CHARACTERIZATION OF SaBR, A NOVEL MURINE
CELL SURFACE PROTEIN

by

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ABSTRACT

Regulation of hematopoiesis is a complex process consisting of multiple interactions involving cell surface proteins and soluble mediators.

SaBR is a novel murine cell surface protein that is differentially expressed within the hematopoietic system in terms of cell lineage, location, developmental stage and activation status and as such is a candidate for a hematopoietic regulator. In the lymphocyte lineage, SaBR is expressed throughout B cell development, but is only found on mature single positive T cells. In vitro activation of purified splenic lymphocytes results in changes in SaBR expression dependent on the activator being used. In B cells, SaBR is downregulated following cross-linking of the B cell receptor in conjunction with a costimulatory stimulus, but is upregulated if two costimulatory signals are used. In T cells, SaBR upregulation is observed upon activation with phorbol ester plus ionophore and phorbol ester plus α-CD3. In addition, B220+SaBR+ sorted B cells were found to have a significantly higher tritiated thymidine incorporation than their negative counterparts in both non-activated and activated populations.

Sequencing analysis of a cDNA encoding murine SaBR revealed an 820 a.a type I transmembrane protein with three putative extracellular domains. The amino terminal portion of SaBR encodes a somatomedin B-like domain and the other two extracellular domains have sequence similarity to a region of von Willibrand factor and the membrane proximal domains of the selectin and complement receptor families. A human cDNA was found to have 73% nucleotide identity to mouse SaBR and Northern analysis revealed the presence of two differentially expressed human SaBR mRNA products as
compared to one for the mouse. When histochemical analysis was performed, unique
SaBR expression patterns were detected in the kidney and stomach, suggesting a
potential role for SaBR in these tissues.

The studies described in this thesis indicate that SaBR is a unique transmembrane
protein expressed in the hematopoietic system and may play a role in lymphocyte
activation. At present, there is not enough data to make conclusions regarding SaBR
function in the mouse; however, a model presenting SaBR as a regulator of lymphocyte
activation is proposed.
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<td>a-</td>
<td>anti</td>
<td>kb</td>
<td>kilobase</td>
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<td>a.a.</td>
<td>amino acid</td>
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<td>lymphocyte functional antigen</td>
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<td>antigen presenting cell</td>
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<td>mean fluorescent intensity</td>
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<td>B cell receptor</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
<td>NK</td>
<td>natural killer (cell)</td>
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<td>base pairs</td>
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<td>cluster of differentiation (antigen)</td>
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<td>Somatomedin B related protein</td>
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<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
<td>sIg</td>
<td>surface immunoglobulin</td>
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<td>granulocyte-macrophage colony stimulating factor</td>
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<td>side scatter</td>
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<td>HBSS</td>
<td>Hanks balanced salt solution</td>
<td>TCR</td>
<td>T cell receptor</td>
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<td>human growth hormone</td>
<td>Th</td>
<td>T helper</td>
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<td>HSA</td>
<td>heat stable antigen</td>
<td>VLA-4</td>
<td>very late activation antigen 4</td>
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<td>ICAM</td>
<td>intracellular adhesion molecule</td>
<td>vWF</td>
<td>von Willibrand's factor</td>
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<td>WGA</td>
<td>wheat germ agglutinin</td>
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<td>IL -</td>
<td>interleukin</td>
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<td>IP3</td>
<td>inositoltriphasphate</td>
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INTRODUCTION

The formation of different types of blood cells is essential for the development of a normal individual. Abnormalities in the developmental program, or hematopoiesis, results in various types of hematological disease. Understanding the molecular control of normal development and mature cell function makes it possible to answer questions about the origin and possible treatment of these diseases.

The overall objective of this thesis is to study novel cell surface proteins that are expressed on hematopoietic cells and determine if these molecules play a regulatory role during hematopoiesis. The protein I have chosen to study is called SaBR. It is a novel type I transmembrane protein expressed on progenitors and mature cells within the hematopoietic system. In this chapter, I will give a brief overview of hematopoiesis and cell surface proteins with a more detailed description of lymphocyte activation and regulation. Finally, I will outline the history behind the initial cloning of SaBR as well as the thesis rationale and objectives. Other non-hematological aspects of SaBR are discussed in Chapters 3, 4 and 5.

1.1 THE HEMATOPOIETIC SYSTEM

Hematopoiesis is the process whereby multiple types of mature blood cells are generated from more primitive precursors in the bone marrow (Figure 1.1). McCulloch (1993) and Dexter and Spooncer (1987) review the evidence showing that the most primitive hematopoietic cells, or stem cells, can be recognized by their ability to reconstitute multilineage hematopoiesis in lethally irradiated recipient mice. Stem cells are pluripotent and will differentiate into multipotential lymphoid and myeloid
Figure 1.1. **Schematic diagram of the hematopoietic hierarchy.** Adapted from Williams *et al.*, 1990.
progenitors. The multipotential lymphoid progenitors remain in the marrow for subsequent differentiation into mature B cells, or migrate into the thymus for differentiation into mature T cells (Ikuta et al., 1992; Aguila et al., 1997). For a more detailed description of lymphoid differentiation refer to Sections 1.3 and 1.4. The myeloid progenitors proliferate within the bone marrow under the stimulus of a variety of growth factors and eventually differentiate into uni or bipotential progenitors with limited self-renewal (Dexter and Spooncer, 1987; McCulloch, 1993). These progenitors can be identified by their ability to form lineage restricted colonies of mature blood cells when placed in semisolid media with a source of specific hematopoietic growth factors and will eventually undergo transformation to non-self-renewing precursor cells destined for maturation into functional blood cells (McCulloch, 1993).

