) The region of the protein with the binding loop is enhanced and the amino acids that are depicted as interacting with the lipid bilayer are highlighted in different colors:

Table 3.1: Amide I region PPP and SPP polarization SFG fitting results of otoferlin C2F domain interacting with a DPPS/DPPC lipid bilayer (phase= 4.57)

	Otoferlin C2F domain DPPS/DPPC lipid bilayer PPP ($\chi^2_{NR} = 0.122$)	Otoferlin C2F domain DPPS/DPPC lipid bilayer SPP ($\chi^2_{NR} = 0.161$)
Amp. 1	0.624	1.864
ω 1	1621 cm ⁻¹	1629 cm⁻¹
Г 1	28.12 cm ⁻¹	60.73 cm ⁻¹
Amp. 2	-5.043	-10.475
ω 2	1672 cm ⁻¹	1670 cm ⁻¹
Г 2	44.88 cm ⁻¹	60.70 cm ⁻¹

Chapter 4

Characterization of Temperature sensitive mutants of Otoferlin

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4.1. Abstract

Otoferlin is a multi-C2 domain protein involved in calcium-triggered exocytosis in cochlear inner hair cells (IHCs). Deletion of a glutamic acid at position 1804 in otoferlin results in a temperature-sensitive mutant in the C2F domain (C2F-TS) that causes temporary deafness in febrile patients. To characterize the structural differences and stability between C2F-WT and C2F-TS as a function of temperature, we applied circular dichroism and tryptophan fluorescence spectra under physiological and denaturing conditions. In addition, co-sedimentation was also performed to determine the ability of C2F-TS to bind liposomes at different temperatures. The results indicate that C2F-TS is incapable of binding liposomes at both 25 °C and 40 °C. However, this change in function does not appear to be due to gross structural changes, due to the lack of significant shifts in tryptophan fluorescence and circular dichroism spectra compared to wild type C2F (C2F-WT). Based on these results, it appears that C2F-TS may have minor structural change localized in the active site.

4.2. Introduction

Temperature sensitive auditory neuropathy (TSAN) was first reported by Starr et al. in 1998 in children who became "deaf" when their core body temperature rose 1° above body temperature . TSAN is a clinical disorder of sensorineural and transient deafness characterized by absence of auditory brainstem response (ABR) and normal optoacoustic emissions (OAE). ABR records the electrical activity between cochlear inner hair cells and the brainstem neural pathway, whereas OAE measures the response of outer hair cells to sound ^{122,123}. The hearing threshold in patients with TSAN oscillate between normal to profound hearing loss based on their core body temperature. Speech recognition in TSAN patients is severely impaired during elevated temperature, particularly in noisy environments but reverts back to normal when they are afebrile ¹²⁴. The prevalence of TSAN ranges between 4 to 11% of Spanish and Pakistani populations including both genetic and non-genetic etiological factors ^{125, 126}. To date, the non-syndromic hearing impairment (NSHI) inherited genes DFNB9 and DFNB59 have been identified to be associated with the auditory neuropathy (AN) phenotype. DFNB9 and DFNB59 are caused by defects in the OTOF and pejvakin genes ^{127, 128}, respectively.

