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Natural killer cells are lymphocytes that play a role against cancer and viral infections. 2B4 is a membrane glycoprotein expressed on natural killer cells. In the present study we characterized 2B4 from mice strains BALB/c, 129/Svj and A.CA. Nucleotide and peptide analysis revealed that polymorphic residues in 2B4 are located in the variable domain. My second project was to determine the amino acids involved in the binding between 2B4 and CD48. Twelve mutations were made in human 2B4 to disrupt their interaction. In the last part of the study, an attempt has been made to elucidate the role of tyrosine and threonine amino acids found in the novel tyrosine motifs (TxYxxI/V) that reside in the cytoplasmic domain.

MOLECULAR BASIS FOR 2B4-CD48 INTERACTIONS

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MOLECULAR BASIS FOR 2B4-CD48 INTERACTIONS

THESIS

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CHAPTER 1

INTRODUCTION-NATURAL KILLER CELLS AND 2B4 RECEPTOR

Natural killer cells are lymphocytes distinct from T and B cells. Natural killer cells spontaneously detect and kill cancerous and virally infected cells through receptors that transduce either activating or inhibiting signals (1). They require no prior stimulation for killing activity. They regulate the immune response by secreting interferon-γ and other cytokines resulting in the migration of other immune cells to the area. Among the cytokines produced by NK cells, interferon-γ may be particularly important as it controls viral replication, activates macrophages, enhances MHC class I and class II antigen presentation, and directs antigen specific immune responses (2). 2B4, an NK cell receptor, was originally identified on mouse NK cells and the subset of T cells that mediate non-major histocompatibility complex (MHC)-restricted killing (3). [Studies] indicate that activation of human NK cells via surface 2B4 induces transcriptional activation of INF-γ. Moreover, mRNA stability may also contribute to the enhanced production of INF-γ by human NK cells following 2B4 activation (4). Natural killer cell killing is not MHC class I restricted in the classical sense, but is influenced by the expression of MHC class I molecules on the target cell surface. NK cells spare target cells expressing self MHC class I molecules while efficiently eliminating MHC class I-deficient normal and tumor cells (5)

Natural killer cell function is mediated by surface receptors. Unlike many other activation and adhesion molecules, 2B4 expression is restricted to cells that mediate NK-like killing (6). This indicates that the 2B4 molecule plays an important role in the regulation of immune cell function. These receptors can transmit either positive or negative signals and belong to the lectin superfamily or immunoglobulin superfamily (IgSF) (7). One important surface receptor is called 2B4. Studies have revealed that human 2B4 leads to the induction of NK cell functions including both cytotoxicity and upregulation of invasive, degradative potential (3). 2B4 is expressed on all NK cells and a subset of T cells that mediate NK-like killing. 2B4 is a member of the immunoglobulin superfamily because of structural characteristics. More specifically the variable and constant domains of the 2B4 gene are what identify it as a member of the Ig superfamily. The other structural domains are the transmembrane domain, which spans the cell surface, and the cytoplasmic domain, where signals are transduced to cause activating or inhibitory effects.

It has been shown that monoclonal antibody against 2B4 enhances killing in various cancer cells. Monoclonal antibody was used to detect 2B4 on protein level. C57BL/6 was the first mouse strain that the gene 2B4 was originally cloned from. Using a mAb generated against C57BL/6, we determined that by FACS analysis only mouse strains C57BL/6 and B10.A were positive. Surprisingly, however, several mouse strains were not detected for 2B4 by mAb. By northern blot analysis, all mouse strains revealed a distinct banding pattern of 2B4 transcript. The 2B4 gene exists in two splice variants, a long form and a short form. One form has a cytoplasmic tail of 150 amino acids (2B4L)

and the other has a tail of 93 amino acids (2B4S) (8). We hypothesize that failure of anti-2B4 to recognize 2B4 in different mouse strains may be due to polymorphism of the 2B4 gene. By DNA sequencing, we were able to determine the entire 2B4 transcript. Upon comparison of the 2B4 gene across four different mouse strains, C57BL/6, A.CA, BALB/c, and 129/Svj, polymorphic residues reside in the variable, constant, and cytoplasmic domains. However, the majority of the polymorphic residues reside in the variable domain.

The data obtained in the polymorphism study forms the basis to determine the binding site for human 2B4-CD48 interactions. Unlike the mouse 2B4 isoforms, the human 2B4 isoforms differ in their extracellular domain (9). The CD48 molecule belongs to a subfamily of the Ig superfamily that also includes the CD2, CD58, 2B4, signaling lymphocyte activation molecule (SLAM), and Ly-9 molecules. Receptor-ligand interactions are known to occur between several members of this family, and these interactions can strengthen cell to cell adhesion (10). CD48 was discovered in humans as well as a cell surface molecule expressed by B lymphocytes in response to EBV (Epstein-Barr virus) infection and cellular activation signals (11). CD48, the natural ligand of 2B4, is highly expressed on the surface of EBV+ B cell lines. Remarkably, NK cells from XLP (X-linked lymphoproliferative) patients could not kill EBV+ B cell lines. This failure was found to be the consequence of inhibitory signals generated by the interaction between 2B4 and CD48, as the antibody-mediated disruption of the 2B4-CD48 interaction restored lysis of EBV+ target cells lacking human histocompatibility leukocyte antigen (HLA) class I molecules (12). CD2 and its ligands, CD58 in humans,

and CD48 in rodents belong to the CD2 subset of the IgSF, which also includes 2B4, CDw150, CD84, and Ly-9, all of which are expressed on hematopoietic cells (13). CD2 is a T lymphocyte cell-adhesion molecule (CAM) belonging to the immunoglobulin superfamily (IgSF) which mediates transient adhesion of T cells to antigen-presenting cells and target cells. Reported ligands for human CD2 include the structurally-related IgSF CAMs CD58 (LFA-3) and CD48 as well as, more controversially, the unrelated cell-surface glycoprotein CD59 (14). The CD2-CD48 interaction is among the best-characterized cell-adhesion systems, interacting in solution with a very low affinity($\approx 10^{-4}$ M^{-1}) and a dissociation rate constant of at least $6s^{-1}$ (15). Human CD2 is a cell surface glycoprotein present on T lymphocytes and natural killer cells and is important in mediating both cellular adhesion and signal transduction through interactions with its counter receptor CD58 (16). Highly elevated soluble CD48 protein is presented in leukemia and autoimmune diseases. In knock-out mice where the CD48 gene has been disrupted, animals have shown to have severe defects in T cell activation. 2B4 may play a major role in the development of the immune response because 2B4 and CD48 are counter-receptors. In our previously mentioned study, we identified that polymorphic residues reside in the variable domain. Studies done by others have also identified that there is polymorphism in the CD48 gene. [Genomic DNA] analysis reveals an allelic polymorphism within the Ig V-like domain of murine CD48 (17). 2B4 is a member of the CD2 subset of the Ig superfamily including CD2, CD48, and CD58. CD2 is a transmembrane cell surface glycoprotein found on all T cells and NK cells. The CD2-CD58 interaction is well characterized and involves the V domains of both.

Polymorphism of receptor 2B4 and CD48 resides in the V domain. The binding [between 2B4 and CD48] is approximately ten-fold greater that the CD48 to CD2 interaction suggesting that 2B4 is the physiologically significant ligand of CD48 (1).

J. H. Wang, Ph.D. from Boston, Massachusetts published his studies titled, "Structure of a heterophilic adhesion complex between the human CD2 and CD58 counter receptors." He identified the binding affinities between the CD2-CD58 interaction by mutating amino acids involved in their binding. The analysis of the molecular interface in the CD2-CD58 complex offers a first consideration of the nature of a receptor-ligand pair involving two Ig supergene family (18). In our lab, we compared the amino acids in the variable domain of genes 2B4, CD48, CD2 and CD58. Using Dr. Wang's work of CD2-CD58 interaction, we chose similar amino acids to mutate in the human 2B4 gene to determine the molecular basis in the 2B4-CD48 interaction. Results from this study will allow us to determine the specific amino acid involved in the binding of counter-receptors 2B4 and CD48 in its functional epitope. We hypothesize that by mutating particular amino acids in the variable domain of the human 2B4 gene will result in the disruption of its binding to its counter-receptor, CD48.

In the final part of my thesis, my focus was to study the basis for 2B4 signaling by mutating specific tyrosine and threonine motifs found in the cytoplasmic tail of the gene. The 2B4 cytoplasmic region contains tyrosine motifs that are potential targets for phosphorylation (13). Human 2B4 contains four tyrosine-based motifs that resemble immunoreceptor tyrosine-based motifs on its cytoplasmic tail (1). It has been shown that following cell treatment with sodium pervanadate tyrosine phosphorylation of 2B4 has

been detected both in normal NK cells (19) (12) and in cell transfectants (20). Experiments performed in 2B4 cell transfectants suggested that 2B4 associates both with the Src homology 2 domain-containing protein (SH2D1A), also termed SLAM-associated protein (SAP), and with the SH2 domain-containing phosphatase (SHP-2) (20). More recent studies in normal NK cells provided evidence that 2B4 associates with SHP-1 (rather than SHP-2) and that cell treatment with sodium pervanadate leads to association of tyrosine-phosphorylated 2B4 with SH2D1A (12). Upon specific engagement of 2B4, LAT becomes tyrosine phosphorylated and recruits downstream elements such as PLCγ and Grb2. This suggests that LAT may function as a central linker protein in the 2B4-dependent activation of human NK cells (21). We hypothesize that amino acid, threonine plays a significant role in the phosphorylation of tyrosine motifs located in the cytoplasmic tail of the 2B4 gene because it is conserved in the novel motifs. This study is significant because it has been shown that protein tyrosine kinase activation is one of the first biochemical events in the signaling pathway leading to activation of NK cell cytolytic machinery (22).

Stimulation of NK cell-mediated cytotoxicity involves the coupling of proximal Src and Syk family protein tyrosine kinases to downstream effectors. LAT is tyrosine phosphorylated upon stimulation of NK cells through FcγRIII receptors and following direct contact with NK-sensitive target cells. This NK stimulation induces the association of LAT with several phosphotyrosine-containing proteins (23). Unlike B and T lymphocytes, NK cells do not express Ag-specific receptors. Rather, their activation is initiated by the FcγRIII (Ab-dependent cell-mediated cytotoxicity (ADCC) or, in a less

defined manner, through a direct interaction with certain malignant or virus-infected target cells ("natural cytotoxicity") (24).

CHAPTER 2

POLYMORPHISM IN THE 2B4 GENE OF INBRED MOUSE STRAINS

Kumaresan R. Pappanaicken, Huynh T. Van, and P.A. Mathew. 2000. "Polymorphism in the 2B4 gene of inbred mouse strains". *Journal of Immunogenetics* Jul, 51 (8-9): 758-61.

2.1 Inbred mouse strains

Four different mouse strains were chosen for this study, C57BL/6, A.CA, BALB/c, and 129/Svj. The nucleotide sequence of the 2B4 gene located in the variable domain of each of the four strains is represented in figure 1 (C57BL/6), figure 2 (A.CA), figure 3 (BALB/c) and figure 4 (129/Svj). The peptide sequences are shown in figure 5 (C57BL/6), figure 6 (A.CA), figure7 (BALB/c), and figure 8 (129/Svj).

2.2 Nucleotide and peptide comparison results

The nucleotide sequence of A.CA-2B4 clone revealed that it is highly homologous to C57BL/6-2B4L and encodes 398 amino acid protein whereas nucleotide sequence of 129/Svj-2B4 clone showed high homology with BALB/c-2B4 and encodes 399 amino acid protein. At the nucleotide level C57BL/6-2B4 and A.CA-2B4 showed

99.4% similarity and BALB/c and 129/Svj have 100% similarity. (Figure 9) (Figure 10). Sequencing was performed at the Automated Sequencing Facility, Molecular Oncology Center, UT Southwestern Medical Center, Dallas.

2.3 Materials and Methods

RT-PCR analysis from each mouse inbred strain

RNA was isolated from each strain. RT-PCR was performed to make cDNA from mRNA.. cDNA synthesis was performed using murine Moloney leukaemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI). Two microgram of mRNA was used in the reverse transcription reaction. Primers, 5X buffer, 0.1 M DTT, 10 mM dNTPs and superscript reverse transcriptase enzyme was mixed and heated at 42°C for 50 minutes. Reverse transcriptase is an RNA-dependent DNA polymerase. Its function is to make a DNA complement of the mRNA molecule. cDNA was amplified from A.CA spleen LAK cells by using 5'-2B4 primer (TAG ACA TCA GAG CAC CTG GAG) and 3'-2B4 primer (GGA GAA CTT TGA TGT CTA CTC CTA GTT GTT) and for 129/Svj spleen LAK cells 5'-2B4 primer (GTA GTT CTG CTG TGT CCT GCT) and 3'-2B4 primer (GGA GAA CTT TGA TGT CTA CTC CTA GTT GTT) designed based on the C57BL/6 2B4L sequence (25).

