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Mutagenic Analysis of SH3 Binding Site in the Avian ChB6 Alloantigen

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“Mutagenic Analysis of SH3 Binding Site in the Avian ChB6 Alloantigen”

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By

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Introduction:

The Immune system is the set of defenses in the body that protects us from becoming ill. It is made up of many different types of cells and substances, all of which work together to help us heal when we have been injured. The immune system recognizes, fights, and remembers foreign invaders like bacteria or viruses, which can cause illness in our bodies. Such invaders are called “pathogens”. Our immune system is responsible for defending the body against a wide and constantly changing range of possible pathogens. The number of cell types, molecular interactions and soluble factors that are involved in the immune response are not only responsible for an organism’s defense against infections but also play an important role in self-maintenance and antitumor responses. When the immune system hits the wrong target or is crippled, it can result in disease, including allergy, AIDS, arthritis and many more (1, 2).

The immune system is amazingly complex; it can target unique features of pathogens by tailoring specific response to that pathogen. In order to do this, cells in the immune system must first have the capacity to recognize a pathogen and convert that recognition into action. This is done by a variety of receptors on the surface of immune cells (1).

Innate immunity is the first line of defense against invading pathogens. The cells of the innate immune system can detect pathogens using pattern recognition receptors. Pattern recognition receptors are encoded within the germline and bind to molecules characteristic of larger groups of pathogens. For instance, Toll Like receptor (TLR) 2 and 4 can bind lipopolysaccharide (LPS). LPS is a component of the outer membrane of all gram negative bacteria and is thus a molecular pattern that is a signature of this group of bacteria, or a Pathogen Associated Molecular Pattern
(PAMP)(3). Upon binding of LPS to the TLR 2/4 dimers on the surface of an innate immune cell, it initiates a cascade of intracellular events leading to the production of cytokines, intercellular signaling molecules, which ultimately results in inflammation. In this way the innate immune system can activate a defense mechanism quickly, but it is not a defense tailored to that specific pathogen, TLR does not distinguish between the LPS of *E. coli* and *P. aeruginosa*, so the innate system, while it can initiate a response, is not capable of a pathogen specific response and it does not provide lasting immunological memory (1, 2).

The adaptive immune system on the other hand does have the capacity to recognize specific features of a pathogen and create immunological memory, producing a life-long memory of our previous infections. The adaptive immune system is made of B and T lymphocytes. B and T lymphocytes are similar in many ways and I will focus here on B-lymphocytes since they are the focus of my research.

To effectively eliminate the variety of pathogens that an individual may encounter in their lifetime, the immune system has evolved to recognize many different antigens. Within adaptive immunity there are also safe guards where the self-reactive or defective cells are eliminated (1). The removal of such cells is by apoptosis, which is a form of cell suicide characterized by an orderly breakdown of the cell to limit the leakage of cytoplasmic components that could cause inflammation. Apoptosis can be initiated by signaling through specific cell surface molecules and is carried out by downstream mediators. While several model systems of apoptosis do exist, one pathway in developing avian B-cells, which is activated by the cell surface molecule chB6, has yet to be elucidated(4,5). The Funk lab has been interested in the role of alloantigen chB6 (Bu1) in the developing of B cells within the bursa and has shown that chB6 can trigger apoptosis. When an agonist antibody
stimulates chB6, rapid cell death follows and it is presumed that there is an endogenous ligand. Funk lab has evidence which suggests that there is certain section of chB6 molecule that is essential in initiating a death signal inside the cell (Funk unpublished). Within this death signaling region is a SH3 binding site and I was interested in testing whether mutating this SH3 binding site affects the cell death. This project furthers our understanding of the biology of chB6.

**Literature review**

**B-lymphocytes** are responsible for the manufacturing of antigen recognition molecules known as immunoglobulin (Ig), also called antibodies. To efficiently identify and eliminate the variety of pathogens that an individual may encounter, the immune system has evolved to recognize many different antigens by creating a vast array of cells, each bearing a unique receptor (the B Cell receptor which includes the antibody molecule) for a particular antigen. When a B cell bearing a receptor that binds to an antigen with high affinity encounters that antigen, the B cell will divide, creating a series of descendants all bearing an antibody of identical specificity and this process is known as clonal selection(1). Some of these descendants become memory cells and some become plasma cells. Plasma cells secrete their antibody and thus help to effect an immune response to the antigen. These secreted antibody molecules have two main functions: to bind to an antigen and recruit different cells and molecules to destroy the foreign antigen after the antibody has bound to it(1).

Since each B cell can make only one type of antibody, millions of B cells are required to counter the vast array of potential pathogens. It has been estimated that
there are \(10^8\) antigens that an individual may be exposed to, thus production of a large variety of antibodies is necessary. This diversity is generated by a process called V(D)J gene rearrangement (1,6).

To understand how antibody diversity is created, it is first necessary to understand the physical structure of an antibody protein. Antibody molecules are Y shaped molecules, which consists of three equal sized portions connected by a flexible tether. All antibodies are constructed in the same way from pairing heavy and light polypeptides and the generic term immunoglobulin is used for all such proteins. Immunoglobulin molecule is composed of two types of protein chain: heavy chains and light chains, each immunoglobulin molecule is made up of two heavy chains and two light chains which are joined together by disulfide bonds so that each heavy chain is linked with a light chain and the two heavy chains are linked together (Figure 1,2). Both the light and heavy chains contain constant and variable regions.

The variable regions of each antibody molecule are different from of all other antibody molecules and this is the specific region which binds to an antigen (Ag), therefore the variable region can have almost infinite number of forms. It is the variable region that determines the specificity to the antigen. In both the light and heavy chains, amino acid sequence variability is concentrated within three segments known as hyper variable loops or complementary determining regions. Thus when an antigen binds to an antibody each will have a shape that is complementary to the other, like a hand fitting a glove. There are almost \(10^8\) antigens that any individual may be exposed to and due to this high number, the production of a large variety of antibodies is necessary. The Human genome contains approximately only 20,000(1) genes and due to this limited amount of genetic material available, B cells undergoes
series of random gene rearrangement within their antibody to create a huge diversity of unique antibodies (1).

Gene rearrangement occurs in the exon that encodes the variable region of an antibody, as this is where the antigen-binding site is situated. The variable region of the heavy chain undergoes gene rearrangement followed by the variable region of a light chain. Three separate gene segments encode the heavy chain variable region: The variable (V\textsubscript{H}) gene segment, the diversity (D\textsubscript{H}) gene segment and joining (J\textsubscript{H}) gene segment. In heavy chain rearrangement, a (D\textsubscript{H}) gene segment first is joined to a (J\textsubscript{H}) segment; then a (V\textsubscript{H}) segment rearranges with a rearranged (DJ\textsubscript{H}) segment to form a complete heavy chain variable region exon. The joining of these regions constructs continuous exons, which encodes the entire heavy chain variable region. (Figure 1,2). The light chain rearranges in a similar fashion, but it contains only V and J segments (1).

Since there are many functional gene segments for immunoglobulin and any of these segments is capable of rearranging with each other, a diverse group of antibodies can be produced. In humans, the heavy chain locus consists of 65 functional (V\textsubscript{H}) segments, 27 (D\textsubscript{H}) segments and 6 (J\textsubscript{H}) segments, therefore about 11,000 different V\textsubscript{H} regions are possible just based on the recombination of multiple gene segments (65 X 27 X 6). Once the heavy chain is rearranged, light chain rearrangement begins, there are two types of light chain: kappa (κ) and lambda (λ), each containing distinct V and J gene segments. In the human Kappa light chain, there are 40 functional V segments and 5 J segments making 200 combinations. In lambda light chains there are 30 functional V segments and 4 J segments making 120 combinations. This sums up to 320 different light chains and 11,000 heavy chains resulting in $3.5 \times 10^6$ antibodies with diverse specificities based solely on the number
of gene segments available(1). Additional mechanisms during the recombination process create diversity by subtly altering the exact recombination points, meaning that even if the same $V_L$ and $J_L$ recombined in different B cells, they would likely create a different sequence across the junction of the gene segments. For example, the enzyme terminal deoxynucleotidyl transferase (TdT) can add non-templated nucleotides during the recombination event. In all, these mechanisms are quite powerful and can create an estimated more than $10^{12}$ distinct antibody molecules.(1,2, 6, 7)

When we encounter an infection, at least a few of the B cells recognize the foreign molecule and are able to mount a response. It is this diversity of B cells that helps us to respond to a wide range of infections (1). It is interesting to note that gene diversification in avian species occurs somewhat differently. In birds, B cells develop in a primary lymphoid organ, the bursa of Fabricius, rather than the bone marrow(8,9). The avian Ig locus has only few available D segments, and only one active V and J segment for the heavy chain gene (8). Similarly there is only one active V and J for the light chain gene. Therefore gene rearrangement can result in limited receptor diversity in chicken. In order to generate diversity among immunoglobulin in avian species, B-cells undergo somatic gene conversion. B cell precursors that have successfully undergone V (D) J rearrangement in the embryonic spleen migrate towards the bursal epithelial basement membrane from the spleen and proliferate in the follicles of bursa (6-8). During the proliferation in the bursa pseudo genes, which are non-functional V gene segments, are introduced into the rearranged gene, thus creating diversity in the receptors. Gene conversion can occur multiple times in multiple positions in the same B cell clone during development in the bursa(7,8)
Rabbits also undergo this type of B cell diversification in order to generate their antibody repertoire, however this process takes place in the appendix(10).