The final stages of mature blood cell development can be identified by morphological changes which are visible at the cellular level under the light microscope. The earlier progenitor stages are more difficult to define in this manner; therefore, other distinguishing characteristics must be used. During differentiation, functionally important cell surface molecules, or markers, are expressed or extinguished; therefore fluorescently labeled antibodies to specific combinations of lineage-restricted and non-restricted markers can be used to identify unique cell populations by flow cytometry. For example, mouse stem cells can be defined by their positive expression of the lineage non-restricted marker Sca-1, their ability to bind WGA (wheat germ aglutinin) and their lack of expression of lineage markers that define uni or bipotential progenitor cells already committed to a specific cell lineage (Rebel et al., 1994; Spangrude et al., 1988).
1.2 REGULATION OF HEMATOPOIESIS

The development of hematopoietic cells is regulated through complex signaling pathways involving soluble growth factors, membrane bound factors, the extracellular matrix and other cell types (Dorshkind, 1990; Rolink and Melchers, 1991a; Ikuta et al., 1992; Schwarzmeier, 1996). Studies have shown that a large number of positive and negative factors are required to regulate the survival and/or proliferation of any single type of developing or mature cell (Dexter and Spooncer, 1987; Metcalf, 1989). Furthermore, it now seems likely that many growth-factor stimulated responses are determined by the target cell, rather than by the factor to which the cell is exposed (Sutherland et al., 1991). Studying hematopoietic regulation is further complicated by the fact that many growth factors affect not only a large range of target cells, but may also be produced by many different cell types of both hematopoietic and non-hematopoietic origin that coexist within the bone marrow (Clark and Kamen, 1987; Malipiero et al., 1990; Schwarzmeier, 1996).

1.2.1 Regulation by growth factors

The regulation of hematopoietic cell proliferation and differentiation by soluble growth factors has been extensively studied (Schwarzmeier, 1996). In vitro experimentation suggests that growth factors fall into two broad categories: 1) multipotential growth factors, such as IL-3 and GM-CSF, act on multipotential progenitor cells. These factors are not lineage specific, are required throughout differentiation and are important for self-renewal and proliferation. 2) lineage-restricted growth factors, such as erythropoietin (EPO), G-CSF and M-CSF act on more mature progenitors and are only required later during the development of specific cell lineages. They influence
proliferation and differentiation of lineage restricted progenitors as well as the survival of mature cells (Whetton and Dexter, 1986; Sieff, 1987; Metcalf, 1989; Schwarzmeier, 1996).

Early experiments showed that in the absence of additional growth factors, the bone marrow stromal cells could not only maintain the pluripotent stem cell in vitro (Schofield and Dexter, 1985), but also promote the proliferation, development and maturation of progenitor cells able to produce both lymphoid and myeloid colonies (Dexter et al, 1984). These stromal cells were shown to be capable of producing growth factors (Sutherland et al., 1991; Gualtieri et al., 1984; Fibbe et al., 1988;), however, intimate contact of the hematopoietic progenitors with the stromal cell layer was also required for survival and proper differentiation of stem cells (Dexter et al., 1990). Some growth factors, such as GM-CSF, bind to extracellular (ECM) components, which may serve as an important mechanism for enhancing their effective concentration at localized sites (Gordon et al., 1987), but interactions between cell surface molecules also play an important role in triggering both the proliferation of progenitors committed to specific lineages and their differentiation into mature cell types (Dorshkind, 1990; Rolink and Melchers, 1991a; Sachs, 1987).

1.2.2 Regulation by cell-cell contact and cell surface proteins

Considerable progress has been made in defining cells that regulate hematopoiesis in the bone marrow, but very little is known about the specific molecules involved in this regulation. The bone marrow microenvironment that regulates hematopoietic cell development is composed of hematopoietic cells, extracellular matrix molecules and stromal cells. Stromal cells can be defined as a non-hematopoietic adherent cell
population composed of adventitial cells, reticular cells, endothelial cells, fibroblasts and adipocytes (Rolink and Melchers, 1991a). It has been known for some time that blood cells and stromal cells are intimately associated and that most immature progenitors are associated with the adherent stromal cell layer (Dorshkind, 1990). Diffusion chamber cultures with immature progenitors have shown that a lack of stromal cell contact results in a loss of CFU-S (Bentley, 1981) and a disruption in B cell differentiation (Kierney and Dorshkind, 1987).

Reticular cells are also involved in regulating hematopoiesis. These cells have extensive cytoplasmic extensions that make intimate contact with hematopoietic cells as well as with endothelial cells. In fact, this contact may serve to regulate emigration of blood cells through the endothelial cell layer into the systemic circulation (Dorshkind, 1990). In addition to producing stimulatory and inhibitory cytokines, macrophages and T cells are also believed to play a role in hematopoietic cell regulation, possibly through cellular contacts with progenitor cells (Rolink and Melchers, 1991a).

It is believed that stromal cells and hematopoietic progenitors are likely to make multiple molecular contacts. In the early 1990's, several molecules including fibronectin (Bernardi et al., 1988), VLA-4 (Miyake et al., 1991), CD44 (Miyake et al., 1990a) and hyaluronate (Miyake et al., 1990b) were shown to mediate these contacts; however identifying new contacts has proven to be difficult due to the complexity of the interactions involved.
1.3 T LYMPHOPOIESIS

In order for T cell development to occur, it is believed that multipotential stem cells from the bone marrow first travel to the thymus (Shortman and Wu, 1996; Aguila et al., 1997). In support of this hypothesis, Wu (1991) found early stem cells within the outer subcapsular zone of the thymus (Mondino et al., 1996) that were capable of forming T cells, B cells, natural killer (NK) cells and dendritic cells. From the subcapsular zone, the immature precursors migrate deeper into the thymus and enter the cortex where they begin to differentiate (Mondino et al., 1996). Further differentiation occurs during their migration into the thymic medulla (Mondino et al., 1996; Williams et al., 1990). During the migration from cortex to medulla more than 95% of the thymocytes will die due to negative selection processes that preserve only those T cells that express self-MHC restricted, foreign antigen-specific T cell receptors (TCRs) (Mondino et al., 1996; Anderson et al., 1996). In fact, it is estimated, in mice, that approximately $5 \times 10^7$ immature cells develop in the thymus each day, but after differentiation and selection, only $1 \times 10^6$ mature cells leave (von Boehmer, 1994; Mondino et al., 1996).

