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Shaking Up the Immunoglobulin Superfamily

Christopher Mendoza

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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ABSTRACT

Shaking Up the Immunoglobulin Superfamily

Christopher Mendoza
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Doctor of Philosophy

The immunoglobulin superfamily (IgSF) is a large protein superfamily of membrane and soluble proteins that influence recognition, binding, and adhesion. Among members of this family are cell adhesion molecules (CAMs), which form cell-cell contact points that play key roles in development, cell polarization, and cellular fate. Cadherins (CADs) are calcium-dependent proteins of the adherens junction (AJ), and polarize epithelium and endothelium. The tight junction (TJ) is a multiprotein junctional complex whose function is to control the permeability of the paracellular pathway. At the membrane level, TJs are composed of three types of proteins: claudins (CLDNs), occludin (OCLN) and junctional adhesion molecules (JAMs). JAMs are members of the IgSF while CLDN and OCLN are 4-α-helix membrane proteins. Although JAMs are part of the TJ and reside in the same ultrastructure, they are similar to CADs in their secondary, tertiary, and quaternary protein structure. Crystallographic studies of CADs in the presence of calcium yielded trans interactions that resulted in cell-cell contacts. In the absence of calcium, CADs form cis interactions that do not form cell-cell interactions. The crystal structure of JAM-A, has a quaternary organization of a cis dimer. In spite of the many similarities, a link between CADs and JAMs remains unclear. Beyond this point, the association between JAMs, CLDNs, and OCLN in the TJ is vaguely understood. The JAM family (JAM-A, -B, -C and 4) and their tissue-specific distribution indicate that they are key to understanding the TJ’s function and the interplay with the AJ. JAM-A has been used as a prototype for the other three members of the family, but based on current evidence we hypothesized that these proteins may display unique properties to support TJ’s function in a given tissue. Are JAMs affected by calcium just as CADs? Do CLDNs and OCLN make direct contact with JAMs? Do JAMs coordinate the interplay between TJ and AJ? We designed a strategy based on recombinant proteins and biophysical methods to answer these questions. First, we fused the extracellular domain of each JAM to maltose-binding protein (MBP). Our results indicate that JAM proteins have similar secondary structures, but unique tertiary structures. Surface Plasmon Resonance experiments showed that JAM proteins favored heterotypic compared to homotypic interactions. Second, we addressed the effects of cations (Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, and Zn^{2+}) on JAM-A. The exposure of JAM-A to the resulted in changes in its secondary, tertiary structure, and homotypic binding affinity. Finally, we addressed whether cations had an effect on the other TJ components and if there is an interplay with E-CAD. We determined that in the assembly of a simple TJ and AJ, JAM-A and E-CAD are calcium-dependent, while CLDN1 and OCLN are calcium independent. We conclude that TJ components such as CLDN1 and OCLN may work as anchors to maintain cell-cell interactions while JAM-A and E-CAD would be regulated by cations in order to accommodate other homeostatic functions.

Keywords: junctional adhesion molecule, claudin, occludin, e-cadherin, surface plasmon resonance
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## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF APPENDICES</td>
<td>xii</td>
</tr>
<tr>
<td>CHAPTER 1: Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Characterization of JAMs</td>
<td>5</td>
</tr>
<tr>
<td>The Effects of Cations on JAM-A Proteins</td>
<td>7</td>
</tr>
<tr>
<td>The Effects of Calcium on the TJ and AJ Components (Interplay)</td>
<td>8</td>
</tr>
<tr>
<td>References</td>
<td>10</td>
</tr>
<tr>
<td>CHAPTER 2: Molecular Characterization of JAM Proteins and their Interactions</td>
<td>13</td>
</tr>
<tr>
<td>Abstract</td>
<td>14</td>
</tr>
<tr>
<td>Introduction</td>
<td>15</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>16</td>
</tr>
<tr>
<td>JAM Expression and Purification in E. coli</td>
<td>17</td>
</tr>
<tr>
<td>Size Exclusion Chromatography</td>
<td>18</td>
</tr>
<tr>
<td>Determination of Conserved Structures by Circular Dichroism (CD)</td>
<td>19</td>
</tr>
<tr>
<td>Homotypic Interactions of JAMs</td>
<td>20</td>
</tr>
<tr>
<td>Heterotypic Interactions of JAMs</td>
<td>21</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>24</td>
</tr>
</tbody>
</table>
CHAPTER 5: Calcium Dependency of the Tight Junctions and the Interplay with Adherens Junction
Homotypic Interactions of TJ Components and AJ Measured by Surface Plasmon Resonance (SPR) .................................................................138

Heterotypic Interactions of TJ Components and AJ Proteins by SPR ..........139

Materials and Methods ........................................................................................................141

Protein Preparation: Cloning, Expression and Purification .................................141

Size-Exclusion Chromatography (SEC) .................................................................142

SDS-PAGE Assay ........................................................................................................143

Circular Dichroism Spectrometry .........................................................................143

Surface Plasmon Resonance ..................................................................................143

Conclusion .................................................................................................................144

References ..................................................................................................................152

CHAPTER 6: Conclusion ..............................................................................................158

Future Directions .......................................................................................................164

References ..................................................................................................................167

CURRICULUM VITAE ..................................................................................................168
LIST OF FIGURES

Figure 1.1: IgSF Protein Family and Structure of the Immunoglobulin (Ig) Domain ....................2

Figure 1.2: The Role of Tight Junctions and Their Protein Composition .................................3

Figure 1.3: The Conserved Ig-like Domain in the Extracellular Components of JAMs, and the cis Dimerization .................................................................4

Figure 2.1: Characterization of Junction Adhesion Molecule (JAM) Family of Proteins .........................................................30

Figure 2.2: Circular Dichroism Analysis of JAM Proteins and E-CAD ...................................31

Figure 2.3: Surface Plasmon Resonance Characterization of Homotypic Interactions of JAMs ........................................................................................................32

Figure 2.4: Surface Plasmon Resonance Characterization of Heterotypic Interactions of JAMs ........................................................................................................34

Figure 2.5: Ranked Strength of Homotypic and Heterotypic Interactions ................................35

Figure 2.A1: Conserved Structures of JAM Proteins ................................................................41

Figure 2.A2: pET28-MBP ...........................................................................................................43

Figure 2.A3: gBlock Sequences ................................................................................................47

Figure 2.A4: Growth Curve .......................................................................................................48

Figure 2.A5: Proteins Thermal Stability by Circular Dichroism ................................................51

Figure 3.1: Similarities Between JAM-A and E-CAD in a Calcium-free Environment ..........77

Figure 3.2: Prediction of Cation-Binding Sites in JAM-A (PDBID 1NBQ) ..............................78

Figure 3.3: The Effect of Examined Cations on MBP JAM-A Oligomerization ......................79

Figure 3.4: Circular Dichroism of MBP and MBP JAM-A Exposed to Different Cations .......................................................................................................................80

Figure 3.5: Cations Affect Homotypic Binding of MBP JAM-A .............................................81

Figure 3.6: Morphology Changes to HEK293 Cells Transfected with JAM-A(GFP) .............83
Figure 3.7: Graphical Ranking of Cations Affecting the Homotypic Binding Affinity (KD) of JAM-A .......................................................... 84

Figure 3.A1: pET28-MBP (Kanamycin resistance) Vector ...................................................... 97

Figure 3.A2: gBlock Sequence ............................................................................................. 98

Figure 3.A3: Primers for PCR Amplification ....................................................................... 98

Figure 3.A4: Prediction Cation-Binding Sites for Human JAM-A ........................................... 101

Figure 3.A5: Prediction Calcium-Binding Sites for Human E-CAD PDBID 2072 ................. 102

Figure 3.A6: Overexpression of E-CAD(GFP) in HEK 293 Cells ........................................... 102

Figure 4.1: Characterization of JAM-A, CLDN1, OCLN, and E-CAD ................................. 117

Figure 4.2: Determination of Secondary Structures of CC1, COC, JAM-A and E-CAD by Circular Dichroism (CD) ................................................................. 118

Figure 4.3: Surface Plasmon Resonance Characterization of Homotypic Interactions of MBP JAM-A, MBP CC1, MBP COC and MBP E-CAD ................................. 119

Figure 4.4: Surface Plasmon Resonance Characterization of Heterotypic Interactions of MBP JAM-A, MBP CC1, MBP COC, and MBP E-CAD ................................. 121

Figure 4.5: Conceptual Ranking of the Interactions of TJ and AJ Components ..................... 122

Figure 5.1: Characterization of MBP-ECAD, MBP-JAMA, MBP-CC1, and MBP-COC Proteins ........................................................................................................ 146

Figure 5.2: Circular Dichroism (CD) Analysis of MBP-ECAD, MBP-JAMA, MBP-CC1, and MBP-COC ........................................................................................................ 147

Figure 5.3: Surface Plasmon Resonance (SPR) Characterization of Homotypic Interactions of MBP-ECAD, MBP-JAMA, MBP-CC1, and MBP-COC .......................... 148

Figure 5.4: Surface Plasmon Resonance (SPR) Characterization of Heterotypic Interactions of MBP-ECAD, MBP-JAMA, MBP-CC1, and MBP-COC .......................... 149

Figure 5.5: Ranking of the Interactions in the Assembly of the AJC and Model of Calcium Dependency of the TJ and AJ Components .................................................. 151

Figure 6.1: Model of the Role Membrane Proteins of the TJs and AJs Play in the Regulation of Cell Adhesion ................................................................. 163
LIST OF TABLES

Table 2.1: Surface Plasmon Resonance Analysis .................................................................33
Table 2.A1: Primers for PCR Amplification ............................................................................52
Table 2.A2: Protein Yields ......................................................................................................52
Table 3.1: Surface Plasmon Resonance (SPR) Analysis of MBP JAM-A when Exposed to Different Cations .......................................................................................82
Table 4.1: Surface Plasmon Resonance Analysis ..................................................................120
Table 5.1: Analysis of the Protein-Protein Interactions was Performed by SPR ..................150
LIST OF APPENDICES

Appendix 2.A: Supplementary Information of the Molecular Characterization of the Extracellular Domain of Human Junctional Adhesion Proteins ........................................40

Appendix 3.A: Supplementary Information of Cations Affect the Folding and Binding of Junctional Adhesion Molecule-A .................................................................................94
CHAPTER 1: Introduction

Protein families are groups of homologous proteins, that is, they have similarities in amino acid sequences and three-dimensional structures. Protein superfamilies are larger groups of proteins that have evolved from a more distant ancestor. They generally have lower sequence homology as compared to a protein family but still have significant structural features in common. The immunoglobulin protein superfamily (IgSF) is one of the largest protein superfamilies, with over 700 cell surface and soluble proteins that are involved in the recognition, binding and adhesion of cells (Figure 1.1A.) [1,2]. All members of the superfamily contain one or more immunoglobulin (Ig) domains (Figure 1.1B) [3]. This domain has a unique three-dimensional structure composed of a sandwich of two anti-parallel β-sheets, and most are involved in cell adhesion or ligand binding. The IgSF contains many subfamilies including antigen receptors, cell adhesion molecules (CAMs), cytoskeletal proteins, growth factors and cytokine receptors [1,2].
Figure 1.1: IgSF Protein Family and Structure of the Immunoglobulin (Ig) Domain. A) Among the IgSF, the junctional adhesion molecules (JAMs) are a subfamily with four members that exist only in vertebrates. These proteins are primarily located in the tight junction (TJ), a proteic structure present in polarized epithelium and endothelium. Additionally, some members of this family are expressed in leukocytes, platelets, and glial cells. B) The IgSF family members have a conserved structure that is known as the Ig domain. In our study, we showed that JAMs have a conserved Ig-like domain [3].
Figure 1.2: The Role of Tight Junctions and Their Protein Composition. A) The role of tight junctions is to work as a fence or barrier to regulate the molecules crossing the cell-to-cell environment. These tight junction components are also important in sensing environmental cues, which results in a cellular response by signal transduction. B) Tight junction components are composed of claudin (CLDN), occludin (OCLN) and junctional adhesion molecules (JAMs). Intracellularly ZO-1, ZO-2, and ZO-3 with MUPP1, MAGIs and Cingulin are the components that allow for the stabilization of these tight junction components by binding to Actin [1].

TJ components are important because they act as gates and barriers that control the paracellular space, dictating what enters and exits the cellular environment [4,5]. These TJ components are responsible for the compartmentalization of the cellular environment as well as separation of tissues from one another (Figure 1.2 A) [6]. TJs are connected directly to the cellular cytoskeleton through intracellular soluble adapter proteins (Figure 1.2 B) [7]. Changes in the environment are sensed by the TJ and transmitted to the intracellular compartment.
Figure 1.3: The Conserved Ig-like Domain in the Extracellular Components of JAMs, and the *cis* Dimerization. A) The structure of JAM proteins share an Ig-like domain that would result in similar folding between JAM-A, JAM-B, JAM-C and JAM4 [6]. B) The crystal structure of JAM-A has been reported to form *cis* dimers, which would not allow cell-to-cell interactions [15].

The composition of membranal TJ components are claudin (CLDN), occludin (OCLN) and JAMs. Much is known about CLDNs (a family of 27 members in mammals) [8] and OCLN but little is known about the role that JAM proteins play in TJs [9-12]. Today most of the research performed regarding TJ’s function is in vitro in cells, with microscopy and fluorescent microscopy, but there are no biophysical studies concerned with protein binding. Much progress has been made by localizing proteins by tagging them with a fluorescent probes in order to understand their localization and potential protein partners [13]. However, these studies do not determine how these proteins might interact with one another and what other interacting partners they may have. Studies have determined the homotypic interaction of JAM proteins such as JAM-A, -B, -C and 4, but have not shown whether these proteins promote heterotypic interactions [14]. There is also a limitation on how these proteins might fold, or interact in a *cis*
or trans manner. As such, studies have shown that JAM-A forms a cis dimer [15] that does not make cell-cell contacts (Figure 1.3 B), and therefore would be against the function of the protein in the TJ. Thus, there must be a switch that would turn JAMs from cis to trans, allowing for cell-to-cell interaction to occur. Other unanswered questions remain such as whether JAM proteins like JAM-A are able to bind to other TJ components such as CLDN and OCLN, as well as whether TJ components bind to Adherens Junction (AJ) components such as epithelial cadherin (E-CAD).

To provide an understanding in the gap of knowledge, our research has primarily focused on the purification process, secondary structure, oligomerization, and protein-protein interactions of these TJ components. In this study we addressed the following three questions: 1. Do JAM proteins have homotypic and heterotypic interactions? To address this, we characterized the JAM proteins using recombinant proteins and biophysical methods. 2. Do cations affect the structure and binding of TJ components such as JAM-A? To answer this question we determined the effects of cations in JAM-A proteins. 3. Do cations such as calcium affect the binding of TJ components (CLDN1, OCLN and JAM-A), and the interplay with AJ (E-CAD)? We addressed this question by determining the effects of calcium on the TJ and AJ components. The following section helps us compile our findings to provide a model of the potential role of these TJ and AJ components.

Characterization of JAMs

One of the first major successes in this study in characterizing the role of JAM proteins was purifying the four members of the JAM family (JAM-A, -B, -C and 4) [16]. JAM-A has been previously purified and crystallized [15,17], but JAM-B, -C and 4 have never been purified. When JAM-A was purified and crystallized in previous studies, it was found to form dimers
With that the knowledge of this JAM-A dimer formation, most studies have been performed with the belief that the remaining JAM proteins (-B, -C and 4) form dimers as well [18]. We found that these proteins form different oligomerization states that are not dimers, they are tetramers and octamers [16]. We predicted this based on amino acid composition and hydrophobic plots [16].

JAM proteins are assumed to bind to one another in a homotypic form, meaning JAM-A would bind to JAM-A, JAM-B would bind to JAM-B and so on [15]. In this study, we found that this is not the case and that heterotypic interactions are more favorable than the homotypic interactions, as determined using Surface Plasmon Resonance (SPR) [16]. This suggests that depending on the type of cell and the types of JAM proteins expressed, there will be a vast combination of homotypic and heterotypic interactions. This will be based on the tissue-specific compartmentalization requirements to exclude molecules as in the case of the blood brain barrier (BBB) [19]. We would expect that environments that need to be less permeable, like the BBB, to produce more hydrophobic interaction with proteins such as JAM-C and JAM 4 either as homotypic, heterotypic or both [6]. Thus, depending on the environment, it is possible that more than one JAM family member would be expressed in cells to allow for both homotypic and heterotypic interactions to occur, further modulating the TJ according to homeostatic requirements. The key findings in this study are that these JAM proteins have more unique oligomerization than what was previously recognized. The conserved secondary structure was based on the fact that these proteins all have Ig-like domains that would allow them to promote cell-to-cell interactions. These structures also allowed for stronger heterotypic interactions compared to the homotypic interactions.
The Effects of Cations on JAM-A Proteins

In our previous study, we found that JAM-A formed a dimer when purified by Size-Exclusion Chromatography (SEC), however, this study was performed using PBS [16]. In the cellular environment cells are exposed to other cations such as Cu^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, and Fe^{3+}. An example of how cations affect protein structure and function has been seen in cadherins (CADs) [20]. Calcium is responsible for causing rigidization of CAD dimers resulting in the increase of cell-cell interactions [21]. Outside the TJ, JAM-A activation in platelets occurs by phosphorylation [22,23]. Other examples of the effect of cations on protein structure have been seen, such as the effect that Zn^{2+} has on N-CAD, in that it mediates cellular adhesion in the central nervous system [24] and accelerates the morphological changes in long term synaptic plasticity [25]. However, detailed understanding on what effect cations have on TJ components such as JAM-A is lacking. To address the effects that these cations have on the crystallized JAM-A protein we purified these proteins with Cu^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, and Fe^{3+}. We determined that these proteins formed oligomerization states other than dimers, as shown with PBS. Cations affected the secondary structure of JAM-A as determined by Circular Dichroism (CD). Additionally, cations affected the quaternary structure of JAM-A as determined by changes in oligomerization. The effect of cations on the secondary structure affected the homotypic binding affinity of JAM-A. Aggregation of HEK293 cells was observed when JAM-A was overexpressed. The increase in homotypic binding was determined to be stronger with Zn^{2+} compared to the other cations, as shown by SPR. This was validated to affect cellular aggregation with the overexpression of JAM-A and exposure to Zn^{2+} compared to Ca^{2+}, Mg^{2+}, and no cation. Together this means that the body regulates the binding of these proteins by using cations as molecular switches. Therefore, we would expect other TJ components to be affected...
by cations, to regulate the distance of the TJs and allow information to be moved in and out of a
cell.

The Effects of Calcium on the TJ and AJ Components (Interplay)

Cations regulate the secondary structure that affects the proteins’ function [26,27]. The
cellular environments can be constantly regulated to increase or decrease interactions between
TJs and AJs. This regulation is of importance in controlling signals that can regulate the cellular
environment and control cell-to-cell communication. In the case of the TJ and AJ components,
there is a misunderstanding of the uniform effect. The regulation of TJ components must be
maintained with certain proteins that would not be affected by cations to allow for the
maintenance of cell-to-cell interactions. Therefore, we need to understand which of the TJ
components work as anchors to maintain the cell-to-cell environment regardless of the changes
in cations that the rest of the proteins are exposed to. Other proteins must be able to react to
cations that affect the interactions between other TJ or AJs. However, there is no understanding
of which of these proteins could be the anchor, meaning that they are not affected by cations or
other proteins that are affected by cations. To address the question of the effects that cations have
on the overall TJ and AJ components we used PBS and calcium. Most of the studies in the
literature have been performed with calcium specifically for activating E-CAD (an AJ
component) [28]. We found that JAM-A and E-CAD were affected the most by calcium
compared to CLDN1 and OCLN. We also determined that these proteins have heterotypic
interactions such as JAM-A vs CLDN1, JAM-C vs OCLN, CLDN1 vs OCLN, JAM-A vs E-
CAD, CLDN1 vs E-CAD, and OCLN vs E-CAD. These heterotypic interactions are stronger
with PBS than with calcium suggesting that cations do affect the binding of these proteins. The
intracellular environment contains a lower concentration of calcium, thus our results with PBS
may reflect heterotypic interactions that occur while these proteins travel to the plasma membrane. We believe that there could be a regulatory signal that allows for opening the space and for transport to occur. One key finding was that calcium had a small effect on the homotypic interactions of CLDN1 vs CLDN1, and OCLN vs OCLN, and also had a small effect on the heterotypic interactions of CLDN1 vs OCLN suggesting that these proteins might be used as anchors to maintain the cell-to-cell interactions, while allowing for JAM-A and E-CAD to relax binding, perhaps affecting cellular signaling.

Our work has clarified details about TJ components and their interactions. We have advanced the knowledge in this field by providing a methodological approach to produce recombinant JAMs, demonstrating that JAM family components have both homotypic and heterotypic interactions. We have also determined that cations such as Cu$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, Fe$^{2+}$, and Fe$^{3+}$ affect the oligomerization, secondary structure, and homotypic binding of JAM-A. Our data provide evidence that cations such as calcium affect the homotypic and heterotypic interaction of TJ components such as JAM-A and AJ (E-CAD), but have little effect on CLDN1 and OCLN. This means that the body controls the interplay between the TJ and AJ components by using cations.
References


CHAPTER 2: Molecular Characterization of JAM Proteins and their Interactions

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Abstract

The junction adhesion molecule (JAM) family of proteins play central roles in the tight junction (TJ) structure and function. In contrast to claudins (CLDN) and occludin (OCLN), the other membrane proteins of the TJ, whose structure is that of a 4α-helix bundle, JAMs are members of the immunoglobulin superfamily. The JAM family is composed of four members: A, B, C and 4. The crystal structure of the extracellular domain of JAM-A continues to be used as a template to model the secondary and tertiary structure of the other members of the family. In this article, we have expressed the extracellular domains of JAMs fused with maltose-binding protein (MBP). This strategy enabled the work presented here, since JAM-B, JAM-C and JAM4 are more difficult targets due to their more hydrophobic nature. Our results indicate that each member of the JAM family has a unique tertiary structure in spite of having similar secondary structures. Surface plasmon resonance (SPR) revealed that heterotypic interactions among JAM family members can be greatly favored compared to homotypic interactions. We employ the well characterized epithelial cadherin (E-CAD) as a means to evaluate the adhesive properties of JAMs. We present strong evidence that suggests that homotypic or heterotypic interactions among JAMs are stronger than that of E-CADs.