PCR amplification of 2B4 gene

Using these specific 2B4 primers, the 2B4 gene was amplified by PCR method using *Taq* polymerase (Promega, Madison, WI), a heat-stable DNA polymerase from bacterium *Thermus aquaticus*. PCR was performed for 25-30 cycles in a Perkin Elmer

thermocycler 2400 (Perkin Elmer, Norwalk, CT). Several factors influence the efficiency of the PCR process. These factors include *Taq* polymerase, deoxynucleotide triphosphates (dNTP), magnesium ions, template DNA, and primers in the reaction. Concentrations of each factor used were 1mM dNTP, 25 mM $MgCl_2$, 10 uM of each primer, 2μg/ml template and 5 units/μl *Taq* polymerase. PCR cycle temperatures were 94°C for 3 minutes and 45 seconds, annealing temperature 50°C for 1 minute and extension temperature 72°C for 2 minutes followed by a final extension for another 7 minutes. Repeated rounds of denaturation, primer annealing, and extension resulted in large quantities of product due to exponential amplification of the desired sequence.

Gel purification by electrophoresis analysis

Gel purification was used to check for 2B4. PCR products were electrophoretically separated on a 1% agarose gel for 45 minutes at 70 volts. 10mM ethidium bromide and 6x concentration tracking dye was added to the samples to visualize separated DNA bands under UV radiation. The tracking dye contains sucrose, bromophenol blue, and distilled water. The 2B4 gene was then cloned into pGem TA easy vector (Promega, Madison, WI). An additional purification step was done using the gel extraction method. QIAquick Gel Extraction Kit was purchased from Qaigen Inc., Valencia, CA. The agarose gel slice was cut and dissolved in three volumes of Buffer QG at 50°C. Buffer QG aids to solubilize the agarose gel slice and provides the optimal conditions for DNA to bind to the silica membrane. One gel volume of isopropanol was added to precipitate the DNA. To bind the DNA, the sample was applied to a QAIquick column and centrifuged for one minute at full speed. To remove all traces of agarose,

0.5ml of Buffer QG was added to the column and centrifuged for one minute. Buffer PE was then added to the column to wash the DNA. In this step all salts, enzymes, unincorporated nucleotides, agarose, ethidium bromide, and detergents are washed away by the ethanol-containing Buffer PE. Next, DNA was eluted from the column with 30 microliters of distilled water. The concentration of DNA was measured by spectrophotometery. Subsequent ligation process was carried out at 16° for 12-16 hours. The ligation reaction contains 50ng/μl plasmid vector DNA, 10x DNA ligase buffer, cDNA, DNA ligase (100 units/μl), 10 mM ATP, and distilled water.

Transformation of the 2B4 gene into *E. coli* cells

Transformation of the 2B4 insert was accomplished using JM109 *E. coli* cells (Promega, Madison, WI). This process was performed to grow larger quantities of the plasmid containing the 2B4 insert. One microliter of the ligation product was incubated in 50 μl of competent cells. Bacterial cells were allowed to sit on ice for 30 minutes. Cells were then heat shocked at 42°C for 45 seconds. Enriched media was added and cells were propagated in a 37°C incubator for 1 hour with shaking at 250 rpm. Enriched medium contains bacto-tryptone, bacto-yeast extract, NaCl, KCl, 10 mM $MgCl_2$, and 20 mM glucose. Cell culture was plated on LB agar plates containing 50mg/ml ampicillin and 100mM IPTG. LB medium contains bacto-tryptone, bacto-yeast extract, and NaCl. Granulated agar was added to the medium to make LB-agar plates. Plates were incubated at 37°C for 16 hours. Isolated colonies were then propagated in 5 ml of LB media with 50mg/ml ampicillin at 37°C for 16 hours at 250 rpm (revolutions per minute).

Plasmid purification and isolation

Plasmid purification and isolation was achieved by using the Wizard Miniprep Kit (Promega, Madison, WI). The mini-prep is a quick method used for isolating small amounts of plasmid DNA, about 1 μg of DNA per milliliter of bacterial culture. The mini-prep also allows for rapid screening of transformants by subsequent restriction digestion of the isolated DNA. Three milliliters of culture were transferred to microfuge tubes and centrifuged at maximum speed for 30 seconds. Medium was discarded. Bacterial pellet was resuspended in alkaline lysis solution I by vigorous vortexing. Freshly prepared alkaline lysis solution II was added to each bacterial suspension. Tubes were gently inverted several times. Alkaline lysis solution II was added and thoroughly mixed with the bacterial lysate by gently inversion. Tubes were stored on ice for 3-5 minutes. Centrifugation of bacterial lysate was performed at maximum speed for 5 minutes. Supernatant is then transferred to new tubes. Equal volumes of phenol and chloroform were added. Organic and aqueous phases were mixed by vortexing followed by centrifugation at maximum speed for 2 minutes. Upper layer of aqueous phase was transferred to a new tube. Two volumes of ethanol were added at room temperature to precipitate the plasmid DNA from the supernatant. Nucleic acids were collected by centrifugation at maximum speed for 5 minutes. One milliliter of 70% ethanol was added to the pellet and a final centrifugation spin was necessary to recover the DNA. Nucleic acids were dissolved in 30 μl of double distilled water and stored at −20° C. The spectrophotometer was used to assess the purity of DNA. Optical density measured at wavelength 260 nm for DNA, and 280 nm for RNA.

Figure 1: Full length nucleotide sequence of the 2B4 gene from inbred mouse strain, C57BL/6.

C57BL/6-2B4

51	CAGTTAGCTG	CGAGCCTATT	GACATCTGAG	CACCTGGAGA	AGACAGGGCC	**100**
101	CCCGTAGTCT	GCTCTGTCCT	GTGGTGATGT	TGGGGCAAGC	TGTCCTGTTC	**150**
151	ACAACCTTCC	TGCTCCTCAG	GGCTCATCAG	GGCCAAGACT	GCCCAGATTC	**200**
201	TTCTGAAGAA	GTGGTTGGTG	TCTCAGGAAA	GCCTGTCCAG	CTGAGGCCTT	**250**
251	CCAACATACA	GACAAAAGAT	GTTTCTGTTC	AATGGAAGAA	GACAGAACAG	**300**
301	GGCTCACACA	GAAAAATTGA	GATCCTGAAT	TGGTNNNATA	ATGATGGTCC	**350**
351	CAGTTGGTCA	AATGTATCTT	TTAGTGATAT	CTATGGTTTT	GATTATGGGG	**400**
401	ATTTTGCTCT	TAGTATCAAG	TCAGCTAAGC	TGCAAGACAG	TGGTCACTAC	**450**
451	CTGCTGGAGA	TCACCAACAC	AGGCGGAAAA	GTGTGCAATA	AGAACTTCCA	**500**
501	GCTTCTTATA	CTTGATCATG	TTGAGACCCC	TAACCTGAAG	GCCCAGTGGA	**550**
551	AGCCCTGGAC	TAATGGGACT	TGTCAACTGT	TTTTGTCCTG	CTTGGTGACC	**600**
601	AAGGATGACA	ATGTGAGCTA	CGCTTTGTAC	AGAGGGAGCA	CTCNNNTGAT	**650**
651	CTCCAATCAA	AGGAATAGTA	CCCACTGGGA	GAACCAGATT	GACGCCAGCA	**700**
701	GCCTGCACAC	ATACACCTGC	AACGTTAGCA	ACAGAGCCAG	CTGGGCAAAC	**750**
751	CACACCCTGA	ACTTCACCCA	TGGCTGTCAA	AGTGTCCCTT	CGAATTTCAG	**800**
801	ATTTCTGCCC	TTTGGGGTGA	TCATCGTGAT	TCTAGTTACA	TTATTTCTCG	**850**
851	GGGCCATCAT	TTGTTTCTGT	GTGTGGACTA	AGAAGAGGAA	GCAGTTACAG	**900**
901	TTCAGCCCTA	AGGAACCTTT	GACAATAAT	GAATATGTCA	AGACTCACG	**950**
951	AGCCAGCAGG	GATCAACAAG	GATGCTCTAG	GGCCTCTGGA	TCTCCCTCGG	**1000**
1001	CTGTCCAGGA	AGATGGGAGG	GGACAAAGAG	AATTGGACAG	GCGTGTTTCT	**1050**
1051	GAGGTGCTGG	AGCAGTTGCC	ACAGCAGACT	TTCCCTGGAG	ATAGAGGCAC	**1100**
1101	CATGTACTCT	ATGATACAGT	GCAAGCCCTTC	TGATTCCACA	TCACAAGAAA	**1150**
1151	AATGTACAGT	ATATTCAGTA	GTCCAGCCTT	CCAGGAAGTC	TGGATCCAAG	**1200**
1201	AAGAGGAACC	AGAACTATTC	CTTAAGTTGT	ACCGTGTACG	AGGAGGTTGG	**1250**
1251	AAACCCATGG	CTCAAAGCTC	ACAACCCTGC	CAGGTGNAG	CCGCAGAGAG	**1300**
1301	CTGGAGAACT	TTGATGTCTA	CTCCTAGTTG	TTAGCAGTGG	CCTCATCCTG	**1350**

Figure 2: Full length nucleotide sequence of the 2B4 gene from inbred mouse strain, A.CA.

A.CA-2B4

51	CAGTTAGCTG	CGAGCCTATT	GACATCAGAG	CACCTGGAGA	AGACAGGGCC	**100**
101	CCCGTAGTCT	GCTCTGTCCT	GTGGTGATGT	TGGGGCAAGC	TGTCCTGTTC	**150**
151	ACAACCTTCC	TGCTCCTCAG	GGCTCATCAG	GGCCAAGACT	GCCCAGATTC	**200**
201	TTCTGAAGAA	GTGGTTGGTG	TCTCAGGAAA	GCCTGTGCACG	CTGAGGCCTT	**250**
251	CCAACATACA	GACAAAAGAT	GTTTCTGTTC	AATGGAAGAA	GACAGAGCAG	**300**
301	GGCTCACACA	GAAAAATTGA	GATCCTGAAT	TGGTNNNATA	ATGATGGTCC	**350**
351	CAGTTGGTCA	AATGTATCTT	TTAGTGATAT	CTATGGTTTT	GATTATGGGG	**400**
401	ATTTTGCTCT	TAGTATCAAG	TCAGCTAAGC	TGCAAGACAG	TGGTCACTAC	**450**
451	CTGCTGGAGA	TCACCAACAC	AGGCGGAAAA	GTGTGCAATA	AGAACTTCCA	**500**
501	GCTTCTTATA	CTTGATCATG	TTGAGACCCC	TAACCTGAAG	GCCCAGTGGA	**550**
551	AGCCCTGGAC	TAATGGGACT	TGTCAACTGT	TTTTGTCCTG	CTTGGCGACC	**600**
601	AAGGATGACA	ATGTGAGCTA	CGCTTTGTAC	AGAGGGAGCA	CTCNNNTGAT	**650**
651	CTCCAATCAA	AGGAATAGTA	CCCACTGGGA	GAACCAGATT	GACGCCAGCA	**700**
701	GCCTGCACAC	ATACACCTGC	AACGTTAGCA	ACAGAGCCAG	CTGGGCAAAC	**750**
751	CACACCCTGA	ACTTCACCCA	TGGCTGTCAA	AGTGTCCCTT	CGAATTTCAG	**800**
801	ATTTCTGCCC	TTTGGGGTGA	TCATCGTGAT	TCTAGTTACA	TTATTTCTCG	**850**
851	GGGCCATCAT	TTGTTTCTGT	GTGTGGACTA	AGAAGAGGAA	GCAGTTACAG	**900**
901	TTCAGCCCTA	AGGAACCTTT	GACAATAAT	GAATATGTCA	AGACTCACG	**950**
951	AGCCAGCAGG	GATCAACAAG	GATGCTCTAG	GGCCTCTGGA	TCTCCCTCGG	**1000**
1001	CTGTCCAGGA	AGATGGGAGG	GGACAAAGAG	AATTGGACAG	GCGTGTTTCT	**1050**
1051	GAGGTGCTGG	AGCAGTTGCC	ACAGCAGACT	TTCCCTGGAG	ATAGAGGCAC	**1100**
1101	CATGTACTCT	ATGATACAGT	GCAAGCCCTTC	TGATTCCACA	TCACAAGAAA	**1150**
1151	AATGTACAGT	ATATTCAGTA	GTCCAGCCTT	CCAGGAAGTC	TGGATCCAAG	**1200**
1201	AAGAGGAACC	AGAACTATTC	CTTAAGTTGT	ACCGTGTACG	AGGAGGTTGG	**1250**
1251	AAACCCATGG	CTCAAAGCTC	ACAACCCTGC	CAGGTGNAG	CCGCAGAGAG	**1300**
1301	CTGGAGAACT	TTGATGTCTA	CTCCTAGTTG	TTAGCAGTGG	CCTCATCCTG	**1350**

Figure 3: Full length nucleotide sequence of the 2B4 gene from inbred mouse strain, BALB/c.