The earliest T lymphoid specific progenitor can be distinguished from NK cell and dendritic cell precursors only by the expression of the $\alpha$ chain of the IL-2R (CD25). This progenitor is CD3'CD4'CD8' and all of the TCR chains are in germline configuration. At this point, differentiation to three different T cell lineages can occur, the $\alpha\beta$ T cells (TCR consists of an $\alpha$ and $\beta$ chain), the $\gamma\delta$ T cells (TCR consists of a $\gamma$ and $\delta$ chain) and the CD4'CD8' $\alpha\beta$ T cells (Shortman and Wu, 1996). Figure 1.2 shows the
Figure 1.2. T cell development in the thymus. Schematic representation of early T cell populations in the thymus and their distinguishing cell surface markers. Adapted from Shortman and Wu (1996).
various stages of T cell development within the thymus and the markers that characterize each stage.

In addition to the adult thymus, γδ T cells can develop in the fetal thymus and intestinal epithelia (Haas, 1993). The cells that develop in the fetal thymus home to and are maintained throughout adult life in the skin and the mucosa of the uterus, vagina and tongue (Haas, 1993). Although the developmental processes for γδ T cells are virtually the same as for αβ T cells, the γδ T cells form monospecific subpopulations due to the preferential use of only 4 Vγ chains (Haas, 1993; Shortman and Wu, 1996). Furthermore, mature γδ T cells recognize non-peptide antigen and while recognition requires a functional TCR, antigen presenting cells expressing MHC are unnecessary (Boismenu and Havran, 1997). Although the function of γδ T cells is still unclear, recent studies suggest that they may complement αβ T cells in the defense against microorganisms and viral infections (Haas, 1993; Welsh et al., 1997).

The developmental pathway for mature CD3+CD4-CD8- T cells is unknown. Their function is also a mystery, but recently Kitano et al., (1997) found that they may play a role in hypereosinophilia syndrome (HES). Examination of T cell subsets within the blood revealed that proliferation of CD3+CD4-CD8- T cells leads to IL-5 production, which in turn has been causally linked to eosinophilia (Kitano et al., 1997).

Since over 90% of mature peripheral T cells are of the αβ lineage, this discussion will concentrate on the development of those cells (Figure 1.2). The first event to occur along the αβ developmental pathway is the rearrangement of the TCR β chain in the CD3+CD4+CD8- progenitors as they migrate into the cortex. During migration, the cells come into contact with thymic epithelial cells which produce IL-7 and stem cell factor.
(SCF), cytokines which are required for proliferation and RAG gene expression (Suda and Zlotnik, 1991; Muegge et al., 1993; Appasamy et al., 1993). The expression of RAG is required for β chain rearrangement (Shortman and Wu, 1996). This stage is also characterized by the upregulation of the marker CD3 and the downregulation of the markers c-kit and CD44 (Shortman and Wu, 1996). At this point the cells are generally referred to as CD3^+CD4^-CD8^-, or double negative cells.

After β chain gene rearrangement, the β chain is expressed on the cell surface in association with a pre-TCR α chain and CD3 (Von Boehmer and Fehling, 1997; Groettrup et al., 1993). Together, the β chain/pre-TCR α chain and CD3 comprise the pre-TCR complex. This complex has been shown to be required for successful α chain gene rearrangement, allelic exclusion and differentiation to form a population that is CD3^+CD4^-CD8^+, or double positive (Shortman and Wu, 1996; Von Boehmer and Fehling, 1997; Saint-Ruf et al., 1994). Interestingly, Brumo et al. (1996) have shown that the pre-TCR complex plays a role in regulating TCRδ expression in TCR transgenic mice. They suggest that even though the γδ population does not express the pre-TCR α chain, the pre-TCR may be involved in the development of γδ T cells by regulating γδ gene rearrangement (Von Boehmer and Fehling, 1997).

α chain rearrangement follows β chain rearrangement and results in the production of unique functional T cell receptors conferring distinct antigen-binding specificity’s (Shortman and Wu, 1996). As the CD4^+CD8^+ cells migrate through the cortex and into the medulla they come into close physical contact with extracellular matrix (ECM) components and a variety of non-lymphoid cells; including epithelial cells, medullary macrophages and dendritic cells (Mondino et al., 1996; Anderson et al., 1996).
This physical contact has been shown to be important for thymocyte maturation and selection (Mondino et al., 1996; Anderson et al., 1996). In fact, several studies have shown that production of ECM in the thymus is mirrored by the expression of receptors for these molecules on developing thymocytes and that ECM receptor expression is highest on immature CD4-CD8- precursors and gradually decreases during maturation (Anderson et al., 1996; Wadsworth et al., 1992).

