CHARACTERIZATION OF A MURINE ACTIVATED LYMPHOCYTE ANTIGEN (MALA-2): THE MURINE HOMOLOGUE OF HUMAN INTERCELLULAR ADHESION MOLECULE 1 (ICAM-1)

by

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ABSTRACT

A previously characterized rat monoclonal antibody, YN1/1.7, recognizes an antigen termed murine activated lymphocyte antigen (MALA-2). MALA-2 is a 95-100 kD monomeric glycoprotein and is expressed on mitogen activated spleen cells but is present at low levels on thymocytes, fibroblasts, and lymph node cells. Interestingly, YN1/1.7, inhibits mixed lymphocyte reaction (MLR) suggesting that MALA-2 is directly involved in lymphocyte activation. In this research project, the gene encoding MALA-2 was characterized by cDNA cloning, genomic cloning, and analysis of an assumed alternatively spliced mRNA. Two cDNA clones were isolated from an NS-1 cDNA library using oligonucleotide probes constructed from amino acid sequences of peptides derived by tryptic cleavage. The two cDNAs, K4-1.1 and K3-1.1, both encode MALA-2 but differ in their 5' untranslated sequences and those encoding the leader and N-terminal nine amino acids. MALA-2 is a transmembrane glycoprotein with five immunoglobulin-like domains. It displays homology with the human intercellular adhesion molecule 1 (ICAM-1), as well as human ICAM-2, human ICAM-3, and murine ICAM-2. Screening of genomic libraries yielded a partial genomic clone (4.0 kb), containing five 3' exons and a pseudo exon. The five exons are common to both cDNAs and have consensus splice donor and acceptor sequences. The pseudo exon lacks these splice donor and acceptor sequences. The exons encoding the 5' region of K4-1.1 were not isolated, but using data from Southern blot analyses a proposed map of the whole gene was constructed. Two 4.0 kb *Bam HI* fragments seem to contain all of the exons, with the 5' region probably consisting of two exons, and being located at least 6.0 kb upstream of the five 3' exons. The K3-1.1 cDNA has not been reported elsewhere, thus it was further analysed for its authenticity. The 5' region unique to K3-1.1 did not seem to be linked to the 5' region of K4-1.1, and Northern blot analysis failed to detect a 3.0 kb message corresponding to the K3-1.1 cDNA. However, PCR analysis using primers spanning the common junction between the two cDNAs detected a fragment. These data suggest that a K3-1.1 transcript can exist but may be expressed at a very low level.

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LIST OF ABBREVIATIONS

Ab	antibody
AC	adenylate cyclase
AMP	ampicillin
APC	antigen presenting cell
ARAM	antigen recognition activation motif
β-ME	2-mercaptoethanol
bp	base pair
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
Con A	concanavalin A
CTL	cytotoxic T lymphocyte
DAG	diacylglycerol
DEAE	diethylaminoethyl
dH_2O	distilled water
DMEM	Dulbecco's modified minimum essential media
DNA	deoxyribose nucleic acid
DTT	dithiothreitol
E-rosettes	erythrocyte rosettes
EC	endothelial cells
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGF-R	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
EtBr	ethidium bromide
FACS	fluorescence activated cell sorter
G proteins	guanine nucleotide binding regulatory protein
G	gauge
g	grams
GAP	GTPase activating protein
GαRIg	goat anti-rat immunoglobulin
GPI	glycosyl-phosphatidylinositol
GTE	glucose/Tris/EDTA solution
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine
HEV	high endothelial venules
Hfl	high frequency lysogeny
hr	hour
IAA	isoamyl alcohol
ICAM	intercellular adhesion molecule
IFNγ	interferon gamma
Ig	immunoglobulin
IL-2R	interleukin 2 receptor
IMF	integrin modulating factor
IP_3	inositol 1, 4, 5-trisphosphate
kb	kilobases
kD	kiloDalton
LAD	leukocyte adhesion deficiency
Ldlr	LDL receptor
LFA	lymphocyte function-associated
LPS	bacterial lipopolysaccharide
MAb	monoclonal antibody
MAG	myelin-associated glycoprotein
MALA-2	murine activation lymphocyte antigen

MAP-K	mitogen activated protein kinase
MαRIg	mouse anti-rat immunoglobulin
MHC	major histocompatibility complex
min	minute
MLR	mixed lymphocyte response
MOI	multiplicity of infection
mRNA	messenger ribose nucleic acid
NCAM	neural cell adhesion molecule
NK	natural killer
NP40	nonidet P-40
nt	nucleotide
O/N	overnight
ORF	open reading frame
PAF	platelet activating protein
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
PHA	phytohaemagglutinin
PI	phosphatidylinositol 4, 5 bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLN	peripheral lymph nodes
PM	plasma membrane
PMA	phorbol myristic acetate
PMN	polymononuclear cell
PP	Peyer's patch
PTK	protein tyrosine kinase
R.T.	room temperature
RBC	red blood cell
RGD	Arg-Gly-Asp
RGE	Arg-Gly-Glu
RNA	ribose nucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second
SH2 or SH3	Src Homology region 2 or 3
SLe	sialyl Lewis
SRBC	sheep red blood cell
SSC	saline sodium citrate buffer
SSPE	saline sodium phosphate EDTA buffer
TAE	Tris acetate EDTA buffer
TAP	T cell activating protein
TBE	Tris borate EDTA buffer
Tc	T cytolytic cell
TCR	T cell receptor
TE	Tris EDTA buffer
Th	T helper cell
TNE	Tris/NaCl/EDTA solution
TNFα	tumour necrosis factor alpha
TR	transferrin receptor
Tris	tris(hydroxymethyl)aminomethane
U	units
VCAM	vascular cell adhesion molecule
VLA	very late antigen

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PREFACE

When this thesis project was started, little was known about the molecules and mechanisms involved in several lymphocyte functions. For example, the molecules involved in lymphocyte activation were just beginning to be identified and their mode of action understood. The T and B cell receptors had been characterized but all of the components had not been identified, and the signal transduction pathways were not totally elucidated. Costimulatory signals provided by accessory cells were also known to be essential for activation, however the molecules responsible for this effect had not been significantly characterized. Additionally, the proteins involved in lymphocyte adhesion and trafficking were just being identified. The interaction between cluster of differentiation 2 (CD2) and lymphocyte function-associated antigen 3 (LFA-3) was known to be an important adhesion system, and with the cloning of human ICAM-1 in 1988, the LFA-1 and ICAM-1 system quickly became the focus of intensive work in the adhesion field. In the next few years, human ICAM-2, ICAM-3, and murine ICAM-2 were characterized and also studied for their role in lymphocyte adhesion. The selectin family of molecules was also identified and their importance in lymphocyte trafficking determined.

The main goal of this work was to characterize the gene encoding the antigen recognized by a rat monoclonal antibody, YN1/1.7. Preliminary studies indicated the antigen, termed MALA-2, was involved in lymphocyte activation. Thus, the antigen was studied by cDNA cloning, genomic cloning, and the analysis of a unique cDNA assumed to represent an alternatively spliced messenger RNA (mRNA).

Since MALA-2 seemed to be involved in lymphocyte activation, several molecules and their role in lymphocyte activation, as well as adhesion, and trafficking are discussed in the first chapter of this thesis. This introduction was designed to give a detailed review of the field at hand and is meant to acquaint the reader with the evergrowing complexity of molecules on the lymphocyte cell surface and their function in cell responses.

CHAPTER 1

INTRODUCTION

1.1 GENERAL CONCEPTS

Within the immune system of vertebrates, lymphocytes patrol the body, recognizing and destroying external invaders. They mediate this protection from infection through interactions of recognition elements in their plasma membrane with the environment. For example, lymphocytes adhere to and migrate through endothelial vessel walls to enter the tissues via specific adhesion proteins, and are stimulated to proliferate and differentiate into effector cells via specialized antigen receptors. These cell surface proteins serve to convert signals from soluble or cell-bound ligands into an appropriate cellular response. This chapter discusses the cell surface proteins expressed on lymphocytes and their role in lymphocyte activation and adhesion .

1.1.1 Cell Surface Proteins

The plasma membrane (PM) of eukaryotic cells has several functional roles, the foremost being that it acts as a barrier to the external environment and monitors the movement of macromolecules into the cell. It is made up of a bilayer of phospholipids with the hydrophobic fatty acid tails oriented together to exclude water molecules, and the polar heads groups oriented peripherally. Water and small hydrophobic molecules permeate through the PM easily, but the transport of ions, metabolites, and proteins is mediated by proteins intrinsic to the membrane.

Intrinsic proteins have regions which are hydrophobic (transmembrane domains) and hydrophilic (extracellular and cytoplasmic domains). The transmembrane region of a protein interacts with the hydrophobic regions of phospholipids thereby passing through the PM, while either end of the protein may be exposed to the aqueous environment of the exterior of the cell (extracellular) or interior of the cell (cytoplasmic). These intrinsic proteins can act as receptors for stimuli from the environment, by binding ligands and transducing signals across the PM to elicit a response in the cell. For example, growth factors interact with their receptors at the cell surface and stimulate the cell to proliferate

and/or differentiate. Additionally, other intrinsic proteins interact with the environment or other cells through adhesive interactions possibly changing the cell's shape or locomotory pattern.

The interaction of surface receptors with their respective ligands or counter-receptors results in the transmission of information through a signal transduction pathway leading to a response in the cell. The receptor itself can initiate a signalling cascade if it possesses an intrinsic tyrosine kinase activity such as the epidermal growth factor receptor (EGF-R) (1), or if it is closely associated with a tyrosine kinase such as the interleukin 2 receptor (IL-2R) (2). Alternatively, GTP binding proteins (G proteins) associated with the receptor can alter the levels of secondary messengers like cAMP, or the activity of other proteins such as phospholipase C resulting in the activation of protein kinase A (PKA) or protein kinase C (PKC), respectively (3). There can also be crosstalk between various receptors and signal transduction pathways allowing the integration of information from different ligands and the fine tuning of the expression of specific genes, ultimately, determining the appropriate response in the cell.

Interactions between cells and the extracellular matrix (ECM) are necessary for tissue maintenance, cell locomotion, and cell communication. The extracellular surface of cells is a complex array of glycoproteins, proteogylcans, and glycolipids. The carbohydrate determinants of these large molecules interact with each other to form the glycocalyx (4). The oligosaccharide side chains of glycoproteins and glycolipids can be complex and sialic acid residues are usually found at the ends of the carbohydrate side chains resulting in a net negative surface charge on the eukaryotic cells. Activated T cells will interact with antigen presenting cells (APC) despite this repulsive charge, however, other cells are inhibited from interacting. For example, erythrocyte rosetting (E-rosetting) is greatly increased when the sialic acid content within the glycocalyx is reduced. Treatment of cells with neuraminidase or de novo synthesis of cell surface proteins will reduce the sialic acid content on cells and allow them to interact more closely (5).

Membrane receptors can bind to identical receptors on a different cell. Examples of these homophilic interactions are the cadherins (a family of adhesion molecules) which bind to each other specifically (E-cadherin binds E-cadherin) (6), and neural cell adhesion molecule (NCAM) which mediates the adhesion between nerve cells (7). These molecules are primarily involved in controlling stable interactions between cells maintaining tissue architecture. Conversely, the complementary interaction between cells involving different molecules is termed heterophilic. These molecules interact with a specific counterpart and mediate more transient adhesion observed during cell communication and cell locomotion. Examples of heterophilic interactions involve the members of the selectin, integrin, and immunoglobulin (Ig) superfamilies. Specifically, members of the Ig family can interact with each other to mediate cell activation. Such is the case with the T cell receptor (TCR) on T lymphocytes which recognizes antigen in association with the major histocompatibility complex (MHC) on the APC (8). Another example of heterophilic interaction is between CD2 on lymphocytes and LFA-3 on sheep red blood cells (SRBC) in cell adhesion (9) . There are also examples of integrins interacting with Igs, such as between LFA-1 and ICAM-1 (10).

Most proteins associated with the PM have a transmembrane domain. However, proteins can also be linked to the PM via a glycosyl-phosphatidylinositol (GPI) moiety (11,12). These proteins have much greater lateral mobility through the membrane as compared with transmembrane domain containing proteins. Thy-1 (13), T cell activating protein (TAP) (14), and LFA-3 (15) are all examples of GPI linked receptors.

The identification and characterization of cell surface proteins has been aided greatly in recent years with the advent of monoclonal antibody (MAb) technology. This technique involves the fusion of B lymphocytes from the spleen of an immunized animal with immortal B lymphocyte myeloma tumor cells. Only those hybrid cells which have the characteristics of producing a single antibody (Ab) and proliferate indefinitely in culture, will grow under selective culture conditions. These hybridomas are propagated as individual clones and each stably produces a single MAb (16).

MAbs have a uniform specificity, can be produced against any antigen, and can be used as a specific probe to localize the antigen to a tissue or to purify the antigen to study its structure and function.

1.2 LYMPHOCYTE ACTIVATION

The purpose of the immune system is to mount an immune response against any type of invading antigen. This is carried out by several specialized cells, in concert with the complement system, and various organs of the immune system. Granulocytes and macrophages are cells derived from the myeloid lineage, and these degrade and phagocytose foreign material. Lymphocytes are more specialized and become activated through specific cell surface proteins when they encounter antigen. Upon activation, they proliferate and differentiate into effector cells. B lymphocytes differentiate into plasma cells for the production of antibodies, and T lymphocytes develop into helper or cytolytic effector cells. Helper T cells (Th) are characteristically $CD4^+/CD8^-$ (17) and interact with B cells to enhance their proliferation and differentiation into antibody-secreting plasma cells, as well as inducing the stimulation of other T cells by the release of cytokines. Cytolytic T cells (Tc) are characteristically $CD4^-/CD8^+$ (17) and act to kill target cells expressing foreign antigens such as virus infected cells or cells of an allograft.

1.2.1 Antigen Specific Activation

Specialized receptors on T and B lymphocytes mediate recognition of antigen. B lymphocytes express Ig molecules on their cell surface which act as receptors for antigen, but these proteins do not have significant cytoplasmic domains to transduce a signal into the cell. Specific heterodimers, termed $\alpha\beta$ or $\alpha\gamma$, have been found to associate with IgM and IgD molecules via the Ig transmembrane domains and it is postulated that this complex of proteins recognizes antigen and transmits a signal to the B cell (18). B cells can recognize antigen in soluble form (19,20), and a tyrosine kinase, lyn, has been implicated in transducing the signal from the antigen/Ig recognition (21). Upon activation, B cells differentiate into plasma cells which are specialized for the production and secretion of

antigen-specific antibodies. Memory B cells are also produced to arm the host against later invasions and to increase the variety of Ig molecules produced. T cells will only recognize antigen in the context of the products of the MHC (8). They have an antigen receptor, TCR, which is a complex of several proteins and after binding antigen/MHC, specific signal transduction pathways tell the T cell to proliferate.

The TCR is a hetero-oligomeric structure composed of six or seven different subunits (22). These proteins can be divided into three distinct subgroups (23). The first subgroup is the Ig-like clonotypic chains, which are responsible for the antigen specificity of the T cell and these exist as heterodimers. Two major forms of receptor heterodimers have been characterized; they are αβ, primarily found on mature T cells, and γδ, which are predominant on epithelial T cells (Table I) (24,25). These heterodimers are noncovalently associated with the monomorphic peptides of CD3 and require association with CD3 for surface expression (26). The CD3 proteins comprise the second subgroup of the receptor, consisting of three subunits. Two subunits are glycoproteins (γ and δ), and one subunit is nonglycosylated (ε); all are encoded by three homologous clustered genes. The CD3 chains are likely to exist in the TCR receptor as two subcomplexes of CD3 γ–CD3 ε and CD3 δ–CD3 ε (27,28). The last subgroup is the ζ family dimers. This family is distinct from CD3 genetically, structurally, and in its range of cellular expression. There are three proteins encoded by two genes; they are ζ, its alternatively spliced form, η, and the γ chain of the multisubunit Ig E FcR receptors. TCR/CD3 complexes must contain a ζ dimer, and three such dimers have been characterized ζ–ζ, ζ–η, and ζ–Fcε γ (29). There is mixing and matching of the three chains into multiple subunits associated with the TCR/CD3 complex creating a diversity of TCR receptor types on a given T cell (such as αβ–γε–δε–ζζ or αβ–γε–δε–ζη).

In vitro studies of T cell clones (helper/cytotoxic) have shown that at least two signals are required to induce T cell activation via TCR/CD3 . The first signal involves the perturbation of the TCR/CD3 complex with antigen/MHC or MAb such that the TCR/CD3 is crosslinked on the cell surface. The second signal is provided by accessory cells and specific

Table I[@]

ANTIGEN SPECIFIC ACTIVATION ANTIGENS

Antigen		mouse	human	Distribution
TCR-αβ	α	44-55 kD	45-60 kD	-most mature T cells
	β	40-55 kD	40-50 kD	
TCR-γδ	γ	45-60 kD	45-60 kD	-epithelial T cells
	δ	40-60 kD	40-60 kD	
CD3	γ	21 kD	25-28 kD	-most T cells
	δ	28 kD	20 kD	
	ε	25 kD	20 kD	
	ς	2x16 kD	2x16 kD	
	η(p21)	21 kD	?	
	ω(TRAP)*	28 kD	28 kD	

[@]taken from Abbas et al.1991(24),Chan 1988(25).
*TRAP-TCR associated protein, not on cell surface.

costimulatory molecules such as B7/BB1 expressed on their surface (30). These signals can be replaced by Ca^{++} ionophores and phorbol esters which act to bypass the TCR receptor complex and activate PKC directly (31). Both signals are required to cause full cell proliferation sustained by IL-2 secretion.

The recognition of antigen in association with MHC by the T cell is translated into biochemical signals that control cellular responses. The biochemical pathways of TCR perturbation are still being delineated; however, the early events of signalling have been worked out using antigen presented with MHC to T cell clones or antigen-specific T cell hybridomas, and MAbs which mimic the changes induced by physiologic ligand. Primary events observed early in the T cell activation cascade are the immediate rise in cytoplasmic Ca^{++} levels (32), and the enhancement of the turnover of phosphatidylinositides (PI) (33). Engagement of the TCR receptor leads to the breakdown of phosphatidylinositol 4, 5 bisphosphate creating secondary messengers inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG) (34). IP_3 is implicated in the release of intracellular Ca^{++} stores (35), and Ca^{++} influx from the environment (36), and DAG is capable of activating the serine/threonine kinase, PKC (37). PKC phosphorylates several substrates including CD3 γ and mitogen activated protein kinase (MAP-K) (38). However, MAP-K is also phosphorylated at tyrosine residues implying the activation of a second kinase pathway (39,40). The hydrolysis of PI occurs within 30 sec of the triggering of the TCR complex (23), and is presumed to be mediated by G proteins and phosphatidylinositol specific-phospholipase C (PLC) (41,42). G proteins are trimeric molecules ($\alpha\beta\gamma$) which have intrinsic GTPase activity, converting GTP to GDP (3). They are usually associated with membrane receptors and serve to amplify the signal from the activated receptor. Upon stimulation, the α subunit releases GDP and binds GTP. It also dissociates from the $\beta\gamma$ complex but remains associated with the PM. Specific α subunits activate or inhibit PLC or adenylate cyclase (AC). PLC β isoform can be stimulated through G proteins and it in turn can initiate PI breakdown. GDP has been

found to partially block TCR-mediated signalling in permeabilized and PHA stimulated blasts and recently, a 55 kD novel G protein was found to associate with TCR (43).

As a result of TCR engagement, at least two kinase pathways are activated and several TCR/CD3 subunits are phosphorylated (23). CD3 γ is phosphorylated on serine residues, and this phosphorylation can be mimicked by the addition of phorbol esters or DAG to cells, and thus can be attributed to PKC. Raf-1, a serine/threonine kinase, is also activated within 5 min of cellular stimulation and this activation is PKC dependent (39). However, CD3 ζ chain is phosphorylated on tyrosine residues and this cannot be mimicked by the addition of phorbol esters or DAG. Furthermore, PLC γ is tyrosine phosphorylated and this precedes the PI breakdown. Therefore, an additional protein tyrosine kinase (PTK) pathway is activated that cannot be explained by the stimulation of PI turnover. The primary sequences of the subunits of the TCR/CD3 complex do not show any homology to recognizable tyrosine kinases so the TCR must be coupled either directly or indirectly to a nonreceptor tyrosine kinase. A T cell specific member of the src tyrosine kinase family, lck, is noncovalently associated with the cytoplasmic domain of either CD4 or CD8 (23), possibly acting to couple tyrosine kinase activity with the TCR. CD4 and CD8 are known to diffuse laterally through the membrane to associate with the TCR/CD3 complex, and bind their respective MHC (CD4 /MHC II; CD8;/MHC I) (44,45,46). The binding of TCR to antigen/MHC brings the TCR in close proximity of CD4 or CD8, allowing indirect coupling with lck. The CD4-lck interaction has been shown to be required for activation of a T cell clone (47); however, lck may not be the only tyrosine kinase involved in T cell activation. Recently, another tyrosine kinase, fyn, has been proposed as the candidate for TCR coupled activation. It is also a member of the src family, and is expressed in a variety of tissues, but the fyn present in T cells is a uniquely spliced form of the gene (48). Under gentle detergent conditions, the fyn has been shown to immunoprecipitate with the TCR complex (49), and no lck is observed in these precipitates. Both fyn and lck are thought to be involved in the initial tyrosine phosphorylation events but they themselves need to be dephosphorylated to become active. Additionally, Chan *et al.*

have isolated a novel tyrosine kinase termed ZAP-70 (50) which is associated with the cytoplasmic portion of CD3 ζ following TCR stimulation (51). ZAP-70 undergoes tyrosine phosphorylation following TCR stimulation and is expressed in T and NK cells. Tyrosine phosphorylation and association of ZAP-70 with CD3 ζ requires the presence of the src family tyrosine kinases (50). COS cells transfected with ZAP-70, fyn, or lck alone result in minimal tyrosine phosphorylation but cotransfection of ZAP-70 with either fyn or lck increases the tyrosine phosphorylation observed including CD3 ζ. Thus, it is probable that fyn or lck phosphorylate ZAP-70 as well as CD3 ζ and PLC γ.

The ras family of G proteins are ubiquitously expressed and within minutes of TCR stimulation, ras is activated in primary T cells. This activation is dependent upon PKC (52), and carried out by the regulation of GTPase activating protein (GAP). GAP has different forms, rasGAP and rhoGAP, which regulate the GTPase activity of p21ras and actin polymerization, respectively (53). Normally, rasGAP stimulates the hydrolysis of GTP to GDP by p21ras, but in stimulated cells rasGAP is phosphorylated, presumably by PKC, and this inhibits it, resulting in the build up of p21ras-GTP.

Recently, it has been shown that the CD3 complex can be divided into two transducing pathways. The CD3 ε and ζ can both act independently from the remaining TCR components to stimulate the cell. Chimeric molecules consisting of the extracellular and transmembrane domains of IL-2R and cytoplasmic tail of CD3 ε expressed without other TCR components have been demonstrated to effect increases in tyrosine phosphoproteins and IL-2 secretion (54). Similarily, crosslinking of chimeric receptors containing the cytoplasmic domain of CD3 ζ results in full proliferation indistinguishable from those mediated by intact TCR (55,56). Specific antigen recognition activation motifs (ARAM) have been identified in several CD3 subunits of the TCR complex as well as the α and β subunits associated with IgM and IgD on B cells, and rat FcεR β and γ chains (57). These ARAM sequences are 17 amino acids long, and are rich in leucine, isoleucine, and tyrosine residues. They have a relatively conserved sequence and are found within the cytoplasmic domains of the CD3 subunits and ζ

chain. All of the CD3 chains contain one ARAM while the ζ chain has a triplication, increasing its efficiency of signal transduction. One ARAM is sufficient to couple chimeric receptors to events associated with T cell activation (57).

Another molecule, CD45, has been shown to have tyrosine phosphatase activity in its cytoplasmic domain (58) and may be involved in the activation of T cells. It is expressed on all lymphoid and myeloid cells, different functional subpopulations of mature peripheral T cells expressing combinations of the four isoforms. CD45 has several isoforms (180, 190, 200, 220 kD) on T cells (59), and B cells (240 kD), also called B220 (60), derived by alternative splicing of 5' exons of the gene (61). Some MAbs to CD45 can induce IL-2R expression and IL-2 production when peripheral resting T cells are costimulated by sepharose-bound anti-CD3 (62,63). Structurally, CD45 and the TCR seem to interact as shown by chemical crosslinking studies on a murine hybridoma (64). CD45-negative T cells are defective in TCR mediated activation of the PTK pathway (65) and do not increase cytoplasmic Ca^{++} or induce cytokine secretion following TCR stimulation (65,66). Reconstitution of these cells with CD45 restores TCR function. It is thought that CD45 activates fyn and lck by dephosphorylation, thereby starting the cascade of signalling events. Interestingly, CD45 mediated activation is restricted to $CD4^+$ T cells whereas anti-CD45 Ab inhibit cytolytic activities by Tc and NK cells (67,68). In mouse T lymphoma cells, fodrin (a spectrin-like protein), has been co-isolated with CD45 (180 kD) suggesting that CD45 may associate with the cytoskeleton (69).

In summary, the TCR is a complex of several molecules and involves different signal transduction pathways. The heterodimeric αβ or γδ associate with CD3 subcomplexes and their interaction with antigen/MHC facilitates CD45 involvement in the receptor complex. CD4 or CD8 become involved, binding to their MHC counterpart on the APC or target cell, and CD45 may interact with their associated lck or independently with fyn to dephosphorylate and activate the kinases. Fyn and lck, once activated, phosphorylate several intracellular substrates including PLC γ, CD3 ζ, and probably ZAP-70. PLC γ activation leads to PI breakdown and PKC activation. PK C activation results in the phosphorylation of CD3 γ, raf-

1, and MAP-K, and rasGTP activity is increased due to the inhibition of GAP. This overall signaling cascade of two intermixing kinase pathways (PTK - tyr; PKC - ser/thr) and the phosphorylation of multiple substrates leads to autocrine cell proliferation through regulated changes in the expression of specific genes (70,71).

1.2.2 Costimulatory Antigens

Perturbation of the TCR/CD3 complex with antigen/MHC or MAb alone is often insufficient to fully activate resting cells (72), and may lead to clonal anergy (73). Optimal activation and proliferation requires an additional stimulatory signal that must be provided in conjunction with engagement of the TCR/CD3 to drive the cell to autocrine proliferation. This additional signal is termed the costimulatory or secondary signal. Accessory cells such as macrophages can provide this second signal, and several molecules have been identified as being involved in enhancing the T cell response via ligation to their ligands or MAbs. These accessory molecules in T cell activation are CD4, CD8, CD28, CD2, CD44, CD45, LFA-1, CD43, VLA-4, CD24, Thy-1, CD5, TAP, Tp45, Tp90, and Ly-6 (Table II) (24).

CD4 and CD8 are antigens expressed on usually mutually exclusive T cell subsets, Th and Tc, respectively (18). Their expression usually correlates with the MHC which the T cell recognizes antigen with, thus $CD4^+$ Th are MHC II restricted, and $CD8^+$ Tc are MHC I restricted (74). CD4 is a 52 kD glycoprotein, while CD8 exists as a disulfide-linked heterodimer (75). There is a family of CD8 heterodimers in the mouse composed of α (34kD), α' (38 kD) and β (30 kD) (76,77). Various combinations of heterodimers of α, α', and β have been detected, and tetramers and hexamers have also been described on thymocytes (78). In humans, CD8 is a disulfide-linked homodimer of two 32 kD glycopeptides. MAbs against CD4 and CD8 have stimulatory and inhibitory effects on effector function, implying that these antigens may be involved in activation by transducing positive or negative signals to the lymphocyte (24, 81,18, 82). Both CD4 and CD8 have also been shown to mediate cell-cell adhesion via binding their respective MHCs (81, 82), thereby stabilizing T cell interaction with the APC or target cell, respectively.

Table II[@]

COSTIMULATORY ANTIGENS

Antigen	Ligand	Molecular Mass	Distribution
CD4	MHC_{II}	52 kD	helper T cells,thymocytes
CD8	MHC_I	mouse: α 34 kD, α 38 kD, β 30 kD; human: α 2x32 kD	most cytotoxic T cells, thymocytes
CD28	B7/BB1	2x44 kD	most $CD4^+$ cells,50% $CD8^+$ cells,thymocytes,B cells
CTLA-4	B7/BB1	2x44 kD	on activated lymphocytes
CD2	LFA-3	50 kD	90% peripheral T cells,some NK cells,70% thymocytes,B
CD44	HA,coll, addressin	80-200 kD	T,thymocytes,B,granulocytes, macrophages,erythrocytes,NK
CD45	CD22?	180,190,200, 220,240 kD	B,T subsets,thymocytes, monocytes,PMNs
LFA-1	ICAMs	α 180 kD β 95 kD	90% thymocytes,leukocytes
CD43	ICAM-1	115 kD 135 kD	T,B,thymocytes,monocytes neutophils,platelets
VLA-4	VCAM-1, FN	α 150 kD β 130 kD	resting B & T,monocytes
CD24	CD24?	60 kD	immature T,B,95% bone marrow
Thy-1		18-25 kD (GPI)	mouse-all T,dendritic,neurons human-only neurons,thymocytes
CD5	CD72?	67 kD	all mature T, some B
TAP		12-19 kD (GPI)	most peripheral T cells some thymocytes
Tp45		45 kD	3-19% peripheral T cells $CD8^+$
Tp90		90 kD	3-14% peripheral T cells $CD8^+$
Ly-6		10-18 kD	dev stages T & B,PMNs

[@]taken from Abbas et al.1991(24),and Chan 1988(25).
HA,hyaluronan;coll,collagen;PMN,polymorphonuclear leukocytes;FN,fibronectin.

CD28 is a homodimer of 44 kD glycopolypeptides expressed on approximately 80% of peripheral blood T cells and defined by MAb 9.3 (83). Stimulation of CD28 with MAb in the presence of TCR crosslinking MAb (84), phorbol myristic acetate (PMA) (85), anti-CD2 (86), and immoblized anti-CD3 (87) results in accessory cell independent proliferation while anti-CD28 alone does not. The ligand for CD28 has been identified as B7/BB1 which is expressed on the APC or target cell. CTLA-4 is a transmembrane glycoprotein homologous to CD28, but it is undetectable on resting cells and increases greatly upon cell activation where it is coexpressed with CD28 (88). CD28 and CTLA-4 comap to mouse chromosome 1 and are most likely the products of a gene duplication event (89). Both CTLA-4 and CD28 bind B7/BB1; however, CTLA-4 has a greater affinity for the ligand and is expressed at a fraction (1/30-1/50) of that of CD28. The interaction of CD28 or CTLA-4 with B7/BB1 may represent the costimulatory signal necessary for full T cell proliferation to occur. Indeed, transfection of B7/BB1 into mouse melanoma cells induces rejection of tumors in vivo (90, 91). CD28 initiates a signalling pathway distinct from TCR. Cyclosporin A has no effect on CD28 stimulated IL-2 production but abrogates the IL-2 produced by TCR stimulation or phorbol ester and Ca^{++} ionophore addition (92, 93). CD28 appears to augment anti-TCR response by increasing IL-2R expression and IL-2 production through stablization of the cytokine mRNA (30).

CD2 plays a dual role in T cell activation. It can act as a costimulatory molecule when bound by two Abs, or it can be adhesive when binding to its ligand LFA-3 (section 1.3.4). CD2 is expressed on both immature and mature T cells and has been identified as the SRBC receptor responsible for the formation of E-rosettes (94,95). Activation via CD2 requires two distinct MAbs and accessory cells or IL-1 (96). CD2 is upregulated 10-20 fold after activation suggesting CD2 is important for subsequent interactions between T cell/APC. Modulation of TCR with anti-Ti or anti-CD3 does not affect the expression of CD2 on the surface; however, the induction of IL-2 production and proliferation via CD2 pathway is inhibited (97).

Therefore, the TCR/CD3 complex seems to regulate the CD2 pathway ensuring that only antigen-specific clonal expansion can occur and polyclonal expansion is inhibited.

Recently, it was observed that CD2 changes its avidity in response to PMA, anti-CD3, or anti-TCR (98). A murine hybridoma transfected with human CD2 increases CD2 avidity for CD58 (LFA-3) after crosslinkage of TCR or exposure to specific antigen. The quantity of CD2 on the cell surface is not changed and the avidity is maximal at 30 min and returns to basal levels by 90 min. PTKs and PKC are involved as demonstrated by the use of inhibitors (98) and the COOH-terminal asparagine residue (#327) is required for TCR and PMA induced CD2 avidity (99).

CD44 is an acidic sulfated integral membrane glycoprotein with several isoforms ranging from 80-200 kD. Anti-CD44 MAbs enhance both CD2 and CD3 mediated T cell activation. Suboptimal but significant activation of peripheral T cells is achieved when high concentrations of CD2 and CD44 Ab are used, causing a 25 fold enhancement of CD2 and IL-2 secretion alone (100). Crosslinking the CD44 resulted in modulation of CD2 as demonstrated by an increased expression of $T11^3$ activation epitope (101). Interestingly, a few anti-CD44 Abs inhibit CD2/LFA-3 mediated rosetting, suggesting that CD44 may be in close proximity to erythrocyte LFA-3 (102). CD44 is also found in the serum, shed from the leukocyte surface as a mode of regulation of adhesion (103). In lymphocytes, CD44 is associated with PKC, and PKC activation by anti-CD44 Ab may be involved in both ankyrin binding (104), and promotion of T cell adhesion via LFA-1 (105). However, a recent report by Neame *et al.* (106) indicated that intact fibroblasts treated with phorbol ester did not show detectable changes in CD44 phosphorylation. Moreover, this group characterized serines 323 and 325 as being necessary for CD44 phosphorylation but suggests they are not targets for any basophilic protein kinases such as PKC or cAMP-dependent PKA.

CD45 has multiple isoforms and its expression is developmentally regulated during maturation. The large cytoplasmic domain has intrinsic tyrosine phosphatase activity and

is implicated in dephosphorylating fyn and lck in T cell activation (section 1.2.1). Anti-CD45 MAbs can either inhibit or enhance various functional responses of T lymphocytes.

LFA-1 is restricted to leukocytes and belongs to the β2 subfamily of the integrin superfamily. It is a heterodimer and has three ligands characterized to date termed ICAM-1(107), ICAM-2 (108), and ICAM-3/R (109, 110). Anti-LFA-1 and anti-ICAM-1 Abs inhibit several lymphocyte functions including cytotoxic T lymphocyte (CTL) killing, and T cell dependent Ab production (10). LFA-1 is associated with the cytoskeleton, and localizes to the region of cell/cell contact during conjugate formation (111). LFA-1 acts as a costimulatory molecule by stablizing T-APC and T-target cell contacts. Transfection of ICAM-1 into MHC_{II} fibroblasts enhanced MHC_{II} restricted T proliferative response in a CD18 dependent fashion (112).