BALB/c-2B4

51	CAGTTAGCTG	CGAGCCTATT	GACATCTGAG	CACCTGGAGA	AGACAGGGCC	**100**
101	CCCGTAGTCT	GCTCTGTCCT	GTGGGGATGT	TGGGGCAAGC	TGTCTCTGTTC	**150**
151	ACAACCGTTTC	TGCTCCTCAG	GGCTCATCAG	GGCCAAGACT	GCTCAGATTC	**200**
201	TCCTGAAGAA	GTGGTTGGTG	TCTCAGGAAA	GCCTGTCCGG	CTGAGGCCTT	**250**
251	CCAACATACA	GACAAAAGAT	GTTTCTGTTC	AATGGAAGAA	GAAAGTACAG	**300**
301	GGCTCACACA	CAAACACTGA	GATCCTGAAT	TCGTGTAATA	ATAATGCTTC	**350**
351	CAGATGCTCA	ACTGTGGTTT	CTAGTGATAT	CTATGGCTTT	GATTATGGGG	**400**
401	ATTTTGCTCT	TAGTATCAAG	TCAGCTCAGC	TGCAAGACAG	TGGTCACTAC	**450**
451	CTGCTGGAGC	TCACCTACAG	AAACGGAACA	GTGTGCACTA	AGAACTTCCA	**500**
501	GATTCTTATA	CTTGATCATG	TTGAGACACC	TCACCTGAAG	GCCCAGTGGA	**550**
551	ACGCCTGGAC	TAATGGGACT	TGTCAACTGT	TTTTGTCCTG	CTTGGTGCCC	**600**
601	AAGGATGACA	ATGTGAGCTA	TGCTTTGTAC	AGAGGGAGCA	CGCTGATGAT	**650**
651	CTCAAATCAA	AGGAATAGTA	CCCACTGGGA	GAACCAGACT	GATGCCAGCA	**700**
701	GCCTGCACAC	ATATACCTGC	AACGTTAGCA	ACAGAGCCAG	CTGGGCAAAC	**750**
751	CACACCCTGA	ACTTCACCCA	TGGCTGTCAG	AGTGTCCATT	TGAATTTCAG	**800**
801	ATTTCTGCCC	TTTGGGGTGA	TCATCGTGAT	TCTAGTTACA	TTATTTCTTG	**850**
851	GGGCCATCAT	TTGCTTCTGT	GTGTGGACTA	AGAAGAGGAA	GCAGTTACAG	**900**
901	TTCAGCCCTA	AGGAACCTTT	GACAATAAT	GAATATGTCA	AGACTCACG	**950**
951	AGCCAGTAGG	GATCAACAAG	GATGCTCTAG	GGCCTCTGGA	TCTCCCTCAG	**1000**
1001	ATGTCCAGGA	AGATGGGAGG	GGACAAAGAG	AATTGGACAG	GCGCGTTTCT	**1050**
1051	GAGGTGCTGG	AGCAGTTGCC	ACAGCAGAAT	TCCCCTGGAG	ATAGAGGCAC	**1100**
1101	CATGTACTCT	ATGATCCAGT	GCAAGCCCTTC	TGATTCCACA	TCACAAGAAA	**1150**
1151	AATGTACAGT	ATATTCAGTA	GTCCAGCCTT	CCAGGAAGTC	TGGATCCAAG	**1200**
1201	AAGAGGAACC	AGAACTCTTC	CTTAAGTTGT	ACCGTGTACG	AAGAGGTTGG	**1250**
1251	AAACCCATGG	CTCAAAGCTC	ACAACCCTGC	CAGGTGAAG	CCGCAGAAAA	**1300**
1301	CTGGAGAACT	TTGATGTCTA	CTCCTAATTG	GTAAGCAGTG	GGCCTCATCC	**1350**

Figure 4: Full length nucleotide sequence of the 2B4 gene from inbred mouse strain, 129/Svj.

129/Svj-2B4

51	CAGTTAGCTG	CGAGCCTATT	GACATCTGAG	CACCTGGAGA	AGACAGGGCC	**100**
101	CCCGTAGTCT	GCTCTGTCCT	GTGGGGATGT	TGGGGCAAGC	TGTCTCTGTTC	**150**
151	ACAACCGTTTC	TGCTCCTCAG	GGCTCATCAG	GGCCAAGACT	GCTCAGATTC	**200**
201	TCCTGAAGAA	GTGGTTGGTG	TCTCAGGAAA	GCCTGTCCGG	CTGAGGCCTT	**250**
251	CCAACATACA	GACAAAAGAT	GTTTCTGTTC	AATGGAAGAA	GAAAGTACAG	**300**
301	GGCTCACACA	CAAACACTGA	GATCCTGAAT	TCGTGTAATA	ATAATGCTTC	**350**
351	CAGATGCTCA	ACTGTGGTTT	CTAGTGATAT	CTATGGCTTT	GATTATGGGG	**400**
401	ATTTTGCTCT	TAGTATCAAG	TCAGCTCAGC	TGCAAGACAG	TGGTCACTAC	**450**
451	CTGCTGGAGC	TCACCTACAG	AAACGGAACA	GTGTGCACTA	AGAACTTCCA	**500**
501	GATTCTTATA	CTTGATCATG	TTGAGACACC	TCACCTGAAG	GCCCAGTGGA	**550**
551	ACGCCTGGAC	TAATGGGACT	TGTCAACTGT	TTTTGTCCTG	CTTGGTGCCC	**600**
601	AAGGATGACA	ATGTGAGCTA	TGCTTTGTAC	AGAGGGAGCA	CGCTGATGAT	**650**
651	CTCAAATCAA	AGGAATAGTA	CCCACTGGGA	GAACCAGACT	GATGCCAGCA	**700**
701	GCCTGCACAC	ATATACCTGC	AACGTTAGCA	ACAGAGCCAG	CTGGGCAAAC	**750**
751	CACACCCTGA	ACTTCACCCA	TGGCTGTCAG	AGTGTCCATT	TGAATTTCAG	**800**
801	ATTTCTGCCC	TTTGGGGTGA	TCATCGTGAT	TCTAGTTACA	TTATTTCTTG	**850**
851	GGGCCATCAT	TTGCTTCTGT	GTGTGGACTA	AGAAGAGGAA	GCAGTTACAG	**900**
901	TTCAGCCCTA	AGGAACCTTT	GACAATAAT	GAATATGTCA	AGACTCACG	**950**
951	AGCCAGTAGG	GATCAACAAG	GATGCTCTAG	GGCCTCTGGA	TCTCCCTCAG	**1000**
1001	ATGTCCAGGA	AGATGGGAGG	GGACAAAGAG	AATTGGACAG	GCGCGTTTCT	**1050**
1051	GAGGTGCTGG	AGCAGTTGCC	ACAGCAGAAT	TCCCCTGGAG	ATAGAGGCAC	**1100**
1101	CATGTACTCT	ATGATCCAGT	GCAAGCCCTTC	TGATTCCACA	TCACAAGAAA	**1150**
1151	AATGTACAGT	ATATTCAGTA	GTCCAGCCTT	CCAGGAAGTC	TGGATCCAAG	**1200**
1201	AAGAGGAACC	AGAACTCTTC	CTTAAGTTGT	ACCGTGTACG	AGGAGGTTGG	**1250**
1251	AAACCCATGG	CTCAAAGCTC	ACAACCCTGC	CAGGTGAAG	CCGCAGAAAA	**1300**
1301	CTGGAGAACT	TTGATGTCTA	CTCCTAGTTG	TTAAGCAGTG	GGCCTCATCC	**1350**

Figure 5: Full length peptide sequence of the 2B4 gene from inbred mouse strain, C57BL/6.

C57BL/6-2B4

1	MLGQAVLFTT	FLLLRAHQGQ	DCPDSSEEVV	GVSGKPVQLR	PSNIQTKDVS	**50**
51	VQWKKTEQGS	HRKIEIINWY	NDGPSW.SNV	SFSDIYGFDY	GDFALSIKSA	**100**
101	KLQDSGHYLL	EITNTGGKVC	NKNFQLLILD	HVETPNLKAQ	WKPWTNGTCQ	**150**
151	LFLSCLVTKD	DNVSYAFWYR	GSTL.ISNQR	NSTHWENQID	ASSLHTYTCN	**200**
201	VSNRASWANH	TLNFTHGCQS	VPSNFRFLPF	GVIIVILVTL	FLGAIICFCV	**250**
251	WTKKRKQLQF	SPKEPLTIYE	YVKDSRASRD	QQGCSRASGS	PSAVQEDGRG	**300**
301	QRELDRRVSE	VLEQLPQQTF	PGDRGTMYSM	IQCKPSDSTS	QEKCTVYSW	**350**
351	QPSRKSGSKK	RNQNYSLSCT	VYEEVGNPWL	KAHNPARLSR	RELENFDVYS	**400**

Figure 6: Full length peptide sequence of the 2B4 gene from inbred mouse strain, A.CA.

A.CA-2B4

1	MLGQAVLFTT	FLLLRAHQGQ	DCPDSSEEVV	GVSGKPVQLR	PSNIQTKDVS	**50**
51	VQWKKTEQGS	HRKIEIINWY	NDGPSW.SNV	SFSDIYGFDY	GDFALSIKSA	**100**
101	KLQDSGHYLL	EITNTGGKVC	NKNFQLLILD	HVETPNLKAQ	WKPWTNGTCQ	**150**
151	LFLSCLATKD	DNVSYAL.YR	GSTL.ISNQR	NSTHWENQID	ASSLHTYTCN	**200**
201	VSNRASWANH	TLNFTHGCQS	VPSNFRFLPF	GVIIVILVTL	FLGAIICFCV	**250**
251	WTKKRKQLQF	SPKEPLTIYE	YVKDSRASRD	QQGCSRASGS	PSAVQEDGRG	**300**
301	QRELDRRVSE	VLEQLPQQTF	PGDRGTMYSM	IQCKPSDSTS	QEKCTVYSW	**350**
351	QPSRKSGSKK	RNQNYSLSCT	VYEEVGNPWL	KAHNPARLSR	RELENFDVYS	**400**

Figure 7: Full length peptide sequence of the 2B4 gene from inbred mouse strain, BALB/c.

