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Characterization Of The Interactions Of Calmodulin With Fas And Death Receptor-5, And Determine The Role Of Calmodulin - Death Receptor-5 Binding In Disc Formation In Breast Cancer.

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CHARACTERIZATION OF THE INTERACTIONS OF CALMODULIN WITH FAS AND DEATH RECEPTOR-5, AND DETERMINE THE ROLE OF CALMODULIN - DEATH RECEPTOR-5 BINDING IN DISC FORMATION IN BREAST CANCER

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ABSTRACT

Breast cancer is one of the most commonly diagnosed cancers. Calmodulin (CaM) is involved in regulating breast cancer growth, proliferation, and transformation to malignancy. CaM has been shown to play a role in regulating Fas and DR-5 mediated apoptosis. The Fas receptor apoptotic pathway has been well characterized in cholangiocarcinoma; however, targeting Fas for anti-cancer therapy has shown hepatotoxicity *in vivo*. Thus, *in vitro* studies with breast cancer will focus on the DR-5 mediated apoptotic pathway. Targeting DR-5 to regulate breast cancer apoptosis is a promising strategy for breast cancer treatment. Understanding the mechanism of Fas and DR-5 interaction with CaM is imperative to unveil CaM-Fas/DR-5 binding as a potential target for novel strategies to modulate Fas or DR-5 apoptosis. Here the interactions of CaM with Fas death domain (DD) were characterized using a combined isothermal titration calorimetry (ITC), and circular dichroism spectroscopy (CD) approach. Furthermore, an interaction of CaM and DR-5 was characterized and the role of CaM-DR-5 binding in DR-5-mediated DISC formation was determined in MCF-7 and MDA-MB-231 breast cancer cells. ITC showed an endothermic and an entropy-driven CaM-Fas DD WT binding interaction at 37^{o}C. The binding of CaM and Fas DD was significantly decreased by the Fas DD V254N mutation. Far UV CD showed that the V254N mutation resulted in secondary structure changes in the Fas DD. The structural changes of Fas DD

V254N could affect CaM and Fas DD interaction. These results provided the structural and thermodynamic evidence to explicate the role of the V254N mutation of Fas in CAM-Fas interaction. For the characterization of CaM and DR-5 interaction, co-immunoprecipitation experiments using a DR-5 antibody demonstrated the interactions of CaM with DR-5. Further, upon DR-5 activation by TRA-8, CaM is recruited into the DR-5 mediated (DISC) and CaM antagonist trifluopherazine (TFP) attenuated DR-5 mediated DISC formation in MCF-7 and MDA-MB-231 breast cancer lines. The results showed the important role of CaM-DR-5 binding in DR-5 mediated DISC formation in breast cancer cells.

Keywords: Breast Cancer, DR-5, DISC, Calmodulin, Fas, Isothermalt titration calorimetry, Circular dichroism spectroscopy.

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LIST OF ABBREVIATIONS

AKT	Protein Kinase B
Apaf-1	apoptotic protease activating factor-1
Bak	Bcl-1 antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell leukemia/lymphoma 2
Bcl-XL	B-cell leukemia/lymphoma X linked protein
Bid	BH-3 interacting death domain agonist
CaM	Calmodulin
CaMKII	Calmodulin Kinase II
CD	Circular Dichroism
DD	Death Domain
DISC	Death Inducing Signaling Complex
DR-5	Death Receptor 5
ER	Estrogen Receptor
ERK	Extracellular Signal-Regulated Protein Kinase
FADD	Fas Associated Death Domain
Fas DD	Fas Death Domain
FLIP	Flice like inhibitor portion
HER2	Human Epidermal Receptor-2
IKK	I kappa B kinase
ITC	Isothermal Titration Calorimetry
JNK	c-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MCL-1	Myeloid leukemia cell differentiation protein
MD	Molecular dynamics
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PARP-1	Poly DP-ribose Polymerase 1
PI3K	Phosphotydle-inositol-3 kinase
PR	Progesterone Receptor
RIP	Receptor interacting protein
siRNA	Small interfering ribonucleic acid
SMAC/DIABLO	second mitochondrial activator of caspases/ direct

	inhibitor of apoptosis-binding protein with low isoelectric point
TAK1	Transforming growth factor activated kinase-1
TRADD	TNF receptor-1 associated dead domain protein
TRAF2	TNF receptor associated factor 2
TRAIL	Tumor Necrosis Factor Receptor Apoptosis Inducing Ligand
V254N	Valine residue 254 substitution to Asparagine
W-7	N-(6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide
XIAP	X-chromosome linked inhibitor for apoptosis protein

CHAPTER 1

INTRODUCTION AND SIGNIFICANCE

Breast Cancer Pathology and the Role of Calmodulin

Breast cancer is the unregulated growth of cells in breast tissue, including carcinomas of breast epithelial tissue layers, adenocarcinoma of breast glandular tissue and sarcomas of breast connective tissue [(1)]. Breast cancer is one of the most commonly diagnosed cancers worldwide accounting for 23% of new cancer cases and 14% all cancer cases per year as of 2008, with nearly 1 in 8 women being diagnosed with breast cancer in their life time [(2, 3)]. Breast cancer accounts for nearly 1 in 4 cancers diagnosed in women in the US and the second leading cause of cancer death in US women [(2, 4)]. Molecular and genetic typing of breast cancer cell has characterized 5 different breast cancer subtypes: Basal-like (triple negative), Normal-like, Luminal A, Luminal B and Human epidermal receptor -2 (HER2) over expressing [(5-8)]. The luminal A and B subtypes are estrogen (ER) and progesterone (PR) hormone receptor positive and classify the majority of breast cancers. The basal subtype is void of the estrogen and progesterone receptors and does not over express HER2. The HER2 over expressing subtype have an amplified expression of HER2 and may be ER and PR positive or negative [(9, 10)]. Conventional cancer chemotherapy nonspecifically induce cytotoxicity in rapidly reproducing cells, and is the preferred treatment in breast cancer cases with receptor-negative tumors [(11)]. Conventional chemotherapy for breast cancer have steadily

increased the survival rate, however, drug resistance, toxicity and poor drug response are critical issues that still hinder the effective treatment of breast cancer [12]. Breast cancer resistance to conventional radiation and chemotherapy has made the development of new treatments necessary [13, 14]. Molecular classification of breast cancer subtypes in clinical research and in clinical practice establish target markers that influence breast cancer cell growth guiding individualized treatment for breast cancer patients [15, 16]. The use of targeted therapy to inhibit the activity of key markers such as tamoxifen for estrogen receptor positive and trastuzumab for HER2 over expressing breast cancer subtypes has decreased toxicity and improved treatment of metastatic breast cancer [17-19]. However, many breast cancer types have developed resistance to targeted therapy or lack the specific targets, hindering the effective use of targeted treatment options. Activation of Fas or DR-5 receptors has been shown to induce caspase dependent apoptosis in breast cancer cell lines [9, 20, 21]. The induction of breast cancer apoptosis by targeting Fas or DR-5 death receptor mediated apoptosis may be a potential strategy for breast cancer treatment. Understanding the mechanism of Fas and DR-5 mediated apoptotic signal regulation in breast cancer is imperative to unveil potential target(s) that can modulate Fas and DR-5 mediated apoptosis and lead to the development of novel strategies for effective targeted breast cancer treatment.

The intracellular protein calmodulin (CaM) functions as a mediator of Ca^{2+} signals and regulates cellular processes including cell motility, chemotaxis, gene expression, and secretion protein synthesis[22, 23]. Increased expression of CaM is associated with breast cancer and increased CaM levels has been correlated to cancerous cellular transformation to malignancy [24, 25]. CaM may play a significant role in the

progression of HER2 over expressing breast cancer subtypes by regulating the activity of the HER2 receptor. It has been reported that CaM directly binds to HER2 in a calcium dependent manner and CaM's interaction with HER-2 has been shown to regulate HER2 stimulated breast cancer cell growth [26-28]. Similarly CaM may also regulate the hormone receptor positive luminal breast cancer subtypes. High expression of CaM have been reported in ER positive breast cancer cells [29]. CaM directly interacts with ER [5, 30-33], and CaM binding to ER has been shown to regulate ER's affinity for the hormone estrogen [22, 23, 34]. Short term treatment (<72 hours) of breast cancer cells with calmodulin antagonists TMX, TFP, W-7 and R24571 inhibits breast cancer cell growth, presumably by CaM's regulation of pathways controlling the cell cycle [35-37]. Extended treatment (≥72 hours) breast cancer cells with CaM antagonists TMX and R24571 can induce breast cancer cell apoptosis [37, 38]. In lymphocytes CaM expression is increased during glucocorticoid mediated apoptosis[39] and ionomycin, an ionopore that increases the influx of Ca^{2+} induced apoptosis in HIV infected T cells[40]. CaM has been shown to regulate HIV induced apoptosis of human T cells as CaM antagonists inhibit Fas-mediated apoptosis of CD4+ T-cells induced by HIV infection [41].

Fas-Mediated DISC and the Role of Calmodulin.

The Fas receptor (CD95 or APO-1) is a member of the tumor necrosis factor receptor (TNF) superfamily [42]. TNF receptor superfamily members are characterized by cysteine rich extracellular domains. Within the TNF receptor super family, the death receptor class members, which includes Fas, all possesses a homologous cytoplasmic death domain (DD) [43-45]. The activation of death receptors present on the cell surface

leads to the initiation of the extrinsic apoptotic pathway via their death domain [46]. The extrinsic and intrinsic apoptotic signaling pathways govern apoptosis at the cellular level. The Intrinsic pathway is mediated and controlled by members of the B-cell leukemia/lymphoma 2 (Bcl-2) protein family and typically responds to sever DNA damage, hypoxia, or other cell stresses [47]. Figure 1 depicts the extrinsic apoptotic signaling pathway mediated by death domain containing death receptors Fas and DR-5. Upon stimulation by binding their respective ligand or agonistic antibody to their extracellular domains, death receptors such as Fas or DR-5 oligomerizes into homotrimers [48]. Fas or DR-5 homotrimers are bound at their death domains (DD) by the adapter protein Fas associated death domain protein (FADD). FADD recruits procaspase-8 forming the death inducing signaling complex (DISC) [49-51]. DISC formation facilitates the autoproteolytic cleavage and activation of procaspase-8 into its active form caspase-8, which is released from the DISC complex [49]. Depending on the amount of caspase-8 produced from DISC, one of two apoptotic pathways may be initiated converging at the activation of caspase-3. In type I cells, a high content of caspase-8 from DISC can activate effector caspases-3 and -7 directly leading to cellular disassembly [52]. However, in type II cells, a low content of capsase-8 is produced from DISC and the intrinsic pathway is activated to amplify the apoptotic signal [53, 54]. In type II cells, caspase-8 mediates the cleavage of BH-3 interacting death domain agonist (Bid) [55]. Activated Bid leads to the activation of pro-apoptotic Bcl-2 family members Bcl-2 associated X protein (Bax) and or Bcl-1 antagonist/killer (Bak) that translocate from the cytosol to the mitochondrial membrane leading to loss of mitochondrial membrane integrity and the release of cytochrome-C and apoptogenic factor second mitochondrial

activator of caspases/direct inhibitor of apoptosis-binding protein with low isoelectric point (SMAC/DIABLO) from the mitochondria [56, 57]. SMAC/DIABLO displaces the X-chromosome linked inhibitor for apoptosis protein (XIAP) from caspase-3, -7, and -9 [57, 58]. Cytosolic cytochrome-C binds apoptotic protease activating factor-1 (Apaf-1), which recruits procaspase-9 forming apoptosomes. The apoptosome complex proteolytically activates caspase-3 and -7 whose activation of downstream caspases directs the apoptotic program into a multitude of sub-pathways to activate specific death substrates inducing cell apoptosis [59].