CD43 has two forms: 115 kD, expressed on lymphocytes, thymocytes, and monocytes, and 135 kD, expressed on neutrophils and platelets (113). It is a heavily sialylated molecule which is defective in T cells of males with the X-chromosome linked immunodeficiency disorder called Wiskott-Aldrich syndrome (114). These patients are susceptible to opportunistic infections and have no response to polysaccharide antigens due to defects in the Th and Tc effector functions. Some anti-CD3 MAbs have a costimulatory effect on lymphocytes, NK, and monocytes. The MAb L10 triggers T cell activation through PI breakdown in the presence of monocytes (115). CD43 has been shown to bind ICAM-1, besides LFA-1 and Mac-1, but interestingly, HeLa cells transfected with CD43 exhibit diminished binding to T cells mediated by CD18 (116).

VLA-4 is a β1 integrin and binds fibronectin in the ECM, as well as vascular cell adhesion molecule (VCAM-1), which is induced on vascular endothelium after exposure to inflammatory cytokines. VLA-4, VLA-5, and VLA-6 increase avidity for their ligands after T cell activation and provide costimulatory signals to T cells. Anti-TCR MAbs and soluble VCAM-1 cause lymphocyte proliferation (24).

CD24, also known as heat stable antigen, has a wide distribution and is highly glycosylated. Anti-CD24 MAbs blocked costimulatory activity of splenic accessory cells for anti-CD3 induced proliferation of $CD4^+$ T cells (112). This treatment induced non-responsiveness in T cells.

Thy-1 is 18-25 kD GPI glycoprotein with an Ig like domain expressed on most peripheral lymphocytes and neurons in mouse but absent from human peripheral blood lymphocytes (PBL) (13). A strong proliferative response is induced in resting T cells by anti-Thy-1 in the presence of anti-Ig or PMA, or the simultaneous addition of two anti-Thy-1 antibodies without PMA (117,118). TCR/CD3 expression seems to be required for the anti-Thy-1 IL-2 response, however the rapid increase in Ca^{++} levels is independent of TCR expression (119,120). Furthermore, the finding that Thy-1 is attached to the membrane via a phophoinositol linkage implicates a direct transduction pathway through phospholipid hydrolysis (121).

CD5 is a 67 kD glycoprotein expressed on all mature T cells and some B cells (122,123). Anti-CD5 Abs appear to enhance IL-2R expression and IL-2 production (84,124) in resting cells stimulated by sepharose-anti-CD3. CD5 seems to associate with the TCR/CD3 complex as demonstrated by immunoprecipitation studies, and the same protein kinase substrates were coprecipitated with anti-CD5 or anti-CD3 (125). This suggests the activation of a similar signal transduction pathway by the two molecules. Simultaneous ligation of CD5 and CD28 by MAb induces polyclonal cell activation (126). Anti-CD5 increases cGMP levels (like anti-CD28) and a rise in intracellular Ca^{++} only via an ion influx from the extracellular environment (127,128). The depletion of PKC seems to uncouple signal transduction between CD5 and the calcium channel.

Another antigen possibly involved in lymphocyte activation is a TAP, a 12-19 kD GPI linked protein expressed on the majority of peripheral T cells (14). Some anti-TAP Abs induce resting T cells to proliferate in the presence of accessory cells or IL-1; however,

immobilized MAb do not induce proliferation indicating that crosslinkage of the protein on the surface is not the only requirement for activation.

More recently, MAbs directed against Jurkat and HPB-ALL cell lines have identified two antigens, Tp45 and Tp90, possibly involved in cell activation (129,130). MX13 MAb identified Tp45, and induces IL-2 production from Jurkat in the presence of PMA, via mobilization of intracellular Ca^{++} stores (129). Modulation of TCR results in the disappearance of Tp45 as well as TCR, and vice versa, indicating that although they are not physically linked they are in close vicinity of each other on the cell surface. Tp45 is expressed on 3-19% PBL which are also $CD2^+$, and seems to be preferentially expressed within the $CD8^+$ Tc subset. In contrast, Tp90, identified by MX20 MAb, is unaffected in its expression by the modulation of TCR. However, like CD2, IL-2 production is inhibited in response to MX20 MAb after TCR modulation (129). MX20 alone induces IL-2 production in Jurkat without PMA, but the levels are enhanced in the presence of PMA. MX20 also has a strong mitogenic effect on PBL in the presence of adherent cells or PMA. Like Tp45, Tp90 expression seems to be preferentially within the $CD8^+$ subset, and 3-14% of total PBL are $Tp90^+$ and $CD2^+$.

Ly-6 is a group of at least 5 molecules of 10-18 kD expressed on various developmental stages of mouse T and B cells, as well as PMNs. These proteins are GPI linked and some MAb specific for Ly-6 can activate T cells to secrete cytokines (24).

Lectins also stimulate polyclonal expansion of T cells, and are assumed to work by binding the carbohydrate groups exposed on surface glycoproteins, somehow triggering the TCR complex in the presence of macrophages or PMA (131).

1.2.3 Postactivation Antigens

As a consequence of activation, specific antigens are newly expressed on the cell surface or upregulated from their basal level of expression. The function of several of these molecules remains to be determined but some act as specific growth factor receptors or transport proteins, rendering the cell sensitive to the local microenvironment, as well as

stimulating the uptake of metals, ions, and proteins necessary for proliferation. The mechanism which drives an activated cell to proliferate is not well understood. Most studies concentrate on the events following growth factor or ligand binding to its specific receptor. The result of growth factor/receptor interaction is assumed to be the creation of secondary messengers which alter various biochemical pathways and focuses the cell on the sole purpose of preparing for and executing mitosis (132,133). Putative secondary messengers, such as protein phosphorylation, increased cAMP levels, activation of Na+/H+ exchange and cytoplasmic alkalinization, and phospholipid hydrolysis, have been postulated as playing a role in driving the cell to proliferate (134). These changes in the cell trigger the activation of specific genes (c-myc, interferon γ (IFNγ)) and increased mRNA and protein synthesis, ultimately leading to DNA synthesis and cell division (135,136).

Specific examples of growth factors that are important to the proliferation of lymphocytes are IL-2 and IL-4. The activation of lymphocytes results in the expression of the IL-2R in both T and B cells, and secretion of IL-2 from T cells (137,26). The IL-2R contains at least two subunits called α (p55/Tac), and β (p70), and exists in three isoforms with high, intermediate, and low activity (138). The high and intermediate affinity receptors, which are thought to consist of the α and β heterodimers and the β chain alone respectively, are effective in IL-2 mediated signal transduction, but the low affinity receptor which consists of the α chain alone is ineffective. Recently, a third component p64, tentatively called γ, has been isolated and cloned (2). It associates with the β chain as demonstrated by immunoprecipitation with an anti-β MAb (139, 140). The γ chain belongs to the cytokine receptor family and may play a role in signal transduction because regions of its cytoplasmic domain are homologous to Src homology region 2 (SH2) which can bind to phosphotyrosine residues of some phosphoproteins. Cotransfection of γ and β chains in L cells results in an IL-2R of intermediate affinity while cotransfection of αβγ in L cells converts a preexisting αβ IL-2R to a high affinity IL-2R. Thus, the high affinity IL-2R may be a heterotrimer of the αβγ subunits. Studies also suggest that the γ chain is required for the receptor mediated

internalization of IL-2 since the high affinity receptors internalized IL-2 more efficiently than the intermediate receptors (2). The interaction of IL-2 with its receptor is necessary for cell proliferation since anti-IL-2R or anti-IL-2 prevents proliferation (141), however, the transduction mechanism is not clear. Activation of Na+/H+ exchange and cytoplasmic alkalinization is observed but not obligatory, and there is no rise in Ca^{++} levels resembling TCR activation with MAb or lectins (142). The breakdown of phospholipids is not detected, however, PKC may be involved as its translocation from the cytosol to the plasma membrane has been observed upon IL-2 induction (143). The tyrosine kinase, lck, has been found to be associated with IL-2R β, and IL-2 can stimulate lck activity when added to lymphocytes (144). It is known that IL-2 decreases AC activity, thus decreasing cAMP levels, while agonists of AC reduce IL-2 induced phosphorylation (141); this implicates a feedback mechanism for regulation of induction of proliferation by either pathway.

IL-4 is a 20 kD glycoprotein that also has mitogenic effects on lymphocytes. It induces resting B cells to upregulate their MHC II expression, promotes the production of IgG and IgE in response to LPS, and induces proliferation of some cell lines (145,146). In addition, it synergizes with erythropoietin and colony stimulating factors to promote the proliferation of various hematopoietic lineages, and stimulates resting T cells to enter S phase in the presence of PMA but without APC (146). The mechanism of action on T cell growth is postulated as being either the stimulation of the IL-2 autocrine pathway, or the activation of expression of its own receptors. Many T cells do not secrete IL-2 but do produce and respond to IL-4 (146). IL-4 receptors have been identified on T and B cells , and seem to be upregulated after activation via Con A or LPS respectively (147). The putative receptor molecule is approximately 60 kD and has a high affinity for IL-4. IL-4 has also been shown to increase CTL activity (148). The signal transduction mechanism is unclear but probably acts through a protein kinase pathway (146).

The transferrin receptor (TR) is upregulated on the cell surface of all proliferating cells, indicating the massive iron requirement of dividing cells (149,150). Iron is required for DNA

synthesis, electron transport chain functions, and hemoglobin synthesis (reticulocytes and erythrocytes) (151). Transferrin protein circulates free in the plasma, binding iron atoms and subsequently delivering them to cells expressing TR. Transferrin is produced by the liver, and can bind either one or two iron atoms and interact with its receptor in either iron loaded form. The transferrin-Fe^{+++}-TR complex is internalized by receptor mediated endocytosis and the iron is released into the cell while the TR and transferrin can either be degraded, stored within an intracellular pool, or recycled to the plasma membrane (149). Apotransferrin is released from TR upon recycling as it has the lowest affinity for TR compared to the iron bound forms. TR expression follows IL-2R expression, and anti-IL-2R will prevent TR expression and cell proliferation, indicating that TR gene is activated as a result of IL-2/IL-2R interaction (149,151).

4F2 antigen (gp40/80) is present at low levels on quiescent cells and its expression is increased after activation, appearing at a higher density within four hours of stimulation (147). It is thought to act as a transport protein like Na+, K+ ATPase, or as a regulatory protein for an exchanger. The large subunit (80 kD) has been recently cloned (152) and only has one transmembrane domain which is not indicative of a normal transport protein (153). However, it is possible the α chain (40 kD) has several transmembrane domains usually found in transport proteins and so conclusions as to the structure and function of this antigen must await further study.

1.3 LYMPHOCYTE ADHESION

The interaction between lymphocytes and their environment primarily involves transient adhesion as the cells patrol the body for foreign antigens and interact with APC and target cells. This transient adhesion can be antigen independent such as homing and extravasation, or antigen dependent such as the interactions between Th-APC and Tc-target cell. Homing is primarily mediated by the selectins, a few integrins, and possibly CD44, while extravasation involves the selectins, as well as the integrins, and the Ig family members. Antigen dependent adhesion is controlled by CD2/LFA-3 and LFA-1/ICAMs.

1.3.1 Superfamilies of Cell Adhesion Molecules

With the advent of MAb technology, several antigens important in lymphocyte functions have been characterized. Specific molecules mediate communication between cells, via binding their counter receptors on the opposing cell, as well as adhesive or locomotory functions seen during lymphocyte migration or homing. MAbs have been useful in understanding the mechanisms involved in transmission of a signal through a receptor from the environment to the interior of a cell. Perturbation of these receptors with MAbs mimics ligand binding thus enhancing or inhibiting specific cellular functions. Three families of adhesion molecules contain proteins involved in lymphocyte function; these families are the selectins, the integrins, and the immunoglobulins.

The selectin family consists of three membrane glycoproteins that mediate leukocyte-endothelial cell interactions by binding to carbohydrate ligands on opposing cells (Table III) (154, 155). Each of the three selectins contains an N-terminal lectin binding domain, an EGF-like domain, two to nine complement binding repeats, a transmembrane domain, and a short cytoplasmic tail (154). Three types have been isolated: L-selectin, E-selectin, and P-selectin (155). L-selectin is expressed on leukocytes and mediates polymorphonuclear (PMN) cell attachment to the endothelial cells (EC) as well as homing of lymphocytes to the

Table III[@]

SELECTIN FAMILY

Antigen	Distribution	Opposing cell
L-selectin (Mel-14,LAM-1,LECAM-1)	-leukocytes (cleaved after act'n)	-PLN endothelium,SLe^X -GlyCAM,inflam sites
P-selectin (CD62,GMP140,PADGEM)	-platelets (α granules) -endothelium (Weibel-Palade bodies (thrombin/histamine act'd)	-monocytes,neutrophils -T cell subsets (SLe^X)
E-selectin (ELAM-1)	-endothelium (IL-1,TNF,LPS act'd)	-monocytes,neutrophils -T cell subsets,SLe^X

[@]taken from McEver 1992(154) and Lasky 1992(155).

peripheral lymph nodes (PLN) (156). E-selectin is expressed by activated endothelial cells, while P-selectin is expressed on activated ECs and platelets.

The integrins are a superfamily of molecules which is involved in cell-cell interactions as well as cell-substrate interactions. The structure and functions of the integrins have been well characterized (157). All of the members of this family have a common heterdimeric structure of noncovalently associated α and β subunits. These subunits are synthesized separately and associate with each other within the Golgi apparatus before being expressed at the cell surface. There are 14 α and 8 β chains characterized to date (Table IV) (10, 157). The α chain has a seven fold repeat, the last 3-4 repeats bind divalent cations such as Ca^{++} or Mg^{++} that are required for integrin binding. The β chain has a four fold repeat of a cysteine rich region believed to be internally disulfide bonded. The members of the integrin family can be categorized into three subfamilies based on three different β chains; these are the β1(CD29, VLA proteins), β2 (CD18, leukocyte integrins), and β3 (CD61, cytoadhesins) subfamilies (158).

The β1 subfamily members are primarily receptors for proteins within the ECM. Several recognize the tripeptide sequence Arg-Gly-Asp (RGD) characterized by Ruoslahti *et al.* (159). However, the very late antigen 4 (VLA-4), which binds fibronectin in the ECM (160), can also mediate cell to cell interactions via its second ligand, VCAM-1, which is expressed on endothelial cells (161). The divalent cation sites seem to play an essential role in determining VLA-4 regulation and ligand specificity (162). The β2 subfamily (also called the leukocyte integrins), is restricted to expression on the leukocytes and are primarily involved in cell to cell interactions. Three molecules are within this subfamily: LFA-1, Mac-1 or CR3, and p150/95. These are extensively referred to by the CD nomenclature , CD11a/CD18, CD11b/CD18, and CD11c/CD18, respectively (163). The β3 family includes the platelet receptors GPIb-IX and GIIb/IIIa which bind von Willebrand factor and are involved in platelet aggregation and blood clotting. A deficiency of these integrins results in defective platelet adhesion and bleeding disorders (164).

Table IV[@]

INTEGRIN FAMILY

Subunits α	Subunits β	Names	Ligand*	Distribution# nonLeukocyte	Distribution# Leukocyte
α_1,CD-	β_1	VLA-1	coll,lam	F,BM	B',T'
α_2,CD49b	(CD29)	VLA-2	coll,lam	P,F,EN,EP	T'
α_3,CD-		VLA-3	fbn,coll,lam	EP,F	
α_4,CD49d		VLA-4	fbn,VCAM-1	NC,F	B,T,M,LGL
α_5,CD-		VLA-5	fbn	F,EP,EN,P	Th,T
α_6,CD49f		VLA-6	lam	P	T
α_7			lam		
α_8			?		
α_v,CD51			vtn,fbn		
α_L,CD11a	β_2	LFA-1	ICAM-1/2/3		B,T,M,G
α_M,CD11b	(CD18)	Mac-1	iC3b,fgn,ICAM-1,fx		M,G
α_X,CD11c		p150/95	fgn,iC3b?		M,G
α_{IIb},CD41	β_3	gpIIb/IIIa	fbn,fgn,vWf	P	
α_v,CD51	(CD61)	VNR	vtn,fgn,vWf,tsp	EN	B',M
α_6,CD49f	β_4		lam?	E	
α_v,CD51	β_5		vtn,fbn	C,F,EP	M
α_v,CD51	β_6		fbn		
α_4,CD49d	β_7 (β_p)	LPAM-1	fbn,VCAM-1		T
α_{IEL}					IEL
α_v,CD51	β_8		?		

[@]taken from Springer 1990(10) and Hynes 1992(157).
*coll,collagen;lam,laminin;fbn,fibronectin;vtn,vitronectin;vWf,von Willebrand factor;tsp,thrombospondin;fx,factor X.
#EN,endothelial cells;EP,epithelial cells;F,fibroblasts;NC,neural crest, melanocytes;P,platelets;C,carcinomas;BM,basement membrane-associated;B,B lymphocytes;T,T lymphocytes;T'B',activated cells only;Th,thymocytes; M,monocytes;G,granulocytes;LGL,large granular lymphocytes;IEL,intraepithelial lymphocytes.

The antibody molecules expressed on the surface of B lymphocytes, and circulating in the serum were the first characterized molecules in what is now known as the Ig superfamily. The members of this family all have at least one characteristic domain made up of approximately 100 amino acids folded in two anti-parallel β-sheets and in most cases held together by a disulphide bond (166). These antigens are involved in a wide range of functions including both cell activation and cell adhesion. Other members of this family include the TCR, CD4, CD8, CD2, CD28, MHC I, MHC II, Thy-1, LFA-3, ICAM-1/2/3, NCAM, VCAM-1, and myelin associated glycoprotein (MAG) (165).

1.3.2 Homing

The mammalian immune system can be divided into primary and secondary units. The primary lymphoid tissues are the bone marrow and the thymus (166). Lymphocytes are ultimately derived from the bone marrow where they differentiate from hematopoietic stem cells (167). B lymphocytes leave the bone marrow and directly enter circulation and are readily responsive to antigen. Conversely, T lymphocytes leave the bone marrow as prothymocytes and migrate to the thymus for further differentiation and "education", developing into mature T cells after specific selection processes. Within the thymus, T lymphocytes progress through several developmental stages characterized by two proteins, CD4 and CD8. Early stage thymocytes are $\alpha\beta^-/CD4^-/CD8^-$, becoming $\alpha\beta^+/CD4^+/CD8^+$, and finally maturing as $\alpha\beta^+/CD4^+/CD8^-$ or $\alpha\beta^+/CD4^-/CD8^+$ to be released into the periphery (168). The $\gamma\delta^+$TCR cells break away from this development early, becoming $CD4^-/CD8^-$ as mature cells. There is also a population of $\alpha\beta^+$TCR cells which are $CD4^-/CD8^-$ but these presumably develop after the $CD4^+/CD8^+$ stage. The secondary organs of the immune system are the lymph nodes, spleen, tonsils, adenoids, Peyer's patches, and appendix. These organs are responsible for collecting and concentrating foreign antigens and are the sites where immune responses are initiated, particularily naive lymphocytes responding to antigen for the first time.

Two circulatory networks, the lymphatic system, and the blood system, connect the secondary lymphoid organs (169). Lymphocytes circulate through the body via the bloodstream and enter the lymph nodes by specifically adhering to high endothelial venules (HEV). The lymph nodes are made up of two compartments, the outer layer (cortex), and the inner core (medulla). The lymphatic system collects the interstitial fluid that bathes the cells of a tissue, and transports the fluid and any lymphocytes in the tissue through the afferent lymphatic vessels to the lymph nodes where they disperse, interacting with any APC. The APC present antigens to the lymphocytes as they traverse through the cortex, and the cells and the lymph are collected within the sinusoids of the medulla and funnelled into the efferent lymphatic vessels to be returned to blood circulation via the thoracic duct. The structure of the lymph node is such that there is maximal exposure of the antigen-specific lymphocyte repetoire with any antigen filtered and trapped in the lymph and presented APC. It also allows optimal interactions between T and B cells to potentiate the immune response. Activation of a specific lymphocyte and subsequent proliferation occurs within the lymph node, accounting for the draining lymph nodes being swollen during infections. B cells differentiate and proliferate in specific regions of the lymph node called germinal centres, while T cells stay within the cortex. The resultant Abs and effector cells are released into circulation.

Specific molecules have been identified as being involved in lymphocyte recirculation. Attachment of lymphocytes to the HEV of lymph nodes is via recognition of specific determinants. These determinants vary between different tissues as shown by organ selective attachment of the lymphocytes to PLN HEV and Peyer's patches (PP) HEV. The MEL-14 MAb exclusively inhibits the attachment of lymphocytes to PLN HEV recognizing a homing receptor (170). In vitro assays involving frozen HEVs and binding lymphocytes revealed that the addition of mannose-6-phosphate could inhibit lymphocyte adhesion to the HEV, suggesting the involvement of a carbohydrate moiety (171). Further studies characterized the antigen recognized by MEL-14 as a transmembrane protein now termed L-

selectin. Sialylated derivatives of Lewis x (SLe^X) oligosaccharides have been idenitified as ligands for the selectins (155), and thus the selectins seem to mediate adhesion via their N-terminal lectin binding domain. However, the adhesive interactions mediated by E- and P-selectin may be more complex. Another non-myeloid specific polysaccharide related to SLe^X is sialyl Lewis a (SLe^a) and it has clear ligand activity for E-selectin (155). Protease experiments demonstrated that P-selectin appears to bind a carbohydrate contained on a protease-sensitive substrate (172). In addition, both E- and P-selectin mediated adhesion of neutrophils is, at least in part, directed by SLe^X presented by L-selectin on the neutrophil surface (156). A complementary DNA (cDNA) for a 50 kD mucin-like protein called glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) has recently been identified as the protein component presenting the carbohydrate determinant to L-selectin (173). Additionally, a mucosal addressin, termed MadCAM-1 and recognized by MAb MECA-367 has recently been cloned. MadCAM-1 is expressed on PP-HEV and mesenteric lymph nodes, and Abs to it block the binding of gut associated lymphocytes to these endothelia (174). MAbs to β7 and α4 inhibited the binding of lymphocytes to purified MadCAM-1. LPAM-1 is a new integrin molecule made up of the α_4 subunit (VLA-4) and a novel β subunit (β_7). This alternative VLA-4 ($\alpha_4\beta_7$) and the normal VLA-4 ($\alpha_4\beta_1$) mediate specific adhesion to PP-HEV (175).

Lymphocytes which have been previously activated (memory cells) differ in their recirculation compared with unstimulated (naive cells) (176). Memory cells selectively migrate back to the tissues where the antigen was first encountered. This makes sense evolutionarily speaking, because it is most probable that the lymphocyte will encounter the same antigen at the same location. Thus, memory cells are more likely to be found in the skin, gut, or lung as opposed to the lymph nodes. Naive cells, however, are channelled through the lymph node allowing maximal exposure of these antigen-specific lymphocytes to any antigen presented by APC. Memory cells express a wide variety of proteins that are absent or expressed at low levels on naive cells. These proteins are general adhesion

molecules like LFA-1, LFA-3, VLA-4, VLA-5, CD45, ICAM-1 and SLe^X. Within the $CD4^+$ lineage, CD45 has been used to differentiate memory cells from naive cells because of the expression of different isoforms on the two subsets. The distinctions between these two subsets is not as distinct in the CD8 lineage (177). Naive cells are $CD45RA^+$ and memory cells are $CD45RO^+$. The $CD45RO^+$ population have been reported to respond to recall antigens and mitogenic antigens (178), and migrate into the tissues (179) to a greater extent then the $CD45RA^+$ population.

CD44 is another molecule which may partially mediate lymphocyte homing. It was originally described by Dalchau *et al.* (174) as a human molecule defined by monoclonal Ab F10-44-2, and is present on T cells, granulocytes, macrophages, and cortical thymocytes (180, 181). It displays some structural homology with cartilage link family members which can form complexes with hyaluronic acid and proteoglycan monomers (182). The gene for CD44 has recently been cloned and has 19 exons spanning a 50 kb region (183). Several isoforms of CD44 have been identified, and arise by alternative splicing of 12 of the 19 exons. The two most common forms are the haematopoietic form with a 37 kD protein core heavily glycosylated to produce a 90 kD surface protein (184), and the epithelial variant with a 135 amino acid insertion within the extracellular domain producing a 180 kD product (185,186). CD44 binds hyaluronan (186), fibronectin (187), and collagen I & VI (188), as well as possibly mediating organ selective attachment of leukocytes during extravasation. Its cytoplasmic domain has been shown to interact with the cytoskeletal protein ankyrin (189). A 58-66 kD murine mucosal vascular endothelial addressin interacts with affinity purified CD44 (190). CD44 has also been implicated in tumour metastasis because certain isoforms are induced on metastatic carcinomas (174).

1.3.3 Extravasation

The selectins are also involved in recruitment of the leukocytes from the blood into the tissues. This is accomplished by initiating interaction of the circulating leukocytes with the EC by tethering the leukocytes under shear stress. Migration of leukocytes into a site of

infection or injury involves four stages i) cell attachment to the endothelial lining, ii) triggering by chemokines or molecules (CD31) to activate integrins, iii) conformational changes of integrins resulting in stronger leukocyte/endothelial adhesion, and iv) cell migration into the surrounding tissue (174). Attachment is most likely to occur in the HEV of post capillary venules which all lymph nodes have. However, under inflammatory stress, specific regions of EC can develop to resemble HEVs. Inflammatory factors such as IFNγ, tumour necrosis factor alpha (TNFα), and IL-1 cause dilation of blood vessels decreasing blood flow and activate ECs to express adhesion molecules like E and P-selectin, ICAM-1, and VCAM-1. Initial attachment of the cells to the ECs is postulated to be mediated by the selectin family. PMNs begin to roll along the endothelium slowing down their rate of movement through the blood vessel. L-selectin on leukocytes, recognizes SLe^X on the endothelial lining but is quickly shed upon attachment to the EC. MAbs to L-selectin inhibits PMN localization at sites of acute inflammation in vivo (191, 192), and block PMN binding to cytokine activated ECs in vitro (193, 194). Activation of EC by cytokines stimulates the upregulation of E-selectin on EC (de novo synthesis) and P-selectin which is stored in the Weibel-Palade bodies (195,196) and is quickly translocated to the plasma membrane. E-selectin is synthesized by activated ECs in response to TNFα, IL-1, and LPS . The expression is maximal at 4-8 hours and is downregulated by 24-48 hours in vitro (197). Anti-E-selectin Abs partially inhibit PMN adhesion to cytokine-activated ECs (198). P-selectin is translocated to the plasma membrane within seconds after stimulation with thrombin or histamine (199), and then rapidly re-internalized. PMN adhesion to the activated EC parallels this transient expression of P-selectin. In contrast, platelets sustain expression of P-selectin on their surface (200, 201). Triggering of the cells to become more adhesive is not well understood but inflammatory factors may contribute to this second stage of extravasation. Cytokines, produced and released at the site of inflammation are postulated to become trapped by proteoglycans expressed on the endothelial surface and may activate cells (202). IL-8 binds selectively to the luminal surface of small vessels and triggers granulocyte adhesion to

endothelial ligands via β2 integrins. Similarily, macrophage inflammatory protein-1 (MIP-β) localizes to HEV of reactive lymph nodes and is most effective at augmenting adhesion of $CD8^+$ T cells to VCAM-1 (203). ECs also express a biologically active phospholipid called platelet activating factor (PAF) on their surface, which is proadhesive for granulocytes (204). PAF is synthesized within minutes of stimulation by thrombin and histamine, but is also quickly degraded, limiting the duration of the signal. Cells which are activated demonstrate increased avidity of β1 and β2 integrins for their respective ligands. PMN adhesion relies on the LFA-1/ICAM-1 system and Mac-1/ICAM-1 system (β2), while lymphocytes are more dependent on the VLA-4/VCAM-1 system (β1) (205). VCAM-1, like ICAM-1, is strongly induced on cytokine-stimulated endothelial cells, and VLA-4 expression is constitutive, like LFA-1, on lymphocytic and monocytic lineages. These molecules mediate increased adhesion and possibly promote the migration of leukocytes through the endothelial layer.

1.3.4 Cell/Cell Adhesion

Antigen dependent adhesion in Th-APC and Tc-target cell interactions primarily involves the CD2/LFA-3 and LFA-1/ICAM adhesion systems. Both systems are regulated in their adhesive states via changes in receptor avidity and when activated they promote cell/cell adhesion and enhance the immune response.

The adhesive function of CD2 is dependent upon T cell activation. Resting T cells do not bind human RBCs, however, activated human T cells adhere tightly to RBCs in vitro in a phenomena called E-rosetting (206). SRBC bind either resting T or activated T cells. Both populations of RBC express LFA-3. This increase in adhesion after activation appears to be regulated by an alteration in the negative charge on the T cell surface (9). The effect of activation on the CD2/LFA-3 interaction can be mimicked by the digestion of the glycocalyx with neuraminidase to reduce the sialic acid content and allow close contact between the cell surfaces (207, 208). T cell blasts have 5x less sialic acid content per cell than resting T cells (5), and therefore a lower negative charge (209). Similarily, cell interactions within the

nervous system are controlled by the level of sialylation; polysialylation of NCAM antagonizes its ability to promote adhesion (210).

MAbs against CD2 or LFA-3 inhibit T cell mediated cytolysis, NK cell activity and proliferation in response to lectins or alloantigens (9). LFA-3 exists in two isoforms as a result of differential mRNA splicing (15); these forms are a GPI linked form and a typical transmembrane domain containing protein. Both forms are fully active in mediating CD2 dependent adhesion and in promoting T cell function (211). Unlike the LFA-1/ICAM-1 interaction, CD2/LFA-3 is temperature and Mg^{++} independent. The CD2/LFA-3 interaction can contribute a 4-30 fold enhancement of the immune response (212, 152), however their interaction alone cannot stimulate T cells (213, 214).

The second adhesion system involves the LFA-1 and ICAM molecules. LFA-1 was originally identified by a MAb which inhibited homotypic aggregation of cells (215). LFA-1 has a wide distribution on most leukocytes and is constitutively expressed on cells. Anti-LFA-1 MAb inhibit several cell functions such as CTL killing, T dependent Ab production, NK cell killing, and adhesion of leukocytes to endothelial cells (10). LFA-1 interacts with ICAM-1, ICAM-2, and ICAM-3/R. These molecules belong to the Ig family and demonstrate a novel interaction between the integrin and Ig families. ICAM-1 is expressed in a wide variety of cell types. Most interestingly, it is low on resting cells but is transcriptionally induced by inflammatory lymphokines TNFα, IL-1, and IFNγ. As a result, activated endothelial cells increase their ICAM-1 expression after activation contributing to the recruitment of leukocytes from the blood. Additionally, the increase in ICAM-1 expression on lymphocytes, monocytes, and B cells enhances the interaction between the cells potentiating the immune response.

Besides LFA-1, ICAM-1 also has other receptors: Mac-1, originally characterized as the iC3b complement receptor (216), and CD43, a defective molecule in Wiskott-Aldrich syndrome (217). Mac-1 recognizes domain III of ICAM-1 (218) and is expressed primarily on monocytes and macrophages as well as granulocytes. ICAM-1 has also been shown to be the

receptor for rhinovirus (219). ICAM-1 has been implicated as being an adhesion molecule involved in Plasmodium falciparum infected RBC binding to capillary endothelium (220). However, this ability to bind ICAM-1 is not a universal feature of all binding parasite lines.

The other ligands of LFA-1, ICAM-2 and ICAM-3, have only recently been characterized. ICAM-2 has two Ig-like domains which are most homologous with the N-terminal domains of ICAM-1, and is expressed at a basal level on endothelium, resting lymphocytes, and monocytes. Little or no ICAM-2 is induced on lymphocytes and ECs after exposure to inflammatory mediators (221). ICAM-3/R has 5 Ig-like domains like ICAM-1 and is found on most leukocyte lineages. ICAM-3 is not expressed on endothelial cells even after stimulation with TNFα, or IL-1 (109) but it is high on resting leukocytes. The role of ICAM-2 and ICAM-3 in adhesion is still unclear, but they may act in the initial binding of cells to the endothelium preceding ICAM-1 induction and increased adhesion (110).

Although cells may express complementary adhesion receptors it does not mean they will necessarily adhere. Adhesion between cells is tightly balanced between prevention of nonspecific aggregation of cells, and the promotion of cell contact to optimize a possible immune response. Examination of the LFA-1/ICAM-1 interaction reveals that it is the LFA-1 which regulates the adhesiveness between cells. Phorbol ester treatment of cells leads to adhesion within an hour and there are observed increases in avidity in LFA-1 for binding purified ICAM-1, while ICAM-1^+ cells (PMA treated) do not increase binding to LFA-1 (10). Perturbation of the TCR/CD3 complex and CD2 can lead to a high increase in avidity of LFA-1 for its ligands (222). This increase in LFA-1 avidity is transient, peaking 10-20 min after TCR stimulation and returning to basal levels by 40 min (182), and may involve a conformational change in the receptor. Comparatively, CD2 crosslinking with MAb causes a persistent high avidity state (223) which could account for Th cells interacting with APCs for hours or days. This inside/outside signaling supports increased adhesion between cells after cell activation enhancing the immune response, as well as deadhesion to allow the Tc to

move to the next target or the Th to go off and proliferate (10). The interactions between LFA-1 and ICAM-2 or ICAM-3 have not been as well characterized.

Recently, integrin modulating factor (IMF-1) was isolated and is postulated to control the avidity of Mac-1 (and possibly LFA-1) on PMNs. (224). Stimulation of PMN increases the level of IMF-1 which parallels a rise in Mac-1 avidity for purified iC3b. IMF-1 purified from PMNs and added back to resting PMNs causes PMNs to increase binding through Mac-1. Analysis of IMF-1 supports it being a fatty acid or isoprenoid acid which is degraded quickly cooresponding to adhesion/deadhesion states. A cDNA designated cell adhesion regulator also has regulatory activity on cell adhesion (225).

A tyrosine kinase, p125 focal adhesion kinase (p125FAK), was isolated and cloned in chicken and mouse recently (226, 227), and is concentrated at focal contacts. It is phosphorylated in src transformed cells (226), and crosslinkage of β1 integrins results in its phosphorylation in human KB epithelial cells (228). Its presence in adherens suggests that phosphorylation of components of adherens may regulate their integrity. The α chain of LFA-1 is constitutively phosphorylated and the β chain is transiently phosphorylated after cell activation, however its phosphorylation does not seem to be required for the increase in LFA-1 avidity (229).

The importance of the leukocyte integrins is exemplified in leukocyte adhesion deficiency (LAD) which is characterized by lack of expression of the β2 family integrins (LFA-1, Mac-1, p150/95). Patients suffering from this disease have recurrent bacterial and fungal infections because of failure to recruit leukocytes to the site of infection. They are categorized as severe (<1% normal surface expression), or moderately deficient (3%-10% normal surface expression) (163). Early studies in mouse-human lymphocyte hybrids showed that interspecies complexes could form, and surface expression of the human α subunit but not the β subunit could be rescued from patient cells (230). This indicated that the α subunit was normal and competent for surface expression. The lack of integrin expression seems due to mutations in the β2 subunit (231, 232). Transfection of the β2 subunit into EBV-

transformed cell lines from four patients rescued the surface expression of the LFA-1 molecule and restored functional binding activity to purified ICAM-1 (233). It was observed that LAD patients accepted grafts more readily than normal individuals probably based on their lack of recruitment of leukocytes to foreign tissue. Consequently, anti-LFA-1 MAb have been administered to people prior to a graft and has proven relatively successful (234).