**Problem of antibodies**

While diversity in the antibody repertoire is essential to counter an ever-changing group of pathogens, it creates certain problems. The rearrangement events described above use varied gene segments and non-templated nucleotides to generate as much diversity in the antibody repertoire as possible. However, this randomness also means that we can create antibodies that would attack our own cells. It also means that some B cells will be defective and unable to produce a functional antibody protein(2). Non–functional proteins may arise when the rearrangement process creates a premature stop codon, for example. This results in production of a non-productive gene rearrangement and will ultimately result in B cells that cannot produce a functional immunoglobulin molecule. Among those cells that successfully generate an antibody molecule, some of them can be auto reactive, binding to self-molecules. These cells bear Ig that could potentially bind to self-molecules and will initiate an attack on one’s own cells. This lack of tolerance to our own tissues could lead to autoimmune diseases where our immune system attacks us. Both non-functional and potentially auto reactive cells must be eliminated. Evidence indicates that they are eliminated via apoptosis to minimize damage to surrounding cells and tissues, and prevent a defective B cell from reaching peripheral circulation where it could become dangerous (11). In studies of both mouse and chicken, it is estimated that 90 - 95% of B cells undergo apoptosis during development and only 5% migrate out to the periphery to become a mature B cell capable of responding to antigen(11,12).
Given the challenge of creating a diverse antibody repertoire, it is not as simple as just deleting potentially dangerous cells. If immature B cells in bone marrow show any kind of specificity for a self-molecule, the problem can be corrected through receptor editing and clonal deletion (13-19). As I will discuss later, B cells receive signals via their surface bound antibody molecules and the nature of this signal can influence how B cells behave. In receptor editing, light chains can make several successive rearrangements. Remember that the antibody specificity is determined by the variable regions of both the heavy and light chains; by replacing the light chain it might make the antibody non-self-reactive and thus save the B cell. (13,14,20). The more common among path is clonal deletion; where self-reactive cells receive a signal to undergo cell death via apoptosis and are eliminated prior to maturing into functional B cells. Escape from apoptosis can lead to the presence of auto reactive lymphocytes and also growth of leukemic cells due to uncontrolled growth of cells or failure to die when given an apoptotic signal. (20,21)

Cell surface molecules such as Fas (CD95) and others in the tumor necrosis factor receptor (TNFR) family are known to be involved in the deletion of the mature B cells in the periphery via initiating apoptotic signals (22,23). Thus when a mature B cell encounters a portion of self-molecule that it shows an affinity for, one of four major mechanisms occurs to prevent autoimmunity. First the cell becomes inactive or anergic which results in the down regulation of surface immunoglobulin expression and inhibition of the signaling pathways. On the other hand cells may be restricted from interacting with the T cells, a signal that is crucial for a proper immune response and also B cell survival, thus fails to interact with T cells can ultimately inhibit any signals given to B cell. In yet other mechanisms, signals that normally prevent apoptosis from occurring may be down regulated, or signals that promote apoptosis
may be up regulated. The end result for all these mechanism is death of auto reactive B cells. If any of these fails, it can result in auto immunity (1). Also it is interesting to note that any deregulation of TNF production, whether it is low or high, can also lead to autoimmune diseases (24). In fact recent studies have shown that dualistic, pro inflammatory and immune or disease suppressive role for TNF in these conditions. Recent studies have also shown that blocking of TNF in autoimmune prone chronic inflammatory diseases can lead to unpredictable outcomes. For example studies have shown that blockade of TNF in human rheumatoid arthritis or inflammatory bowel disease could be beneficial for majority of the patients. Thus controlling the amount of TNF in our body is very crucial to reduce the chances of autoimmune diseases (24, 25,26,27).

An autoimmune disorder occurs when the body’s immune system attacks and destroys healthy body tissue by mistake, thus the ability to discriminate between self and non-self antigens is vital to the functioning of the immune system as a specific defense against invading microorganisms. Failure of the immune system to "tolerate" self-tissues can result in pathological autoimmune states leading to debilitating illness and sometimes death (1). It's estimated that 5 to 8 percent of people in the United States are living with an autoimmune disease. Researchers aren't sure why, but the prevalence of autoimmune diseases seems to be increasing. (24).

Cell signaling

Cells, particularly lymphocytes, are subject to a variety of external stimuli and their responses to those stimuli are responsible for a cell responding in a particular way. For instance, the same stimulus, antigen binding with high affinity to a cell surface antibody, can cause an immature B cell to either attempt receptor editing or undergo apoptosis (1). In a mature B cell this stimulus would trigger cell division and
subsequent metabolic changes to make the daughter cells into antibody secreting plasma cells. But the exact number of divisions, the antibody-secreting capacity of the plasma cell, the life-span of the plasma cell, and a host of other factors are all ‘tuned’ by the signaling machinery activated in individual cells. So, in order to understand the immune system and B cell function, we must understand cell signaling.

In general, cells that do not undergo proper gene rearrangement do not receive specific developmental signals from their surrounding environment and therefore undergo a programmed cell death also called apoptosis(23). Cells that undergo apoptosis loose contact with their surrounding tissue. Their cytoplasm and nucleus condense resulting in cell shrinkage. Their mitochondrial membranes become more permeable and loose membrane potential, releasing cytochrome c into the cytoplasm (28). The nucleolus and nuclear envelope break down, resulting in condensation of chromatin. The plasma membrane ultimately divides the cell into apoptotic bodies by forming invaginations and protrusions. These dying cells signal their apoptotic state to the surrounding tissue, which assists in the degradation of apoptotic bodies by phagocytosis (28).

Necrosis is also a form of cell death, where cells die in response to physical and chemical injury. Necrotic cells take up water and swell up. Swelling causes the cell to burst open releasing all its contents into the surrounding environment (28). The physiological effects differ greatly from those of apoptosis. Since a necrotic cell releases its contents, there is a high chance that infected cells may release pathogens that end up spreading infections. Additionally, the release of cytochrome c by mitochondria could cause tissue damage and activate auto reactive lymphocytes. Thus apoptosis is the preferred mechanism to eliminate B cells (28).
There are two major pathways through which a cell can initiate apoptosis; loss of signal transduction, or receptor initiated signal transduction in which healthy cells are instructed to die (23). As stated earlier, the process of gene rearrangement and gene conversions frequently results in errors. Cells that do not produce in-frame Ag receptor end up undergoing apoptosis. Additionally even activated lymphocytes can undergo this type of cell death at the end of an immune response once the antigen and inflammatory response have been removed from the system (23).

In contrast, a healthy cell whose death is induced by cell surface receptors requires caspase activation (23). Apoptosis initiated by the death domain (DD) containing death receptors of the Tumor necrosis factor receptor family (TNFR) is an example of this type of cell death receptor (29,30). Once the death receptor is bound by its respective ligand, the death domain of the cytoplasmic tail binds to the death domain of an adaptor molecule. An adaptor molecule plays a key role in linking death receptor and downstream members of a signaling pathway. For example, the adaptor protein FADD links the surface receptor to intracellular procaspase, activating the caspase and initiating cell death (1,31).

Fas (CD95), a member of the TNFR family, is also an example of a death receptor. Its role is to trigger apoptosis in cells. Fas is activated by its ligand or agonist Ab (antibody) which results in the recruitment of Fas- associated death domain protein (FADD) via their death domain. This results in trimerization of the FAS molecules. Fas and FADD interact via their C terminal death domain, which exposes FADD’s N terminal death effector domain (DED). The DED of FADD in turn interacts with the DED domain in procaspase 8. This binding of procaspase 8 and FADD results in cleavage of procaspase 8, forming active caspase 8 and initiating a protease cascade. In this cascade, caspase 8 activates downstream caspases such as
caspase 3 and 7. The final stage is when caspase activated DNase enters the nuclease and cleaves the DNA resulting in DNA fragmentation, one of the main characteristics of an apoptotic cell (29, 32-34)

Immune cell responses are controlled by many receptors that are capable of curbing the activity of the cell. The first signal originates from the antigen receptor in T and B cells and these early signals are tempered by a number of activating and inhibitory receptors, which ultimately play an important role in determining the immune response. It is important to note that in many cases inhibitory and activating receptors can be from the same receptor family. The immunoreceptor tyrosine based inhibition motif (ITIM) is one of the significant features of many inhibitory receptors. It consists of a six amino acid sequence containing tyrosine(Y) with conserved N terminal(Y-2) and c terminal (Y-3) residues (35). On the other hand, activating forms contain immunoreceptor tyrosine based activation motifs (ITAM), where tyrosine phosphorylation forms a docking site for kinases (36). The activating receptors have a short cytoplasmic tail and have transmembrane residues that help in the association with adaptor molecule containing ITAM(31). Immunoreceptor families with inhibitory and activating members include Fc receptors (36). An example to explain this process would be CD40 co stimulatory molecule. CD40 is constitutively expressed on APC’s and CD40 ligand (CD40L) is induced on T cells upon Ag recognition. Thus the interaction between CD40 and CD40 L also enhances T cell responses. Even though the mechanism remains unclear, it is believed that CD40:CD40L interactions serve to upregulate interactions of other CD’s like CD 80 on APC’s and to induce the production of cytokines such as IL-12 which ultimately promotes T cell differentiation. Researchers have shown that CD28 and CD40L play distinct and complimentary roles
in T cell activation and also suggests why blocking both these costimulatory pathways may have distinct effects on T cell response to Ag (37).