OTOF mutations were first identified by Yasunaga et al. in 1999 in patients with prelingual or congenital non-syndromic hearing loss ⁸⁴. Pathogenic mutations in OTOF gene are associated with two phenotypes including non-syndromic hearing loss, DFNB9 and a less severe and temperature sensitive auditory neuropathy (TSAN). The autosomal recessive non-syndromic hearing loss is characterized by severe (71-90 Db loss) to profound (>90 Db loss) congenital deafness. Inherited hearing impairment is the most frequent hereditary sensory defect with overall incidence of more than 1 in 1000 children. Studies in Spanish and Pakistani population indicated that 3-8% of these children suffer from congenital non-syndromic hearing loss because of mutations in otoferlin¹²⁸. There are more than 60 pathogenic mutations have been found in otoferlin ⁷⁶. Among these, there are 15 pathogenic missense mutations of which 10 are found in the C-terminal domains of otoferlin, C2C to C2F. Also, there are missense and deletion mutants found in otoferlin associated with temperature sensitive auditory neuropathy TSAN. Varga et al., 2006 have reported a missense mutation in the C2C domain of otoferlin (substitution of isoleucine with Threonine) I515T causing TSAN ¹²⁹. In 2010, Wang et al. reported a TSAN associated missense mutation in C2E the domain of otoferlin (R1607W) in the Chinese population ¹³⁰. In 2010 Marlin *et al.* found a deletion in the calcium binding C2F domain of otoferlin (Glutamic acid 1804) causing TSAN ¹²⁸.

The phenotype of temperature sensitive deafness may be due to defects in normal protein trafficking. To support this hypothesis, documented studies can be found on temperature sensitive albinism and cystic fibrosis. For instance, a temperature sensitive missense mutation in the tyrosinase gene resulted in protein trafficking defect as it is retained in the endoplasmic reticulum (ER) and degraded by the proteasomes instead of translocating into melanosomes ¹³¹. A missense mutation in the cell surface cyclic AMP dependent chloride channel also lead to ER- associated degradation pathway. Using *in vitro* experiments, Wang et al. 2008 have shown that the channel localizes to the cell surface and is functional at lower temperatures of 27 - 32° C. Thus, the temperature sensitive mutants of otoferlin can also exhibit temperature sensitive trafficking ¹³². At higher temperatures, the protein may be degraded by ER- associated degradation pathway whereas at lower temperatures, the protein could be translocated to the plasma membrane.

Moreover, we also hypothesize reduced activity of otoferlin due to structural changes as the temperature increases. All temperature sensitive mutations exist in the Ca²⁺ binding domains. For instance, G1804del is found in the C2F domain and C2F has been reported to bind Ca²⁺, lipids and directly interact with the neuronal SNAREs, SNAP-25 and syntaxin-1 ⁶. Mutations in the C2F domain diminished calcium and SNARE binding ⁷¹. Thus, the binding of temperature sensitive mutants to Ca²⁺, lipids and its proteome may be temperature sensitive.

In our present study, we report the loss of lipid binding activity for C2F-TS at both 25 °C and 40 °C. We compared the structural differences between C2F-WT and C2F-TS and to try to determine the underlying reason for the lack of lipid binding. However, we found no major difference in structure between C2F-WT and C2F-TS

and conclude that small, localized structural changes at the active site lead to loss of lipid binding.

4.3. Materials and Methods

4.3.1. Materials.

POPS (1-palmitoyl-2-oleoyl-sn-glycero-3-phos- pho-L-serine), POPC (1-palmitoyl-2oleoyl-sn-glycero-3-phos-phocholine) were purchased from Avanti Polar Lipids, Inc. Glutathione Sepharose beads were purchased from GE-Hamersham Biosciences (Pittsburgh, PA). Other common reagents were obtained from Sigma Aldrich (St. Louis, MO).

4.3.2. Protein constructs, expression and purification.

The C2F domain of otoferlin was cloned as a GST-fusion construct into PGEX-6P3 vector. The sub cloned C2F domain was verified by DNA sequencing. The PGEX-6P3 vector containing the C2F domain was transformed into BL21 *Escherichia coli* cells. For generating Glu1804 del (C2F-TS) mutant of C2F domain, primers for mutation were designed as mismatch primers to the nucleotide site of interest. C2F-TS domain was constructed using Stratagene Quickchange site-directed mutagenesis kit with PGEX-6P3-oto-C2F-WT plasmid template. The construct generated was sequenced and verified by Genscript.