Opposite to LAD, patients with Down's syndrome (trisomy 21), seem to have increased adhesiveness as a result of overexpression of β2 subunit on the additional chromosome (235). Studies on EBV-immortalized cells from Down's syndrome patients (236, 237) reveals that these cells aggregate in response to phorbol esters more readily than normal cells. The aggregation was inhibited by MAb against both the LFA-1 α and β subunits.

The downregulation of several adhesion molecules is implicated in tumor cells becoming more invasive and metastatic. Tumor cells detach from the tumor mass at higher propensity and spread (238, 239). Cells chemically mutated to select for LFA-1 deficient cells have a higher evasive potential that the parental nonmutated cells (240) The low expression of LFA-1 characteristic of Burkitt's lymphoma may allow the cells to escape immunological surveillance (241). Some Burkitt's lymphoma cells were shown to be deficient in ICAM-1 and LFA-3 expression as well (242).

1.4 THESIS OBJECTIVES

The objective of this thesis study was to characterize the antigen recognized by a rat MAb, called YN1/1.7. The YN1/1.7 MAb was raised against NS-1 cells (mouse myeloma) in a previous study, and was reactive with mitogen stimulated mouse lymphocytes. The antigen recognized by YN1/1.7 MAb was termed murine activated lymphocyte antigen-2 (MALA-2), and was biochemically characterized as a glycoprotein monomer with an apparent molecular weight of 95 kD (243). MALA-2 is expressed on mitogen activated spleen cells, but is absent or present in very low quantities on normal spleen cells, lymph node cells, and thymocytes. The YN1/1.7 MAb partially inhibited Con A stimulation of spleen cells, and

almost completely abrogated mixed lymphocyte response (MLR) (243). Thus, it seemed that MALA-2 was involved in lymphocyte activation, and possibly played an important role in the direct contact between cells (as in MLR) as opposed to mitogen stimulated activation of cells. In the following thesis study, the gene encoding MALA-2 was further characterized by cDNA cloning, genomic cloning, and analysis of a unique cDNA, assumed to be alternatively spliced.

Specifically, the first phase of this research (described in chapter 3) involved the construction and screening of a NS-1 cDNA library, and the subsequent isolation of two cDNAs. The nucleotide (nt) sequences of these cDNAs (K4-1.1 & K3-1.1) differ only in their 5' ends, and are assumed to be products of alternative splicing from the same gene. Significant sequence identity was discovered when the amino acid sequences of the open reading frames (ORFs) were compared with a protein previously characterized in the human system, termed ICAM-1(244).

In the second phase of this research (described in chapter 4), a partial genomic sequence was characterized, and the authenticity of the larger cDNA clone (K3-1.1) was determined. The isolated genomic sequence spans a region of 4 kb and contains 5 exons in the 3' region of the gene. A partial restriction map of the gene was derived from Southern blot analyses. A possible mRNA transcript corresponding to K3-1.1 was analyzed by Northern blot and polymerase chain reaction (PCR) analyses. Additionally, an antisera against the 5' end of the protein translated from the K3-1.1 cDNA was developed using synthetic peptides, and NS-1 cell lysates were examined by Western blot and immunoprecipitation for the detection of a protein encoded by the K3-1.1 cDNA.

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CHAPTER 2

MATERIALS AND METHODS

2.1 SOURCES OF MATERIALS

2.1.1 Animals

C57BL/6 (B6) and BALB/c mice were obtained from Charles River Canada, Quebec, Canada. Fisher rats were also obtained from Charles River Canada.

2.1.2 Cells and Antibodies

NS-1 cells (BALB/c myeloma) (American Type Culture Collection, ATCC, Rockville, MD) and P388D$_1$ cells (a monocyte-macrophage cell line)(a gift from N. Reiner, Infectious Diseases, VGH) were maintained in tissue culture in Dulbecco's modified minimum essential media (DMEM) supplemented with 5% heat-inactivated fetal bovine serum, 50 U/ml penicillin, and 50µg/ml streptomycin. TF-1 cells (an erythroleukemia cell line)(a gift from J.D. Thacker,Terry Fox Lab), and L cells (a gift from K. Humphries, Terry Fox Lab) were maintained in tissue culture in DMEM supplemented as above with the addition of human IL-3 (5ng/ml) to the TF-1 cell line. The rat monoclonal antibody (MAb) YN1/1.7 that recognizes the murine activated lymphocyte antigen 2 (MALA-2), was previously generated in our laboratory by immunization of Fisher rats with NS-1 cells and fusion of recovered immune cells with the rat myeloma Y3 , and screening of the hybridomas for activity against activated lymphocytes. MAb YN1/1.7 was purified from ascites fluid by $(NH_4)_2SO_4$ precipitation (50% saturation) followed by DEAE affi-gel blue (Bio-Rad Laboratories, Richmond, CA) chromatography.

2.1.3 Materials for Genetic Studies

The oligo dT-cellulose was obtained from Pharmacia (Uppsala, Sweden). All DNA reaction enzymes and restriction enzymes used in this study were obtained from Pharmacia, Bethesda Research Laboratories (BRL) (Burlington, Ontario), or New England Biolabs (Mississauga, Ontario). Reactions were carried out as per the manufacturers instructions. Nitrocellulose filters were obtained from Schleicher and Schull (Keene, NH), and nylon membranes (Zetaprobe) were obtained from BioRad Laboratories. All ^{32}P-nucleotides were

purchased from New England Nuclear (Boston, MA) , or ICN Biomedicals (Canada, Ltd). The ^{125} I nucleotides were purchased from Amersham (Oakville, Ontario).

2.2 cDNA CLONING

2.2.1 Synthetic Oligonucleotide Probes

From tryptic peptide sequences obtained prior to this thesis study, three antisense oligonucleotides were synthesized. Two oligonucleotide probes (27mer, 42mer) were nonredundant based on a preferred codon usage table (1), while the third (17mer mix) had a redundancy of 64. All oligonucleotide probes were synthesized by an Applied Biosystems DNA synthesizer (Dr. M. Smith's laboratory, University of British Columbia, Vancouver, BC), and were 5' end-labelled for screening purposes. Initially, test reactions using various amounts of [γ^{32}P]-dATP (4500 Ci/mmol) were analyzed in a polyacrylamide gel to determine the optimal amount of labelled nucleotide to use. Briefly, 10 pmol of probe was mixed with T4 kinase buffer (10x=500mM Tris pH 7.4,100mM $MgCl_2$, 50mM DTT), 10 units (U) T4 polynucleotide kinase, and 5,10, or 20 pmol (15,30, 60 µCi) [γ^{32}P]-dATP in a total volume of 20 µl and incubated at 37^{o}C 1 hour (hr). The reaction was stopped with 200 µl CH_3COONH_4 (2M) and analysed on a 20% acrylamide gel (38:2 acrylamide:bis, 10M urea, 1% ammonium persulphate, TEMED(N,N,N',N'-Tetra-methylethylenediamine) in 1x TBE=0.1M Tris-HCl, 0.09 boric acid, 2mM EDTA) electrophoresed at 350V 0.5 hr and exposed to X-ray film. The reaction which still had a slight excess of unincorporated labelled nucleotide was used to label the probe routinely. The labelled probe was separated from unincorporated [γ^{32}P]-dATP on a Sephadex G50 column (Pharmacia)(bed volume 3 ml), heat denatured, and added to the hybridization solution.

2.2.2 Library Construction

A nonsize-selected cDNA library of NS-1 was constructed in the λgt10 vector in our laboratory. Briefly, total RNA was extracted from NS-1 cells ($2x10^8$) with urea/lysis buffer (7M urea, 2% sarkosyl, 350mM NaCl, 10mM Tris-HCl pH 7.9, 1mM EDTA) and homogenized for 20 minutes (min) on ice (2). One gram (g) of CsCl was added per 2.5 ml lysate and the

mixture was laid over a 3.5 ml CsCl cushion (5.7M CsCl, 0.1M EDTA), and centrifuged at 28,500 revolutions per minute (rpm) at 20°C for 20 hrs (SW 28.5 rotor, Beckman L8-60M Ultracentrifuge). The supernatant was carefully decanted and the RNA pellet was resuspended in 10mM Tris-HCl pH 7.5, 5mM EDTA, 1% SDS, microfuged 1 min, and the supernatant collected. The pellet was redissolved in the above Tris/EDTA/SDS solution, centrifuged, and the supernatants pooled. This RNA solution was extracted with 4:1 $CHCl_3$:butanol, centrifuged at 2500 rpm for 10 min (JA-20 rotor, Beckman J2-21 centrifuge), and the aqueous phase was collected. The organic phase was reextracted with the above Tris solution, the aqueous layers were pooled, and reextracted with equal volume of 4:1 $CHCl_3$:butanol. The aqueous layer was collected and ethanol precipitated, centrifuged at 5000 rpm for 20 min at -10°C (JA-20 rotor, Beckman J2-21 centrifuge), and the pellet was resuspended in distilled H_2O (dH_2O), and quantitated spectrophotometrically ($1OD_{260}$=40μg/ml).

Poly A^+ RNA was isolated by two passages over an oligo dT-cellulose column. Typically, the total RNA sample was centrifuged, resuspended in 1 ml dH_2O, heated to 65°C for 5 min, cooled on ice, mixed with 1 ml of 2x loading buffer (1x=0.5M NaCl, 20mM Tris pH 7.6, 1mM EDTA, 0.1% SDS) and loaded onto the column preequilibrated with the same buffer. The eluate was immediately collected, reheated and reapplied to the column. The column was washed 4x with 4 ml of 0.5M NaCl loading buffer (0.5M NaCl, 20mM Tris pH 7.6, 1mM EDTA, 0.1% SDS), and 4x with 4 ml of 0.1M NaCl loading buffer (0.1M NaCl, 20mM Tris pH 7.6, 1mM EDTA, 0.1% SDS) to remove the poly A^- fractions. Poly A^+ RNA (mRNA) was eluted with 5x 2 ml elution buffer (10mM Tris-HCl pH 7.6, 1mM EDTA, 0.05% SDS), and quantitated spectrophotometrically at OD_{260}. The high OD_{260} fractions were pooled.

Synthesis of cDNA was done according to the method of Gubler and Hoffman (3). Ten μg of poly A^+ RNA was used as a template for first strand cDNA synthesis. Briefly, the poly A^+ RNA was dissolved in 10 μl dH_20 and 4μg of oligo dT was added in a total volume of 40 μl. This mixture was heated at 65°C for 10 min then cooled on ice, and added to 60 μl of a

premixed cocktail containing (0.5M Tris-HCl pH 8.3, 1M KCl, 0.1M $MgCl_2$, 20mM of each dNTP, 0.1M DTT, 0.1M NaPPi, 8Uof reverse transcriptase). This cDNA synthesis reaction was incubated for 30 min at 42°C, then additional reverse transcriptase (2U) was added and the solution was incubated for a further 15 min. For second strand cDNA synthesis the reaction mixture was cooled on ice, and then mixed with 40 µl of 5x second strand synthesis buffer (1x= 20mM HEPES pH 6.9, 20mM KCl, 4mM $MgCl_2$), 20mM of each dNTP, 2 µl [α^{32}P]-dCTP (800 Ci/mmol), 1M $(NH_4)_2SO_4$, bovine serum albumin (BSA) (1mg/ml), 1mM β–NAD, RNase H (1U/µl), E.coli Polymerase I (5U/µl), and E.coli ligase (1U/µl) in a total volume of 200 µl and incubated at least 24 hrs at 15°C. The synthesis reaction was stopped by the addition of 0.5M EDTA, and was extracted with 1:1 Tris saturated phenol:chloroform ($CHCl_3$), collecting the aqueous phase and reextracting the organic phase with TE pH 7.6. The pooled aqueous phase was then extracted with 24:1 $CHCl_3$: isoamyl alcohol (IAA), the organic phase was reextracted with TE pH 7.6, again pooling the two aqueous phases.

The recovered cDNA was precipitated with CH_3COONH_4 and ethanol. The length of the first and second strands was analysed by alkaline agarose gel electrophoresis by standard methods (4).

Throughout the synthesis of the first and second strand of cDNA, aliquots of the reaction mixture were taken at different time points and mixed with [α^{32}P]-dCTP (800 Ci/mmol) and spotted onto Whatman glass microfibre filter papers for the purpose of tracing the synthesis of cDNA. These filters were later analyzed by trichloroacetic acid precipitation, and the incorporation of the [α^{32}P]-dCTP was used as a measure to calculate the amount of cDNA produced from the poly A^+ RNA.

The synthesized cDNA was methylated and the ends were filled in by standard methods (4). *Eco R1* linkers, endlabelled with [γ^{32}P]-dATP (4500 Ci/mmol) , were ligated to cDNA in a solution containing 0.5M Tris-HCl pH 7.6, 0.1M $MgCl_2$, and dH_2O, which was heated at 45°C for 10 min and then mixed with 0.1M DTT, 10mM ATP, and T4 ligase, and further incubated at 15°C for 60 hrs. The cDNA and *Eco R1* linker construct was digested with *Eco R1* to yield

sticky ends and passed over a A5M column to purify the cDNA/linker from unbound linkers. Labelled cDNA/linker fractions were pooled, ligated into λgt10 arms, and packaged (Gigapack II, Stratagene, La Jolla, CA). The resulting library was plated on E.coli C600 high frequency lysogeny (Hfl) and the phage number in the library calculated. The titre of the cDNA library was approximately $1x10^6$ plaque forming units (pfu) in total.

2.2.3 Screening of λgt10 library

The cDNA library was plated on E.coli C600 Hfl at $5x10^4$ pfu per 22x22 cm^2 plate, and the plaques were lifted twice on to separate nitrocellulose filters and lysed in situ (0.5M NaOH,1.5M NaCl,denaturation;1M Tris-HCl, 3M NaCl, neutralization). The filters were baked for 2 hrs at 80°C under vacuum. They were prewashed 1 hr at 42°C in 50mM Tris-HCl (pH 8.0), 1M NaCl, 1mM EDTA, and 0.1% SDS as described by Maniatis *et al.* (4), followed by prehybridization in 6xSSC (1xSSC= 0.15M NaCl and 0.015M sodium citrate, pH 7.0), 5x Denhardt's (1x Denhardt's= 0.2 mg/ml Ficoll, 0.2 mg/ml polyvinylpyrrolidone, and 0.2 mg/ml BSA), 0.5% SDS, and 0.1 mg/ml denatured salmon sperm DNA in polyethylene envelopes for 3-4 hrs at the hybridization temperatures (see below).

Probe 1 (27mer) was used for the initial screening of the cDNA library. Hybridization was carried out overnight (O/N) in 6xSSC, 5x Denhardt's, 0.01M EDTA, 0.5% SDS, and 0.1 mg/ml salmon sperm DNA, at 45°C as derived from the formula T_m= 16.6 log $[Na^+]$ + 0.41 (GC%) + 81.5 - P_m - B/L - 0.65(P_f) where [Na+]=0.9M, GC%=67, P_m= 25% mismatch, B= 675, L = 27, and $P_{formamide}$=0 (5). The filters were then washed 15 min at R.T. in 2xSSC and 0.1% SDS, 2 hrs at 30°C in 1xSSC and 0.5% SDS, and 30 min at 30°C 1xSSC and 0.5% SDS. Filters were airdried, wrapped in Saran Wrap, and exposed to Kodak XAR film O/N at -70°C with an intensifying screen (Cronex, Dupont).

Positive clones were identified, isolated, and further analyzed by *Eco R1* digestion and Southern blot analysis to ensure that cross-hybridization of the probe with λgt10 vector was not occurring. Briefly, a 5 ml culture was incubated O/N, lysed with $CHCl_3$, and centrifuged at 10 000 rpm for 10 min at 4°C (JA-20 rotor, Beckman J2-21 centrifuge). RNase A and DNase I

(1μg/ml final) were added to the lysate and it was incubated at 37°C 30 min. The phage particles were precipitated by the addition of 4 ml 20% polyethylene glycol (PEG) and 2M NaCl in SM (50mM Tris-HCl pH 7.5, 0.1% gelatin, 0.1M NaCl, 8mM $MgSO_4 \cdot 7H_2O$) on ice for greater than 1 hr. The phage particles were pelleted at 10 000 rpm 20 min 4°C (JA-20 rotor, Beckman J2-21 centrifuge), resupended in 500 μl SM and lysed by 5 μl 10% SDS, and 5 μl 0.5M EDTA pH 8.0 at 68°C 15 min. This was followed by extraction with an equal volume of i) Tris saturated phenol, and ii) 24:1 chloroform ($CHCl_3$):IAA. The phage DNA from the aqueous phase was isopropanol precipitated, resuspended in TE (10mM Tris-HCl pH 8.5, 1mM EDTA pH 8.0) and quantitated by agarose gel electrophoresis with ethidium bromide (EtBr) (5μg/ml) in Tris-borate buffer (TBE)(0.05M Tris, 0.05M Boric acid, 1mM EDTA). A suitable amount of phage DNA was digested with *Eco R1* to liberate the cDNA insert and this digestion mixture was electrophoresed in a 1% agarose gel with EtBr (5μg/ml) in TBE, and alkaline blotted O/N onto a nylon membrane (Zetaprobe). The phage Southern blot was hybridized in 1.5xSSPE (1xSSPE= 0.18M NaCl, 10mM NaH_2PO_4 , 1 mM EDTA pH 7.4), 1% SDS, 0.5% (w/v) non-fat powdered milk (Blotto) and 0.5 mg/ml denatured salmon sperm DNA with probe 1 (27mer) at 45°C (25% mismatch), and with probe 2 (42mer) at 49°C (30% mismatch). Hybridization with probe 3 (17mer mix) was done at 43°C as derived from the formula (GC x 4°C + AT x 2°C) - 5°C = T_{hyb} (6) and in 6xSSC, 10x Denhardt's, 0.1% SDS, 50mM sodium phosphate (pH 6.5), and 0.1 mg/ml denatured salmon sperm DNA.

The library was rescreened after it was discovered that clone K1-8 (2.0 kb) lacked a 5' translation initiation site. Plaque lift hybridizations were done at 65°C and 50°C initially using cDNA fragments (without poly A tail) generated from clone K1-8 digested with *Pvu II*. The 5' most fragment of clone K1-8 digested with *Hind III* was used for subsequent screenings. Both cDNA probes were isolated and purified by agarose gel electrophoresis, electroelution, and labelled by nick translation (Nick Translation Reagent Kit, BRL). Typically, 1 μg of probe, 50 μCi of [α^{32}P]-dCTP (800 Ci/mmol), and nucleotides dATP, dGTP, dTTP were incubated 1 hr at 15°C with Polymerase I. The labelled probe was purified by passage over

Sephadex G-50 nick column (Pharmacia) equilibrated with TE, and heat denatured prior to addition to the hybridization mix.

2.2.4 cDNA Sequencing

The cDNA inserts were excised by *Eco R1* digestion from the phage DNA of the positive clones, K1-8, K3-1.1, and K4-1.1, and were subcloned by standard methods (4) into the *Eco R1* sites of plasmids pTZ19R (K1-8, K3-1.1) and pUC19 (K4-1.1) (United States Biochemical Corporation, Cleveland, OH). Typically, a large amount of cDNA insert was isolated from 1L of culture as follows. The O/N culture (multiplicity of infection=0.005) was fully lysed by 5 ml $CHCl_3$, centrifuged 8 000 rpm 20 min at 4°C (JA-20 rotor, Beckman J2-21 centrifuge) to pellet the debris. Sixty grams of NaCl (1M final) and 70g PEG (7% final) was added to the lysate and the phage precipitated by incubation on ice for about 5 hrs. The phage were centrifuged at 8 000 rpm 30 min at 4°C (JA-20 rotor, Beckman J2-21 centrifuge) and resuspended in a low volume of phi 80 buffer (0.1M NaCl, 0.2M Tris-HCl pH 7.4, 10mM $MgCl_2$), extracted with equal volume of $CHCl_3$, and centrifuged at 5 000 rpm 15 min at 4°C (JA-20 rotor, Beckman J2-21 centrifuge). CsCl (0.5 g/ml) was added to the aqueous phase and the solution was placed over a CsCl step gradient and centrifuged O/N at 22 000 rpm 15°C (SW28.1 rotor, Beckman L8-60M Ultracentrifuge). Using a 21G needle, the blue band of phage particles was recovered, and 1.5g/ml CsCl in phi 80 buffer was added to fill an SW41 ultracentrifuge tube. This continuous CsCl gradient was centrifuged 32 000 rpm 15°C O/N (SW41 rotor, Beckman L8-60M Ultracentrifuge), the blue band collected, and then diluted with 1x dialysis buffer (1M Tris-HCl pH 7.9, 3M NaCl) to a total volume of 40 ml and the phage were pelleted out at 22 000 rpm 15°C for 2 hrs (SW28.1 rotor, Beckman L8-60M centrifuge). The phage recovery was titrated by plating on host bacteria and then extracted as follows i) Tris saturated phenol (2x), ii) 1:1 phenol:$CHCl_3$ (1x), and iii) 24:1 $CHCl_3$:IAA (1x). The resulting phage DNA was precipitated with ethanol and digested with*Eco R1* to liberate the cDNA insert, which was then ligated into the appropriate vector.

Typically, the vector was precut with the appropriate restriction enzyme, ligated to the cDNA insert, and the resulting construct was transformed into the host cells (NM522 for pTZ19R, and DH5α for pUC 19). White transformed colonies were picked and a plasmid miniprep was done as follows. Two ml cultures were incubated O/N at 37°C with constant shaking. Bacteria were pelleted at 9000 rpm 15 min at 4°C (JA-20 rotor, Beckman J2-21 centrifuge), and 100 µl TE glucose buffer (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0) was added and mixed by inversion. Then 200 µl of fresh 0.2N NaOH/1% SDS was added, placed on ice for 5 min, and 150 µl of 3M CH_3COOK, 2M CH_3COOH pH 4.8 (ice cold) was added and mixed by inversion. The lysate was microfuged 10 min at 4°C, the supernatant removed to a new tube, and extracted once with Tris saturated phenol and once with 24:1 $CHCl_3$:IAA. The aqueous phase was recovered and precipitated with isopropanol, microfuged, washed with 70% ethanol, and resuspended in TE pH 7.5. Aliquots (1 µl) were tested on 0.8% agarose/EtBr/TBE gels. These templates were used for sequencing the ends of the cDNA inserts. For the purpose of generating deletion clones, the 5' piece of K3-1.1 (*Eco R1/Hind III* fragment of pTZ19R/K3-1.1) was subcloned into the *Hinc II* site of pUC 19.

Large plasmid preparations were done to obtain a large amount of plasmid DNA for making deletion clones. Plasmids were isolated from 1L culture. Bacteria were centrifuged at 5000 rpm 4°C (JA-20 rotor, Beckman J2-21 centrifuge) and resuspended in 25% sucrose and 0.05M Tris-HCl pH 8.0. Lysozyme (10mg/ml) in 0.25M Tris-HCl pH 8.0 was added to lyse the bacterial cell wall, and then 250mM EDTA pH 8.0 was also mixed in. Triton X-100 buffer (1% Triton X-100, 62.5mM EDTA pH 8.0, 50mM Tris-HCl pH 8.0) was slowly added dropwise to the lysate with constant stirring. This viscous solution was centrifuged 19 000 rpm 4°C 30 min (JA-20 rotor, Beckman J2-21 centrifuge), and the cell lysate recovered. CsCl and EtBr (10mg/ml) were added at 0.9mg/ml and 1/20 volume respectively, and the solution was centrifuged O/N at 46 000 rpm (VTi50 rotor, Beckman L8-80M Ultracentrifuge). The lower band was recovered by side puncture and centrifuged again O/N at 62 000 rpm (VTi80 rotor, Beckman L8-80M Ultracentrifuge). The lower band was again recovered by side puncture and

extracted with NaCl saturated isopropanol. Plasmid DNA was dialyzed against 2L of TE pH 7.5 with several changes over 3 days. The yield and purity of the plasmid DNA was assessed by spectrophotometrically (A260/A280, 1 OD_{260}=50μg/ml).

The recovered plasmid DNA was digested with the appropriate restriction enzymes to yield a linear form of the plasmid, and timed digestions with exonuclease III (7) were carried out. Insert size decreased with increased digestion time and the ends were filled in, religated, and the constructs were retransformed into host cells. The deletion transformants were analysed by a plasmid miniprep, *EcoR1* digestion, and electrophoresis on a 1% agarose gel with EtBr (5μg/ml) in TBE buffer.

Both pTZ19R and pUC19 plasmids have priming sites on either side of the multiple cloning sites allowing double-strand DNA sequencing. The ends of the cDNA inserts were intially sequenced from templates (plasmid miniprep) from positive transformants, and then deletion clones spanning across the cDNA insert. cDNA sequencing was carried out by dideoxy chain termination method (8) using sequencing kits (Sequencing kit, Pharmacia). Typically, the template was alkaline denatured (2N NaOH,2mM EDTA) for 5 min at R.T., precipitated with ethanol, and annealed to the primer at 37°C 15 min in *Hin I* buffer (60mM NaCl, 6mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 6mM β–mercaptoethanol (β-ME)). [α^{32}P]-dATP (800 Ci/mmol) was added to each template/primer sample, and aliquoted into four reaction tubes containing either ddATP, ddTTP, ddGTP, or ddCTP. Klenow fragment of DNA polymerase (2U) was added and the reactions were incubated at 48°C for 10 min. At this point, a chase solution (dNTPs, Klenow) was added and the mixture further incubated at 48°C 10 min. Formamide stop solution (0.1% xylene cyanol, 0.1% bromophenol blue, 10mM EDTA, 95% formamide) halted the reactions. The extended fragments were heat denatured at 70°C 3 min, placed on ice, and electrophoresed in a prerun 6% acrylamide gel (19:1 acrylamide:bis , 8.32M urea in 1x TBE=0.1M Tris-HCl, .09M boric acid, 2mM EDTA) for 3-5 hrs at 55W using a sequence gel apparatus (International Biotechnologies, New Haven, CT). The gel was lifted from the glass plate on to Whatman #3 paper and exposed to Kodak XK film

O/N at -70°C without an intensifying screen. Regions rich in G/C were resequenced using a Deaza sequencing kit (Pharmacia), and later, T7 polymerase was used in place of Klenow for better resolution.

2.3 PURIFICATION OF MALA-2

2.3.1 Large Scale Preparation of Cell Membranes and Lysates

NS-1 cells ($5.7x10^9$) were washed 3 times in sterile phosphate buffered saline (PBS), resuspended in 10 mM Tris-HCl pH 8.0, and lysed by shearing the cells by passage through a 26Gauge (G) needle 6-7 times. The lysate was centrifuged twice 10 min 4°C at 2000 rpm (Beckman TJ-6 centrifuge), to remove nuclei and insoluble materials, and the supernatants were pooled and centrifuged 30 min 4°C at 18 000 rpm (JA-14 rotor, Beckman J2-21 centrifuge). The pellet was drained and stored at -20°C until further use.

The crude cell membranes were resuspended in 10 ml cold lysis buffer (10mM Tris-HCl pH 7.5, 1% Triton X-100, 0.15M NaCl, and 0.01% NaN_3) and further broken up using a tissue homogenizer. The solution was split in two and diluted to 600ml each with 1% Triton X-100 lysis buffer (10mM Tris-HCl pH 7.5,1% Triton X-100, 0.85% NaCl, 0.01% NaN_3) and phenylmethylsulfonylfluoride (PMSF) was added to final concentration of 0.1mM. In general, 1.5 L of 1% Triton X-100 lysis buffer was used for every 10^{10} cells processed. The lysis mixture was stirred on ice for 30 min and then centrifuged for 60 min at 10 000 rpm 4°C (JA-20 rotor, Beckman J2-21 centrifuge). The lysate was then immediately used in affinity purification. For the purpose of tracing the course of the antigen purification, a 2 ml lysate prepared from $1x10^7$ ^{125}I surface labelled iodinated cells, was added to the large scale lysate just prior to affinity chromatography.

2.3.2 Affinity Chromatography

The YN1/1.7 MAb was purified from ascites fluid by $(NH_4)_2SO_4$ precipitation (50% saturation) followed by dialysis against 1 L 0.1M $NaHCO_3$. The dialyzed solution was analysed for specific antibody activity by i) indirect binding assay using NS-1 cells as targets, and ii) inhibition of MLR. The dialyzed YN1/1.7 was then coupled to a 4 ml volume of

preactivated Affi-gel 10 agarose beads (Bio-Rad Laboratories) at 4°C with constant agitation. A control affinity column was made using an irrelevant MAb YE1/48 purified from ascites by $(NH_4)_2SO_4$ precipitation followed by DEAE-affigel 10 (BioRad) column chromatography, and coupled with 4 ml of Affigel 10 agarose beads. Both sets of coupled beads were washed extensively with elution buffer (see below) followed by lysis buffer before each use.

The YN1/1.7 antigen (MALA-2) was isolated by three cycles of affinity chromatography as described below. In the first cycle, the large scale NS-1 cell lysate was incubated with about 4 ml of YN1/1.7 MAb coupled agarose beads on ice for 4 hrs with constant agitation. The beads were then packed into a column and were thoroughly washed O/N with 10mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 0.15M NaCl and 0.01% NaN_3 until no radioactivity could be detected in the flow through. The column was briefly washed with 10mM Tris-HCl buffer (pH 7.5) containing 0.1% Triton X-100, 0.15M NaCl and 0.01% NaN_3 before the adsorbed antigen was eluted with 100mM glycine-HCl buffer (pH 2.9) containing 0.05% Triton X-100, 0.15M NaCl and 0.01% NaN_3. The radioactive fractions were pooled and immediately neutralized with a few drops of 1M Tris-HCl buffer (pH 8.0). In the second cycle, an irrelevant Ab, YE1/48, was coupled to 4 ml of Affi-gel 10 agarose beads, and the semipurified MALA-2 fractions were incubated with these beads for 1.5 hr on ice with constant stirring. The flow through was immediately collected and the column washed with 3 ml lysis buffer which was pooled with the first fraction. Finally, the third cycle involved the incubation of the column fraction with 0.5 ml of YN1/1.7 coupled beads for 2 hrs on ice with constant agitation. The beads were then packed into a column and were washed with 50 ml lysis buffer prior to elution with the same glycine-HCl buffer as above. The resulting radioactive fractions were pooled and immediately neutralized with 1M Tris-HCl pH 8.0.

2.3.3 Assessment of Purity and Yield

The amount of MALA-2 was quantitated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to standard methods (4) using a Mini-slab discontinuous gel apparatus (8cmx10cmx1mm) (BRL). The buffer in the separating gel consisted of 375 mM

Tris-HCl pH 8.6 and 0.1% SDS, while the running buffer was 192 mM glycine, 250 mM Tris-HCl pH 8.8, 1% SDS . The molecular weights were determined from pre-stained standard markers (BRL). The gel was fixed by washing 2x in 25% methanol, 10% acetic acid for 30 min, followed by rinsing 3x in dH_2O for 20 min, and washing again in 10% glutaraldehyde, with a final rinse 8x in dH_2O for 10 min. The gel was silver stained in 0.074% NaOH, 1.4% NH_4OH, 0.81% $AgNO_3$, 14.8% ethanol for 15 min, followed by a rinse 3x in dH_2O for 15 min, and was developed in 10% ethanol, 0.005% citric acid, 0.019% formaldehyde. The intensities of the purified antigen bands were visually compared to those of BSA loaded on the same gel at 0.03-0.5 μg quantities. The purified MALA-2 protein was subsequently used for a binding assay done by F. Takei (9). The soluble MALA-2 used in Western blots as a positive control was a gift from C. Welder, Terry Fox Lab (because there was not enough purified MALA-2). Soluble MALA-2 was purified by affinity chromatography and quantitated by SDS-PAGE .

2.4 GENETIC ANALYSES

2.4.1 cDNA Inserts as Probes

Complementary DNA inserts K3-1.1 and K4-1.1, were excised by *Eco R1* digestion from subcloned plasmids, pTZ19R and pUC19, respectively, and were used for the genetic analyses described in the following. Both cDNA inserts and any probes derived from them (K4-EN, K4-Xho, K3-Asp, Xho-Nco, Bgl I, Sal I) were isolated and purified by agarose gel electrophoresis and electroelution. The probes were labelled by random primer extention (10) (Oligolabelling Kit, Pharmacia) using [α^{32}P]-dCTP (3000 Ci/mmol). Typically, 50 ng of probe was added to 200 ng hexanucleotides in a final volume of 28 μl, heated at 95°C 3 min and cooled on ice 1 min. To this was added 4 μl of 10x HLB (500mM HEPES pH 6.9, 100mM $MgCl_2$ and 60mM β-ME)), 4 μl of nucleotide mix (dGTP, dATP, dTTP at 2.5M each), 4 μl of [α^{32}P]-dCTP (3000 Ci/mmol) and 4U of Klenow. The reaction was incubated at R.T. for 1 hr and then stopped by the addition of 5 μl 4M NaOH. A Sephadex G-50 DNA Grade nick column was used for size separation of the labelled probe from unincorporated [α^{32}P]-dCTP . An average reaction

would yield 10^7cpm/400 µl of probe recovered. The probes were heat denatured prior to addition to the hybridization solution.

2.4.2 Genomic Southern Blot Analysis

Genomic DNA was prepared from cultured cells and tissue cells by the SDS/proteinase K method (11). Briefly, the cells were washed 3 times in sterile PBS, the red blood cells (RBCs) were lysed in Tris NH_4Cl, and the remaining cells resuspended in 2 ml TNE buffer (20mM Tris-HCl pH 7.4, 10mM NaCl, 0.10 mM EDTA-Na_2), and lysed by the addition of 20 µl 20% SDS and 10 µl proteinase K (stock 10mg/ml; Sigma Chemical Co., Mississauga, Ontario). This mixture was incubated at least 6 hrs at 37°C, followed by extraction with 2 ml i) Tris saturated phenol (3x), ii) 1:1 Tris phenol:$CHCl_3$ (3x), and iii) 24:1 $CHCl_3$:IAA (2x). The aqueous phase was recovered and dialyzed against 2 L of TE buffer with 3 changes over 36 hrs. The DNA was then quantified spectrophotometrically (1 OD_{260}=50µg/ml) and stored in sterile aliquots at 4°C.

Approximately 10 µg of DNA was digested with various restriction enzymes, precipitated with ethanol, dried, and redissolved in 1xTAE buffer (40mM Tris-HCl pH 7.2, 20mM $CH_3COONa \cdot 3H_2O$, 1mM EDTA-$Na_2 \cdot 2H_2O$) with Ficoll loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% ficoll type 400). The DNA fragments were electrophoresed in a 0.8% agarose gel containing 5 µg/ml EtBr in TAE buffer for 16 hrs at 12 volts (V). The gel was depurinated with 0.1M HCl for 15 min. and treated with 0.5M NaOH and 1.5M NaCl for 30 min. The gel was rinsed with dH_2O and neutralized with 1M Tris-HCl pH 7.0 and 3M NaCl for 30 min. It was blotted onto nylon membranes with 20x SSC O/N. The DNA was crosslinked to the filter by exposure to UV light and the filter was prehybridized in 6x SSC, 10% deionized formamide, 1% SDS, 2mM EDTA, 1% Blotto, and 0.5 mg/ml denatured salmon sperm DNA for 1-2 hrs at 60°C. The filter was hybridized in the same solution with the addition of 10% dextran sulphate O/N at 60°C (12). After hybridization, the filter was washed twice for 30 min at 60°C in 0.3xSSC, 0.1% SDS, and 0.1% Na pyrophosphate, wrapped in Saran Wrap and exposed to Kodak XAR film at -70°C with an intensifying screen.