BALB/c-2B4

1	MLGQAVLFTT	FLLLRAHQGQ	DCSDSPEEVV	GVSGKPVRLR	PSNIQTKDVS	**50**
51	VQWKKKVQGS	HTNTEIIISC	NNNASRCSTV	VSSDIYGFDY	GDFALSIKSA	**100**
101	QLQDSGHYLL	EITYRNGTVC	TKNFQLLILD	HVETPNLKAQ	WNAWTNGTCQ	**150**
151	LFLSCSVPKD	DNVSYAL.YR	GSTLMISNQR	NSTHWENQTD	ASSLHTYTCN	**200**
201	VSNRASWANH	TLNFTHGCQS	VPSNFRFLPF	GVIIVILVTL	FLGAIICFCV	**250**
251	WTKKRKQLQF	SPKEPLTIYE	YVKDSRASRD	QQGCSRASGS	PSDVQEDGRG	**300**
301	QRELDRRVSE	VLEQLPQQNS	PGDRGTMYSM	IQCKPFDSTS	QEKCTVYSW	**350**
351	QPSRKSGSKK	RNQNYSLSCT	VYEEVGNPWL	KAHNPARLNP	QKTGELDVYS	**400**

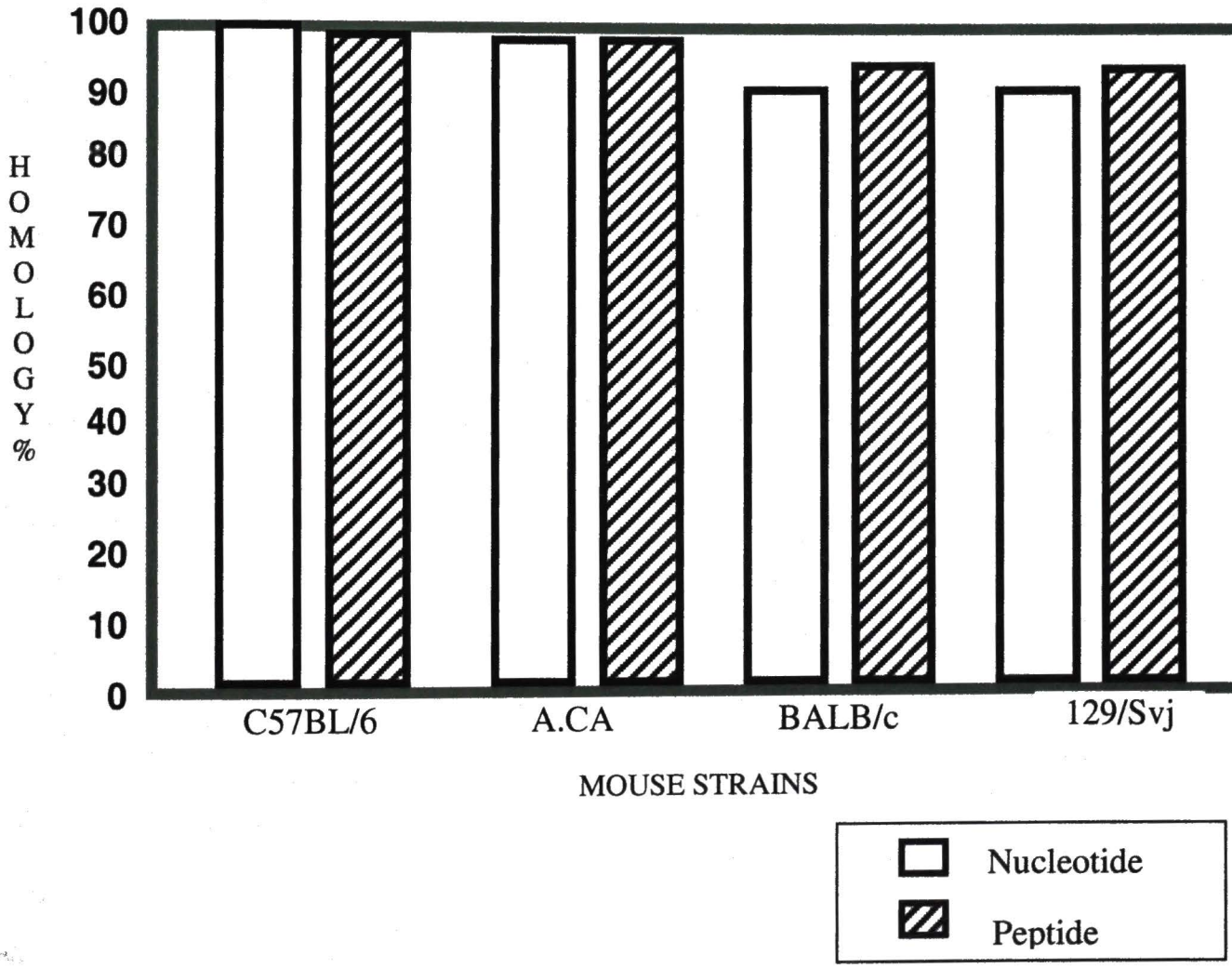
Figure 8: Full length peptide sequence of the 2B4 gene from inbred mouse strain, 129/Svj.

129/Svj-2B4

1	MLGQAVLFTT	FLLLRAHQGQ	DCSDSPEEVV	GVSGKPVRLR	PSNIQTKDVS	**50**
51	VQWKKKVQGS	HTNTEIIISC	NNNASRCSTV	VSSDIYGFDY	GDFALSIKSA	**100**
101	QLQDSGHYLL	EITYRNGTVC	TKNFQLLILD	HVETPNLKAQ	WNAWTNGTCQ	**150**
151	LFLSCSMPKD	DNVSYAL.YR	GSTL.ISNQR	NSTHWENQTD	ASSLHTYTCN	**200**
201	VSNRASWANH	TLNFTHGCQS	VPSNFRFLPF	GVIIVILVTL	FLGAIICFCV	**250**
251	WTKKRKQLQF	SPKEPLTIYE	YVKDSRASRD	QQGCSRASGS	PSDVQEDGRG	**300**
301	QRELDRRVSE	VLEQLPQQNS	PGDRGTMYSM	IQCKPFDSTS	QEKCTVYSW	**350**
351	QPSRKSGSKK	RNQNYSLSCT	VYEEVGNPWL	KAHNPARLNP	QKTGELDVYS	**400**

Figure 9: Chart representation of the nucleotide and peptide homology of mouse strains compared to mouse strain C57BL/6.

Homology to 2B4



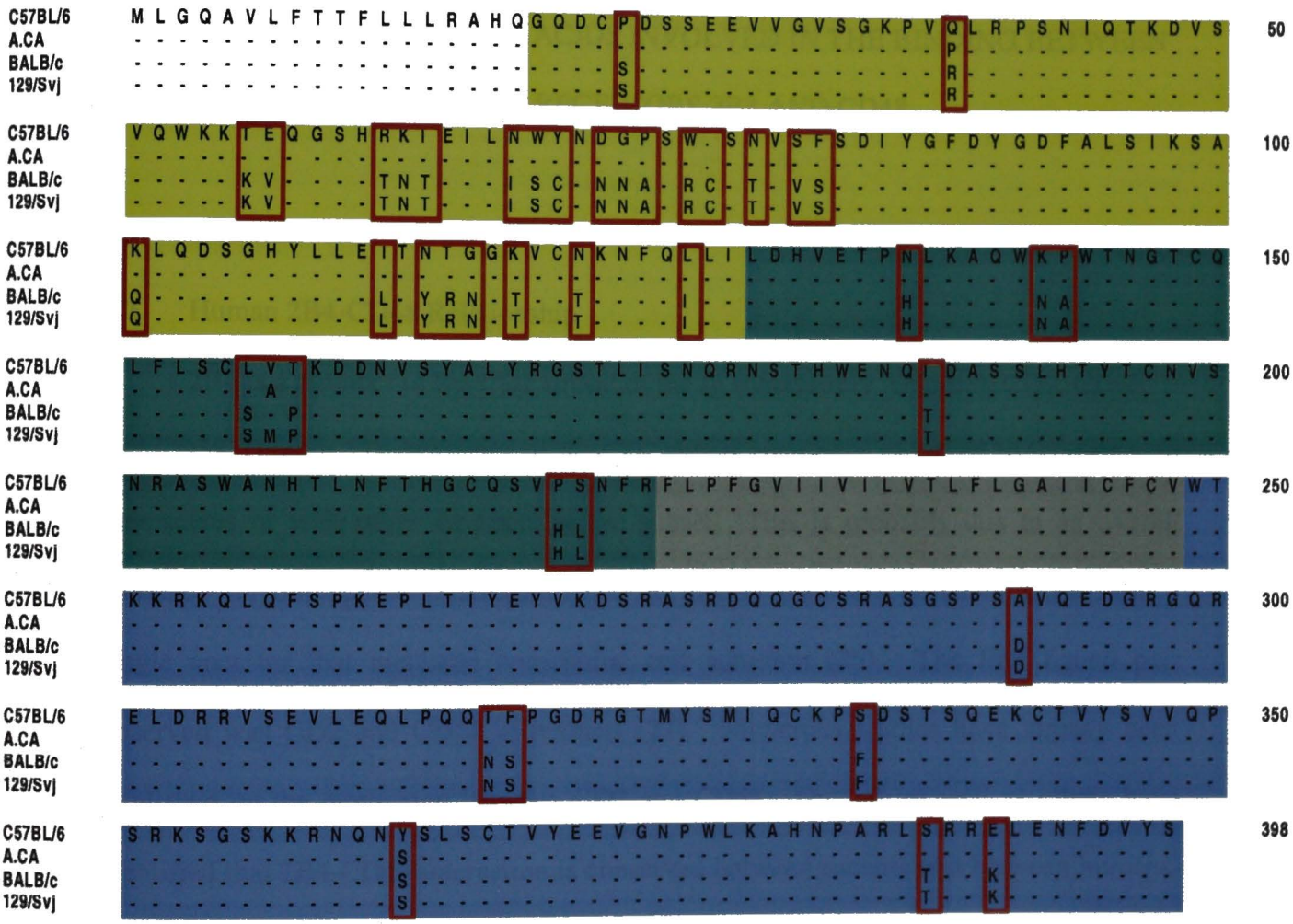
In summary, we have cloned and sequenced the entire 2B4 transcript from four different mouse strains. Comparison of the sequence revealed 2B4 is polymorphic. The polymorphic residues are located in the Ig V domain. (Figure 11) The polymorphism reported in this study may explain the differential recognition of mAb.

Figure 10: Percentage similarity of 2B4 at the nucleotide and protein level among different mouse strains.

Strain	C57BL/6	A.CA	BALB/c	129/Svj
				Peptide Similarity
C57BL/6	**100.0**	99.2	91.7	91.7
A.CA	99.4	**100.0**	91.7	91.7
BALB/c	95.4	94.6	**100.0**	100.0
129/Svj	95.1	94.7	99.7	**100.0**
	Nucleotide Similarity			

Figure 11: Comparison of the predicated polypeptide sequences of 2B4 from C57BL/6, A.CA, BALB/c, and 129/Svj. A dash denotes sequences identical with C57BL/6 at that position. Red boxes indicate amino acids that are different among the mouse strains at that position.

Comparison of 2B4



CHAPTER 3

IDENTIFICATION OF AMINO ACIDS INVOLVED IN THE BINDING BETWEEN COUNTER-RECEPTORS 2B4 AND CD48

3.1 Human 2B4-CD48 Relationship

2B4 is a member of the CD2 subset of the Ig superfamily including CD2, CD48, and CD58. (Figure 12) Members of the CD2 subset have been observed to interact with themselves or with other members of the subset. This is probably due to an overall tendency for homophilic interaction since the family members probably arose from a single ancestor that mediated homophilic cell adhesion (26). The first ligand pair observed was CD2 and CD58 in humans (27). Numerous studies have already been published on the interaction between CD2 and CD58. Our lab as well as others have identified that 2B4-CD48 interaction is conserved between rodents and between humans, and that polymorphism of receptor 2B4 and CD48 resides in their variable domains. Studies have shown that 2B4 is expressed not only on NK cells and CD8+ T cells, but also on monocytes and basophils, indicating a broader role for 2B4 in leukocyte activation(19). In T cells and monocytes, ligation of 2B4 does not lead to T cell or

monocyte activation. Thus it appears that the primary function of 2B4 is to modulate other receptor-ligand interactions to enhance leukocyte activation (19).

3.2 Mutations

We did a comparison of human 2B4, CD48 and choose twelve potential amino acids that may play a role in their binding. We choose these amino acids based on the amino acids that play a role in the binding of CD2 and CD58. (Figure 13) We used the Quikchange Site-directed Mutagenesis (Stratagene, La Jolla, CA) to make our twelve mutations. (Table 1)

Table 1: Human 2B4 Mutations

* Mutations	Original amino acid	Mutant amino acid
H2B4-47KA	Lysine (+)	Alanine
H2B4-49DA	Aspartic acid (-)	Alanine
H2B4-50SA	Serine (n)	Alanine
H2B4-54KA	Lysine (+)	Alanine
H2B4-55KA	Lysine (+)	Alanine
H2B4-61NA	Asparagine (n)	Alanine
H2B4-62GA	Glycine (n)	Alanine
H2B4-68KA	Lysine (+)	Alanine
H2B4-70EA	Glutamic acid (n)	Alanine
H2B4-110TA	Threonine (n)	Alanine
H2B4-114GA	Glycine (n)	Alanine
H2B4-115KA	Lysine (+)	Alanine

(+) = positive charge (−) = negative charge n = neutral

* Mutations are named as human 2B4 (H2B4) at position number of amino acid (47-115) single letter abbreviation of the original amino acid (K, D, S, etc.) followed by single letter abbreviation of mutant amino acid (A).