The PGEX-6P3 vector containing the C2F and C2F-TS domains were transformed into BL21 *Escherichia coli* cells. The bacterial culture ($OD_{600} = 0.6$) was induced for 12 hrs at 18°C with 1mM IPTG. Cells were pelleted at 4,000 rpm and re-suspended in lysis buffer containing 40mM HEPES (pH = 7.5), 200mM NaCl and 1mM DTT. The cells were lysed by sonication in lysis buffer containing protease inhibitors (1mM PMSF, 1-2 µg/ml aprotinin, leupeptin and pepstatin A). The soluble fraction of lysate was incubated with Glutathione resin for 2 hours at 4°C and the resin was washed with lysis buffer before the bound protein was eluted with elution buffer containing HEPES (pH = 7.5) , 200mM NaCl and 200mM reduced glutathione. Purified protein was extensively dialyzed in buffer containing 40mM HEPES (pH 7.5), 200mM NaCl, 1mM DTT and concentrated using an Ultrafree-10 centrifugal filter unit (Pall Corporation, MI, USA). The protein was cleaved from the GST-tag with precission protease enzyme overnight at 4°C and the cut GST tag was removed by incubating with Glutathione Sepharose resin. The pure oto-C2F and C2F-TS domains concentrations were determined by UV absorbance at 280 nm and calculated using extinction coefficients.

4.3.3. Phospholipid Vesicles

The preparation of small unilamellar vesicles (SUVs) was performed according to reported methods ¹⁰⁶. Briefly, chloroform solutions composed of 50% POPS and 50% POPC were mixed and dried under a stream of liquid nitrogen gas and then dried under vacuum for 3 h. The dried lipids were resuspended in buffer and extruded 20 times through a 50 nm filter (Avanti Polar Lipids, Inc.) to SUVs.

4.3.4. Sedimentation Assay

For the liposome binding assay, the C2F-WT and C2F-TS domains of otoferlin (5 µg) were mixed with SUVs (100 µg) in buffer [20 mM HEPES (pH 7.5), 200 mM NaCl and 1mM DTT with calcium (10, 100, and 1 mM) or EDTA (1 mM). The mixture was incubated for 1 h at 25 °C and 40 °C and centrifuged at 85000g for 45 min in a TA-100 ultracentrifuge (Beckmann Instruments). SDS–PAGE gel data presented for calcium
titration experiments consist of total protein control (total input), supernatant (soluble fraction), and pellet (lipid-bound fraction).

4.3.5. Tryptophan Fluorescence

Intrinsic tryptophan fluorescence was measured using a PTI QuantaMaster fluorimeter with 5 nm excitation and emission slit widths. The fluorescence intensity of oto-C2F-WT (5 μ M) and C2F-TS (5 μ M) were measured at 25 °C , 37 °C and 40 °C. The protein samples were excited at 295 nm and fluorescence emission was recorded from 310-460 nm with 1nm steps and an integration time of 0.2 s, averaging 3 samples. Autofluorescence was also measured for both oto-C2F-WT and C2F-TS at different urea concentrations at 25 °C. The reported values represent mean \pm standard deviation for the average of 3 samples.

4.3.6. Circular Dichroism

The protein samples Oto-C2F-WT and C2F-TS (5 μ M) were dialyzed in phosphate buffer, pH 7.5. Circular Dichroism spectra were recorded at 25 °C , 30 °C , 37 °C and 40 °C with a Chirascan CD spectrometer (Applied photophysics Ltd) from 190-260 nm using for both oto-C2F-WT and C2F-TS.

4.4. Results

4.4.1. Lipid binding properties of wild type Oto-C2F and Oto-C2F-TS mutant

We sought to determine the effect of temperature on the lipid binding properties of oto-C2F-WT and the C2F-TS (Glu1804del) mutant by applying a co-sedimentation assay. 50% PS : 50% PC liposomes were mixed with C2F-WT or C2F-TS in the presence of calcium or EDTA at 25 °C and 40 °C. The C2F-WT bound liposomes both in the presence of EDTA or Ca²⁺ at both temperatures, however the temperatures sensitive mutant did not bind liposomes at either temperature regardless of presence of EDTA or Ca²⁺ (Figure 4.1).