**SLAM (Signaling lymphocyte activation molecule)**

A particular family of molecules is called SLAM (signaling lymphocyte activation molecule) and is known to be related directly to B cell biology. It is a family of nine immunoglobulin like receptors, which includes SLAM F1, SLAMF3, SLAMF4, SLAMF5, SLAMF6 and SLAMF7 (38,39). These receptors are known to be closely related to CD2 family and are expressed in various immune cell types. SLAM/CD46 is also immune cell specific cell receptor for measles virus in humans (40). The SLAM family is characterized by having two extracellular Ig domains and an intracellular sequence of amino acids called the immunoreceptor tyrosine switch motif (ITSM) (41). SLAM family members have shown to be been involved in the deletion of lymphocytes and recent studies suggest that SLAM is associated with autoimmune diseases in humans and mice (40,42). X linked lymphoproliferative disease (XLP) 1 is a type of immunodeficiency caused by mutation or deletion in SH2 DIA gene which encodes SLAM associated protein (SAP) (41). Loss of SAP activity is associated with severe natural killer (NK), T and B cell abnormalities and reduced antibody production (40). Studies have shown that SLAM F genes play a major role in mediating the loss of tolerance to nuclear antigens and also in the development of auto immunity in Sleb mice (43). Recent studies have also shown that polymorphisms in SLAM family genes are implicated in both murine and human lupus (43). A recently published report found that the LY 108/SlamF6 gene is implicated in the loss of early B cell as well as T cell tolerance. Ly108 regulates T and B cell interactions.
by modulating positive and negative signals through SAP(43). Collectively this data indicates that SLAM/CD2 family members can have a variety of important impacts on lymphocyte function and survival(43) and underscores the complexity of lymphocyte signaling during both development and during an ongoing immune response.

Recent studies have identified chicken SLAM family of receptors and characterized five members of the family as well as the Sap adaptors (38). Three chicken SLAM genes were identified on chromosome 25 in the chicken genome. The researchers have confirmed that out of five chicken SLAM genes, three of them could be assigned to human counterparts and therefore named as SLAMF1, SLAMF2 and SLAMF4 (38). Although they share only 24 to 28% identity with the mammalian genes, they possess highly conserved cytoplasmic motifs in the case of SLAMF2 and SLAMF4. Studies have shown that chicken SAP is similar to mouse SAP; they are identical in length and both feature a SRC homology domain. Src family protein tyrosine kinases are known to participate in signaling through cell surface receptors that lack intrinsic tyrosine kinase domain. However it is interesting to note that human SAP is much shorter. These results suggest that chicken SLAM receptors employ similar signal transduction pathways as described for mammals(38). Thus suggesting that SLAM receptors in chicken have the ability to modulate the immune response in a similar fashion as in mammals. Scientists are still testing to what extent these molecules impact cell death signaling (39,40,44).

Studies need to be done on how these molecules activate death signaling. Recently scientists have also reported that SLAM receptors play important roles in regulating both innate and adaptive immune response by virtue of their ability to transduce tyrosine phosphorylation signals through immunoreceptor tyrosine based
switch motif sequences (45-51). The process by which cells respond to signals is an interesting area of research. In general during signaling, proteins interact in a specific way, moving the signal from membrane bound proteins to cytoplasmic proteins. Work on the src kinase family of protein tyrosine kinase have defined two types of protein interacting domains; SH2 (src homology 2) domains interact with phosphorylated tyrosine residues and SH3(src homology3) domains interact with proline rich sequences (PXXP) about which I am going to talk in the next couple paragraphs (52).

**Src Family Kinases(SFK):**

The Src family of protein kinases (SFK) plays a crucial role in regulating signal transduction pathways via diverse cell surface receptors. The name src derives from the discovery of the prototypical src gene in the Rous sarcoma virus, a chicken tumor virus discovered in 1911 by Peyton Rous (53) Src family kinases are controlled by receptor protein tyrosine kinases, integrin receptors, G protein coupled receptors, antigen and Fc coupled receptors, cytokine receptors and steroid hormone receptors. Src kinases are usually anchored in the cell membrane by a glycophosphatidyl inositol (GPI) linkage, tethering the kinase to the inner leaflet of the plasma membrane and associating the kinase with lipid rafts in the membrane. Studies of src proteins identified three major domains common in src family members, SH2 (src homology 2), SH3 (src homology 3) and SH1 (kinase catalytic domain). (54,55). Subsequently, SH2 and SH3 domains have been identified in a wide variety of non-src proteins. The SH3 domain consists of approximately 60 amino acids and has been recognized in several proteins(56,57);many of these are involved in signal transduction such as phospholipase C and phosphatidylinositol 3 kinase (PI 3-K) (55).

Src homology 3, which is also known as SH3 domain, binds to target protein through sequence containing proline and hydrophobic amino acids. (55). The SH3
domain is known to have a characteristic fold which consists of five or six beta strands arranged as two tightly packed anti parallel beta sheets (58). The surface of the SH3 domain consists of a flat, hydrophobic ligand binding sites consists of three shallow grooves defined by conservative aromatic residues in which the ligand adopts an extended left handed helical arrangement. The region bound by SH3 domain is proline rich and contains PXXP as a core conservative binding motif. The function of the SH3 domain is not very well understood but it may mediate many diverse processes such as increasing local concentration of proteins, altering their subcellular location and mediating the assembly of large multiprotein complexes (59). It is interesting to note that the SH3 domain is conserved throughout evolution from yeast to mammals and different lines of evidence suggest that SH3 domain would be involved in protein-protein interactions. Scientist have tried hard to resolve the structure of many SH3 domains (60) and all of them show similar folds. The SH3 domain is perhaps the best-characterized member of the growing family of protein-interaction modules. (22, 45, 52, 54, 56, 61)

An important feature to note in any signal pathway is the ability to interact with each other in some sort of specific way, which allows signals to move from a membrane bound protein to cytoplasmic proteins and it is also important to note that signaling processes involves protein conformational change and which involves exposing areas like PXXP (SH3) motifs (52). Research in signaling transduction can be challenging because different proteins have different pathways and they all might interact in additive, synergistic or antagonistic ways (61) and thus SH3 domain becomes an important part of my project.

To summarize, we know that B cells can bear potentially auto reactive antibodies and that these cells must be eliminated early in B cell development. We
also know that in order to receive a signal for elimination B cells must use signal transduction pathways. Yet, signal transduction is inherently complex and we must have clear experimental models to tease apart these pathways. So to understand how signal transduction pathways might trigger the death of B cells we need a model system to study B cells during the deletion event.

**Bursa of Fabricius.**

The chicken has a unique organ, the bursa of Fabricius, which is completely devoted to producing B cells. The bursa epitomizes a model system where we can ask questions about signals monitoring the life and death of B cells at this stage of development (62). The bursa of Fabricius also provides the necessary microenvironment for diversification of avian Ig genes (63). Chemical or surgical ablation of the bursa early in development results in poorly diversified Ig genes and a consequent defect in humoral immunity with reduced antigen specific Ig responses (64). The bursa begins developing between day 3 and 5 embryonic life and forms from epithelial invaginations from a dorsal appendage of the cloaca. It remains connected to the gut by bursal duct. Because of this connection, external gut antigens might be able migrate to bursa. Between days 8 and 10 of embryonic development mesenchyme cells are able to migrate and adhere to the epithelium. There they form epithelial buds by increasing in volume. These buds ultimately develop into bursal follicles at 10 and 11 days; between days 8 and 14 B cell precursors migrate from spleen to the bursa. Precursor B cells undergo Ig gene arrangement while in the embryonic spleen (6), so they enter the bursa as B cells with rearranged V_{H} and V_{L} loci. Once the precursors B cells reach the bursa, they start to proliferate and form approximately 10^4 lymphoid follicles that will eventually hold about 10^5 lymphocytes each. However it is important to note that there is no migration from one follicle to
another (6,9). B cells also undergo a series of gene conversion events while
undergoing proliferation in the bursa to develop a wide variety of antigen specificities
as mentioned earlier. Proliferation of B cells and gene conversion of Ig genes in the
bursa continues until about 16 weeks after hatch (63).

Most of the B cells within the bursa die and only 5% successfully leave the
bursa and participate in the immune system (11). Chickens lacking the bursa
microenvironment exhibit only a few mature B cells that had differentiated from B
cell precursors and a low rate of gene conversion. The low diversity of the antibody
genes in these animals indicated the important role of the bursa in the proliferation
and diversification of the chicken B cell repertoire (64). Chicken gene conversion was
initially believed to occur only in birds but now it is clear that a number of animals,
such as rabbit and sheep, also use this mechanism to develop an immunoglobulin
gene repertoire (66). Bursectomized chickens also exhibited low level of surface
immunoglobulin positive (sIg) B cells in the periphery compared to their non
bursectomized counterparts at 10 weeks of age (64). However they were able to
produce some Ig but they lacked the ability to produce specific antibodies upon
repeated immunization. Even upon in vitro exposure to bursal epithelial cells or bursal
epithelial conditioned media at 10 weeks of age, B cells that had matured in the
absence of the bursa were still unable to produce specific antibodies. This indicates
that not only bursa, but also the early timeframe in which developing B cells interact
with bursal epithelial cells is critical for proper gene conversion as well as B cell
proliferation. Furthermore, B cells in the bursa are more or less at the same stage of
development, they possess antibody on their surface (8,9).

Interestingly, birds can also develop antibody-mediated autoimmune disease
just like humans, indicating that self-reactive lymphocytes are normally censored
within the bursa (63). Loss of Ig expression appears to precede apoptosis in the bursa, which would explain the death of cells incapable of translating Ig transcripts (6,9).