CD4+CD8+ cells expressing low levels of the TCRαβ must interact with MHC class I or II expressing macrophages, dendritic or epithelial cells in order for positive and negative selection to occur, such that only those cells bearing receptors with the potential to recognize foreign peptides in the context of self-MHC molecules are allowed to mature (Anderson et al., 1996; Anderson et al., 1993; Von Boehmer, 1994; Kappler et al., 1987; Nossal, 1994). Low avidity binding of MHC class I and II molecules to the TCRs of developing thymocytes results in positive selection whereas high avidity interactions lead to negative selection and death by apoptosis (Anderson et al., 1996; Von Boehmer, 1994; Kappler et al., 1987; Nossal, 1994). Recently, studies have indicated that in addition to TCR-mediated signals, accessory signals supplied by thymic stromal components are also required for positive and negative selection (Anderson et al., 1996; Anderson et al., 1994; Page et al., 1993). Furthermore, Anderson et al. (1996) have shown that cell contact between epithelial cells and thymocytes is required for positive selection and maturation to the CD4+ or CD8+ stage, while contact with dendritic cells is the most important for negative selection.

Finally, positively selected T cells that recognize class I MHC will differentiate to single positive CD8+ T cells and those that recognize class II MHC will differentiate to
single positive CD4$^+$ T cells ready for exit to the periphery (Shortman and Wu, 1996; Anderson et al., 1996).

Peripheral CD4 and CD8 T cells play different functional roles. The majority of CD8 cells play a cytotoxic role and kill virally infected cells or tumor cells (Collins et al., 1993; Kemeny et al., 1994). CD4 cells are generally helper T cells and serve to activate B cells during an immune response (Croft, 1997; Collins et al., 1994).

Following activation, CD4 T helper cells undergo further differentiation events to form two distinct subsets; Th1 and Th2 cells (Belardelli, 1995; Constant and Bottomly, 1997). The subsets formed depend on the cytokines present in the microenvironment; IFN-γ and IL-12 promote the differentiation of Th1 subsets, while IL-4, IL-5 and IL-10 promote differentiation to Th2 cells (Belardelli, 1995; Constant and Bottomly, 1997).

Th1 cells secrete cytokines usually associated with inflammation, such as IFNγ, and TNFβ and induce cell-mediated immune responses (Constant and Bottomly, 1997). Moreover, Th1 cytokine production is increased in response to intracellular pathogens and this increase is associated with macrophage activation, the delayed-type hypersensitivity response and IgG2a production (Belardelli, 1995).

Th2 cells, on the other hand, produce cytokines such as IL-4 and IL-5 that help B cells to proliferate and differentiate and are associated with humoral immunity (Constant and Bottomly, 1997). Th2 cells have been found to play a role in allergic reactions and helminth infections by increasing IgE production as well as eosinophil and mast cell production (Belardelli, 1995).
1.4 B LYMPHOPOIESIS

During embryogenesis, B lymphopoiesis occurs in waves; first in the placenta and embryonic blood, then in the fetal liver and finally in the spleen and bone marrow (Melchers, 1979; Rolink and Melchers, 1993). In the adult mouse, $5 \times 10^7$ cells per day are produced from B cell progenitors in the bone marrow. Of these, $2-3 \times 10^6$ survive to enter the peripheral pool of mature, recirculating cells (Rolink and Melchers, 1991a).

The generation of mature B cells from multipotent progenitor cells consists of a complex series of differentiation steps that occur in close physical association with the bone marrow microenvironment. During B lymphopoiesis, B lineage progenitors can be found within the extravascular spaces between sinusoids in the shaft of long bones (Rolink and Melchers, 1991a). Proliferation of the most immature B cell progenitors is highest near the inner surface of the bone where the progenitors can come into direct contact with stromal cells. The stromal cell influence is mandatory for B cell development (Kincade et al., 1989; Dorshkind and Landreth, 1992) and is exerted through cell-cell interactions and through cytokine secretion (Cushley and Harnett, 1994; Dorshkind and Landreth, 1992). As the B cells differentiate, they move away from the stroma toward the center of the marrow, either to die or to exit into the peripheral circulation via the sinuses (Rolink and Melchers, 1991a).

Figure 1.3 is a schematic diagram of B cell development using the nomenclature of Melchers et al. (1995). The first descendant of the multipotent progenitor cell that is committed to the B lineage is the pro-B cell. This cell is dependent upon the stroma and the cytokine, IL-7, for survival, it does not express the B lineage-specific marker
Figure 1.3. **B cell development**. Schematic diagram of B cell development showing the cell surface markers and characteristics that distinguish different developmental stages. Adapted from Rolink and Melchers (1993).
BP-1, but does express B220(CD45R), c-kit, CD43 and Thy-1 (Melchers et al., 1995; Dorshkind and Landreth, 1992; Rolink and Melchers, 1991a; Rolink et al., 1991b). Furthermore, it has not yet begun to rearrange its immunoglobulin genes, although it does express surrogate light chain (Melchers et al., 1995).

The next stage, pre-BI, is also stromal cell and IL-7 dependent and shares some of the same cell surface markers as pro-B cells, such as B220 and c-kit expression, but these cells also express the marker BP-1 (Dorshkind and Landreth, 1992; Melchers et al., 1995; Rolink and Melchers, 1991a). Furthermore, many of these cells have undergone $D_HJ_H$ heavy chain gene rearrangement at this stage (Melchers et al., 1995).