The Fas receptor is constitutively expressed in various tissue throughout the human body [60, 61] and the Fas death receptor regulates cell apoptosis in a variety of normal cells and control malignant transformation in cancers [62-64]. Fas is expressed at high levels in normal breast epithelial tissue, however, Fas expression is significantly lower in primary breast cancer cells and breast cancer cell lines representing the 5 breast cancer subtypes [20, 65, 66]. Dysregulation of the Fas receptor and mediators of its signaling pathways has been associated several diseases in humans and mice and contribute breast cancer immune system resistance [65, 67]. The expression of mutant Fas with an altered structure and impaired function has been observed in numerous types of human disease states and cancers [20, 68-71]. Deficiency in Fas function is frequent in invasive breast carcinomas and correlates to larger breast cancer tumor size [65]. Additionally breast cancer cells have been shown to develop resistance to Fas mediated apoptosis, which could be due to loss of function of Fas and or downstream mediators of the Fas apoptotic signal [20, 72].

A Ca^{2+} dependent interaction between CaM and Fas has been shown in cholangiocarcinoma [73], Jurkat [74], and osteoclast [62] cells. Activation of Fas leads to the recruitment of CaM into the Fas-mediated DISC and Ca^{2+} chelator, EGTA, and CaM antagonist, trifluoperazine (TFP), can inhibit CaM recruitment into the Fas-mediated DISC in cholangiocarcinoma cells [73]. Furthermore, TFP treatment can decrease Fas-mediated DISC induced cleavage of caspase-8 in cholangiocarcinoma [73]. CaM binds to the Fas death domain (Fas DD) with helices 1, 2 and 3 of the Fas DD shown to associate with CaM. The CaM binding site in Fas DD has been localized to the helix-2 and connecting loops of the Fas DD. Fas mutations that cause an alteration of the structure and or function of Fas have been detected in many cancers [70, 71]. The expression of mutant Fas with impaired function has been observed in numerous types of cancers [20, 68, 69]. Dysregulation of the Fas receptor and mediators of its signaling pathways has been associated several diseases in humans and mice such as autoimmune lymphoproliferative syndrome (ALPS) in humans [75-77]. Lymphoproliferation complementing Gld (Lpr-cg) mice have a mutation in Fas causing defective Fas signaling [78]. The valine 254 to asparagine (V254N) mutation of the Fas DD is analogous to the mutant allele of Fas in lpr-cg mice [74]. The lpr-cg mice mutant Fas has reduced binding to FADD, which could be due to an altered structure [79]. Understanding the protein interactions underlying Fas-mediated apoptotic pathways is potentially important for identification of novel targets and agents for the effective initiation of cancer cell death.

Our lab has previously investigated the binding thermodynamics and conformation of CaM-Fas DD complexes using explicit solvent molecular dynamics simulations and implicit solvent binding free-energy calculations presenting structural

evidence for CaM-Fas DD binding and the role of Fas DD V254N mutation in CaM-Fas DD interaction [(80)]. Additionally our lab has shown the structural and molecular mechanisms involved in the role of TFP in regulating CaM-Fas binding using molecular dynamics simulations, indicating that the number of TFP bound to CaM directly contributed to conformational changes in CaM conformation and these changes affected CaM binding to Fas DD [(81)]. These computational results provided insight into the structural and molecular mechanisms for the role of CaM and Fas interaction in regulation of Fas-mediated DISC formation and the mechanism by which CaM antagonist TFP, in a concentration-dependent manner, affects CaM and Fas interaction and modulates Fas-mediated DISC formation. A quantitative understanding of CaM and Fas interaction is important for the elucidating the mechanism of CaM's regulation of Fas mediated DISC formation and apoptotic signaling. This knowledge may lead to the design of optimized antagonists for CaM or Fas to regulate CaM and Fas interaction; thus modulating Fas-mediated DISC formation for apoptosis.

Research targeting the Fas-receptor for anti-cancer therapy has provided divisive results. Activation of the Fas-receptor using a Fas agonistic antibody induced apoptosis in malignant human lymphocytes *in vitro* and B cell tumor regression *in vivo* [(82)]. However, Fas agonistic antibody induced Fas activation also induced apoptosis of normal liver cells within the liver of mice [(83-86)]. Furthermore Fas activation has been show to induce Fas mediated apoptosis in normal cells of the spleen, intestine, several reproductive system organs, thymus, skin, and lymph nodes of *in vivo* [(84)]. Fas receptor induced liver injury is the most critical issue against targeting the Fas-receptor for anti-cancer therapy [(83)]. Therefore, until the development of strategies that may circumvent the issue of Fas

receptor mediated hepatotoxicity, our research focus will shift to the investigation of targeting the Death Receptor-5 mediated apoptotic signaling for breast cancer therapy. TRA-8 activation of DR-5 induces apoptosis in breast cancer cells [87], and does not exhibit cytotoxicity against normal liver cells [88, 89].

DR-5–Mediated Apoptotic Signaling and the Role of Calmodulin.

Breast cancer cell lines representing the various subtypes express Death Receptor-5 (DR-5 or TRAIL receptor-2) [87, 90]. DR-5 is another member of the TNF receptor superfamily and is one of five receptors including TRAIL receptor 1 (TRAIL-R1or DR-4) [91]. TRAIL receptor 3 (DcR1) [92] , TRAIL receptor 4 (DcR2)[93], and osteoprotegerin (OPG)[94] bound by TNF receptor apoptosis inducing ligand (TRAIL). DR-5 and DR-4 are the two agonistic receptors to TRAIL that can mediate apoptosis [95]. TRAIL has shown strong antitumor activity with nominal systemic toxicity to most normal cells and tissues [88, 96, 97]. However, TRAIL can induce apoptosis of normal human hepatocytes [98]. It has been speculated that TRAIL requires multiple receptors to induce apoptosis of normal hepatocytes [89]. The activation of DR-5 by TRA-8, DR-5 agonistic antibody, has shown tumoricidal activity *in vitro* and *in vivo* without inducing normal hepatocyte apoptosis [89]. TRA-8 activation of DR-5 can induce apoptosis via a caspase-dependent mechanism in human breast cancer cell lines including triple negative cell lines MDA-MB-468 and MDA-MB-231 [87]. However, sensitivity to DR-5 mediated apoptosis is dependent on the breast cancer subtype [87]. TRA-8 resistant breast cancer cell lines BT474, SKBR3, T47D, and ZR-75-30 can be sensitized to TRA-8 induced apoptosis when in combination with radiotherapy or chemotherapy [9, 87, 99]. Breast cancer

cells can develop resistance to DR-5 mediated apoptosis by sustained expression of anti-apoptotic proteins [100] such as Bcl- XL, FLIP, XIAP, Mcl-1 and down regulation of pro-apoptotic proteins Bid, Bax, Bak, and Smac-DIABLO [101, 102]. Furthermore differential activation and expression of proteins involved in proliferative and anti-apoptotic signaling pathways including PI3K/AKT pathway, MAPK pathway, NF-kB pathway may regulate DR-5 mediated apoptosis sensitivity [102-105].

DR-5 contains a cytoplasmic death domain [96] and similar to the Fas receptor transduces its apoptotic signal via DISC formation and activation of caspase signaling [50] (Figure 1). Formation of the DR-5 mediated DISC signifies the inception of the DR-5 mediated apoptotic signaling pathway [49, 50]. DR-5 mediated DISC includes DR-5, FADD, and caspase-8 [50]. The adaptor molecule FADD contains a DD and a death effector domain (DED) and has the essential role of binding to DR-5 and recruiting capsase-8 to form DISC leading to caspase-8 activation [106]. Dysfunction in any of the DISC components can lead to resistance of DR-5 mediated apoptosis [50, 106]. Mutations to the death domain of DR-5 have been identified in breast cancer [107]. Further, impeding DR-5 mediated DISC formation and post DISC signaling cascade could lead to DR-5 mediated apoptosis resistance [91, 103, 105, 108]. Breast cancer cells may become resistant to TRA-8 induced DR-5 activation by prolonged exposure of TRA-8 and the developed resistance to TRA-8 is due to blockade of DISC formation at the death domain of DR-5[109]. The formation of an anti-apoptotic complex after TRA-8 activation of DR-5 involving glycogen synthase kinase-3 (GSK3), DDX3 and cellular inhibitor of apoptosis protein-1 (cIAP-1) has been identified in breast cancer cells [105]. The characterization of the mechanisms regulating DR-5 mediated DISC formation in breast cancer may

potentially lead to the identification of key protein regulator(s) that modulated DR-5 mediated apoptosis in breast cancer.

Recently a role of CaM in DR-5 mediated apoptotic signaling regulation has been presented for human lung cancer cells[(110)] and hepatocellular carcinoma cells [(111)]. CaM antagonist fluphenazine-N-2-choroethane has been demonstrated to sensitize human lung cancer H1299 to TRAIL-induced apoptosis [(110)]. Fluphenazine-N-2-choroethane enhances TRAIL induced caspase-8 activity and increased caspase-3 and its substrate PARP-1 cleavage. It was indicated that CaM inhibition increased caspase activity by down regulating anti-apoptotic proteins Bcl-XL, c-IAP1, c-IAP2 and XIAP. This observation was reinforced by the results that CaM siRNA, which down regulates the expression of CaM, decreased cell viability when in combination with TRAIL versus CaM siRNA alone. Furthermore CaM siRNA was shown to decrease the expression of c-IAP2 and induce pro- caspase-8 cleavage in the presence of TRAIL[(110)]. Additionally CaM antagonist fluphenazine-N-2-choroethane was shown to inhibit CaM and FLIP binding in the presence of TRAIL, which inhibited ERK (1/2) protein phosphorylation a mediator of the MAPK pathway[(110)]. Preliminary results from our lab showed that combination treatment of tamoxifen, a calmodulin antagonist, with TRA-8 resulted in antagonism of TRA-8 induced apoptosis in ER-positive MCF-7 and T47D and triple negative MDA-MB-231 breast cancer cell lines without estradiol treatment (*unpublished data*). The observed antagonism between TMX and TRA-8 treatment for ER inactivated MCF-7 and T47D and triple negative MDA-MB231 cells could be resulted from TMX's antagonism of CaM to inhibit CaM-DR-5 interaction and further impede DR-5 mediated DISC formation apoptosis. These preliminary results also provide the basis for the further

investigations of the role of CaM-DR5 binding in DR-5 mediated DISC formation and apoptosis, which may lead to the novel therapeutic strategies for breast cancer. Evidence indicates that CaM plays a role in regulating DR-5 induced signaling and a complete understanding of the mechanism of CaM regulation of DR-5 mediated apoptotic signaling needs investigation. Characterization of the interactions of CaM with DR-5 and the role of CaM-DR-5 binding in regulation of DR-5 mediated DISC formation and apoptosis in breast cancer may potentially lead to the identification of key regulators for DR-5 mediated apoptosis in breast cancer. Understanding CaM's regulation of DR-5 mediated signaling would establish a potential target, CaM-DR-5 binding, for modulating DR-5 mediated apoptosis and lead to the development of novel strategies targeting CaM-DR-5 binding as effective targeted therapy for breast cancer treatment.