Table 2: Synthetic oligonucleotide primers containing mutant amino acid alanine

Primer nomenclature	Forward or Reverse Primer	5' to 3' orientation
H2B4-47KA	Forward	a-aac-agc-ata-cag-acg-gcg-gtt-gac-agc-att-gca-tgg-aag
H2B4-47KA	Reverse	ctt-cca-tga-aat-gct-gtc-aac-cgc-cgt-ctg-tat-gct-gtt-t
H2B4-49DA	Forward	aac-agc-ata-cag-acg-aag-gtt-gcc-agc-att-gca-tgg-aag-aag
H2B4-49DA	Reverse	ctt-ctt-cca-tgc-aat-gct-ggc-aac-ctt-cgt-ctg-tat-gct-gtt
H2B4-50SA	Forward	aac-agc-ata-cag-acg-aag-gtt-gac-gcc-att-gca-tgg-aag-aag
H2B4-50SA	Reverse	ctt-ctt-cca-tgc-aat-ggc-gtc-aac-ctt-cgt-ctg-tat-gct-gtt
H2B4-54KA	Forward	tt-gac-agc-att-gca-tgg-gcg-aag-ttg-ctg-ccc-tca-c
H2B4-54KA	Reverse	g-tga-ggg-cag-caa-ctt-cgc-cca-tgc-aat-gct-gtc-aa
H2B4-55KA	Forward	tt-gac-agc-att-gca-tgg-aag-gcg-ttg-ctg-ccc-tca-c
H2B4-55KA	Reverse	g-tga-ggg-cag-caa-cgc-ctt-cca-tgc-aat-gct-gtc-aa
H2B4-61NA	Forward	tg-ctg-ccc-tca-caa-gct-gga-ttt-cat-cac-ata-ttg-aag-tgg-g
H2B4-61NA	Reverse	c-cca-ctt-caa-tat-gtg-atg-aaa-tcc-agc-ttg-tga-ggg-cag-ca
H2B4-62GA	Forward	tg-ctg-ccc-tca-caa-aat-gcg-ttt-cat-cac-ata-ttg-aag-tgg-g
H2B4-62GA	Reverse	c-cca-ctt-caa-tat-gtg-atg-aaa-cgc-att-ttg-tga-ggg-cag-ca
H2B4-68KA	Forward	ttt-cat-cac-ata-ttg-gcg-tgg-gag-aat-ggc-tct-ttg-cct-tcc
H2B4-68KA	Reverse	gga-agg-caa-aga-gcc-att-ctc-cca-cgc-caa-tat-gtg-atg-aaa
H2B4-70EA	Forward	ttt-cat-cac-ata-ttg-aag-tgg-gcg-aat-ggc-tct-ttg-cct-tcc
H2B4-70EA	Reverse	gga-agg-caa-aga-gcc-att-cgc-cca-ctt-caa-tat-gtg-atg-aaa
H2B4-110TA	Forward	ggc-ctc-tac-tgc-ctg-gag-gtc-gcc-agt-ata-tct-gg

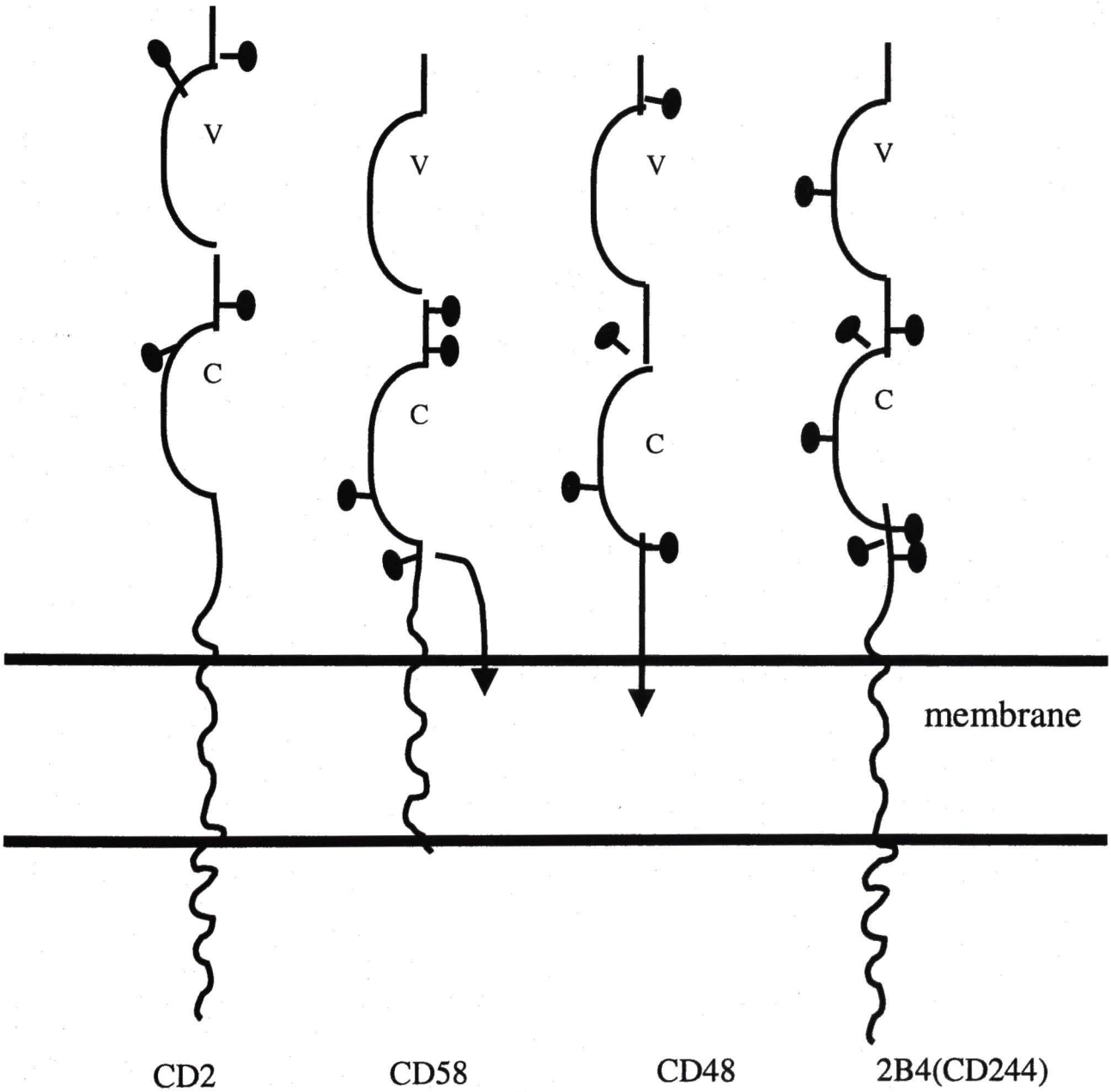
H2B4-110TA	Reverse	cc-aga-tat-act-ggc-gac-ctc-cag-gca-gta-gag-gcc
H2B4-114GA	Forward	c-acc-agt-ata-tct-gca-aaa-gtt-cag-aca-gcc-acg-ttc-c
H2B4-114GA	Reverse	g-gaa-cgt-ggc-tgt-ctg-aac-ttt-tgc-aga-tat-act-ggt-g
H2B4-115KA	Forward	c-acc-agt-ata-tct-gga-gca-gtt-cag-aca-gcc-acg
H2B4-115KA	Reverse	g-gaa-cgt-ggc-tgt-ctg-aac-tgc-tcc-aga-tat-act-ggt-g

3.3 Materials and Methods

PCR amplification of single point mutations

PCR reactions were performed in the Perkin Elmer thermocycler 2400 (Perkin Elmer, Norwalk, CT). The supercoiled double stranded DNA was denatured to anneal the primers containing the desired mutation. Using the nonstrand-displacing action of the *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA), extension was followed by the incorporation of the mutagenic primers resulting in nicked circular strands. *Pfu* DNA polymerase comes from the deep-sea hyperthermophilic archaea *Pyrococcus furiosis*. PCR conditions were 95°C for 30 seconds, primer annealing at 55°C for 1 minute, and an extension at 68°C for 14 minutes, for 14-16 cycles. The advantages of using the *Pfu* Turbo DNA polymerase are shorter extension times, fewer cycles, and lower concentrations of DNA template for polymerase chain reactions. 10x buffer used during the amplification reaction includes 100 mM KCl, 100 mM ammonium sulfate, 200 mM Tris, 20 mM $MgSO_4$, 1% Triton X-100, and 1 mg/ml nuclease-free bovine serum albumin. (Figure 14)

Figure 12: Schematic representation of CD2 subset members depicting their structural similarities.


V
C
V
C
V
C
V
C
membrane
CD2
CD58
CD48
2B4(CD244)

Dpn I digestion of mutant clones

The PCR product was then treated with restriction enzyme, Dpn I (Stratagene, La Jolla, CA) to digest the methylated, nonmutated parental DNA template. One microliter of restriction enzyme was added directly to the PCR product in a microfuge tube. The digestion was carried out in a water bath at 37°C for 1 hour. Restriction enzyme, Dpn I, specifically cleaves methylated sequences. Therefore, Dpn I will digest DNA used as template during PCR amplification, but it will not digest DNA synthesized during the course of the reaction. Digested products were checked by gel electrophoresis. Samples were separated according to size on a 1% agarose gel.

Transformation into supercompetent cells

The nicked double stranded DNA was then transformed into supercompetent Epicurian (*E. coli*) cells (Stratagene, La Jolla, CA). The bacterial cells repairs the nicks in the mutated plasmid. One microliter of the digested product was incubated in 50 µl of competent cells. Bacterial cells were allowed to sit on ice for 30 minutes. Cells were then heat shocked at 42°C for 45 seconds. Enriched media was added and cells were propagated in a 37°C incubator for 1 hour with shaking at 250 rpm. Cell culture was plated on LB agar plates containing 50mg/ml ampicillin , and 100mM IPTG. Plates were incubated at 37°C for 16 hours. Isolated colonies were then propagated in 5 ml of LB media with 50mg/ml ampicillin at 37°C for 16 hours at 250 rpm.

Plasmid purification and isolation

Plasmid was purified and isolated by the Wizard Miniprep kit (Promega, Madison, WI). (Figure 15) Three milliliters of culture were transferred to microfuge tubes and centrifuged at maximum speed for 30 seconds. Medium was discarded. Bacterial pellet was resuspended in alkaline lysis solution I by vigorous vortexing. Freshly prepared alkaline lysis solution II was added to each bacterial suspension. Tubes were gently inverted several times. Alkaline lysis solution II was added and thoroughly mixed with the bacterial lysate by gently inversion. Tubes were stored on ice for 3-5 minutes. Centrifugation of bacterial lysate was performed at maximum speed for 5 minutes. Supernatant is then transferred to new tubes. Equal volumes of phenol and chloroform were added. Organic and aqueous phases were mixed by vortexing followed by centrifugation at maximum speed for 2 minutes. Upper layer of aqueous phase was transferred to a new tube. Two volumes of ethanol were added at room temperature to precipitate the plasmid DNA from the supernatant. Nucleic acids were collected by centrifugation at maximum speed for 5 minutes. One milliliter of 70% ethanol was added to the pellet and a final centrifugation spin was necessary to recover the DNA. Nucleic acids were dissolved in 30 µl of double distilled water and stored at -20°C. The spectrophotometer was used to assess the purity of DNA. The plasmid miniprep procedure contains agents such as EDTA, which aids in the disruption of bacterial outer membranes. Other agents include sodium dodecyl sulfate (SDS), a detergent that denatures proteins, and NaOH, which serves to denature bacterial chromosomal DNA and cell debris that is separated by centrifugation from the plasmid DNA.