The filters were stripped of probe by washing 2x 300ml each in boiling 1% SDS for 20 min. The filters were rinsed in 2xSSC briefly, and exposed to film overnight to be sure the signal was gone.

2.4.3 Northern Blot Analysis

Total cellular RNA was extracted from cell lines using the acid guanidine isothiocynate/CsCl method of Davis *et al.* (4) . Briefly, the cells were washed three times in sterile PBS and pelleted into microfuge tubes at $5x10^7$cells/tube. The cells were lysed in 7.0 ml of 4M guanidine isothiocyanate, 0.025M CH_3COONa pH 6.0, and 0.8% β-ME. This was laid over a 4 ml CsCl cushion (5.7M CsCl, 0.025M CH_3COONa pH 6), and spun O/N at 32 000 rpm at 20°C (SW41 rotor, Beckman L8-60M Ultracentrifuge). The RNA pellet was resuspended in 300 µl of 0.3M CH_3COONa pH 6 and ethanol precipitated with 750 µl ethanol. The pellet was centrifuged 10 min at 14 000 rpm at 4°C (JA-20 rotor, Beckman J2-21 centrifuge), and washed with 80% ethanol. The RNA was quantified spectrophotometrically ($1OD_{260}$=40µg/ml) and stored at -70°C under ethanol.

The quality of RNA was checked in a 1.0% agarose gel containing EtBr (5µg/ml) in TBE buffer electrophoresed at 100V for 1.5 hrs. Poly A^+ RNA was obtained by passage over an oligo dT-cellulose column as previously described (section 2.2.2). Approximately 10 µg of RNA per sample was resuspended in RNA loading buffer (72% formamide, 1x MOPS, 26% formaldehyde, 8% glycerol, 8% bromophenol blue), and electrophoresed at 100V for 2 hrs in 1.0% agarose/0.66M formaldehyde/1xMOPS buffer (20mM 3-[N-morpholino] propanesulphonic acid pH 7.0, 5mM CH_3COONa, 1mM EDTA) (4). The gel was rinsed twice for 20 min in 500 ml of 10xSSC and transferred to nylon membrane O/N with 10xSSC. The filter was UV crosslinked, prehybridized and hybridized for 1-2 hrs and overnight respectively, in 3x SSPE, 1% SDS, 0.5% Blotto, and 0.5mg/ml denatured salmon sperm DNA. After hybridization, the filters were washed three times for 15 min at R.T. as follows i) 2x SSC, 0.1% SDS, ii) 0.5xSSC, 0.1%SDS, iii) 0.1x SSC, 0.1% SDS. The final stringency wash was done for 30 min at 55-60°C in 0.1x SSC, 1% SDS. The filters were wrapped in Saran

Wrap and exposed to XAR film at -70°C with an intensifying screen. The filters were stripped of probe by washing in 10 ml 50% formamide, 10mM Na_2PO_4 pH 6.7 at 65°C for 30 min, and then rinsed with 0.1xSSC, 0.1% SDS at 65°C for 30 min. The filters were exposed to film O/N to ensure the signal was gone.

2.4.4 Polymerase Chain Reaction Analysis

RNA was extracted from NS-1 and P388D1 cells ($5x10^7$) as previously described (section 2.4.3). Approximately 40 µg was resuspended in 125 µl of dH_2O and denatured at 65°C for 10 min and quenched on dry ice. The cDNA synthesis reaction used in experiment 1 was as follows, 5x reverse transcriptase buffer (1x=50mM Tris-HCl pH 8.3, 40 mM KCl 1mM DTT, 6mM $MgCl_2$), acetylated BSA (0.1mg/ml), oligo dT (25ng/µl), dNTP 500ng, RNasin 0.7U/µl (Promega-Fisher Scientific, Vancouver, Canada), and reverse transcriptase (20U/µl) were added to the RNA in a total volume of 300 µl. The synthesis reaction was incubated at 42°C for 1 hr and stored at 4°C O/N. The cDNA synthesis reaction used in experiment 2 and 3 employed random primers (17ng/µl)(hexanucleotides, Pharmacia) instead of oligo dT . Reactions were also run without reverse transcriptase in all experiments as a control for contamination of cDNA reagents.

PCR reactions were carried out with 5 µl of the cDNA reaction mixture by mixing PCR 10x buffer (1x=50mM KCl, 10mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$), dNTP 300ng/µl, and Taq polymerase (5U) (BRL) together with the primers (10ng/µl final) in a total volume of 50 µl. The PCR reaction was performed using 30 cycles of 94°C 1 min, 55°C 2 min, and 72°C 3 min, with a final fill-in step of 72°C 4 min. Ten µl of the reaction was analysed on a 2% agarose gel with EtBr (5µg/ml) in TAE buffer electrophoresed at 100V for 1.5 hrs. The gel was photographed and blotted under alkaline conditions (0.4M NaOH) to nylon membrane O/N. The filter was prehybridized, and hybridized with the K3-Asp probe 1-2 hrs and O/N respectively in 3x SSPE, 1% SDS, 0.5% Blotto, 0.5mg/ml denatured salmon sperm DNA. After hybridization, the filter was washed three times for 15 min at R.T. as follows i) 2xSSC/0.1% SDS, ii) 0.5XSSC/0.1%SDS, and iii) 0.1xSSC/0.1%SDS. The final stringency

wash was done at 60°C for 30 min in 0.1xSSC/1% SDS. Three sets of primers were used to generate different PCR products. The first two sets of primers were internal controls for K3-5' and K4-5' regions respectively. The third set of primers was to amplify the K3-1.1 product if it existed. An actin control was also run to assess the quality of cDNA synthesis. These four reactions were done on cDNA synthesized from both NS-1 and P388D1 cells. Negative controls with cDNA were done to ensure there was no contamination of the primers. Positive controls using the isolated cDNAs as templates were also done to illustrate the expected size of the products.

2.5 GENOMIC CLONING

2.5.1 Genomic Libraries

The genomic library, size selected for *Bam HI* 4.0 kb fragments was made in our laboratory (F.Takei) from EL-4 cells in Charon 27 vector (*Bam HI* sites). The BALB/c embryo library purchased from ATCC, was constructed from a *Mbo I* partial digest, and had an average insert size of 16-20 kb in Charon 28 vector (*Bam HI* sites). The BALB/c liver library purchased from BioCan Scientific Inc (Mississauga, Ontario), was constructed from a *Sau 3A* partial digest with an average insert size of 8-21 kb in EMBL-3 SP6/T7 vector (*Bam HI* sites). Two other size selected libraries were also constructed in the process of this thesis study. One library was made from C57BL/6 spleen DNA cut with *Dra I* and size selected for 3.5-5.5 kb fragments. The other library was made from BALB/c spleen DNA cut with *Bam HI* and size selected for 15-23 kb fragments. DNA fragments were separated by agarose gel electrophoresis, electroeluted, and ligated into the indicated vectors (pUC19, Pharmacia; lambdaGEM, Promega) cut with the same enzymes, respectively.

2.5.2 Genomic Library Screening

The EL-4 and BALB/c embryo libraries were plated on E. coli C600 at $5x10^4$ pfu per 22x22 cm^2 plate. The BALB/c liver library was plated on E. coli NM539 at $5x10^4$ pfu per 22x22 cm^2 plate. All plaques were lifted twice onto separate nylon membranes and lysed in situ (0.5M NaOH, 1.5M NaCl, denaturation; 1M Tris-HCl, 3M NaCl, neutralization). The phage

DNA was crosslinked to the filter by exposure to UV light. The filter was prewashed 1 hr at 42°C in 50mM Tris-HCl (pH 8.0), 1M NaCl, 1mM EDTA, and 0.1% SDS, followed by prehybridization in 3x SSPE , 1% SDS, 0.5% Blotto, and 0.5mg/ml denatured salmon sperm DNA at 55°C in polyethylene envelopes for 1-2 hrs. Hybridization was done in the same solution with the addition of ^{32}P labelled probe O/N at 55°C. The filter was washed three times for 15 min at R.T. as follows i) 2xSSC, 0.1% SDS ii) 0.5xSSC, 0.1% SDS, and iii) 0.1x SSC, 0.1% SDS. The final stringency wash was done at 55°C in 0.1x SSC, 1% SDS for 30 min. The filters were airdried, wrapped in Saran Wrap, and exposed to Kodak XAR film O/N at -70°C with an intensifying screen. Control filters with K4-1.1 in λgt10 were also prepared and included in some of the hybridizations (Tables X & XI) to ensure lifting of the plaque DNA and hybridization with the probe was adequate in the library screening.

Positive plaques were identified, isolated, and further analysed by *Bam HI* (EL-4, BALB/c embryo libraries), and *Xho I* (BALB/c liver library) digestion, and Southern blot analysis to ensure that cross-hybridization of the probe with the respective vectors was not occurring. Briefly, a phage miniprep was prepared as previously described (section 2.2.3), and the phage DNA was digested with *Bam HI* or *Xho I* to liberate the insert. The digestion was electrophoresed in a 1% agarose gel with EtBr (5μg/ml) in TAE buffer and alkaline blotted O/N onto nylon membrane. Hybridization of the phage Southern blot was done in 3x SSPE as per library screening protocol (section 2.5.1) but the final stringency wash was done at 60°C.

2.5.3 Genomic Sequencing

The genomic inserts were excised by *Bam HI* digestion from the phage DNA of the positive clones J4 (Table XI) , G1 and G3 (Table XII), and were subcloned by standard methods (3) into the *Bam HI* sites of pTZ19R and pTZ18R. A large phage preparation was done to isolate a large amount of the J4 insert (section 2.2.4), both for subcloning purposes and restriction site analysis. The J4 insert was subcloned into both pUC19 and pUC18, and the ends were sequenced. However instead of generating deletion clones with exonuclease III, the

J4 insert was cut with *Pst I* and the resulting fragments were subcloned into pUC18 and sequenced. The G1 and G3 inserts were isolated from plate lysates. Briefly, the phage clones were plated on host bacteria at a density that resulted in confluent plaques. Six to ten 8.5x8.5 cm^2 plates were prepared per clone and 3 ml of SM was incubated on the plate O/N at 4°C. The SM/phage solution was collected and spun at 5000 rpm 10 min 4°C (JA-20 rotor, Beckman J2-21 centrifuge) to remove the debris. DNase and RNase were added to the recovered lysate (1μg/ml final) and it was incubated at 37°C for 30 min. The phage particles were precipitated with 20% PEG, 2M NaCl in SM on ice for at least 1 hr. The phage were recovered by centrifugation at 10 000 rpm 20 min 4°C (JA-20 rotor, Beckman J2-21 centrifuge). The phage were resuspended in 500 μl SM and lyzed with 5 μl 10% SDS and 5 μl 0.5M EDTA at 68°C for 15 min. The aqueous phase was extracted once with Tris saturated phenol, once with 24:1 $CHCl_3$:IAA, and precipitated with an equal volume of isopropanol. The G1 and G3 inserts were partially liberated from the phage arms by a *Bam HI* digest, and a resulting 4 kb fragment was subcloned into pUC18 and the ends were sequenced. The G3 4.0 kb fragment was also cut with *Pst I* and the fragments were subcloned and sequenced similar to the J4 clone. Comparison of the G1 and G3 sequences showed that they were identical to the J4 clone.

Sequencing of these genomic inserts was carried out by dideoxy chain termination method (7) using sequencing kits (Pharmacia). All of the plasmids used have priming sites on either side of the multiple cloning sites allowing double stranded DNA sequencing. The ends of the genomic inserts were initially sequenced by the isolation of templates (plasmid miniprep, section 2.2.4), and then partial sequences were obtained from the *Pst I* fragments subcloned into pUC19 vector. Oligonucleotide primers (D. Freeman, Terry Fox Lab) were used to span the remaining gaps and allow the complete sequence to be delineated. Typically, the template was alkaline denatured (2N NaOH, 2mM EDTA) for 5 min at R.T., precipitated with ethanol, and annealed to the primer at 37°C 15 min in Pharmacia annealing buffer (Tris buffer containing $MgCl_2$ and DTT). [α^{32}P]-dCTP (3000 Ci/mmol), labeling mix (dNTPs

without dCTP), and T7 polymerase (3U total) were added to each template/primer sample, incubated at R.T. for 3 min (total volume 20 µl). Then each sample was divided into four and added to the termination mix containing either ddGTP, ddCTP, ddATP, or ddTTP and incubated at 37°C for 5 min. The reactions were halted with formamide stop buffer and the extended fragments were heat denatured at 92°C 5 min, and separated in a prerun 6% acrylamide gel as previously described (section 2.2.4).

2.6 ANTISERA STUDIES

2.6.1 Synthetic Peptides

Two synthetic peptides (9 amino acids each) were obtained from the Tripartite Microanalytical Center in the Department of Biochemistry and Microbiology at the University of Victoria. One was unique to the N-terminal of K3-1.1 and the other was common to both K3-1.1 and K4-1.1 coding sequences. Approximately 10 mg of each peptide was obtained, and dissolved in a small volume of DMSO and brought up to 200 µl with dH_2O. Each synthetic peptide was conjugated to keyhole limpet hemocyanin (KLH)(Sigma Chemical Co.) at 1:1 weight ratio by the addition of glutaraldehyde (0.2%) (Fisher Sci.), and constant stirring for two hours at R.T. Rats were prebled to obtain preimmune serum, and then injected intraperitoneally with 200 µg of peptide/KLH conjugate in PBS with an equal volume of Freund's adjuvant (Gibco/BRL). Rats were injected initially with complete Freund's adjuvant and peptide, and then four times with incomplete Freund's adjuvant and peptide. Sera was collected ten days post-injection. Sera was tested by enzyme linked immunosorbent assay (ELISA) to assess the titre against the peptides.

2.6.2 ELISAs

96 well microtitre plates (Nunc-Immuno, Gibco/BRL) were coated O/N at 4°C, with 100 µl of Blotto (5%), KLH (200 µg/ml), control peptide (200 µg/ml), or specific peptide (200 µg/ml) in 10mM Tris-HCl pH 8.0. The control peptide was a 15mer encoding a partial sequence of the β subunit of IL-3 receptor (WERSLAGAEETIPLQ)(a gift from Rob Cutler, Terry Fox Lab). Unbound sites on the wells were blocked with 450 µl of Blotto (5%) 1 hr at R.T. Serum from

the rats was diluted serially by 1/3 and 100 µl was added to each well in duplicate for 30 min at R.T. The wells were washed twice with Hank's balanced Salt Solution (HBSS) and the excess fluid was flicked off. 100 µl of goat anti-rat Ig (GαRIg)-horseradish peroxidase (BRL) ($7.5x10^{-6}$ dilution) in 9:1 HBSS:Blotto (1%) was added for 30 min at R.T. after which the wells were washed twice with HBSS. 50 µl of the substrate (o-Phenylenediamime Dihydrochloride, Sigma Chemical Co.), at 2mg/ml in 0.1M Na_2P0_4 pH 7.1 with 0.03% H_2O_2 was added for 15 min at R.T. , and the reaction was stopped by the addition of 3M HCl (50 µl). The absorbance was read at 490 nm and the values were averaged and the background was subtracted.

2.6.3 Western Blot Analysis

NS-1 and LTK- (nontransfected L cells; without thymidine kinase) were harvested and washed with PBS. They were counted and resuspended in 2x SDS sample buffer (125mM Tris-HCl pH 6.8, 5% glycerol, 2% SDS, 0.001% bromophenol blue, and +/- β-ME) The cells were lysed and the DNA sheared by drawing the solution through 18G and 26G needles repeatedly. The cell lysate was stored at -20°C and 10 µl ($2.5x10^5$ cells) was loaded onto a 10% SDS-PAGE gel for Western blot analysis.

Cell lysate and soluble MALA-2 (500 ng) samples were heated at 65°C (-β-ME; nonreduced), or 95°C (+β-ME;reduced) for 5 min and loaded onto gels as previously described (section 2.3.3). The samples were electrophoresed at 100V for 2 hrs, and either stained with Coomassie Blue R-250 to visualize the proteins or blotted to nitrocellulose for sera detection. Coomassie Blue R-250 staining involved immersing the gel in 0.1% Coomassie Blue R-250, 50% methanol, 7% acetic acid solution for 15 min, and destaining O/N in 50% methanol and 7% acetic acid. The gel was then covered in Saran Wrap and dried. For antigen detection, proteins were blotted to nitrocellulose membrane in transfer buffer (25mM Tris-HCl, 192mM glycine, 20% methanol) at 300mA (90V) for 1 hr (Hoefer Scientific Instruments). The quality of transfer of the prestained markers was used as an indicator of protein transfer overall. The filter was preblocked O/N with 5% Blotto/1% BSA in PBS at 4°C. Rat sera was diluted to 1/100 in 1% Blotto/PBS (2 ml total volume) and added to the filter for two hrs at R.T. with

constant shaking. The YN1/1.7 tissue culture supernatant (2 ml) was used directly on the filter for one hr at R.T. The filter was washed twice with HBSS, and GαRIg-horseradish peroxidase (1/3000 dilution) in 1% Blotto/PBS was added for one hr at R.T. The filter was washed twice in HBSS and the protein bands were detected by enhanced chemiluminescence (ECL)(Amersham).

2.6.4 Immunoprecipitation

Affigel 10 beads (2ml bed volume) (Bio-Rad) were prewashed with cold dH_20 (3x bed volume) and combined with mouse anti-rat Ig (MαRIg) (3mg), and 0.1M $NaHC0_3$ in a total volume of 14 ml and was shaken O/N at 4^oC. The remaining active sites were blocked with 5 ml 1M Tris-HCl pH 8.0 one hr at R.T. with constant shaking. The MαRIg-beads were washed with PBS and stored at 4^oC in PBS, 0.02% azide.

Cells were surface labelled with ^{125}I by mixing $2x10^7$ cells in PBS with 5 µl ^{125}I (Amersham) with an iodogen bead (Pierce-Professional Diagnostic Inc., Edm, Alta) for 10 min on ice. The labelled cells were recovered, lysed, and precleared three times with MαRIg-beads as described above. Ten µl of YN1/1.7 supernatant, 1C preimmune sera, or 1C Aug 31 sera was added to the precleared ^{125}I cell lysate for 1 hr on ice, and then 100 µl bed volume MαRIg-beads were added. This mixture was incubated O/N at 4^oC . The beads were washed successively with i) lysis buffer (3x), ii) PBS, 1mM EDTA, 0.1% NP40 (1x), iii) PBS alone (1x). The beads were then split into two for each sample and 50 µl of 2x SDS loading buffer (+/- β-ME) was added. The samples were boiled for five min and loaded onto a 10% SDS-PAGE gel . The gel was run for two hrs at 100V , dried, and exposed to film.

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CHAPTER 3

CLONING OF MALA-2 CDNA

3.1 INTRODUCTION

The elucidation of lymphocyte antigens involved in cell activation and cell adhesion is important in understanding the immune response. Through the use of MAbs, the identification, characterization, and function of such antigens can be determined. A rat MAb, YN1/1.7, previously characterized in our laboratory recognizes a 95 kD protein called MALA-2 expressed on activated lymphocytes (1). This MAb partially inhibited Con A stimulated proliferation of spleen cells, and almost completely abrogated MLR (1). Thus, it was suggested that this antigen MALA-2 may be involved in lymphocyte activation. The first goal of this research was to clone the cDNA encoding the MALA-2 protein. Once the amino acid sequence of MALA-2 was elucidated, it could be compared to other known proteins to possibly define the secondary and tertiary structure of the protein as well as its function in the cell.

3.2 RESULTS

3.2.1 Isolation and analysis of MALA-2 cDNA

MALA-2 was purified by affinity chromatography and its tryptic peptide sequences determined previously (2). Three tryptic peptide sequences confirmed by repeated purification and amino acid sequencing experiments were used to synthesize oligonucleotide probes (Table V). Two non-redundant oligonucleotides were constructed based on a preferred codon usage table (3). The third oligonucleotide had a redundancy of 64.

A λgt10 cDNA library constructed from NS-1 mRNA was initially screened with the short non-redundant probe at low stringency (1X SSC, 30^{o}C) allowing 25% mismatch. From 10^5 plaques, 45 positive phage clones were isolated and screened with the other two probes. One phage clone, K1-8, hybridized with all three probes. The 2.0 kb cDNA insert was subcloned into pTZ19R plasmid vector and sequenced.

The sequence had a long ORF and polyadenylation signal but lacked an initiation codon (Figure 1a). Northern blot analysis of NS-1 RNA detected a transcript of >2.0 kb.

Table V

OLIGONUCLEOTIDE PROBES

amino acid	^{5}AspHisGlnAlaAsnPheSerCysArg^{3}	fraction #15a[@]
codon	2 2 2 4 2 2 6 2 6	(9216)[$]
mRNA[*]	gaccaccaggccaacuucuccugccgc	
probe	GCGGCAGGAGAAGTTGGCCTGGTGGTC	27mer
amino acid	PheGluSerLeuGluGlyLeuProAlaSerGluAlaArg	fraction #49[@]
codon	2 2 6 6 2 4 6 2 4 4 6 2 4 6	(6.37×10^{7})[$]
mRNA[*]	uucgagucccuggagggccuguucccugccucugaggcccgc	
probe	GCGGGCCTCAGAGGCAGGGAACAGGCCCTCCAGGGACTCGAA	42mer
amino acid	GlnMetProThrGlnGlu	fraction 28[@]
codon	2 1 4 4 2 2	(64)[$]
mRNA	caaaugccuacucaagaa g c c g g a a g g	
probe	TCTTGAGTAGGCATTTG C G G C T T C C	17mer mixture of 64

[*]nonredundant probes-based on preferred mammalian codon usage(3).
[@]number of fraction collected from HPLC purification of tryptic peptides of MALA-2 performed by Baker 1989(2).
[$]the codon redundancy.

Therefore, the K1-8 cDNA insert was considered incomplete and the library was rescreened, initially using K1-8 cDNA insert deleted of the poly A tail (*Pvu II* digestion) and subsequently the 5' *Hind III* fragment of K1-8 as probes. The screening identified 41 additional phage clones (16 positive with the 5' *Hind III* fragment), all with inserts of 2.2-3.0 kb. Two phage clones, K4-1.1 and K3-1.1, were selected for their long cDNA inserts (2.5 and 3.0 kb, respectively). The K3-1.1 was subcloned and both strands were fully sequenced. Conversely, the K4-1.1 was subcloned and only the 5' end was sequenced until the sequence was found to be fully identical to K3-1.1 after nt 132 in K4-1.1 (4). The K4-1.1 clone is 2525 bp long with a 5' untranslated sequence of 29 bp, a 1611 bp ORF, and a 3' untranslated region of 851 bp (Figure 1b). The 3' untranslated region contains a polyadenylation signal and a poly A tail. The K3-1.1 clone is 3031 bp long and has a 5' untranslated sequence of 553 bp (Figure 1c), and a long ORF of 1593 bp. The two clones have an identical sequence with the exception of their 5' ends. They each have unique untranslated sequences, and those encoding leader and N-terminal sequences. Both clones contain AUUUA sequences implicated in degradation of mRNA (5). K3-1.1 has two such sequences in the 5' untranslated region, one in the ORF, and both clones have four such sequences in the 3' untranslated region. The K4-1.1 clone encodes a typical type I transmembrane protein with a highly hydrophobic N-terminal amino acid sequence which probably functions as a leader sequence, and a hydrophobic region of 24 residues typical of transmembrane domains (underlined by a bold line in Figure 1b), followed by a cluster of highly charged amino acids. In contrast, the K3-1.1 clone has a long 5' untranslated region (553 bp) with multiple initiation codons (underlined in Figure 1c). Their ORF protein sequences are shown in Table VI. The codon at position 14 best corresponds to the consensus sequence proposed by Kozak (6), but the ORF terminates at position 134. Two other codons (positions 205 and 411) also partially satisfy the criteria, however these lack long ORFs. The amino acid sequence immediately following the initiating codon encoding MALA-2 contains mainly charged or polar amino acids lacking hydrophobicity commonly found in leader sequences of type I transmembrane proteins (7). Therefore, the K4-1.1 clone is

a)

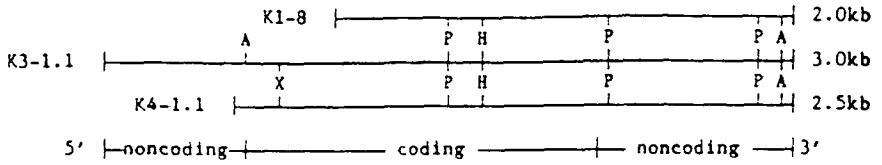

Figure 1 Sequence of MALA-2 cDNAs. **a)** A partial restriction map of the MALA-2 cDNA clones is shown. A-*Asp 700I*, P-*Pvu II*, H-*Hind III*, X-*Xho I*. The cDNA clone K1-8 was initially isolated. Subsequently, the full length cDNA clones K3-1.1 and K4-1.1 were isolated and sequenced. **b)** Complete nucleotide sequence of MALA-2 cDNA (K4-1.1) and predicted amino acid sequence are shown. Cysteine residues are in bold letters, and the potential glycosylation sites are marked by -CHO-. The transmembrane domain is underlined by a bold line. The sequences identified by the tryptic peptide sequencing are underlined, and discrepant amino acid residues are marked by asterisks. The polyadenylation signal sequence in the 3' untranslated segment is also underlined, and the ATTTA sites are boxed. The amino acid sequence is numbered from the predicted cleavage site of the signal peptide. **c)** The nucleotide sequence of K3-1.1 cDNA and the deduced amino acid sequence is shown. Only the 5' end of the sequence is shown. The sequence that is identical to those of the K4-1.1 clone are in bold and underlined. The ATG codons are also underlined and the ATTTA sites are boxed.

b)

```
K4-1.1 cDNA          CGCTACCTAGCACTTTGCCCTGGCCCTGCAATGGCTTCAACCCGTGCCAAGCCCACGCTACCTCTGCTCCTGGCCCTGGTCACCGTTGTGATCCCT  95
                                                    MetAlaSerThrArgAlaLysProThrLeuProLeuLeuLeuAlaLeuValThrValValIlePro
                                                    -25
GGGCCTGGTGATGCTCAGGTATCCATCCATCCCAGAGAAGCCTTCCTGCCCCAGGGTGGGTCCGTGCAGGTGAACTGTTCTTCCTCATGCAAGGAGGACCTCAGCCTGGGCTTGGAGACT  215
GlyProGlyAspAlaGlnValSerIleHisProArgGluAlaPheLeuProGlnGlyGlySerValGlnValAsnCysSerSerSerCysLysGluAspLeuSerLeuGlyLeuGluThr  37
        +1                                                                  ---CHO---
CAGTGGCTGAAAGATGAGCTCGAGAGTGGACCCAACTGGAAGCTGTTTGAGCTGAGCGAGATCGGGGAGGACAGCAGTCCGCTGTGCTTTGAGAACTGTGGCACCGTGCAGTCGTCCGCT  335
GlnTrpLeuLysAspGluLeuGluSerGlyProAsnTrpLysLeuPheGluLeuSerGluIleGlyGluAspSerSerProLeuCysPheGluAsnCysGlyThrValGlnSerSerAla  77

TCCGCTACCATCACCGTGTATTCGTTTCCGGAGAGTGTGGAGCTGAGACCTCTGCCAGCCTGGCAGCAAGTAGGCAAGGACCTCACCCTGCGCTGCCACGTGGATGGTGGAGCACCGCGG  455
SerAlaThrIleThrValTyrSerPheProGluSerValGluLeuArgProLeuProAlaTrpGlnGlnValGlyLysAspLeuThrLeuArgCysHisValAspGlyGlyAlaProArg  117

ACCCAGCTCTCAGCAGTGCTGCTCCGTGGGGAGGAGATACTGAGCCGCCAGCCAGTGGGTGGGCACCCCAAGGACCCCAAGGAGATCACATTCACGGTGCTGGCTAGCAGAGGGGACCAC  575
ThrGlnLeuSerAlaValLeuLeuArgGlyGluGluIleLeuSerArgGlnProValGlyGlyHisProLysAspProLysGluIleThrPheThrValLeuAlaSerArgGlyAspHis  157
                          R  G  E                                                                                R  G  D
GGAGCCAATTTCTCATGCCGCACAGAACTGGATCTCAGGCCGCAAGGGCTGGCATTGTTCTCTAATGTCTCCGAGGCCAGGAGCCTCCGGACTTTCGATCTTCCAGCTACCATCCCAAAG  695
GlyAlaAsnPheSerCysArgThrGluLeuAspLeuArgProGlnGlyLeuAlaLeuPheSerAsnValSerGluAlaArgSerLeuArgThrPheAspLeuProAlaThrIleProLys  197
***    ---CHO---                                                    ---CHO---
CTCGACACCCCTGACCTCCTGGAGGTGGGCACCCAGCAGAAGTTGTTTTGCTCCCTGGAAGGCCTGTTTCCTGCCTCTGAAGCTCGGATATACCTGGAGCTGGGAGGCCAGATGCCGACC  715
LeuAspThrProAspLeuLeuGluValGlyThrGlnGlnLysLeuPheCysSerLeuGluGlyLeuPheProAlaSerGluAlaArgIleTyrLeuGluLeuGlyGlyGlnMetProThr  237
                                                  ***
CAGGAGAGCACAAACAGCAGTGACTCTGTGTCAGCCACTGCCTTGGTAGAGGTGACTGAGGAGTTCGACAGAACCCTGCCGCTGCGCTGCGTTTTGGAGCTAGCGGACCAGATCCTGGAG  835
GlnGluSerThrAsnSerSerAspSerValSerAlaThrAlaLeuValGluValThrGluGluPheAspArgThrLeuProLeuArgCysValLeuGluLeuAlaAspGlnIleLeuGlu  277
            ---CHO---
ACGCAGAGGACCTTAACAGTCTACAACTTTTCAGCTCCGGTCCTGACCCTGAGCCAGCTGGAGGTCTCGGAAGGGAGCCAAGTAACTGTGAAGTGTGAAGCCCACAGTGGGTCGAAGGTG  955
ThrGlnArgThrLeuThrValTyrAsnPheSerAlaProValLeuThrLeuSerGlnLeuGluValSerGluGlySerGlnValThrValLysCysGluAlaHisSerGlySerLysVal  317
                                                                                                    ---CHO---
GTTCTTCTGAGCGGCGTCGAGCCTAGGCCACCCACCCCGCAGGTCCAATTCACACTGAATGCCAGCTCGGAGGATCACAAACGAAGCTTCTTTTGCTCTGCCGCTCTGGAGGTGGCGGGA  1075
ValLeuLeuSerGlyValGluProArgProProThrProGlnValGlnPheThrLeuAsnAlaSerSerGluAspHisLysArgSerPhePheCysSerAlaAlaLeuGluValAlaGly  357
                                                  ---CHO---
AAGTTCCTGTTTAAAAACCAGACCCTGGAACTGCACGTGCTGTATGGTCCTCGGCTGGACGAGACGGACTGCTTGGGGAACTGGACCTGGCAAGAGGGGTCTCAGCAGACTCTGAAATGC  1195
LysPheLeuPheLysAsnGlnThrLeuGluLeuHisValLeuTyrGlyProArgLeuAspGluThrAspCysLeuGlyAsnTrpThrTrpGlnGluGlySerGlnGlnThrLeuLysCys  397
          ***                                                                   ---CHO---
CAGGCCTGGGGGAACCCATCTCCTAAGATGACCTGCAGACGGAAGGCAGATGGTGCCCTGCTGCCCATCGGGGTGGTGAAGTCTGTCAAACAGGAGATGAATGGTACATACGTGTGCCAT  1315
GlnAlaTrpGlyAsnProSerProLysMetThrCysArgArgLysAlaAspGlyAlaLeuLeuProIleGlyValValLysSerValLysGlnGluMetAsnGlyThrTyrValCysHis  437
   ***              *********                                                                  ---CHO---
GCCTTTAGCTCCCATGGGAATGTCACCAGGAATGTGTACCTGACAGTACTGTACCACTCTCAAAATAACTGGACTATAATCATTCTGGTGCCAGTACTGCTGGTCATTGTGGGCCTCGTG  1435
AlaPheSerSerHisGlyAsnValThrArgAsnValTyrLeuThrValLeuTyrHisSerGlnAsnAsnTrpThrIleIleIleLeuValProValLeuLeuValIleValGlyLeuVal  477
                  ---CHO---
ATGGCAGCCTCTTATGTTTATAACCGCCAGAGAAAGATCAGGATATACAAGTTACAGAAGGCTCAGGAGGAGGCCATAAAACTCAAGGGACAAGCCCCACCTCCCTGAGCCTGCTGGATG  1555
MetAlaAlaSerTyrValTyrAsnArgGlnArgLysIleArgIleTyrLysLeuGlnLysAlaGlnGluGluAlaIleLysLeuLysGlyGlnAlaProProPro                512

AGACTCCTGCTGGACCCCCTGCAGGCAACAGCTGCTGCTGCTTTTGAACAGAATGGTAGACAGCATTTACCCTCAGCCACTTCCTCTGGCTGTCCACAGAACAGGATGGTGGCCTGGGGG  1675
ATGCACACTTGTAGCCTCAGAGCTAAGAGGACTCGGTGGATGGAGCAAGACTGTGAACACGTGTGACCCGGACCCACCTACAGCCCGGTGGACCTTCAGCCAAGAAACGCTGACTTCGTT  1795
CTCTATTGCCCCTGCTGAGGGGTCCTGCTAAGGAAGACATGATATCCAGTAGACACAAGCAAGAAGACCACACTTCCCCCCGACACAGGAAAGCTGAGACATTGTCCCCATCTCTTCTTG  1915
ATGTATTTATTAATTTAGAGTTTTACCAGCTATTTATTGAGTACCCTGTATATAGTAGATCAGTGAGGAGGTGAATGTATAAGTTATGGCCTGGACCCTGCTGCAGATGCTGTGAGAGTC  2135
TGGGGAAAGATCACATGGGTCGACGGTTTCTCTACTGGTCAGGATGCTTTTCTCATAAGGGTCGACTTTTTTCACCAGTCACATAAACACTATGTGGACTGGCAGTGGTTCTCTGCTCCT  2255
CCACATCCTGGAGCGTCCCAGCACCTCCCCACCTACTTTTGTTCCCAATGTCAGCCACCATGCCTTAGCAGCTGAACAATCGAGCCTCATGCTCATGAAATCATGGTCCCAGGCGGCTCC  2375
ACCTCAAAGAGAAAGCCTGGAAGGAAATGTTCCAACTCCTTAGAAGGGTCGTGCAAGCTGCTGTGGGAGGGTAAGCACCCCTCCCAGCACAGAAACCTTTCCTTTGAATCAATAAAGTTT  2495
TATGTCGGCTGAAAAAAAAAAAAAAAAAAA                                                                                        2525
```

c)

K3-1.1

```
AATTCCTTTCACGATGGCAAATATTAGGAATTTATTAAAACCACTGTCTCCAACGGTACAAAAATTAAATATTAAGGCATCTTATACTCTCATCCTGACCTATGCAGCAAAGAGAATTATC  121
 TTAAATAAGAGCTAGCACTTATTCAAATGCTTTTCTAACGTATTTGGCAAATTTGTTGTTTTTGTTTTGTTCGGTGTACGGTCATGCATGCAAGTGTACCTGGTATTTGTATGTGTGTGC  241
 AAACGGGTGTATGCACATGTGCTTGCCTGTTGAGGCCAGAGATTTATTGTTCTACATCTTCCTCTATTGCTCTCTACCTGAACCAGGTGCTCAGTGACTCAGCCAGACTGTCAGGGCAGCA  361
 AGCTGGGGAATCCTCCTGTCTCTGCCTCCCCAGCTCTGCGTCTCAGGTCATGCCCCGCTTTTTATGTGGCTGTTGGGGATCTGAACTCAGGACCTCAGGCTTGCACAGCAAACCTTTACT  481
 GACTGAGCCACTTCTCCGGCCCCTCACTTTTCTTCTTATATTCTTTTATTATTCACATAACTGAAAGCATTCATGATCACACACCGGCATCCAGTCCGAGAGAAAAGCATAAACAGTTAT  601
                                                                                                  MetIleThrHisArgHisProValArgGluLysSerIleAsnSerTyr
                                                                                                  -19
CAATTTATTAAGGAGAAGCAGTTTCCTGCTGAAAATGAAGCCTTCCTGCCCCAGGGTGGGTCCGTGCAGGTGAACTGTTCTTCCTCATGCAAGGAGGACCTCAGCCTGGGCTTGGAGACT  721
GlnPheIleLysGluLysGlnPheProAlaGluAsnGluAlaPheLeuProGlnGlyGlySerValGlnValAsnCysSerSerSerCysLysGluAspLeuSerLeuGlyLeuGluThr
+1
```

Table VI[@]

AMINO ACID SEQUENCES OF HYPOTHETICAL PROTEINS TRANSLATED FROM INITIATION CODONS IN 5' REGION OF K3-1.1 cDNA CLONE

ATG Position(nt)	Amino acid sequence
14	MANIRNLLKPLSPTVQKLNIKASYTLILTYAAKRIILNKS
102	MQQRELS
148	MLF
205	MHASVPGICMCVQTGVCTCACLLRPEIYVLHLPLLLST
209	MQVYLVFVCVCLRVYAHVLAC
232	MCVQTGVCTCACLLRPEIYVLHLPLLLST
252	MHMCLPVEARDLCSTSSSIALYLNQVLSDSARLSGQQAGESSCLCLPSS ASQVMPRFLCGCWGSELRTSGLHSKPLLTEPLLRPLTFLLIFFYYSHN
258	MCLPVEARDLCSTSSSIALYLNQVLSDSARLSGQQAGESSCLCLPSSAS QVMPRFLCGCWGSELRTSGLHSKPLLTEPLLRPLTFLLIFFYYSHN
411	MPRFLCGCWGSELRTTSGLHSKPLLTEPLLRPLTFLLIFFYYSHN
425	MWLLGI

[@]taken from Carpenito 1990(4).

considered to code for a functional transmembrane protein, whereas K3-1.1 clone may represent an alternative splicing product, whose function is unknown.