Within the bursa B-lymphocytes are particularly susceptible to apoptosis, yet appear to be kept viable by interactions with epithelial cells within lymphoid follicles. This is the reason why bursa represents a model system where we can question the signaling molecule(s) that control the life and death of B cells at an early stage in their development and prior to encounter with foreign antigens (67,68).

Though there are some clear differences between B cell development in humans and chickens, the overall signaling process remain the same and thus the chicken remains an interesting and informative model for the study of lymphocyte development.

chB6

Understanding B cell development in mammals has been assisted by the development of monoclonal antibodies, which can identify unique protein expression patterns on developing B, cells, allowing scientists to narrow down the different stages within a cell lineage. Gilmore et al. identified a marker present on avian B cells, initially known as Bu-1 (Bursal antigen 1) now called chB6 (69) In these studies two allelic forms of Bu-1 were identified, Bu1a and Bu1b, these alleles being co-dominant. However the function of Bu1 was never identified. Studies by Houssaint et al (70) found out that chB6 is expressed on the surface of virtually all B-cells throughout ontogeny with the exception of plasma cells in the Harderian gland. Low levels of this molecule are also found in a subset of macrophages in bursa, intestine and liver. chB6 is found on the early B cell precursor in the day 8 embryonic spleens, which makes it a B cell marker (70,71). The presence of chB6 on chicken B cells
suggests a physiological function, yet no known function was reported until the late 1990’s.

Cloning of the chB6 cDNA molecule showed that it is a 70 kDa type 1 transmembrane protein with a highly glycosylated extracellular region and a 105 amino acid intracellular component notably rich in proline and acidic residues. chb6 resides of the surface of cells as a disulfide liked homodimer (5, 72) During the initial cloning it was found that there are actually at least three alleles, of chB6; chB6.1 (Bu1a), chB6.2 (Bu1b) and chB6.3 (4,72,73). The majority of the differences between these alleles of chB6 lie in their extracellular domain and they are equally distinct from one another. The intercellular domain is highly conserved, suggestive of cell signaling function (73).

Initially chB6 did not appear to have any homology with known mammalian counterparts and was thought to represent a unique B cell marker for chicken (73). In fact, it was described as a molecule of ‘unique structure’. Researchers have shown some similarities between chB6 and CD2 family, both the molecules possess two Ig like domain in their extracellular domain (44). The gene which encodes chB6 lies within 20Kb of the CD2 gene on chicken chromosome1, in agreement with the clustering of CD2/SLAM genes noted in mammals. However chB6 has no intracellular tyrosine residues, and thus no ITSM (Immune tyrosine switch motif) . The chB6 cytoplasmic domain however shows slight similarity to SLAM/CD2 family members since both the molecules have two extracellular Ig domains and also have conserved proline rich cytoplasmic domains and research indicates that this is an intrinsically disordered protein. Intrinsically disordered domains are highly characteristic of proteins with signaling functions (44)
In 1997 it was determined that chB6 was capable of triggering apoptosis when stimulated with anti-chB6 antibody(4). This response was also replicated when the molecule was transfected into murine cell lines, which implies a conservative death mechanism among species. Binding to chB6 leads to rapid cell death suggesting that chB6 could act as a death receptor. (4, 74). Our lab (Funk Lab) has shown that chB6 can mediate a death signal that can activate caspase 8 and caspase 3, both integral cysteine proteases commonly activated in apoptosis pathways. This apoptosis could be inhibited by overexpression of the anti-apoptotic bcl-2 family member bcl-xL(5).

In murine cells signals from growth factor also inhibits chB6 mediated apoptosis(74). Inhibitors of caspase 3,8 and 9 activity inhibit apoptosis in cells treated with anti chB6(5).

All these studies suggest that there are other death pathways which involves activation of caspase 8(33, 75) but function independently of death domains. The association of chB6 with the CD2/SLAM family of proteins raises interesting hypothesis about a possible role as a co-stimulator or ‘tuner’ of immune signaling. The Funk lab has also shown that chB6 induces apoptosis in a transfected mammalian cell line and apoptosis is inhibited by over expression of the anti-apoptotic protein Bcl-xL(5). The data also showed that when Ab binds to chB6 results in cleavage of a receptor associated caspase, caspase 8, and results in a subsequent rise in the relative amount of the active form of caspase3(74)

Even though there is no obvious homolog to chB6 based on nucleic acid or overall protein sequence, there has to be an underlying conserved function which enables chB6 to trigger apoptosis in both avian and mammalian cells. Funk lab has also shown that chB6 triggers cell death in human Jurkat T cells (P.E Funk
unpublished data). As presented here, the dynamics of B cell development in the bursa continues to be an viable model of lymphocyte development (76)

**Statement of Proposed Research:**

The process of signaling via the novel avian molecule chB6, which leads to a rapid cell death, became the main motive for this research project. This cell death was via apoptosis. In fact, recent work at Dr. Funk’s lab indicated that chB6 has some similar structure with the SLAM/CD2 protein family (44). Past work in the Funk lab identified four new alleles of chB6 in chicken and one allele in turkey, all showed little variation in the cytoplasmic domain, further suggesting an important function (Funk, unpublished observations). Additionally, chB6 is found on the surface of B cells in lipid rafts, a membrane domain associated with signaling function, in close association with the B cell antigen receptor (44). In fact crosslinking chB6 resulted in stronger up regulation of integrin adhesion than cross-linking of the BCR. Collectively, this strongly suggests that chB6 is an integral part of BCR signaling in B cells, and one outcome of that signaling is apoptosis (44, 63, 74).

The Funk lab has characterized a series of truncations of the cytoplasmic domain of chB6 and identified the critical death-signaling region to a 15 amino acid stretch (figure 4). If this region is eliminated chB6 can no longer trigger apoptosis. Initial work has focused on a sequence of charged amino acids in this region, the sequence DHVEA (Figure 3)(Funk unpublished). In fact a single amino acid change of E to Q within this region can reduce the cell death up to 50% (Funk, unpublished observation). At the C terminal region of this 15 amino acid is a consensus SH3 binding site (PPMP). Knowing that SH3 binding sites are critical in a variety of signaling contexts we proposed that this SH3 binding site is required for apoptosis signaling by chB6. Since previous studies have used mutagenesis of SH3 binding
sites to study their function we proposed to use this method to study the role of the
SH3 binding site. Mutation of this binding site (SH3) to disrupt the protein-protein
interaction has been used in other studies(61).

We generated a single amino acid (proline to alanine) change in the SH3
binding site in chB6. Since there are two prolines in an SH3 binding site (PXXP), we
generated mutations with sequences PXXA, AXXA. These mutants were introduced
into BK3a cells and single cell clones were isolated. We used site directed
mutagenesis to introduce point mutations and clones were generated. To test the
ability of these mutant chB6 molecules to induce apoptosis, we introduced them into
BK3a cell line isolated single cell clones. These single cell clones which were
incubated in the presence of antibodies to chB6 for two hours and cell death then will
be measured with the of the vital dye trypan blue (dead cells stain blue )(4).

Our hypothesis was “mutation in SH3 binging site will have an effect on cell
death” our results showed that mutation in SH3 binding side reduces cell death
significantly and this study is one of the first evidence which showed that SH3
binding side is an important area for cell signaling. This work is continuation of the
Funk lab’s previous work to understand death signaling by chB6 and its regulation in
B cells.
**Materials and Methods:**

Primer Design

Mutagenic Oligonucleotide primers for this project were designed individually according to the need of the project. The SH3 binding motif PPMP at position 279 to 282 in the polypeptide chain was targeted to create alanine substitutions at position 279, 282, and 279 plus 282. Primers designed were 30 bases in length for P272A, P282A and 25 bases for P279A with a melting temperature (TM) of 70.7°C and 68°C using Agilent technology. The following formula were used to estimate the TM of primers; TM= 81.5 +0.41(%GC) - (675/N) -% mismatch where N is the primer length in bases. The desired mutation was inserted in the middle of the primer with ~ 10-15 bases of correct sequence on both sides. The sequences of the primers are shown in appendix 1.

PCR amplification

Primers (P279A sense-P279A antisense, P282A sense- P282 antisense) were designed to amplify the sequence including the cytoplasmic tail portion of chB6. PCR reactions were prepared using the Quik Change Mutagenesis kit (Agilent technology, Santa Clara, CA). Primers were diluted to 50pmol per PCR reaction. ChB6.2 template DNA was diluted to a concentration of .7μg/ul per PCR reaction. The reaction mix consisted of 0.1 mM of each dNTP, 1.5 mM of MgCl2, and 2.5 U Taq Polymerase in a total volume of 50μl. The PCR cycle parameters were set as follows: the first cycle held at 94.0°C for 3 minutes, followed by 29 cycles of 30 second periods at 94.0°C, 30 seconds at 62.0°C, and 30 seconds at 72.0°C; the final two cycles held at 72.0°C for 5 minutes each. The amplification products were then
digested with Dpn 1 for 1 hour at 37 degrees C. This procedure digests the parental (i.e. the non-mutated supercoiled dsDNA).

The experimental reactions were transformed into XL1 blue super competent cells and plated on LB agar plates containing 50μg/ml ampicillin. Colonies were selected from these plates and added to tubes each containing 5ml LB broth and 5ul ampicillin. These mixtures were incubated overnight at 37.0°C.