Further proliferation of pro-B and pre-BI cells pushes them from the stromal cell contacts, thereby releasing these cells into $V_H$ to $D_HJ_H$ heavy chain gene rearrangements and differentiation to pre-BII cells (Rolink and Melchers, 1991a). In fact, it has been shown that expression of a successfully rearranged $\mu$ heavy chain interferes with proliferation of developing B cells on the stroma (Melchers et al., 1992). Pre-BII cells can be distinguished from pre-BI cells by the absence of the marker c-kit and the appearance of the $\alpha$ chain of the IL-2R (Melchers et al., 1995; Rolink et al., 1994; Rolink et al., 1991b). Pre-BII cells are also larger than pre-BI, actively cycling, stromal cell and IL-7 independent and have undergone $V_H$ to $D_HJ_H$ rearrangement (Melchers et al., 1995). Light chains are still in germline configuration at this stage, but pre-BII cells still express surrogate light chain, which in combination with the heavy chain comprises the pre-B cell receptor (Melchers et al., 1995). The expression of the pre-B cell receptor signals inhibition of proliferation and inhibition of further heavy chain rearrangements (Melchers et al., 1995; Karasuyama et al., 1996).
The transition from pre-BII to immature B cells is characterized by light chain rearrangement, stromal cell independence and the appearance of surface immunoglobulin (sIgM) (Rolink and Melchers, 1991a; Rolink and Melchers, 1997). During this stage, the immunoglobulin repertoires of immature B cells are screened for their ability to bind soluble autoantigen (Nossal, 1994). Cells that bind strongly to autoantigen will either be deleted, inactivated or undergo secondary light chain gene rearrangements (receptor editing) (Melchers et al., 1995; Gay et al., 1993; Tiegs et al., 1993; Nemazee, 1996). During receptor editing V genes coding for antiself receptors are replaced with V genes coding for nonself receptors (Gay et al., 1993; Nemazee, 1996). Gay et al., (1993) have shown that V gene replacement can occur at both the heavy and light chain \( \kappa \) loci and that light chains of autoreactive antibodies can be displaced by different light chains to yield nonautoreactive specificities.

Mature B cells express both surface IgM and IgD and are fully responsive to antigen. Following activation, mature naïve B cells will proliferate and either differentiate into plasma cells capable of secreting antibodies or migrate into primary follicles within the lymphoid organs to initiate the formation of germinal centers. In the germinal centers the B cells will undergo proliferation, isotype switching and somatic hypermutation (Berek et al., 1991; Von Boehmer, 1994; Liu and Arpin, 1997).

1.5 THE IMMUNE RESPONSE-AN OVERVIEW

The specific immune response to foreign antigens is a highly coordinated series of cellular interactions between antigen-presenting cells (APC), lymphocytes and other effector cells and their soluble cytokine products (Belardelli, 1995; Uradhl and Jenkins, 1994).
The immune response is initiated by the binding of foreign peptide antigen to specific receptors on antigen-presenting cells. Examples of cells that are capable of processing and presenting antigen include monocytes, B cells, and dendritic cells. Depending on the source, antigen may be broken down and processed into small peptides by two different pathways. Exogenous antigen, such as bacterial proteins, are endocytosed and processed by the class II pathway and subsequently presented in association with MHC class II molecules on the cell surface. Endogenous antigens, such as viral peptides, which are already present within the cell, are processed by the class I pathway and presented in association with MHC class I molecules. If the MHC/antigen complex is recognized by a TCR and the appropriate accessory signals are passed from the antigen presenting cell (APC) to the T cell (see section 1.7 for more details on accessory signals), the T cell will proliferate and differentiate into an effector cell (Germain, 1994; Uradhl and Jenkins, 1994).

CD4 cells will only recognize MHC class II and will differentiate into helper T cells, while antigen presentation to CD8 cells is primarily restricted to interactions involving class I MHC recognition. These CD8 cells will differentiate into cytotoxic T cells. Helper T cells interact with B cells to stimulate B cell differentiation and antibody production and cytotoxic T cells kill virally infected cells or tumor cells (Lane, 1995; Collins et al., 1993).

Like T cells, the majority of naïve B cells must not only recognize and interact with a specific foreign antigen for activation to occur, but must also receive signals from accessory cells. In the case of B cells, these accessory signals are received from activated T helper cells. B cells obtain help from T cells by acting as antigen presenting cells for
the T cells. As a result of helper T cell recognition of antigen on the B cell surface, the T cell becomes activated and in turn activates the B cell. T cell help has two components; secreted lymphokines which act as growth and differentiation factors for B cells, and additional signals which require cell contact and involve interactions between B and T cell-surface molecules (Parker, 1993; Paul and Seeder, 1994; Lane, 1995).

1.6 SIGNALING EVENTS DURING LYMPHOCYTE ACTIVATION

T and B cells have structurally different antigen receptors that recognize distinct forms of antigen; however the signal transduction events that result from the interaction of their antigen receptors with antigen are quite similar (DeFranco, 1997; Kurosaki, 1997; Cantrell, 1996; Campbell et al., 1990; Lane et al., 1990; Weiss and Littman, 1994). Many signaling pathways have been implicated in lymphocyte activation, but a detailed description of the pathways and their interactions is beyond the scope of this thesis; therefore, I will only include a brief summary of the main signaling pathways involved.

Lymphocytes are generally quiescent and require antigen stimulation to progress from the G₀ stage of the cell cycle. Once antigenic stimulation occurs, the cells enter the cell cycle, enlarge and begin to divide (Berridge and Irvine, 1989; Weiss and Littman, 1994).

Biochemically, triggering of T and B lymphocytes via their antigen receptors with antigen initiates a number of events including protein tyrosine kinase activity, ras activation and inositol lipid metabolism (Kurosaki, 1997; Szamel and Resch, 1995). Neither the TCR nor the BCR has intrinsic protein tyrosine kinase (PTK) activity, but both activate cytoplasmic PTKs that are important for the initiation of signal transduction. In T cells, TCR engagement stimulates the kinase activity of the src
kinases, fyn and lck (Weiss and Littman, 1994; Szamel and Resch, 1995), while in B cells, lyn, blk, fyn and lck have been reported to associate with the BCR (Kurosaki, 1997; Cambier, 1992). In both B and T cells these activated src family members bind to specific tyrosine containing motifs (TAMs) in the cytoplasmic tails of CD3, Igα or Igβ, which then serve as substrates for tyrosine phosphorylation. Once phosphorylated, additional effector molecules, such as syk (B cells) and zap-70 (T cells), can then be recruited to the antigen receptor complex via SH2 interactions and subsequently tyrosine phosphorylated and activated (Weiss and Littman, 1994; Weil et al., 1995; Kurosaki, 1997; Szamel and Resch, 1995). Other cellular substrates, which may include other receptor molecules, cytoskeletal proteins, phospholipase C (PLC), vav and shc are then activated (Cantrell, 1996). The functions of many of these substrates in activation is unclear; however the hydrolysis of phosphoinositides by PLC is relatively well characterized.