Overall Goal of the Study

The goal of this study is to characterize the interactions of calmodulin with death receptors Fas and DR-5 and determine the role of CaM-DR-5 binding in DR-5 DISC formation in breast cancer cells. The interaction of CaM and Fas and the role of CaM/Fas binding on the regulation of Fas mediated DISC formation and apoptotic signaling has been well characterized in cholangiocarcinoma [62, 74, 112, 113]. Here an isothermal titration calorimetry and circular dichroism spectroscopy are used to quantitatively characterize the interactions of CaM and the death domain of Fas. Inducing Fas activation for anti-cancer therapy can also induce apoptosis on normal cells of the liver and other tissues. Therefore, our studies for breast cancer will focus on the targeting DR-5 mediated apoptosis using the DR-5 agnostic antibody, TRA-8, which does not display

hepatotoxicity. CaM and DR-5 interaction was characterized in ER-positive MCF-7 and triple negative MDA-MB231 breast cancer cell lines and the role of CaM/DR-5 binding in DR-5 mediated DISC formation and the modulation of DR-5 mediated DISC formation by CaM antagonists in MCF-7 and MDA-MB231 breast cancer cell lines were determined.

Specific Aim 1

To quantitatively characterize the interactions of CaM and the death domain of the Fas receptor using a combined circular dichroism spectroscopy and isothermal titration calorimetry approach.

Hypothesis: The V254N mutation in Fas DD will decrease CaM-Fas binding affinity, which could be resulted from secondary structure and conformational changes in Fas DD by V254N mutation. The interactions of CaM with wild type Fas DD (Fas DD WT) and Fas DD V254N were quantitatively characterized using isothermal titration calorimetry (ITC) and Fas DD WT and Fas V254N secondary structures were evaluated using circular dichroism spectroscopy (CD). This study quantitatively characterized CaM-Fas DD interactions and provided the structural and thermodynamic basis for CaM-Fas DD interactions. The results could potentially help for the identification of novel strategies to modulate Fas-mediated DISC formation for apoptosis and for potential cancer chemotherapy.

Specific Aim 2

To characterize a direct interaction of CaM and DR-5, and determine the role of CaM and DR-5 interaction in DR-5-mediated DISC formation.

Hypothesis: CaM directly binds to DR-5 and CaM-DR-5 Interaction plays a key role in regulating DR-5 mediated DISC formation. The interactions between CaM and DR-5 and the role of CaM and DR-5 binding in DR-5 mediated DISC formation in breast cancer were determined using protein complex immunoprecipitation experiments and western blotting analysis.

Study Significance

- A Fas mutant with altered secondary structure and conformation could affect Fas interaction with CaM, and thus alter CaM's regulation of Fas mediated DISC formation and apoptosis. A quantitatively characterization of CaM and Fas DD interactions would provide the structural and thermodynamic evidence for CaM – Fas DD interaction. Further, results could lead to the development novel strategies to modulate Fas-mediated DISC formation for apoptosis.
- The combined approach of ITC, CD and the computational modeling (modeling study not shown here) to quantify CaM – Fas interactions is novel.
- Targeting the Fas receptor for anti-cancer therapy has demonstrated hepatotoxicity *in vivo*. Activation of DR-5 has been shown to induce apoptosis in cancer cells. Thus, the characterization of CaM and DR-5 binding and determination of the role of CaM-DR-5 binding in DR-5 mediated DISC formation in breast cancer could present potential target(s) for regulation of DR-5 mediated apoptosis, and lead to the development of novel strategies for effective targeted breast cancer treatment to

overcome the issue of hepatotoxicity as observed with Fas activation. The characterization of CaM-DR-5 binding and its role in DR-5 mediated apoptosis presents a novel target that might overcome the clinically observed drug resistance of breast cancer treatment, including hormone resistance phenomena in breast cancer treatment.

CHAPTER 2

MATERIALS AND METHODOLOGY

General Methods

Preparation and Purification of Recombinant Proteins.

To quantitatively characterize CaM and Fas DD interaction using ITC and CD experiments, recombinant CaM and Fas DD proteins were produced and purified as follows. *Escherichia coli(E.coli)* BL21 (DE3) (pLysS) strain cells containing 6xHis-SUMO-CaM or 6xHis-SUMO-Fas DD WT (191-335) genes cloned into pET28 vectors were kindly provided by Dr. Jay M. McDonald of the Department of Pathology at the University of Alabama at Birmingham. The Fas DD mutation Fas DD V245N was created by site-directed mutagenesis of pET28 6xHis-SUMO-Fas DD WT (191-335) using the Quik-change II ® site-directed mutagenesis kit (Agilent Technologies). Transformed *Escherichia coli* BL21 (DE3) (pLysS) cells were incubated in LB broth media with 50 µg/mL Kanamycin and 10 µg/mL Chloramphenicol (all Fisher Scientific) on a shacking incubator at 30 ^{o}C. *E.coli* cell population growth was monitored by measuring the optical density of the E.coli culture at 600 nm (OD_{600}) hourly. When E. coli cell culture attained an OD_{600} of 0.6–0.8, recombinant protein expression was

induced by the addition of 0.1 mm isopropyl β-D-thiogalactopyranoside (ITPG) (Promega). The culture was then incubated for an additional 4 hours at 30 ^{o}C and expression of the 6xHis-SUMO fusion proteins were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE). After protein expression E. coli cultures were pelleted by centrifugation and stored at -80 ^{o}C until the time for lysis. Cells were lysed using an EmulsiFlex-C3 french press (Avestin, Ontario Canada) and then lysates were centrifuged and the supernatant collected. 6xHis-SUMO fusion proteins were purified by nickel ion affinity chromatography according to the manufacturer's direction for 6xHis-SUMO fusion protein purification system (Qiagen). The 6xHis-SUMO tag was cleaved off by overnight incubation with the SUMO tag protease and the 6xHis-SUMO tag and SUMO protease were removed by nickel ion affinity chromatography.

Size Exclusion Chromatography.

Recombinant CaM and Fas DD WT and Fas DD V254N were further purified by size exclusion chromatography using a HiLoad 16/600 Superdex 75 prepgrade column on an AKTA purifier (GE life sciences). Column flow rate was 0.8 mL/min, 5 mL of protein solution was injected per chromatography run and 2 ml per fraction of eluted protein solution was collected. Final purity of protein solutions were evaluated by SDSPAGE.

Protein Quantification.

CaM, Fas DD WT and Fas DD V254N concentration was determined by UV absorbance at 280 nm using a Cary 300 UV-Vis spectrophotometer (Varain Inc., Palo Alto, CA). The extinction coefficients 8480 M^{-1} cm^{-1} for Fas DD WT and Fas DD V254N and 2980 M^{-1}

cm^{-1} for CaM were determined from their primary residue sequence using the edelhoch method [(114)] adjusted with the extrication coefficients determined by Pace et. al. [(115)] in the Protpram software (ExPASy SIB Bioinformatics Recourse Portal).

Isothermal Titration Calorimetry.

For ITC experiments CaM, Fas DD WT and Fas DD V254N were expressed and purified as described in the preparation and purification of recombinant proteins and the size exclusion chromatography sections of the general methods. CaM, Fas DD WT and Fas DD V254N proteins were dialyzed into a 50 mM sodium acetate pH 5.0, 150 mM NaCl, 20 mM $CaCl_2$ and 0.5 mM TCEP buffer for ITC experiments. Calorimetry studies were performed using an Auto-ITC_{200} microcalorimeter (MicroCal Corp., Northhampton, MA). Data acquisition and analysis were performed using the Origin software supplied by MicroCal. A typical titration was performed by 20 or 25 sequential injections, 1.6 µL or 2 µL each, of ligand solution from the syringe into a calorimeter cell containing the macromolecule protein solution. Successive injections were separated by 240 second intervals to allow the endothermic peak resulting from the reaction to return to the baseline. A constant stirring speed of 500 rpm and temperature of 37 ^{o}C was maintained throughout the titration.

Circular Dichroism Spectroscopy.

CaM, Fas DD WT and Fas DD V254N proteins were expressed and purified as described in the preparation and purification of recombinant proteins and the size exclusion chromatography sections of the general methods. Following purification CaM, Fas DD

WT and Fas DD V254N proteins were dialyzed into a 50 mM sodium acetate pH 5.0, 150 mM NaCl, 20 mM $CaCl_2$ and 0.5 mM TCEP buffer. Circular dichroism spectroscopy (CD) studies were performed using a JASCO J-810 spectropolorimeter (JASCO Corporation, Japan) fitted with a thermostatic cell holder and a thermostatic water bath. The Far UV CD (260 nm -190 nm) spectra were obtained at 1 nm bandwidth using a 0.1 mm path length quartz cell at 37^oC and each spectrum is reported as an average of four scans. Far UV CD secondary structure percentages were determined from CD spectra using the SELCON3 CD spectra analysis software. The SP43 protein reference sets containing 43 soluble proteins having known structures and CD spectra ranging from 190 to 240 nm were used to determine percent secondary structure content.

Breast Cancer Cell Culture.

To determine an interaction between CaM and DR-5 and role of CaM/DR-5 binding in DR-5 mediated DISC formation in breast cancer. The ER-positive MCF-7 and triple negative MDA-MB-231 human breast cancer cell lines were kindly gifted by Dr. Tong Zhou (University of Alabama at Birmingham, Birmingham, AL). MDA-MB-231 cells were cultured in DMEM supplemented with MEM vitamins, 1X nonessential amino acids, and 1 mM sodium pyruvate. MCF-7 cells were culture in MEM media supplemented with 1 mM sodium pyruvate, 10 g/mL insulin, and 1X nonessential amino acids. Both cell lines were maintained in 1% penicillin, 1% streptomycin and 1% amphotericin B antibiotic medium with 10% FBS at 37°C, 5% CO_2 and 95% relative humidity atmosphere with media changes every 2-3 days, and routinely screened for contamination.

Protein Complex Immunoprecipitation (Co-IP).

To determine an interaction between CaM and DR-5 and the role of CaM/DR-5 binding in DR-5 mediated DISC formation in breast cancer, the CaM-DR-5 complex and DR-5 mediated DISC were isolated from MCF-7 and MDA-MB231 lysates using Co-IP. MCF-7 or MDA-MB-231 cells were lysed using a 50 mM Tris-HCL pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1:100 Halt protease inhibitor from thermo fisher scientific (Waltham, MA) buffer at 4^{o}C for 30 minutes. The total protein concentration, which is the concentration of all soluble proteins in the cell lysate, was determined using Bicinchoninic acid (BCA) assay from thermo fisher scientific (Waltham, MA) and a total protein concentration of 5 mg/mL was used for Co-IP experiments. Lysates were precleard with 40 µL of 4B-sepharose beads from Sigma-Aldrich (St. Louis MO) at 4 ^{o}C for 1 hour. TRA-8 or 2B9 conjugated sepharose beads were kindly gifted from Dr. Tong Zhou at the University of Alabama at Birmingham and were incubated overnight at 4^{o}C in cell lysates. Following overnight incubation Co-IP beads were washed using a 50 mM Tris-HCL pH 7.5, 150 mM NaCl, 0.5% Triton 100 X wash buffer five times, followed by the addition of 40 µL of 2x laemmli sample buffer.