Keywords: junctional adhesion molecule, tight junction, adherens junction, cadherin
Introduction

Tight junctions (TJs) are cell–cell promoting structures localized to the apical region of endothelial and epithelial cells. TJs function as barriers, controlling the paracellular space, and forming an apical/basolateral intramembrane diffusion barrier in the outer leaflet of the plasma membrane, referred to as the fence function [1,2]. Dysfunction of the TJ is relevant to edema, jaundice, diarrhea, inflammatory bowel disease, and metastasis among others conditions [3–6]. TJs are proteic structures represented by a complex mixture of three membrane proteins: claudins (CLDNs), occludin (OCLN), and junctional adhesion molecules (JAMs). Additionally, adapter and effector proteins anchor the TJ to the cytoskeleton, indicating its relevance in mechanotransduction [7,8]. The role of CLDN, compared to that of OCLN, appears to be crucial for the barrier function of TJs [7–10]. The role of JAMs in controlling permeability has been discussed in terms of its function as a gatekeeper to prevent ions or molecules such as water from crossing through the paracellular space [11]. JAMs are members of the immunoglobulin super family. Four members of the JAM family have been identified as members of TJs: JAM-A, JAM-B, JAM-C and JAM 4 [12–15]. JAMs are important in the control of vascular permeability and immune cells transmigration across endothelial–cell barriers by engaging in homotypic, heterotypic interactions [16]. JAM-A interactions assure strong cell–cell adhesion, playing important roles in proliferation and epithelial cell barrier functions [17]. Alterations to the barrier integrity caused by the disruption of JAM-A can indirectly modulate immune responses [18]. JAMs are expressed by a large variety of cell types and tissues, including epithelial and endothelial barriers, cells of the male reproductive system, and cells of the central and peripheral nervous systems [19]. Recent studies of JAM-A and JAM-C have expanded their roles to include tumorigenic functions, the inhibition of apoptosis and promoting proliferation, and epithelial-to-
mesenchymal transition [20,21]. JAM proteins’ relevance in cell and tissue physiology, and pathophysiology, may be obscured by the lack of characterization of their interactions. The purification of JAM proteins has been a bottleneck in their structural characterization due in part to their transmembrane domain. However, employing the extracellular domain of JAM-A, Prota et al., 2013 [22] successfully obtained a crystal structure. This study provided information of the quaternary structure of JAM-A, a homodimer, linked through its first extracellular immunoglobulin domain. However, there is a gap of understanding in the specific case of JAM-B, -C or 4. Additionally, heterotypic interactions of JAMs may play a relevant role in physiological events [19]. Nevertheless, there are no literature reports regarding these heterotypic interactions, remaining unclear if they are energetically favored in nature, and their possible role for cellular and tissue physiology. To address this gap in knowledge requires that we purify all JAM proteins while maintaining their native adhesive properties, in order to determine, through structural studies, their oligomerization state, as well as homotypic and heterotypic interactions. Here, we present such a study, where through maltose-binding protein (MBP) fusion strategies we have purified all extracellular domains of the TJ’s JAM proteins. This strategy enabled yields of sufficient quantities of material for protein–protein interactions studies, circular dichroism and surface plasmon resonance.

Results and Discussion

In the classical sense, cell adhesion is classified under several subcategories. Thus, cell–cell interactions and cell–basal membrane anchorage are examples of well recognized fields of study. Proteins responsible for cell–cell adhesion are membrane proteins. Most membrane proteins are naturally of low abundance and require a unique platform for expression and purification [23]. However, yields of proteins with a native-like structure and function following
overexpression in bacteria, yeast, insect cells or cell-free systems are often still inadequate. Protein engineering techniques, for example those employing fusion partners, are used to increase expression and stability [24]. In the case of adhesion molecules or other membrane proteins that have a single transmembrane helix, a reliable strategy is to study the intracellular or extracellular domains separately [25]. A classic example is that of the cadherin family of cell adhesion molecules [26]. Epithelial cadherin (E-CAD) has been studied by multiple biophysical methods [27]. E-CAD serves as an excellent standard for cell adhesion since its constant of affinity (KD) has been well established [28]. JAM proteins are also cell adhesion proteins, a subfamily of the immunoglobulin superfamily. JAM4 has only been reported recently [15]. JAMs are integral part of TJs. Their structural difference, when compared to CLDN or OCLN, both 4-α helix membrane proteins, is striking. How do these structurally different proteins interact with each other? How do they support the structure and function of the TJ? Our research strategy aims to elucidate the structural properties of JAMs that may qualify these proteins to be part of the only protein structure that controls the paracellular space [29].

**JAM Expression and Purification in E. coli**

Web-based amino acid sequence analysis (see Materials and Methods) was key in the design of a single expression strategy for all JAM proteins, later extended to E-CAD, for consistency. We confirmed that all the members of the JAM family in humans have two conserved immunoglobulin folds in the extracellular domain, in spite of the low amino acid sequence homology (less than 15%) (Figure 2.A1). In the JAM family, JAM-A was the most hydrophilic molecule while JAM4 was the most hydrophobic. JAM4 is also the protein that presents the most disordered regions, especially in the cytosolic region (Figure 2.A1). Protein modeling also revealed similarities in the extracellular domain of these proteins (Figure 2.A1).
Following the *in silico* evaluation of the extracellular domain of human JAMs, we examined the direct expression of all the targets. JAM-B and JAM-C were successfully expressed with low yields compared to the other proteins in this study (Table 2.A2). Other designs contained a TEV protease cleavage site between MBP and the JAM, but unfortunately resulted in mixed species and very low yields (data not shown). Our final strategy was the use of plasmid pET28-MBP, with MBP N-terminal to the gene of interest (Figure 2.A2). This was based on the merits described in the literature to drive high protein expression and stability of the fused target, enabling the targets to retain their individual structure and function [30,31]. The pET28-MBP was subcloned to contain the extracellular domains of JAM-A, JAM-B, JAM-C, JAM 4 and E-CAD (see Materials and Methods and Figures 2.A2 and 2.A3). To produce high yields of proteins and allow proper disulfide bond formation and cytosolic expression, we used the SHuffle T7 bacterial strain [32]. Cell growth was monitored for a 24-h period (Supplemental Information, Figure 2.A4). Plasmids hosting JAM targets hampered the growth of the cells at 37°C in LB containing both kanamycin (required by pET28-MBP) and spectinomycin (required by SHuffle cells) [32]. Bacterial growth was determined to have to reach an OD600 of 0.8–1 the before addition of 0.1 M of IPTG. Culture continued at 16°C overnight. Protein purification with amylose resin, followed by size exclusion, produced sufficient yields of >95% pure protein for structural studies (Figure 2.1.B).

**Size Exclusion Chromatography**

Size exclusion chromatography identified a unique feature among JAMs, their quaternary structure. In the case of E-CAD (data not shown) formed dimers as described in the literature[26], similar to the published oligomeric state of JAM-A[16]. This led to determination of whether the other JAMs (-B, -C and 4) had a similar oligomeric state. Here we report that for
the first time that this is not the case. JAM-C, JAM-B and JAM 4 form higher order of organization. Through Size Exclusion we determined that JAM-C forms tetramers, octamers in the case of JAM-B and decamers for JAM4 (Figure 2.1.D). Our research strategy did not identify the exact organization of these oligomers. Nevertheless, considering that the basic organization for adhesion molecules is *trans* interactions, we suggest that JAMs may form higher order of structure seeded on *trans* dimers[16,26].

**Determination of Conserved Secondary Structures by Circular Dichroism (CD)**

Based on the dimerization results obtained in size exclusion chromatography, we decided to determine whether the JAM proteins shared a conserved secondary structure. In Figure 2.2, we present two pieces of evidence that seem to indicate that beyond the *in silico* analysis and protein modeling, the extracellular domain of JAMs is composed of high β-sheet structures. The crystal structure of MBP [34], as well as CD [35] data, found in the literature, and performed at 21°C has been reported to have an α-helix content of 36%, and a β-sheet content of 17%. We used these values to compare our results. Our CD data indicates that under the conditions of our experiment (22°C), MBP has 38.1% of α-helix content and 19.1% of β-sheet content, consistent with the literature. In Figure 2.2.A, we plotted the CD data for all MBP-fused extracellular domains. This graph indicates that these proteins, with their immunoglobulin domain render similarities in the final fusion protein composed of MBP and JAMs. The CD values were compared according to the percentage of α-helix, and β-sheet. Figure 2.2.B shows that these fusion proteins had a higher β-sheet content compared to the unfused MBP at only 19.1%. MBPfused JAMs were examined and compared to MBP alone. JAM-A increased the β-sheet content of the fusion to over 50%, which could be the result of the increased thermal stability of JAM-A (Figure 2.A5). The least thermal stable JAMC (Figure 2.A5) influenced its fusion with
MBP to adopt to a greater coil–coil structure (Figure 2.2.B). On the other hand, the extracellular domain of JAM 4 greatly increased both the α-helix and β-sheet content of the fusion. This could be due to the hydrophobicity of JAM 4 (Figure 2.A1) or its quaternary organization (Figure 2.1.D) or a combination of both.

Based on both the size exclusion chromatography and CD, we asked whether based on the difference in aggregation and of secondary structures these proteins produced tighter binding in homotypic or heterotypic interactions. To address this question, we employed surface plasmon resonance (SPR). This technique is used to measure the binding of molecules in real-time without the need of labels [37,38]. Using this technique, we determined both the homotypic and heterotypic interactions of JAM-A, JAM-B and JAM-C that have been reported [19,38–40] and compared it to JAM4, which remains understudied.

**Homotypic Interactions of JAMs**

Vendome and colleagues [27], discussed the formation of homotypic interactions in the case of E-CAD. While exploring affinity constants obtained by multiple methods (SPR and analytical ultracentrifugation) the authors conclude that rather than obtaining absolute values, they observed that the data are in agreement of the behavior of E-CAD. Vendome and colleagues explain that each technique offers enough differences to produce unequal values even when measuring the same properties of the same protein [27]. Our research strategy circumvented this paradigm by measuring both E-CAD and JAMs’ protein–protein interactions using SPR, with E-CAD values serving as a standard parameter. Finally, rather than interpret the absolute value determined by SPR, we normalized the values presented (affinity constant, KD) to that of the better studied E-CAD. In addition to the comparison of protein–protein interactions, normalizing KD to that of E-CAD enables a simple estimation of the adhesion contributions of the AJ and the
TJ to the intercellular interactions. Figure 2.3 offers the normalized affinity values (KD) for the members of the JAM family (see also Table 2.1). Compared to E-CAD vs. E-CAD (in the presence of Ca$^{2+}$) all JAM proteins displayed a higher affinity (KD) for the homotypic interactions. JAM4 demonstrated over 1000-fold higher affinity than E-CAD. JAM-A, -B, and -C presented 5-, 25-, and 8-fold stronger affinity than E-CAD.

**Heterotypic Interactions of JAMs**

When cells expressing more than one JAM protein in the TJ establishes contact with a neighboring cell, a variety of heterotypic interactions between JAMs may occur [19]. If JAMs interact in a *cis* fashion, we would not see an interaction with SPR. However, if JAMs interact in a *trans* fashion, the proteins involved would interact with SPR. Based on our SPR data, we were able to determine that JAMs interact in a *trans* fashion. Sodium caprate is a detergent known for disrupting the TJ and increasing the paracellular permeability [41]. Our SPR experimental design included the use of caprate to eliminate the *trans* interactions (mimicking the effects of caprate *in vitro* and *in vivo*) [41]. Figure 2.4 offers the normalized affinity values (KD) for the members of the JAM family (see also Table 2.1). Each graph is normalized according to the homotypic interactions of the corresponding JAM. Figure 2.4.A suggests that heterotypic interactions between JAM-A and JAM-B or JAM4 may be favored over JAM-A homotypic interactions. This corresponds with reports where JAM-A and JAM-B interact during embryonic development [13]. In the case of JAMB, its preferred heterotypic interaction should be with JAM-C (Figure 2.4.B). JAM-B and JAM-C regulate the migration of cerebellar granule neurons during development of the cerebellum [42]. Even though they have not been reported to form heterotypic interactions *in vivo* or *in vitro*, our data suggest that these heterotypic species may play a key role in the brain. Both JAM-C and JAM4 experiments (Figure 2.4.C,D) show that the
recorded data suggest these interactions are highly favored. Unfortunately, the lack of research regarding JAM4 makes it difficult to further evaluate the observed results. Finally, JAM-A and JAM-C are expressed on the surface of platelets [43]. Their role in the coagulation cascade is unclear. Considering that platelets under homeostatic conditions do not aggregate, data collected here seem to be in agreement that JAM-A and JAM-C may not interact with each other if platelet aggregation was triggered. Their participation in the coagulation cascade might yet remain related to their adhesive properties but involving other proteins, for example, integrins [12].

Our study provides insight into the vast interactions of JAM proteins. It is not surprising that the JAM protein family performs homotypic and heterotypic interactions. The dimerization of JAM-A was validated in the crystal structure obtained by Prota [22]. Here, we determined that there are different oligomeric states formed by JAM-B, JAM-C and JAM 4. Specifically, we found that JAM 4, the most hydrophobic member of the family, forms a higher quaternary order, compared to the least hydrophobic JAM-A, that forms a dimer. These results suggest that there is a difference in binding between these proteins, and that promiscuous interactions among other members of the family may be equally relevant. We present evidence that the JAM proteins form homotypic and heterotypic interactions with members of the JAM family. Unlike previously published work, we have seen that the heterotypic interactions tend to have a lower KD value, suggesting that there is a tighter binding. Our data indicate that the formation of heterotypic interactions may be more favored when compared to homotypic interactions. The relevance of our findings could indicate that JAMs play a major role in controlling the paracellular space, and thus tissue barriers. JAMs may also play a key role in hemostasis. Interestingly, we confirmed through circular dichroism that these proteins share a similar secondary structure. Furthermore, this could be crucial for the function of these proteins in the tight junctions. The hydrophobic
profiles of JAMs demonstrated a striking difference among members; these can be a major
difference in oligomeric assembly in both homotypic and heterotypic interactions. Circular
dichroism revealed that all of these proteins lost their ability to retain secondary structure, and
did not fold at 50 or 75°C. This is due to the breakdown of hydrophobic and Van der Waals
interactions. This result indicates that these proteins would fold correctly at physiological
temperature, while at higher temperatures they would become dysfunctional. Finally, our work
might shed light on the fundamental question of why there are four different JAM proteins and
what their specific roles are in the formation of tight junctions in specific tissues. The tissue-
specific expression of JAMs is only partially known, but well established in other tissues. Our
evidence suggests a greater role of JAMs in permeability than previously suggested [40,44].

Based on our findings, heterotypic interactions could result in stronger intercellular interactions,
leading to further control of the TJ permeability, thus conforming to the tissue homeostatic
needs. We might imagine a scenario in which there needs to be an interaction of the most
hydrophobic JAMs, either homo- or heterotypic, to regulate permeability in tissues such as the
blood–brain barrier. Such a scenario can be responsible for the trafficking of ions, water and
other hydrophilic molecules through the tight junction barrier. Opposite to the blood–brain
barrier is the case of the kidney, where certain regions support the reabsorption of ions and
water. In this case, control of the tight junction’s permeability may rely on JAM expression of
the less hydrophobic JAMs, such as JAM-A. Future work would investigate these ideas, and
what is clear from our research is that there is a difference in how these JAMs oligomerize, and
that they form homotypic and heterotypic interactions. Understanding these differences may
result in unveiling the specific inner workings in tight junctions and its control of the paracellular
permeability. In Figure 2.5, we summarize the homotypic and heterotypic interactions of JAMs,
and rank them according to their strength, or in other words, the strong adhesive contributions to
the tight junction.

Materials and Methods

Materials

All cloning and PCR reagents were obtained from New England Biolabs (Ipswich, MA, USA. https://www.neb.com/ , accessed on 10 March 2021). Amylose resin was purchased from NEB and used according to manufacturer’s protocol. All chemicals were purchased from Sigma–Aldrich (St. Lois, MO, USA. https://www.sigmaaldrich.com/united-states.html, accessed on 10 March 2021). pET28a empty vector was obtained from Sigma–Aldrich, catalog number 69864. pMAL c2x plasmid (discontinued from New England Biolabs) was used to generate maltose binding protein (MBP) as a gene of interest to clone into pET28a between restriction sites NcoI and NdeI (Figure 2.A2).

Web-based Analysis Tools

Amino acid sequence alignment was performed using phylogeny, https://www.phylogeny.fr/ , accessed on 10 March 2021, which uses a MUSCLE amino acid sequence alignment. The generation of hydropathy plots was carried out using Expasy ProtScale: https://web.expasy.org/protscale/ , accessed on 10 March 2021. The order, and disorder plots were obtained using: http://www.pondr.com/ , accessed on 10 March 2021. Bestsel circular dichroism analysis was carried out using http://bestsel.elte.hu/index.php , accessed on 10 March 2021.
Protein Models

Models and molecular graphics images were produced using the UCSF Chimera v. 1.15 package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) [45].

Cloning, Expression, and Purification of Proteins

gBlocks for the extracellular domains of human proteins JAM-A (Gly27-Arg228, accession number Q9Y624), JAM-B (Gly28-Ser238, accession number P57087), JAM-C (Gly30- Glu236, accession number Q9BX67), JAM4 (Gly34-Arg286, accession number Q9NSI5), and E-CAD (Val102-Asp312, accession number P12830) were obtained from IDT DNA Technologies (Iowa City, IA, USA. https://www.idtdna.com/pages, accessed on 10 March 2021) (Figure 2.A3), codon optimized for E. coli K-12 (IDT DNA Technologies Codon Optimization Tool). The gBlocks were amplified with forward and reverse primers (Table 2.A1), followed by restriction enzyme digestion (XhoI and NdeI, New England Biolabs). Fragments were subcloned in pET28a-MBP plasmid, kanamycin resistant (Figure 2.A2). The final product produces an N-terminal MBP-fusion protein of the target with a C-terminal 6xHis tag. Cloning and subcloning transformations were performed in NEB 5-alpha (New England Biolabs). Plasmids for protein expression were transformed into SHuffle T7 Express (New England Biolabs), spectinomycin resistant. Protein expression and purification (amylose resin) were performed following manufacturer’s instructions. Eluate was concentrated by using Microsep Advance with 10k Omega centrifugal devices from Pall Corporation (Port Washington, NY, USA. https://www.pall.com/, accessed on 10 March 2021).
Size Exclusion Chromatography (SEC)

All size exclusion chromatography was performed using the NGC Chromatography System and its accompanying software (Hercules, CA, USA. https://www.bio-rad.com/, accessed on 10 March 2021). The SEC column used to purify proteins of interest was ENrich™ SEC 650 10 × 300 (BioRad, Hercules, CA, USA). Protein concentration was determined by using the Nanodrop Onec from Thermo Scientific. PBS was employed as a running buffer for SEC. Fractions were pooled and concentrated as mentioned above. Product peaks were compared for the position to the size exclusion standards from BioRad Catalog Number 151-1901.

Circular Dichroism (CD) Spectrometry

CD measurements were performed on the Spectrophotometer Model 420 AVIV (Biomedical Inc. Lakewood, NJ USA), calibrated with PBS. The far UV–CD spectra of 0.100 µM target protein was equilibrated with PBS (pH 7.4–8.0) and recorded in 100 mm QS glass cuvette cell. For the analysis of thermal stability and changes in the secondary structure 0.100 µM of protein sample was incubated at 4°C, 22°C, 37°C, 50°C, and 75°C, and monitored by measuring changes in ellipticity at 260 nm to 195 nm using 20 s scans. A secondary structure consisting of alpha helix or beta sheet percentages was performed by the usage of Bestsel circular dichroism analysis, http://bestsel.elte.hu/index.php, accessed on 10 March 2021. The experimental design for CD was performed with the MBP fusion protein since there was a low yield of protein if there was a TEV cleavage site or with other constructs.
**SDS-PAGE Assay**

Two µg of either boiled or not boiled MBP, E-CAD, JAM-A, JAM-B, JAM-C and JAM4 were electrophoresed on 8% SDS-PAGE gel (BioRad). Gel staining was performed using standard protocols [46].

**Surface Plasmon Resonance (SPR)**

SPR was performed using Open SPR by Nicoya Lifesciences (Kitchener, ON, Canada https://nicoyalife.com/, accessed on 10 March 2021. We assayed protein-protein interactions by loading 0.100 mg of each protein as ligand into the Carboxy sensor chip (Nicoya Lifesciences). The proteins are immobilized in the Carboxy sensor chip through the exposed primary amine groups that are both found on the lysine residues and the N-terminus of the amino acid residues. As a result, the amines can form a covalent bond with the carboxyl surface after it is activated by EDC/NHS [47]. Following the blocking step (manufacturer’s buffer) 200 µL of 1 M sodium caprate was administered to disrupt the preformed protein-protein interactions. All proteins analyzed formed at least dimers; these species needed to be disrupted in order to determine new protein-protein interactions kinetics. Triplicate injections of the analyte protein in concentrations of 12.5 µg, 25 µg, 50 µg and 100 µg per 200 µL injections. Caprate injections were performed after each analyte interaction was concluded. The close curve fitting to the sensograms were calculated by global fitting curves (1:1 Langmuir binding model). The data was retrieved and analyzed with TraceDraw software (Kitchener, ON, Canada).