Thus, MALA-2 is a type I transmembrane protein with an extracellular domain of 461 amino acids and a cytoplasmic domain of 28 amino acids. It has nine potential N-linked glycosylation sites and both an RGD and an Arg-Gly-Glu (RGE) sequence which are found within the extracellular domain of the protein. All of the tryptic peptide sequences are accounted for within the deduced amino acid sequence (underlined in Figure 1b), although a few discrepant residues were noted (marked by asteriks in Figure 1b). None of the discrepancies can be explained by a single base pair change in the cDNA sequence. In light of the low signals of these amino acid residues in the amino acid sequencing experiments (40pmol and below) we consider the cDNA-deduced sequence more reliable than those determined by peptide sequencing. Comparison of the cDNA sequence with the non-redundant probes based on tryptic peptides 2 and 7 (using a preferred codon usage criteria) revealed a 78% (peptide 2) and 69% (peptide 7) identity.

3.2.2 Sequence Similarity Studies

Comparison of amino acid and nucleotide sequences with those of published lymphocyte surface proteins revealed a striking similarity between MALA-2 and human ICAM-1 (HICAM-1). The similarity was evident both at the nucleotide level (Figure 2a) and the protein level (Figure 2b). The overall amino acid sequence identity of MALA-2 with HICAM-1 is 54% and both molecules share a common overall protein structure. All of the cysteine residues are conserved, and MALA-2 displays the internal repeat motif of HICAM-1. MALA-2 (MICAM-1) was also compared with human ICAM-2 (HICAM-2)(8), human ICAM-3 (HICAM-3)(9), and murine ICAM-2 (MICAM-2)(10) exhibiting amino acid sequence identities of 28%, 43%. and 30% respectively. All of the ICAMs and MALA-2 exhibit a repetitive motif approximately every 100 amino acid residues, indicating the presence of Ig like domains (Figure 3). The sequences surrounding the cysteine residues are highly conserved between the ICAMs and MALA-2, and demonstrate sequence similarity to other members of the Ig gene

a)

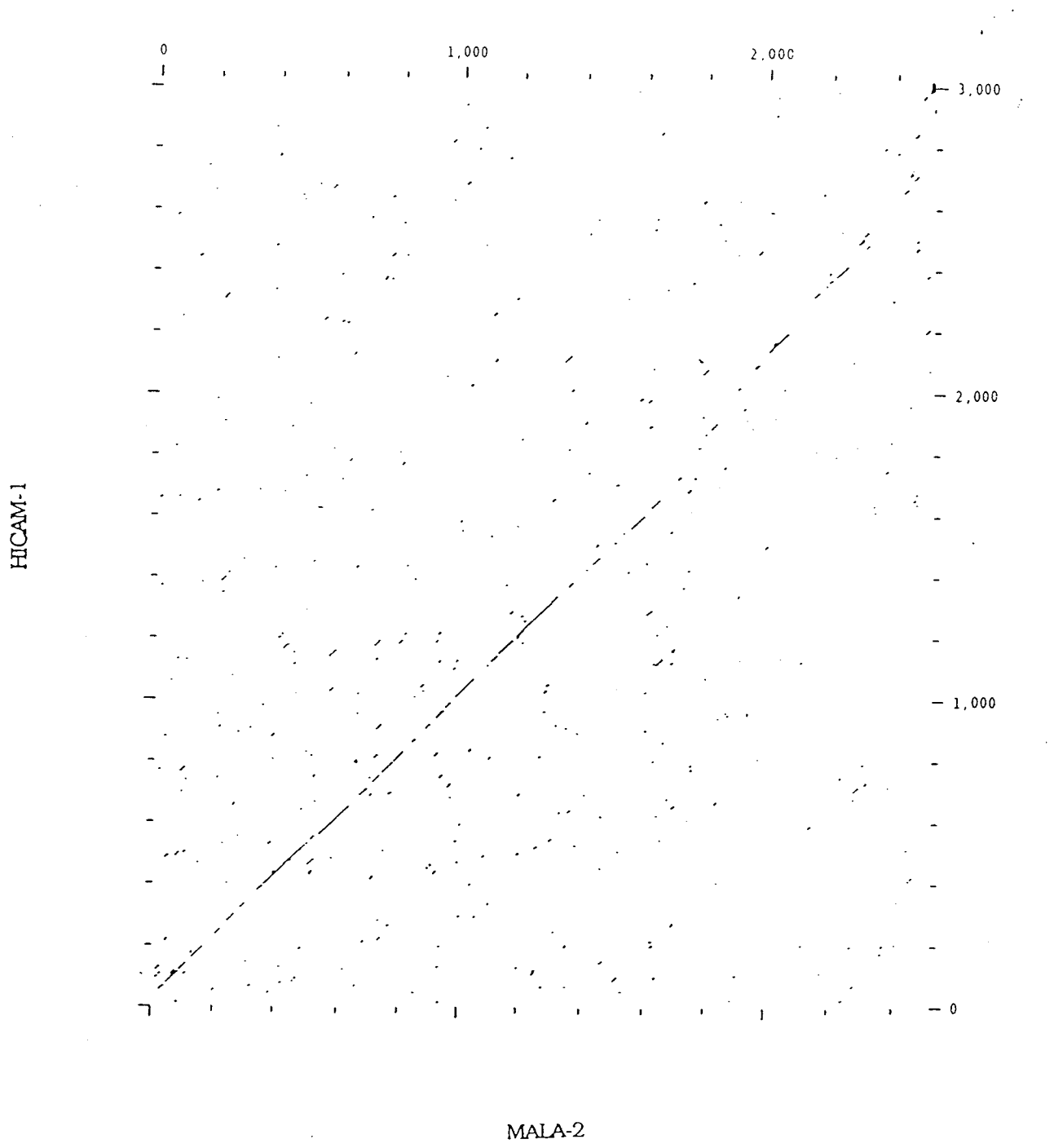

Figure 2 Similarity between MALA-2 and HICAM-1 sequences. **a)** The nucleotide sequence of MALA-2 is compared with the human ICAM-1 cDNA sequence (24). Diagonal dot-matrix comparison was used as follows, a window of 21 nucleotides was examined between each of the sequences, and when 14 nucleotides were identical a dot was plotted. **b)** The amino acid sequence of MALA-2 is aligned with that of the human ICAM-1. The third line shows the amino acid residues shared by the two sequences. Conserved cysteine residues are in bold and underlined.

b)

```
                                                1
MALA-2    MASTRAKPTLPLLLALVTVVIPGPG-DAQVSIHPREAFLPQGGSVQVNCSSSCKEDLSLGL   35
ICAM-1    MAPSSPRPALPALLVLLGALFPGPGGNAQTSVSPSKVILPRGGSVLVTCSTSCDQPKLLGI   35
           A      P LP LL L      PGPG  AQ S  P    LP GGSV V CS SC     LG

MALA-2    ETQWLKDE-LESGPNWKLFELSEIGEDSSPLCFENCGTVQSSASATITVYSFPESVELRPL   95
ICAM-1    ETPLPKKELLLPGNNRKVYELSNVQEDSQPMCYSNCPDGQSTAKTFLTVYWTPERVELAPL   96
          ET   K E L  G N K  ELS   EDS P C  NC   QS A    TVY  PE VEL PL

MALA-2    PAWQQVGKDLTLRCHVDGGAPRTQLSAVLLRGEEILSRQPVGGHPKDPKEITFTVLASRGD  156
ICAM-1    PSWQPVGKNLTLRCQVEGGAPRANLTVVLLRGEKELKREPAVG---EPAEVTTTVLV-RRD  153
          P WQ VGK LTLRC V GGAPR  L  VLLRGE  L R P  G    P E T TVL  R D

MALA-2    HGANFSCRTELDLRPQGLALFSNVSEARSLRTFDLPATIPKLDTPDLLEVGTQQKLFCSLE  217
ICAM-1    HGANFSCRTELDLRPQGLELFENTSAPYQLQTFVLPATPPQLVSPRVLEVDTQGTVVCSLD  214
          HGANFSCRTELDLRPQGL LF N S    L TF LPAT P L  P  LEV TQ    CSL

MALA-2    GLFPASEARIYLELGGQMPTQESTNSSDSVSATALVEVTEEFDRTLPLRCVLELADQILET  278
ICAM-1    GLFPVSEAQVHLALGDQRLNPTVTYGNDSFSAKASVSVTAEDEGTQRLTCAVILGNQSQET  275
          GLFP SEA   L LG Q      T   DS SA A V VT E   T  L C   L  Q  ET

MALA-2    QRTLTVYNFSAPVLTLSQLEVSEGSQVTVKCEAHSGSKVVLLSGVEPRPPTPQVQFTLNAS  339
ICAM-1    LQTVTIYSFPAPNVILTKPEVSEGTEVTVKCEAHPRAKVT-LNGVPAQPLGPRAQLLLKAT  335
            T   Y F AP   L   EVSEG  VTVKCEAH   KV  L GV   P  P  Q  L A

MALA-2    SEDHKRSFFCSAALEVAGKFLFKNQTLELHVLYGPRLDETDCLGNWTWQEGSQQTLKCQAW  400
ICAM-1    PEDNGRSFSCSATLEVAGQLIHKNQTRELRVLYGPRLDERDCPGNWTWPENSQQTPMCQAW  396
           ED  RSF CSA LEVAG    KNQT EL VLYGPRLDE DC GNWTW E SQQT  CQAW

MALA-2    GNPSPKMTCRRKADGALLPIGVVKSVKQEMNGTYVCHAFSSHGNVTRNVYLTVLYHSQNNW  461
ICAM-1    GNPLPELKC-LKDGTFPLPIGESVTVTRDLEGTYLCRARSTQGEVTREVTVNVL--SPRYE  454
          GNP P   C  K     LPIG    V     GTY C A S  G VTR V   VL  S

MALA-2    TIIILVPVLLVIVGLVMAASYVYNRQRKIRIYKLQKAQEEAIKLKGT-APPP            512
ICAM-1    IVIITVVAAAVIMGTAGLSTYLYNRQRKIKKYRLQQAQKGTAMKPNTQATPP            506
            II V    VI G      Y YNRQRKI  Y LQ AQ        T A PP
```

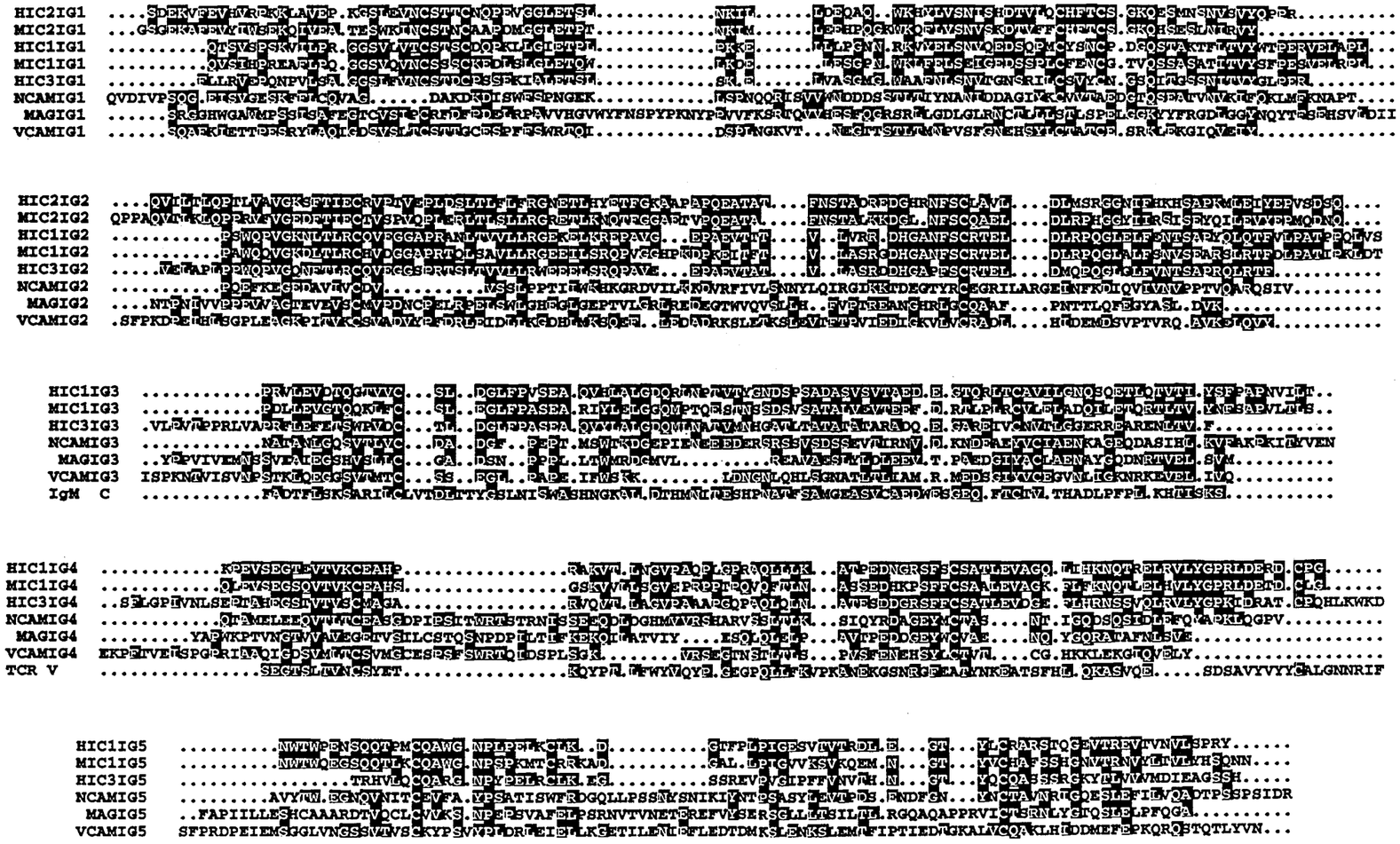

Figure 3 Ig Domain Homology of MALA-2, the ICAMs, and Members of the Ig Superfamily. Sequences were aligned using the Genetic Computer Group (University of Wisconsin) sequence analysis programs and by eye. The dots are gaps that have been introduced into the amino acid sequence to optimize alignment. Conserved residues between the Ig domains are boxed. HIC1-Human ICAM-1 (24), HIC2-Human ICAM-2 (8), HIC3-Human ICAM-3 (9), MIC1-Mouse ICAM-1 (MALA-2) (23), MIC2-Mouse ICAM-2 (10), NCAM-Neural CAM (13), MAG-Myelin Associated Glycoprotein (14), VCAM-Vascular CAM (15), TCR V (12), IgM C (11).

superfamily (11,12). Two neural proteins, NCAM (13) and MAG (14), show the most sequence similarity to with the ICAMs, as well as an endothelial adhesion protein, VCAM-1 (15). Table VII shows the percent amino acid sequence identity between various Ig domains of the ICAMs, NCAM, MAG, and VCAM-1. An arbitrary amino acid identity of $\geq$30% was chosen as being significant because it was 3x the standard deviation above the mean of a randomized sequence comparison (equivalent to $p \leq 0.01$). Amino acid sequence identity is highest between Ig domains of the same molecule from different species, for example, HICAM-1 with MALA-2 (MICAM-1), or HICAM-2 with MICAM-2. Comparing individual domains, domain II seems most highly conserved within the ICAMs as in HICAM-1 and HICAM-3 (77%), and HICAM-1 and MALA-2 (MICAM-1) (66%). There are several unexpected similarities (>30%) but the importance of these is difficult to determine. VCAM-1 has very high internal similarity between domains I and IV, II and V, and III and VI.

3.3 DISCUSSION

MALA-2 is a 95 kD monomer antigen expressed on murine activated lymphocytes and lymphoid cell lines (1). The MAb YN1/1.7 that detects this antigen inhibits MLR, suggesting that MALA-2 is involved in the activation of T cells. Two cDNA clones encoding MALA-2 were isolated and characterized in this thesis study. The amino acid sequence deduced from the cDNA clones contains all of the tryptic peptide sequences generated from purified MALA-2 (2) indicating that the isolated cDNA clones indeed code for MALA-2. Comparison of the amino acid sequences of the cDNA clones with known proteins demonstrates significant similarity to HICAM-1.

The size and distribution of HICAM-1 and MALA-2 are virtually identical (1,16,17). Both proteins are approximately 95-110 kD, and are expressed on vascular endothelium, dendritic cells, macrophages, HEV of lymphoid tissues, epithelial cells of the thymus, as well as mitogen activated lymphocytes (16,17,18). Variations in molecular weights are due to different glycosylation patterns (19). HICAM-1 is inducible on endothelial cells by inflammatory cytokines such as IL-1, IFNγ, and TNFα (20). Similarily, murine lymphocytes

Table VII

MATRIX OF IG DOMAIN SEQUENCE SIMILARITY (% amino acid identity) (≥30% significant*)

	HICAM-1					HICAM-2		HICAM-3					MICAM-1					MICAM-2		NCAM					MAG					VCAM						
HICAM-1	I	II	III	IV	V	I	II	I	II	III	IV	V	I	II	III	IV	V	I	II	I	II	III	IV	V	I	II	III	IV	V	I	II	III	IV	V	VI	VII
I																																			
II	13																																		
III	13	22																																	
IV	28	21	26																																
V	19	22	20	23																															
HICAM-2																																			
I	**35**	17	19	21	16																														
II	15	**34**	24	27	14	24																													
HICAM-3																																			
I	**38**	15	27	23	19	**37**	17																												
II	14	**77**	21	28	23	23	**38**	16																											
III	26	21	**47**	23	21	25	24	**30**	22																										
IV	15	19	14	**55**	24	9	22	15	22	20																									
V	14	16	18	9	**37**	16	13	19	20	15	12																								
MICAM-1																																			
I	**51**	20	26	29	14	**31**	17	**37**	22	22	16	13																							
II	16	**66**	20	27	28	22	**33**	16	**67**	27	21	19	23																						
III	14	26	**46**	23	19	17	22	19	**31**	**36**	13	16	18	21																					
IV	23	29	22	**61**	22	26	27	16	**32**	18	**45**	16	28	**35**	20																				
V	22	21	12	**30**	**52**	15	14	17	10	16	23	**35**	13	19	13	19																			
MICAM-2																																			
I	**30**	16	15	21	14	**57**	21	**31**	13	20	19	13	**34**	15	21	15	14																		
II	17	**38**	20	27	23	20	**58**	16	**36**	22	23	14	16	**36**	19	23	**30**	25																	
NCAM																																			
I	21	22	9	17	15	16	14	13	20	20	15	15	24	22	18	15	19	10	12																
II	14	13	20	16	13	16	16	10	18	14	14	20	17	19	16	15	17	13	22	25															
III	9	18	16	21	21	19	14	20	21	14	23	23	15	22	18	20	26	16	16	24	22														
IV	17	16	21	12	17	19	18	16	19	13	17	23	13	18	22	21	27	13	22	27	20	**31**													
V	15	23	28	25	29	16	19	19	10	18	18	19	15	13	21	16	26	20	18	23	20	20	**31**												
MAG																																			
I	21	18	21	26	23	16	20	21	19	15	20	12	21	18	15	17	18	18	19	13	15	17	13	20											
II	17	18	22	27	20	23	22	16	16	25	20	10	17	18	23	20	20	20	24	17	14	19	15	17	13										
III	25	20	16	24	**31**	16	17	11	16	16	26	23	22	13	27	27	**36**	14	27	20	26	**32**	**33**	28	28	18									
IV	21	16	27	23	29	18	19	17	13	19	25	27	24	8	24	17	26	20	13	18	20	23	**32**	**31**	26	15	**45**								
V	16	24	17	24	14	13	13	15	24	20	24	27	20	20	22	20	12	18	21	20	20	23	26	19	22	19	19	21							
VCAM																																			
I	21	15	23	18	23	20	18	21	24	21	20	25	22	16	18	19	20	18	17	25	23	27	26	**33**	20	20	20	20	18						
II	18	26	19	22	17	23	27	16	23	18	21	14	18	23	26	21	19	27	26	18	14	24	19	15	15	25	21	11	19	16					
III	27	18	16	28	26	19	21	27	21	25	17	18	19	22	27	17	28	16	17	25	27	**30**	**34**	27	24	22	24	28	19	25	22				
IV	24	21	26	15	19	22	22	23	17	20	17	17	28	21	25	28	24	22	20	26	24	25	**30**	29	21	17	21	21	25	**71**	14	30			
V	25	23	24	22	15	27	24	17	24	18	25	19	20	22	22	24	12	16	24	22	19	25	17	12	11	**34**	22	20	18	16	**60**	21	20		
VI	**30**	26	15	27	26	20	20	20	17	25	17	28	15	22	22	19	**30**	10	17	21	25	26	**32**	**30**	27	19	29	24	25	24	19	**61**	28	24	
VII	15	13	27	22	23	23	19	19	20	14	26	22	16	19	17	16	24	15	21	24	29	27	25	19	22	19	17	23	20	16	21	28	15	32	27

*amino acid identity ≥30% was chosen as being significant because it was 3x the standard deviation above the mean of a randomized sequence comparison. This is equivalent to a 1% probability of it being a random event or $p \leq 0.01$.

exposed to Con A and lipopolysaccharide (LPS) increase MALA-2 expression (1). HICAM-1 is regulated at least partly at the level of transcription (21), and promoter analysis by deletion constructs of the human gene suggests the involvement of cis-acting elements. Indeed, studies on MALA-2 mRNA indicate there are also sequences in the cytoplasmic and 3' untranslated region important for post-transcriptional regulation by IFNγ (M. Ohh, Terry Fox Lab, personal communication). Variable mRNA species are observed with both MALA-2 and HICAM-1 (22,23), but these are not easily explained on the basis of different poly A tails and may be due to the formation of secondary structures within the RNA.

Two cDNA clones were isolated in this thesis study. Both encode MALA-2 but vary in their 5' untranslated sequences and those which encode the leader and N-termini of the proteins. K3-1.1 has a large 5' untranslated region with ten start sites and a signal sequence containing charged residues. Conversely, K4-1.1 has a short 5' untranslated region and a typical hydrophobic signal sequence. COS cells transfected with K3-1.1 cDNA or K3-1.1 cDNA lacking the 5' untranslated region, stained negatively for the expression of MALA-2 as detected by fluorescence activated cell sorting (FACS) with YN1/1.7 MAb (4). Conversely, 23% of COS cells transfected with K4-1.1 cDNA were positive for MALA-2 expression. Northern blot analysis of these COS cells demonstrated that mRNA was present from all three cDNA constructs (4). The K4-1.1 translated protein was detected in transfected COS cells by Western blot analysis and immunoperoxidase staining with YN1/1.7 MAb, while the K3-1.1 translated protein was not. Therefore, it is possible that a K3-1.1 transcript is not properly processed to produce a protein, or that the K3 protein may not be recognized by YN1/1.7 MAb.

The nucleotide (K4-1.1) as well as the deduced amino acid sequences of MALA-2 have striking similarities with those of HICAM-1. The similarity between MALA-2 and HICAM-1 is particularily evident in the overall structures of these two proteins. All of the cysteine residues are conserved, and both molecules consist of five similar segments, each having a size of ~100 amino acid residues. In HICAM-1, these 5 Ig-like domains are arranged in an unpaired manner creating a rod-like structure (24). There is a short proline rich stretch of

amino acids between domains II and III creating a bend in the extracellular region. Conceivably, this configuration allows a larger surface area to be available to the LFA-1 receptor for binding, thereby enhancing the kinetics of the interaction (24). The proline rich stretch of amino acids at the possible hinge region is conserved in MALA-2.

The receptor for HICAM-1, LFA-1, belongs to the integrin family of adhesion molecules (25). Some integrins recognize the tripeptide sequence RGD (26). HICAM-1 lacks the RGD sequence but has a similar RGE sequence located within domain II (24). MALA-2 has both a RGD and a RGE sequence also located in domain II. The conservation of the RGE sequence suggests it may be involved in the binding of these ligands to LFA-1. However, deletion and mutation analysis of HICAM-1 has revealed that amino acid residues important to LFA-1 binding do not involve the RGD or RGE sites. Furthermore, the RGD peptides have been shown to have no effect on LFA-1/HICAM-1 interaction (25). Domains I and II of HICAM-1 have been identified as having the binding sites for LFA-1(27). Purified MALA-2 immobilized on microtiter plates binds Con A stimulated spleen cells and binding is specifically inhibited by antibody to MALA-2 or murine LFA-1 (23). The summation of this evidence strongly supports MALA-2 as the murine homologue of HICAM-1.

HICAM-1 plays an important role in the immune system. It is involved in antigen-dependent and antigen-independent interactions (28). The ICAM-1/LFA-1 interaction can enhance TCR activation and is important for several effector cell functions. MAbs to either ICAM-1 or LFA-1 inhibit CTL killing, T cell dependent Ab production, NK cell activity, and leukocyte/endothelial adhesion(28). The binding of LFA-1 to ICAM-1 stabilizes the formation of conjugates between cells and can act as a costimulatory signal to enhance lymphocyte activation (29). Similarily, the YN1/1.7 MAb inhibits MLR implying cell contact is necessary for cell activation (1). Mitogen activation of lymphocytes is only weakly affected by YN1/1.7 suggesting that ICAM-1 mediated cell contact may not be crucial. This can be explained by the fact that Con A is an agglutinating agent and a specific antibody would not affect cell to cell binding.

The ICAMs, NCAM, MAG, and VCAM-1 all display Ig-like domains and have conserved regions surrounding the cysteine residues. Domain II is the most highly conserved between HICAM-1, HICAM-3, and MALA-2, indicating it may be important to the function of these molecules. Indeed, the binding site of LFA-1 has been defined in domains I and II of HICAM-1(27) and specific amino acids crucial to LFA-1 binding are conserved between HICAM-1 and MALA-2. VCAM-1 demonstrates an internal sequence similarity between domains I, II, and III, and domains IV, V, and VI. This may be due to a duplication event involving the first three exons of the gene (15).

The characterization of MALA-2 as the murine homologue of HICAM-1 has important implications. The development of an in vivo mouse model will allow the importance of ICAM-1 in various immune responses to be defined. Manipulation of the K4-1.1 cDNA will allow derivatives of MALA-2 such as soluble MALA-2, and chimeric molecules to be created and these will be useful in further studies to understand the specifics of the LFA-1/ICAM-1 interaction.

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CHAPTER 4

GENOMIC CLONING AND K3-1.1 ANALYSES

4.1 INTRODUCTION

In the second phase of this thesis study, the genomic structure of the MALA-2 gene was partially characterized and the authenticity of the K3-1.1 transcript was examined. Genomic cloning was undertaken to: i) study the exon/intron organization of the MALA-2 gene, ii) determine whether the two cDNA clones represent alternative splicing products of a single gene, and iii) identify and study the regulatory elements of the MALA-2 gene. The K3-1.1 cDNA, one of the two cDNA clones isolated, has only been reported by our laboratory (1), and does not seem to be translated into a protein recognizable by YN1/1.7 MAb (2). Thus, the existence of a K3-1.1 transcript was examined by Northern blot and PCR analyses. In addition, an attempt was made to develop an antisera against a synthetic peptide within the N-terminal of the translated K3-1.1 protein to examine its possible existence in cells.