4.4.2. Determining effect of the deletion mutation on the secondary structure of the C2F domain using circular dichroism and intrinsic tryptophan fluorescence.

Analysis of our sedimentation data indicates that the C2F-TS is incapable of binding 50%POPS:50%POPC liposomes regardless of temperatures. Thus, we sought to determine if deletion of the glutamic acid (Glu1804del) induced structural changes in the C2F-TS relative to the wild type C2F domain. We found a very slight difference in CD spectra between the C2F-TS and C2F-WT at 25 °C , 30 °C, 37 °C and 40 °C suggesting that there are minor structural changes between wild type and TS mutant (Figure 4.2).

We also used intrinsic fluorescence spectroscopy to characterize structural differences between the C2F-WT and C2F-TS constructs. We observed a very slight shift in the fluorescence emission maxima for C2F-TS and C2F-WT, 336 nm and 338 nm respectively. Thus, there was only a small differences fluorescence spectra at all tested temperatures between C2F-WT and C2F-TS (Figure 4.3).

We included a linker of around 10 kDa for solubility purposes, thus structural changes may be overshadowed by otherwise disordered structure and also the presence of several tryptophan residues (four) accounting for circular dichroism and tryptophan fluorescence data respectively.

We next performed chemical denaturation measurements at various urea concentrations to characterize any differences in structural stability between C2F-WT and C2F-TS. In agreement with CD and intrinsic fluorescence data, we observed no significant change in structural stability between C2F-TS and C2F-WT (Figure 4.4).

4.5. Discussion

Temperature sensitive mutations have been reported in several human diseases including albinism, cystic fibrosis and respiratory tract infections ^{133,131}. Recently, a set of missense mutations were found in OTOF, a gene encoding a mutli-C2 domain protein called otoferlin that lead to temperature sensitive auditory neuropathy

(TSAN). Elevation of body temperature by as little as 1° C leads to profound hearing loss, whereas patients have normal speech recognition at normal body temperature. These mutations may result in defects that prevent specific subcellular localization and reduced protein levels in the cell due to temperature induced protein unfolding and proteasome degradation. Supporting our hypothesis, Strenzke et al., reported that a TS mutation in the C2C domain of otoferlin (Ile 515 Thr) resulted in diminished levels of otoferlin by 65% at the plasma membrane and also affected the size of the synaptic vesicles ¹³⁴. In addition, temperature sensitive mutations could affect protein-protein interactions. For instance, the temperature sensitive mutation in neuronal SNARE protein syntaxin blocked synaptic neurotransmission in Drosophila melanogaster due to decrease in binding to synaptobrevin and it did not affect localization of the protein to synaptic vesicles ¹³⁵. Our results from the sedimentation assay indicate that the mutation leads to loss of function regardless of temperature changes. This loss of function appears to be not due to major structural changes, since no difference in both the circular dichroism and tryptophan fluorescence spectra of both mutant and wild type-C2F when detected. In addition, chemical denaturation results suggest no difference in structural stability between wild type-C2F and C2F-TS. Based on our findings, the lack of lipid binding could be due to localized structural changes in the active site of the domain. The glutamic acid is predicted to present in one of the calcium binding loop of the C2F domain. The acidic residues coordinate Ca²⁺ and enabling the C2 domain to interact with anionic phospholipid,

phosphatidylserine. Hence, the deletion of glutamic acid (E1804del) might perturb the anionic phospholipid binding which we found in our study.