**Surface Plasmon Resonance Statistics**

SPR for each run was performed 3 times per sample, and analyzed by using the TraceDraw software (Kitchener, ON, Canada) according to the suggestions by Nicoya.
(Kitchener, ON, Canada). The data in Figure 2.3 were normalized by using the KD values of each combination run (samples) by dividing each by the E-CAD vs. E-CAD KD value. For Figure 2.4, we normalized the heterotypic KD values with that of the homotypic interaction for each JAM protein. Thus, all heterotypic interactions of JAM-A were normalized to the KD value of the homotypic JAM-A interaction. We performed a similar analysis with JAM-B, JAM-C and JAM4.

Conclusion

JAMs are an integral component in the formation of TJs, but very little is known about their specific role in those TJs. In this study, we determined that it is possible for these JAM proteins to have homotypic and heterotypic interactions. Our contributions expand the understanding that each member of the JAM family may have a different quaternary organization, beyond what was previously reported for JAM-A. Here, JAM-B, -C, and 4, form tetramers and multimers. Additionally, these JAM proteins have similar secondary structures that could represent the basis of similar function. Based on these results, we propose a model where it could be possible for these proteins to interact in combinations of JAMs based on the tissue specificity and tissue environment. All authors have read and agreed to the published version of the manuscript.
Author Contributions

C.M.: experimental design, experiment performance, data analysis, manuscript writing. This author contributed to every aspect of the research and manuscript preparation. Conceptualization, methodology, \textit{in silico} analysis, formal analysis, data curation and original draft preparation with its accompanying review and editing. This author mentored and supervised S.H.N. experimental performance of surface plasmon resonance (SPR), data analysis. Aided in sample preparation. D.M.: experimental design, data analysis, manuscript writing. This author also contributed to manuscript preparation. Conceptualization, methodology, formal analysis, data curation and original draft Preparation with its accompanying review and editing. This author mentored all other co-authors. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.
Figure 2.1: Characterization of Junction Adhesion Molecule (JAM) Family of Proteins. A) Extracellular domains of human epithelial cadherin (E-CAD) or JAM proteins (Figure 2.A3) were cloned C-terminal to maltose binding protein (MBP) in pET28a backbone plasmid, the red line indicates a 6xHIS tag C-terminal to the target protein. B) Proteins are purified with amylose resin and size exclusion chromatography. Proteins are purified to >95% purity, Coomassie blue stain gel. C) Additional characterization was performed through in silico protein models (see Materials and Methods) of JAMs, the crystal structure of JAM-A (PDB ID: 1F97) is next to the models for comparison. D) Size exclusion profiles are overlapped to show how each JAM protein forms unique quaternary structures.
Figure 2.2: Circular Dichroism Analysis of JAM Proteins and E-CAD. A) Circular dichroism analysis, comparison of all MBP fusion, extracellular domain of E-CAD or JAM proteins. Folding similarities are observed for the behavior of the fusion proteins. B) The analysis of each curve (Materials and Methods) for the target proteins, including non-fused MBP, is presented in a table that describes the distribution of secondary structure. The content of alpha helix, beta sheet or other (coiled) is presented. Our non-fused MBP protein displayed a similar distribution of the secondary structure as previously reported in the literature [35,36].
Figure 2.3: Surface Plasmon Resonance Characterization of Homotypic Interactions of JAMs. Homotypic interactions of JAMs were determined by surface plasmon resonance (SPR) (see Materials and Methods). The homotypic interaction of E-CAD was also determined. Considering the large amount of evidence for E-CAD [27] we normalized the affinity (KD) by that of E-CAD. Thus, the Y-axis represents the normalized affinity, JAM/E-CAD as a ration. The X-axis describes the homotypic interactions tested. These values are based on taking the KD values from each sample and dividing it by the KD value of E-CAD vs. E-CAD shown on Table 2.1.
Table 2.1: Surface Plasmon Resonance Analysis. Protein–protein interactions were analyzed using SPR (see Materials and Methods). The data were analyzed with TraceDraw software. Here, we present values for constant of association, $K_a$ ($1/(M^*s)$); constant of dissociation, $K_d$ (1/s); and constant of affinity, $K_D$ (M). Standard deviations are reported. * E-CAD experiments conducted in the presence of 3 mM CaCl₂.

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Figure 2.4: Surface Plasmon Resonance Characterization of Heterotypic Interactions of JAMs. Heterotypic interactions of JAMs were determined by SPR (see Materials and Methods and Table 2.1). We studied the affinity of each JAM protein for all other members of the family. For each JAM analyzed, we normalized affinity (KD) by that of the homotypic interaction of said JAM. The Y-axis represents the normalized affinity, heterotypic JAM/homotypic JAM, as a ration. The X-axis describes the homotypic interactions tested. (A) Heterotypic interactions of JAM-A, normalized to KD of JAM-A vs. JAM-A. (B) Heterotypic interactions of JAM-B, normalized to KD of JAM-B vs. JAM-B. (C) Heterotypic interactions of JAM-C, normalized to KD of JAM-C vs. JAM-C. (D) Heterotypic interactions of JAM4, normalized to KD of JAM4 vs. JAM4.
Figure 2.5: Ranked Strength of Homotypic and Heterotypic Interactions. In this figure, we summarize the major findings of our research. Graphically, we display the homo- and heterotypic interactions of JAMs, and the homotypic interactions of E-CAD. The ranking of these interactions is in order of strength.
References


Appendix 2.A: Supplementary Information of the Molecular Characterization of the Extracellular Domain of Human Junctional Adhesion Proteins
(Kanamycin resistance)

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**Number of amino acids:** 374. **Molecular weight:** 40992.61

Sites for NdeI (HM amino acids) and XhoI (LE amino acids) are present in the plasmid.

**Example pET28-MBP-JAM-A-6xHIS**

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430 440 450 460 470 480
TTRLVCYNNK ITASYEDRTV FLPTGITFKS VTREDTGTYT CMVSEGGNS YGEVKVKLIV

490 500 510 520 530 540
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Number of amino acids: 588. Molecular weight: 64250.35

Figure 2.A2: pET28-MBP

(IDT DNA Technologies, Codon Optimized for E. coli K-12)

- JAM-A
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- JAM-C
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45
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- JAM4
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- E-CAD
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CACTACGGCGACAGCCGTGATTACGGGTGACCGACGctcgagCCACCGCTGAGCAATAAC
TA

Figure 2.A3: gBlock Sequences.
Figure 2.A4: Growth Curve. Cells are grown in LB from a 1:1000 dilution of overnight culture of SHuffle cells. Cell growth is monitored (OD$_{600}$) every hour for 20 hours.
Determining Structural Changes by Temperature of MBP

![Graph showing ellipticity vs. wavelength for different temperatures of MBP.]

Determining Structural Changes by Temperature of CAD

![Graph showing ellipticity vs. wavelength for different temperatures of CAD.]

49
Determining Structural Changes by Temperature of JAM-A

Determine Structural Changes by Temperature of JAM-B
Figure 2.A5: Proteins Thermal Stability by Circular Dichroism
Table 2.A1: Primers for PCR Amplification

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Table 2.A2: Protein Yields

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CHAPTER 3: Cations as Molecular Switches for Junctional Adhesion Molecule-A

Christopher Mendoza¹, Sai Harsha Nagidi², Keegan Peterson¹ and Dario Mizrachi¹*

¹Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, Utah.

²Department of Molecular Microbiology, College of Life Sciences, Brigham Young University, Provo, Utah.

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Abstract

Junctional adhesion molecules (JAMs) are key structural and functional cell adhesion components of tight junctions (TJs), that are also expressed in platelets, leukocytes, and progenitor cells. An interplay between TJs and adherens junctions (AJ) has been reported. Cadherins (CADs), components of the AJ, are calcium-dependent adhesion molecules while the TJ as a cell adhesion unit is considered calcium-independent. Structurally speaking, CADs and JAMs are members of the Immunoglobulin superfamily (IgSF). Knockdown of epithelial CAD (E-CAD) or JAM-A results in increased cell proliferation, which is the opposite of that observed in other TJ membrane proteins such as claudins (CLDNs) or occludin (OCLN). The crystal structure of JAM-A yielded a U-shape cis-dimer, stabilized by extensive ionic and hydrophobic contacts between two N-terminal domains. Calcium-free CADs form cis-dimers. There is not a clear understanding of how or the extent to which the ionic microenvironment regulates JAM interactions and enables cell-cell interactions. We hypothesized that cations may play that role, the way that calcium-bound CADs form trans-interactions effectively resulting in cell-cell adhesion. A cation-binding site algorithm was used to predict JAM-A binding regions and identified calcium, copper, iron (II), iron (III), magnesium, and zinc as possible molecular switches. In this article, we use recombinant protein and biophysical methods to study the effects of these cations on JAM-A. We present evidence suggesting these cations play key roles in regulating JAM-A secondary, tertiary, and quaternary structure. Additionally, the effects of cations result in changes to the homotypic affinity of JAM-A.

Keywords: junctional adhesion molecule, surface plasmon resonance, circular dichroism, cadherin
Introduction

Tight junctions (TJs) are cell-cell promoting structures localized to the apical region of endothelial and epithelial cells. TJs function as barriers, control the paracellular space, and form an apical intramembrane diffusion barrier in the outer leaflet of the plasma membrane, referred to as a fence function[1,2]. TJs are proteic structures represented by a complex mixture of three membrane proteins: claudins (CLDNs), occludin (OCLN), and junctional adhesion molecules (JAMs). The CLDN family of membrane proteins play central roles in TJ structure and function[3,4]. However, recent literature revealed claudin-independent aspects of the TJ’s function related to JAMs and the TJ’s microenvironment[4]. Additionally, adapter and effector proteins anchor the TJ to the cytoskeleton, indicating its relevance in mechanotransduction[5,6]. The role of CLDNs compared to that of OCLN appears to be crucial for the barrier function of TJs[5-7]. The role of JAMs in controlling permeability has been discussed in terms of its function as a gatekeeper to prevent ion and molecules such as water from crossing through the paracellular space[4]. proteins can work as signaling components that are influenced by phosphate and perhaps other ions.

JAMs comprise a small subfamily of the immunoglobulin superfamily of adhesion receptors with a multitude of physiological functions in vertebrate development and homeostasis[8]. The four members of the JAM subfamily (JAM-A, -B, -C and 4) localize to the TJ in a tissue specific manner[9]. Dysfunction of the TJ is relevant to edema, jaundice, diarrhea, inflammatory bowel disease, and metastasis, among other conditions[10]. Moreover, the first indications for a role of JAM-A in the formation of the epithelial barrier arose from observations in patients suffering from Crohn's disease (CD) or ulcerative colitis (UC), the two major forms of inflammatory bowel disease (IBD)[11]. Some proteins comprising TJs work as receptors for
viruses and extracellular stimuli, pathogenic bacteria and viruses targeting TJ functions[10]; JAM-A in particular engages all reovirus serotypes[12,13].

In the case of platelets, signals such as JAM-A phosphorylation are transmitted during platelet activation[14]. JAM-A acts in a protein kinase dependent manner after phosphorylation [15,16]. Studies have shown that loss of JAM-A resulted in a prothrombotic phenotype, and in murine platelets lacking JAM-A there was an impairment of outside signaling[17]. Thus, phosphorylation of JAM-A results in a conformational change leading to proper signaling[14]. This suggests that TJ proteins can work as signaling components that are influenced by phosphate and perhaps other ions.

Purification of JAMs has been challenging. Purification of JAM-A was successfully performed in the study by Prota et al. [18], which determined that JAM-A forms cis-dimers in a crystallographic experiment. However, in this crystalized form of JAM-A it is unclear how changes in secondary structure could affect the protein’s function[18]. Prota et al. [18] attributed the dimer formation to electrostatic and hydrophobic forces, but do not suggest how these can be influenced to alter oligomeric states or adhesive properties[19]. For example, it is not clear how JAM-A’s dimer contributes to the formation of TJ in endothelial or epithelial cells[11,18]. Charged and polar groups are responsible for protein properties[20,21]. Modulation of the charges on the amino acids, by pH or by phosphorylation and dephosphorylation or by altering the concentration of cations in the microenvironment, have significant effects such as protein structural changes and switch-like responses leading to protein function[22-24]. An example of how cations affect protein structure and function is that of Ca\(^{2+}\), which is important for the cell-adhesion in cadherins (CAD)[25-27]. Calcium causes the rigidization[28] of the CAD dimers, resulting in the increase of cell-cell interactions[29]. Calcium levels in plasma can range from
1.8-2.7 mM [30]. In contrast, intracellular levels of Ca^{2+} range from 0.3 to 1 mM[31]. In structural studies using epithelial CAD (E-CAD), the low Ca^{2+} concentrations (<1 mM) caused the protein to form cis-dimers[27,32]. In the case of high Ca^{2+} concentration (>1 mM), E-CAD formed trans-dimers[27]. These experiments seem to indicate CADs may form cis-dimers intracellularly but switch to trans-dimers once exposed to the higher levels of Ca^{2+} in the extracellular microenvironment, resulting in cell-cell interactions. Another example of cations’ effect on protein structure was seen in the effect that Zn^{2+} has on neural cadherin (N-CAD) by mediating cell adhesion in the central nervous system[33]. In synaptic studies it was found that released Zn^{2+} might have a strong accelerating effect on morphological changes that are involved in long term synaptic plasticity[34]. The synaptic vesicles were found to have a 1 mM concentration of Zn^{2+}[34]. The presence of JAM-A on the surface of platelets has been described as well as the importance of JAM-A phosphorylation during platelet aggregation[15-17]. The role of cations such as Ca^{2+} and Zn^{2+} are well characterized during platelet activation[35-37]. There is no evidence in the literature of the role of cations as structural switches on JAM-A leading to its cell-adhesion function. Nevertheless, the literature recognizes that both JAMs and CADs have similar folding of their extracellular domains, that of immunoglobulin-like domains[38,39].

Furthermore, an interplay between the TJ and Adherens Junction (AJ), formed by CADs has been described[40]. A plausible question is the role of Ca^{2+} in that interaction. If CADs are Ca^{2+}-dependent cell adhesion molecules[41], would their counterparts in the TJ, with similar protein folding, also be Ca^{2+}-dependent or at least Ca^{2+}-sensitive? Taken together there is still no clear understanding of the role of Ca^{2+} or Zn^{2+}, or other physiologically relevant cations, on proteins such as JAM-A.
Additionally, Cu\(^{2+}\) concentrations in the brain range from 0.1 to 0.5 mM\[42\]. Copper toxicity can result from chronic or long-term exposure to high levels of Cu\(^{2+}\) through contaminated food and water sources. Copper intoxication in Wistar rats is observed when concentrations reach 1 to 2 mM\[42\]. The average intracellular iron concentration is 1.3 mM for astrocytes, 1.8 mM for microglia and 3 mM for oligodendrocytes\[43\]. Iron toxicity is observed in the aging brain and in cases of Alzheimer’s disease\[44,45\], in part due to brain cell necrosis\[46\].

Addressing this gap in understanding of the role that cations have on proteins such as JAM-A requires that we purify JAM-A and expose it to different cations (zinc, calcium, magnesium, copper, phosphate or iron) monitor oligomerization and secondary structure, and perform protein-protein affinity experiments. In our previous work\[47\], we have shown that JAMs purified in PBS buffer combine with different binding affinities for homotypic and heterotypic interactions. In this study, we used recombinantly purified JAM-A protein. Exposing purified JAM-A to different cations enabled us to better understand its protein folding, binding, and oligomerization properties.

In this study we address the following questions: First, do cations affect the oligomerization of JAM-A? To address this question, we purified JAM-A in the presence of different cations using Size-Exclusion Chromatography. Second, do cations have an effect on the conformational change of JAM-A? To answer this question, we used the product from the Size-Exclusion Chromatography to perform Circular Dichroism. Third, do the cations increase the binding affinity of homotypic JAM-A interactions? To answer this question, we performed Surface Plasmon Resonance. We found that cations had an effect on the oligomerization,
secondary structure, and binding affinity of JAM-A. Which also showed which cations had the greatest effect on folding, binding and protein oligomerization.

Material and Methods

**Study System**

We purified JAM-A protein in its most native state by tagging it with Maltose Binding Protein (MBP)\[47\]. We then determined whether this protein had cation-binding sites. To do this we used JAM-A’s (PDB ID: 1NBQ) crystal structure to predict the cation-binding sites using an online docking tool. Next, we purified the protein by column chromatography, followed by size-exclusion chromatography that used a 2 mM solution of each cation. This allowed us to determine whether there would be changes to the oligomeric state of MBP JAM-A. Then we used Circular Dichroism to determine whether exposure to different cations causes a change in secondary structure. To determine whether the structural changes resulted in changes in binding affinity, we performed Surface Plasmon Resonance analysis. To study the effects of overexpressing JAM-A in HEK 293 cells we prepared pcDNA3.1 JAM-A(GFP) and pcDNA3.1 E-CAD(GFP) (Figure 3.A1).

**Protein Preparation by Cloning, Expression and Purification of MBP JAM-A**

In order to clone, express, and purify the JAM-A protein, we used a synthetic DNA gBlock obtained from IDT (Integrated DNA Technologies, Iowa City, IA, US). The gBlock was amplified with forward and reverse primers using PCR (Figure 3.A2 and 3.A3). The amplified PCR product was incubated with restriction enzymes (XhoI and NdeI), and incubated at 37°C for 8 hours. The restriction enzymes were inactivated by incubation at 65°C for 20 minutes. The PCR product was cloned into pET28a vector at the XhoI and NdeI sites by ligation. Maltose
binding protein (MBP) was cloned N-terminal of JAM-A using NcoI and NdeI sites on plasmid pET28a (Millipore Sigma, Burlington, MA, US). An amino acid sequence of these plasmids is provided in Figure 3.A1.

To express the plasmid and produce enough copies for purification, we transformed the plasmid into the bacterial strain DH5α. Purification of the plasmid was performed by using Zyppy Plasmid Miniprep Kit from Zymo Research. Sanger sequencing was performed by Genewiz (New Jersey) to determine whether the plasmid cloning of JAM-A was correct and that no mutations were present. After verification of the plasmid sequence, we transformed the plasmid into SHuffle bacterial cells[48,49] that would express MBP JAM-A. We followed our previously described protocol for protein expression of MBP JAM-A[47]. Then we lysed the bacterial cells by addition of 100 µL of 0.5 M EDTA in lysis buffer (500 mM NaCl, 30 mM Tris pH 7.4) followed by French Press. Resuspended bacteria were loaded onto the Thermo Spectronic French Pressure Cell Press Model FA-078. Lysis was performed at 1500-2000 psi and the lysate was collected in a new 50 mL tube. The lysate was centrifuged for 30 minutes at 10,000 RPM using a F15-8x50cy rotor from Thermo Scientific. The supernatant was decanted into a 50 mL Eppendorf tube containing Amylose resin from New England Bio Labs (E8021L) and incubated while rotating for 1 hour at 4°C. Column chromatography was performed to collect the protein from the amylose beads. Then the column was washed with 100 column volumes of wash buffer containing 500 mM NaCl and 30 mM Tris pH 7.4. The elution was incubated for 3 minutes each time. The eluate was concentrated by using the Microsep Advance with 10k Omega centrifugal devices (Reference # MCP010C41) from Pall Corporation and centrifuged at 10,000 RPM for 10 minutes until reaching 2 mL of final elution volume.
**Size-Exclusion Chromatography (SEC)**

Determination of the shift of oligomerization was performed using 0.250 mg of protein in one of the solutions of 30 mM HEPES, 100 mM NaCl with 2 mM of each cation (calcium, copper, magnesium, zinc, phosphate, iron II, or iron III) as chloride salt. The protein was incubated in the buffer with the cation of interest for 2 hours. The sample was injected into the injection valve of the NGC Chromatography System (Bio-Rad) with a syringe. The elution peaks and change in the area under the curve was calculated with SEC ChromLab 4.0 software (Bio-Rad).

**Circular Dichroism Spectrometry**

Circular dichroism (CD) measurements were performed on Spectropolarimeter Model 420 (AVIV Biomedical Inc., Lakewood, NJ USA). Changes in ellipticity were performed from 250 nm to 190 nm using 20 second scans at a concentration of 100 µM protein in a 10 mm QS glass cuvette at 22°C. The secondary structure which consisted of alpha helix, antiparallel, parallel, turn, or other was determined by using the online Bestel circular dichroism analysis software: [https://bestsel.elte.hu/index.php](https://bestsel.elte.hu/index.php).

**SDS-PAGE Assay**

Protein samples were prepared as follows: 1 µg of either boiled or unboiled MBP, JAM-A with the various cations were electrophoresed on 8% SDS-PAGE gel with loading dye containing 1% SDS, 0.125 M Tris (pH 6.8), and 40% glycerol. Protein bands were visualized after staining with Coomassie Brilliant Blue for 2 hours. The Coomassie Brilliant Blue was distained with distain buffer (40% MeOH, 10% Acetic Acid, 50% H2O) for 2 hours.
Native Gel Assay

Native gel consisted of 15% resolving gel recipe containing 2.5 mL ultra-pure H2O, 5 mL 30% polyacrylamide/Bis Solution (29:1), 2.5 mL 1.5 M Tris HCl pH 8.8, 5 µL TEMED and 50 µL 10% APS (Ammonium Persulfate) added as the last step. The 5% stacking gel consisted of the following: 2.3 mL ultra-pure H2O, 62 µL 30% Acrylamide/Bis Solution (29:1), 1 mL 0.5 M Tris HCl pH 6.8, 5 µL TEMED, and 30 µL 10% APS (added last to allow for gel solidification to occur). The difference between this gel and the SDS gel was that the native gel did not contain SDS, which allowed for the protein to preserve different oligomeric states. Protein was loaded 1 µg per well and ran at 100 volts for 110 minutes. Protein bands were visualized after staining with Coomassie Brilliant Blue for 2 hours. The Coomassie Brilliant Blue was distained with distain buffer (40% MeOH, 10% Acetic Acid, 50% H2O) for 2 hours.