Minipreps of chB6 Transformants

DNA was isolated from overnight culture using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). To verify the PCDNA 3 vectors, which contain the chB6.2 insert, DNA was digested with BamH1 restriction enzymes and resolved on an agarose gel. The presence of single nucleotide changes was confirmed by sequencing at SeqWright Genomic Services Sequences, Houston Texas. Translations of each mutation are given in Appendix 1.

Cell lines

The bursa lymphoma cell lines were used which included LSCC-DT40 and LSCC-BK3a. DT40 Cells were grown in DMEM (Life Technologies Laboratories, Grand Island NY) supplemented with 6% tryptose phosphate broth (Life Technologies), 10% New born bovine serum (NBS) (Hyclone) 4% chicken serum(sigma chemical co.), and 10mM HEPES ph 7.3 (life technologies). BK3a cells were grown in DMEM supplemented with 6 % tryptose phosphate broth, 6% NBS,1% chicken serum and 10mM HEPES, ph 7.3. The cells were split approximately thrice per week.
Transfection

BK3a cells were transfected with the help of electroporation (250 V, 950uF) with pcDNA3 vector (Invitrogen, San Diego, CA) containing either the cDNA encoding chB6.2 (accession no. X92867), mutants p282A, p279Ap282A or with no insert served as our negative control. After electroporation the mixture was rested for 10 minutes at room temperature. Next the transfected cells were transferred into culture flask with BK3a media. Stable transfected cells were selected using G418, neomycin at 1 unit/mL.

Immunofluorescence

To confirm the presence of chB6 on the surface of transfected cells, Immunofluorescence was performed.

10^6 cells were suspended with primary antibody FU5-11G2 (mouse anti-chB6.2 ascites) (4), diluted 1:200 and incubated for 20 minutes on ice. The cells were then washed twice and resuspended in FITC-conjugated anti mouse IgG secondary antibody 1:200 (Millipore, Temecula, CA) and incubated on ice for 20 minutes. After 20 minutes the cells were washed twice and resuspended in PBS. Immunofluorescence was viewed via epifluorescence microscope.

Single cell cloning

Single cell cloning was performed as described by (John A Ryan PhD, Corning incorporated Life science). Cell suspension at 2x10^4 cells/mL in BK3a media were used. Using an 8 channel micro pipettor 100 uL of the medium were added to the wells in the 96 well plate except A1 which was left empty. 200 uL of the cell suspension were added to the A1 well, then using a single channel pipettor 100ul were
transferred quickly from the first well to the well B1 and was mixed by gently pipetting. Using the same tip, 1:2 dilutions were repeated down the entire column disregarding 100ul from H1 so that it ends up with same volume as the wells above it. With the 8 channel micro pipettor an additional 100 uL of medium were added to each well in column 1 making the final volume of the cells and medium up to 200 uL/well. Then using the same pipettor 100 uL were quickly transferred from wells in the first column (A1 through H1) to those in the second column (A2 through H2) and were mixed with gently pipetting. Using the same tips, 1:2 dilutions were repeated across the entire plate and discarding 100ul from each wells in the last column (A12 through H12) so that all the wells end up with 100 ul of cell suspension. The final column was brought up to 200Ul by adding 100 ul medium to each wells. The plates were incubated at 37° C in a humidified CO₂ incubator. After 7 to 10 days, clones were identified and detectable by microscopy. Single cell clones were transferred to flasks with 10ml media plus G418 for expansion. Single cell clones were confirmed by immunofluorescence.

Western Blot

Cell lysate:

10⁷ cells were resuspended in lysis buffer (RIPA buffer) which contains protease inhibitor. The mixture was incubated in ice for 30 minutes and spun for 30 minutes. Supernatant was transferred to a clean tube for western blotting. To best compare protein expression levels in different clones the cells were lysed at 5 X 10⁷ cells per milliliter. Proteins concentrations were determined by the BCA method (Pierce Chemical, Rockford, IL). 50ug of protein were loaded per lane.
Protein samples were resolved in a 10% SDS PAGE gel. Proteins were transferred to supported nitrocellulose membrane at 100mA for 1.5 hour. Where needed, membranes were stained for 45 minutes in Ponceau S solution (Research Products International, Mount Prospect, IL) to confirm equal loading of samples. The membrane was blocked overnight in a blocking/incubation buffer of 4% Bovine Serum Albumin (Fraction V) (Fisher scientific) in TBS at room temperature with gentle agitation. The membrane was washed with TBS (tris buffer saline) with 0.1% Tween for 5 minutes. Wash was repeated thrice. Membrane was then incubated overnight at room temp with gentle agitation in primary antibody (anti EMI) 1:200 with blocking buffer. The primary antibody was raised in rabbits using the chB6-specific peptide EMIADVESQENASNC. The blot was then washed three times with TBS (tris buffer saline) tween solution also known as wash solution. Secondary antibody, goat anti rabbit IgG conjugated (Roche, Indianapolis, IN) with either horseradish peroxidase or alkaline phosphatase, was added at a 1:5000 dilution in blocking/incubation buffer for 2 hours at room temp with gentle agitation. This was poured off and again membrane was washed three times with TBS (Tris buffer saline). Peroxidase conjugate was visualized with 1-Step TMB substrate (Pierce). Alkaline Phosphatase conjugate was visualized with 1-step NBT/BCIP substrate (Pierce) for colorimetric visualization.

Cell death assay:

Cell death assays were performed as previously described (4). Briefly, transfected B cell clones or DT40 cells were placed in 24 well plate (10^6 cells/ well) and cultured with or without anti-chB6.2 antibody (FU5-11G2) ascites 1:200 or culture supernatant 1:40 for 2 H. After two hours viable cells were determined by a trypan blue dye
exclusion test. Percentage of non viable cells were determined by the formula: (Non viable cells/ total number cells) * 100. The results were represented as mean +_ SD for triplicate cultures.

**Results:**

Previous studies are consistent with the hypothesis that chB6 serves as a death receptor. chB6 exists on the cell surface of most avian B cells throughout their ontogeny and, when stimulated with anti chB6 antibody, it executes rapid cell death consistent with apoptotic cell death (4, 70,71). The Funk lab has characterized a series of truncations of the cytoplasmic domain of chB6 and identified the critical death-signaling region to a 15 amino acid stretch (Introduction, Funk, unpublished observations). If this region is eliminated chB6 can no longer trigger apoptosis. Initial work has focused on a sequence of charged amino acids in this region, the sequence DHVEA (Introduction). At the C terminal region of this 15 amino acid is a consensus SH3 binding site (PPMP). Knowing that SH3 binding sites are critical in a variety of signaling contexts, I propose that this SH3 binding site is required for apoptosis signaling by chB6 and mutation of this site will result in a decreased percentage of cells undergoing apoptosis after crosslinking chB6.

In order to test this hypothesis, single amino acid changes (proline to alanine) were generated using site directed mutagenesis. A similar strategy was employed by Pawson (77, 78) to define the role of SH3 binding sites, thus site directed mutagenesis was our best approach since this technique allow us to introduce point mutation by changing a single amino acid. Following PCR with the mutagenic primers (Appendix 1) colonies were selected for isolation of
plasmid DNA. Plasmid DNA was screened by restriction digestion with Bam HI and Sac I to confirm the recovery of appropriately sized fragments (Figure 4). Plasmid DNA from two colonies containing appropriately sized inserts was sequenced to confirm that interested sites were mutated correctly and to confirm the translation of the mutated chB6 (Appendix 1 and 2). SH3 binding site located in the cytoplasmic tail of chB6. Two mutations were used, a single P to A substitution at position 282 (P282A) and a substitution of both of the consensus prolines at positions 279 and 282 (P279A,P282A), both mutations were made in the chB6.2 form.

Once plasmids encoding mutated chB6.2 proteins were identified, the mutants were introduced into BK3a cell line by electroporation and stable transfectants were isolated by resistance to G418. The BK3a lymphocyte line is derived from a Japanese line of chickens and does not express either the chB6.1 or chB6.2 alleles of chB6 as identified by the allele-specific antibodies we have in the lab (4, 79). This makes BK3a an ideal cell line for our work: we can transfect chB6-encoding DNA into BK3a cells and confirm that our DNA is expressed (4). Previous work published by the Funk laboratory has used this system to study chB6 triggered apoptosis (4). Immunofluorescence was performed to confirm the presence of chB6.2 on the surface of BK3a cells after growth of stable transfectants. Subsequently, single cell clones were isolated by limiting dilution. To confirm the presence of the mutated chB6 on the surface of the B cell immunofluorescence was performed again. Table 1 represents the percentage of immunofluorescence positive cells among a group of single cell clones. 100% of the clones expressed chB6 on its surface as identified by the chB6.2 specific monoclonal antibody FU5-11G2. DT40 served as the positive control. E12, G7,
D9 and B 12 were the double mutant (P279A, P282A) clones (Table 1) and D10, D12 and C10 were the three single mutants (P282A) that were selected for further analysis. A total of 16 clones of P279A P282A and 15 clones of P282A were isolated.