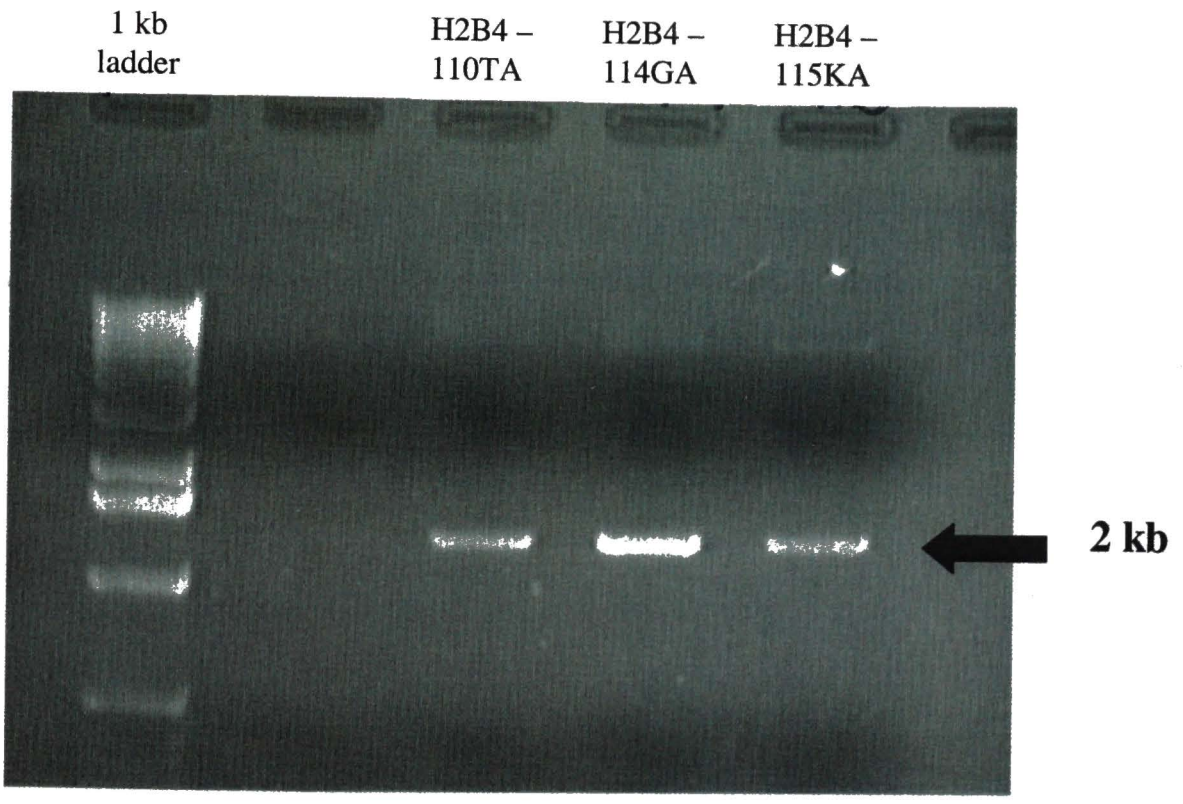
Cesium chloride density gradient purification

The mutant clones were confirmed by DNA sequencing. This final step was to confirm that the human 2B4 gene contained the desired point mutation. Once the sequence was confirmed, cesium chloride density gradient was performed. Cesium chloride is a heavy concentrated solution that forms density gradients after a few hours of high-speed ultracentrifugation. Amounts of ethidium bromide, an intercalating agent, were added to the isolated plasmid DNA. Staining with low concentrations of ethidium bromide serves as the method to detect quantities of DNA, due to its fluorescent properties and its ability to intercalate between the base pairs of double-stranded DNA. Ethidium bromide also binds to RNA, single-stranded DNA, and closed circular DNA. Thus the cesium chloride density gradient results in effectively separating plasmid and chromosomal DNA that are used in subsequent experiments. The nucleic acid extraction steps were performed with water-saturated butanol. This solvent is used to reduce the volume of dilute solutions so that the nucleic acid can be recovered easily by precipitation with ethanol.

Figure 13: Comparison of the amino acids in the variable domain of the CD2 subset members CD48, Human 2B4, CD58, and CD2. The red boxes indicate the amino acids that were mutated in the human 2B4 gene.

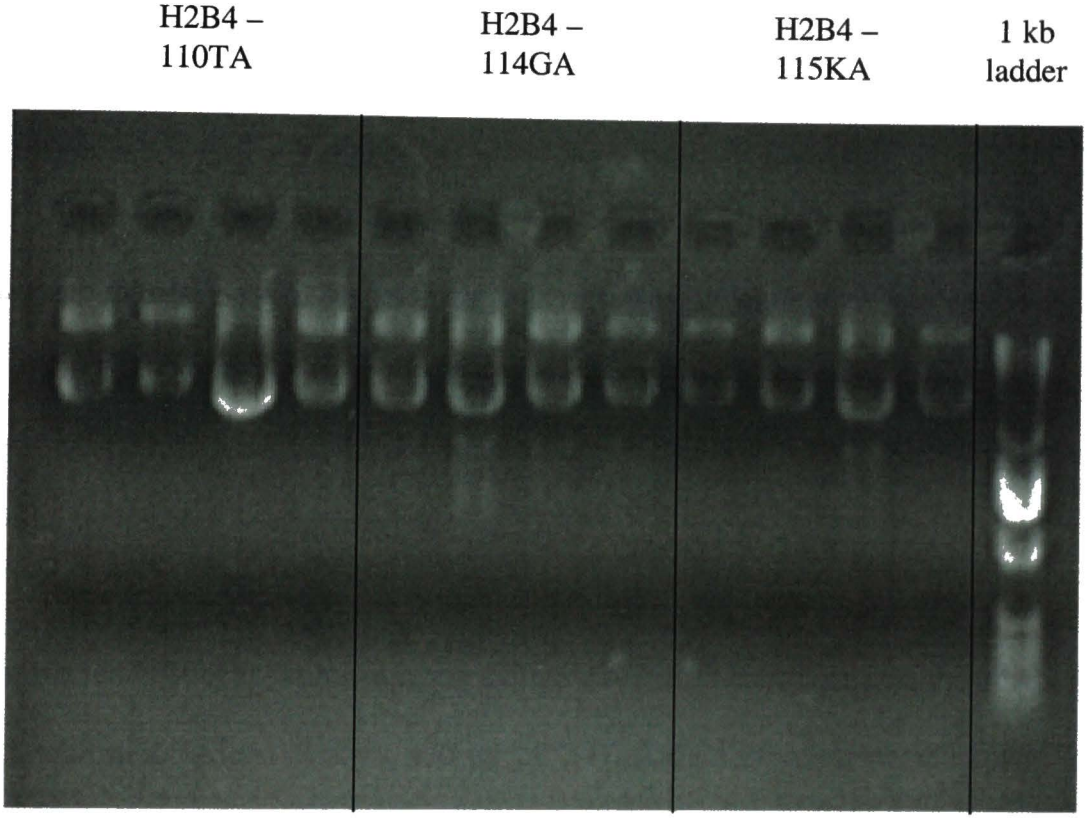

CD48 51 S L P E N Y K Q L T W . . F Y T F D Q K
H2B4 S I Q T K V D S I A W K K L L P S Q N G
CD58 . . . V P L K E V L W K K . . . Q K D K
CD2 Q M S D D I D D I K W E K T S D . K K K
CD48 I V E W D S R K S K Y F E S K . F K G R
H2B4 F H H I L K W E N G S L P S N T S N D R
CD58 V A E L E N S E F R A F S S . . F K . .
CD2 I A Q F R K E K E T F K E K D T Y K N R
CD48 R V R L D P Q S G A L Y I S K V Q K E D
H2B4 R F S F I V K N L S L L I K A A Q Q Q D
CD58 R V Y L D T V S G S L T I Y N L T S S D
CD2 . . . . L F K N G T L K I K H L K T D D
CD48 S T Y I M R V L K K T G . N E Q E W K I
H2B4 G L Y C L E V T S I S G . K V Q T A T F
CD58 D E Y E M E S P N I T D . . . . T M K F
CD2 D I Y K V S I Y D T K G K N V L E K I F 130

Figure 14: 1% agarose gel of PCR products. Mutations shown here represent human 2B4 amino acid at position 110 (H2B4110TA), 114 (H2B4114GA), and 115 (H2B4115KA).



1% agarose gel

Figure 15: 1% agarose gel of plasmid isolated from mutant clones H2B4110TA, H2B4114GA, H2B4115KA.



1% agarose gel

Stable transfections into BW cells

Mutant clones were transfected into BW cells, a strain of T cell lymphoma. These stable transfectants will be used in binding assays to determine if the mutation has disrupted the binding between 2B4 and CD48.

Binding assays by FACS analysis

The binding assays will consist of flow cytometry by FACS analysis. BW cells transfected with wild-type 2B4 and BW cells transfected with mutant 2B4 will be flowed first with mAb (anti-2B4) C1.7 and polyclonal serum. This step will confirm that transfected cells express 2B4. The second flow will be with CD48 IgG fusion protein, followed by mAb against IgG tagged with FITC.

Prior to performing the transfections with BW cells, a growth curve of untransfected BW cells with neomycin sulfate (G418) (Gibco, BRL, Gaithersberg, MD) was established. After 10 days, 400 μg of G418 killed all untransfected cells. Thus electroporation of 50 million cells with 100 μg of linearized plasmid and selection with G418 for three weeks resulted in only stable mutant transfectants surviving through G418 selection.

3.4 Results

With our 2B4 gene in vector pCI-neo as the target for the mutation, we designed primers each containing one point mutation (Table 2). Each of the amino acids was changed to alanine. Sequencing was performed at the Automated Sequencing Facility, Molecular Oncology Center, at UT Southwestern Medical Center, Dallas.

CHAPTER 4

ANALYSIS OF AMINO ACIDS THREONINE AND TYROSINE IN THE NOVEL MOTIF (TXYXXV/I) WITHIN THE CYTOPLASMIC DOMAIN OF THE HUMAN 2B4 GENE

4.1 Novel tyrosine motifs

The 2B4 cytoplasmic region contains tyrosine motifs that are potential targets for phosphorylation. Human 2B4 contains four tyrosine-based motifs (TxYxxV/I) that resemble immunoreceptor tyrosine-based motifs on its cytoplasmic tail (Figure 16). We hypothesize that amino acid, threonine plays a significant role in the phosphorylation of the tyrosine motifs because it is conserved in the novel motifs. Phosphorylation of the tyrosine embedded within the motif sequence permits binding to src homology 2 (SH2) domains, while the amino acid sequence surrounding the tyrosine controls the specificity of SH2 domain binding. SH2 domains are found in a variety of kinases, phosphatases and adaptor proteins involved in signaling cascades (28). One importance of studying the signaling of the 2B4 gene involves effector proteins. An effector called SHP-1 has been shown to inhibit the level of tyrosine phosphorylation in key signaling molecules in NK cells. Multiple sequence alignments revealed the presence of this TxYxxV/I motif not only in CD2 subfamily members but also in the cytoplasmic domains of members of the

SHP-2 substrate 1, sialic acid-binding Ig-like lectin, carcinoembyronic Ag, and leukocyte-inhibitory receptor families (29) Many other proteins such as transmembrane adaptor proteins also play a role in signal transduction. They serve as signal integrators to dictate immune responses. The cytoplasmic domains of 2B4, CS1, SLAM, CD84, and Ly9 all contain at least two instances of a novel tyrosine motif (TxYxxI/V) (1, 20, 30-37). The similarity of the novel tyrosine motifs to an immuno-tyrosine-inhibiting-motif (ITIM) suggests that it may play a role in modulating protein interactions (38, 39). In this study, I hoped to determine the significant importance of the amino acids, tyrosine and threonine, and its role in the signaling function of the human 2B4 gene, which studies have shown, to lead to the induction of NK cell functions including both cytotoxicity and upregulation of invasiveness of tumor and virally-infected cells.