Surface Plasmon Resonance (SPR)

We employed Open SPR by Nicoya Lifesciences to assay protein-protein associations of JAM-A with the incubated cations. SPR is an optical effect that can be utilized to measure the binding of molecules in real time without the use of labels. SPR instruments are primarily used to measure the binding kinetics and affinity of molecular interactions. SPR can be used to measure interactions such as the binding between two proteins, a protein and an antibody, DNA and a protein, and many other molecules. SPR can be thought of as the following equation:

\[
\text{Analyte} + \text{Ligand} \rightleftharpoons \text{Analyte-Ligand (Complex)}.
\]

This equilibrium equation shows that not all of the ligands will be bound to the protein. When the ligand is bound to the protein forming the complex this is considered to be \(K_{\text{on}}\) (M\(^{-1}\)s\(^{-1}\)) or the speed of association. \(K_{\text{off}}\) is the speed of dissociation (s\(^{-1}\)). A final calculation enables the experiment to reveal the Analyte-Ligand affinity:

\[
K_D = \frac{K_{\text{off}}}{K_{\text{on}}} = \frac{[\text{Analyte}]x [\text{Ligand}]}{[\text{Analyte-Ligand}\text{Complex}}. \]

The \(K_D\) is the
dissociation constant where half the ligand binding sites of the protein are bound to the ligand and half of the ligand is not bound to the protein at equilibrium. Thus, a smaller $K_D$ value means that the analyte and the ligand have higher binding affinity for one another (Nicoya Lifesciences, users manual). In our experiments, the ligand and the analyte is MBP JAM-A. To determine the binding affinity, we used 0.050 mg of each protein as a ligand into the Sensor Carboxy Chip, for coupling to any amine group on the ligand (Nicoya Lifesciences). The proteins were immobilized in the Carboxy Sensor Chip through the exposed primary amine groups that are found in the lysine residues and at the N-terminus. These primary amines form covalent bonds with the carboxyl surface after it is activated by the EDC/NHS (Nicoya Life Sciences)[69]. The blocking step (manufacturer’s buffer) followed by 200 µL of 1 M sodium caprate was administered to disrupt the preformed protein-protein interactions[50]. Triplicate injections of the analyte protein were made in the following concentrations: 12.5 µg, 25 µg, 50 µg and 100 µg per 200 µL injection. Caprate injections were performed after each analyte interaction to be sure that there were no other interactions occurring before the next analyte injection was performed.

After conducting the experiments, the close curve fitting to the sensograms was calculated using global fitting curves using the 1:1 Langmuir binding model. The data was retrieved and analyzed with TraceDrawer software (Kitchener, ON, Canada). SPR for each sample was performed in triplicate and analyzed using the TraceDrawer software (Kitchener, ON, Canada) according to the recommendations by Nicoya (Kitchener, ON, Canada).

**Tissue Culture**

HEK 293 cells (ATCC catalog# CRL-1573) were cultured following standard procedures in RPMI media with 10% FBS. CAL 27 cells (ATCC catalog# CRL-2095) were cultured using
DMEM-F12 media with 10% FBS. We followed ATCC culture guides
(https://www.atcc.org/resources/culture-guides).

Confocal Microscopy

A confocal microscope (Olympus FluoView FV1000) was used to observe the
transfected HEK 293 cells. The microscope is equipped with an argon laser with excitation light
at wavelengths of 405nm, 458 nm, 488 nm, and 515 nm. In addition, it provides green Helium-
Neon and red Helium-Neon laser sources with respective excitation wavelengths of 543 nm and
633 nm. The cells were observed and imaged at 20x magnification using standard procedure. The
Olympus FluoView FV1000 software was used to obtain images of the samples.

ATP Proliferation Assay

Using Cal 27 cells we performed proliferation assays using ATPlite Luminescence Assay
System (PerkinElmer, Akron, Ohio) following the manufacturer’s instructions.

DNA and siRNA Transfections

HEK 293 cells were transfected with plasmids following standard procedures[51] in 6-
well plates with 2 ug of DNA per well using jetPRIME transfection reagent (Polyplus, New
York), according to manufacturer’s instructions. CAL 27 cells (2×10⁵) were seeded in 12-well
plates and transfected after 24 h with 30 nM siRNA duplexes using JetPrime transfection
reagent, according to the manufacturer's instructions. siRNA duplex oligonucleotides were
synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa), control siRNA (IDT
cat#51-01-14-03); against human E-CAD[52], against human JAM-A[53].
Statistical Analysis

Student’s t-tests were applied for comparisons. Data are expressed as mean±SD. The significance threshold was 5% (*P<0.05). All experiments were repeated four times.

Results

Proteins are organized in families. The categories depend on primary amino acid sequence homology, conserved residues, domain folding, and other tertiary and quaternary structural elements. The biggest protein family is that of the Immunoglobulin Superfamily (IgSF) with close to 750 members[54,55]. Among the functions observed for the different subfamilies of the IgSF we find pattern recognition and cell-adhesion molecules[56,57]. CADs are calcium-dependent cell-adhesion molecules that form the membranal structure of the AJ. Calcium-free CADs form cis-dimers and do not result in cell-cell adhesion. JAM-A is a protein from the IgSF that also has cell-adhesion properties. JAM-A’s crystal structure[18,58], was obtained in the absence of additional cations like calcium. This calcium-free structure results in a U-shape cis dimer. We proceeded to design a few simple experiments to characterize similar structure and function between E-CAD and JAM-A.

Figure 3.1 indicates important similarities between E-CAD and JAM-A in a calcium-free environment. Both proteins are members of the IgSF. E-CAD and JAM-A form cis-dimers in the absence of calcium. Both E-CAD and JAM-A use EC1 for binding and glycosylation occurs in domains away from the binding site. Figure 3.1.C reveals that upon transfection of E-CAD or JAM-A, as GFP fusion proteins, resulted in a rounding phenotype in HEK 293 cells, which is a feature described in the literature for overexpressing cells that affect morphology[59,60]. A final level of similarity is revealed by the ATP proliferation assay (Figure 3.1.D). CAL 27 cells (epithelial tongue Squamous Cell Carcinoma) contain a very simple TJ, composed of JAM-A,
CLDN1, and OCLN. Additionally, as an epithelial cell-derived product CAL 27 cells express E-CAD. In the literature, the knockdown of E-CAD in human ovarian cancer cells is related to increased proliferation[52].

In our experiments with CAL 27 and siRNA specific for E-CAD or JAM-A, we observed that both knockdown experiments resulted in increased cell proliferation when compared to control (non-specific) siRNA primers. Considering the multiple levels at which E-CAD and JAM-A are similar in structure and function we proposed to study if JAM-A, like E-CAD, could be calcium-dependent. JAM-A’s structure is dissimilar to that of the other membrane proteins of the TJ, namely CLDNs and OCLN (4-α-helical structured). Their combined ultrastructure defines the function of the TJ. Nevertheless, an interplay between the TJ and AJ has been described in a recent review[40]. Thus we propose to understand if the similarities between E-CAD and JAM-A may extend to calcium-dependency, indicating that JAM-A may be the molecule that coordinates the structure and function of TJs with those of the AJ.

Cations act as switches in the CAD subfamily of the IgSF. Most CADs are recognized as calcium-dependent[25,61], and others are recognized to be magnesium-dependent[62], nickel-dependent[63], and zinc-dependent[34]. Finally, CAD desmosomes adhesive properties are decreased by calcium[64]. At least 30% of proteins bind metal ions. Bound ions are essential for protein folding, subunit assembly, interaction with other macromolecules, and protein function[65]. In this study we determined that cations had an effect on the secondary structure, binding affinity and oligomerization of MBP JAM-A. Our previous work[47] showed that each JAM proteins had a unique tertiary and quaternary structure but they had similar secondary structures. We also determined that JAMs are involved in homotypic or heterotypic interactions[47]. Our previous studies were performed in the presence of Phosphate saline
solution (PBS)[47]. We did not address whether changes in ionic interactions due to buffer conditions, including cations, could affect JAM protein properties. Similarly, the study by Prota and colleagues[18] reported the crystal structure of the extracellular domain of JAM-A but did not address whether cations would affect dimerization and higher order oligomerization as established for CADs[66]. This has perpetuated a number of misconceptions about JAM proteins. One misconception was that JAM-A only formed a dimer, as did other members of the JAM family. In this study, we designed experiments to challenge this concept. We determined that MBP JAM-A forms different oligomeric forms depending on the cation to which it is exposed in solution. Changes in aggregation have been demonstrated for proteins and nanoparticles when exposed to different cations or different ionic strengths[19,67]. We determined that the cations changed the conformation, oligomerization, and the energetics as seen in the homotypic binding affinity of MBP JAM-A.

Prediction of Cation Binding Sites in JAM-A

In order to predict the cation-binding sites the crystal structure of the protein of interest must be known. Therefore to perform the prediction we used JAM-A crystal structure PDB ID 1NBQ and the MIB: Metal Ion-Binding Site Prediction and Docking server (http://bioinfo.cmu.edu.tw/MIB/). The metal cations used in this docking server were: Ca$^{2+}$, Mg$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Fe$^{2+}$, and Fe$^{3+}$. The software gave us ranking scores of potential cation binding sites for JAM-A, where the cation docking sites with the highest scores represent the probability of being a cation binding site[68,69]. The docking sites with the highest scores were used for visualization of the different cations using UCSF Chimera (Figure 3.2.A-F). The lesser ranked predicted sites can be found in the supplementary file, Figure 3.A4. The amino acid sequence of JAM-A (PDB ID 1NBQ) was used to label the potential docking sites with the highest score for
the cations (Figure 3.2.G). After the determination of cation docking sites, we purified the MBP JAM-A protein to perform the experiments in the next section.

All the binding sites correspond to unstructured amino acids in the structure of JAM-A[18]. As a way to validate the utility of the data obtained from the MIB webserver, we predicted calcium-binding sites of E-CAD (Figure 3.A5). The MIB server adequately predicted the sites observed in E-CAD calcium crystal structure[26]. This result highlights the relevance of MIB as a predictive of the approach we have taken with JAM-A.

Expression System, Cloning, and Purification in E. coli

In this study we recombinantly expressed the two extracellular immunoglobulin domains of JAM-A. We used Maltose Binding Protein (MBP) as a fusion partner in order to allow for the generation of high yield of proteins, and to maintain consistency with our previous work[47]. Other designs that contained a TEV protease cleavage site between MBP and JAM-A resulted in low protein yields. To address the issue of low yield, we used the plasmid pET28-MBP, containing the MBP in the N-terminus of JAM-A[47] (see Figure 3.A1). This design, based on the literature[72], allowed for high protein expression and stability. The fusion partner enabled the target protein to maintain its structure and function[48]. The pET28-MBP-JAM-A (extracellular domain) was subcloned (see Materials and Methods). The pET28-MBP-JAM-A plasmid was expressed in the SHuffle T7 bacterial strain to allow for the proper folding, disulfide formation and cytoplasmic expression of the JAM-A extracellular protein[48]. The growth of pET28-MBP-JAM-A transformed bacterial cells was monitored until they reached an OD600 of 0.8-1.0, then IPTG was added to a final concentration of 1 mM. The culture continued at 16°C overnight.
Protein purification was performed with Amylose resin and finalized with Size-Exclusion Chromatography with 2 mM of each cation (\(\text{Cu}^{2+}, \text{Ca}^{2+}, \text{Mg}^{2+}, \text{Zn}^{2+}, \text{Fe}^{3+}, \text{Fe}^{2+}\)) in HEPES buffer (100 mM NaCl, 30 mM HEPES at pH 7.4). The protein had high yields with a purity of >95% (Figure 3.2.B). Additionally, oligomerization changes of JAM-A were observed for the different cations, which was shown using a native gel (Figure 3.2.C) without SDS, which maintained the protein in its native structure and allowed oligomers to be observed.

**Determination of Protein Shift by Size Exclusion Chromatography**

Following the *in silico* prediction of cation-binding sites (Figure 3.A4), the extracellular domain of human JAM-A, was purified as a fusion protein of MBP JAM-A[47]. The function of cell-adhesion molecules such as JAM-A depends on their ability to dimerize or multimerize. Size-exclusion chromatography (SEC) has been established as a powerful tool to determine oligomeric state and changes in oligomeric state[73] and has been used to study protein denaturation[74]. Further purification of MBP JAM-A was performed using SEC. Size-exclusion peak shift and oligomerization were determined using 2 mM of each cation (\(\text{Cu}^{2+}, \text{Ca}^{2+}, \text{Mg}^{2+}, \text{Zn}^{2+}, \text{Fe}^{2+}, \text{Fe}^{3+}\)) in HEPES buffer (100 mM NaCl, 30 mM HEPES at pH 7.4). As a control, we used Phosphate Buffered Saline (PBS) and HEPES buffer (100 mM NaCl, 30 mM HEPES at pH 7.4) without any of the cations. Among all the cations, we determined that the greatest shift from dimer to oligomer was observed in the presence of \(\text{Zn}^{2+}\), which was not observed with the rest of the cations (Figure 3.3). SEC columns cannot accurately predict size beyond MBP JAM-A tetrameric form. The term oligomer was used for \(\text{Zn}^{2+}\) and it could represent an octamer or higher order of quaternary organization. Our data suggests a correlation between the formation of oligomers and the increased homotypic binding of MBP JAM-A. Further, we investigated whether the increase in binding was the result of changes in the secondary structure of MBP.
JAM-A. To determine whether cations affected the secondary structure and as a result would affect self-association, we used Circular Dichroism.

**Determination of Changes in the Secondary Protein Structure when Exposed to Different Cations by Circular Dichroism (CD)**

In Figure 3.4.A and B the MBP unfused protein was used as a control where the CD spectra was determined for this protein. Figure 3.4.C and D show a change in the formation of antiparallel folding in MBP JAM-A based on cation exposure, from Zn\(^{2+}\) (55.2%) at 22°C compared to Mg\(^{2+}\) (51.9%), Ca\(^{2+}\) (49.4%), Cu\(^{2+}\) (37.3%), and Fe\(^{3+}\) (28.8%), HEPES (26.1%), Fe\(^{2+}\) (19.4%) and PBS (7.7%). The shift in the parallel folding in MBP JAM-A based on cation exposure resulted in the percentages of PBS (46.7%), Fe\(^{2+}\) (20.5%), HEPES (14.1%), Ca\(^{2+}\) (12.8%), Cu\(^{2+}\) (5.5%), Mg\(^{2+}\) (0.0%), Zn\(^{2+}\) (0.0%), and Fe\(^{3+}\) (0.0%). These results suggest that there is a change in the secondary structure of the protein that is dependent on cation exposure affecting the percentage of parallel and antiparallel of JAM-A (Figure 3.4). Thus, the cation affects the formation of \(\beta\)-sheets, and other folding properties of JAM-A. In some cases, these cations, such as Ca\(^{2+}\) and Mg\(^{2+}\), caused an increase in the \(\alpha\)-helical content of MBP JAM-A, while affecting its oligomeric state (Figure 3.4.C). To address the effect that cations had in the homotypic binding (self-binding) of MBP JAM-A, we performed Surface Plasmon Resonance (SPR).

Koch and colleagues studied changes to CD spectra of E-CAD extracellular domains 1 and 2[26]. The authors suggested that calcium binding to E-CAD resulted in conformational changes that affected the rigidization of the protein structure in preparation for binding[26]. In contrast to what we observe in JAM-A, magnesium had no effect on E-CAD CD spectra[26]. Cations’ change to secondary structure is observed with MBP JAM-A and indicates that as they
bind the flexible regions of JAM-A (Figure 3.2), they create switches that ultimately result in changes in binding. These changes were not observed in MBP alone (Figure 3.4.A and B). Finally, the two cations that produce the larger extent of oligomerization, zinc and iron (III) have different effects on MBP JAM-A. Zinc greatly increases the content of antiparallel structure while iron (III) increased greatly the unstructured content. Similarly, the energy landscape of E-CAD[75] includes the formation of cis-dimers in multiple conformations (x-dimer and S-dimer) that can be transitioned by calcium acting as molecular switch to trans state triggering cell-cell interactions through rigidization[66,75]. The NMR structure of E-CAD[76] also suggests that the structure is largely dynamic, depending on protein concetration and calcium binding. This can be equated to protein expression and abundance, and the electrostatic contributions of the environment.

*Determination of the Effects of Cation on MBP JAM-A Homotypic Interactions by SPR*

The homotypic interaction of MBP JAM-A was observed to increase with exposure to cations. The largest effect was due to Zn$^{2+}$ when compared to Mg$^{2+}$, HEPES, and PBS. Based on these results (Figure 3.4), KDs for homotypic interactions were ranked from greatest to least binding: Zn$^{2+}$ > Cu$^{2+}$ > Mg$^{2+}$ > Ca$^{2+}$ > Fe$^{3+}$ > PBS > Fe$^{2+}$ >HEPES. Our results mirrored the shifts found with SEC and suggest that binding between these proteins changed depending on the cation it was exposed to. The protein experienced conformational changes, as determined by CD, which resulted in changes in binding, measured by SPR. The increased binding affinity for homotypic MBP JAM-A could be based on the conformational change that the cations produced in the secondary structure of the protein. Zinc produced the highest binding affinity, compared to the other cations and HEPES, which may originate on the conformation of the β-sheet (parallel and anti-parallel), α-helix, turn, and other structures (Figure 3.3). MBP JAM-A in HEPES had a
constant of affinity of approximately 48 µM, but when exposed to Zn\(^{2+}\), the affinity increased to 28 nM. Higher affinity is related to an increased buried contact surface[77], which is in agreement with Zn\(^{2+}\)'s effects on MBP JAM-A, increased oligomeric state and increased homotypic affinity. The metal cation binding sites of the protein may have become closer when bound, which could lead to conformational changes[78-80] in the MBP JAM-A protein. Calcium and magnesium had different binding affinities that caused different conformational changes in the binding of target proteins. The different cation binding sites predicted from JAM-A (Figure 3.A4) may result in a regulatory switch leading to the conformational changes and ultimately contribute to the TJ formation and cell-cell adhesion.

**Effects of Cations on JAM-A Associated Morphology and Proliferation**

Overexpressing JAM-A in HEK 293 cells results in cell rounding (Figure 3.1). Our JAM-A construct was tagged with C-terminal GFP (Figure 3.A1). We observed that 48 hours post-transfection and in the absence of cations, cells remain mostly round and do not aggregate. This result has been shown in studies where the knockdown of JAM-A accelerates the proliferation and migration of human keratinocytes [53]. Our data indicates that different cations increase cellular aggregation (Figure 3.6, panels 2-4). For example, the influence of zinc, transfected cells aggregated the most. In Figure 3.6.B we show the cellular aggregation between two adjacent cells expressing JAM-A (GFP). In the literature, E-CAD overexpression results in similar cell-cell adhesion structures[83]. We obtained similar results using our E-CAD(GFP) construct in HEK 293 cells exposed to calcium (Figure 3.A6).
Discussion

In our previous study[47], we determined that JAMs bind to other members of their family with high affinity. Our experiments were conducted using PBS and did not alter electrostatic interactions of the targeted proteins. In this study, we decided to determine the effect that cations might have on JAM-A. We used JAM-A because this protein has been crystalized[18] and enabled the *in silico* prediction of cation-binding sites. In the literature, JAM-A’s crystal structure has been used to extrapolate the behavior of all other members of the family, leading to misconceptions that our research is currently addressing. Therefore, we decided to use the available crystal[18] structure to first determine whether there could be potential cation-binding and found binding sites for Ca$^{2+}$, Mg$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Fe$^{2+}$, and Fe$^{3+}$. The purified JAM-A in the presence of these cations had different shifts in oligomerization shown by SEC, with Zn$^{2+}$ having the most drastic effect. This led us to further determine the effects that cations had on the secondary structure of JAM-A.

Using CD, we determined that the cations had an effect on the secondary structure of JAM-A. Strikingly, it was determined that Zn$^{2+}$ resulted in more β-sheet content when compared to the other cations. This could be due to the effect calcium has on CADs, triggering further stabilization of the flexible regions of the extracellular Ig domains, a process called rigidification[28]. Other examples of the effects of cations have on the changes in protein structure and stability are seen in calcium-binding protein 1, prothymosin α, avian thymic hormone, hepatitis C virus NS3 protease, and calcium and integrin-binding protein[84-89]. Avian thymic hormone (ATH), which is expressed in the chicken, consists of two β-parvalbumin isoforms[85,87]. It has been found to have cation-binding sites with the following dissociation constants: 4-10 nM for Ca$^{2+}$ and 40-80 µM for Mg$^{2+}$[86]. Studies to determine the effect on the
secondary structure of ATH showed that Ca\(^{2+}\) did not produce distinguishable changes in secondary structure, but Mg\(^{2+}\) exposed the hydrophobic regions to the solvent, shown by fluorescence emission spectra\[88,89\]. In the case of calcium-binding protein 1 (CaBP1), it has been found that it not only binds to three Ca\(^{2+}\) ions, but also binds to one Mg\(^{2+}\) ion with a dissociation constant of 300 \(\mu\)M[90]. This is linked to the conformational changes of the CaBP1 protein, because when exposed to cations it formed a dimeric conformation, but in the absence of cations it formed a molten globule-like structure, shown by dynamic light scattering[90]. This suggests that cation binding induces homodimerization and structural stability of CaBP1[90]. This leads to CaBP1 promoting the opening of the L-type Ca\(^{2+}\) channel when compared to Calmodulin (CaM)[91-93].