Western Blot Analysis.

CaM-DR-5 complex and DR-5 mediated DISC proteins were evaluated following Co-IP using western blot analysis. Samples from Co-IPs were heated at 100 ^{o}C for 5 minutes and loaded for western blot analysis. Protein samples were resolved by SDS-PAGE and transferred onto a 0.45 um nitrocellulose membrane from Bio-Rad (Hercules, CA). For samples from Co-IP of CaM-DR-5 complex membranes were incubated overnight at 4^{o}C

with primary antibodies against DR-5 from Abcam (Cambridge, MA) and CaM from Milipore (Billerica, MA). For samples from Co-IP of DR-5 mediated DISC proteins membranes were incubated overnight at 4^{o}C with primary antibodies against DR-5 from Abcam (Cambridge, MA), FADD from Thermo Fisher Scientific (Waltham, MA), caspase-8 from cell signaling technologies (Beverly, MA), or CaM from Milipore (Billerica, MA).Following overnight antibody incubation membranes were washed 3 times with TBST and then a 1 hour incubation with horseradish peroxidase-conjugated secondary antibodies from southern biotechnologies (Birmingham, Alabama) at room temperature. Proteins were evaluated using enhanced chemiluminescence western blotting detection reagents from Thermo Fisher Scientific (Waltham, MA) according to manufacturer's instructions.

Specific Aim 1: Quantitatively characterize the interactions of CaM and the Death Domain of the Fas receptor using a combined circular dichroism spectroscopy and isothermal titration calorimetry approach.

CaM with Fas DD Binding from Isothermal Titration Calorimetry.

Fas DD WT and Fas DD V254N were dialyzed into a 50 mM sodium acetate pH 5.0, 150 mM NaCl, 20 mM $CaCl_2$ and 0.5 mM TCEP buffer for ITC experiments. Blue native PAGE of CaM, Fas DD WT, and Fas DD V254N proteins at pH5 were evaluated for monomer or dimer existence. For CaM to Fas DD WT ITC experiments, 600 µM or 750 µM or 1000 µM CaM solution was loaded into the syringe and titrated into the calorimeter cell containing 50 µM Fas DD WT. For CaM to Fas DD V254N ITC experiments 750 µM or 1000 µM CaM solution was loaded into the syringe and titrated

into the calorimeter cell containing 50 µM Fas DD V254N. Control experiments were performed by titrating CaM from the syringe into the calorimeter cell containing buffer with the resulting heat changes subtracted from the measured heats of binding of CaM to Fas DD WT or Fas DD V254N. The thermodynamic binding parameters: enthalpy change (ΔH), association constant (K_a), and stoichiometry (n) were derived from the nonlinear least-squares one site binding model fit to the isotherm plot of CaM and Fas DD binding and the entropy change (ΔS) was calculated from the enthalpy change (ΔH) and association constant (K_a) by the Origin software (MicroCal, Northampton, MA). Gibbs free energy (ΔG) was calculated from the equation $\Delta G = \Delta H - T\Delta S$, where T is the absolute temperature. The values of the enthalpy change (ΔH), association constant (K_a) and stoichiometry (n) are calculated as the error-weighted mean of replicate ITC trials [(116)]. The entropy ($-T\Delta S$) and free energy (ΔG) value is the average with standard deviation of replicate ITC trials**.**

Far UV Circular Dichroism Spectroscopy of Fas DD.

Fas DD WT and Fas DD V245N proteins were dialyzed into a 50 mM sodium acetate pH 5.0, 150 mM NaCl, 20 mM $CaCl_2$, and 0.5 mM TCEP buffer. To evaluate Fas DD secondary structure the Far-UV CD spectra (260 nm – 190 nm) of 50 µM Fas DD WT, and 50 µM Fas DD V245N were obtained at 1 nm bandwidth using a 0.1 mm path length quartz cell at 37^{o}C and each spectra is reported as an average of four scans.

Specific Aim 2: Characterize a direct interaction of CaM and DR-5, and determine the role of CaM and DR-5 interaction in DR-5-mediated DISC formation in breast cancer.

Characterize the interactions between CaM and DR-5 in breast cancer cells using complex-immunoprecipitation (Co-IP) experiments.

Three to four 100 mm dishes were seeded with 3 x 10^6 MCF-7 or MDA-MB-231 cells each and grown to 85-90% confluence. Cells were washed three times with PBS and then lysed and prepared according to the Co-IP procedure described in the general methods. 40 µL of TRA-8 conjugated 4B-sepharose beads (kindly provided by Dr. Tong Zhou, University of Alabama at Birmingham) were used for Co-IP of the DR-5-CaM complex and 40 µL of mouse iso-IgG conjugated 4B-sepharose beads from cell signaling technologies (Beverly, MA) were used as the negative control. Western blot analysis was performed using primary antibodies to detect DR-5 (DR-5 antibody from Abcam (Cambridge, MA) and CaM (CaM antibody from Milipore (Billerica, MA).

Determine the role of CaM-DR-5 binding in DR-5-mediated DISC formation.

The role of CaM/DR-5 binding in DR-5-mediated DISC formation and the effect of CaM antagonist trifluopherazine (TFP) on DR-5-mediated DISC formation were determined. Three to four 100 mm dishes were seeded with 3 x 10^6 cells and grown to 85-90% confluence. Breast cancer cells were treated under four conditions: 1) no TRA-8 or TFP treatment, 2) treatment with 1 µg/mLTRA-8 only for 3 hours, 3) preincubated with 10 µM TFP for 15 minutes after which 1 µg/mL TRA-8 was introduced to the cells for 3

hours, and 4) preincubated with 10 μM TFP for 30 minutes after which 1 μg/mL TRA-8 was introduced to the cells for 3 hours. Cells were lysed and prepared according to the Co-IP procedure described in the general methods. 2B9 conjugated 4B-sepharose beads were kindly gifted from Dr. Tong Zhou at the University of Alabama at Birmingham. The monoclonal antibody 2B9 is an anti-DR-5 antibody which does not compete with TRA-8 for binding DR-5 allowing for the Co-IP of TRA-8 activated DR-5 mediated DISC complex [(105)]. 2B9 conjugated 4B-sepharose beads were added to cell lysates and incubated overnight at 4 ^{o}C. Following Co-IP Western blot analysis was performed using primary antibodies to detect FADD from Thermo Fisher Scientific (Waltham, MA), DR-5 from Abcam (Cambridge, MA), CaM from Milipore (Billerica, MA) and Caspase-8 from cell signaling technology (Beverly, MA).

CHAPTER 3

RESULTS AND DISCSSION

CaM with Fas DD Binding from Isothermal Titration Calorimetry

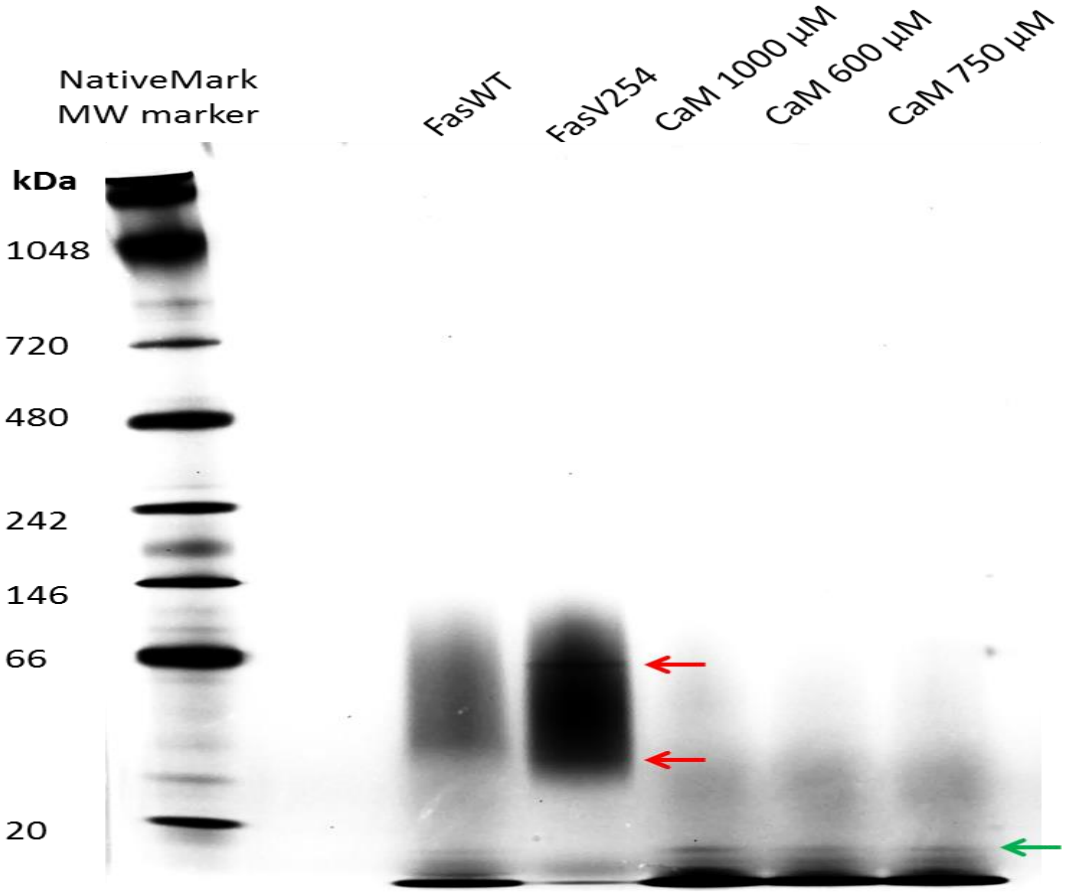

FIGURE 1. Native PAGE of Fas DD WT, Fas DD V254N, and CaM in 50 mM sodium acetate pH 5.0, 150 mM NaCl, 20 mM $CaCl_2$ and 0.5 mM TCEP buffer used for ITC. Blue native PAGE was performed to evaluate the dominant dimer or monomer state of Fas DD WT, Fas DD V254N, and CaM proteins.

Native PAGE of Fas DD WT shows the Fas DD WT protein (17 KDa) is predominantly in a monomer state, a band was observed just below the 20 KDa marker (Green arrow Figure 1). Further, light bands at 35 - 40 KDa and 66 KDa for Fas DD WT indicate some Fas DD WT is in a dimer state and a potential tetramer state (red arrows Figure 1). The blue native PAGE of Fas DD V254N protein (17 KDa) shows stronger bands at 35 - 40 KDa and 66KDa, with a lighter band just below 20 KDa compared to the Fas DD WT demonstrating Fas DD V254N protein is predominantly in a dimer state and tetramer state (red arrows of Figure 1). For the different concentrations of CaM (17 KDa) used for ITC bands were observed just below the 20 KDa mark, showing the CaM protein is in a monomer state (green arrow Figure 1).