Moreover, Stenzke et al. 2016 found that elevated cochlear temperature exceeding 38 °C did not fully abolish sound encoding in mouse oto-C2C temperature sensitive mutant (Ile515Thr), but exocytosis was abolished in human patients. The less pronounced effect in a mouse mutant is due to the lack of 20 amino acid stretch that contained RXR motif which was found in a human otoferlin. Stenzke et al. found that RXR motif in human otoferlin rendered protein more heat sensitive. Thus, the absence of structural difference between mouse TS-C2F and WT-C2F with increase in temperature might be due to the lack of RXR motif which was found in human otoferlin¹³⁴. However, we included a linker of around 10 kDa to increase the solubility of the domain, so the structural changes may be overshadowed by otherwise disordered structure and also the presence of several tryptophan residues (4) accounting for circular dichroism and tryptophan fluorescence. Hence, future work should include to characterize the C2F-WT and C2F-TS by removing the linker region. We also yet to determine the effect of TS mutation on other molecular interactions (Ca²⁺, SNARE proteins) compared to C2F-WT.

4.6. Abbreviations

POPS, 1-palmitoyl-2-oloeyl-sn-glycero-3-phospho-L-serine; POPC, 1-palmitoyl-2-oloeyl-sn-glycero-3-phosphocholine; CD, Circular Dichroism; TSAN, Temperature sensitive auditory neuropathy.



Figure 4.1: Association of C2F-WT and C2F-TS with liposomes in the presence or in the absence of calcium

Association of C2F-WT (5 µM) and C2F-TS (5 µM) with 50% POPS: 50%POPC liposomes at

different temperatures @ 25 °C and 40 °C (S= supernatant, P= Pellet).



Figure 4.2. Circular dichroism spectroscopy of C2F-WT (5 $\mu M)$ and C2F-TS (5 $\mu M)$ at

25 °C , 30 °C, 37 °C and 40 °C.



Figure 4.3. Intrinsic fluorescence spectra of both C2F-WT and C2F-TS. C2F-TS (5 μ M) and C2F-WT (5 μ M) in HEPES buffer at 25 °C, 37 °C and 40 °C.





(5 μ M) at increasing concentrations of urea (1, 2, 3, 4, 5, 6, 7, 8 M).

Chapter 5

Conclusions, Impacts and Future directions

Hearing loss is one of the most common defects, affecting 360 million people worldwide. This can be due to several factors including congenital, present at or soon after birth or acquired with age. Approximately one in three people over 65 years has hearing loss. Congenital hearing loss affects 32 million children in the world. The prevalence of hearing loss in children is greatest in the South Asia, Asia Pacific and Sub-Saharan Africa regions. The economic impact of hearing loss is estimated to cost society an average of 300, 000 dollars over the lifetime of a person. It also has serious impacts on quality of life including the literacy rate in children. Although, hearing loss is clearly a major health problem its genetic basis for the pathology is poorly understood. To date, over 60 pathogenic mutations in otoferlin have been found to be associated with inherited, non-syndromic congenital hearing loss. Otoferlin has been proposed to regulate the release of neurotransmitters at IHCs in cochlea and any defects in otoferlin manifest hearing loss. However, the function of otoferlin, how it works and what it looks like structurally have yet to be fully determined.