Calcium- and integrin-binding protein (CIB) is another example of a protein with Ca\(^{2+}\) -binding sites. Studies have shown that CIB has an affinity for Ca\(^{2+}\) with dissociation constants of 0.5 and 2 \(\mu\)M[94]. Therefore, Ca\(^{2+}\) leads to a conformational change that stabilizes the secondary and tertiary structures of the protein. Through CD experimentation it was found that CIB had a helical content and random coil structures. This was caused by the exposure to Ca\(^{2+}\), which affected the \(\alpha\)-helical content, unstructured protein regions and tertiary structure of the protein[94]. The results of our study are similar to a previous study [85] that showed that there is an effect of cations on the \(\alpha\)-helical content, unstructured protein regions, and tertiary structure of the protein, which is similar to our results where the exposure of cations has a change on the secondary and tertiary structures of JAM-A. These cations therefore, affect the secondary structure, and produce changes in the oligomeric state. The observed effect of the cations on the secondary structure of JAM-A led us to question whether they affected binding.
The homotypic binding of JAM-A was affected by cations. When exposed to Zn\(^{2+}\), the constant of affinity was the highest, as shown by SPR (Table 3.1). Overall, the changes in conformation of JAM-A, driven by the cations, would coordinate the binding and oligomerization of JAM-A. Taken together, this suggests that JAM-A may play a role in how TJs are assembled, leading to changes in binding and oligomerization that can either increase or decrease the TJ function. This novel finding suggests that the TJ components are tightly regulated by changes in extracellular cation concentrations. Another consequence could be that the ultra-structure of the TJ may be controlled by cation concentration or other microenvironment events that alter the electrostatic properties surrounding it. Future studies should be performed to determine whether cations have an effect in the binding of other TJ components with these cations. JAM-A’s unique ability to be part of the TJ while mirroring CADs behavior in the presence of calcium may result in JAM-A orchestrating the interplay between the TJ and the AJ. New strategies to understand which amino acids are responsible for cation-binding in JAM-A are needed. Studying cations effect on other JAMs is also key to understanding the interconnection between TJ and AJ. Finally, considering that the crystal structure of JAM-A has been determined, future crystallographic studies may include cations of interest.

Conclusion

JAM-A is a protein important to the formation of TJs but very little is known about the role that cations play in regulating its structure, oligomeric state, and binding properties. In this study, we determined that cations affect the secondary, tertiary, and quaternary structure of JAM-A, and its binding affinities in homotypic interactions. Our contribution brings to light the key role that cations play in regulating the homotypic interactions of JAM-A[18,47,95]. The
exposure of JAM-A to different cations resulted in changes in the secondary structure, which may be linked to the effects on binding as seen in our SPR analysis. Based on our results, we present a graphical ranking (Figure 3.7) that highlights the effects that cations have on the homotypic binding affinity of JAM-A. These cations, of physiological importance can reach high concentrations in the plasma or extracellular fluids and thus act as molecular switches regulating JAM-A homotypic interactions and ultimately cell adhesion. The role that cations may play in the secondary structure, oligomerization, and binding of JAM-A could influence TJ formation in different cell types and tissues and orchestrate the interactions between TJ and AJ.

Authorship Contributions


Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Figure 3.1: Similarities Between JAM-A and E-CAD in a Calcium-free Environment. A) Schematic structure of E-CAD and JAM-A. SP, signal peptide; PP, pro peptide; EC, extracellular domains of Ig folding; TMD, transmembrane domain; CPD, cytoplasmic domain. Red circles denote glycosylation sites, the solid black line indicates the binding Ig domain for cis- and trans interactions. B) Models of cis-dimer formation based on crystallographic studies. Two E-CAD monomers interact through EC1 (blue and orange). JAM-A in the absence of calcium forms a U-shaped dimer (green and yellow monomers). C) HEK 293 cells transfected with Empty vector (pcDNA 3.1), pcDNA 3.1 JAM-A(GFP), or pcDNA3.1 E-CAD(GFP). D) ATP proliferation assay of CAL 27 cells. Gene silencing was achieved by siRNA primer transfection. Untreated cells (None) were not transfected; siCTRL, cells were transfected with non-specific primers; cells were transfected with specific primers to knockdown JAM-A (siJAM-A) or E-CAD (siE-CAD). The (*) denotes statistically significant (p<0.05).
Figure 3.2: Prediction of Cation-Binding Sites in JAM-A (PDBID 1NBQ). A) Ca$^{2+}$ binding sites were predicted to be ASP85 and GLU84. B) Mg$^{2+}$ predicted binding sites were GLY219 and THR220. C) Cu$^{2+}$ predicted binding sites were ASP85 and ARG86. D) Zn$^{2+}$ predicted binding sites were ASP65, ASP68 and THR70. E) Fe$^{2+}$ predicted binding sites were GLU42 and GLU102. F) Fe$^{3+}$ predicted binding sites were CYS153 and CYS212. Predicted binding sites were generated by the MIB webserver (http://bioinfo.cmu.edu.tw/MIB/). Models were visualized using UCSF chimera[70,71]. G) Amino acid sequence of the JAM-A crystal structure PDB ID 1NBQ shows the cation binding sites labeled in the color similar to the cation color in Figures 3.2 A-F.
Figure 3.3: The Effect of Examined Cations on MBP JAM-A Oligomerization. A) The shift of MBP JAM-A with the cations was seen as follows: 1) Zn$^{2+}$ produced multimers, 2) Ca$^{2+}$ produced dimers and slight monomers. 3) Mg$^{2+}$ produced dimers and slight monomers, 4) Phosphate buffer produced dimers, 5) Cu$^{2+}$ produced dimers and shifted to a slight tetramer. 6) HEPES buffer produced a dimer and slight tetramer. B) SDS PAGE of purified MBP JAM-A exposed to different cations. All of the proteins in the presence of 2 mM cations, where purified by size-exclusion chromatography were determined to be JAM-A at > 95% purity. Maltose Binding Protein (MBP) was used as a control in lane 1. All other samples were MBP JAM-A with different cations following size exclusion, lanes 2 to 9, in the following order: PBS, HEPES, Ca$^{2+}$, Mg$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Fe$^{2+}$, Fe$^{3+}$. C) Native gel of purified MBP JAM-A exposed to different cations. MBP JAM-A homotypic interactions were observed when exposed to the different cations. Samples were loaded in the same order as in Panel A, with MBP as control. For Figure 3.3.B and 3.3.C the relevant molecular weights are shown in the ladder.
Figure 3.4: Circular Dichroism of MBP and MBP JAM-A Exposed to Different Cations. A) Changes to the secondary structure of MBP exposed to different cations at 22°C. B) The changes in the secondary structure from Panel A, are shown in the table highlighting percentages of Alpha, Beta (Antiparallel, and Parallel), Turn, and Other. C) Changes to the secondary structure of JAM-A when exposed to different cations at 22°C. D) The changes of the secondary structure, seen in Panel C, are shown in the table highlighting percentages of Alpha, Beta (Antiparallel and Parallel), Turn, and Other content found in the structure.
Figure 3.5: Cations Affect Homotypic Binding of MBP JAM-A. Binding affinity (KD) of JAM-A under the effect of different cations was normalized to the value obtained with HEPES. The ranking of affinities is presented in the following order, with the smallest value in the graph as the highest binding affinity and the largest number as the lowest binding affinity: Zn$^{2+}$ > Cu$^{2+}$ > Mg$^{2+}$ > Ca$^{2+}$ > Fe$^{3+}$ > PBS > Fe$^{2+}$ > HEPES. We present the data obtained from studying JAM-A under the influence of different cations. See Table 3.1, containing $K_a$ (association constant), $K_d$ (dissociation constant), and KD (binding affinity calculated by $K_a/K_d$).
Table 3.1: Surface Plasmon Resonance (SPR) Analysis of MBP JAM-A when Exposed to Different Cations. All experiments had a Chi$^2$ value less than 10% of $R_{max}$[81,82]. The values obtained by SPR are $K_a$ (association constant, that measures the rate at which the two partners bind during the association phase), $K_d$ (dissociation constant that measures the rate at which the protein called the anylate separates from the ligand attached to the sensor chip), and $K_D$ (binding affinity calculated by $K_a/ K_d$).

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<tr>
<th>PPI evaluated</th>
<th>$K_a$ (1/(M*s))</th>
<th>$K_d$ (1/s)</th>
<th>$K_D$ (M)</th>
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<tr>
<td>MBP JAM-A vs. MBP JAM-A HEPES</td>
<td>$1.26 \times 10^3 \pm 1.03 \times 10^2$</td>
<td>$4.80 \times 10^{-4} \pm 4.24 \times 10^{-6}$</td>
<td>$4.78 \times 10^{-7} \pm 3.48 \times 10^{-9}$</td>
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<tr>
<td>MBP JAM-A vs. MBP JAM-A PBS</td>
<td>$3.21 \times 10^3 \pm 9.73 \times 10^1$</td>
<td>$3.84 \times 10^{-4} \pm 5.77 \times 10^{-6}$</td>
<td>$1.55 \times 10^{-7} \pm 1.83 \times 10^{-9}$</td>
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<tr>
<td>MBP JAM-A vs. MBP JAM-A Ca$^{2+}$</td>
<td>$5.92 \times 10^3 \pm 1.53 \times 10^3$</td>
<td>$6.24 \times 10^{-4} \pm 6.27 \times 10^{-6}$</td>
<td>$1.05 \times 10^{-7} \pm 4.56 \times 10^{-8}$</td>
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<tr>
<td>MBP JAM-A vs. MBP JAM-A Mg$^{2+}$</td>
<td>$4.53 \times 10^3 \pm 5.75 \times 10^1$</td>
<td>$2.42 \times 10^{-4} \pm 4.13 \times 10^{-6}$</td>
<td>$5.39 \times 10^{-8} \pm 1.57 \times 10^{-9}$</td>
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<tr>
<td>MBP JAM-A vs. MBP JAM-A Zn$^{2+}$</td>
<td>$6.52 \times 10^4 \pm 6.56 \times 10^2$</td>
<td>$2.84 \times 10^{-5} \pm 4.22 \times 10^{-6}$</td>
<td>$4.34 \times 10^{-10} \pm 6.70 \times 10^{-11}$</td>
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<tr>
<td>MBP JAM-A vs. MBP JAM-A Cu$^{2+}$</td>
<td>$4.09 \times 10^3 \pm 2.75 \times 10^1$</td>
<td>$4.77 \times 10^{-6} \pm 4.64 \times 10^{-6}$</td>
<td>$1.09 \times 10^{-8} \pm 1.10 \times 10^{-9}$</td>
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<td>MBP JAM-A vs. MBP JAM-A Fe$^{2+}$</td>
<td>$5.25 \times 10^4 \pm 4.39 \times 10^2$</td>
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<td>MBP JAM-A vs. MBP JAM-A Fe$^{3+}$</td>
<td>$1.75 \times 10^3 \pm 6.29 \times 10^1$</td>
<td>$1.78 \times 10^{-6} \pm 6.29 \times 10^{-10}$</td>
<td>$1.51 \times 10^{-7} \pm 1.86 \times 10^{-8}$</td>
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Figure 3.6: Morphology Changes to HEK293 Cells Transfected with JAM-A(GFP). A) HEK 293 cells were transfected with JAM-A(GFP) plasmid. 24 hours post-transfections cells were exposed to buffer (panel 1) or cations (calcium, panel 2; magnesium, panel 3; zinc, panel 4). Below each bright field image is the corresponding capture using GFP/FITC filter. The (*) denotes the 2 cells used in the next panel. B) The 2-cell structure from panel 4 (zinc) was further studied using Z-stack (4 nm increments). This gallery shows 5 optical sections taken from the whole length of the cell, top to bottom (left to right). Comparing the bright field image with this sequence of images we observe that JAM-A(GFP) is distributed through the interface.
Figure 3.7: Graphical Ranking of Cations Affecting the Homotypic Binding Affinity (KD) of JAM-A. In vitro (recombinant protein) ranking suggests that cations affect JAM-A binding, with the greatest amount of binding at the top and decreasing toward the bottom.
References


Appendix 3.A: Supplementary Information of Cations Affect the Folding and Binding of Junctional Adhesion Molecule-A
Number of amino acids: 374. Molecular weight: 40992.61

Sites for NdeI (HM) amino acids and XhoI (LE amino acids) are present in the plasmid.

pET28-MBP-JAM-A-6xHIS construct

Number of amino acids: 588. Molecular weight: 64250.35

Human JAM-A (GFP) fusion. In bold is GFP sequence.
MGGLYKQSWF SEPSDQIFVD PTIHSTHPA AAAKLMGTKA QVERKLLCLF ILAILLCSLA

LGSVTVHSSE PEVIPENNP VKLSCAYSFG SFSPRVWKVD QGDTTRLVCY NNKITASYED

RVTLPLTGT FKSVTREDTG TYTCMVSEEQ GNSYGEVK VKLIVLVPSSP TVNIPSSATI

GNRAVLTCSE QDGSPPEY QT FKGDGIYMPT NPKSTRAFSN SSVLNPPTG ELVDPLSAS

DTGEYSCEAR NGYGTPM TSN AVRMEAEVNV VGVIVAALV TLIIILYLGF GIVFAYSRGH

FDRTKKGTSK KKVLYSQP SRSSEGFQKQTS SFLVLEGAAA GMADPVSGKE EJFTGVVPIL

VELDGDVNGH KFSVSJEGEE DATYGLKTLK FICTTGKLPV PWPTLVTTLT YGQQCFSRYP

DHMKQHXDFK SAMPEGVQ ERIFFKDDGN YKTRAEVKFE GDTILVNRIEL KGIDFKEDGN

ILGHKLEYN YSHHNYIMAD KQKNGLKVF VIGHTNEDGS VQLADHYQQN TPIGDGPVLL

PDNHYLSTQS ALSKDPNEKR DHMVLLFVT AAGITLGME LYK

Human E-CADHERIN(GFP) fusion

MGPSRSRLSA LLLLQVSSW LCQEPEPCHP GFDAEYFTFT VPRHLRGER VLGRVFEDC

TQRQRTAYFS LDTRFKVGT DGVTVKRPRL FHPQIHELV YAWSTYRFK STKVTLTVG

HHHRPPPQHA SVSGLQAEW TFPNSSGLR RQKRDWVIPP ISCPENEKGP FPKNLVQIKS

NKDKEGKVFS ISITGQGADTP PVGVIIFER TGLWKVTEPL DRERIATYL FSHAVSSGN

AVEDPMELT VTDQDNKPF EFTQVFKQS VMEGALPGTS VMEVTATDAD DDVNTYNAI

AYTILSQDE LPDKNMFTIN RNTGVISVVT TGGLRESFTP YTLVVQAADDL QGEGLSTTA

AVITVTDNND NPFRPNPTY KGQVPENAEV VVITTLKVD ADAPNTPAWE AVYTILNDDG

GQFVVTTPNV NNDGILKTAK GLDFEAQQY ILHVAVTNVV PFEVSLTST ATTVTVDLV
Figure 3.A1: pET28-MBP (Kanamycin resistance) Vector
Figure 3.A2: gBlock Sequence

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<td>atataCATATGggaaagtgaaggggaag</td>
<td>tatataCTCGAGggacagcattggaagtcat</td>
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Figure 3.A3: Primers for PCR Amplification
### Human JAM-A Calcium Potential Docking Binding Sites

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### Human JAM-A Magnesium Potential Docking Binding Sites

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### Human JAM-A Zinc Potential Docking Binding Sites

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### Human JAM-A Copper Potential Docking Binding Sites

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### Human JAM-A Iron II Potential Docking Binding Sites

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### Human JAM-A Iron III Potential Docking Binding Sites

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Figure 3.A4: Prediction Cation-Binding Sites for Human JAM-A
### E-CAD Calcium Potential Docking Binding Sites

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**Figure 3.A5:** Predicted Calcium-Binding Sites for Human E-CAD PDBID 2O72

![Image](image1.png)

**Figure 3.A6:** Overexpression of E-CAD(GFP) in HEK 293 Cells. Cells display a high concentration of E-CAD(GFP) in the interface between cells.
CHAPTER 4: Interaction of Tight Junction Membrane Proteins and the Interplay with Epithelial Cadherin

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Abstract

The interplay between the Tight Junction (TJ) and Adherens Junction (AJ) has been described briefly in the literature. Maturation of the TJ is believed to occur post assembly of the AJ. Cadherins (CADs) are calcium-dependent adhesion membrane proteins of the AJ whereas the TJ membrane proteins are considered calcium-independent. Here we investigate the interaction between the membrane proteins of the TJ (claudin, junctional adhesion molecule, and occludin) and epithelial cadherin (E-CAD) in the absence of calcium. E-CAD cell-cell adhesive properties are optimal in the presence of high concentrations of calcium (extracellular milieu) while in the intracellular environment (low calcium) they form cis-dimers. We report structural changes measured by Circular Dichroism and protein-protein interactions measured by Surface Plasmon Resonance.

Keywords: junctional adhesion molecule, claudin, e-cadherin, circular dichroism, surface plasmon resonance
Introduction

Epithelia act as barriers that separate the external environment from the internal environment within the human body. Epithelial cells are polarized to allow for the transport of molecules across the membranes in an asymmetric manner[1]. The components responsible for the formation of complex partitions separating the internal and external environments are the tight junctions (TJs). The TJ is a macromolecular complex formed by membrane proteins and soluble adapter proteins that anchor and stabilize it by their physical association with cytoskeletal networks of actin, microtubule and intermediate filaments[2]. In the TJ, membrane components are important for maintaining cell-cell adhesion that allows compartmentalization to occur between tissues[3,4]. TJs act as barriers to control the paracellular space and the formation of the apical/basolateral intermembrane in the outer leaflet of the plasma membrane[5-7]. Examples of dysfunction of the TJs include edema, jaundice, diarrhea, inflammatory bowel disease, and metastasis [8-10].

At the membrane level, TJs are composed of claudins (CLDNs), occludin (OCLN) and junctional adhesion molecules (JAMs)[5]. The role of CLDN has been thought to be of high importance compared to OCLN in maintaining the function of barrier formation[11,12]. Junctional adhesion molecules (JAM) have been shown to be important components in the formation of TJs[13]. JAMs are a subfamily of the immunoglobulin superfamily (IgSF) of adhesion receptors[14] that play roles in vertebrate development and homeostasis[15]. There are four members of the JAM subfamily: JAM-A, -B, -C and 4. Our previous studies have shown that the JAMs may display a more dynamic behavior with high affinity for other members of the family that enables them to form both homotypic and heterotypic interactions[16]. It remains unanswered the extent to which JAMs interact with CLDNs or OCLN. JAM proteins belong to
the TJ, but their structure is more similar to that of CADs, a member of another subfamily of IgSF[17]. CADs remain universally acknowledged as calcium-dependent adhesion molecules while the TJ is recognized as calcium-independent[18].

Studies at the cellular level, in vivo or in vitro, remain inefficient in characterizing the forces that regulate the interplay between TJ and AJ. The use of recombinant proteins has characterized the binding affinities of CADs[19] and we have recently reported values for JAMs[16]. CADs have been reported to alter their homotypic interactions from cis to trans when extracellular levels of calcium reach 2-3 mM[20]. Intracellular calcium concentrations are near 100 nM in the cytosol[21]. Some compartments like the endoplasmic reticulum (ER), Golgi and traffic vesicles maintaining calcium levels below 200 μM[21].

Many challenges to the study of the TJ components are related to the purification process. The classical approach for expressing single transmembrane proteins is to cleave the hydrophobic domain[22]. This method allows for the solubilization and purification of proteins such as JAM-A[23] or CADs[24]. The studies performed by Prota et al. used recombinant JAM-A to crystalize the protein[25]. These studies demonstrated the secondary, tertiary, and quaternary structures of JAM-A[23,25]. In the case of CLDNs, these have been successfully purified and crystallized[26]. On the other hand, only a partial structure of the intracellular C-terminal domain of OCLN has been determined[27]. Studies conducted with only soluble domains of TJ and AJ membrane proteins or where the hydrophobic interactions are decreased or eliminated by the use of detergents[18] offer no real answer on whether these proteins interact with one another.

Overexpression studies of TJ components such as CLDNs reconstitute TJ strands in fibroblasts[28]. Knockout experiments of CLDN binding fragment in Clostridium perfringens
enterotoxin reduced TJ formation, suggesting that claudins are a key component of TJ formation[29]. The complete knockout of CLDNs in epithelial cells such as MDCK II cells lacked TJ suggesting that claudins are essential for TJ formation[30].

To present evidence of the direct interaction between membrane proteins of the TJ and AJ can be a complex task. CLDNs (a family of over 25 members in mammals), OCLN, and JAMs (a family of four members) form complex structures[2,30,31]. To simplify the task, we focused on a simple TJ, formed by JAM-A, CLDN1, and OCLN. Such a TJ is observed in CAL27 cells, human epithelial tongue (Squamous Cell Carcinoma)[32]. In epithelial cells, we find E-CAD[33], and we will use it to study the interplay between epithelial AJ and this simple TJ. Our strategy requires the production of recombinant proteins: JAM-A, CLDN1, OCLN and E-CAD. The literature contains protocols to produce the two extracellular domains of E-CAD responsible for its adhesive properties[34,35]. We have recently published protocols for the expression of the extracellular domain of JAM-A[16] as well as a synthetic approach to express the adhesive domains of CLDN1 and OCLN[36]. In our previous studies we successfully expressed and purified our proteins to have >95% purity by fusing our protein of interest with maltose-binding protein (MBP)[16,36]. Having access to these recombinant proteins that display native adhesive properties, we set out to address the following questions: 1. Do these TJ components interact with other TJ components? 2. Do the TJ components interact with the AJ membrane protein E-CAD? Here we will answer these questions by using Size Exclusion Chromatography (SEC), Circular Dichroism (CD) and Surface Plasmon Resonance (SPR).