PLC mediates the breakdown of phosphatidylinositol bisphosphate (PIP₂) to the second messengers, inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). DAG activates protein kinase C (PKC) and IP₃ induces an increase in cytoplasmic free calcium (Berridge and Irvine, 1989; Weiss and Littman, 1994). The increased intracellular Ca²⁺ concentration and activation of PKC have been causally linked to cellular responses in both B and T lymphocytes. The best characterized of these responses is the transcriptional activation of the IL-2 gene in T cells (Taniguchi and Minami, 1993), believed to be mediated in part by the induction of the transcription factor NFκB by PKC (Williams et al., 1995). The binding of IL-2 to the IL-2 receptor signals the cells to
transition from G1 to the S phase of the cell cycle, resulting in cellular proliferation and
differentiation (Taniguchi and Minami, 1993).

Ras has also been implicated in lymphocyte activation. In T cells it has been
shown that multiple transcription factors are required to regulate the IL-2 gene, including
AP-1, NFκB, Oct-1 and NFAT (Cantrell, 1996). Activated ras can induce AP-1 in T
cells and also synergize with a calcium signaling pathway to activate NFAT (Rayter et
al., 1992; Woodrow et al., 1993). Once activated, NFAT proteins will translocate to the
nucleus and combine with AP-1 to form a functional transcriptional factor complex
which will activate the IL-2 gene (Rao, 1994). Furthermore, there are binding sites for
NFAT in the enhancers of a number of cytokine genes, including IL-2, IL-4 and TNF,
therefore ras likely plays a role in the activation of these genes as well (Rao, 1994).

Activation ultimately results in the induction or inhibition of specific genes, many
of which encode cell-surface molecules that play a role in activation itself or in effector
functions (Janeway and Bottomly, 1994; Bireland and Monroe, 1997). Which genes are
induced or inhibited depends on the signal transduction pathways activated, which in turn
depend on the differentiation state and the soluble and cell-mediated signals received by
the lymphocyte. Table 1.1 lists some of the cell surface proteins that are upregulated
upon T and B cell activation.