4.2 RESULTS

4.2.1 Genomic Southern Blot Analyses

The digestion of BALB/c spleen DNA with *Bam HI* resulted in the detection of a single fragment (4.0 kb) using the entire K4-1.1 cDNA as a probe (Figure 4 & 5a). Digestion of C57BL/6 spleen DNA under the same conditions detected two additional fragments (6.6, 2.1 kb), as well as the 4.0 kb fragment. However, these were not visible on other Southern blots (1) and were considered nonspecific crosshybridization. The *Dra I* digestion detected three fragments (4.5, 3.0, 1.8kb) in both mouse strains (data gathered from several Southerns). Extra fragments were detected in the BALB/c DNA but these did not appear in the NS-1 cell line Southern blot and were also considered nonspecific crosshybridization. The *Eco RI* digestion detected three fragments in the C57BL/6 DNA (6.6, 3.1, 2.4 kb) but the BALB/c DNA exhibited extra fragments. These extra fragments may also be nonspecific crosshybridization because they have not been reported elsewhere (1). Neither *Bam HI* or *Eco RI* cuts the K4-1.1 cDNA, and *Dra I* only cuts once, so other restriction sites are presumed to be within introns to account for the banding pattern seen. The Southern blot of cell lines

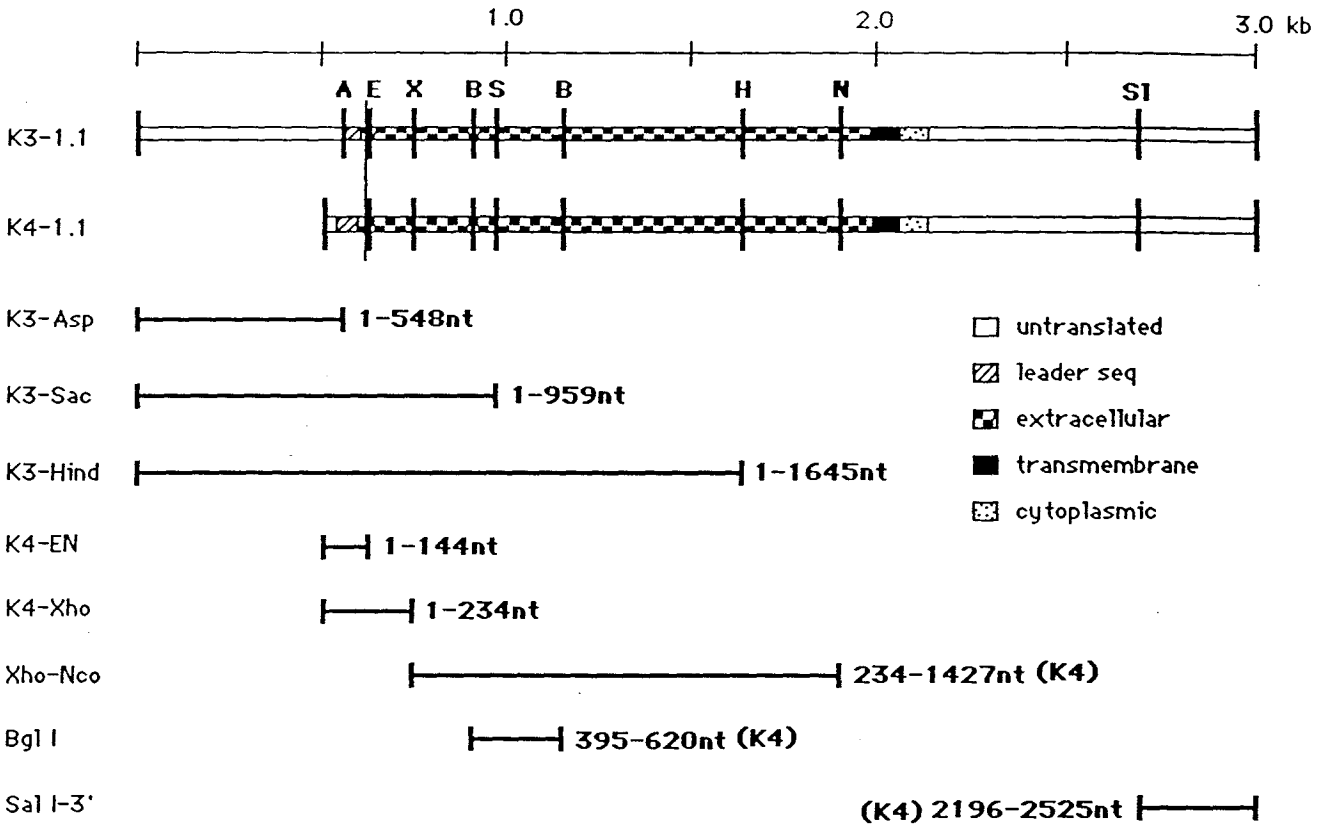


Figure 4 cDNA Probes. The K3-1.1 and K4-1.1 cDNAs and probes derived from them are shown. Restriction enzyme sites are marked (A-*Asp 700I*, E-*Eco NI*, X-*Xho I*, B-*Bgl I*, S-*Sac II*, H-*Hind III*, N-*Nco I*, Sl-*Sal I*) and smaller probes were obtained by restriction enzyme digestion, gel separation, and electroelution The sizes of the probes are designated in nucleotides numbered with reference to the K3-1.1 or K4-1.1 cDNAs.

a)

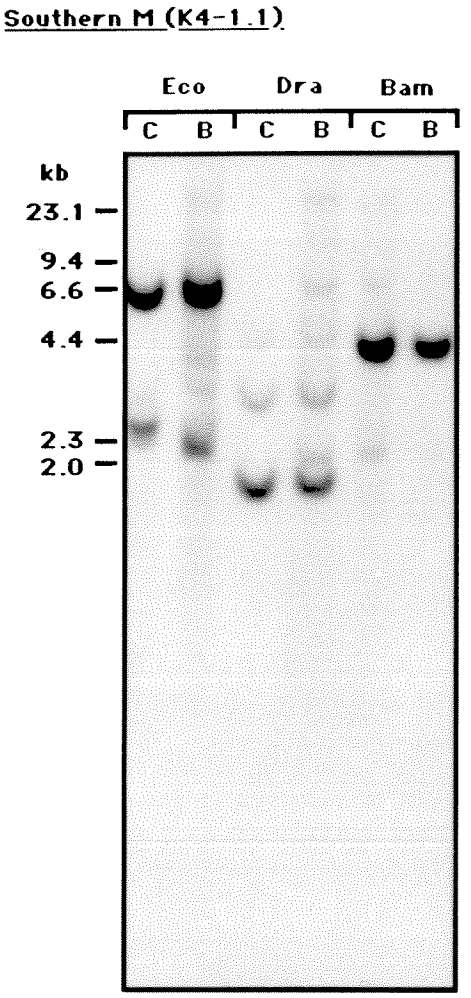
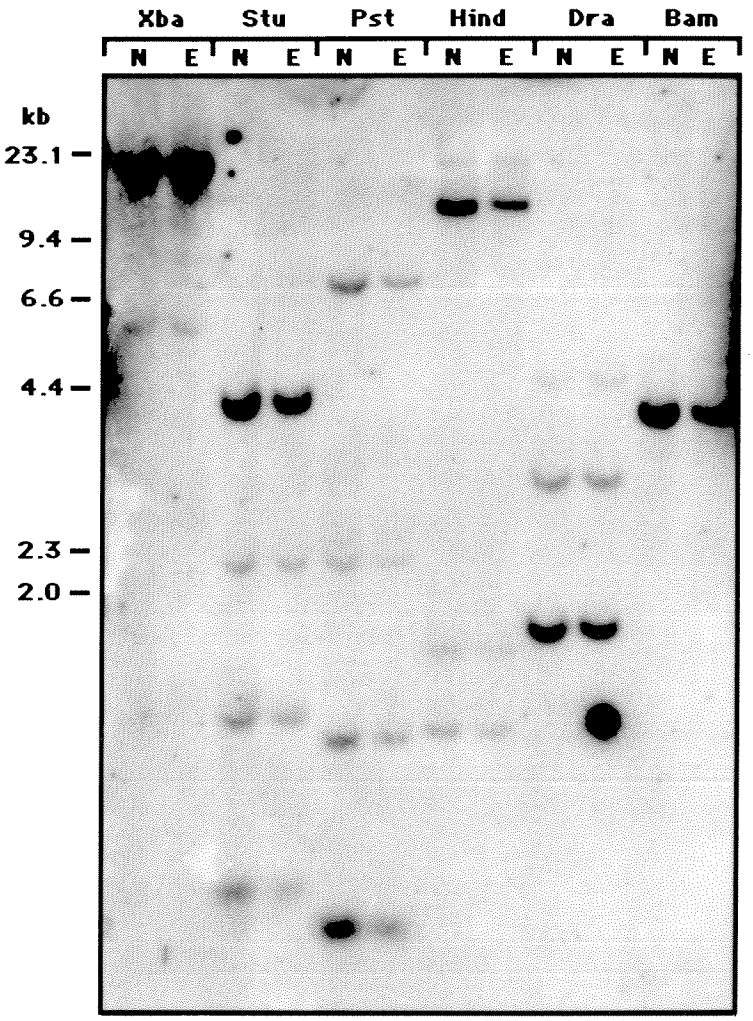


Figure 5 Genomic and Cell Line Southern Blots. **a)** Genomic DNA was isolated from BALB/c (B), and C57BL/6 (C) spleen cells. Approximately 10 μg was digested with *Eco RI* (lane 1,2), *Dra I* (lane 3,4), or *Bam HI* (lane 5,6) and separated on 0.8% agarose gel, blotted, and probed with K4-1.1 cDNA. **b)** Genomic DNA was isolated from NS-1 (N) and EL-4 (E) cells. Approximately 10 μg was digested with various restriction enzymes (*Xba I, Stu I, Pst I, Hind III, Dra I, Bam HI*), separated on 0.8% agarose gel, blotted, and probed with K4-1.1 cDNA.

b)

Southern H (K4-1.1)



derived from C57BL/6 and BALB/c mouse strains, EL-4 and NS-1, respectively, showed no variation between fragment sizes generated with different enzymes (Figure 5b).

In order to obtain a partial restriction enzyme map of the gene and determine the optimal fragments to characterize when cloning the gene, several Southern blots using various restriction enzymes were analysed (Figure 6 & 7). Several probes specific to different regions of the K4-1.1 gene were used and double digests were also employed to map the gene. Table VIII summarizes the fragments detected with various probes from all of the Southern blots and Figure 8 shows a partial restriction map of the gene. Data was gathered from several Southern blots and only fragments that were consistantly detected under the same conditions were recorded. A 4.0 kb *Bam HI* fragment was always detected in any Southern blot no matter what K4-1.1 probe was used (Table VIII & Figure 6). Therefore, most of the gene is likely to be contained within at least this 4.0 kb *Bam HI* fragment. However, double digestions indicated that there were possibly two 4.0 kb *Bam HI* fragments containing the gene. This was implied because of the detection of three fragments using the entire K4-1.1 cDNA as a probe (3.7, 2.25, & 1.4 kb) (Figure 7b), that together added to a value greater than 4.0 kb, and only the detection of the 3.7 kb with the K4-EN probe (*Bam HI / Hind III* digest) (Figure 7a). This suggested the 5' region was contained within the 3.7 kb fragment, and the remaining exons were within the other fragments.

It was assumed for the restriction map that the K4-1.1 cDNA was encoded by exons arranged in a 5' to 3' orientation with the first exon encoding the 5' region of the cDNA and the other exons following a similar manner. Restriction sites were located on the map (Figure 8) using the K4-1.1 specific probes to associate specific fragments of a digestion with certain regions of the cDNA. Briefly, a *Hind III* digestion probed with the entire K4-1.1 cDNA detected four fragments (23, 13, 1.7, 1.4 kb) (Figure 6f & Table VIII). However, only the 23 kb fragment was detected using the K4-Xho probe thereby orienting this 23 kb fragment to the 5' region of the gene (Figure 6a). The Bgl I probe only detected the 1.7 kb fragment, associating this fragment with the middle of the gene (Figure 6d). Similarily, the Sal I probe oriented the

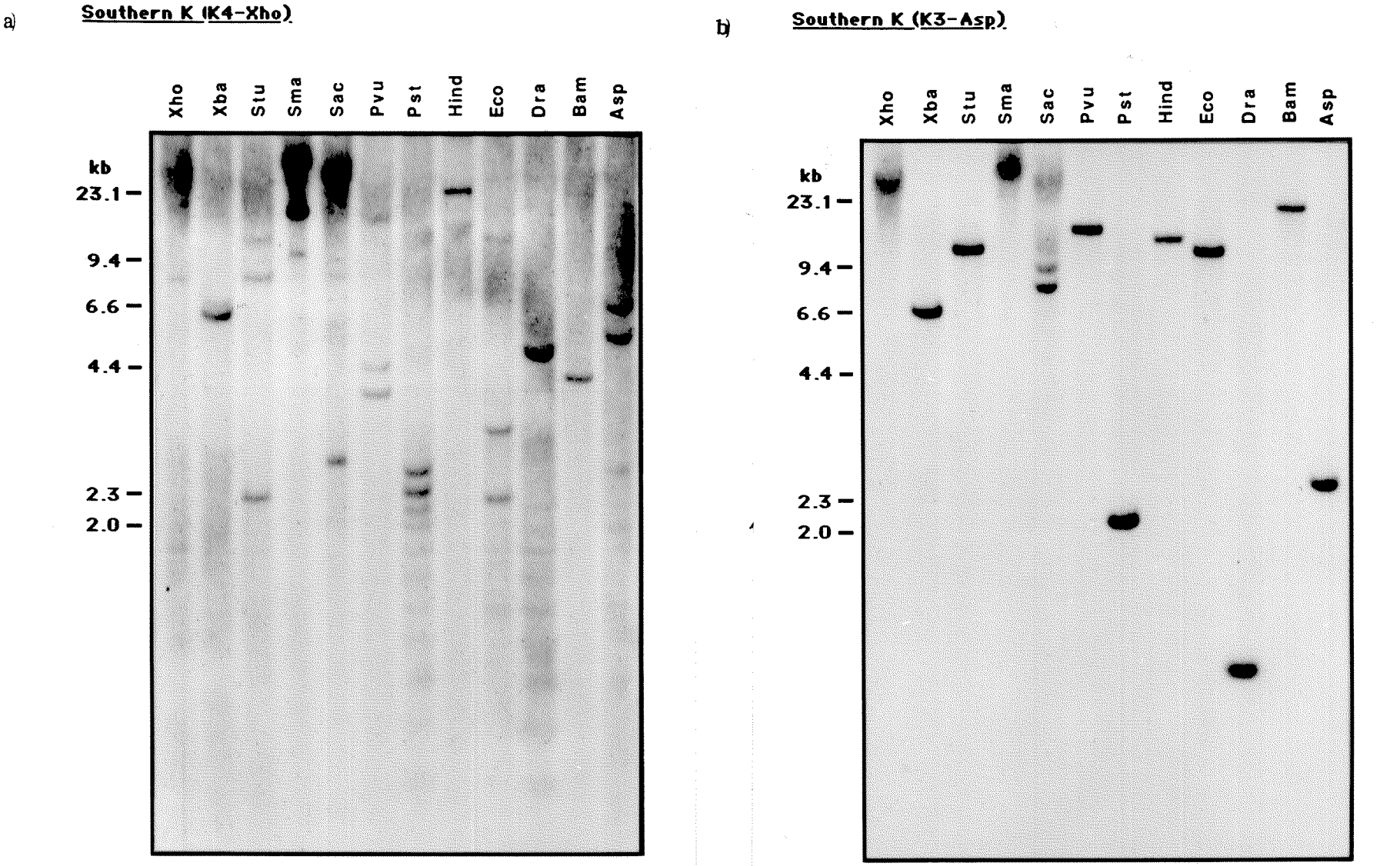
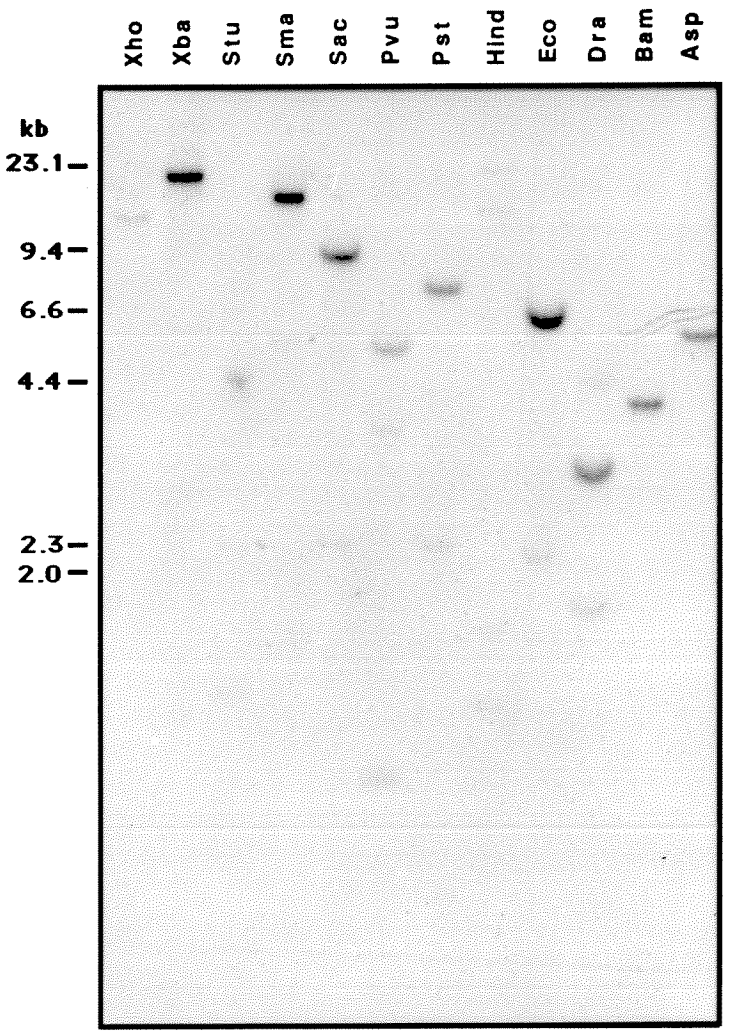


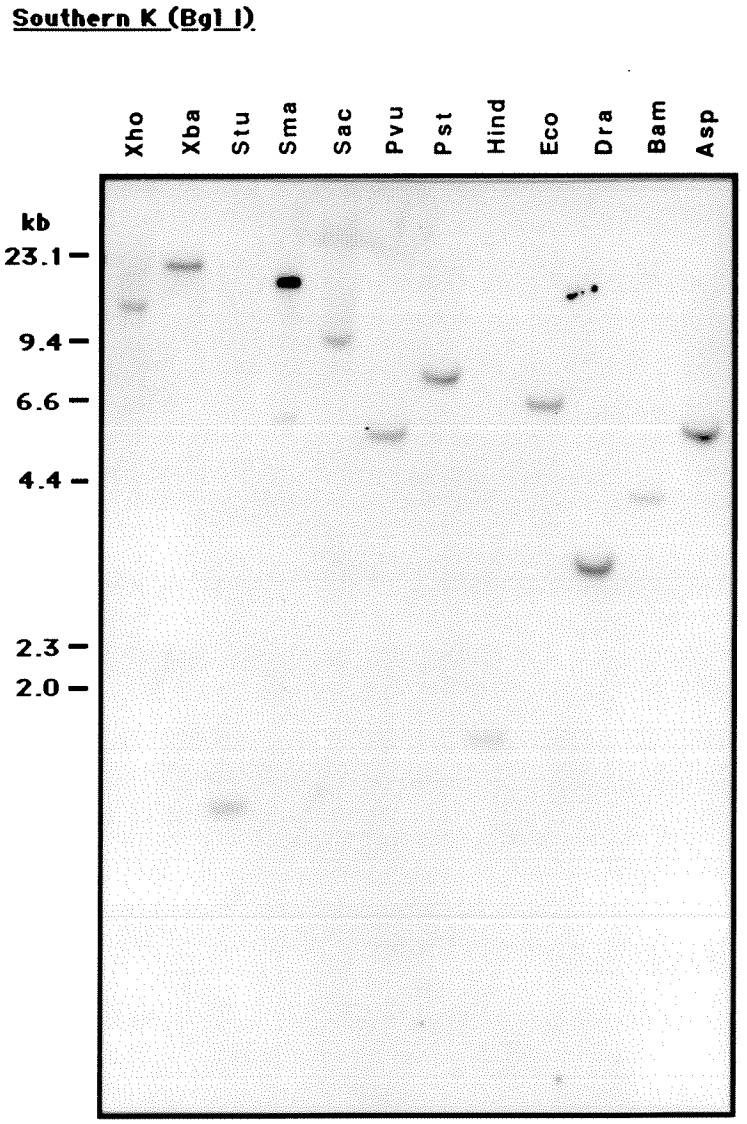
Figure 6 Genomic Southern Blot Analyses. Genomic DNA was isolated from C57BL/6 spleen cells and approximately 10 µg was digested with various restriction enzymes (*Xho I, Xba I, Stu I, Sma I, Sac I, Pvu II, Pst I, Hind III, Eco RI, Dra I, Bam HI, Asp 700I*). The fragments were separated on 0.8% agarose gels, blotted, and probed with **a**) K4-Xho, **b**) K3-Asp, **c**) Xho-Nco, **d**) Bgl I, **e**) Sal I, or **f**) K4-1.1. The Southern blots are designated K, J, and F to indicate that different blots were employed.

c

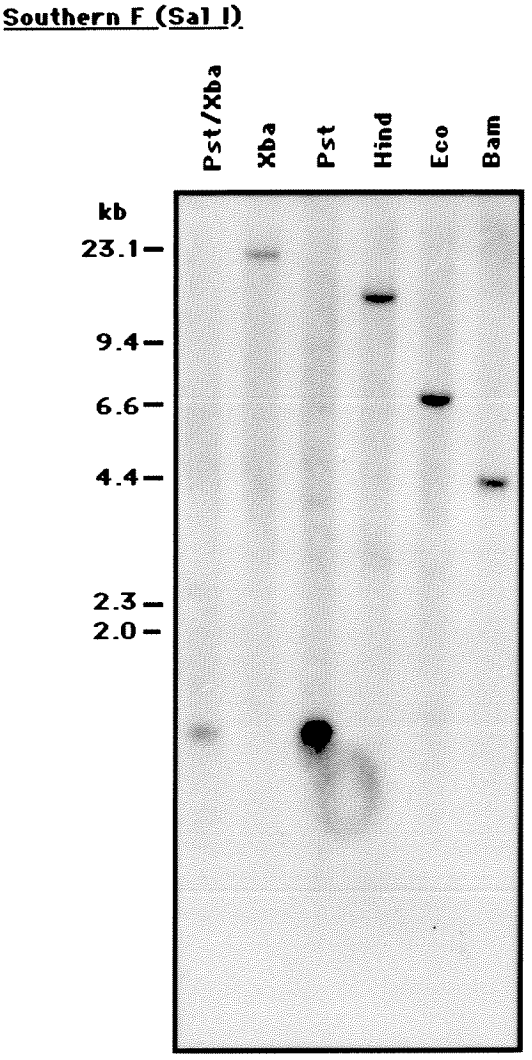
Southern J (Xho-Nco)



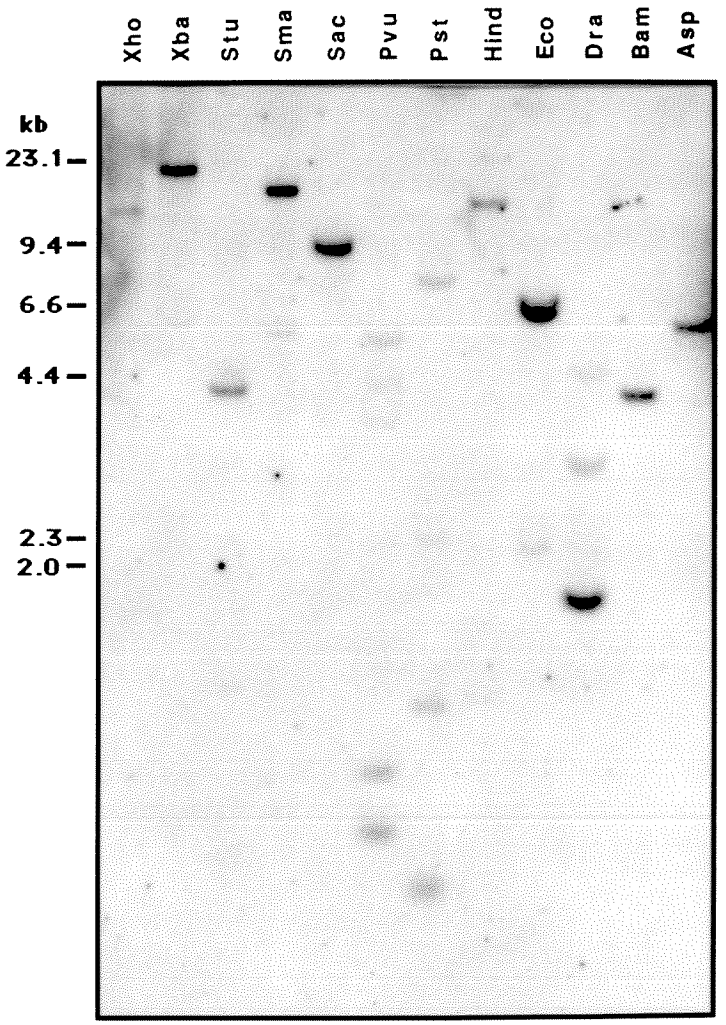
d


Southern K (Bgl I)
Xho
Xba
Stu
Sma
Sac
Pvu
Pst
Hind
Eco
Dra
Bam
Asp
kb
23.1
9.4
6.6
4.4
2.3
2.0

e)


Southern F (Sal I)
Pst/Xba
Xba
Pst
Hind
Eco
Bam
kb
23.1
9.4
6.6
4.4
2.3
2.0

Southern J (K4-1.1)



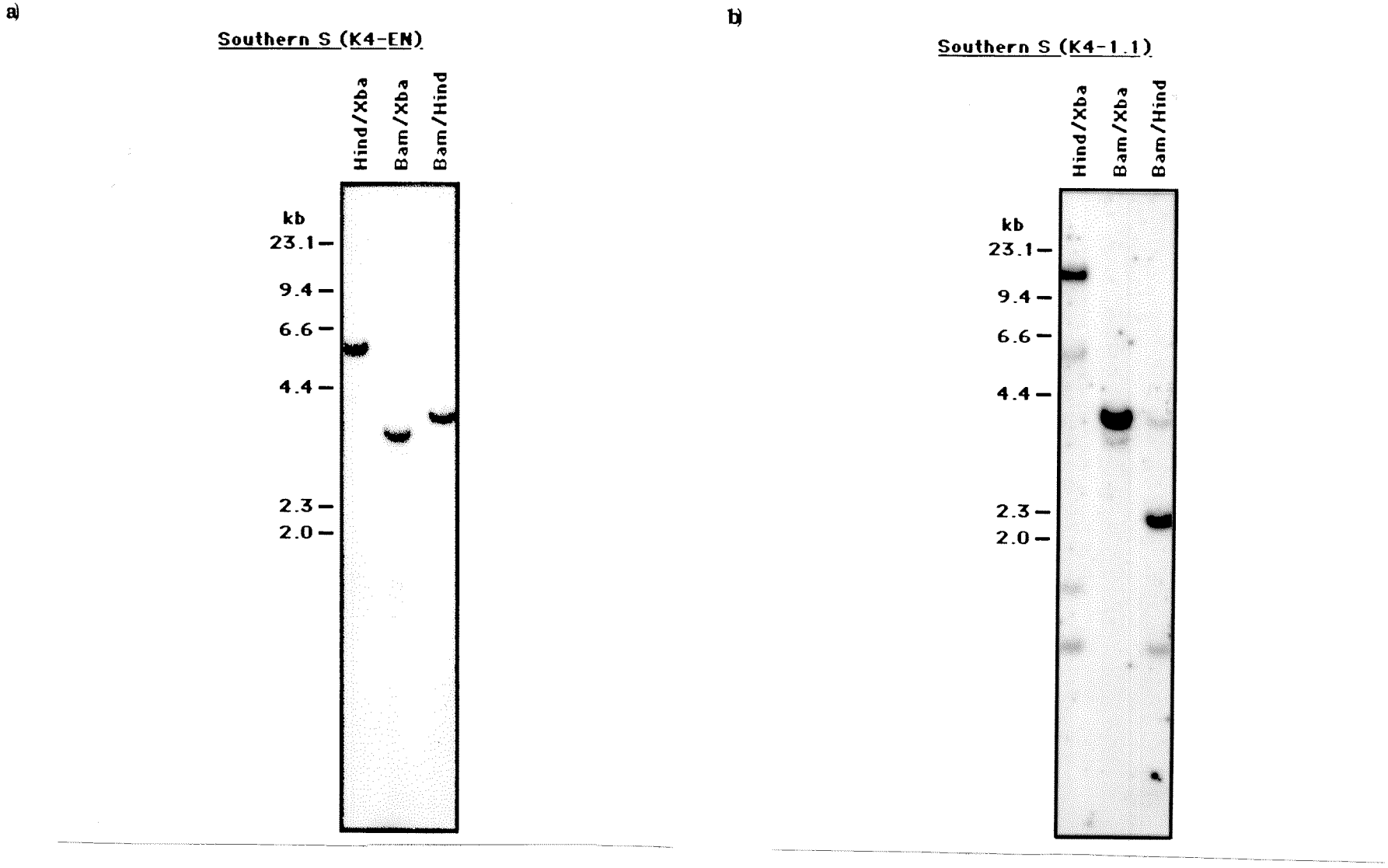


Figure 7 Double Digested Genomic Southern Blot Analyses. Genomic DNA was isolated from C57BL/6 spleen cells and approximately 10 µg was digested with various combinations of enzymes (*Hind III* and *Xba I*, *Bam HI* and *Xba I*, *Bam HI* and *Hind III*). The fragments were separated on a 0.8% agarose gel, blotted, and probed with **a**) K4-EN, and **b**) K4-1.1.

Table VIII

SIZES OF DNA FRAGMENTS DETECTED IN
SINGLE DIGEST GENOMIC SOUTHERN BLOT ANALYSES*

enzyme	South K (K4-Xho)	South J (Xho-Nco)	South K (Bgl I)	South F (Sal I)	South J (K4-1.1)	South K (K3-5')
Asp 700I	6.3/5.1	5.6	5.3	ND@	6.3	2.6
Bam HI	4.0	4.0	4.0	4.0	4.0	20
Dra I	4.6	4.6/3/1.8	3.0	ND	4.5/3/1.8	1.3
Eco RI	3.1/2.3	2.2/6.2	6.3	6.6	6.5/2.2/3.1	11
Hind III	23	23/13/1.7/1.4	1.7	12.3	23/13/1.7/1.4	12.5
Pst I	2.6/2.3	7.4/2.3	7.2	1.7	7.2/2.5/2.3/1.7/.4	2.1
Pvu II	3.8	5.4/3.8/1.2	5.4	ND	5.4/4.4/3.8/1.1/.8	14.3
Sac I	2.7	9/2.3/1.9	9.3	ND	9.0	9/7.5
Sma I	16.7	14.7/5.7	15.5/5.7	ND	16/5.7	-
Stu I	2.3	4.7/2.3/1.4	1.4	ND	4.1/2.3/1.4/.6	11
Xba I	6.1	20/6.0	19	20	20/6	6.6
Xho I	8.2	12.3	12	ND	12	-

@ND-not done.
*fragment sizes listed are gathered from several Southern blots.

Table IX

SIZES OF DNA FRAGMENTS DETECTED IN
DOUBLE DIGEST GENOMIC SOUTHERN BLOT ANALYSES

enzyme	South S (K4-EN)	South S (K4-1.1)
Bam/Hind	3.7	3.7/2.25/1.4
Bam/Xba	3.35	3.7/3.35
Hind/Xba	5.8	12.5/5.8/1.8/1.4

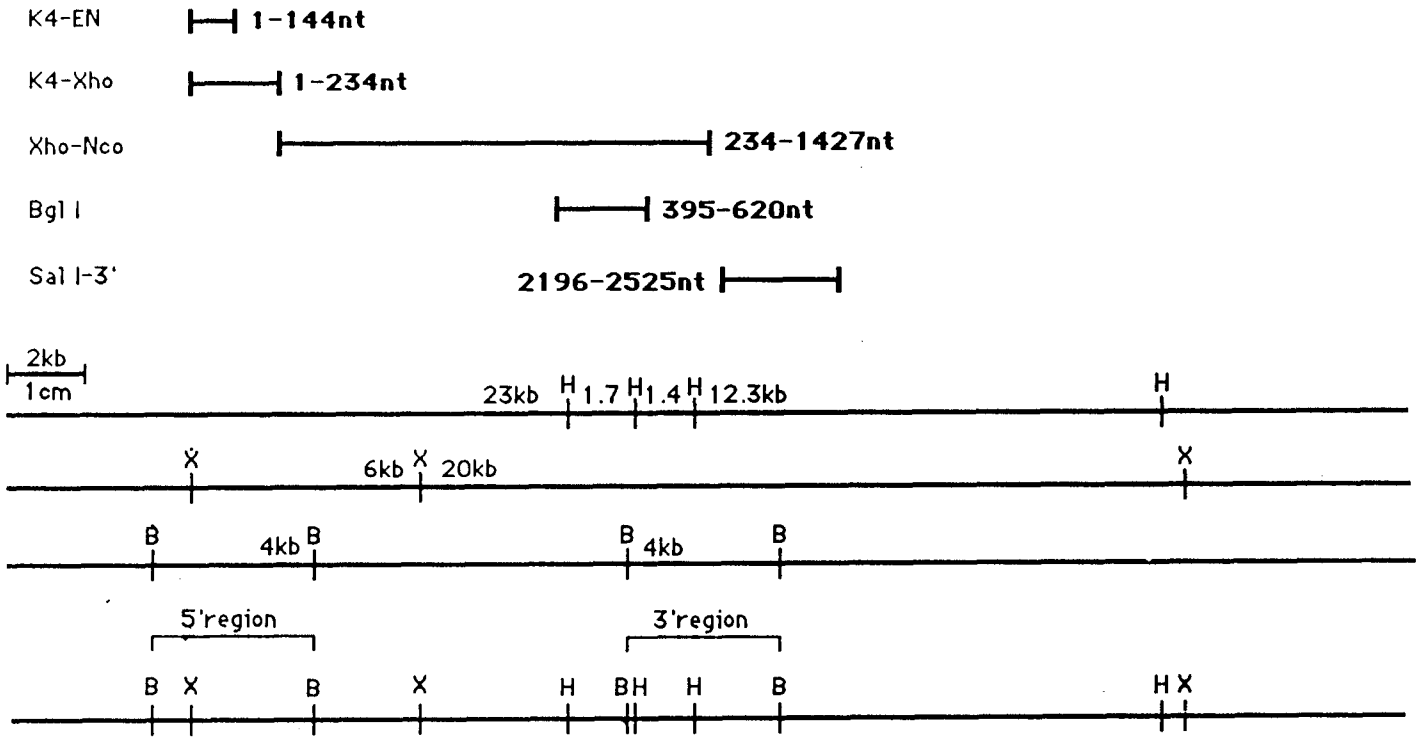


Figure 8 Partial Restriction Enzyme Map of the MALA-2 Gene. The K4-1.1 cDNA probes used in Southern blots analyses are shown along with a partial restriction enzyme map of the MALA-2 gene (H-*Hind III*, X-*Xba I*, B-*Bam HI*). The *Hind III*, *Xba I*, and *Bam HI* digests are shown individually, each of the fragments aligned with the cDNA probes detecting them. The probes are numbered with reference to the K4-1.1 cDNA, and the sizes of the fragments are marked.

13 kb fragment to the 3' region of the gene (Figure 6e). The 1.4 kb fragment was arbitrarily placed on the map. A *Xba I* digestion probed with the entire K4-1.1 cDNA only detected two fragments (20 and 6 kb)(Figure 6f). Only the 6kb fragment was detected with the K4-Xho probe (Figure 6a), thereby associating this fragment with the 5' region of the gene. A *Hind III/Xba I* double digest revealed a 12.5 kb fragment detected with the K4-1.1 probe that was associated with the 3' region of the gene (Figure 7b), and this was similar to the fragment detected in *Hind III* digest (13kb). Thus, the 12.5 kb *Hind III/Xba I* fragment could be placed in the 3' region of the gene, and an *Xba I* site was placed 20 kb upstream from this point within the 23 kb *Hind III* fragment. The *Bam HI* sites were oriented by the fragments detected in double digests using K4-EN and K4-1.1 as probes (Figure 7). A *Bam HI/Xba I* fragment of 3.35 kb was detected with the K4-EN probe so this was drawn as part of the 5' *Bam HI* fragment, and the *Bam HI/Hind III* digest detected a 2.25 kb fragment in the 3' region which could be drawn incorporating the *Hind III* sites. Thus, through examination of the individual fragments detected with specific probes in the single digests and double digests, a restriction map could be constructed for each enzyme and these maps could be combined to yield a more complete map incorporating several restriction sites (Figure 8).

The 5' region of K3-1.1 cDNA was also detected on Southern blots confirming that it is endogenous to the mouse genome. However, it did not appear to be on a fragment of DNA common to the 5' region of the K4-1.1 cDNA (Figure 6a/b). The Southern blot probed with K3-Asp and K4-Xho detected *Xba I* fragments of a similar size but these were not identical and were probably not the same fragment. Thus, the K3-5' region was not included in the restriction map.

4.2.2 Genomic Cloning of MALA-2

Since there were possibly two 4.0 kb *Bam HI* fragments containing most of the gene, an EL-4 genomic library, size selected for *Bam HI* fragments of approximately 4.0 kb, was screened with the entire K4-1.1 cDNA (Table X). Two phage clones, both containing a 4.0 kb insert, were isolated. The insert from one clone (J4) was subcloned into pTZ19R and the ends

Table X

SUMMARY OF CLONES RECOVERED FROM
EL-4 LIBRARY SCREENING

Pfu	Probe	Control	Positives
$2x10^5$	K4-1.1	ND[@]	2[*]
$4x10^5$	K4-EN	ND	0
$2x10^5$	K4-Xho	ND	0
$2x10^5$	K4-Xho	+	0
	K4-1.1	+	0
	K4-Xho(.5xSSC)	+	0

[@]ND-not done.
[*]BamHI insert (4.0kb) containing five 3' exons.

were sequenced. The insert was then cut with *Pst I* and the resulting fragments were subcloned and sequenced individually. The second clone (J3) was further analyzed by probing a phage Southern blot with the K4-Xho probe. No signal was detected , so J3 was not characterized further. The J4 sequence contained five exons corresponding to 358-2525 nucleotides of the K4-1.1 cDNA (Figure 9 & 10). Four exons are approximately 300 nucleotides in length each, and partially correlate to domains II, III, IV, and V of MALA-2. Domains IV and V are not totally encoded by one exon and extend slightly into the next exon, so the divisions between exons is not exactly in conjunction with the protein domains. The fifth exon within the 4.0 kb *Bam HI* fragment is larger, containing the transmembrane, cytoplasmic, and untranslated regions. Comparison of the J4 sequence with the K4-1.1 cDNA revealed a region of 118 bp between the first and second exons (Figure 9 & 10) that showed 78% nucleotide sequence identity with part of the third exon. This region is illustrated in Figure 11 as the second diagonal line set slightly apart from the other five diagonal lines. This pseudo exon is about one half an Ig domain but lacks the typical splice acceptor and donor sequences suggesting it is not expressed. All of the introns contain the splice acceptor and donor consensus sequences following the GT-AG rule (Figure 12) (3) and have phase I breakage points to allow correct in frame translation.