Our experimental design will show an interplay between membrane proteins of the TJ and AJ in the absence of calcium, representing the intracellular environment and more
particularly the traffic route these proteins share while traveling to the plasma membrane prior to creating fully functional junctions.

Results and Discussion

**JAM-A, CC1, COC, and E-CAD Expression and Purification in E. coli**

Extracellular domains of JAM-A and E-CAD[16], and chimera CLDN1 (CC1) and chimera OCLN (COC)[36] were expressed as a fusion with maltose binding protein (MBP). This strategy was designed according to the literature which demonstrated that MBP drives high protein expression and stability of the fused target protein, thus enabling target proteins to retain their individual structure and function[16,37,38]. The pET28-MBP was subcloned to contain the target proteins JAM-A, CC1, COC and E-CAD. All of our targeted proteins require proper disulfide formation to allow for the proteins of interest to fold and function[26,33,39]. The SHuffle T7 bacterial strain[40] is a tool that allows cytosolic expression of targets while enabling proper disulfide bond formation, both properties resulted in high protein yields[16,41]. Plasmids hosting MBP JAM-A, MBP CC1, MBP COC, and MBP E-CAD were transformed in Shuffle T7 bacterial and grown at 37°C in LB containing both Ampicillin (required by pET28-MBP) and spectinomycin (required by SHuffle cells). Bacterial growth was allowed to reach an OD600 of 0.8-1.0 before addition of IPTG to a final concentration of 0.1 M. After addition of IPTG the bacterial culture continued at 16°C overnight. Protein purification was performed using Amylose resin, followed by Size-Exclusion Chromatography (SEC). The purified protein that was used to perform the studies in this manuscript was determined to be >95% pure (Figure 4.1). Proteins were kept in Phosphate buffered saline (PBS) to mimic the calcium-free conditions of the intracellular environment.
Size Exclusion Chromatography (SEC)

The purification process and oligomerization analysis were performed by using SEC. Each of the TJ components had unique oligomeric states. MBP E-CAD formed dimers as described in the literature[16,24,42], while the dimeric result observed with MBP JAM-A was consistent with published dimers for JAM-A[23,25,43]. This led to the determination that other TJ components such as MBP CC1 and MBP COC had similar oligomeric states. We determined that both MBP CC1 and MBP COC resulted in higher orders of organization with values above 20 monomeric units (Figure 4.1.C).

Determination of Secondary Structures by Circular Dichroism (CD)

Based on the oligomerization results obtained in SEC, we decided to determine whether there were conserved secondary structures with these TJ and AJ components Questions of what a protein does inside a living cell is not a simple to answer. Protein crystallography often represents a single state of the target protein but doesn’t show the changes in the secondary protein structure [44]. On the other hand, CD is recognized as a leading technique to the study dynamic secondary structure of proteins without being restricted by size[45]. In Figure 4.2, we determined the CD spectra of all targeted proteins. We also present a table that shows the percentages of each secondary structure of the studied protein.

The analysis of the CD spectrum of non-fused MBP resulted in approximately 75% structure: 45% Alpha, 18.8% Antiparallel, and 13.4% Parallel. Non-fused MBP was only 22.8% unstructured (coiled). We proceeded to analyze each MBP-fusion with the structure displayed by the non-fused MBP. As expected, the IgSF proteins (MBP E-CAD and MBP JAM-A) had an increase in β-sheet content (Figure 4.2). The structure of MBP JAM-A was determined to be more β-sheet compared to the MBP E-CAD (Figure 4.2.B), that contained approximately 10% of
unstructured regions. MBP CC1 also had an increased β-sheet content, which is related to the native structure of the extracellular loops of CLDNs where 50% are found to be β-sheets[36]. MBP COC increased α-helical content over 7% compared to MBP CC1. In the absence of a crystal structure of full length OCLN we suggest that the extracellular loops of OCLN contain more α-helical structure compared to CLDN1.

Based on both the SEC and CD data, we asked whether the observed secondary structure and oligomeric states (Figure 4.1 and 4.2) produced differences in homotypic and heterotypic interactions. To address this question, we performed Surface Plasmon Resonance (SPR). This technique was used to measure the binding of proteins in real-time without the need to use labels[46].

_**Homotypic Interactions of JAM-A, CC1, COC, and E-CAD**_

The studies performed by Vendome and colleagues [19], described the formation of homotypic interactions of E-CAD. In our previous study, we determined that JAM proteins formed homotypic interactions, leading us to ask whether other TJs do as well. CLDN dimers as well as higher order of oligomers for OCLN have been described [3,28,47,48], but without the quantification of protein-protein interactions. To address this question, we measured homotypic interactions by using SPR, with MBP E-CAD values serving as a standard. Instead of interpreting the absolute constant of affinity (KD) value (Table 4.1) determined by SPR, we normalized the values presented, to the better-studied homotypic E-CAD.

Figure 4.3 shows the normalized affinity values (KD) for the homotypic interactions of the TJ components. All of the TJ components displayed a higher affinity (KD) for homotypic interactions compared to MBP E-CAD. MBP CC1 values for CLDN1, showed a 10,000-fold
higher affinity than MBP E-CAD. MBP JAM-A and MBP COC presented 4- and 72-fold stronger affinity than MBP E-CAD.

*Heterotypic Interactions of JAM-A, CC1, COC and E-CAD*

Cells express multiple proteins that make up the TJ, and AJ, however, what is not well understood is whether these TJ and AJ components interact with one another. Studies assume that the TJ components bind to members of the TJ, but they do not show whether they interact with AJ components. Our SPR data helps in understanding the heterotypic interactions of TJ components and the interplay with AJ (E-CAD).

Figure 4.4 suggests that heterotypic interactions between TJ components are favored over homotypic interactions. Additionally, Figure 4.4 shows that TJ components also interact with the E-CAD an AJ components. This sheds light onto what has been reported in the literature, showing that there is an interplay between TJ and AJ components[18]. This may suggest that cell-to-cell interactions are more complicated and would also differ depending on the cellular environment, intra- or extracellular, which could result in different interactions between TJ and AJ components. Protein-protein interactions between these components will depend on transcription regulation, mRNA stability, protein translation and abundance, and other factors[49].

Finally, as our experiments represent TJ and AJ membrane protein interactions in the absence of calcium, we interpret the formation of the different homotypic and heterotypic interactions in a small model ranking them according to their KD (Figure 4.5). All of the proteins involved in these interactions traffic through the ER and Golgi[50-53]. Our experimental design to study these proteins in PBS and in the absence of calcium represents the unique opportunity of these proteins to interact without external forces like the ones offered by
intracellular adapter networks, zonulae occcludentes (ZO), and zonulae adhaerentes (ZA)[43]. It has been suggested that CADs are expressed first and aid in the formation and maturation of the TJ[54]. Finally, CLDNs and OCLN are believed to form only cis interactions while trafficking to the plasma membrane[54] enabling them to interact with other proteins present in the vesicles. It is possible that the additional interactions help CLDNs and OCLN to maintain homotypic cis interactions, preventing vesicle collapse or invaginations. In Figure 4.5, we show that both homotypic and heterotypic interaction rankings seem to suggest that a mixture of these species can be found inside trafficking vesicles. Once these traffic vesicles reach the plasma membrane new interactions with the intracellular adapters[43] may take place to aid the formation of the TJ and AJ. Our findings suggest that the interplay between the TJ and AJ may be greater intracellularly than at the plasma membrane. Based on these data, we suggest that maturation of the TJ in the plasma membrane, its separation into a separate membranal micro domain than that of the AJ[55,56], and migration away from the AJ may depend on other factors that require further investigation. We suggest that future work may examine the effects of calcium as a molecular switch on CADs[57,58] as an important player in the changes needed for functional assembly of AJ and TJs.

Materials and Methods

 Protein Preparation by Cloning, Expression and Purification

In order to clone, express and purify the MBP JAM-A, MBP CC1, MBP COC and MBP E-CAD proteins, we used g Blocks obtained from IDT. The gBlocks were amplified with forward and reverse primers using PCR. The amplified PCR product was incubated with Xhol and Ndel and incubated at 37°C for 8 hours. Inactivation of the restriction enzymes was
performed by incubating the reaction at 65°C for 20 minutes. The PCR product was then cloned into pET28 vector at the XhoI and NdeI sites by ligation to express the JAM-A, CC1, COC and E-CAD proteins containing an MBP at the C-terminus.

Plasmid transformation was performed in the bacterial strain DH5α. Plasmid purification was performed from single bacterial colonies using the Zyppy Plasmid Miniprep Kit from Zymo Research. Sanger sequencing was performed by Genewiz (New Jersey) to determine whether the plasmid coding for JAM-A, CC1, COC and E-CAD was correct and that no mutations were present. After the verification of the plasmid sequence, we transformed the plasmid into SHuffle bacterial cells[40,41] that would express the protein. French Press was performed with the bacterial pellet with the addition of 100 µL of 0.5 M EDTA. The re-suspended bacterial cells were loaded into the Thermo Spectronic French Pressure Cell Press Model FA-078. Lysis was performed at 1500-2000 psi and the lysate was collected into a 50 mL tube. The lysate was centrifuged for 30 minutes at 10,000 RPM using a F15-8x50cy rotor from Thermo Scientific. The supernatant was decanted into a 50 mL tube containing Amylose resin from New England Bio Labs (E8021L), and incubated while rotating for 1 hour at 4°C. Column chromatography was performed to collect the protein from the amylose beads. The column was washed with 100 mL of wash buffer containing 500 mM NaCl and 30 mM TRIS. The elution was performed for 3 minutes each time using 100 mM Maltose. The elution was concentrated using the Microsep Advance with 10k Omega centrifugal device (Reference # MCP010C41) from Pall Corporation and centrifuged at 10,000 RPM for 10 minutes until reaching 2 mL of final elution volume.

Size Exclusion Chromatography (SEC)

Size exclusion chromatography was performed using the NGC Chromatography System and its accompanying software (BioRad, Hercules, CA, USA. https://www.bio-rad.com/). The
SEC column used in to purify the proteins of interest was ENrichTM SEC 650 10 × 300 (BioRad, Hercules, CA, USA). Protein concentration was determined by using the NanUSA). Nanodrop Onec from Thermo Scientific. PBS was employed as a running buffer for SEC. Product peaks were compared for the position to the size exclusion standards from BioRad Catalog Number 151-1901.

Circular Dichroism (CD) Spectrometry

CD measurements were performed on the Spectrophometer Model 420 AVIV (Biomedical Inc. Lakewood, NJ USA). Changes in ellipticity at 260 nm to 195 nm using 20 second scans were measured with a 100 µM concentration of protein in a 10 mm QS glass cuvette cell at 37°C. The secondary structure consisting of alpha helix, antiparallel, parallel, turn, or other was determined by the usage of Bestel circular dichroism analysis, https://bestsel.elte.hu/index.php.

SDS-PAGE Assay

Two µg of boiled MBP, E-CAD, JAM-A, CC1 and COC were electrophoresed on 8% SDS-PAGE gel (BioRad). Gel staining was performed using standard protocols.

Surface Plasmon Resonance (SPR)

SPR was performed using Open SPR by Nicoya Lifesciences (Kitchener, ON, Canada. https://nicoyalife.com/, accessed on 10 March 2021). We assayed protein–protein interactions by loading 0.050 mg of each protein as a ligand into the Carboxy sensor chip (Nicoya Lifesciences). The proteins are immobilized in the Carboxy sensor chip through the exposed primary amine groups that are both found on the lysine residues and the N-terminus of the amino acid residues. As a result, the amines can form a covalent bond with the carboxyl surface after it is activated by
Following the blocking step (manufacturer’s buffer) 200 µL of 1 M sodium caprate was administered to disrupt the formed ligand-analyte complex. All proteins analyzed formed at least dimers; these species needed to be disrupted in order to determine new protein–protein interactions kinetics. Triplicate injections of the analyte protein were performed in concentrations of 12.5 µg, 25 µg, 50 µg and 100 µg per 200 µL injections. Caprate injections were performed after each analyte interaction was concluded. The close curve fitting to the sensograms was calculated by global fitting curves (1:1 Langmuir binding model). The data was retrieved and analyzed with TraceDraw software (Kitchener, ON, Canada).

Conclusion

The membrane proteins of the TJ and AJ are key in the formation of the Apical Junction complex and the maintenance of homeostasis. In this study, we used recombinant proteins that preserve the adhesion properties of E-CAD, JAM-A, CLDN1, and OCLN to answer questions that pertain to their interactions in the absence of calcium. Our data suggest that, under these conditions, the TJ components interact with each other, and that TJ membrane proteins interact with the AJ membrane protein E-CAD. Our results extrapolate to the intracellular environment, where calcium concentration can reach >1,000-fold those of the extracellular calcium concentration. Using recombinant proteins and biophysical methods, we have unveiled the intracellular interplay between the TJ and AJ. Future research may examine the extent of interaction between the TJ and AJ at the plasma membrane level, where these components are exposed to high concentrations of calcium.
Author Contributions

C.M. Experimental design, experimental performance, data analysis, manuscript writing, conceptualization, methodology, data curation, original draft, protein purification. This author contributed to every aspect of the research and manuscript preparation. S.H.N. Experimental performance of surface plasmon resonance (SPR), data analysis and sample preparation. K.C. Bacterial culture, inoculation, bacterial cell lysis by French Press. J.M Bacterial culture, inoculation, bacterial cell lysis by French Press. D.M. Experimental design, data analysis, manuscript writing, methodology, data curation, and original draft preparation with its accompanying review and editing. This author mentored all other co-authors. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interests: The authors declare no conflict of interest.
Figure 4.1: Characterization of JAM-A, CLDN1, OCLN, and E-CAD. A) Extracellular domains of human epithelial cadherin (E-CAD), JAM-A, and chimeras CC1, or COC were cloned C-terminal to maltose binding protein (MBP) in pET28a backbone plasmid. B) Proteins were purified with Amylose resin and Size-exclusion chromatography. Proteins were purified to >95% purity, Coomassie blue stain gel. C) Size-exclusion Chromatography showed that JAM-A and E-CAD elute as dimers, and that CC1 and COC elute as larger multimers.
Figure 4.2: Determination of Secondary Structures of CC1, COC, JAM-A and E-CAD by Circular Dichroism (CD). A) Circular dichroism analysis comparison of all MBP fused proteins, extracellular domains of E-CAD, JAM-A, CC1 and COC compared to non-fused MBP. B) The analysis of the Circular Dichroism results for the target proteins, including non-fused MBP are presented in a table, corresponding to the secondary structure. The table represents α-helix (Alpha), β-sheet (Antiparallel, Parallel), turn or other (coiled).
Figure 4.3: Surface Plasmon Resonance Characterization of Homotypic Interactions of MBP JAM-A, MBP CC1, MBP COC and MBP E-CAD. Homotypic interactions of MBP JAM-A, MBP CC1, MBP COC, and MBP E-CAD were determined by surface plasmon resonance (SPR). Due to the large amount of evidence for the homotypic interaction of MBP E-CAD, we normalized the affinity value of homotypic MBP E-CAD. Thus, the Y-axis represents the normalized affinity, for example, MBP JAM-A/MBP E-CAD as a ratio. The X-axis describes the homotypic interactions of the samples. These values are based on taking the $K_D$ value from each sample and dividing it by the $K_D$ values for MBP E-CAD v. MBP E-CAD shown in Table 4.1.
Table 4.1: Surface Plasmon Resonance Analysis. Protein-protein interactions were analyzed using SPR (see Materials and Methods). The data was analyzed with TraceDraw software. The table represents the values for constant of association, $K_a$ ($1/(M*seconds)$); constant of dissociation, $K_d$ ($1/seconds$); and constant of affinity, $K_D$ ($M$).

<table>
<thead>
<tr>
<th>PPI evaluated</th>
<th>$K_a$ ($1/(M*seconds)$)</th>
<th>$K_d$ ($1/seconds$)</th>
<th>$K_D$ ($M$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP E-CAD v. MBP E-CAD</td>
<td>7.90E+02 ± 2.14E+01</td>
<td>3.29E-04 ± 2.72E-06</td>
<td>2.28E-06 ± 4.43E-07</td>
</tr>
<tr>
<td>MBP JAM-A v. MBP JAM-A</td>
<td>8.79E+02 ± 4.36E+01</td>
<td>4.70E-04 ± 1.42E-06</td>
<td>5.57E-07 ± 3.74E-08</td>
</tr>
<tr>
<td>MBP CC1 v. MBP CC1</td>
<td>8.88E+04 ± 1.53E+02</td>
<td>2.42E-05 ± 3.09E-06</td>
<td>2.70E-10 ± 3.64E-11</td>
</tr>
<tr>
<td>MBP COC v. MBP COC</td>
<td>3.19E+04 ± 2.84E+02</td>
<td>3.17E-05 ± 3.80E-06</td>
<td>3.14E-08 ± 1.18E-09</td>
</tr>
<tr>
<td>MBP JAM-A v. MBP CC1</td>
<td>2.45E+04 ± 1.69E+02</td>
<td>3.76E-04 ± 2.24E-06</td>
<td>1.34E-07 ± 4.45E-09</td>
</tr>
<tr>
<td>MBP JAM-A v. MBP COC</td>
<td>9.04E+04 ± 4.15E+02</td>
<td>1.22E-05 ± 3.55E-06</td>
<td>1.34E-10 ± 4.06E-11</td>
</tr>
<tr>
<td>MBP JAM-A v. MBP E-CAD</td>
<td>1.13E+05 ± 1.92E+02</td>
<td>3.41E-05 ± 3.27E-06</td>
<td>3.41E-10 ± 4.00E-11</td>
</tr>
<tr>
<td>MBP CC1 v. MBP COC</td>
<td>1.27E+03 ± 9.05E+00</td>
<td>1.32E-04 ± 2.66E-05</td>
<td>1.33E-07 ± 1.84E-08</td>
</tr>
<tr>
<td>MBP CC1 v. MBP E-CAD</td>
<td>3.16E+04 ± 7.61E+01</td>
<td>5.18E-05 ± 4.40E-06</td>
<td>1.48E-08 ± 3.71E-09</td>
</tr>
<tr>
<td>MBP COC v. MBP E-CAD</td>
<td>2.20E+03 ± 1.16E+02</td>
<td>6.82E-05 ± 7.33E-06</td>
<td>1.02E-07 ± 3.42E-08</td>
</tr>
</tbody>
</table>
Figure 4.4: Surface Plasmon Resonance Characterization of Heterotypic Interactions of MBP JAM-A, MBP CC1, MBP COC, and MBP E-CAD. The heterotypic interactions were determined by SPR. We studied the affinity of TJ vs TJ and TJ vs AJ components. For each analyzed heterotypic SPR experiment we normalized the affinity (KD) by that of MBP JAM-A v. MBP CC1. The Y-axis represents the normalized affinity, heterotypic interaction sample/MBP JAM-A v. MBP CC1, as a ratio. The X-axis represents the homotypic interaction tested.
Figure 4.5: Conceptual Ranking of the Interactions of TJ and AJ Components. The model generated here represents the possible interactions of TJ and AJ components that were obtained from the SPR results. This model shows the homotypic and heterotypic interactions as well as the final combination of the two (on the right) between MBP JAM-A, MBP CLDN1, MBP OCLN, and MBP E-CAD. The strength of the interactions is ranked from the strongest on top to the weakest on the bottom.
References


CHAPTER 5: Calcium Dependency of the Tight Junctions and the Interplay with Adherens Junction

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Abstract

The Apical Junction Complex (AJC) is a membranal proteic ultra-structure that allows for the formation of complex environments that regulate homeostasis and cellular structure and fate. The tight junction (TJ) and the adherens junction (AJ) are elements of the AJC. Claudins (CLDNs), occludin (OCLN) and the junction adhesion molecules (JAMs) play a key functional role in the cell-cell adhesion function of the TJ. Cadherins (CADs) are calcium-dependent membrane protein residents of the AJ. The assembly of a TJ has been attributed mostly to the formation of CLDN strands. Nevertheless, the coordination of CLDN, OCLN, and JAMs needed to achieve TJ function remains unclear. In this article, we have weighed the structural and functional similarities between TJ and AJ components, and suggest that an interplay between these structures exists. We employed synthetic biology strategies to express the adhesive extracellular domains of JAM-A, epithelial CAD (E-CAD), CLDN1, and OCLN. We examined the homotypic and heterotypic interactions between these TJ and AJ components by means of Surface Plasmon Resonance (SPR) in the presence of calcium. Based on SPR data and evidence present in the literature, we propose a model of a simple TJ, composed of CLDN1, OCLN, and JAM-A. We examine evidence that may explain how the TJ and AJ may interact in order to assemble individually and collectively to form the AJC.

Keywords: junctional adhesion molecule, claudin, e-cadherin, circular dichroism, surface plasmon resonance
Introduction

Epithelial cells form a barrier against the environment but are also tasked to regulate the exchange of molecules between an organism and the environment. Epithelial cells are characterized by polarization of their plasma membrane, resulting in a unique appearance of structurally, compositionally, and functionally distinct surface domains[1]. The Apical Junction Complex (AJC) is a membranal ultra-structure that allows for the formation of compound environments that regulate homeostasis, cellular structure, and fate[2]. The AJC of vertebrate epithelial cells includes tight junctions (TJ), also known as zonulae occludentes (ZO), and adherens junction (AJ), named zonulae adhaerentes (ZA)[3]. Both the TJ and the AJ form continuous circumferential belts around the apicolateral region of polarized epithelial cells and coordinate a highly organized network of cytoskeletal structures[3]. The TJ is important for maintaining cell-cell adhesion while displaying a fence (asymmetry in protein and lipid composition between apical and basolateral cell surfaces) and a barrier function (restriction in the transport of ions and nonelectrolytes through the extracellular clefs between cells)[4,5]. TJ dysfunction has been reported in conditions such as edema, jaundice, diarrhea, inflammatory bowel disease, metastasis, deafness, and more[6-9]. At the AJ, the extracellular domains of cadherins (CADs), calcium-dependent cell-adhesion molecules, mediate adhesion of cells to their neighbors[10]. The intracellular domain interacts with catenins to control the assembly and dynamics of the AJ and modulate connections with the actin cytoskeleton and stimulating signaling pathways[11-14].