1.7 REGULATION OF LYMPHOCYTE ACTIVATION

When a naïve lymphocyte encounters antigen, several distinct outcomes can
occur: proliferation, cytokine secretion, differentiation into effector cells, inactivation or
cell death. The final outcome depends on signals received through the antigen receptor
Table 1.1. **Lymphocyte activation markers.** Characteristics of cell surface proteins upregulated on activated T and B lymphocytes.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Characteristics</th>
<th>Molecule</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td></td>
<td>B cells</td>
<td></td>
</tr>
<tr>
<td>*CD25 (IL-2R)</td>
<td>required for T cell proliferation (Taniguchi and Minami, 1993)</td>
<td>*CD9</td>
<td>associated with beta-1 integrins; may confer cell motility (Shaw et al., 1995)</td>
</tr>
<tr>
<td>*CD26 (dipeptidyl peptidase IV)</td>
<td>costimulates T cell proliferation (Tanaka et al., 1993)</td>
<td>*CD11a (LFA-1)</td>
<td>adhesion; inside out signaling (Branden and Lundgren, 1993)</td>
</tr>
<tr>
<td>CD28</td>
<td>costimulates T cell activation (June et al., 1994)</td>
<td>*CD11c (integrin α chain)</td>
<td>involved in adhesion and activation (Akiyama, 1996)</td>
</tr>
<tr>
<td>*CD30</td>
<td>transduces death signals; stimulates IL-5 production in T cells (Bowen et al., 1996)</td>
<td>*CD19</td>
<td>co-receptor for B cell activation (Tedder et al., 1994)</td>
</tr>
<tr>
<td>*CD37</td>
<td>function unknown; member of the transmembrane 4 superfamily (Tomlinson and Wright, 1996)</td>
<td>*CD20</td>
<td>co-receptor for B cell activation (Tedder et al., 1994)</td>
</tr>
<tr>
<td>*CD38</td>
<td>costimulation (Lund et al., 1996)</td>
<td>*CD23</td>
<td>costimulates B cell activation (Gordon, 1994a)</td>
</tr>
<tr>
<td>*CD39</td>
<td>adhesion, costimulation (Kansas and Tedder, 1991)</td>
<td>*CD25 (IL-2R)</td>
<td>required for B cell proliferation and activation (Taniguchi and Minami, 1993)</td>
</tr>
<tr>
<td>CD40L</td>
<td>counter-receptor for CD40; involved in lymphocyte activation (Grewal and Flavell, 1996)</td>
<td>*CD38</td>
<td>costimulation (Lund et al., 1996)</td>
</tr>
<tr>
<td>CD44</td>
<td>adhesion, costimulation (Huet et al., 1989)</td>
<td>*CD39</td>
<td>adhesion, costimulation (Kansas and Tedder, 1991)</td>
</tr>
<tr>
<td>CD49a (VLA-1 α chain)</td>
<td>adhesion to collagen, laminin; costimulation (Bank et al., 1994)</td>
<td>CD40</td>
<td>costimulates B cell activation; adhesion; isotype switching (Gordon et al., 1994b)</td>
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<tr>
<td>CD49b (VLA-2 α chain)</td>
<td>adhesion to ECM, collagen R (Kamata and Takada, 1994)</td>
<td>CD46 (MCP)</td>
<td>regulation of complement activation (Seya, 1996)</td>
</tr>
<tr>
<td>CD51</td>
<td>receptor for vitronectin, fibrinogen, von Willebrand factor; involved in T cell stimulation (Halvorson et al., 1996)</td>
<td>CD48</td>
<td>counter-receptor for CD2 (Kato et al., 1992)</td>
</tr>
<tr>
<td>CD54 (ICAM-1)</td>
<td>adhesion; T cell activation (Branden and Lundgren, 1993)</td>
<td>CD49a (VLA-1 α chain)</td>
<td>adhesion to collagen, laminin; costimulation (Bank et al., 1994)</td>
</tr>
<tr>
<td>CD55 (DAF)</td>
<td>regulation of complement activation (Nicholson-Weller and Wang, 1994)</td>
<td>CD54 (ICAM-1)</td>
<td>adhesion molecule (Branden and Lundgren, 1993)</td>
</tr>
<tr>
<td>CD58 (LFA-3)</td>
<td>ligand for CD2; adhesion and T cell activation (Deckert et al., 1992)</td>
<td>CD55 (DAF)</td>
<td>regulation of complement activation (Nicholson-Weller and Wang, 1994)</td>
</tr>
<tr>
<td>CD63</td>
<td>neutrophil activation and adhesion (Skubitz et al., 1996)</td>
<td>CD58 (LFA-3)</td>
<td>ligand for CD2; isotype switching (Diaz-Sanchez et al., 1994)</td>
</tr>
<tr>
<td>CD69</td>
<td>lymphocyte proliferation (Lopez-Cabrera et al., 1993)</td>
<td>CD62L (L-selectin)</td>
<td>adhesion, lymphocyte rolling (Frenette and Wagner, 1997)</td>
</tr>
<tr>
<td>CD70</td>
<td>ligand for CD27; costimulation in T cells (Hintzen et al., 1995)</td>
<td>CD70</td>
<td>ligand for CD27; cytokine production and B cell activation (Agematsu et al., 1995)</td>
</tr>
<tr>
<td>CD71 (transferrin R)</td>
<td>Iron metabolism; proliferation and differentiation (Brekelmans et al., 1994)</td>
<td>CD71 (transferrin receptor)</td>
<td>Iron metabolism; proliferation and differentiation (Brekelmans et al., 1994)</td>
</tr>
<tr>
<td>CD74</td>
<td>class II MHC invariant chain; activation of protein kinase C (Shih and Floyd-Smith, 1995)</td>
<td>CD72</td>
<td>receptor for CD5; costimulation; involved in inhibition of apoptosis (Nomura et al., 1996)</td>
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<tr>
<td>CDw75</td>
<td>sialyl transferase (Erikstein et al., 1992)</td>
<td>CD74</td>
<td>class II MHC invariant chain; activation of protein kinase C (Shih and Floyd-Smith, 1995)</td>
</tr>
<tr>
<td>CDw78</td>
<td>forms part of the MHC class II molecule (Slack et al., 1995)</td>
<td>CDw6</td>
<td>function unknown</td>
</tr>
<tr>
<td>CD80</td>
<td>counter-receptor for CD28; T cell activation (Branden and Lundgren, 1993)</td>
<td>CDw78</td>
<td>forms part of the MHC class II molecule (Slack et al., 1995)</td>
</tr>
<tr>
<td>CD81</td>
<td>regulates proliferation of lymphocytes (Miyazaki et al., 1997)</td>
<td>CD80</td>
<td>counter-receptor for CD28; involved in activation (Stack et al., 1994; Lane, 1997)</td>
</tr>
<tr>
<td>CD82</td>
<td>costimulates T cell proliferation (Lebel-Binay et al., 1995)</td>
<td>CD81</td>
<td>function unknown</td>
</tr>
<tr>
<td>CD83</td>
<td>function unknown</td>
<td>CD82</td>
<td>function unknown</td>
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<td>CD84</td>
<td>function unknown</td>
<td>CD83</td>
<td>function unknown</td>
</tr>
<tr>
<td>CD85</td>
<td>function unknown</td>
<td>CD84</td>
<td>function unknown</td>
</tr>
<tr>
<td>CD86</td>
<td>counter-receptor for CD28; costimulatory molecule (Lane, 1997)</td>
<td>CD97</td>
<td>counter-receptor for CD28; costimulatory molecule (Lane, 1997)</td>
</tr>
<tr>
<td>CD97</td>
<td>CD55 ligand; associated with inflammation (Gray et al., 1996)</td>
<td>CD109</td>
<td>CD55 ligand; associated with inflammation (Gray et al., 1996)</td>
</tr>
<tr>
<td>CD98</td>
<td>function unknown</td>
<td>CD109</td>
<td>involved in lymphocyte development (Warren et al., 1996)</td>
</tr>
<tr>
<td>CD99</td>
<td>function unknown</td>
<td>CD98</td>
<td>function unknown</td>
</tr>
<tr>
<td><strong>CD120b</strong> (TNFR)</td>
<td>involved in lymphocyte activation (Gruss et al., 1996)</td>
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<tr>
<td><strong>CDw128</strong></td>
<td>IL-8 R</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MHC class II</strong></td>
<td>antigen recognition (Baluyut and Subbarao, 1988)</td>
<td></td>
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<tr>
<td>CTLA-4</td>
<td>counter-receptor for B7; inhibitory role in T cell proliferation (Walunas et al., 1994)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ox40</td>
<td>costimulates T cell proliferation; induces IL-2 and IL-4 secretion (Calderhead et al., 1993)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-1BB</td>
<td>costimulates T cell activation (Hurtado et al., 1995)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSA</td>
<td>costimulates T cell proliferation (Liu et al., 1992)</td>
<td></td>
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</tr>
</tbody>
</table>

| **CD100**         | involved in adhesion and differentiation (Dorfman et al., 1996) |
| **CDw108**        | function unknown                                              |
| **CD120b** (TNFR) | involved in lymphocyte activation (Gruss et al., 1996)         |
| **CDw8**          | MHC class II antigen recognition                               |
| Thy-1             | homotypic adhesion, T cell proliferation (Louie et al., 1995) |