Because the J4 clone was incomplete, library screening was continued with probes spanning the 5' region of the K4-1.1 cDNA (K4-Xho or K4-EN). A total of $8x10^5$ plaques of the EL-4 library, $8x10^5$ plaques of the embryo library, and $6x10^5$ plaques of the liver library were screened with K4-Xho or K4-EN (Table XI). Additionally, $1x10^6$ plaques were reprobed with K4-1.1, however, in all cases no positive plaques were detected. A size selected plasmid library of *Dra I* fragments of approximately 3.5-5.5 kb was also screened with K4-Xho, but no positive clones were isolated. Control filters of K4-1.1 cDNA in λgt10 were included as indicated in these hybridizations.

To assess the quality of the libraries, the filters were also screened with two other cDNAs, YE1/48 (4) and CD43 (5), as well as λ*Hind III* and C57BL/6 spleen DNA. YE1/48 is the

Figure 9 Sequence of the J4 clone. The nucleotide sequence of the J4 clone (4.0 kb *Bam HI* insert) is shown. Introns are in lower case letters, exons are in upper case bold letters and are marked (E-exon), and the unexpressed 1/2 Ig exon (118nt) is underlined.

```
                                E3...
   1  cccaaccccg tttccttgca gCGTTTCCGG AGAGTGTGGA GCTGAGACCT CTGCCAGCCT GGCAGCAAGT AGGCAAGGAC CTCACCCTGC GCTGCCACGT
 101  GGATGGTGGA GCACCGCGGA CCCAGCTCTC AGCAGTGCTG CTCCGTGGGG AGGAGATACT GAGCCGCCAG CCAGTGGGTG GGCACCCCAA GGACCCCAAG
 201  GAGATCACAT TCACGGTGCT GGCTAGCAGA GGGGACCACG GAGCCAATTT CTCATGCCGC ACAGAACTGG ATCTCAGGCC GCAAGGGCTG GCATTGTTCT
 301  CTAATGTCTC CGAGGCCAGG AGCCTCCGGA CTTCGgtgag gtccttcaca gcgggcaagt gggttcttgg gaggaagaag accccagcaa tggggaaagg
 401  tgctgagaag atagtaatcc agatagtggg agttttgggg atggtgagct ggctctgtgg gtaaaggaag ggacttcatc caagcttgat gacctgagtt
 501  caattcccag cttccaaagg agaaactgac tcctgaaagt tgtcttttga cctccacaag tgagtcacat acatatatac acagcaaata aatgaataaa
 601  aagaaagaaa aagggtggtg tgtggggtgg accccagagg tccaactcat actgagtggc agcctccagg atcacaaaca acacttcttt tgttctgcgg
                                                                                                        E4...
 701  ccctggaggt ggctgggcag ttgctgttaa aaaaaacaac ccaggacccc ggaactccac tttgcttcca tggccctgtc cttctccccc aagcATCTTC
 801  CAGCTACCAT CCCAAAGCTC GACACCCCTG ACCTCCTGGA GGTGGGCACC CAGCAGAAGT TGTTTTGCTC CCTGGAAGGC CTGTTTCCTG CCTCTGAAGC
 901  TCGGATATAC CTGGAGCTGG GAGGCCAGAT GCCGACCCAG GAGAGCACAA ACAGCAGTGA CTCTGTGTCA GCCACTGCCT TGGTAGAGGT GACTGAGGAG
1001  TTCGACAGAA CCCTGCCGCT GCGCTGCGTT TTGGAGCTAG CGGACCAGAT CCTGGAGACG CAGAGGACCT TAACAGTCTA CAgtaagaag gggcggggct
1101  tagtccagag atagcgatgt tgagacctga ctgaccccaa tggaggggta ctaaaggggt aggactgaca agggtggcct tcggggcgta gcctaacttg
1201  gtatctcatt caggagtaag aactggcacc agggcacgac tagcgctgtg gtggcacttt caagagagag aactgaaggg aggcttagca ctgctttaat
1301  cccagcacaa gggtggcaga ggtagtaggt agagtcctta tgagttccag gccagccagg gctacacaat gagaccctgt ctcaaaaagt gtggggctag
1401  gcctttcaga aggcctagtt tcagctaggc ccagttcctg gatggccagt gctggagatt ggggctgaca ctaaaggtcc atcttgtact tgaggctgag
                                                    E5...
1501  tggtcatagg cggggtttgc actgggcggg gctcacggtg tgtccgctcc cagACTTTTC AGCTCCGGTC CTGACCCTGA GCCAGCTGGA GGTCTCGGAA
1601  GGGAGCCAAG TAACTGTGAA GTGTGAAGCC CACAGTGGGT CGAAGGTGGT TCTTCTGAGC GGCGTCGAGC CTAGGCCACC CACCCCGCAG GTCCAATTCA
1701  CACTGAATGC CAGCTCGGAG GATCACAAAC GAAGCTTCTT TTGCTCTGCC GCTCTGGAGG TGGCGGGAAA GTTCCTGTTT AAAAACCAGA CCCTGGAACT
1801  GCACGTGCTG Tgtgagttgg ctgcagtgct gagtgatagt catttcctac tgccccatgt gccttcggag ctttccctga tctctgtctc tgacactatt
          E6...
1901  tctccagATG GTCCTCGGCT GGACGAGACG GACTGCTTGG GGAACTGGAC CTGGCAAGAG GGGTCTCAGC AGACTCTGAA ATGCCAGGCC TGGGGGAACC
2001  CATCTCCTAA GATGACCTGC AGACGGAAGG CAGATGGTGC CCTGCTGCCC ATCGGGGTGG TGAAGTCTGT CAAACAGGAG ATGAATGGTA CATACGTGTG
2101  CCATGCCTTT AGCTCCCATG GGAATGTCAC CAGGAATGTG TACCTGACAG TACTGTgtga gtatcccagg atataacggc tggatgagga gtgggctagg
                                                    E7...
2201  tgccagggcc tcaagtctca ccattccctt ctgtctctac cacacagACC ACTCTCAAAA TAACTGGACT ATAATCATTC TGGTGCCAGT ACTGCTGGTC
2301  ATTGTGGGCC TCGTGATGGC AGCCTCTTAT GTTTATAACC GCCAGAGAAA GATCAGGATA TACAAGTTAC AGAAGGCTCA GGAGGAGGCC ATAAAACTCA
2401  AGGGACAAGC CCCACCTCCC TGAGCCTGCT GGATGAGACT CCTGCTGGAC CCCCTGCAGG CAACAGCTGC TGCTGCTTTT GAACAGAATG GTAGACAGCA
2501  TTTACCCTCA GCCACTTCCT CTGGCTGTCC ACAGAACAGG ATGGTGGCCT GGGGGATGCA CACTTGTAGC CTCAGAGCTA AGAGGACTCG GTGGATGGAG
2601  CAAGACTGTG AACACGTGTG ACCCGGACCC ACCTACAGCC CGGTGGACCT TCAGCCAAGA AACGCTGACT TCGTTCTCTA TTGCCCCTGC TGAGGGGTCC
2701  TGCTAAGGAA GACATGATAT CCAGTAGACA CAAGCAAGAA GACCACACTT CCCCCCGACA CAGGAAAGCT GAGACATTGT CCCCATCTCT TCTTGATGTA
2801  TTTATTAATT TAGAGTTTTA CCAGCTATTT ATTGAGTACC CTGTATATAG TAGATCAGTG AGGAGGTGAA TGTATAAGTT ATGGCCTGGA CCCTGCTGCA
2901  GATGCTGTGA GAGTCTGGGG AAAGATCACA TGGGTCGACG GTTTCTCTAC TGGTCAGGAT GCTTTTCTCA TAAGGGTCGA CTTTTTTCAC CAGTCACATA
3001  AACACTATGT GGACTGGCAG TGGTTCTCTG CTCCTCCACA TCCTGGAGCG TCCCAGCACC TCCCCACCTA CTTTTGTTCC CAATGTCAGC CACCATGCCT
3101  TAGCAGCTGA ACAATCGAGC CTCATGCTCA TGAAATCATG GTCCCAGGCG GCTCCACCTC AAAGAGAAAG CCTGGAAGGA AATGTTCCAA CTCCTTAGAA
3201  GGGTCGTGCA AGCTGCTGTG GGAGGGTAAG CACCCCTCCC AGCACAGAAA CCTTTCCTTT GAATCAATAA AGTTTTATGT CGGCTGAgtg tcttgtgagt
3301  ttgtgtacag tcatcacatc agttaggcaa agcctaagga ctgccgactc ccataatgcc tcagggttgt ctggtaacct aaccctaact ctgagtctgt
3401  ggatcaggtt ggtccccacc cccacccccc ttcttttttg agacaggttc tctttgtggc catggatgtc ctgaaatctg ctatgtggaa tgggctggcc
3501  ttgacttcac aaagatgggc caacctgtcc tgaatgctag gactaaatga caaagccact gccatgtcta gtaaaatcta cgtacataga cagggtttcc
3601  cagtgtagac aggatggcct tgaacttaca gagactgcct ccctgggagt gcgggacaag gcagtgcaca ccaagcgtaa ctcctatagg ccgcgagcat
3701  ctggtgcagg tgctctatct ctgcgccagc taactgcctg ttgcgcactt cattgccatg gagtctgccc ttctgctccc gtcgcttttg ctggtggctg
3801  cctatccgag gggtgggagc ccccagcaag agtggatgca aagtcctccc gcgccttccg tgacctcagc acctttctgg gtgcgtctta atccagagct
3901  agaggccgtg cctcccgggg ctcagcgtgg cttaactgca gccacaactg ccctgccggt gcattccagc ctgcgcaccc aactgcggca gggaaagata
4001  gttaatg
```

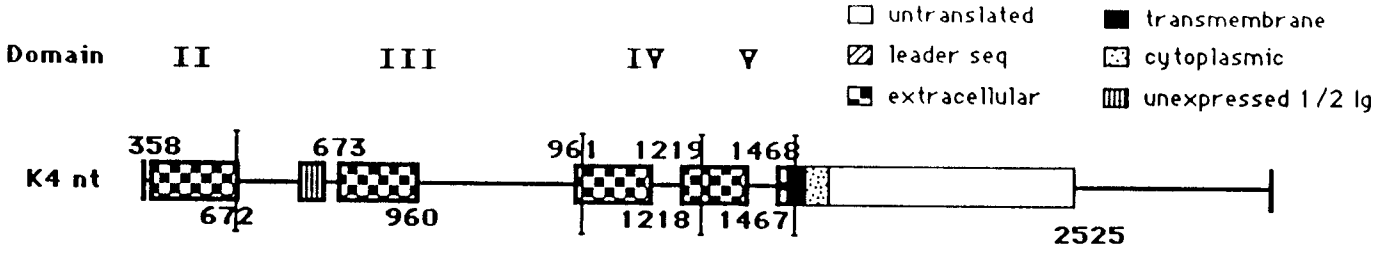


Figure 10 Partial Structure of MALA-2 Gene. The exon/intron organization of the isolated J4 clone (4.0 kb *Bam HI* insert) is shown. Numerals represent nucleotides of K4-1.1 cDNA. Protein domains are separated by verticle lines and are denoted in roman numerals.

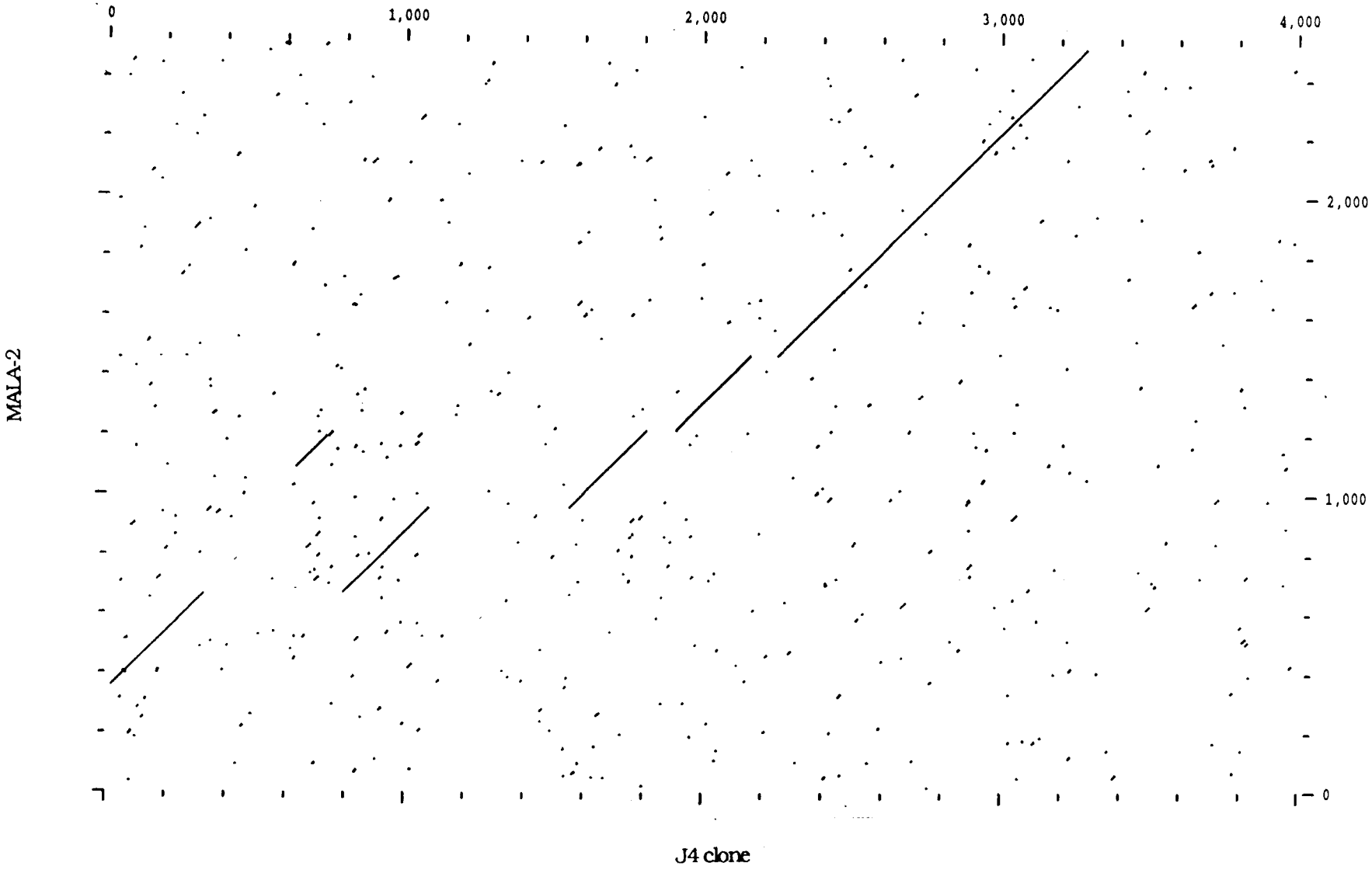


Figure 11 Comparison of the isolated J4 sequence with MALA-2 cDNA. The sequence of the isolated J4 clone was compared to K4-1.1 cDNA. Diagonal dot-matrix comparison was used as follows, a window of 21 nucleotides was examined between each of the sequences, and when 14 nucleotides were identical a dot was plotted.

Intron/Exon Boundaries

intron	exon	intron
...gtttccttgcagCGTTTC...	(3=315 bp)	...ACTTCGgtgaggtccttc...
...ttctcccccaagATCTTC...	(4=288 bp)	...TCTACAgtaagaaggggcg...
...gtccgctcccagACTTTT...	(5=258 bp)	...TGCTGTgtgagttggctgc...
...ctatttctccagATGGTC...	(6=249 bp)	...TACTGTgtgagtatcccag...
...tctaccacacagACCACT...	(7=1040bp)	...GGCTGAgtgtcttgtgagt...

Figure 12 Partial Intron/Exon Boundaries of MALA-2 Gene. Exon sequences are in uppercase letters; intron sequences are in lowercase letters.

Table XI

SUMMARY OF CLONES RECOVERED FROM
BALB/C EMBRYO,LIVER, AND SIZE SELECTED LIBRARY SCREENING

	Pfu	Probe	Control	Positives
embryo lib	$4x10^5$	K4-EN	ND[@]	0
	($2x10^5$)	K4-Xho	ND	0
	$4x10^{5\#}$	K4-Xho	ND	0
	($2x10^5$)	K4-Xho(.5xSSC)	+	0
	($4x10^5$)	K4-1.1	+	0
embryo lib	$2x10^5$	K3-1.1	ND	2*
	$2x10^5$	K3-Asp	ND	0
	$2x10^5$	K3-Sac	ND	0
liver lib	$2x10^5$	K4-Xho	ND	0
	($2x10^5$)	K4-1.1	ND	0
	$4x10^{5\$}$	K4-Xho	+	0
	($2x10^5$)	K4-Xho(.5xSSC)	+	0
	($4x10^5$)	K4-1.1	+	0
liver lib	$2.5x10^5$	K3-Sac	ND	2[x]
pUC19 C57BL/6** Dra I:3.5-5.5kb	$1.4x10^5$	K4-Xho	+	0
lamGEM BALB/c** BamHI:15-23kb	$6x10^4$	K3-Asp	ND	0

[@]ND-not done
()number of pfu rescreened with indicated probes.
[#]$2x10^5$ pfu also probed with YE1/48(no positives),and CD43(8 positives).
*further analysis showed a BamHI fragment(4.0kb) containing five 3' exons.
[$]$2x10^5$ pfu also probed with λHindIII(+),and C57BL/6 spl DNA(+).
[x]phage Southern blot was K3-Asp$^+$ but K4-Xho$^-$.
**two size selected libraries constructed in this thesis study.C57BL/6 spleen DNA,DraI cut,size selected for 3.5-5.5 kb in pUC19 vector, and BALB/c spleen DNA,BamHI cut, size selected for 15-23 kb in lambdaGEM vector.
-all stringency washes done at 55°C in 0.1xSSC/1%SDS for 30 min except where indicated.

Ly-49A antigen belonging to the recently characterized NK specific multi-gene family (6), and CD43 is also termed large sialylglycoprotein which is deficient in Wiscott-Aldrich syndrome. The probe for CD43 detected two positives per $5x10^4$ plaques in the embryo library, but when the same filters were probed with the YE1/48 probe no positives were detected. The liver library was screened with labelled *λHind III* DNA, and C57BL/6 spleen DNA confirming that phage DNA was bound to the filters. Thus, the conditions for lifting the phage DNA and hybridizing the filters with probes were adequate to detect positive plaques. The embryo library was representative for the CD43 gene, but not the MALA-2 gene. Controls were not done for YE1/48 screening so it is difficult to assess this negative result.

The BALB/c embryo and liver libraries were also screened with K3-1.1, K3-Asp, and K3-Sac probes (Table XI). From a total of $8.5x10^5$ plaques, four phage clones were isolated. Two of the phage clones liberated a *Bam HI* fragment which was identical to the previously characterized J4 clone. The other two phage clones contained large inserts (12 and 14.5 kb) which were positive with the K3-Asp probe but negative with the K4-Xho probe on a phage Southern blot. These clones were not characterized further at the time, as the primary objective was to characterize the K4-1.1 gene.

Since the 5' region of K4-1.1 was not isolated, the Southern blot data and the J4 sequence were combined to construct a more complete proposed gene structure (Figure 13). The J4 clone has 5 exons encoding the 3' region of the K4-1.1 cDNA and this was oriented as the 3' *Bam HI* fragment. The 5' region of the gene (1-357 nt) is at least 6 kb upstream of the five characterized exons on the basis of the preliminary restriction map. Additionally, when genomic DNA is cut with *Pst I* or *Eco RI* and probed with the K4-Xho probe, two fragments are detected by Southern blot analysis . The lack of these restriction sites within the 5' region of the K4-1.1 cDNA supports them being within an intron, therefore separating the 5' region into at least two exons.

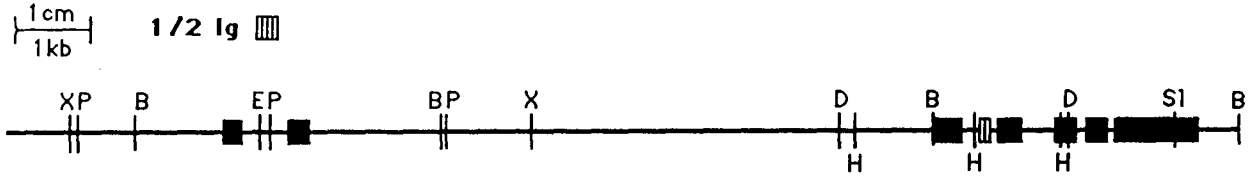


Figure 13 Proposed Structure of MALA-2 Gene. Based on genomic Southern blot analyses and the J4 sequence, the exon organization and partial restriction map of the MALA-2 gene is shown (X-*Xba I*, P-*Pst I*, B-*Bam HI*, D-*Dra I*, E-*Eco RI*, H-*Hind III*). The 1/2 Ig domain is marked. The exact location of the *Eco RI* and *Pst I* sites is not defined, and are arbitrarily placed. All other restriction sites are placed according to the Southern blot data and known sites within the K4-1.1 cDNA.

4.2.3 Analysis of a K3-1.1 Transcript

The K3-1.1 cDNA has not been previously reported. It has a large 5' region containing 10 initiation sites besides the one which encodes MALA-2, however, this start site does not conform to the consensus sequence proposed by Kozak (7). Similarily, the signal sequence of K3-1.1 is atypical, encoding several charged amino acid residues and not the usual hydrophobic residues. Therefore, K3-1.1 was analyzed further by Northern blotting and PCR to study its authenticity.

Northern blot analysis with the K3-Asp probe detected a low molecular weight mRNA species (0.3 kb) in NS-1 cells (Figure 14). Conversely, the K4-Xho probe detected a 2.5 kb species and a low molecular weight species (<0.24kb). The 2.5 kb species corresponds in size to the K4-1.1 cDNA. When Xho-Nco or K4-1.1 were used as probes, the 2.5 kb as well as a 2.1 kb species appeared. The 2.1 kb species has not been previously detected and its origin is unknown. A mRNA corresponding to the K3-1.1 cDNA in size (3.0 kb) was not detected.

For PCR, mRNA was extracted from NS-1 or P388D1 cells, cDNA was synthesized using oligo dT (expt 1) or random primers (expt 2 & 3) and reverse transcriptase, and PCR was performed using specific oligonucleotide primers (Figure 15a). Two internal PCR controls were done, the first within the unique 5' region of K3, and the second within the 5' region of K4. The test reaction involved a 5' primer within the 5' region of K3-1.1 and a 3' primer within the common region in both cDNAs . If the K3-1.1 transcript existed within cells, a PCR product should have been detected in the PCR test reaction. An actin control was also run to assess the quality of cDNA synthesis. These four reactions were done on cDNA synthesized from both NS-1 and P388D1 cells. Negative controls without cDNA were run to ensure there was no contamination of the primers. A reaction using the test primers was also run without reverse transcriptase to ensure no contamination of the cDNA synthesis reagents. And finally, reactions were run with the isolated cDNAs as templates to illustrate the expected size of the PCR products. Figure 15b/c shows the results of the first experiment.

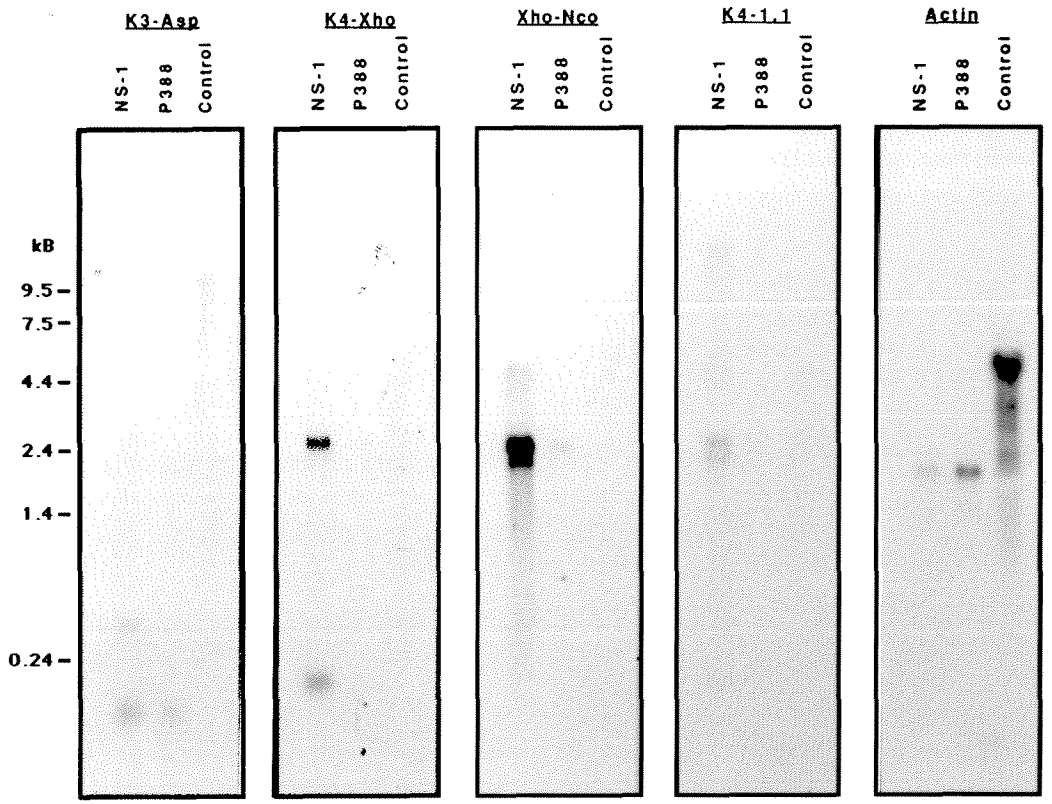


Figure 14 Northern Blot Analysis to search for K3 RNA. Poly A^+ RNA was isolated from NS-1, P388D1, and TF-1 cells (negative control). Approximately 10 μg of poly A^+ RNA was run on a formaldehyde gel, blotted, and probed with K3-Asp, K4-Xho, Xho-Nco, K4-1.1 or Actin.

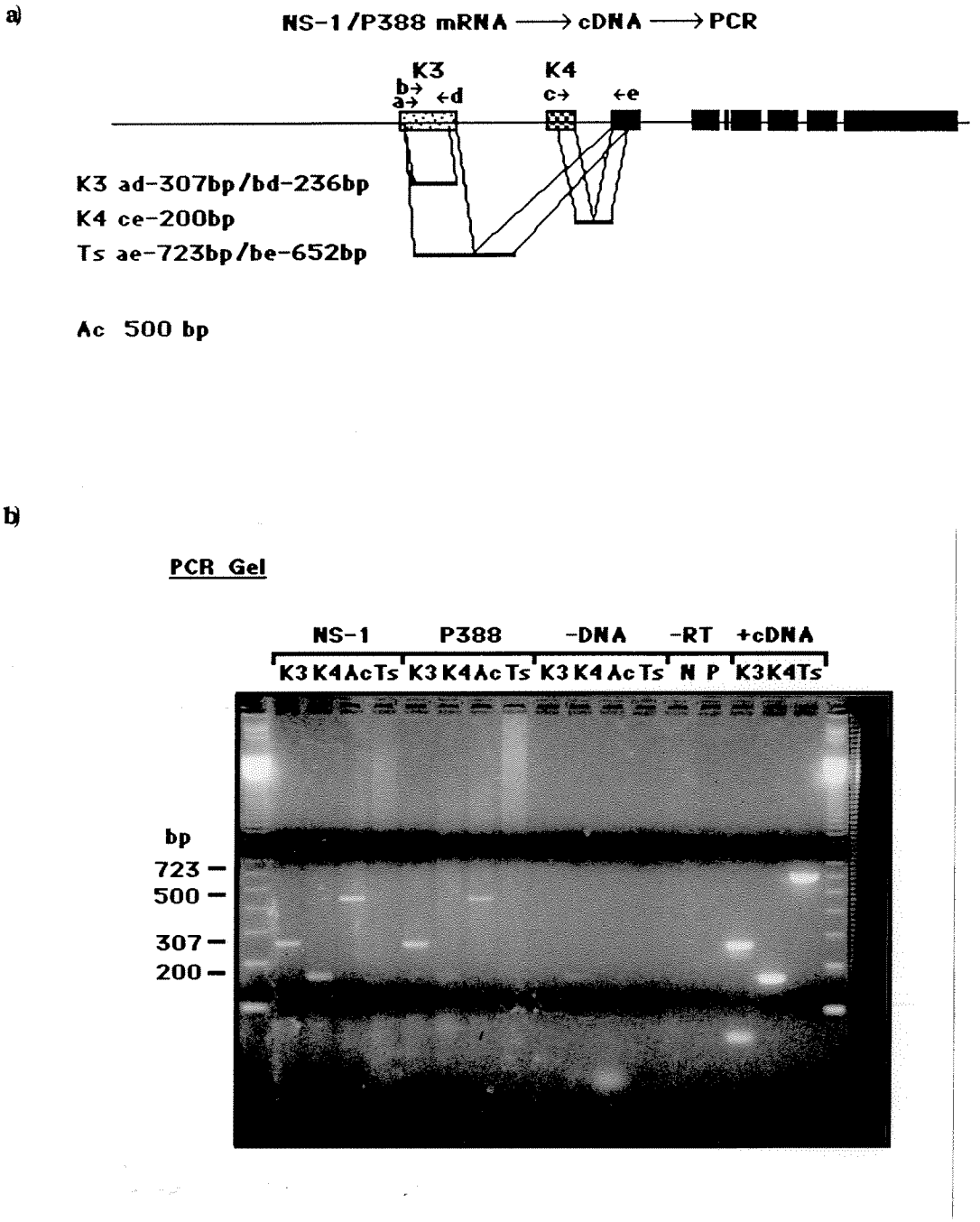
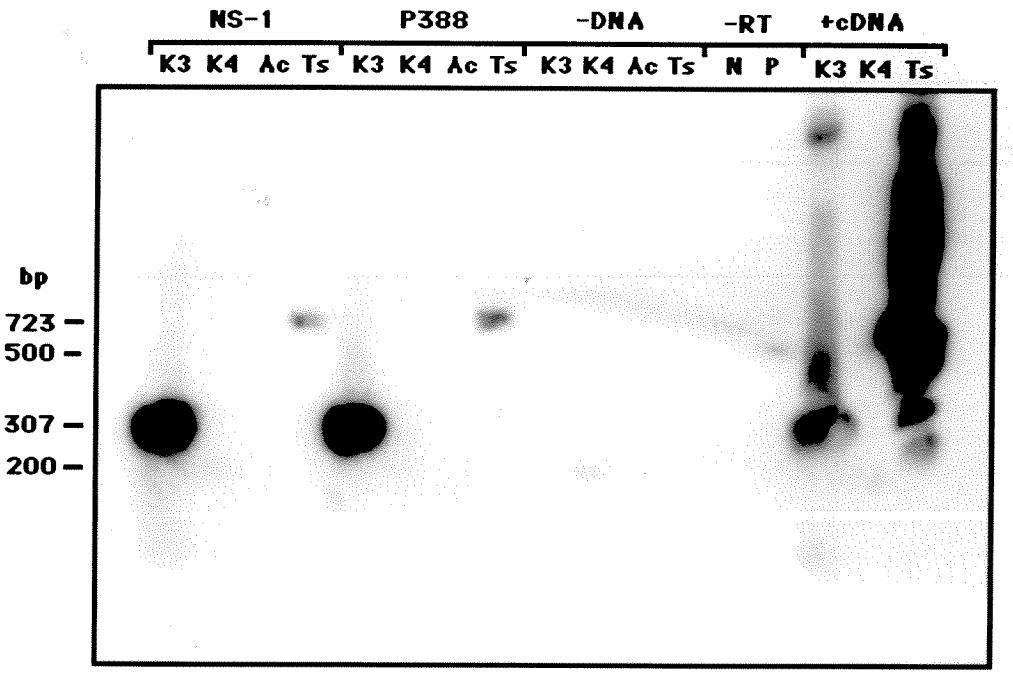


Figure 15 K3-1.1 PCR Analyses. **a)** Expected PCR product sizes using different primers. K3 internal control (expt 1: 307 bp; expt 2: 236 bp), K4 internal control (200 bp), K3 test reaction (expt 1: 723 bp: expt 2: 652 bp), and actin control (500 bp). **b)** Experiment 1: EtBr stained agarose gel of PCR products (one fifth of reaction loaded). K3, K3 internal control (lanes 1,5,9,15); K4, K4 internal control (lanes 2,6,10,16); Ac, actin control (lanes 3,7,11); and Ts, K3 test reaction (lanes 4,8,12,13,14,17). -RT, Reverse Transcriptase negative control. N, NS-1 cells. P, P388D1 cells. **c)** Experiment 1: PCR Blot. The EtBr gel of PCR products was blotted and probed with K3-Asp (unique to K3-1.1). **d)** Experiment 2: EtBr stained agarose gel of PCR products (one fifth of reaction loaded). PCR analysis was only done on NS-1 cDNA. RT-Reverse Transcriptase negative control. **e)** Experiment 2: PCR Blot. EtBr gel of PCR products was blotted and probed with K3-Asp (unique to K3-1.1).