The CaM binding to Fas DD WT isotherm illustrated an endothermic binding characteristic between CaM and Fas DD WT at 37 ^{o}C (Figure 2 A). The heat absorbed per injection of CaM to Fas DD was highest at low molar ratios and decrease as the molar ratio of CaM to Fas DD increased until only the CaM dilution enthalpy is observed. In addition tothe endothermic heat change of CaM binding to Fas DD, an exothermic heat trend was observed in conjunction with CaM-Fas DD binding. The exothermic heat trend is masked by the high endothermic heat change of CaM binding to Fas DD WT at the beginning of the titration but becomes more evident towards the end of CaM-Fas DD WT binding as the Fas DD WT becomes saturated. The exothermic peaks were observed at injections 15-18 and then gradually become more positive until the CaM dilution enthalpy is observed. Blue native gel electrophoresis of Fas DD WT protein showed some Fas DD WT is in a dimer and possible tetramer state (red arrows of Figure 2). Fas DD WT could be undergoing a CaM binding induced dissociation resulting in an

exothermic heat change simultaneously with the endothermic CaM binding to Fas DD WT. To obtain a more accurate estimation of the thermodynamics of CaM binding to the Fas DD an approximated enthalpy curve for the CaM binding induced Fas DD dissociation was calculated. After the CaM dilution enthalpy (Figure 2B) was subtracted from the CaM to Fas DD WT isotherm (Figure 2A), a plot of the enthalpy values from the last 7 to 8 injections were fit to a polynomial line (Figure 2C). The approximated CaM binding induced Fas DD dissociation enthalpy plot was calculated from the equation of the polynomial line (Figure 2D) and is similar to reported dissociation enthalpy curves [(117-119)]. The Fas DD dissociation enthalpy was subtracted from the CaM to Fas DD isotherms with the ensuing isotherm fit to the one site model (Figure 2E) provided in the Origin software (MicroCal, Northampton, MA).

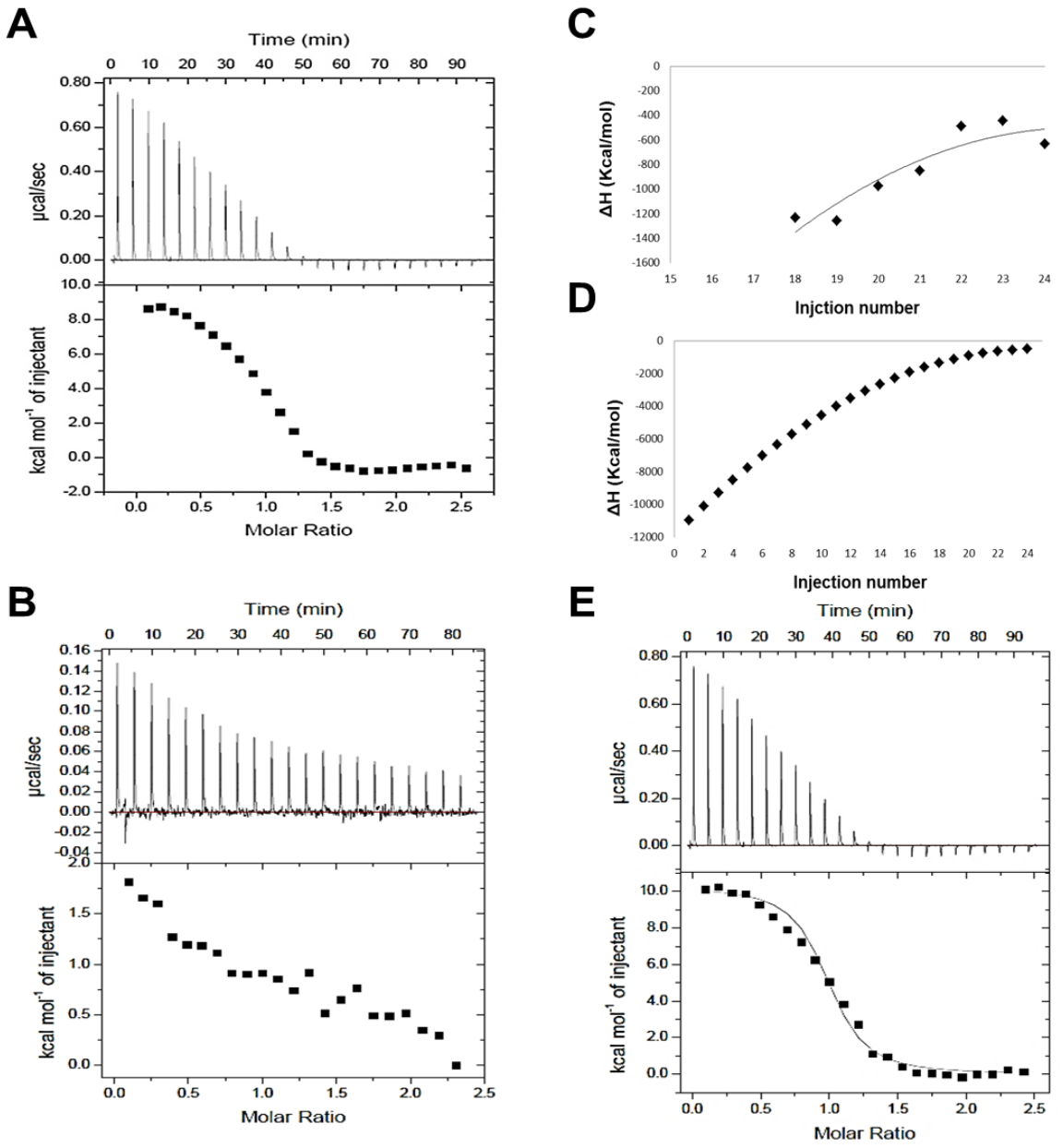

FIGURE 2. CaM to Fas DD WT isotherm and calculation of the approximate CaM binding induced Fas DD dissociation enthalpy. (A) 600 µM CaM titrated to 50 µM Fas DD WT raw isotherm. (B) 600 µM CaM dilution isotherm. (C) Experimental enthalpy points from injections 18 to 24 of CaM to Fas DD WT binding isotherm after subtracting the CaM dilution enthalpy. (D) Calculated approximate Fas DD WT dissociation enthalpy. (E) Final CaM to Fas DD WT binding isotherm after subtraction of the CaM dilution enthalpy and the Fas DD WT dissociation enthalpy.

Table 1. Thermodynamic parameters of CaM-Fas DD binding determined by ITC at 37 °C and pH 5

	K_a (M^{-1})	ΔG (kcal/mol)	ΔH (kcal/mol)	-TΔS (kcal/mol)	N
CaM/Fas DD WT	(9.88± 1.04) x10^5	-8.51± 0.02	9.97± 0.11	-18.40± 0.36	1.00± 0.01
CaM/Fas DD V254N	(4.89 ± 0.95) x10^5	-8.07± 0.03	9.94± 0.27	-18.12± 0.55	0.93± 0.02

K_a is association constant; ΔG is the changes in free energy; ΔH is the changes in enthalpy; $T\Delta S$ is the changes in entropy; n is stoichiometry.

The one site model fit to the CaM to Fas DD WT isotherm yielded an association constant (Ka) of (9.88 ± 1.04) x10^5 $M^{-1,}$ a positive enthalpy value (ΔH) of 9.97 ± 0.11 kcal/mol and an entropy value (-TΔS) of -18.40 ± 0.36 kcal/mol resulting in an entropy driven interaction between CaM and Fas DD WT and a free energy (ΔG) of -8.51 ± 0.02 at 37^{o}C (Table 1). Ca^{2+} binding to CaM regulates CaM's binding activity by modulating the amount of exposed apolar surface area available to interact with various CaM binding substrates [(120)]. Ca^{2+} bound CaM assumes a conformation that has a hydrophobic surface area exposed to the aqueous solvent [(121, 122)]. The entropy driven interactions between CaM and Fas DD WT observed from ITC could be due to the burial of the apolar surface area of CaM to the apolar CaM binding site on Fas DD, with 7 non-polar residues in a binding site of 13 residues [(74)]. This is known as the hydrophobic effect and would explain the favorable -TΔS value of CaM and Fas DD WT binding at 37^{o}C observed in ITC. The hydrophobic effect is the energetics involved in transferring non-polar compounds from a liquid organic phase to aqueous phase [(123)]. When applied to residue interactions with in a protein the interactions of non-polar residues in the interior of a

protein or at a protein-protein interface, as during binding, could be considered as similar to the interactions of compounds undergoing the organic to aqueous phase transfer [(124)].