4.2 pFLAG-human 2B4 clones

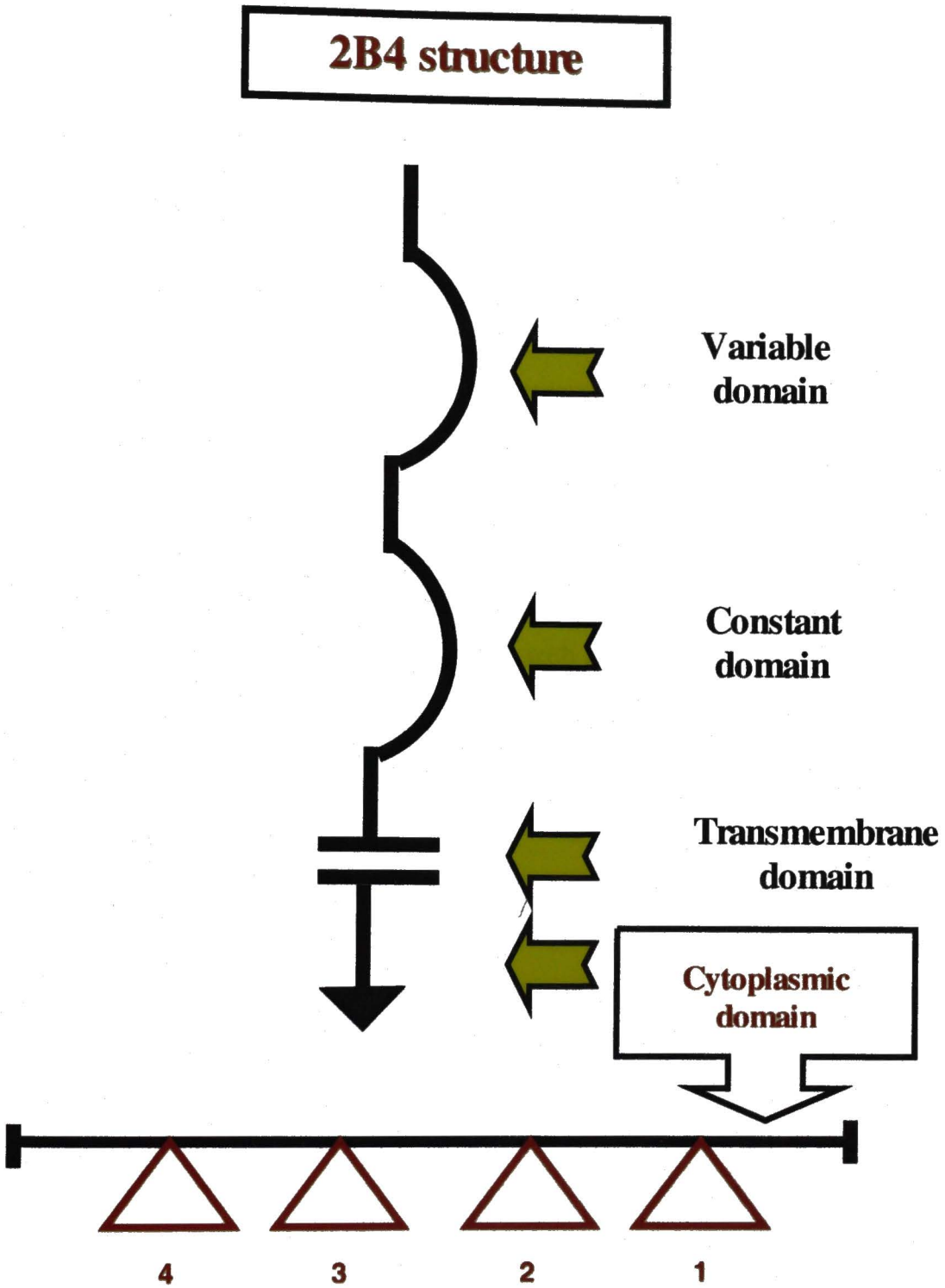
Human 2B4 receptor contains four tyrosine-based motifs (TxYxxV/I) that resemble immunoreceptor tyrosine-based motifs on its cytoplasmic tail. It has been shown that phosphorylation of the tyrosine in the motif is required for its interaction with SAP, a modulator of 2B4 signaling. We hypothesize that the, amino acid, threonine plays a significant role in 2B4 signal transduction because it is conserved in the novel motifs. Therefore, we will explore the importance of the threonine and tyrosine residues in 2B4 signaling by mutagenesis.

4.3 Material and Methods

Ligation of human 2B4 gene into pFLAG-CMV vector

My experimental strategy involves ligating the full human 2B4 gene into an expression vector. Ligation process was carried out at 16° for 12-16 hours. The ligation reaction contains 50ng/μl plasmid vector DNA, 10x DNA ligase buffer, cDNA, DNA ligase (100 units/μl), 10 mM ATP, and distilled water. The expression vector that I chose was the pFLAG-CMV-3 vector (Sigma-Aldrich, St. Louis, MO). This vector is 6.3 kb in size and is used for transient or stable expression and secretion of N-terminal FLAG®

Figure 16: Schematic representation of the 2B4 gene and the four tyrosine motifs (TxYxxV/I) located in its cytoplasmic tail.



Four tyrosine motifs in the cytoplasmic tail
(TxYxxV/I)

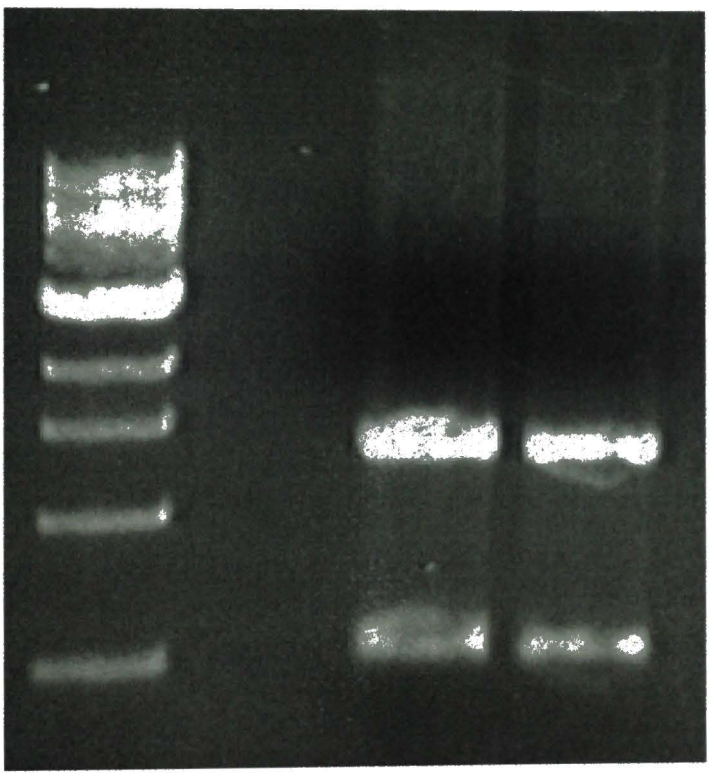
Figure 17: pFLAG ligated with human 2B4.

PCR product digested with Hind III and Xba I

and then separated on a 1% agarose gel.

Marker

PCR product digested with HIND III and Xba I



1.3 kb

0.6 kb

Figure 18: pFLAG-Human 2B4 PCR products separated on a 1% agarose gel. Numbers correspond to primers used for each reaction.

Primer nomenclature:

1- Y motif 1 T-A

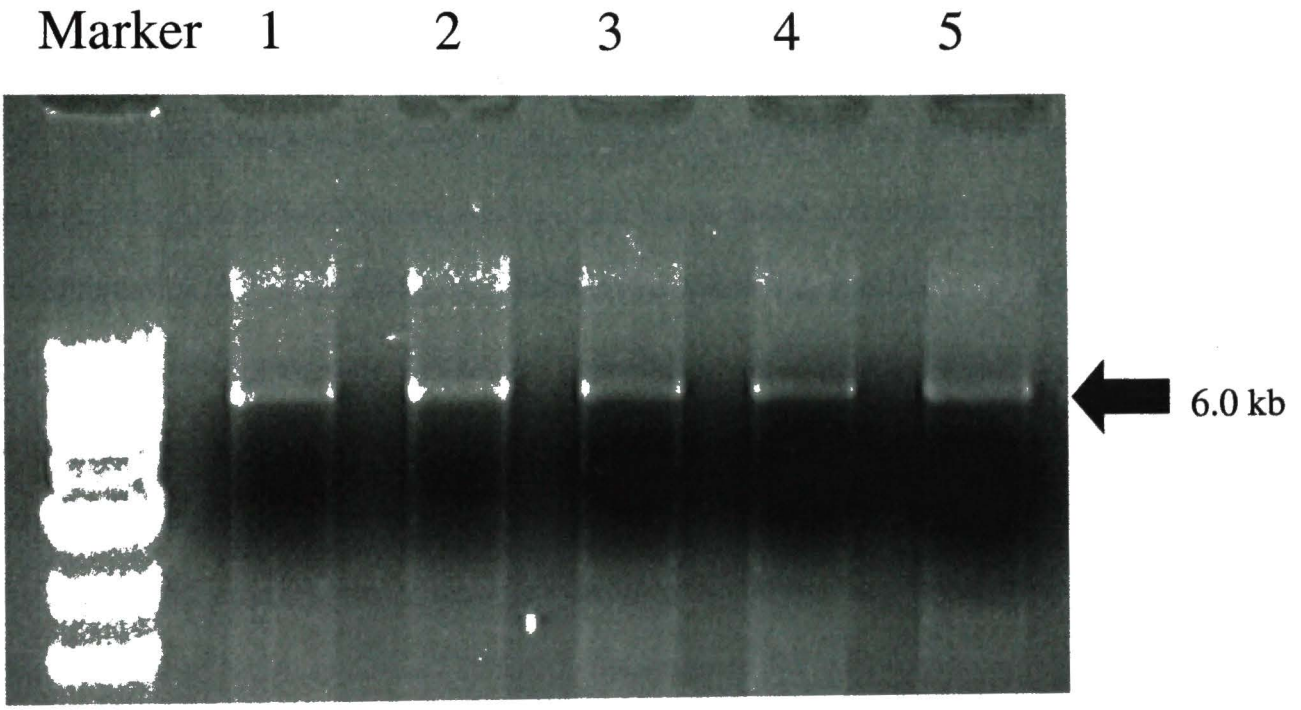
2- Y motif 1 Y-F

3- Y motif 2 stop

4- Y motif 2 stop T-A

5- Y motif 2 stop Y-F

PCR products



fusion proteins in mammalian cells. It is also a shuttle vector for *E. coli* and mammalian cells. I have designed vector primers and by PCR, amplified the human 2B4 gene into the pFLAG vector. To confirm the amplification of the 2B4 gene into the pFLAG vector, the 2kb in size PCR product was digested with HIND III and Xba I for one hour at 37°C. Restriction digestion resulted in a band corresponding to 1.3 kb which correlates to the insert size of human 2B4 gene (Figure 17).

PCR amplification of mutations

Vector primers were designed and amplified by PCR to incorporate the human 2B4 gene into the pFLAG vector. My next aim was to delete two of the four tyrosines in the cytoplasmic tail of the 2B4 gene. The last two tyrosines found in the cytoplasmic tail of the human 2B4 gene were deleted by introducing an early stop codon after the second tyrosine amino acid. The two amino acids that I would like to focus on are tyrosine and threonine. (Figure 18) Synthetic oligonucleotide encoding the desired mutation is annealed to the target region of the wild-type template DNA where it serves as a primer for initiation of DNA synthesis (Table 3). Extension of the oligonucleotide by a DNA polymerase generates a double-stranded DNA that carries the desired mutation. The mutated DNA is then inserted at the specific location of the target gene, and the mutant protein is expressed.

Immunoprecipitation and Western blot analysis

In order to detect our protein of interest, 2B4, mammalian cell cultures were cultivated in RPMI 1640 (Gibco BRL, Gaithersberg, MD) rich media. Growth factors added to the media include 1M HEPES, pH 7.8, 0.1 mM non-essential amino acids, 1

mM sodium pyruvate, 10% fetal bovine serum, antibiotic/antimitotic and sodium bicarbonate. Cells were grown one million cells per milliliter and split 1:2 every few days. Immunoprecipitation is used to detect and quantitate target antigens in mixtures of protein. The high selectivity of this technique is based on the specificity of the immunoglobulin for its ligand. The cells were lysed in buffer containing 2.5 ml 1M HEPES, pH 7.5, 1.5 ml 5M NaCl, 100 ul 0.5M EDTA, 5 ml glycerol, and 2.5 ml 20% NP-40. Following the lysis procedure, the beads containing the protein and antibody were washed in buffer containing 0.5 ml HEPES, 0.3 ml 5M NaCl, 20 ul 0.5M EDTA, 1 ml glycerol, and 0.5 ml 20% NP-40. The cleared lysate is rotated overnight with Protein G plus A agarose beads (Oncogene) and monoclonal antibody clone C1.7.1. The isotype of this monoclonal antibody is IgG1 K (mouse) (Immunotech, Marseille, France). The analysis of the protein is followed by the detection of the target protein after its dissociation from the antibody.

An SDS-polyacrylamide gel electrophoresis gradient gel was set up to separate proteins according to size. Electrophoresis is performed in the presence of the detergent sodium dodecyl sulfate (SDS), a strong protein denaturant. When SDS binds to protein, it causes the proteins to unfold. In the presence of a reducing agent such as 2-mercaptoethanol, the disulfide bonds between cysteine residues, which serve to stabilize secondary structures, are broken. An SDS gel contains distilled water, 40% acrylamide mix, 1.5 M Tris (pH 8.8), 10% SDS, 10% ammonium persulfate, and TEMED for the separating portion of the gel. The 5% stacking gel which is pour onto of the separating gel contains distilled water, 40% acylamide mix, 1.0 M Tris (pH 6.8), 10% SDS, 10%

ammonium persulfate, and TEMED. The stacking gel contains buffers of lower ionic strength and lower pH than the separation gel. Ammonium persulfate is used to provide the free radical molecules that ultimately drive the polymerization process. While TEMED (N,N,N',N'-tetramethylethylenediamine) accelerates the polymerization of acrylamide and bisacrylamide. The 1X SDS gel-loading buffer is made of 50 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% bromophenol blue, and 10% (v/v) glycerol. The kalideoscope marker serves as size standards to determine the molecular weight of the protein of interest. The MultiMark Multi-Colored Standard was purchased from Invitrogen, Carlsbad, CA. The migration of the proteins is a result of the amount of SDS bound and thus the molecular weight of the protein. Generally, the size of the pores in the gel allow smaller molecules to migrate more rapidly because they are better able to penetrate the gel. Thus a 10% gel was used to separate larger molecules such as antibodies that may be at 250-150 kDa, and smaller molecules such as 2B4 protein that weigh 66 kDa. The separated components are transferred from the gel to a solid membrane and then probed with specific antibodies that react with antigenic epitopes displayed by the target protein attached to the membrane. When the proteins are immobilized on the membrane, antibodies are used to detect the transferred protein. The membrane is submerged in a blocking solution that prevents the antibody from reacting nonspecifically with the membrane. The primary antibody used was the C1.7 mAb, which binds to the specific protein of interest. A second antibody recognizes the primary antibody and is conjugated to an enyzme. Once a substrate is added the coupled enzyme

yields a colored, precipitated product at the site of the protein of interest on the membrane.