My dissertation work presents the biophysical characterization of otoferlin and tests the hypothesis that otoferlin acts as Ca^{2+} sensor for neurotransmitter release in IHCs of the cochlea. If otoferlin is to act as a calcium sensor, we would expect the C2 domains of otoferlin to bind Ca^{2+} with an affinity constant that quantitatively matches the maximal intracellular presynaptic calcium concentrations of 30~50 µM. Using isothermal titration calorimetry and co-sedimentation, I have found that the calcium binding affinities of otoferlin indeed fall within the the physiological concentrations found in presynapses. Also, I determined Ca^{2+} and liposome binding using isolated C2 domains, future studies should include combination of multiple C2 domains and full length protein to demonstrate cooperativity of of the C2 domains of otoferlin in binding to liposomes. The cooperative behavior of the C2 domains of otoferlin can be studied using stopped-flow fluorescence spectroscopy. For instance, synaptotagmin tandem C2AB domains exhibit slower disassociate rate than either of the single domains, whereas synaptotagmin 7 C2 domains were found to interact with membranes independently ¹³⁶. I also found that the C2C and C2F domains of otoferlin preferentially bind plasma membrane enriched lipid (PI(4,5)P2), and PI(4,5)P2 might serve to target otoferlin to the presynapse in a calcium independent manner. My finding that otoferlin binds to PI(4,5)P2 has led to the proposed mechanism in which this lipid serves to target otoferlin to the presynapse, positioning otoferlin for regulating fast calcium dependent exocytosis at the hair cell synapse. In this study, I also found a PI(4,5)P2 conserved motif in the C2C domain of otoferlin and PI(4,5) P2 binding was abolished when conserved lysine residues L478A and L480A were mutated. However, the conserved PI (4,5) P2 binding motif does not exist in the C2F domain of otoferlin and future studies like alanine-scanning mutagenesis should be performed to map the PI(4,5)P2 binding site of the C2F domain. Future studies should also characterize the lipid binding specificity of the other C2 domains of otoferlin to determine if they bind other subcellular organelles enriched lipids like PI4P, PI3P and To further characterize the otoferlin-lipid interface, I conducted sum PI(3,5)P2. frequency generation spectroscopy (SFG) on the C2F domain and determined the orientation of the domain when bound to lipid bilayers. Similar to other C2 domain

proteins including C2 domains of synaptotagmin that acts as a Ca²⁺ sensor in neurons, I found that the C2F domain orients on the membrane with a tilt angle of 32°± 2° with calcium binding loops interacting with the lipid bilayer. The SFG spectra of the domain has inflections near 1621cm⁻¹ and 1672 cm⁻¹ consistent with β-barrel structures. This study represents the first structural view of the C2 domain of any ferlin family member docked at the membrane interface. The experiment was performed with a lipid bilayer composed of phosphatidylserine and phosphatidylcholine. In case of synaptotagmin 1, C2B domain interaction with PI(4,5)P2 predisposes synaptotagmin to penetrate lipid bilayer in response to Ca²⁺ with rapid kinetics. Also, the docking angle of PKCα increased from 35° to 44° when PI(4,5)P2 is included in the membrane. This change in the angle of the C2 domain could modulate the activity of membrane docked C2 domain as it diffuses between membrane regions with different local PS and PI (4,5)P2 concentrations. We have yet to determine the orientation of the C2F domain in the presence of PI(4,5)P2. Mutations that abolish calcium binding in the C2F domain do not disrupt liposome binding. Thus, it will be interesting to determine how calcium binding mutants orient at the membrane interface to understand C2F mechanistic view at the membrane interface. Also, since otoferlin is a 6-C2 domain protein, it would be interest to determine the how the full length protein orients on the bilayer, perhaps by coupling SFG experimental spectra with molecular dynamic simulations. It is also known that C2 domains increase lipid acyl chain disorder and distort the membrane, which is believed to help facilitate fusion and SFG could also be applied to examine the effects

of otoferlin C2 domains on lipid ordering. In addition to membrane docking studies, the membrane bending and penetration properties of individual and multiple C2 domains of otoferlin is yet to be determined. Many C2 domains, including synaptotagmin bend and penetrate lipid bilayer with calcium binding loops of the domains in the presence of Ca²⁺. This penetration induces changes in the curvature of the lipid bilayer which is an important mechanistic step of membrane fusion. Thus, it is crucial to determine if C2 domains of otoferlin penetrate lipid bilayer to drive curvature changes. To characterize the membrane penetration properties of otoferlin, several methods can be used, including labeling of the predicted membrane penetrating amino acid with the environment sensitive fluorophore (AEDANS) or by incorporating an unnatural amino acid. For instance, burial of the AEDANS label in the lipid bilayer during penetration results in AEDANS fluorescence spectra shift towards higher wavelength ¹⁰¹. Also, the membrane penetration can be determined by directly imaging liposomes using electron microscopy to observe the resulting lipid tubules that are produced due to the changes in the curvature of the lipid bilayer ¹³⁷. Temperature sensitive mutations in otoferlin result in hearing loss when patients are febrile. I have characterized a temperature sensitive mutant in the C2F domain of otoferlin in an attempt to determine the underlying cause of loss of function. I worked under the hypothesis that temperature sensitive mutant is structurally less stable compared to wild-type protein and is more temperature sensitive. However, results indicated no major difference in structural stability between the WT-C2F and TS-C2F domain as the temperature increased. I did find however that the TS-C2F domain lost