Previous studies have shown that crosslinking chb6 with monoclonal antibody induces rapid cell death by apoptosis. Using this method we determined whether our mutated chB6 molecules could induce cell death. We selected three clones of each mutant for further analysis. Clones were incubated with anti chB6.2 for two hours at 37 °C, as previously described (4), and aliquots were removed to assess cell viability by trypan blue exclusion. Consistent with previous work in the Funk lab (4), DT40 and a BK3a clone transfected with un-mutated 6.2 undergo rapid cell death when treated with anti-chB6.2 antibody as compared to cells incubated without antibody (Figure 5). However, BK3a clones expressing the P282A mutant of chB6.2 (clones D10, D12, C10) show no significant difference in the percentage of dead cells between wells incubated with anti-chB6.2 and treated and wells without anti-chB6 antibody (Figures 6,7,8). This result was consistent through three separate experiments. Likewise, BK3a clones expressing the double mutation (P279A,P282A) showed similar results; DT40 control wells show rapid cell death following treatment with anti-chB6.2 but the mutants (E12,B12,G7 and D9) show no significant difference in the amount of cell death following by the treatment with anti-chB6.2 as compared to untreated cells (Figure 9,10,11,12). These results are consistent throughout four experiments. It was interesting to note that one of the double mutant clones, clone E12, showed some experiment to experiment variability. In one experiment there was an increase in cell death after anti-chB6.2 in another
experiment wells treated with anti-chB6.2 actually showed reduced cell death. In two other experiments there was no difference between the groups. This was not seen in any of the other clones. Collectively, the results presented here are consistent with the hypothesis that mutation in SH3 binding site disrupts the protein – protein interaction and thus disrupts the death signals reducing the ability to cause rapid cell death.

One possible explanation for the inability of the mutant chB6 molecules to induce cell death is that the mutation altered intracellular trafficking, resulting in either inappropriately glycosylated, non-dimerized, or non-raft localized chB6. ChB6 is normally a heavily glycosylated disulfide-bonded dimer on the surface of avian B cells and localizes to the lipid raft microdomains of the cell membrane (5, 44). Since we saw excellent immunofluorescence with the conformation dependent FU5-11G2 antibody we thought it likely the protein was properly folded. Western blotting using a chB6-peptide specific antiserum (Figure 13, 14) revealed that all chB6 transfectants had a disulfide-bonded chB6 as seen by lanes run in non-reducing conditions. Furthermore, the level of glycosylation as assessed by the size range of chB6 was similar in all mutants as well as an un-mutated chB6 transfectant and the DT40 control cell line. Collectively this argues that the mutant chB6 molecules are glycosylated normally, disulfide bonded normally, and traffic to the membrane normally, so any alteration is cell death can be ascribed to the mutation in the PPMP motif rather than a defect in protein folding or trafficking.

It is possible that the defects in triggering cell death that we observe could be due to lower levels of chB6 expression in the transfectants. To address this we performed a western blot against equal proteins quantities of cell lysate
to compare expression levels. To confirm equal loading the blot was stained with ponceau S solution (Figure 15). The blot was then probed with primary antibody (anti chB6 peptide) and secondary antibody (alkaline phosphatase conjugate). The result confirms that all of the P282A mutants had similar levels of expression to the control chB6.2 transfectant and were likely expressing more chB6 than DT40 control cells (Figure 16). Notably, the un-mutated chB6.2 transfectant and DT40 show similar levels of cell death after treatment with anti-chB6 so the defect in death of the P282A mutants cannot be ascribed to level of expression. Likewise, all of the P279AP282A mutants had higher levels of expression than the chB6.2 transfectant. The parent cell line for the transfection, BK3a, had barely detectable levels of chB6 in these experiments (Figure 14).

**Discussion:**

The majority of B cells die while still in the primary lymphoid organ in both mammalian and avian species (1, 12). This controlled cell death occurs in order to maintain a healthy population of B cells. It is crucial that self-reactive and non-functional developing B cells are not able to migrate into the periphery and cause harm to the organism. Earlier research has mainly focused on death by neglect as a means to eliminate cells that fail to correctly assemble either a pre B cell receptor (pre-BCR) or a mature BCR. These cells do not receive extrinsic signals from their surroundings environment which are necessary for them to survive (1).
The kinetics of B cell loss in mammals appear to be more rapid than in typical death by neglect models (1). In addition, the clonal deletion of autoreactive B cells in transgenic mouse models is more consistent with a death by instruction mechanism which is caused by receptor initiated signal transduction (1). Thus it is interesting to know the different signals that govern the fate of a B cell are integrated and can result in either cell survival or cell death (1). Recent work in mice has implicated CD2/SLAM family proteins in maintaining B cell tolerance both in the periphery and in the bone marrow (40, 43). The Funk lab have proposed that chB6 could function as a death receptor during B cell development in chickens to mediate a death by instruction response (63). The work presented here supports the hypothesis that chB6 interacts with a receptor initiated apoptotic pathway and extends previous work by showing that the PPMP motif of chB6 is essential in this pathway.

**ChB6**

Despite the majority of work currently being done in the mouse model system, avian models still provide unique insight into the life and death decisions made by developing B cells because they can be monitored during the development stages in which these events occur. chB6 is a protein molecule which is found in all chicken B cells throughout their ontogeny and, when chB6 is stimulated by agonist antibodies, rapid cell death follows, leading to the proposal that chB6 might function as a death receptor during B cell development (469, 70, 71,). Signals from chB6 result in the cleavage of caspase 8 and caspase 3 into their active forms (5, 74). Caspase 8 and 3 are both cysteine proteases commonly activated in death receptor pathways (28). Furthermore, the ability of chB6 to trigger apoptosis can be regulated by other signals within the cell and
by the anti-apoptotic BCL–xL protein (4, 5, 74,). Collectively this argues that chB6 uses a signaling pathway very like known death receptors. Furthermore, transfection of the chB6 cDNA into mammalian cells replicates these features, arguing for a conserved mechanism of action (4, 74).

However chB6 has little similarity to known death receptors so the mechanism which propagates this cell death is still being explored. chB6 is a heavily glycosylated transmembrane protein (5). There are multiple alleles of chB6, all of which seem equally distinct from each other in the extracellular domain (73, Funk unpublished). Notably, all of these alleles are highly conserved in the cytoplasmic domain. The chB6 protein is 335 amino acids in length, with 105 predicted to reside in the cytoplasm. This cytoplasmic domain is notably rich in proline residues (73) and acidic residues and has no tyrosine residues. If portions of the cytoplasmic domain of chB6 are truncated, chB6 drastically loses its ability to transmit a death signal (Funk, unpublished). Again, this data is consistent with the hypothesis that chB6 can function as a death receptor. It was also interesting to note that within this death signaling region is SH3 binding site that was targeted in this study. SH3 binding site is known to mediate wide variety of signals which includes different growth signals and are also known to be involved in apoptosis (55, 56, 59, 61). Our lab wanted to know whether mutating SH3 binding site affects cell death and if does then it would take a step closer to understand the biology behind chB6 molecule.

**ChB6 and CD2**

While chB6 was initially reported as a molecule of ‘unique structure’, more recent work has suggested similarity between chB6 and CD2/SLAM
family (44, 73). ChB6 appears to have two extracellular Ig like domains that are characteristic of the CD2/SLAM family of proteins. ChB6 lies about 20kB from the CD2 gene (44). CD2 is an adhesion and costimulatory molecule expressed by T cells and natural killer cells (83). In humans the CD2 protein consists of 327 amino acids, of which 116 are located at the C terminus in a long cytoplasmic domain which is characterized by the presence of at least four well conserved proline rich regions and the striking absence of tyrosine residues (80). The CD2 cytoplasmic domain is rich in proline residues like ChB6, and these proline residues have been shown to be critical in CD2 signaling (80, 83). Researchers studying CD2 protein also found out that CD2 cytoplasmic domain is highly conserved between murine and human’s CD2 suggesting similarities in the signal transduction pathways. CD2 together with CD 28 co-receptor can help in T cell activation thus CD2 has also been considered as a co stimulatory molecule. CD2 triggering can also deliver mitogenic signals when T cells are cultured with combinations of anti CD 2 monoclonal antibodies. Early biochemical events during CD2 dependent T cell activation include translocation of protein kinase c (PKC) from cytosol to the cell membrane and an increase in intracellular free Ca++ levels (81). This latter phenomenon is likely to be involved in both activation and cell death by apoptosis and this has been demonstrated in glucocorticoid treated human thymocytes as well as in human cell lines. Studies have confirmed that stimulation of CD2 receptor by monoclonal antibody induces a rapid high peak in intracellular calcium levels which are able to induce apoptotic death in target cells (82). Although T cells from mice lacking expression of CD2 were initially reported to have normal activation parameters, a recent study suggests that CD2 may have a small role in enhancing T cell
activation. In fact, studies have shown that in knockout mice models where they lack CD2/CD28 show some profound defect in activation and proliferation of cells thus suggesting that knocking out CD2, disrupts CD2 clustering which plays an essential role in helping to form a specialized cell contact called the immunological synapse, this study also suggested that CD28 can induce protein clustering and segregation and thus functions’ very similar to CD2 (83).

Furthermore the cytoplasmic tails of CD2 and CD28 interact with the same or similar intracellular protein.

**SLAM**

The SLAM (signaling lymphocyte activation molecule) sub-family of the CD2/SLAM family of proteins can also mediate either positive or negative signals (42). The SLAM family is related to CD2 in having two extracellular Ig domains but is distinct in having the ITSM motif (41). In humans and mice CD2 and SLAM genes are clustered on the same chromosome, suggesting that they arose from gene duplications. The SLAM family of receptors and their associated signaling adaptors play an important role in the regulating of various stages of autoimmunity (42). They are known to regulate lymphocyte lymphocyte interactions which are ultimately involved in cell mediated and humoral mediated immune response. Recent studies have also shown that SLAMF receptors function as disease modifiers and susceptibility factors of systemic autoimmunity. SLAM F6 has been implicated in maintaining B cell tolerance in the periphery in mouse models of lupus as well as in the control of autoreactive cells in the bone marrow (40, 43). There is some evidence that the SLAM family of receptors does play important roles in immunity. They provide key effects in multiple immune cell types. Recent studies have also shown that SLAM family
receptors can either promote or inhibit the functions of primary receptors and these alternative activities are controlled by whether or not SLAM related receptors co-exist with members of SLAM associated protein SAP family of intracellular adaptor molecules (84, 85).