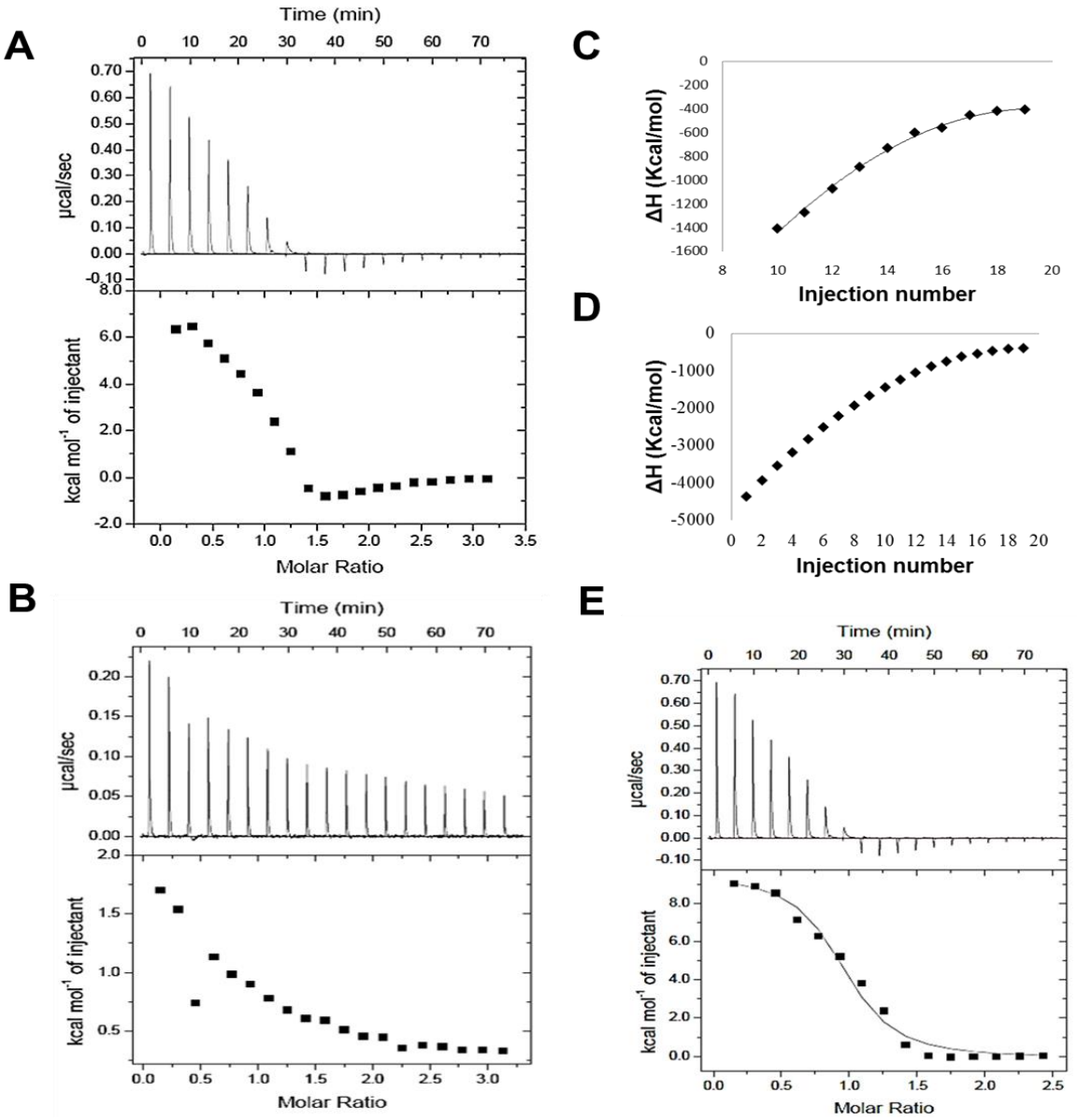

FIGURE 3. CaM to Fas DD V254N isotherm and calculation of the approximate CaM binding induced Fas DD dissociation enthalpy. (A) 750 µM CaM titrated to 50 µM Fas DD V254N raw isotherm. (B) 750 µM CaM dilution isotherm. (C) Experimental enthalpy points from injections 10 to 19 of CaM to Fas DD V254N binding isotherm after subtracting the CaM dilution enthalpy. (D) Calculated approximate Fas DD dissociation enthalpy. (E) Final CaM to Fas DD WT binding isotherm after subtraction of CaM dilution enthalpy and Fas DD dissociation enthalpy.

To investigate the effect of a Fas DD mutation on CaM interaction with Fas DD, CaM binding to the Valine 254 to asparagine (V254N) mutation of the Fas DD was also evaluated using ITC. The Fas DD V254N mutant is analogous to the identified mutant allele of Fas in MRL/lpr−/− mice that are homozygous for Fas mutations and deficient in Fas- mediated apoptosis [(125)]. Further, the Fas DD V254N mutant has reduced binding to FADD, which could be due to an altered protein structure [(79)]. CaM binding to Fas DD V254N resulted in a similar endothermic calorimetric titration profile (Figure 3 A) as the CaM to Fas DD WT isotherm. Blue native gel electrophoresis shows Fas DD V254N is predominantly in a dimer and tetramer state (red arrows Figure 2); further CaM binding to Fas DD V254N isotherm exhibited a greater magnitude exothermic heat trend than observed in the CaM to Fas DD WT isotherm. Thus, the Fas DD V254N protein could also be undergoing a CaM binding induced dissociation similar to the Fas DD WT. Therefore, an approximated enthalpy isotherm for the CaM binding induced Fas DD V254N dissociation was calculated and the Fas DD V254 dissociation enthalpy was subtracted from the CaM to Fas DD V254N isotherms with the resultant isotherms fit to the one site model (Figure 3E). CaM binding to Fas DD V254N resulted in a free energy (ΔG) of -8.07 ± 0.04 kcal/mol, with a positive enthalpy value (ΔH) of 9.94 ± 0.27 kcal/mol, and an entropy value ($-T\Delta S$) of -18.12 ± 0.55 kcal/mol resulting in an entropy driven interaction the similar to CaM binding to the Fas DD WT at 37^{o}C. However, the V254N mutation to the Fas DD resulted in 2 fold decrease in the association constant (Ka) 4.89 ± 0.95 x10^{5} M^{-1}. The reduction in the CaM to Fas DD binding association compelled further investigation into what possible change in the Fas DD structure caused

by the V254N mutation that could lead to the decreased binding association observed in ITC experiments.

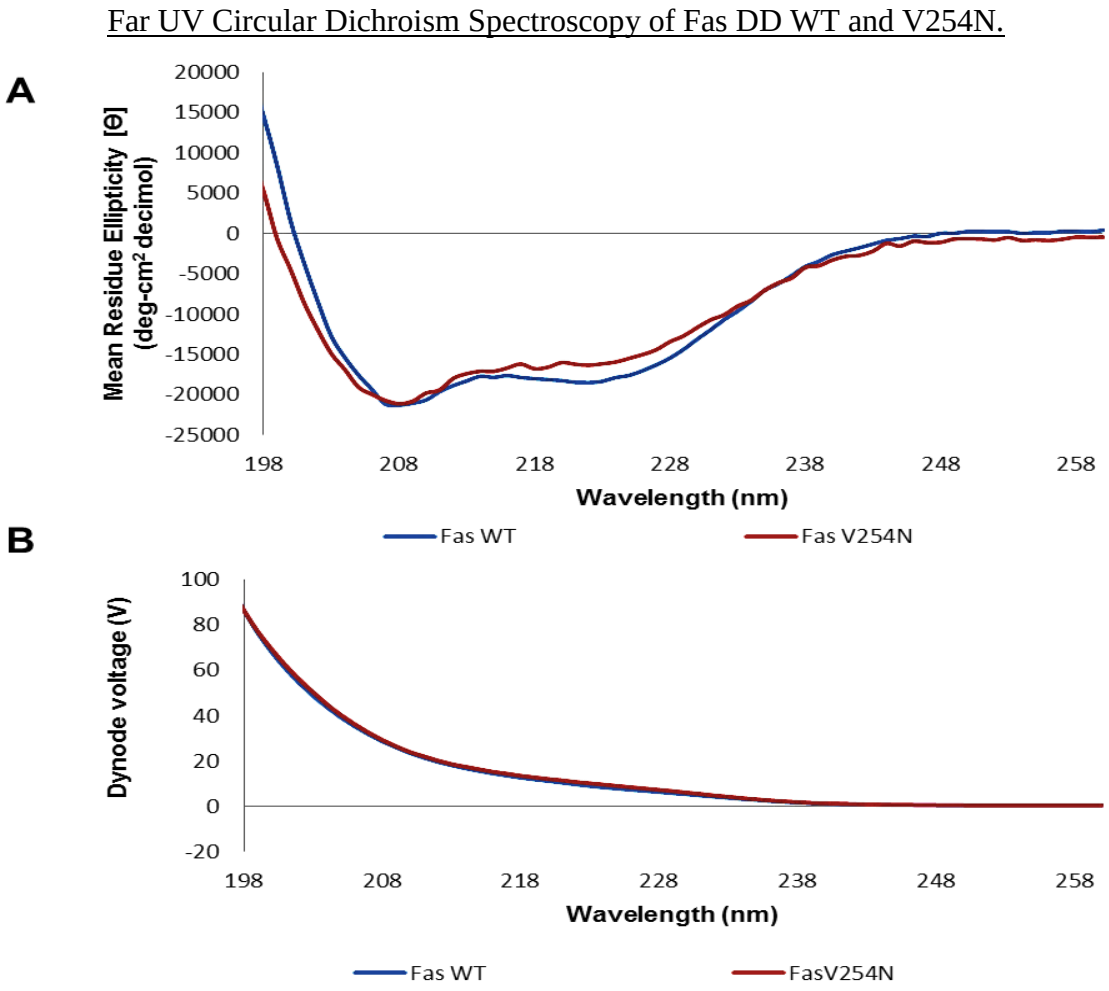

FIGURE 4. (A) Far UV CD spectra of Fas DD WT and Fas DD V254N mutant at 37^{o}C and (B) dynode voltage from Fas DD WT and Fas DD V254N Far UV CD scan. The CD spectra (A) revealed that the V254N mutation of Fas DD resulted in secondary structural changes of the Fas DD. Dynode voltage (B) shows that differences in Fas DD WT and Fas V254N CD spectra are not due to differences in Fas DD WT and Fas DD V254N concentration.

Table 2. Fas DD WT and Fas DD V254N Secondary Structure Composition Approximation from SELCON3 Software Analysis.

	% α-Helix	% β-sheet	% Turn	% Random
Fas DD WT from NMR [79]	48	0	22	35
Fas DD WT	62	0	14	24
Fas DD V254N	51	9	21	28

Circular dichroism spectroscopy (CD) was used to evaluate a structural basis for the decreased binding affinity between CaM and the Fas DD caused by the V254N mutation observed in ITC. Figure 4 shows the circular dichroism spectra of the Fas DD WT and Fas DD V254N. The Far UV CD spectrum of Fas DD WT was near baseline at wavelengths >245 nm, then exhibits a negative peaks at 222 nm and 208 nm wavelengths and then the CD signal becomes large and positive towards 198 nm wavelength consistent with a protein containing a majority helical content [126]. For the far UV CD spectrum of Fas DD V254N the magnitude of the negative peak at 222 nm wavelength decreased by -2154 $deg \cdot cm^2 \cdot decimol^{-1}$. Further there is a noticeable decrease in magnitude of the negative CD signal between 232 nm to 215 nm and 205 nm to 198 nm wavelengths for Fas DD V254N compared to the Fas DD WT, indicating a reasonable decrease in alpha helical secondary structure of the Fas DD caused by the V254N mutation[126] .

The secondary structure content of Fas DD WT and Fas DD V254N was estimated from their respective Far UV CD spectra using SELCON 3 [127]. Table 2 reports the percentage of secondary structure elements for Fas WT and Fas V254N from

SELCON3. The SELCON 3 estimated the Fas DD WT to be of 62% α-helical, 0% β-sheet, 14% turn, and 24% random secondary structure content. Comparison to DSSP secondary structure percentage content determined from the NMR minimized average structure of Fas DD (201-321) PDB ID: 1DDF [79] (Table 2) shows that the α-helical, β-sheet, turn, and random structure percentage content are within acceptable margins of error as determined by root mean square deviation for the SELCON 3 calculation method [127]. SELCON 3 estimated Fas DD V254N to be of 51% α-helical, 9% β-sheet, 21% turn, and 28% random secondary structure content. The V254N mutation to the Fas DD resulted in a decrease in α-helical content and increases in β-sheet, turn, and random secondary structure content compared to Fas DD WT which is consistent with the observed Far UV CD spectra (Figure 4A). Far UV CD analysis of the Fas DD suggests the V254N mutation to the Fas DD resulted in subtle to reasonable secondary structural changes of the Fas DD. The V254N mutation could be altering the proper formation of the turn structure and or adjacent helices 2 and 3 structures resulting in reasonable changes in secondary structure of the Fas DD, as the V254N mutation is located in the CaM binding site in the Fas DD on the loop between helices 2 and 3 [74]. However, the overall secondary structure of the Fas DD V254N is similar to the Fas DD WT. The V254N mutation could result in changes in the orientation of the helical elements in the tertiary structure of the Fas DD. Our computational study showed Fas DD V254N mutation resulted in difference in conformational flexibility and the degree of correlated motion between residues in Fas DD indicating tertiary structure differences between the Fas DD WT and Fas DD V254N mutant (not shown here). The structure changes in the Fas DD V254N mutant could influence the Van der Waals interactions and electrostatic

interactions between CaM and Fas DD of the CaM – Fas DD complex (R. Fancy et al. (2013) under revision). The structure changes of Fas DD caused by the V254N mutation could contribute to the decrease in binding affinity between CaM and the Fas DD observed in ITC experiments.