The membrane proteins composing the TJs are: claudins (CLDNs), occludin (OCLN), and junctional adhesion molecules (JAMs)[15-17]. Adapter and effector proteins anchor the TJ components to the cytoskeleton to maintain stability of these cell-cell interactions[18]. The role
of CLDN has been thought to be of high importance compared to OCLN in maintaining the barrier integrity of the TJ[19]. JAMs play roles in vertebrate development and hemostasis[20]. Four members of the JAM subfamily, JAM-A, -B, -C, and 4, localize to the TJ[21,22]. Our previous studies showed that the JAMs have both homotypic and heterotypic interactions[23]. Little is known regarding JAMs interaction with other TJ components. In addition, a relationship between TJ and AJ has been suggested where they are physically united by the ZO adapter proteins, signaling molecules, and actin cytoskeletal modifiers [12]. This interplay between TJ and AJ has not been defined in terms of their membrane components, namely CADs, CLDNs, OCLN, and JAMs.

Structurally, CLDNs and OCLN are 4-α-helical membrane proteins, while JAMs are a subfamily of the immunoglobulin superfamily (IgSF) of adhesion receptors[20,24-26]. In the AJ, CADs are calcium-dependent adhesion molecules with a secondary structure homologous to that of the IgSF[26]. However, despite the proximity between the TJ and the AJ, and the fact that JAMs and CADs have similar folding, the literature offers no direct evidence for calcium-dependency of TJ membrane proteins or their function. Understanding if the interplay between TJ and AJ is calcium-dependent can be of great importance. Calcium levels in plasma can range from 1.8-2.7 mM [27]. In contrast, intracellular levels of Ca\(^{2+}\) range from 0.3 to 1 mM[28]. In structural studies using epithelial CAD (E-CAD), the low Ca\(^{2+}\) concentrations (<1 mM) caused the protein to form cis-dimers[29,30]. In the case of high Ca\(^{2+}\) concentration (>1 mM), E-CAD formed trans-dimers[30]. These experiments indicate CADs may form cis-dimers intracellularly and at low Ca\(^{2+}\) concentrations in the extracellular milieu but switch to trans-dimers once exposed to the higher levels of Ca\(^{2+}\), resulting in cell-cell adhesion. Calcium, via CADs, acts as a molecular switch that controls cell-cell interactions, cytoskeleton remodeling, and cellular
Intracellular calcium plays an important role in the reassembly of the TJ and was suggested to be necessary for the dissociation of TJ-cytoskeletal complexes, thus influencing TJ reassembly and paracellular permeability barrier restoration[34]. One final piece of evidence that Ca\textsuperscript{2+} may influence the AJC, not simply the AJ, is its role in the viral breach of epithelial barrier. Viruses are proficient at utilizing Ca\textsuperscript{2+} signal to create a cellular environment that meets their own demands[35]. CLDNs have been implicated in the infection process of several medically important human pathogens, including the hepatitis C virus, dengue virus, West Nile virus and HIV, among others[35,36]. Many cell-adhesion molecules that belong to the IgSF, JAM-A among them, have been identified as viral receptors[37]. Epithelial CAD (E-CAD) acts as an entry factor for hepatitis C virus[38]. Mechanistic studies demonstrated that E-CAD is intimately associated with CLDN1 and OCLN on the cell membrane, and that depletion of E-CAD drastically diminished the cell-surface distribution of these two TJ proteins, indicating that E-CAD plays an important regulatory role in CLDN1/OCLN localization on the cell surface and that together, as an ultrastructure, may play a role in viral entry[38].

Many challenges to the study of the TJ components have been based on the purification process. The classical approach for expressing single-transmembrane membrane proteins is to eliminate the hydrophobic domain and work directly with the soluble domain that is normally responsible for function[39]. This method allows for the solubilization and purification of proteins such as JAM-A[40,41] or CADs[29,30,42]. Prota et al., and previously Kostrewa et al., demonstrated that JAM-A could be successfully crystalized while maintaining tertiary and quaternary organization[40,41]. In the case of CLDNs there has been a successful purification and crystal structure of these proteins, but unfortunately, only the secondary structure is preserved[43]. Finally, a partial crystal structure of the c-terminal intracellular domain of OCLN
is available[44]. Studies examining the hydrophobic interactions for TJ and AJ continue to yield no useful answers as to the interactions or interplay between these structures in the AJC[12]. Additionally, studies examining the interactions between TJ and AJ as a result of electrostatic alterations to extracellular environment, such as calcium concentrations, have been challenging and yielded no reports, to the best of our knowledge. If CADs are calcium-dependent, we may ask if Ca^{2+} transport can be affected at the TJ level. Paracellular calcium absorption is a passive process occurring down an electrochemical gradient and it is considered the major route of absorption when calcium intake is high[45]. At least three ways to control Ca^{2+} absorption have been suggested: 1) by changes in the protein composition of TJ, 2) by controlling the driving forces for Ca^{2+} absorption across the TJ, and finally 3) by direct biophysical interference with either CLDNs or transcellular transport proteins[46]. Some CLDNs are cation-permeable and indeed their dysfunction alters calcium homeostasis and results in disease[47]. Characterizing TJ dependency of Ca^{2+} in cell-adhesion could be crucial to understand the role of Ca^{2+} as a molecular switch for the AJC.

To address this gap in knowledge, we studied the interactions of a very simple AJC composed of JAM-A, CLDN1, OCLN, and E-CAD. This study required that we purify proteins in a fashion that preserves structure-function relationships. Our previous studies used synthetic biology strategies to produce soluble extracellular domains of JAM-A[23], chimeric CLDN1, and chimeric OCLN[48]. All of these proteins preserve the structure-function properties of the native proteins[23,48]. Crystallographic and mechanistic studies have demonstrated that E-CAD switches between cell adhesion-inactive to a cell adhesion-active state based on Ca^{2+} concentration[38,42,49]. Thus, we sought to determine whether other membrane components of the AJC, namely the members of the TJ, JAM-A, CLDN1, and OCLN, are similarly calcium-
dependent. As stated, JAM-A\cite{41,50} and a complex of CLDN1/OCLN/E-CAD\cite{38} are involved in viral entry. Considering that these proteins are components of the AJC and that they play similar roles as part of this structure, we propose that as E-CAD is a calcium-dependent adhesion molecule it is possible that CLDN1, OCLN, and JAM-A may also respond to calcium as a molecular switch. With access to highly pure recombinant proteins representing all components of our system (JAM-A, CLDN1, OCLN, and E-CAD) we decided to address the following questions: 1. Do these TJ components interact with other TJ components? 2. Do the TJ components interact with E-CAD? 3. Is there an effect of Ca\textsuperscript{2+}, acting as a molecular switch, that affects the binding of both TJs and AJs and thus their interplay in the AJC?

Results and Discussion

Cell adhesion is classified by several categories such as cell-cell interactions and cell-basal membrane anchorage. The proteins that are responsible for cell-cell adhesion are membrane proteins that are naturally found in low abundance and require a unique platform for expression and purification\cite{51}. Therefore, the yields of these proteins are difficult to obtain from overexpression experiments in bacteria, yeast, insect cells, or in a cell-free system. To address this low yield, targeted proteins are fused with proteins that will increase yield and maintain stability\cite{52}. In the case of adhesion molecules and other membrane proteins that have a single transmembrane helix, a reliable strategy is to separately study the intracellular or extracellular domains. One example of the use of this strategy is with E-CAD\cite{53}. Crystallographic studies of E-CAD focus on the two most N-terminal IgG domains responsible for adhesion, while neglecting the other three extracellular domains\cite{29}.
JAM-A, CLDN1, OCLN and E-CAD Expression and Purification in Escherichia coli

Extracellular domains of JAM-A and E-CAD (extracellular domains 1 and 2) were expressed as a fusion with maltose binding protein (MBP)[23], here will be named MBP-JAMA and MBP-ECAD, respectively. Chimeric proteins, similarly, fused to MBP, for CLDN1 (MBP-CC1) and OCLN (MBP-COC) were produced. Initially, the designs contained a TEV protease cleavage site located between MBP and the targeted protein, resulting in mixed species and very low yields. To address these limitations, we used the pET28-MBP plasmid, where MBP is located N-terminus of the gene of interest. This strategy was selected according to the merits described in the literature where MBP drives high protein expression and stabilizes the fused target protein, thus enabling proteins to retain their individual structure and function[54,55]. In order to produce proper disulfide bond formation in our targets and obtain high yields of purified targets, we used the SHuffle T7 bacterial strain that is engineered to perform such in the bacterial cytosol instead of the periplasmic compartment[54-57].

Plasmids hosting MBP-JAMA, MBP-ECAD, MBP-CC1, and MBP-COC were transformed into SHuffle cells and where grown at 37°C in LB containing both Ampicillin (required by pET28-MBP) and spectinomycin (required by SHuffle cells)[57]. When bacterial growth reached an OD600 of 0.8-1.0, 0.1 M of IPTG was added. After the addition of IPTG, the bacterial culture continued at 16°C overnight. Protein purification was performed using Amylose resin (New England Biolabs (NEB), Ipswich, MA, USA), followed by Size-exclusion chromatography (SEC). All purified proteins for this study were determined to be >95% pure (Figure 5.1.B).
Size Exclusion Chromatography

SEC identified unique features for the oligomerization of each of our targets: MBP-ECAD, MBP-JAMA, MBP-CC1, and MBP-COC (Figure 5.1.C). MBP-ECAD formed dimers and tetramers as described in the literature[42,53]; similarly, MBP-JAMA displayed an equilibrium between dimeric and tetrameric structure (Figure 5.1.C). The crystal structure of both murine and human JAM-A, in the absence of calcium, results in a dimer as the basic unit of this protein[40,41]. MBP-CC1 and MBP-COC had higher order oligomerization, perhaps in orders beyond 20-monomers[48] (Figure 5.1.C). The unique quaternary organization of these proteins suggest that the differences in oligomerization could arise from differences in folding and binding properties, unique to each to achieve cell-adhesion.

Determination of the Effects of Calcium on the Secondary Structure of Our Targets by Circular Dichroism (CD)

Based on the effects of oligomerization results of the TJ components (JAM-A, CLDN1, OCLN) and AJ (E-CAD), we decided to determine whether there were differences in the secondary structure of the proteins. Figure 5.2 represents two pieces of evidence where the MBP tagged proteins had unique secondary structures. In Figure 5.2.A, we plotted the CD data for all the MBP-fused extracellular domains. The graph indicates that the proteins had unique secondary structures with the fused MBP protein. The CD values were compared with free MBP by looking at the percentages of \(\alpha\)-helix, \(\beta\)-sheet (antiparallel and parallel), turn, and other, which are seen in Figure 5.2.B. Calcium, is responsible for increasing rigidity in E-CAD[58]. Additionally, CD of E-CAD resulted in an increase of the mean molar residue ellipticity suggesting its ability to bind calcium[49]. Compared to our previous report of MBP-ECAD and MPB-JAMA calcium-free experiments [23] we observe that both proteins decrease in \(\alpha\)-helix
and β-sheet content, while doubling their % of Other. These results are not observed for MBP-CC1 or MBP-COC.

Based on both the SEC and CD data, we asked whether the difference in aggregation and secondary structure of these proteins would produce tighter binding in homotypic or heterotypic interactions. To address this question, we performed surface plasmon resonance (SPR). This technique allowed us to measure the binding of these proteins in real time without the use of labels. Using SPR, we determined both the homotypic and heterotypic interactions of E-CAD, JAM-A, CLDN1, and OCLN in the next section.

**Homotypic Interactions of TJ Components and AJ Measured by Surface Plasmon Resonance (SPR)**

The studies performed by Vendome and colleagues demonstrated the properties of homotypic interactions of E-CAD[59]. In our previous study we determined that JAM proteins formed homotypic interactions[23], and we reported homotypic JAM-A interactions to have at least 5-fold greater affinity than homotypic E-CAD interactions. Thus, we decided to quantify the strength of affinity of other TJ components. To accomplish this, we measured homotypic interactions by using SPR, with MBP-ECAD values serving as a standard. Instead of interpreting the absolute value determined by SPR, we normalized the values presented (affinity constant, $K_D$) to the better-studied homotypic E-CAD.

Figure 5.3 shows the normalized affinity values ($K_D$) for the homotypic interactions of the TJ components (MBP-JAMA, MBP-CC1, and MBP-COC). All of the TJ components displayed a higher affinity ($K_D$) for homotypic interactions than MBP-ECAD. MBP-CC1 represented 16-fold higher affinity than MBP-ECAD. MBP-JAMA and MBP-COC presented 3- and 4-fold stronger affinity than MBP-ECAD.
Below we present the SPR data obtained for studies conducted in this study. SPR can be thought of as the following equation: Analyte + Ligand $\leftrightarrow$ Analyte-Ligand (Complex). This equilibrium equation shows that not all of the ligands will be bound to the protein. When the ligand is bound to the protein forming the complex this is considered to be $K_{on}$ (M$^{-1}$s$^{-1}$) or the speed of association. $K_{off}$ is the speed of dissociation (s$^{-1}$). The $K_D$ is the dissociation constant where half the ligand binding sites of the protein are bound to the ligand and half of the ligand is not bound to the protein at equilibrium. Thus, a smaller $K_D$ value means that the analyte and the ligand have higher binding affinity for one another (Nicoya Lifesciences, users manual).

**Heterotypic Interactions of TJ Components and AJ Proteins by SPR**

Cells express multiple proteins that make up the TJ and AJ, however, what is not well understood is whether these TJ and AJ components interact with one another. Studies assume that the TJ components bind to members of the TJ, but they do not show whether they interact with AJ components. Our SPR data helps in understanding the heterotypic interactions of TJ components and the interplay with AJ (E-CAD).

Figure 5.4 suggests that heterotypic interactions between TJ components are favored over homotypic interactions. Additionally, Figure 5.4 shows that the TJ components also interact with E-CAD and AJ components. This sheds light into the belief that there is an interplay between TJ and AJ components. This could suggest that cell-to-cell interactions are more complicated and would differ among cellular environments, resulting in different interactions between TJ and AJ components.

Influenced by the data collected here, the literature accumulated for CADs, and our own previous reports[23,48], we propose a model (Figure 5.5) to explain the interplay between TJ and AJ. While assembling the AJC, initial formation of the AJ leads to assembly of the TJ but E-
CAD is not required to maintain TJ organization[60]. Thus, we propose a model (Figure 5.5) in which nascent components of the AJC (Traffic Vesicle) in the presence of low calcium concentration may form all possible combinations since the lowest affinity measured here is ~250 nM and average intracellular protein concentration is nearing 1-10 µM[61]. Thus, the formation of these complexes may depend on gene expression profiles, mRNA stability, and protein abundance[62]. As TJ and AJ proteins arrive to the plasma membrane, they encounter the ZO and ZA Adapter Networks that, according to the literature [34,46], help the assembly of the TJ and AJ in low calcium concentrations, in the range of 0.3 to 1 mM[28]. This step can sort the different species that will prevail in the formation of the TJ, the AJ, and ultimately the AJC.

Considering that CLDN1 and OCLN are the two species with the highest KD and are independent of calcium, they may act as the two proteins responsible for the barrier function of the TJ. Traffic of CLDNs and OCLN from the endoplasmic reticulum (ER) mobilizes them in cis-interactions, furthermore, when inserted at the plasma membrane cell-cell interactions aid the maturation of CLDN and OCLN trans-interactions[63]. When levels of calcium increase in the extracellular environment (2-3 mM) it acts as a molecular switch to turn on JAM-A and E-CAD to form trans-oligomeric states. In structural studies using the extracellular domains of E-CAD, the low Ca\(^{2+}\) concentrations (<1 mM) caused the protein to form cis-dimers[29,30]. In the case of high Ca\(^{2+}\) concentration (>2 mM), E-CAD formed trans-dimers[30]. Finally, we considered the membrane-to-membrane distance (MTMD) within the AJC. The TJ[64] and AJ[65] reside in separate membrane microdomains. Lipids are responsible for membrane curvature and membranal stress[66]. The distance between two cells at the AJ is ~20 nm[11]. Examining several crystal structures of CLDN proteins[43], the distance between membranes could be estimated to be close to 5 nm. Due to the similarity of structure and function between CLDN and
OCLN it is conceivable that the same is true for OCLN-OCLN or OCLN-CLDN \textit{trans} interactions. JAM-A homotypic \textit{trans} interactions, based on its crystal structure\cite{40,41}, may create membrane distances of \textasciitilde10 \text{nm}. The highly favored JAM-A-OCLN interactions (Table 5.1) may be an intermediary between the shorter CLDN-CLDN contact points and greater gap between membranes created by E-CAD to E-CAD interactions (from \textasciitilde5 \text{nm} to \textasciitilde20 \text{nm}).

Our model combines reports from the literature with quantitative measurements the binding affinity of protein-protein interactions by SPR generated in this study, and previous work performed in our laboratory [23,48] to further our original hypothesis that calcium may act as a molecular switch on both the TJ and AJ membrane protein components. We confirmed that calcium is indeed a molecular switch for E-CAD. We identified JAM-A as a calcium-dependent adhesion molecule, and present evidence to demonstrate that CLDN1 and OCLN’s adhesive properties create calcium-independent tight cell-cell interactions.

Materials and Methods

\textit{Protein Preparation: Cloning, Expression and Purification}

Cloning, expression, and purification of targets (JAM-A, CLDN1, OCLN, and E-CAD) were prepared as described previously\cite{23,48}. Proteins are cloned in pET28 as C-terminal to maltose-binding protein (MBP). Both CLDN1 and OCLN are synthetic chimeras\cite{48} containing full length extracellular domains and 30\% of the transmembrane helices. Plasmids thus prepared are named MBP-ECAD, MBP-JAMA, MBP-CC1 (chimeric CLDN1), and MBP-COC (chimeric OCLN). Plasmids were preserved in glycerol stocks of the bacterial strain DH5\textalpha. After the verification of the plasmid sequence, we transformed the plasmid into SHuffle bacterial cells \cite{27,68} that would express the protein. French Press was performed on the bacterial pellet with
the addition of 100 µL of 0.5 M EDTA. The re-suspended bacterial cells were loaded into the Thermo Spectronic French Pressure Cell Press Model FA-078. Lysis was performed at 1500-2000 psi and the lysate was collected into a new 50 mL tube. The lysate was centrifuged for 30 minutes at 10,000 RPM using a F15-8x50cy rotor from Thermo Scientific. The supernatant was decanted into a 50 mL tube containing Amylose resin from New England Bio Labs (E8021L), and incubated while rotating for 1 hour at 4°C. Column chromatography was performed to collect the protein from the amylose beads. The column was washed with 100 mL of wash buffer containing 500 mM NaCl and 30 mM TRIS. The elution was performed for 3 minutes each time using 100 mM Maltose. The elution was concentrated using the Microsep Advance with 10k Omega centrifugal device (Reference # MCP010C41) from Pall Corporation and centrifuged at 10,000 RPM for 10 minutes until reaching 2 mL of final elution volume.

Size-Exclusion Chromatography (SEC)

Determination of the shift of oligomerization was performed using 0.250 mg of protein in one of the two solutions of 30 mM HEPES, 100 mM NaCl with 3 mM Ca²⁺. The protein was incubated with the buffer for 2 hours before SEC was performed. The sample was injected into the injection valve of the NGC Chromatography System (Bio-Rad). The ENrich SEC 650 column: 10 x 300 mm, 24 ml, prepacked high-resolution SEC 650 size exclusion column, size range 5k–650k Da (BioRad) was used for size exclusion chromatography. The change in the area under the curve was calculated with SEC software (Bio-Rad).
**SDS-PAGE Assay**

Two µg of either boiled MBP, MBP-ECAD, MBP-JAMA, MBP-CC1, or MBP-COC in HEPES containing 3 mM CaCl₂ were electrophoresed on an SDS-PAGE gel (BioRad). Gel staining was performed using standard protocols.

**Circular Dichroism Spectrometry**

Circular Dichroism (CD) measurements were performed using the Spectropolarimeter Model 420 (AVIV Biomedical Inc., Lakewood, NJ USA). Changes in ellipticity at 260 nm to 195 nm using 20 second scans were measured with a 100 µM concentration of protein in a 10 mm QS glass cuvette cell at 37°C. The secondary structure consisting of alpha helix, antiparallel, parallel, turn, or other was determined by the usage of Bestel circular dichroism analysis, [https://bestsel.elte.hu/index.php](https://bestsel.elte.hu/index.php).

**Surface Plasmon Resonance**

Open SPR by Nicoya Lifesciences was performed to determine protein-protein associations of MBP-ECAD, MBP-JAMA, MBP-CC1, or MBP-COC with HEPES with 3 mM Ca²⁺. We performed protein-protein interactions by loading 0.050 mg of ligand protein onto the Carboxy sensor chip. The proteins were immobilized onto the Carboxy sensor chip through the exposed primary amine group found in both the lysine residues and the N-terminal of the amino acid residues. This leads to the amines forming a covalent bond with the carboxyl surface after activation by EDC/NHS[67] The blocking step (manufacturer’s buffer) followed by 200 µL of 0.5 M sodium caprate was administered to disrupt the preformed protein-protein interaction[68,69]. Triplicate injections of the analyte protein were made in the following concentrations: 12.5 µg, 25 µg, 50 µg and 100 µg per 200 µL injections. Caprate injections were
performed after each analyte interaction to be sure that there were no other interactions occurring before the next analyte injection was performed. After the experiments were conducted, the close curve fitting to the sensograms was calculated using global fitting curves using the 1:1 Langmuir binding model. The data was retrieved and analyzed with TraceDraw software (Kitchener, ON, Canada).