Collectively, this provides strong evidence that chB6 is a member of the CD2/SLAM family and suggests that it may have roles in B cell biology beyond promoting apoptosis. However, the uniqueness of the chB6 cytoplasmic tail poses interesting challenges to our experiments. The work presented here suggests parallels to CD2's proline dominated signaling leading to Ca^{++} flux (80, 83, 81). It has been shown that chB6 signaling in DT40 B cells leads to an upregulation of integrin affinity, an effect often dependent on Ca^{++}, and that chB6 is located in intimate association with the BCR, which can also signal via Ca^{++}(44). Thus experiments to explore if chB6 signals lead to Ca mobilization and if the mutations created here have an impact on that Ca mobilization would seem well justified. Further parallels to CD2 signaling, including the activation of PKC, seem obvious aims for future work. Thus this study takes a step closer to understand the structure and characteristics of this chB6 molecule.

Importantly, the mutant chB6 molecules here seem to traffic to the cell surface and be glycosylated normally. Consequently the inability to trigger apoptosis is not due to aberrant folding or trafficking of chB6. Subsequent studies to confirm that these chB6 mutants enter the lipid rafts are warranted, but not within the scope of this project. However, Li et al. show that the mature, disulfide bonded, chB6 is exclusively in the rafts of DT40 cells (44). Since our mutants are disulfide bonded on the cell surface and the raft targeting CCC motif is not altered we expect that our mutants in fact traffic to the rafts.
The Funk lab has already identified a 15 amino acid stretch that is crucial for cell death and within this stretch; there lies a SH3 binding site. The region bound by SH3 domain is proline rich and contains PXXP as a core conservative binding motif. SH3 domain comprises of 60 residues and typically plays an important role in assembly or regulatory function (55, 56, 57, 58). An assembly role is exemplified by the adaptor protein Grb2 which is involved in the p21 ras dependent growth factor signaling pathway. However, the main function of the SH3 domain is not very well understood (45, 56, 57). They may mediate many diverse processes such as increasing local concentration of proteins, altering their subcellular location and/or mediating the assembly of large multiprotein complexes. Proline recognition domains are usually found in the context of larger domain signaling proteins. Their binding events often direct the assembly and targeting of protein complexes which are involved in cellular growth. The domain that bind proline rich motifs are critical to assembly of many intracellular signaling complexes and pathways (52). Studies have shown that Proline rich motifs are usually favored in a cell and there are numerous reasons for that. Firstly, for any peptide sequence to function in a binary interaction, it has to get exposed to the solvent and have the accessibility to the binding partner (61). Of the 20 naturally occurring amino acids, proline might be best suited for such a role. It is a well-known fact that a regular secondary structures such as α-helices and β-sheets that are essential for protein folding and topology. Consequently, proline-containing sequences are often found on the surface of a protein, as opposed to being buried within the core (60, 58). A typical question that remains unknown whether meaningful biological outputs necessarily
depend on specific pairwise SH3 ligand interactions or is it more realistic to think of these interactions in a similar way, i.e. does a particular SH3 domain or a ligand site simultaneously engage in multiple interactions in dynamic equilibrium and thus SH3 binding site became an important part of my project.

SH3 domains which is located in the cytoplasmic domain are small protein modules that mediate protein-protein interactions in signal transduction pathways which are usually activated through protein tyrosine kinases. Mutation of SH3 binding site disturbs the protein–protein interaction thus disturbing the signal transduction pathways downstream (86). In general signal transduction refers to the ordered flow of cellular information by sequential engagement of regulatory molecules in order to modulate a biological response. In this study we have shown that SH3 binding site is important in the apoptotic signaling pathway, as mutation of this site eliminates the induction of apoptosis in BK3a cells. The present study also provides further evidence supporting the role of Src kinase activity in the regulation of chB6 induced killing.

Notably BK3a has little to no endogenous chB6, confirming it’s utility in transfection experiments such as this. However, BK3a is reported as a B lineage cell so the absence of the B cell marker chB6 is somewhat puzzling. However, chB6 is not seen on a subset of B cells in the chicken, notably plasma cells. BK3a was derived by viral infection of chickens, resulting in the transformation of cells into tumor cell lines. BK3a is reported as a bursal lymphoma, so it is possible that BK3a transformation altered the ability to produce chB6 (79).
**Future direction**

Previous studies in the Funk lab have also shown that when chB6 is cross linked, it activated cell binding to integrin substrate. In DT40 cells, the cross linking BCR activates integrin dependent cell binding (44). Real time cell binding assay confirms that cross linking chB6 on DT40 initiates cell binding to both laminin and gelatin. CD2 molecules also act as integrin regulators which co stimulate integrin mediated cell binding during lymphocyte activation. All these data and studies suggest that chB6 could be a distant paralog of CD2 molecule and disrupting the proline residues could disturb the calcium influx thus reducing the cell death.

This research provides the groundwork for numerous other investigations into the function of chB6 and its importance in B cell physiology. The reduction in cell death supports our hypothesis that chB6 indeed works as a death receptor and mutating the SH3 binding site disrupts the protein: protein interaction and hence reduces the cell death by apoptosis. It is also possible that mutation in proline residues in chB6 decreases the intracellular calcium influx thus saving the cells from dramatic cell death. The next step would be to mutate the SH3 binding site and to stimulate the chB6 with anti chB6 monoclonal antibody and to monitor the intracellular calcium influx. Ca 2+ ATPase activity assay could be performed on the targeted cells. This activity can be measured on the membranes by sonication of the cells and then evaluation of apoptotic cell death could be performed. Since many death receptors propagate a death signal via caspase cascade with caspase 8 often being one of the first mediators involved in the process. It would be interesting to note whether this particular mutation has any effect on the caspase cascade. This would give us more insight
about the chB6 molecule act as a death receptor. Another interesting aim would be to study the integrin activation within these chB6 SH3 mutants. The importance of characterization chB6 as a death receptor and understanding the means by which this molecule initiates a death signal is extremely important. It would also be interesting to examine the calcium levels after chB6 crosslinking. This project takes us to one step closer to understand the function of this molecule.

**Conclusion**

This work solidifies the hypothesis that chB6 interacts with a physiological cell death pathway and offers new experimental insights to the role of this molecule in birds as well as parallels to mammalian counterparts. The life of a B cell is coordinated by a continuous series of checks that are mediated by signaling within the cell and between the cell and its surroundings. These signals will determine if the cell will continue development within the primary lymphoid organ and if it will progress to a mature B cell allowed to circulate in the periphery. Failure in any of these signals can have a detrimental effect on the life of the organism. The evidence we obtained supports our hypothesis that there is a relationship between chB6 SH3 binding site and cell death. This study also gives an early indication that chB6 could belong to CD2 family since the structures and functions have a number of similarities. This study also provides insight to the signaling pathway of developing B cells in the bursa of a chicken. From this, the knowledge of this pathway can be expanded so that we fully understand the structure and function of chB6 and what shapes the existence of
a healthy B cell repertoire during both development and in the periphery. A proper immune response is critical to the survival of all organisms.

By better understanding how the signals that result in cell death work, we can better understand why the signals fail to work and potentially what can be done to correct or prevent these issues.
References

References:


73. Tregaskes, C. A., N. Bumstead, T. F. Davison, and J. R. Young, Jr. 1996. Chicken B-cell marker chB6 (Bu-1) is a highly glycosylated protein of unique structure. *Immunogenet*. 44: 212-217


Figure legends.

Figure 1: VDJ recombination

The VDJ recombination is described in the following image, Antibodies which are consist of two heavy and light chain are covalently connected with the help of disulphide bond. (1)

Figure 2. Structure of an Antibody.

This image describes the structure of an antibody and the constant and variable domains. (1)

Figure 3. chB6 sequence:

The underlined portion is the apoptotic sequence and the bolded letters (PPMP) is the SH3 binding site.

Figure 4. P282A and P279A P282A enzyme digest

PCR primers described in the appendix were used to obtain chB.2 mutants. ChB6 mutants were tested by running the samples on 1.2% agarose gel alongside molecular weight markers. Lane 1 is the lambda marker. Lane 2 : chB6 plasmid DNA undigested and digested with restriction enzyme which served as the positive control. Lane 8 and Lane 9 represents the plasmid DNA of the single mutant (P282A) and lane 17, 18 represents the plasmid DNA of the double mutants (P279A,P282A).
Figure 5. Cell death of DT40 cells and BK3a cells transfected with unmutated chB6.

DT40 or a single cell clone of BK3a lymphocytes transfected with unmutated chB6 (6.2) were incubated with anti chB6.2 or no antibody for 2 hours at 37°C. 100 uL aliquots were removed and cell death were determined by trypan blue exclusion. Data presented as mean ± standard deviation of triplicate samples. Data are representative of three experiments. * p < 0.05 by paired T test.

Figure 6. Cell death of BK3a cells transfected with P282A mutant chB6

Single cell clones of BK3a lymphocytes transfected with chB6 mutant (P282A) were incubated with anti-ChB6.2 or no antibody for 2 hours at 37°C. Aliquots were removed and cell death determined by trypan blue exclusion. Data are presented as mean ± SD of triplicate cultures. DT40 served as the positive control, D10, D12, C10 were the three different clones. * p < 0.05 by paired T test. N.S. p > 0.05.