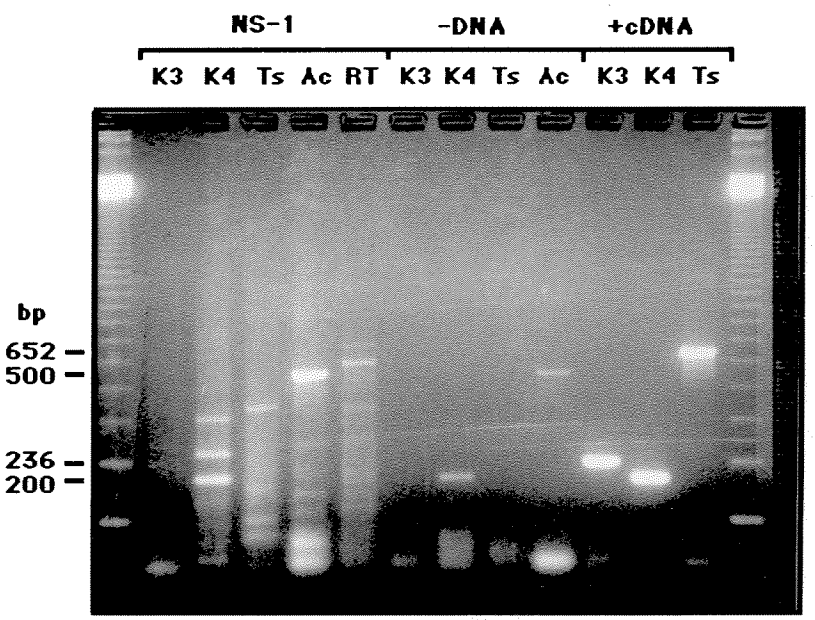
c)

PCR Blot (K3-Asp)

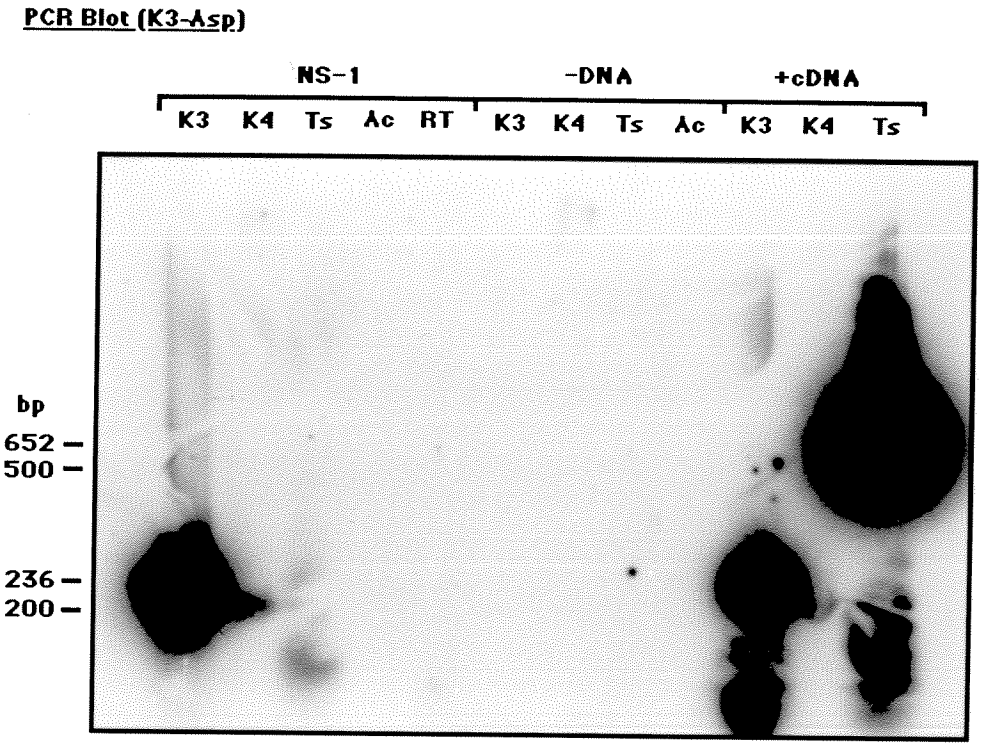


d)

PCR Gel



e)


PCR Blot (K3-Asp)
NS-1
-DNA
+cDNA
K3 K4 Ts Ac RT K3 K4 Ts Ac K3 K4 Ts
bp
652
500
236
200

The EtBr stained agarose gel illustrated the expected sizes of the internal controls, actin control, and positive controls, however, the test lanes did not show any visible products. When the gel was blotted and probed with K3-Asp, however, a product of the expected size was visible in the K3 test lanes of both cell lines. A second experiment (Figure 15d/e) was performed with only NS-1 mRNA and a different 5' primer for the K3 reactions. A K3 test PCR product was not visible in the EtBr stained agarose gel and the hybridization of the blotted filter with the K3-Asp probe did not detect any product in the test lane. A third experiment was done using cDNA made with random primers and the same PCR primers as expt 2, and a positive result was obtained (data not shown). Therefore, two out of three experiments support the existence of a K3-1.1 transcript. The PCR product was not sequenced due to limiting amount of material obtained.

4.2.4 Detection of Possible K3 Protein Using Anti-peptide Antisera

An effort was made to identify a protein possibly translated from the K3-1.1 transcript by the development and use of a polyclonal sera against the K3-1.1 N-terminal. Two synthetic peptides (9 amino acids) were obtained (from the Tripartite Microanalytical Center in the Department of Biochemistry and Microbiology at the University of Victoria.) (Figure 16). One peptide was unique to the nine amino acids of the N-terminal of the K3-1.1, while the second peptide was within the region common to both K3-1.1 and K4-1.1 translated proteins. This second peptide was to act as an internal control. These peptides were conjugated to KLH and injected into six Fisher rats (3 per peptide). After four or five injections, the sera from two control rats, and one test rat (because these were the only remaining rats) were tested by ELISA against KLH alone, specific peptide, and control peptide. All sera demonstrated activity against the injected peptide (Figure 17).

Cell lysates from L cells (negative control) and NS-1 cells (test) were examined by Western blot analyses. The filters were exposed to various antisera in an effort to detect MALA-2 and a K3-1.1 translated protein (Figure 18). Purified soluble MALA-2 was included as a positive control. The YN1/1.7 MAb detected MALA-2 in NS-1 cell lysate (~100kD) only

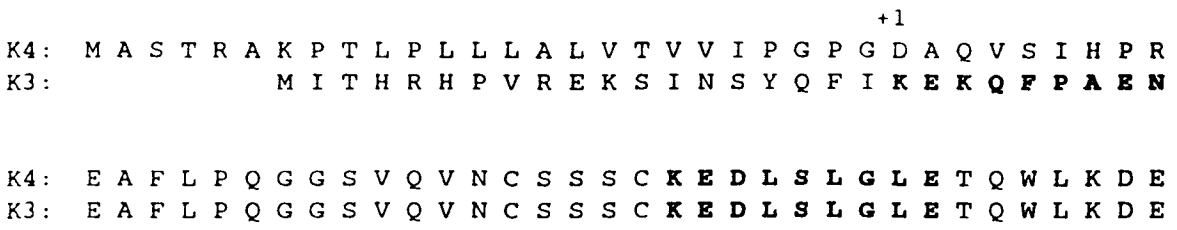
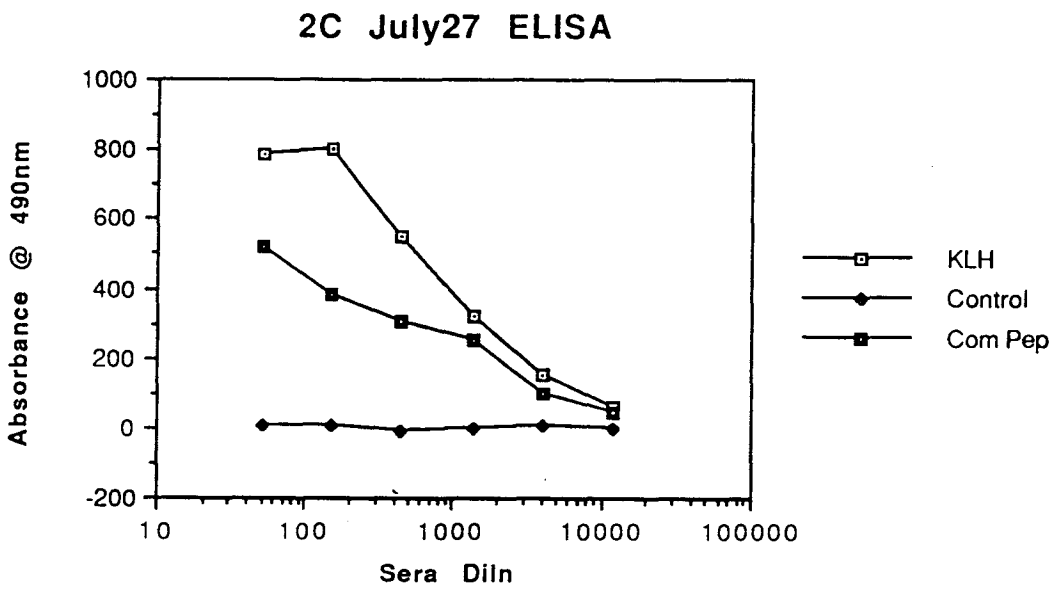


Figure 16 Synthetic Peptides (K3 Unique and K4 Common). The leader and N-terminal sequences of K4-1.1 and K3-1.1 cDNAs are shown. The synthetic peptides used in developing antisera are in bold letters.

a)



b)

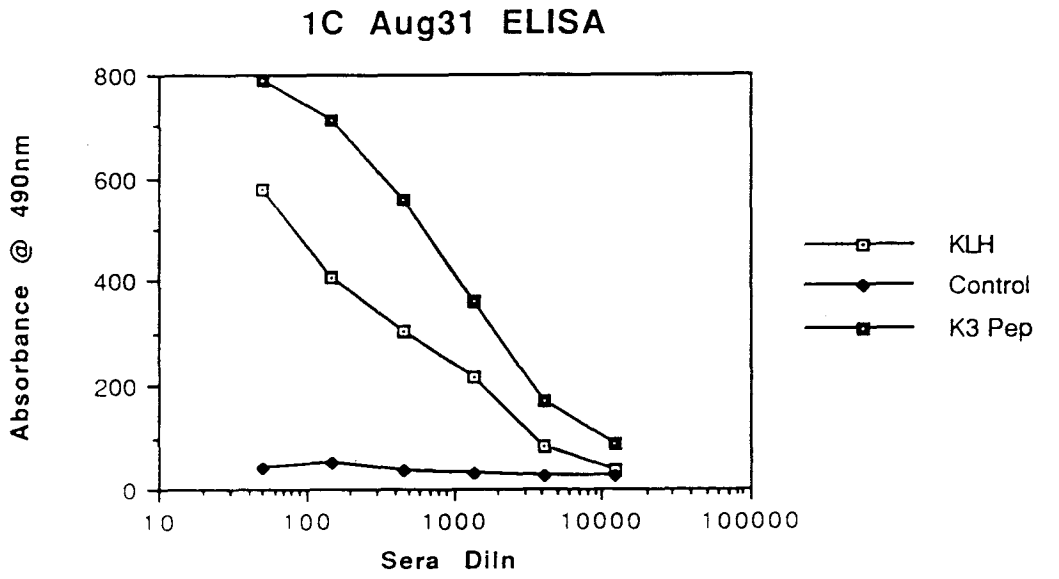
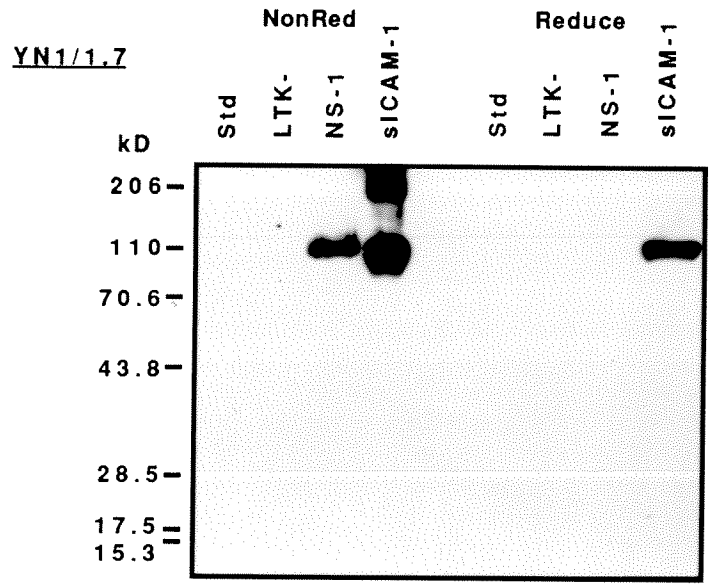


Figure 17 Anti-peptide AntiSera Titrations. Sera (from rats injected with synthetic peptides conjugated to KLH) were diluted and tested by ELISA against KLH alone, specific peptide, and control peptide. Background levels were subtracted. **a)** 2C July 27 antisera (common peptide), **b)** 1C Aug 31 antisera (K3 test peptide).

a)



b)

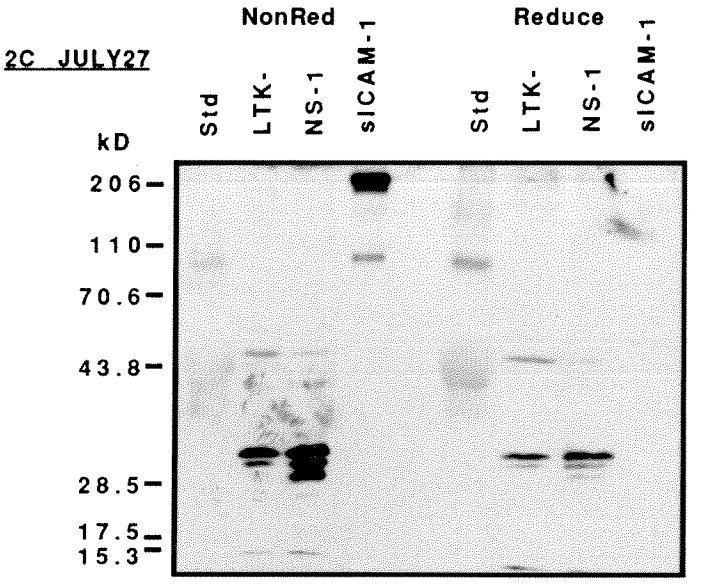
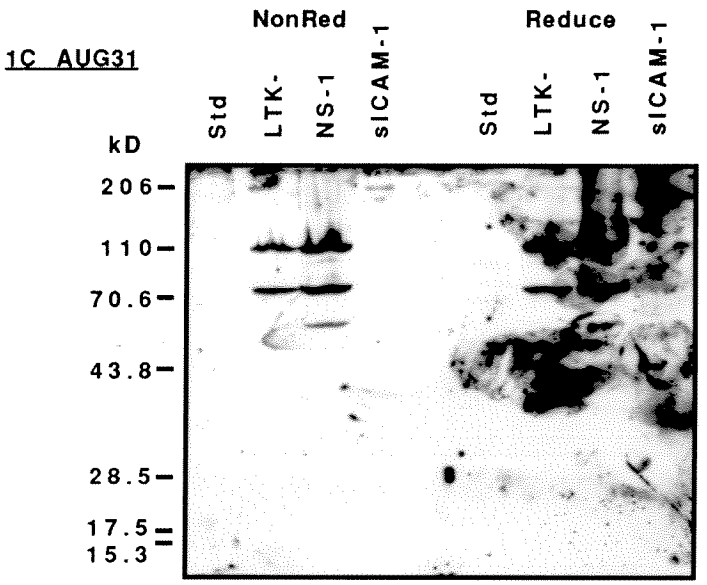


Figure 18 Western Blot Analyses Using Anti-peptide AntiSera. NS-1 and L cell lysates were separated by SDS-PAGE (10%), blotted to nitrocellulose filters, and detected with the respective antiserum or MAb. Std, molecular weight standards; LTK-, L cell lysate (negative control); NS-1, NS-1 cell lysate (test); sICAM-1, soluble MALA-2, 500 ng (positive control) **a)** YN1/1.7 MAb, **b)** 2C July 27 antisera (common peptide), and **c)** 1C Aug 31 antisera (K3 unique peptide).

c)


1C AUG31
NonRed
Reduce
Std
LTK-
NS-1
sICAM-1
Std
LTK-
NS-1
sICAM-1
kD
206
110
70.6
43.8
28.5
17.5
15.3

under nonreducing conditions, but detected soluble MALA-2 (~93 kD) under nonreducing and reducing conditions (Figure 18a). A large protein of 200 kD was also visible but this was thought to be the YN1/1.7 MAb contaminating the immunoaffinity purified soluble MALA-2 preparation as the secondary Ab (anti-rat Ig) specifically reacted against this 200 kD protein. Both control sera (2B and 2C against the common peptide) only recognized soluble MALA-2 under nonreducing conditions and not MALA-2 in the NS-1 cell lysate (Figure 18b: 2B control sera not shown). Thus, although the control antisera demonstrated activity against the injected peptide, it did not recognize the native MALA-2 protein. The test sera (1C against K3 unique peptide) did not detect soluble MALA-2 under nonreducing or reducing conditions (Figure 18c). However, two unique proteins, 90 kD and 63 kD, were detected under both nonreducing and reducing conditions in the NS-1 cell lysate. The 90 kD was closest to the expected size of the K3-1.1 translated protein.

To further analyze these possible K3 proteins, immunoprecipitation was done on ^{125}I labelled cells (Figure 19). Only the positive control YN1/1.7 MAb precipitated a protein, which corresponded to the expected size of MALA-2. The preimmune sera or K3 test sera did not precipitate any labelled K3 protein (Figure 19). Immunoprecipitation was also done on unlabelled cells (data not shown), however, no unique K3 proteins were detected.

4.3 DISCUSSION

Southern blot analysis of the MALA-2 gene indicates it is a single copy gene with no obvious differences between different strains of mice or cell lines derived from the same strains. A 4.0 kb *Bam HI* fragment on Southern blots is positive for the 5' and 3' region of K4-1.1 cDNA however double digests indicate the existence of two 4.0 kb *Bam HI* fragments. One clone, J4 (4.0 kb), was isolated from a genomic library screened with K4-1.1 cDNA, and contained five exons in the 3' region of the MALA-2 gene. Although several libraries were extensively screened, attempts to isolate the 5' region of the MALA-2 gene were not successful. A restriction map and exon organization of the gene was surmised from Southern blot data and the J4 sequence. The 5' region of the K3-1.1 cDNA is endogenous to the mouse genome but

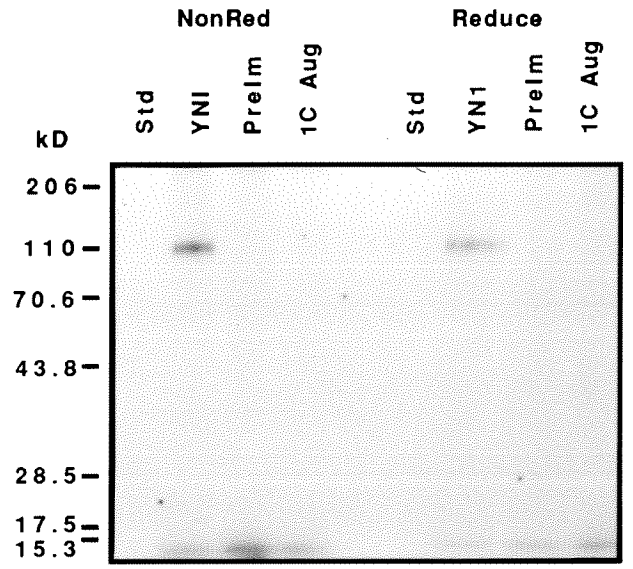


Figure 19 Immunoprecipitation of a possible K3 Protein. NS-1 cells were surface labelled with ^{125}I and lysed. The lysate was precleared with MαRIg-Affigel 10 beads, and incubated with YN1/1.7 MAb, preimmune sera, or 1C Aug 31 antisera. MαRIg-Affigel 10 beads were added and the Ag-Ab complexes were collected by centrifugation. The recovered proteins were separated by SDS-PAGE and exposed to film.

is not closely linked to the K4-1.1 5' region. Two clones positive with the K3-Asp probe did not contain sequences within the K4-Xho probe. A K3-1.1 transcript is not detectable by Northern blot analysis but PCR results support the existence of a K3-1.1 transcript. The attempt to make an antisera that bound a possible K3-1.1 protein did not yield conclusive results.

Genomic cloning resulted in the isolation of a *Bam HI* fragment containing five exons corresponding to the 3' region of the gene, as well as a pseudo exon encoding one half an Ig domain between the first and second characterized exons. Four exons correlate somewhat to the domains II-V of the MALA-2 protein although the separation is not exact. This correlation of the exons with the domains of the protein is common to other members of the Ig family (8). The last exon encodes for the transmembrane, cytoplasmic, and untranslated regions similar to the VCAM-1 gene (9). The pseudo exon, between the first and second exons, lacks the splice acceptor and donor consensus sequences, and ICAM-1 transcripts containing this one half Ig domain in the liver or lung RNA of mice stimulated by LPS have not been detected by PCR (10). Similar one half exons are found in the VCAM-1 gene (designated ψ1 and ψ2) but these are only expressed in rabbit and not in human (9). The ancestoral immunoglobulin has been postulated to have been a half domain structure that evolved to form a homodimer and it is possible this ψ sequence is leftover from exon shuffling (8). Further screening of genomic libraries failed to isolate a clone containing the 5' region of the MALA-2 gene although it can be surmised that this is also contained within a 4.0 kb *Bam HI* fragment.

The inability to isolate a clone encoding the 5' end to the MALA-2 gene may have been due to the libraries used in the screening or more specifically the representation of the wanted gene in the libraries. Screening of the library with K4-1.1 should have yielded the some clones, however no positives were isolated from screening $1x10^6$ plaques. Phage DNA was present on the filter (detected by C57BL/6 spleen DNA & λ *Hind III* DNA), and the hybridization conditions were adequate (K4-1.1 cDNA in λgt10 controls were positive).

Similarily, screening the libraries with an unrelated cDNA (YE1/48) to assess the quality of the library also failed to isolate any clones, however proper controls were not done in this case. Conversely, when the CD43 cDNA was used as a probe, 8 positive clones per $2x10^5$ plaques were detected. Thus, the embryo library seemed to be representative for the CD43 gene, but the MALA-2 gene is not present at a comparable frequency.

The representation of a particular sequence of interest in a genomic library is dictated by the size of the cloned fragments and the size of the genome. If the typical mammalian genome is $3x10^9$ bp and the average clonable fragment is $1.2x10^4$ bp, these values can be used in an equation to calculate the number of clones to screen to ensure a 99% chance of isolating an individual sequence (10). When this calculation is performed, a value of $1x10^6$ clones is obtained. A total of $2.2x10^6$ plaques were screened from all of the libraries, however the number of unique clones within these plaques is not known, and several plaques may contain the same insert. The gene for MALA-2 seems to be at a lower frequency than the above calculated estimate (1 positive per $1x10^6$pfu). More screening may have resulted in the isolation of a clone. This low frequency of the MALA-2 gene in the libraries may have been due to the techniques used to construct the libraries although both the embryo and liver libraries were purchased and expected to be standardized in their construction, or have something to do with the DNA itself. Highly repetitive DNA may be difficult to clone due to its instability, and the formation of secondary structures. Examples of such elements that are difficult to clone are present in the literature (11).

Recently, Ballantyne *et al.* published the full genomic structure of murine ICAM-1 (12). From screening $2x10^6$ plaques (NIH3T3 genomic library), they isolated two clones spanning the same 4 kb *Bam HI* fragment that was characterized in this thesis study, encoding 5 exons and the pseudo exon (ψ). Neither of these clones hybridized with the K4-Xho probe and a total of $2x10^6$ plaques from a second genomic library (BALB/c) was subsequently screened isolating a single clone. This clone still lacked the 5' most region of K4-1.1. Oligonucleotides were used to generate a 90 bp 5' probe by PCR, and a Southern blot was probed detecting a 3.1

kb *Eco RI* fragment, and a *Bam HI* 4.3 kb fragment. A size selected library of genomic DNA (*Eco RI*: 2.5-3.5 kb fragments) was constructed and four identical clones with 3.1 kb inserts were subsequently isolated from screening approximately $4x10^5$ plaques. One clone was further characterized.

In summary, three overlapping clones were isolated encoding 6 exons of murine ICAM-1, and one nonoverlapping clone contained the 5' most exon and upstream regulatory elements. Ballantyne *et al.* concluded that the murine ICAM-1 gene spans over 13 kb and is composed of seven exons and six introns (12) . Exon 1 contains the 5' untranslated and signal peptide, exons 2-6 contain Ig domains I-V respectively, and exon 7 has the transmembrane, cytoplasmic and 3' untranslated regions. The most 5' clone **is** 2.1 kb upstream of exon 1. Probes from exon 1 and 2 hybridize to a single 4.3 kb *Bam HI* and a 6 kb *Xba* I fragment.

Examination of methods and results of Ballantyne *et al.* indicates that a large number of plaques were screened to obtain the four clones that contained the whole gene (12). Two $x10^6$ plaques were screened to isolate two clones which covered the same region as the J4 clone characterized in this thesis study, and another $2.4x10^6$ plaques were screened to isolate 2 clones containing the 5' most exons. This low representation of the gene is in agreement with the present study. Additionally, characterization of the regulatory regions of the MALA-2 gene show the presence of 3 AP-1, and 2 SP-1 sequences (12), and these repeats may contribute to the instability of the gene and the ability to clone it. More suspicious though, is the inability to isolate a clone which contained both exons 1 and 2. Thus, it is conceivable that the intron between these exons may contain sequences which make the 5' region unstable and difficult to clone. Sequences which are direct repeats could recombine and delete portions of the gene, or the sequences may be toxic to the phage vector.

The proposed structure of the MALA-2 gene put forward in this study was confirmed by Ballantyne *et al.* Two 4.0 kb *Bam HI* fragments encompass all of the exons of the gene (12): one *Bam HI* fragment contains the two 5' exons, and the other *Bam HI* fragment contains the remaining five exons and the ψ sequence. *Eco RI* fragments of 2.3 and 3.1 kb, and a *Xba I*

fragment of 6 kb were detected on Southern blots when probed with K4-Xho, and these fragment sizes are similar to those reported by Ballantyne *et al* .(12). Interestingly, the human ICAM-1 gene is also made up of seven exons and six introns, and maps to chromosome 19 near the LDL receptor (Ldlr) gene (13). The gene for murine ICAM-1 has been mapped to the centromeric end of chromosome 9 near the Ldlr and one of three genes involved in insulin-dependent diabetes, Idd-2 (14). These data establish a new conserved segment between human chromosome 19 and proximal mouse chromosome 9.

The 5' region of the K3-1.1 cDNA characterized in this thesis study does not appear to be closely associated with the K4-1.1 sequences. Four clones were isolated from genomic libraries by screening with K3-1.1, K3-Asp, and K3-Sac. Two clones contained a 4.0 kb *Bam HI* fragment identical to the J4 clone, and were probably detected because of contaminating 3' sequences with the probes, although the probes were checked by agarose gel for impurities. The other two clones were quite large (12 & 14.5 kb) but were only positive with the K3-Asp probe on a phage Southern blot and not the K4-Xho probe. Southern blot analyses with K3-Asp and K4-Xho also do not detect a common fragment.

The two cDNAs isolated in this work show the same sequences beginning at nt 638 of K3-1.1 and nt 132 of K4-1.1, and this common point is located in the middle of the second exon of the gene as characterized by Ballantyne *et al.* (12). This organization of the introns and exons does not favor normal alternative splicing for the production of the K3-1.1 transcript; however, the remainder of a cryptic splice site is present at nt 636 (AT/G) and its use would support the creation of K3-1.1 (Chapter 3, Figure 1c). CD44 has two cryptic sites in exons 5 and 7 which are used in certain cell types, although they conform to the consensus sequence fully (AGG)(15).

Northern blot analysis done previously (1) uniquely detected a 3.0 kb mRNA with the K3-Asp probe in NS-1 cells, while the K4-1.1 probe detected the 3.0 kb as well as a 2.5 kb message. It was concluded from this data that the K3-1.1 cDNA was representative of the 3.0 kb message. However, this Northern blot analysis has not been confirmed. Moreover, on

numerous occasions a smaller mRNA (0.3 kb) has been detected with the K3-Asp probe. Similarily, in this thesis study, Northern blot analysis detected a 0.3 kb message with the K3-Asp probe and not a 3.0 kb message that would correspond to the K3-1.1 cDNA. The origin of this 0.3 kb message is unknown.

The existence of K3-1.1 as a transcript in cells is supported by the PCR analysis done in this study. A K3 PCR product was detected in two out of three experiments. PCR has much greater sensitivity than Northern blots, therefore it may be that the K3-1.1 transcript is at a very low level and is only detected by PCR part of the time.

The attempt to detect a K3 protein using an antisera developed against a predicted unique K3 N-terminal peptide was inconclusive. The control antisera had activity against synthetic peptide but failed to detect the native MALA-2 in NS-1 cell lysate. The K3 test sera detected two proteins but it is difficult to say whether one of these represents a possible K3-1.1 translated protein.

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CHAPTER 5

SUMMARY AND PERSPECTIVES

5.1 DISCUSSION

Immune responses are primarily mediated by lymphocytes. These cells recognize foreign antigens through specific receptors on the cell surface, and become activated, subsequently differentiating, and proliferating into effector cells. Lymphocyte activation occurs because of the transduction of signals from the cell membrane into the cell and the alteration of expression of specific genes. The elucidation of the cell surface molecules involved in activation as well as other lymphocyte functions, such as adhesion and homing, is important to understanding how an immune response is elicited. MAbs have been invaluable as a tool in the identification and characterization of such antigens besides possibly delineating the function of a particular molecule by mimicking ligand binding.

In a previous study, a rat MAb (YN1/1.7) was raised against NS-1 cells and selected for activity against activated lymphocytes (1). The antigen it recognizes, termed MALA-2, is a 95 kD monomeric protein which is highly expressed on mitogen activated spleen cells but expressed at low levels on normal spleen cells, lymph node cells, and thymocytes. Interestingly, YN1/1.7 MAb inhibits MLR, suggesting that MALA-2 plays a direct role in lymphocyte activation. For this reason the characterization of the gene encoding MALA-2 was studied in this thesis.

Initially, the primary sequence of MALA-2 was studied by cDNA cloning and sequencing. Two cDNAs encoding MALA-2 were isolated from a NS-1 cDNA library using oligonucleotide probes made from tryptic peptide sequences. These cDNA clones (K3-1.1 & K4-1.1) differ in their 5' ends, each having unique untranslated sequences and those encoding the signal and N-terminal sequences. K3-1.1 is 3031 bp in length with a large 5' untranslated region containing 10 initiation sites. It encodes an atypical signal sequence with charged amino acids. K4-1.1 has a short 5' untranslated region with a start site conforming to the consensus sequence proposed by Kozak (2) and encodes a typical hydrophobic signal sequence. MALA-2 is a type I transmembrane protein with a large extracellular region made

up of five Ig-like domains. Comparison of the MALA-2 protein sequence with published proteins revealed significant homology with human ICAM-1. Specifically, the ORF of the K4-1.1 cDNA displays 54% amino acid sequence identity with HICAM-1 with stretches up to 19 residues being identical. The size and distribution of HICAM-1 and MALA-2 are virtually the same. Both molecules are differentially glycosylated accounting for different molecular weights observed (95-115 kD), and are expressed on vascular endothelium, dendritic cells, macrophages, HEV of lymphoid tissues, and epithelial cells (3,4,5). Inflammatory cytokines such as IFNγ and IL-1 greatly increase ICAM-1 expression on vascular endothelium supposedly acting at the level of gene transcription (6). Both molecules have been shown to bind LFA-1. Based on these data it can be concluded that MALA-2 is the murine homologue of HICAM-1. MALA-2 also displays sequence similarity with ICAM-2 (mouse & human), ICAM-3 (human), NCAM, MAG, and other members of the Ig family. All of these molecules have Ig-like domains of about 100 amino acids, and conserve the same cysteine residues.

Having identified MALA-2 as a HICAM-1 homologue, the genomic cloning of the gene was undertaken. This was studied in order to determine the regulatory elements of the gene and to determine if the two isolated cDNAs (K3-1.1 & K4-1.1) are products of alternative splicing. Preliminary Southern blot analyses indicated the gene exists as a single copy with no obvious differences between strains of mice, and was most likely contained within two 4.0 kb *Bam HI* fragments. A partial restriction map was constructed from the Southern blot data. Screening of one size selected library (EL-4, *Bam HI*: 4.0 kb fragments) resulted in the isolation of a 4.0 kb *Bam HI* fragment containing five exons and a pseudo exon. Four of the five exons correspond to domains II-V of the MALA-2 protein, and the last exon encodes the transmembrane, cytoplasmic, and 3' untranslated region. The pseudo exon is about one half an Ig domain in size and is located between the first and second characterized exons. It has 78% sequence identity with part of the third exon but lacks the splice donor and acceptor sequences. The function of the pseudo exon is not known, although it may be a remainder of the primodial gene postulated to have consisted of two one half Ig domains (7).

The sequences encoding the 5' region of the gene (1-357 nt in K4-1.1 cDNA) were not characterized although libraries were extensively screened with the K4-Xho probe. Controls were included in these screenings, indicating the hybridizations were adequate. Probing the embryo library with another gene, CD43, detected several positive plaques indicating the library was representative for this gene. However when both the embryo and liver libraries were screened with the full K4-1.1 cDNA, no positives were detected. It was concluded that the frequency of the MALA-2 gene in the libraries was below what one would expect accounting for average gene length and mouse genome size. Thus, there may be sequences within the gene, probably within the introns, which makes the gene unstable and difficult to clone.

Combining Southern blot data with the sequence of the J4 clone, a more complete picture of the gene structure was deduced. Further analyses of Southern blots allowed a partial restriction map of the gene to be constructed. It was surmised that the 5' region of the gene consisted of at least two exons on another 4.0 kb *Bam HI* fragment, and were located at least 6.0 kb upstream of the other five exons. Thus the whole gene spanned about 16 kb, and the exons were contained within two *Bam HI* 4.0 kb fragments. These data were confirmed by Ballantyne *et al.* (8).

PCR analyses support the existence of the K3-1.1 transcript as an alternatively spliced product from the same gene. However, it is not detectable by Northern blot analysis suggesting it is expressed at a low level.

Since the K3-1.1 cDNA has not been previously reported, and it is at a relatively low level in cells, it is plausible it is created through a rare splicing event. Interestingly, Southern blots indicated the 5' region unique to K3-1.1 was not closely linked to the 5' region of K4-1.1. Additionally, screening genomic libraries with K3-Asp detected two phage clones with large inserts that were not positive with the K4-Xho probe on a phage Southern however this does not exclude them from being near the K4-1.1 sequences. The characterization of the exons by Ballantyne *et al.* suggests that a cryptic splice site would need to be employed to produce a K3-1.1 transcript (8).

An attempt was made to develop an antisera against a protein produced from K3-1.1 by using synthetic peptides. Both the control and K3 test antisera had activity against the synthetic peptides bound to plastic, however, the control sera failed to detect native MALA-2. The K3 antiserum did detect two proteins in NS-1 cell lysate; however, it is difficult to assess whether these represent a possible K3-1.1 translated protein.

The original aim of this research project was to characterize the gene encoding MALA-2. cDNA cloning of MALA-2 determined it as the murine homologue of HICAM-1, a molecule involved in lymphocyte adhesion, and costimulation. This discovery corresponds well to the inhibition of MLR by YN1/1.7 MAb previously documented. The interaction between ICAM-1 and its receptor, LFA-1, is critical to several immune responses. The genomic structure of the gene encoding MALA-2 was partially characterized, and has the characteristic one exon per protein domain known in the Ig superfamily. The existence of a K3-1.1 transcript isolated in this project was supported by PCR analyses, although the generation of such a transcript is most likely a rare event and involves the use of a cryptic splice site.

Experiments that would be interesting to do in the future involve further investigation of the K3-5' sequences. Southern blots indicate that K3-5' is not closely linked to the K4-Xho however analysis of larger fragments of DNA by pulse field electrophoresis may provide some information as to how proximate they are. Furthermore, the characterization of the two phage clones isolated which were positive with K3-Asp but not with K4-Xho may generate probes which may be useful for mapping. More PCR analysis, to see if a K3-1.1 transcript is consistantly found would also be appropriate. Sequencing of PCR products would of course be necessary to confirm their identity.

Analysis of a possible protein produced by K3-1.1 may be better examined by the use of the K3-1.1 antiserum against COS cells transfected with K3-1.1 cDNA. In this case there may be more protein in these cells allowing easier detection as compared to NS-1 cells. The result still may be negative, but at least the probability of obtaining a positive result would be higher.

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