Taken together the CD and ITC results quantitatively characterize CaM and Fas DD interactions and provided the structural and thermodynamic evidence to explicate the role of the V254N mutation of the Fas DD in CaM and Fas DD interaction. Further, these results could help for the identification of novel strategies to modulate Fas-mediated DISC formation for apoptosis.

CaM and DR-5 interaction and role of CaM-DR-5 binding in DR-5-mediated DISC formation in ER-positive MCF-7 and triple negative MDA-MB-231 cell lines.

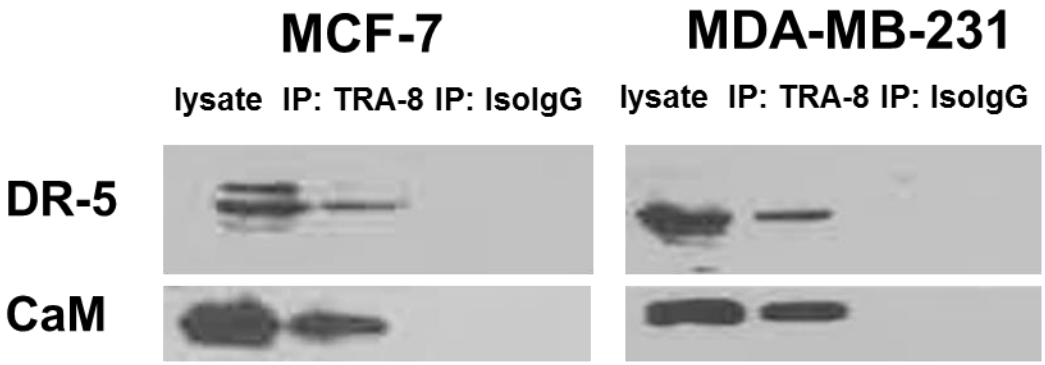

FIGURE 5. Co-IP of CaM-DR-5 complex in MCF-7 and MDA-MB-231 cell lines. lysate lane: whole cell lysate of breast cancer cell showing expression of DR-5 and CaM, IP TRA-8 lane: IP using TRA-8 conjugated beads demonstrating CaM-DR-5 binding, IP Iso-IgG lane: IP using a mouse Iso-IgG conjugated beads demonstrating that CaM or DR-5 do not bind to the sepharose beads or the mouse IgG portion of TRA-8.

The interaction of endogenous CaM and DR-5 was demonstrated by complex-immunoprecipitation (Co-IP) of DR-5 from lysates of MCF-7 and MDA-MB-231 breast

cancer cells using TRA-8 conjugated sepharose beads and mouse Iso-IgG conjugated sepharose beads. The presence of DR-5 and CaM in the cell lysate before Co-IP was assessed by taking a sample of the whole cell lysate and performing a western blot analysis to detect the presence of DR-5 and CaM using primary antibodies against DR-5 and CaM (Figure 5 lysate lane). Results show CaM and DR-5 are detected in the IP: TRA-8 lane demonstrating that TRA-8 binds to DR-5 which is then bound by CaM (Figure 5). However, DR-5 and CaM are not present in the control IP: Iso-IgG lane. Therefore, CaM or DR-5 is not binding to the sepharose bead nor mouse IgG portion of the TRA-8 antibody, confirming a DR-5-CaM interaction. Results demonstrated that CaM binds to DR-5 in both the ER-positive MCF-7 and triple negative MDA-MB-231 breast cancer cell lines.

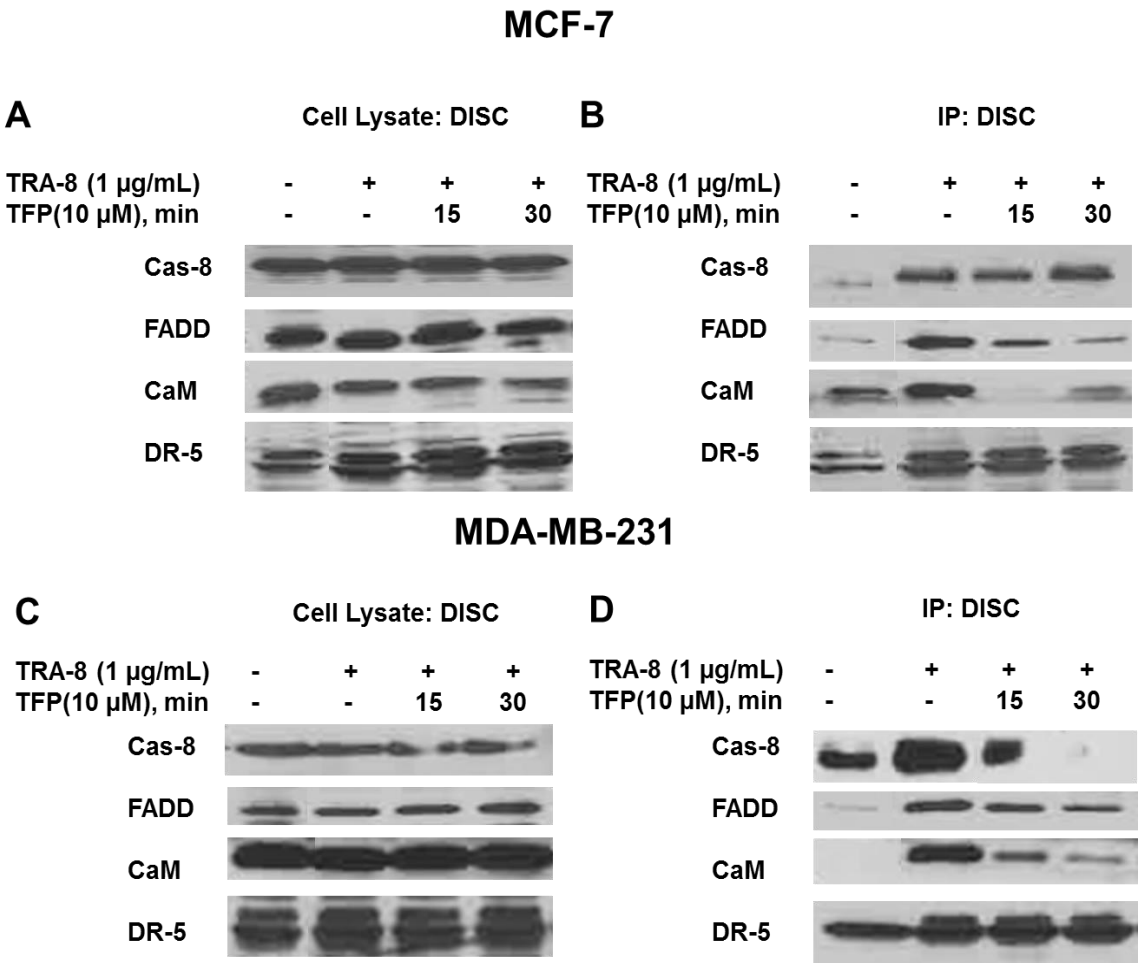

Figure 6. Co-IP of DR-5 mediated DISC complex in MCF-7 and MDA-MB231 cell lines. Upon DR-5 activation, CaM was recruited into DISC and CaM antagonist: TFP inhibited CaM recruited to DISC and attenuated DR-5 recruitment of FADD and Caspase-8 for DISC formation. MCF-7 and MDA-MB-231 cells were untreated, treated with 1 μg/mL TRA-8 only for 3 hours, or preincubated with 10 μM TFP for 15 or 30 minutes followed by TRA-8 treatment for 3 hours.

CaM recruitment into DR-5 mediated DISC by TRA-8 induced DR-5 activation and the role of CaM-DR-5 binding in DR-5-mediated DISC formation were determined by complex-immunoprecipitation (Co-IP). Co-IP of DR-5-mediated DISC was performed using an anti DR-5 antibody (2B9) conjugated beads as described in general method section. Figure 6 shows the cell lysate and Co-IPs of the DR-5 mediated DISC complex in MCF-7 and MDA-MB-231 cells. Co-IP (Figure 6 B and D) results show TRA-8

activation of DR-5 in MCF-7 and MDA-MB-231 cells increased the presence of FADD, caspase-8 and CaM, indicating CaM is recruited into DR-5 mediated DISC (lane 2 of Figure 6 B and D). Recruitment of FADD and caspase-8 for DISC formation mediates DR-5 apoptotic signal by activation of the caspase apoptotic signaling pathway [(50)]. Pretreatment of MCF-7 and MDA-MB-231 cells with 10 μM TFP for 15 or 30 minutes decreased CaM recruitment into DISC and attenuated DR-5 recruitment of FADD and caspase-8 for DISC formation (lanes 3 and 4 in Figure 6 B and D). Western blot of MCF-7 and MDA-MB-231 cell lysates show treatment with TRA-8 alone or both TRA-8 and TFP did not alter the expression of DR-5, FADD, caspase-8 or CaM in both MCF-7 and MDA-MB-231 cells (Figure 6 A and C). Therefore, TFP's regulation of DR-5 mediated DISC formation is not due to an altered expression of DR-5, FADD, caspase-8, or CaM. Preliminary results from a cell apoptosis assay of MCF-7 cells that were not treated with estradiol then treated with CaM antagonist TMX only, TRA-8 only and combined TMX and TRA-8 treatment showed antagonism between TMX and TRA-8 (group unpublished data). These observations could have resulted from TMX serving as a CaM antagonist to inhibit CaM-DR5 interactions and thus inhibit DR5-induced apoptosis. CaM antagonist TFP inhibition of DR-5 mediated DISC formation (Figure 6) could help to interpret the observed antagonism between TMX and TRA-8 treatment in ER inactivated MCF-7 cells in cell apoptosis assay. These results demonstrate CaM binds to DR-5 and CaM-DR5 binding regulates DR-5-mediated DISC formation, which could further affect DR-5 mediated apoptosis.

CHAPTER 4

CONCLUSION

In this study the interactions of CaM with Fas DD were quantitatively characterized using combined ITC and CD experiments. As well an interaction between CaM and DR-5 was established and the role of CaM and DR-5 binding was determined in DR-5 mediated DISC formation. The role of CaM in Fas-mediated DISC formation and Fas apoptotic signaling regulation has been well characterized qualitatively [62, 69, 73, 74, 80, 81, 112, 113, 128-131]. To augment current knowledge on CaM and Fas DD interaction and further explicate the mechanism of CaM and Fas DD binding a quantitative characterization of CaM and Fas DD was undertaken. For the quantitative characterization study ITC results illustrate an endothermic binding characteristic between CaM and Fas DD WT at 37^{o}C and an entropy-driven interaction between CaM and Fas DD. The main contribution to the favorably entropy could be due to the removal of nonpolar residues on the surface of CaM and of the CaM binding site on Fas DD from aqueous solution during CaM to Fas DD binding known as the hydrophobic effect [124]. CaM binding to Fas DD V254N resulted in a similar endothermic binding characteristic as CaM binding to Fas DD WT, however, the binding affinity of CaM and Fas DD was significantly decreased by the V254N point mutation of the Fas DD as shown by ITC. Far UV CD showed that the V254N mutation resulted in change in the secondary structure of the Fas DD by a decrease in the α-helical secondary structural

content of the Fas DD. Conformational and dynamical motion analyses based on MD simulations performed in our lab show that the V254N mutation resulted differences in conformational flexibility and the changed degree of correlated motion between residues in Fas DD compared to the Fas DD WT. These secondary structure and conformational changes by Fas DD V254N mutation could directly affect the Van der Waals interactions, electrostatic interactions between CaM and Fas DD and the entropy of CaM – Fas DD complexes, further decreasing CaM – Fas DD binding as observed in ITC experiments (R. Fancy et. al, manuscript in revision). Results from the quantitative characterization of CaM and Fas DD interactions using CD and ITC provide the structural and thermodynamic evidence to explicate the role of the V254N mutation of the Fas DD in altering CaM and Fas DD interaction.