The enzyme-linked affinity purified antibody was combined with a sensitive chemiluminescent substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) resulting in a signal that is measured on X-ray hyperfilm.

Transient transfections into BW cells

Establishing transient transfections is a method used to transfer DNA into eukaryotic cells. Recombinant DNA is introduced into a recipient cell line to obtain a temporary, but high levels of expression of the target gene. The transfected DNA does not become integrated into the host chromosome, and expression is usually lost after a few days.

4.4 Preliminary results

Each of the mutated clones was confirmed by DNA sequencing at the Automated Sequencing Facility, Molecular Oncology Center, UT Southwestern Medical Center, Dallas. Once the mutations are confirmed the mutated clones will be transiently transfected into BW cells. Preliminary results of an immunoprecipitation and western blot with BW cells stably transfected with wild-type human 2B4 (BW + h2B4) gene is shown in figure 19. The larger band at 66 kDa represents the human 2B4 gene. Lower bands may be products of degradation. Bands at the 250 kDa size are representative of the antibody and agarose beads sizes. Subsequent experiments will be to perform

immunoprecipitation and western blots on the mutated clones and killing assays to analyze cytolytic capability of the transfectants.

Table 3: Synthetic oligonucleotide primers containing tyrosine and threonine mutations

*Primer nomenclature	Forward or Reverse Primer	5' to 3' orientation
Y motif 1 T-A	Forward	ccc-aag-gaa-ttt-tta-gca-att-tac-gaa-gat-gtc-aag-gat-c
Y motif 1 T-A	Reverse	gat-cct-tga-cat-ctt-cgt-aaa-ttg-cta-aaa-att-cct-tgg-g
Y motif 1 Y-F	Forward	ccc-aag-gaa-ttt-tta-aca-att-ttc-gaa-gat-gtc-aag-gat-c
Y motif 1 Y-F	Reverse	gat-cct-tga-cat-ctt-cga-aaa-ttg-tta-aaa-att-cct-tgg-g
Y motif 2 stop	Forward	ga-ggg-ggg-agc-acc-atc-tac-tct-atg-atc-cag-tcc-tag-tct-tct-gct-c
Y motif 2 stop	Reverse	g-agc-aga-aga-cta-gga-ctg-gat-cat-aga-gta-gat-ggt-gct-ccc-ccc-tc
Y motif 2 stop T-A	Forward	ga-ggg-ggg-agc-gcc-atc-tac-tct-atg-atc-cag-tcc-tag-tct-tct-gct-c
Y motif 2 stop T-A	Reverse	g-agc-aga-aga-cta-gga-ctg-gat-cat-aga-gta-gat-ggc-gct-ccc-ccc-tc
Y motif 2 stop Y-F	Forward	ga-ggg-ggg-agc-acc-atc-ttc-tct-atg-atc-cag-tcc-tag-tct-tct-gct-c
Y motif 2 stop Y-F	Reverse	g-agc-aga-aga-cta-gga-ctg-gat-cat-aga-gaa-gat-ggt-gct-ccc-ccc-tc

*Primer nomenclature:

Y motif 1 T-A = Tyrosine motif one, changing threonine to alanine

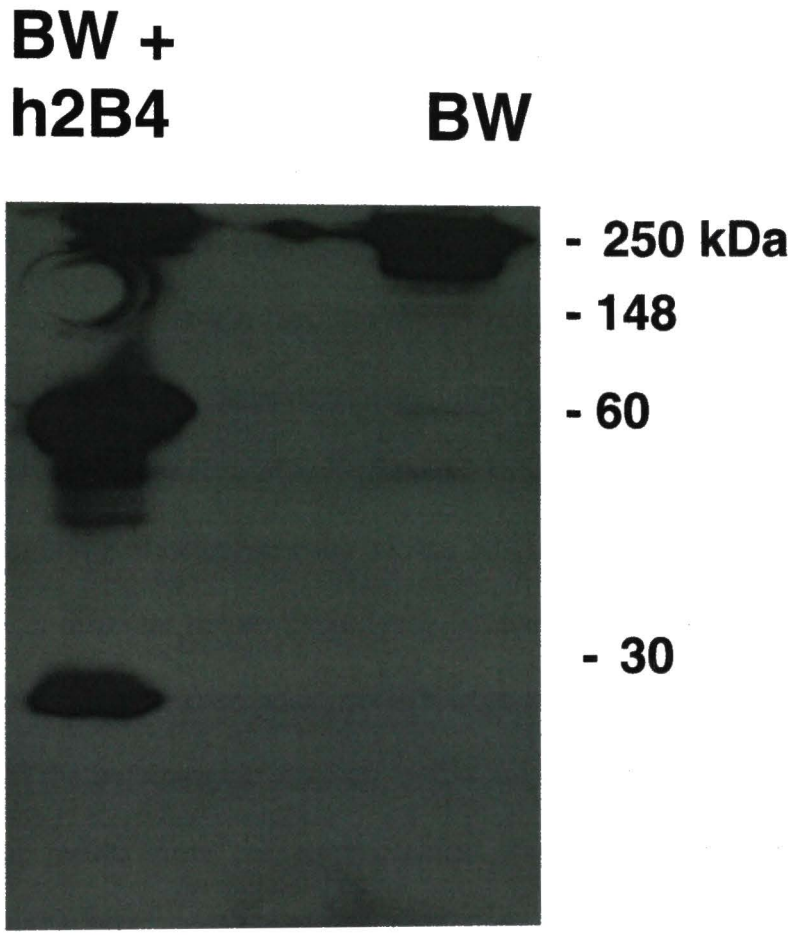
Y motif 1 Y-F = Tyrosine motif one, changing tyrosine to phenylalanine

Y motif 2 stop = Tyrosine motif two, introducing an early stop codon after this motif

Y motif 2 stop T-A = Tyrosine motif two, introducing an early stop codon after this motif and changing threonine to alanine

Y motif 2 stop Y-F = Tyrosine motif two, introducing an early stop codon after this motif and changing tyrosine to phenylalanine

Figure 19: Immunoprecipitation with anti-2B4 antibody, followed by western blot analysis. Samples were fractionated on an 8% SDS-PAGE gel. The first lane is BW cells stably transfected with human 2B4 gene, and the second lane is BW cells alone. Marker sizes are indicated in kDa (kilodaltons).


BW +
h2B4
BW
- 250 kDa
- 148
- 60
- 30

SUMMARY AND SIGNIFICANCE

Earlier studies have shown that anti-2B4 mAb specifically recognizes the epitopes expressed on NK cells derived from mice strains C57BL/6 and C59/J. However, our lab has shown, by northern analysis, that 2B4 transcripts are expressed in all mouse strains. Northern blot analysis has shown at least three transcripts for 2B4 in adherent lymphokine-activated killer cells of several mouse strains...three mRNA are the products of differential splicing of heterogeneous nuclear RNA (31). Failure of anti-2B4 mAb to recognize 2B4 in different mouse strains may be due to the polymorphism of the 2B4 gene. The molecular characterization of 2B4 from mice strains BALB/c, 129/Svj and A.CA show that the polymorphic residues in 2B4 are located in the immunoglobulin V-domain. Our results from the polymorphism studies indicate that the nucleotide sequence of A.CA-2B4 clone is highly homologous to C57BL/6-2B4, whereas the nucleotide sequence of 129/Svj-2B4 clone is highly homologous to BALB/c-2B4. In addition, the peptide homology between the strains showed that C57BL/6 and A.CA have 99% similarity and BALB/c and 129/Svj have 100% similarity.

2B4 was chosen to bind CD48 by molecular binding assays and surface plasmon resonance (10, 13). Importantly, 2B4 bound purified soluble CD48 with six to ninefold higher affinity than CD2, which was previously proposed to be a ligand of CD48 (40).

Molecular binding studies [showing] that 2B4 can interact with CD48 with a relatively high affinity, suggesting that 2B4 may trigger NK cell cytotoxicity by interacting with CD48 (19). NK cell-target cell recognition is highly complex and involves multiple interactions between complementary sets of molecules on NK cells as well as target cells (19). To determine the physical interaction between 2B4 and its counter receptor, CD48, specific amino acids must be identified on both receptors that are responsible for their binding. By mutating these amino acids to a neutral amino acid, such as alanine, disruption of their binding can lead to a better understanding of how NK cell receptor, 2B4, is able to recognize its ligand and enhance cytotoxicity. Completion of the twelve mutant clones were confirmed by DNA sequencing. Subsequent experiments will be to perform binding assays with each of the mutant clones and determine if single or multiple mutations will cause a disruption between receptor and ligand interaction.

The molecular basis of the stimulatory capacity of 2B4 is still unknown. 2B4 may mediate a unique pattern of signal transduction, as suggested by the presence in its cytoplasmic tail of four tyrosine-based motifs that resemble ITIM motifs (19). A commonality of NK-cell receptors that transduce inhibitory signals is the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tails (41, 42). Similar motifs have been previously shown in SLAM, a 2B4 homolog, and have been shown to bind protein tyrosine phosphatase SHP-2 and the SH2-containing adaptor molecule SAP(30, 43). SLAM (CD150), signaling lymphocyte activation molecule, is a member of the CD2 subset and is expressed on T cells and B cells and regulates T cell activation and production of Ig by B cells (44),(30). Both SLAM and 2B4 appear to

produce several splice variants from the same gene (31, 45). SLAM has four different transcripts including a cytoplasmic, a soluble, and two membrane forms...the difference in the membrane form is due to splicing within the cytoplasmic tail; this leads to the loss of the two distal of the four tyrosine motifs in the longer form to produce the shorter form (1). The extracellular domains of murine short and long isoforms are identical while the shorter form is missing the two distal tyrosines at the C terminal. Thus the murine 2B4 follows the same splicing pattern as that seen in SLAM molecules (1). Defective signaling via 2B4 and SLAM may contribute to the pathogenesis of X-linked lymphoproliferative disease due to mutations in SAP, SLAM-associated protein (31).

Natural killer cells express receptors that can either activate or inhibit their effector functions (1). In recent years a new family of NK cell–expressed genes have been identified as killer cell inhibitory receptors (KIR). Individual KIR can selectively bind various HLA class I allotypes and consequently transduce inhibitory signals that block NK cell lysis of ligand-bearing target cells. A distinct subset of related and linked genes express truncated versions of KIR that are otherwise highly homologous in amino acid sequence...these receptors appear to transmit stimulatory signals into NK cells and have been termed killer cell activating receptors (KAR) (46). These killer cell Ig-like receptors (KIRs) are a family of HLA class I-specific receptors that can inhibit or activate NK cells and a subset of T lymphocytes. Inhibitory KIRs prevent activation of NK cells upon binding to HLA class I molecules expressed on the target cells. The mechanism of KIR-mediated inhibition involves recruitment of the tyrosine phosphatase SHP-1 (47) (48). Many lymphocyte signaling pathways are regulated by protein tyrosyl

phosphorylation, which is controlled by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) (49). Studies have shown that crosslinking of 2B4 on NK cells results in its rapid tyrosine phosphorylation, implying that this initial step in 2B4 signaling does not require colligation of other receptors. Ligation of 2B4 in the context of an NK cell-target interaction leads to 2B4 tyrosine phosphorylation, target cell lysis, and IFN-γ release (50). In another recent report, studies showed that a single NK cell can be in contact with a sensitive and a protected target cell at the same time and kill the sensitive target while sparing the protected one (51). By introducing these specific amino amid mutations into the cytoplasmic domain of the human 2B4 receptor, we hope to determine the role of threonine in tyrosine phosphorylation. Further progress in the molecular characterization of 2B4 and its interactions with other members of the CD2 subset will provide a better understanding of their role in our immune system.

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