its ability to bind anionic lipid phosphatidylserine at both 25° and 40°. Based on these results, I proposed that the C2F-TS may have minor structural differences localized in the calcium binding region of the domain that results in loss of lipid binding. Future work should include characterizing the C2F-WT and C2F-TS using differential scanning calorimetry which provides thermodynamic parameters of protein unfolding. Moreover, we are yet to characterize other temperature sensitive mutations of otoferlin present in C2C, C2D and C2E domain of otoferlin and the effect of TS mutation on other molecular interactions (Ca²⁺, SNARE proteins) compared to wild-type protein.

Overall, our results reveal fascinating parallels between otoferlin, synaptotagmins, and extended synaptotagmins. Extended synaptotagmins (E-Syt) similar to synaptotagmin in that they are multi-C2 domain proteins, with 5 in E-Syt 1 and 3 in E-Syt 2 and E- Syt 3. E-Syt's are required for forming an endoplasmic reticulum-plasma membrane (ER-PM) contact to exchange phospholipids between membranes. The C2E and C2C domain of E-Syt 1 and E-Syt 2/3 bind to the plasma membrane phospholipid PI(4,5)P2 in a calcium independent manner similar to otoferlin ^{138,139,140,141,142}. Ca²⁺ influx mediates the interaction of other C2 domains of the protein with the plasma membrane brining two membranes to the close proximity for efficient phospholipid transfer between them. Similar to Otoferlin and E-synaptotagmins, the neuronal Ca²⁺ sensor synaptotagmin also tethered to the plasma membrane in a calcium independent manner through its C2B domain.

Our findings from otoferlin research can serve as a model for understanding the ferlin family of proteins. Ferlins are implicated in regulating membrane trafficking and vesicle fusion ⁶¹. There are six mammalian ferlins including dysferlin, otoferlin and myoferlin, Fer1l4, Fer1l5 and Fer1L6 and two invertebrate ferlins fer-1 in Caenorhabditis elegans and misfire in Drosophila melanogaster. In 1997, Achanzer et al., have found the first ferlin fer-1 in *Caenorhabditis elegans* (*C.elegans*), a fertilization factor required for fusing membranous organelles with the plasma membrane in a calcium dependent manner, hence the name ferlins ¹⁴³. Mutations in Fer-1 causes infertility in *C.elegans*. Similarly, defects in misfire causes infertility in Drosophila melanogaster ^{144, 145, 146}. Dysferlin null mice showed defects in membrane resealing in sarcolemma resulting in dysferlinopathy phenotype ¹⁴⁷. Myoferlin is implicated in breast cancer ⁶⁵. Whereas Fer1L4, Fer1L5 and Fer1L6 are not characterized yet. Similar to otoferlin, the C2 domains of dysferlin exhibit Ca²⁺ dependent and independent modes of anionic phospholipid binding to mediate membrane resealing. However, the lipid specificities of the C2 domains of dysferlin have not yet determined. My studies on otoferlin suggest that some C2 domains within a ferlin proteins may interact with organelle specific lipids that target the protein to a specific subcellular location. In conclusion, the structure functional analysis of otoferlin could shed light if ferlin family of proteins share a common mechanism in mediating membrane fusion reactions.

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