Conclusion

In this study we determined the homotypic and heterotypic binding of JAM-A protein to other TJ components such as CLDN1 and OCLN in the presence of calcium. We also determined that JAM-A binds to E-CAD, a member of the AJ, with high affinity in the presence of calcium. We also addressed our initial question of whether calcium acts as a molecular switch regarding cell-adhesion properties of the components of the TJ, considering it does affect the structure and function of CADs. The strongest homotypic interactions were with chimeric CLDN1. MBP-CC1 homotypic interactions reported by our group[48] are similar to those obtained in this study in the presence of calcium. We found that the same applied to MBP-COC homotypic interactions. Our data suggest that CLDN1 and OCLN may be calcium-independent cell-adhesion molecules. On the other hand, MBP-JAMA homotypic and heterotypic affinities are influenced by calcium. Little is known about JAM-A’s specific role in the TJ. We have presented a strategy for the study of the extracellular domains of JAM proteins, enabling future studies to better understand TJ assembly and function. Finally, we proposed a model for a simple TJ, the composition of the TJ in the membrane, and the interactions between the different membrane proteins. Our present data suggest that high extracellular calcium levels may be another step in the regulation of the AJC and help provide a strict barrier between the AJ and the TJ, in spite of reports of their association. Thus, protein-protein interaction, intracellular adapter networks, intracellular and
extracellular calcium levels, and further membrane-to-membrane distance may be a few of the elements responsible for the AJC assembly and the TJ and AJ’s function.
Figure 5.1: Characterization of MBP-ECAD, MBP-JAMA, MBP-CC1 and MBP-COC Proteins. A) Extracellular domains of target proteins were cloned C-terminal to maltose-binding protein (MBP) in the pET28a backbone plasmid, ampicillin resistant. B) Coomassie stain of purified proteins followed our Amylose-SEC tandem protocol (see Materials and Methods). The proteins had >95% purity. C) SEC shows that each protein has a unique quaternary structure in the presence of 3 mM CaCl₂. MBP-ECAD forms an equilibrium between dimers and tetramers, similar to MBP-JAMA. MBP-CC1 and MBP-COC form large oligomeric states.
Figure 5.2: Circular Dichroism (CD) Analysis of MBP-ECAD, MBP-JAMA, MBP-CC1, and MBP-COC. A) CD analysis comparison of all MBP fused proteins. Folding differences were observed for all of the fusion proteins. CD experiments were performed at 37°C (see Materials and Methods). B) The analysis of each curve for the proteins of interest including non-fused MBP is presented in the table. Data is presented as the distribution of the secondary structure elements. The content of α-helix, β-sheet (antiparallel and parallel), Turn, or Other is presented.
Figure 5.3: Surface Plasmon Resonance (SPR) Characterization of Homotypic Interactions of MBP-ECAD, MBP-JAMA, MBP-CC1, and MBP-COC. Homotypic interactions were determined by SPR. The homotypic interaction $K_D$ was normalized by that of MBP-ECAD. The Y-axis represents the normalized affinity for example CLDN1/E-CAD as a ratio. The X-axis represents the homotypic interactions that were tested. These were calculated by obtaining the $K_D$ values from each sample and dividing them by that of the homotypic MBP-ECAD interaction (see Table 5.1).
Figure 5.4: Surface Plasmon Resonance (SPR) Characterization of the Heterotypic Interactions of MBP-ECAD, MBP-JAMA, MBP-CC1, and MBP-COC. Heterotypic interactions were determined by using SPR. We studied the combinations of the targeted proteins and normalized the affinity (K_D) by that of MBP-JAMA v. MBP-ECAD. The Y-axis represents the normalized affinity such as Sample/JAM-A v. E-CAD as a ratio. The X-axis represents the heterotypic interaction examined.
Table 5.1: Analysis of the Protein-Protein Interactions was Performed by SPR. The data was analyzed by using TraceDraw software. In this table, we present the values for constant of association, $K_a$ (1/M*s); constant of dissociation, $K_d$ (1/s); and constant of affinity, $K_D$(M). Experiments were conducted using 3 mM CaCl$_2$.

<table>
<thead>
<tr>
<th>PPI evaluated</th>
<th>$K_a$ (1/(M*s))</th>
<th>$K_d$ (1/s)</th>
<th>$K_D$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP-ECAD v. MBP-ECAD</td>
<td>2.37E+03 ± 2.71E+02</td>
<td>5.13E-04 ± 1.18E-05</td>
<td>2.18E-07 ± 3.19E-08</td>
</tr>
<tr>
<td>MBP-JAMA v. MBP-JAMA</td>
<td>4.23E+03 ± 1.21E+02</td>
<td>3.36E-04 ± 4.90E-06</td>
<td>7.92E-08 ± 3.31E-09</td>
</tr>
<tr>
<td>MBP-CC1 v. MBP-CC1</td>
<td>8.88E+04 ± 1.53E+02</td>
<td>2.42E-05 ± 3.09E-06</td>
<td>2.70E-10 ± 3.64E-11</td>
</tr>
<tr>
<td>MBP-COC v. MBP-COC</td>
<td>2.02E+03 ± 6.73E+01</td>
<td>1.07E-04 ± 7.78E-06</td>
<td>5.71E-08 ± 6.41E-09</td>
</tr>
<tr>
<td>MBP-JAMA v. MBP-CC1</td>
<td>4.19E+03 ± 4.28E+01</td>
<td>1.74E-04 ± 3.85E-06</td>
<td>6.61E-08 ± 4.03E-09</td>
</tr>
<tr>
<td>MBP-JAMA v. MBP-COC</td>
<td>2.98E+03 ± 1.38E+02</td>
<td>2.66E-04 ± 6.31E-06</td>
<td>8.96E-08 ± 6.32E-09</td>
</tr>
<tr>
<td>MBP-JAMA v. MBP-ECAD</td>
<td>2.68E+03 ± 7.00E+02</td>
<td>7.12E-04 ± 2.78E-05</td>
<td>2.63E-07 ± 8.48E-08</td>
</tr>
<tr>
<td>MBP-CC1 v. MBP-COC</td>
<td>2.32E+03 ± 2.13E+02</td>
<td>2.72E-04 ± 1.69E-05</td>
<td>1.36E-07 ± 4.26E-08</td>
</tr>
<tr>
<td>MBP-CC1 v. MBP-ECAD</td>
<td>1.69E+03 ± 2.48E+02</td>
<td>1.04E-03 ± 4.05E-06</td>
<td>6.06E-07 ± 8.62E-08</td>
</tr>
<tr>
<td>MBP-COC v. MBP-ECAD</td>
<td>2.54E+03 ± 1.73E+02</td>
<td>4.64E-04 ± 9.29E-06</td>
<td>2.34E-07 ± 3.29E-08</td>
</tr>
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</table>
Figure 5.5: Ranking of the Interactions in the Assembly of the AJC and Model of Calcium Dependency of the TJ and AJ Components. A) Rankings (high $K_D$ on the top) of the possible interactions based on the binding affinities obtained from SPR (Table 5.1). The data is presented as the ranking of Homotypic, Heterotypic, and a Combination of both. The highest binding is that of homotypic CLDN1. B) TJ and AJ assembly. Low calcium in traffic vesicles and extracellularly (orange), act as molecular switch, preserving molecules in an cell-adhesion inactive state. Trafficked molecules reach the plasma membrane where they meet with ZO or ZA adapter network proteins (orange and green triangles, respectively) where they form a complex with actin. Mature CLDN1 and OCLN strands form cell-cell contacts. C) In the presence of high concentrations of calcium, cell-cell interactions form for JAM-A and E-CAD. CLDN1 and OCLN are not affected by calcium levels.
References


Junctional adhesion molecules (JAMs) are important components of the tight junctions (TJs) that govern cell-cell interactions, form tissue barriers, membrane fences (asymmetry), and signal transduction [1]. The purpose of the research performed in our study summarized in Chapter 2 was to determine the role of JAM proteins on TJ formation and function. Much of what is presently in the literature was gained from experiments in cells and tissue. We anticipated that to move the field forward it was crucial to have access to recombinant proteins and to use crystal structures and biophysical methods. The lack of understanding on JAM proteins is based on the assumptions that all of the JAM proteins behave as dimers, similar to what was determined with the JAM-A crystal structure.

The lack in understanding that the JAM proteins produced only homotypic interactions is based on studies performed without the use of recombinant proteins for JAM-B, JAM-C and JAM 4 [2]. Once we normalized our recombinant expression of the extracellular domains of JAMs, the following strategy enabled us to determine that JAMs had homotypic interactions with all four members of the family. In chapter 2, we addressed the differences in the oligomerization of JAM-B, JAM-C and JAM 4. These JAM proteins had higher-order quaternary organization than those of the dimer reported in the literature.

We also showed that these JAM proteins had both homotypic and heterotypic binding that could present in different tissues and environments based on tissue-specific expression, mRNA stability, and protein abundance. Our strategy was designed to better understand how these proteins would bind in vitro in order to determine how they would interact.

These data demonstrate that there is more favorable binding between the heterotypic interactions as compared to homotypic interactions. This indicates that there could be
interactions of JAM protein family members such as the most hydrophobic JAM-C and JAM 4, that could pave the way to an understanding of regions that are impermeable, such as the blood brain barrier (BBB) [3]. Therefore, there could be the possibility for interactions between JAM-C vs JAM 4 to occur as well as JAM 4 vs JAM 4 and JAM-C vs JAM-C. Other interactions such as JAM-A vs JAM-A, JAM-A vs JAM-B and JAM-B vs JAM-B could be found in tissues such as the kidney where the TJ barriers are more permeable for the transport of ions and water [4]. This tissue might need to have weaker interactions, to allow for transport of solutes and water through the cellular environments. Due to the lack of information on the effects of cations on JAM proteins, we decided to use cations to learn more about these interactions.

There are misconceptions in the literature that JAM-A would only form dimers, as observed in solution in the absence of cations [5]. The addition of cations could affect the oligomerization and folding of JAM-A by acting as a molecular switch, to allow for strengthening of the protein-protein interactions that in turn lead to an increase in cell-to-cell interaction. Since JAM-A is part of the TJ and is structurally similar to CADs [6-8], we determined the effects that cations would have on JAM-A. We hypothesized that these proteins would share similar functions in the increase in binding and rigidization as a molecular switch through the use of cations.

This led us to ask the following questions: What are the molecular switches that regulate protein-protein interactions? Could cations such as calcium regulate these protein-protein interactions? By understanding the effects that cations had on JAM-A as a molecular switch we determined the effects that the cations had on other TJ components (CLDN1 and OCLN). Finally, we determined the interplay between TJ and AJ (E-CAD) components and how JAM-A is at the center of these events.
In chapter 3 we demonstrated that JAM-A was affected by cations, in such a way that changed its secondary structure, oligomerization and binding. The secondary structure was determined to be affected dramatically by the different cations, affecting α-helix, β-sheet, and unstructured content as determined by Circular Dichroism. These effects were also seen in oligomerization experiments performed by Size-Exclusion Chromatography that showed in differences in oligomerization states such as tetramers and dimers in PBS. Cations also affected the homotypic binding affinity of JAM-A, shown by Surface Plasmon Resonance (SPR). The effect of cations increased cell-to-cell interactions as seen in our JAM-A overexpression experiments, when exposing these cells to zinc, calcium, and magnesium increased cellular aggregation. Comparing this result with overexpressed JAM-A resulted in no homotypic binding of JAM-A and no cell-to-cell contacts. Therefore, we determined that cations act as a molecular switch to JAM-A that affect the secondary structure, oligomerization, and binding of JAM-A. Due to the results of the effects of cations on binding, we decided to determine whether other TJ components were affected by calcium.

In chapter 4, we addressed whether there is an interplay between the TJ and AJ components. Based on the different cell types and the possibilities that TJ and AJ components could have on expressing different CLDNs, and JAMs, we used a simple TJ strategy based on the composition in CAL27 cells [9] that contain CLDN1, OCLN and JAM-A. The AJ E-CAD was also used in this study since Cal27 cells are epithelial squamous tongue cells that are known produce this protein [9]. Therefore, we purified TJ components (CLDN1, OCLN, JAM-A) and AJ (E-CAD) in the absence of cations in order to study their interactions. The results showed that without the presence of cations the TJ components have both homotypic and heterotypic interactions, and that these TJ membrane proteins interact with the AJ membrane protein E-
CAD. For example, the strongest homotypic interactions were seen with CLDN1 and OCLN compared to JAM-A and E-CAD. The strongest heterotypic interactions were seen with JAM-A vs. OCLN, JAM-A vs E-CAD and CLDN1 vs E-CAD. This suggests that there is an interplay between TJ and AJ proteins. This led us to determine whether cations such as calcium would affect the binding between TJ and AJ proteins.

In chapter 5, we addressed the question of what effect calcium had on the TJ and AJ proteins. To do so, we purified the TJ (CLDN1, OCLN, JAM-A) and the AJ (E-CAD) components in the presence of calcium, at a concentration mimicking its extracellular concentration. The results showed that calcium affects the binding of TJ components such as JAM-A, but has a smaller effect on CLDN1 and OCLN. This would mean that CLDN1 and OCLN would work as anchors to stabilize the cell-to-cell interactions and therefore may be calcium independent. CLDN1 and OCLN did not produce a significant peak shift in Size-Exclusion Chromatography (SEC) experiments. However, there was more of an effect on the peak shift seen in SEC with JAM-A and E-CAD, reinforcing the concept that these two proteins are calcium-dependent, and that CLDN1 and OCLN are calcium-independent.

The conclusion of this work is that there are homotypic and heterotypic interactions between the JAM proteins (JAM-A, JAM-B, JAM-C, and JAM 4). These interactions are dependent on whether the cellular environment is hydrophobic or hydrophilic and seems to correlate the presence of certain JAMs with the homeostatic permeability required by the endothelial or epithelial tissue. The effects of cations would play a role in the secondary structure, oligomerization, and binding of JAM proteins which was observed with JAM-A. It is possible that the other JAM proteins are also affected by cationic switches. In preliminary
experiments with JAM-C, which is more hydrophobic, cations seem to prevent the formation of higher order oligomers as observed with PBS.

Taken together, our studies show that cations such as calcium affect the members of the TJ such as JAM-A and the AJ component E-CAD. However, calcium does not affect CLDN1 and OCLN as much since there is no shift in the peak of these proteins shown in SEC, and the binding affinity is not affected. This suggests that CLDN1 and OCLN could be anchors for maintaining the cell-to-cell interaction while JAMs and E-CAD react to cations to increase or decrease permeability.

Collectively, these data show that proteins such as CLDN1 and OCLN are the anchors that maintain cell-to-cell adhesion, while the rest of the proteins such as JAM-A and E-CAD are affected by cations that would allow for the cells to move away from one another slightly (relaxing contact points), allowing for communication, metabolite transport, or signaling to occur.

Translating these finding to my experiences in the US Navy, combined with information provided by the staff at the USS Midway helped me further understand how TJs and AJs work to produce cell-to-cell interactions. Our data suggest that JAM-A and E-CAD proteins are similar to the lines on a ship that are placed at an angle in order to maintain the ship in place while the water is continuously moving. The angle of these lines stabilizes the ship in its docking position. When there is tension in the lines, they are relaxed to prevent them from snapping off and stabilize the ship. Additionally the crossing over of the lines allows to stabilize the ship and therefore, decrease the tension. Therefore, it is possible that JAM-A and E-CAD are also positioned at an angle to allow the cells to be kept together while at the same time decreasing tension between the other TJ components. In the case of CLDNs and OCLN, these two proteins
may be the anchors that maintain the position of the cells in place while the dynamic changes in JAM-A and E-CAD can be represented as the lines tying the ship to the dock.

Figure 6.1: Model of the Role Membrane Proteins of the TJs and AJs Play in the Regulation of Cell Adhesion. The cell-to-cell environment would be similar to that of a Naval ship docked at a pier. The ship is held in place while being connected to the pier through lines (ropes) and is anchored to the sea floor by the chain and anchor. Based on the findings in our study, we determined that CLDN and OCLN correspond to the anchor and chain, enabling cells to maintain cell-to-cell interactions without interruption. The proteins that are sensitive to cations such as calcium are JAM-A and E-CAD. These proteins are responsible for regulating the dynamic cell-to-cell interactions, like the ropes between the pier and the boat. JAM-A and E-CAD would increase or decrease binding between cells to allow for a response to the changes produced by neighboring cells (metabolites and signaling molecules) and the changing environment.

The anchor is not what keeps the ship in place, it is actually kept in place by the weight of the chain. If the weights of CLDN1 (24 kDa), OCLN (57 kDa), JAM-A (33 kDa) and E-CAD (82 kDa) are compared, we could erroneously assign E-CAD the role of maintaining cell-to-cell interactions, not CLDN1 or OCLN. However, when we consider that the chain maintains the ship in place, in this case the intracellular adapter proteins of the TJ, ZO-1 would be 200 kDa bound to CLDN1 and OCLN. E-CAD has internal connections to α- and β-catenin which
together weigh approximately 180 kDa. Therefore the 200 kDa chain would be heavier than that of the 180 kDa combined α- and β-catenin, reflecting the function of the chain of the anchor.

Looking at the Size Exclusion-Chromatography data in our study and considering that CLDN1 and OCLN form large oligomers that result in an increase in weight, this would mean that these two proteins are the anchors. As for JAM-A and E-CAD, each has a more discrete oligomeric number such as dimers and tetramers, which make a smaller ultrastructure compared to that of CLDN1 and OCLN, and therefore, would not have as much weight in increasing the cell-to-cell interaction. The smaller JAM and E-CAD structures are necessary to allow for a more dynamic interplay, as well as allow for the local passage of signals between cells. If CLDN1 and OCLN were affected by cations they could completely destroy the cell-to-cell interactions leading to cell death. Collectively, this means that JAM-A and E-CAD are affected by extracellular calcium and act as lines on a ship in order to maintain the cell-to-cell interaction, but these interactions are dynamic and allow for a decrease in tension depending on the molecular switch or cation that they are exposed to. In comparison, CLDN and OCLN act as the heavy chain and anchor between cells thus being responsible for the integrity of the tissue, while JAM and E-CAD allow for cellular communication to occur. CLDN1 and OCLN would act as the anchors that would maintain the cell-to-cell interactions regardless of the cations that are present (Figure 6.1).

Future Directions

With the confirmation that cations such as calcium affect the oligomerization and homotypic and heterotypic binding of JAM-A and E-CAD, there needs to be a better understanding on how other proteins involved in cell adhesion such as Connexins, CD9, CD81, and MARVELD2 are affected by calcium or other cations. Based on the effects that cations have
on the oligomerization, secondary structure, and binding of JAM-A, we still need to understand whether cations such as zinc, copper, iron II and iron III have an effect on CLDNs, OCLNs, Connexin, CD9, CD81 and MARVELD2. Studies need to be performed both in vitro and in vivo to understand the cues that these proteins respond to. This is based on the understanding that these cell-to-cell interactions have “kissing points” (very close contact points) that are regulated and bring the membrane of two cells to close contact where negative charge may repel the two cells. It is possible that cations such as zinc would allow for tighter binding to occur in TJ components such as JAMs and E-CAD, but they may not have an effect on CLDNs and OCLN as these components need to be anchored. These signals would therefore come from either the blood or vesicles packed with cations and signaling molecules that would secrete calcium into the cells and perhaps even to the TJ components, and therefore charge the membrane, triggering conformational changes to increase or decrease TJ or AJ formation. Another point to consider would be the type of cell and cellular environment where these TJ and AJs co-exist. For example, we would expect that neurons would have a different population of TJ components that may be more hydrophobic, but this would also depend on what brain regions these TJ and AJ components are located. For example, the synaptic cleft could have vesicle signals with calcium that would affect the TJs and AJs in the targeting cell, which would lead to either an increase or decrease of these cell-to-cell interactions. Therefore, we would need to understand the signals that affect these TJs in different regions of the brain, BBB, blood, and water exposed regions such as the kidney.

To summarize, the TJs could be regulated by the following: 1. Cations present in the blood or the extracellular milieu. These cations may act directly on JAMs, coordinating the interplay between the TJ and AJ. 2. Vesicles and granules. These JAMs could allow the
movement of vesicles and granules from one cell to another. 3. Tissue-specific expression of TJ and AJ components. The tissue-specific expression of TJ and AJ components could change depending on the requirement of the cellular environment such as having more hydrophobic JAMs (JAM-C or JAM4) in the blood brain barrier. As for other tissues that are exposed to water and calcium these TJ components would need to be more permeable, JAM-A and JAM-B. Other possibilities would be that the expression of JAMs would require either homotypic and heterotypic TJ components such as JAM-A vs JAM-A, JAM-A vs JAM-B or they may also have different types of CLDNs, and CADs. 4. Cation toxicity due to cellular death. Iron and other cations can reach toxic levels in the brain upon cell death, leading to concentration changes in the extracellular environment.

In conclusion depending on the type of cell and cellular environment there may be different levels of expression of TJ and AJ components that are regulated by cations such as zinc, calcium, iron, copper and magnesium. This regulation would allow for the strengthening or relaxing the TJ and AJ components, while maintaining CLDNs and OCLN as anchors to maintain cell-to-cell interactions. Therefore, it is important to determine the possibility of the interaction between these TJ and AJ components, their interplay, and regulation by cations in order to understand the different cellular environments and allow for tissue-specific drug design to allow for future treatments such as cancer, and mental health conditions such as Post-Traumatic Stress Disorder (PTSD).
References


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