For the CaM and DR-5 interaction study, an interaction between CaM and DR-5 and a role for CaM and DR-5 binding in DR-5-mediated DISC was determined in the estrogen positive MCF-7, and the triple negative MDA-MB-231 breast cancer cell lines. Co-IP of DR-5 demonstrated CaM binds to DR-5 and CaM is recruited into TRA-8 induced DR-5 mediated DISC formation in MCF-7 and MDA-MB-231 cells. Further CaM antagonist TFP modulates DR-5 mediated DISC formation by inhibiting the recruitment of CaM, FADD and caspase-8 to form DR-5 mediated DISC. These results provide a basis for the continued investigation of characterizing CaM and DR-5 interaction and elucidating the role of CaM-DR-5 binding in DR-5 mediated apoptosis signaling in breast cancer cells. The understanding of CaM-DR-5 binding and its role in DR-5 mediated apoptosis could present a novel target and lead to the identification of therapeutic strategies to for breast cancer treatment. Targeting CaM-DR-5 binding could

overcome the clinically observed drug resistance of breast cancer treatment including hormone resistance phenomena observed in ER positive breast cancer types (MCF-7) and present a target for triple negative breast cancer types (MDA-MB-231).

Collectively the quantitative characterization of CaM with Fas DD, the established interaction of CaM and DR-5 and the role of CaM in DR-5-mediated DISC formation in breast cancer cell lines augment current knowledge of the interaction of CaM with the death receptors Fas and DR-5. The Fas and DR-5 receptors are commonly the focus of anti-cancer therapy studies and an increased knowledge of their signaling regulation may lead to the development of novel strategies to modulate death receptor-mediated DISC formation and death receptor-mediated apoptosis for the treatment of breast cancer.

CHAPTER 5

FUTURE STUDIES

Characterization of CaM and DR-5 interaction

The development of strategies targeting CaM-DR-5 binding for the effective targeted therapy of breast cancer requires an in-depth characterization of CaM and DR-5 interaction. To further characterize CaM and DR-5 interaction, several specific aspects of CaM and DR-5 interaction will be addressed in the near future. A direct interaction between CaM and DR-5 will be determined by examining CaM and DR-5 binding in an *in vitro* pull down assay using purified recombinant CaM and the cytoplasmic region of DR-5, this would exclude the possibility that CaM and DR-5 association is mediated by other DR-5 binding proteins and confirm that CaM regulates DR-5-mediated DISC and

apoptosis via direct interaction with DR-5. CaM is a Ca^{2+} binding protein and many of CaM's interactions with CaM-regulated proteins are Ca^{2+}-dependent [(132)], thus the Ca2+ dependence of CaM binding to DR-5 will be examined. The CaM's interaction with the Fas death receptor has been localized to the Fas DD [(74)]. Recently CaM has been show to interact with FADD in cervical cancer and lymphatic cells with recombinant CaM pull down assay and the binding site of CaM in FADD has been localized to the death domain of FADD [(133)]. Hence, it is anticipated that CaM may bind to the death domain region of DR-5. The binding site of CaM in DR-5 cytoplasmic death domain will be determined. CaM-regulated proteins possess conserved CaM recognition binding motifs within their CaM-binding domains [(132)]. Using computational modeling, the CaM binding site on the cytoplasmic region of DR-5 and the critical residues in DR-5 DD for CaM-DR-5 binding will be predicted. Further, the CaM binding site on DR-5 will be determined using in vitro binding assays involving DR-5 peptide sequences containing the predicted CaM binding site and key residues involved in CaM and DR-5 DD interaction will be determined by designing mutations within the CaM binding site on DR-5 that could perturb CaM and DR-5 DD interaction. Mutations to the death domain of DR-5 have been identified in multiple cancer types including breast cancer [(107, 134-137)] and these mutations to DR-5 may serve as a mechanism of DR-5 apoptosis resistance in cancer cell types. Recently our lab has quantitatively characterized the interactions of the Fas DD and CaM in combined isothermal titration calorimetry (ITC), circulardicroism spectroscopy (CD), and molecular dynamics simulations (MD) study. Similarly to our quantitative characterization of Fas DD and CaM interaction, the quantitative characterization of CaM and DR-5 DD interaction will be examined using ITC, CD

spectroscopy, and MD. ITC will be used to evaluate the thermodynamics of CaM and DR-5 DD binding and the change in CaM and DR-5 binding caused by mutation to the CaM binding site on DR-5. CD spectroscopy will be performed to examine changes in the structure of DR-5 DD caused by mutations at the CaM binding site on DR-5 and the change in structure in CaM and DR-5 DD caused by the formation of the CaM-DR-5 DD complex. Lastly MD simulations will be conducted to reveal the changes in conformational and structural properties caused by mutations to the CaM binding site on the DR-5 DD that could lead to changes in CaM and DR-5 DD binding. Results would provide thermodynamic and structural evidence for CaM and DR-5 DD interaction. Taken together completing these tasks would provide an in-depth characterization of CaM and DR-5 interaction and would augment current knowledge of the interaction of CaM with the death domain of the death receptors Fas and DR-5.

CaM regulation of DR-5 mediated anti-apoptotic complex signaling

Counteractive to DISC formation and induction of apoptotic signaling, DR-5 activation can induce signaling pathways for cell survival and proliferation [138, 139]. As illustrated in Figure 7, DR-5 activated pro-survival signaling may be mediated by the formation of an anti-apoptotic signaling complex which may be composed of TNF receptor-1 associated dead domain protein (TRADD), which like FADD binds to the death domain of the death receptors. TRADD then recruits receptor interacting protein (RIP), TNF receptor associated factor 2 (TRAF2) and transforming growth Factor-beta activated kinase (TAK1) [140]. TAK1 activation leads to the activation of the IKK alpha/beta-IkB/NF-kB pathway, and c-Jun NH2-terminal kinase (JNK) MAPK pathways,

which mediate resistance to DR-5 mediated apoptosis [102, 141]. Perhaps CaM's interaction with DR-5 may regulate DR-5's association with TRADD and the formation of this anti-apoptotic complex.

Alternately, DR-5 activation could mediate the formation of a cytosolic secondary complex not bound to the death domain of DR-5 including FADD, caspase-8, RIP-1 and possibly TRAF2, NEMO, TRADD and IKKα [140, 142]. Formation of the secondary complex could lead to the activation of the MAPK and NF-KB anti-apoptotic pathways. CaM has been shown to interact with FADD and the CaM binding site on FADD has been localized to the death domain of FADD [133]. Therefore, could CaM binding to DR-5 and or FADD regulate the formation of the secondary complex containing caspase-8, RIP, and TRAF2 and NEMO or TRADD and IKKα, thus leading to the activation of the MAPK and NF-KB anti-apoptotic pathways in breast cancer cell lines? The formation of anti-apoptotic complex(s) could play a role in attenuating DR-5 apoptotic signaling after activation, and contribute to the development of resistance to death receptor-induced signaling in breast cancer cells. Understanding the mechanism of the role of CaM/DR-5 binding in DR-5 mediated anti-apoptosis complex(s) formation and how they contribute to resistance to DR-5 mediated apoptosis in breast cancer cells could be imperative for the development strategies for promoting DR-5 mediated apoptosis.

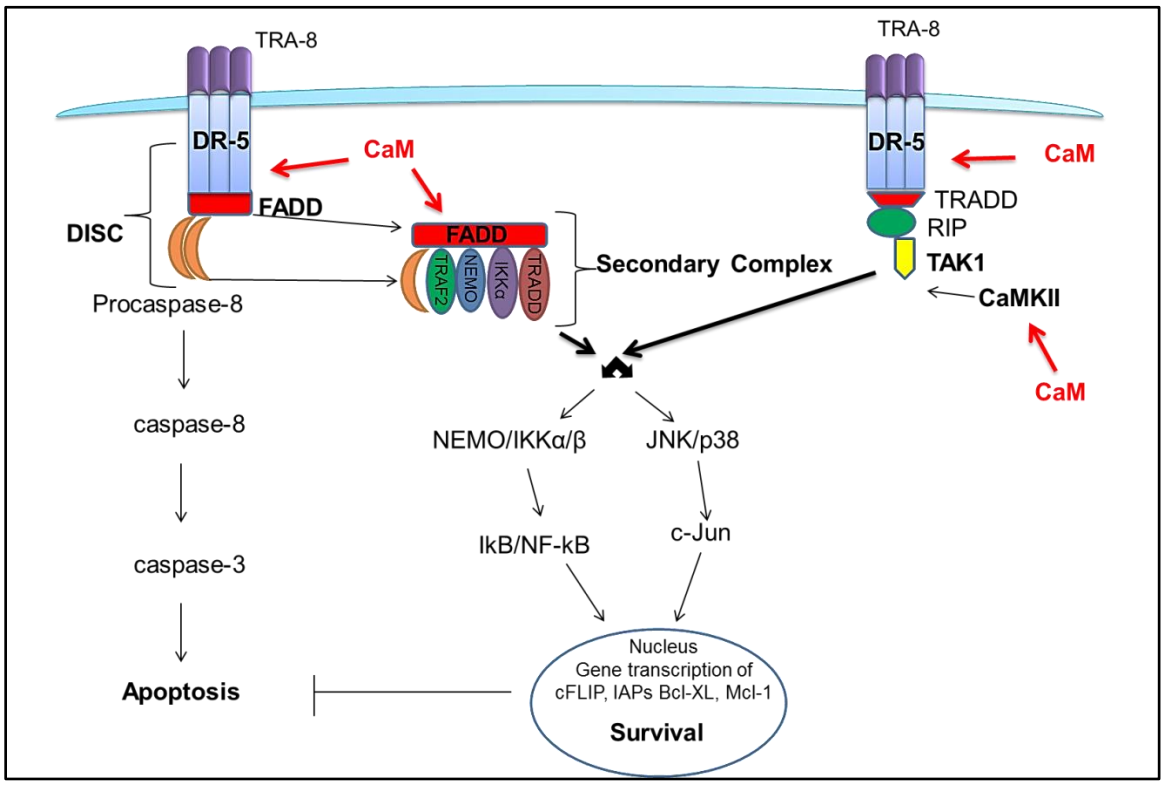

Figure 7. A graphic representation of the potential mechanism of DR-5 mediated DISC and subsequent anti-apoptotic / secondary complex formation and the potential role of CaM in DR-5 DISC and anti-apoptotic complex signaling regulation.

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