Figure 7. Cell death of BK3a cells transfected with P282A mutant chB6

Single cell clones of BK3a lymphocytes transfected with chB6 mutant (P282A) were incubated with anti-ChB6.2 or no antibody for 2 hours at 37°C. Aliquots were removed and cell death determined by trypan blue exclusion. Data are presented as mean ± SD of triplicate cultures. DT40 served as the positive control, D10, D12, C10 were the three different clones. * p < 0.05 by paired T test. N.S. p > 0.05.
Figure 8. Cell death of BK3a cells transfected with P282A mutant chB6

Single cell clones of BK3a lymphocytes transfected with chB6 mutant (P282A) were incubated with anti-Chb6 or no antibody for 2 hours at 37°C, Aliquots were removed and cell death determined by trypan blue exclusion. Data are presented as mean ±SD of triplicate cultures. DT40 served as the positive control, D10, D12, C10 were the three different clones. * p < 0.05 by paired T test, N.S. p> 0.05.

Figure 9. Cell death of BK3a cells transfected with P279A,P282A mutant chB6

Single cell clones of BK3a lymphocytes transfected with chB6 mutant (P279A,P282A) were incubated with anti-ChB6 or no antibody for 2 hours at 37°C, Aliquots were removed and cell death determined by trypan blue exclusion. Data are presented as mean ± SD of triplicate cultures, DT40 served as the positive control, G7, E12 and B 12 were the three different clones. * p < 0.05 by paired T test, N.S. p> 0.05.

Figure 10. Cell death of BK3a cells transfected with P279A,P282A mutant chB6

Single cell clones of BK3a lymphocytes transfected with chB6 mutant (P279A,P282A) were incubated with anti-ChB6 or no antibody for 2 hours at 37°C, Aliquots were removed and cell death determined by trypan blue exclusion. Data are presented as mean ± SD of triplicate cultures, DT40 served as the positive control, G7, E12 and B 12 were the three different clones. * p < 0.05 by paired T test, N.S. p> 0.05.
Figure 11. Cell death of BK3a cells transfected with P279A,P282A mutant chB6
Single cell clones of BK3a lymphocytes transfected with chB6 mutant (P279A,P282A) were incubated with anti-Chb6 or no antibody for 2 hours at 37°C. Aliquots were removed and cell death determined by trypan blue exclusion. Data are presented as mean ± SD of triplicate cultures, DT40 served as the positive control, G7, E12 and B 12 were the three different clones. * p < 0.05 by paired T test, N.S. p > 0.05.

Figure 12. Cell death of BK3a cells transfected with P279A,P282A mutant chB6 single cell clones of BK3a lymphocytes transfected with chB6 mutant (P279A,P282A) and un-mutated chB6 (6.2) were incubated with anti-ChB6.2 or no antibody for 2 hours at 37°C. Aliquots were removed and cell death determined by trypan blue exclusion. Data are presented as mean ± SD of duplicate cultures. DT40 served as the positive control, E12 and D9 were different clones that were tested. * p < 0.05 by paired T test, N.S. p > 0.05.

Figure 13. Western blot of P82A cell
Western blot probed with anti-chB6 peptide antiserum was performed. The figure shows an immunoblot of a gel on which 5 ul of cell lysates have been loaded. The gel was immunostained with an antibody that recognizes chB6 protein. Values are indicated in kDa. + and – indicated the presence and absence of Beta mercaptoethanol. D10,D12,C10 are the single mutants. + or – shows the presence or absence of B mercaptoethanol.
Figure 14 Western blot of p279A p282A cell

Western blot probed with anti-chB6 peptide antiserum was performed. The figure shows an immunoblot of a gel on which 5 ul of cell lysates have been loaded. The gel was immunostained with an antibody that recognizes chB6 protein. Values are indicated in KDA. + and – indicated the presence and absence of Beta mercaptoethanol. B12,G7 and E12 are the double mutants. + or – shows the presence or absence of Beta mercaptoethanol.

Figure 15. Ponceau staining corresponding to the western blot.

Ponceau staining corresponding to the western blot is shown in figure 13. The blot of incubated in Ponceau for 45 minutes. The brightness of the band shows the equal loading. The Gel was loaded in the order of from left to right G7,E12, D9, RB5, D12, C10, RBG C10,6.2F7,Bk3a,DT30.

Figure 16. Western Blot

Protein samples were loaded in 10%SDS and the figure shows an immunoblot of a gel on which 50ul of cell lysates have been loaded. The gel was then immunostained with an antibody that recognizes chB6 protein. The order of the band as follows from left to right DT40, BK3a, 6.2F7, RBG C10, D10, D12, RB5 D9, E12, G7
Figure 1.

Germline configuration

V segments  D segments  J segments  Constant region exons

D to J recombination

V to DJ recombination

transcription, splicing

translation, assembly

(adapted from Janeway 2001)
Figure 2
Figure 3.

FIYILAGCAGAAAILVAVVSSLICCCMRRHKFLPVSDEEKDDGITMSVSVSEGVSPPNGDHVEAQAAQIACPPMPDA

ARPDSGIVPQPMVEENFPVAEPMPDEMIADVESQENASNCFPDPIDN

ChB6 Sequence, Transmembrane and cytoplasmic sections
Figure 4.
Table 1
Percent immunofluorescences after anti chB6 and FITC staining. This is the representative of three experiments

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Figure 5

![Bar graph showing cell death percentage](image)

- **DT40**: 30% (No Antibody), 40% (Anti-chB6.2)
- **6.2 F7**: 50% (No Antibody), 50% (Anti-chB6.2)

*Significant difference*
Figure 6
Figure 7.

![Graph showing cell death percentage for different cell lines.](image)
Figure 8

Cell death %

DT40               D10                  D12                   C10

* N.S N.S N.S

NoAB  AB
Figure 9.

Cell death %

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N.S.  *  N.S.
Figure 10.

Cell death %

- DT40
- G7
- E12
- B12

N.S.                        N.S.                  N.S

*
Figure 11.
Figure 12.
Figure 13

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MW, 

- 205
- 114
- 76.7
- 47
Figure 14.

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MW, 205
114
76.7
47
Figure 15
Appendix
Appendix 1
ChB6.2 sequence:
The complete chB6.2 allele sequence (Genbank).

Appendix 1

Sequence 1

Double mutation P279A, P282A

nnncngnnnnaangctctttagtgctgaggaggtgggaagaaggtgagagaagaagaacta  
X X X X L F S A E E G G K R G - X K L tgagtttttttcaacgatcccaagagactctttatcctgagagtatttgagccggttcatt  
- V F F N D T K V L F T L E V F E P F I gatgctcttgctctcccaatggaactcgacactttgctgtgcaacagtggacaccaagag  
D A W C F P T A D L T C N V D S N E aacatgacacacctgagtttcagctgtcctgggaatttgattagtaag  
N M T F E W K L N N S W P N A N G A C V aagagatggtgcacaagagtttcagctgtcctgggaattttgattagtaag  
K D G G K V R L E K T V P G E F V C K gttacataaacaactggctctttgttgaccagccccattgtgtctctctctgcagctatgga  
V T Y N N G F V W T R P I V L S C S Y G gaccttctgcaagcaacctgtgctctcatctacatctgacagaggtgtgcagtgctcgcgccc  
D L L Q Y W F P I Y I L A G C A G A A A atctctgtgctgtgtctctctacatctgtgcgtcatgagaggagaagcaagatttt  
I L V A V V S L I C C C M R R K H K F ctccccgatccccctcagatgaaagaaggtgatggaataaacaatctgctgtgctccagt  
L P V S D E E K D D G I T M S V V S S gaaggtgtgaagaccccccccccaatgagacacacgactgtggaggtctcagctgcag  
E G V K S P P N G D H V E A Q A A Q I A tgctctccatgcacagctccccccagctgccagcttcgccacccaatgtgcctccag  
C A F M A D A A R F D S G I V P Q P M V gaagaaaaattttccacagttggagtagagcctgggaaaatgccagccccagagatagct  
E E N F P V A V E F G E M P D P E M I A gacgtggaagccccaggaaaacgctttgcaactgtttccctctgaccaaatgataactgattgt  
D V S E Q N A S N C F P D P I D N - C gacacgcctcactgtcctcccacctgtctgtgctgactccgacccccatcttgcacccata  
D T L T V P P C P E S R D T H P L H P I gtctttgctgtagaaatttgtgcttgacaaaaatttagcagggagaagagctgtgtgtctcaggt  
V L L - E L V - K N L A G G R R D V S G ctcgaagctctttacaggggaaaaaasatacatgtggctcagagacctnggatcc  
L L S S F T G K K K Y M W L Q D X G S atcacaacngggccgcgcgcgctgcagaaaaaaaaaaaaaaaatacatgtggctcagagacctnggatcc  
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Sequence 2

Single mutation (P282A)

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Primers:

Sequence – P 279A sense

5 – TCT GGC ATT GGA GCG CAG GCG ATA TGG- 3

Sequence P279A antisense

5- CCA GAT CGC CTG CGC TCCAAT GCC AGA- 3

Sequence P282A sense

5-GGG CAG CGT CTG CCA TTG GAG GGC- 3

Sequence- P282A antisense

5-TGC CCT CCA ATG GCA GAC GCT GCC -3