* from the Leukocyte Differentiation Antigen Database (5th Workshop)
and other accessory molecules (Janeway and Bottomly, 1994; Croft and Dubey, 1997). Of these, the signals provided by the accessory molecules play a large part in the outcome of the interaction.

Accessory molecules fall into three classes; adhesion molecules, co-receptors and costimulatory molecules (Zuckerman et al., 1995). Adhesion molecules bring cells into close contact with one another, thus allowing signals to be passed either by the adhesion molecule itself or by other accessory molecules (Collins et al., 1994). Adhesion molecules often act in a costimulatory capacity as well. Co-receptors serve to signal cells through the antigen receptor complex either directly (Veillette et al., 1988) or indirectly (Bierer et al., 1988) and costimulatory molecules provide independent signals to the lymphocyte distinct from the antigen receptor complex (Zuckerman et al., 1995) (Table 1.2).

Accessory molecules and their counter-receptors are expressed on lymphocytes and on cells presenting the foreign antigen (antigen presenting cell, APC). Expression of these molecules on the APC depends on the APC itself, the presence of cytokines and the activation state of the APC (Uradhl and Jenkins, 1994; Dubey et al., 1995). Hughes (1993) has shown that different APC’s provide different combinations of accessory signals and may preferentially activate different T cell subsets at different stages of activation. Memory T cells, for example, are considerably less dependent on APC accessory signals than naïve cells after α-CD3 stimulation (Croft et al., 1994). Well known examples of the three classes of accessory molecules are described in the following sections.
Table 1.2. **Accessory molecules involved in lymphocyte activation.** Characteristics of some of the cell surface proteins involved in lymphocyte activation.

<table>
<thead>
<tr>
<th>T lymphocytes</th>
<th>B lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecule</strong></td>
<td><strong>Characteristics</strong></td>
</tr>
<tr>
<td>CD2</td>
<td>costimulation (Sen <em>et al.</em>, 1992)</td>
</tr>
<tr>
<td>TCR/CD3</td>
<td>antigen recognition; activation (Weiss and Littman, 1994)</td>
</tr>
<tr>
<td>CD4</td>
<td>co-receptor; activation (Janeway, 1992)</td>
</tr>
<tr>
<td>CD5</td>
<td>costimulation (Hollander, 1982)</td>
</tr>
<tr>
<td>CD8</td>
<td>co-receptor; activation (Janeway, 1992)</td>
</tr>
<tr>
<td>CD11a (LFA-1)</td>
<td>adhesion molecule; costimulation (Branden and Lundgren, 1993)</td>
</tr>
<tr>
<td>CD26</td>
<td>costimulation (Tanaka <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td>CD28</td>
<td>costimulation; enhances TCR mediated signals (Lenschow <em>et al.</em>, 1996)</td>
</tr>
<tr>
<td>CD38</td>
<td>costimulation (Lund <em>et al.</em>, 1996)</td>
</tr>
<tr>
<td>CD39</td>
<td>adhesion, costimulation (Kansas and Tedder, 1991)</td>
</tr>
<tr>
<td>CD45</td>
<td>inhibits T cell activation (Alexander <em>et al.</em>, 1992)</td>
</tr>
<tr>
<td>CD48</td>
<td>costimulation (Kato <em>et al.</em>, 1992)</td>
</tr>
<tr>
<td>CD49a</td>
<td>costimulation; adhesion to ECM (Bank et al., 1994)</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>CD54</td>
<td>adhesion molecule; costimulation (Branden and Lundgren, 1993)</td>
</tr>
<tr>
<td></td>
<td>(ICAM-1)</td>
</tr>
<tr>
<td>CD58</td>
<td>adhesion and costimulation (Deckert et al., 1992)</td>
</tr>
<tr>
<td>(LFA-3)</td>
<td></td>
</tr>
<tr>
<td>CD70</td>
<td>costimulation (Hintzen et al., 1995)</td>
</tr>
<tr>
<td>CD80</td>
<td>costimulation (Branden and Lundgren, 1993)</td>
</tr>
<tr>
<td>(B7-1)</td>
<td></td>
</tr>
<tr>
<td>CD86</td>
<td>costimulation (Lane, 1997)</td>
</tr>
<tr>
<td>(B7-2)</td>
<td></td>
</tr>
<tr>
<td>CD106</td>
<td>inhibits T cell activation (Kitani et al., 1996)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>HSA</td>
<td>adhesion, costimulation, synergizes with B7 (Liu et al., 1992)</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>inhibits T cell activation (Walunas et al., 1994)</td>
</tr>
<tr>
<td>4-1BB</td>
<td>costimulation (Hurtado et al., 1995)</td>
</tr>
<tr>
<td>Ox40</td>
<td>costimulation; induces IL-2 and IL-4 secretion (Calderhead et al., 1993)</td>
</tr>
</tbody>
</table>
1.7.1 Co-receptors

Co-receptors participate in antigen recognition and signal transduction thus increasing the sensitivity of the cell to activation by receptor ligation (Janeway and Bottomly, 1994). In order to determine if a novel cell surface protein is acting as a co-receptor, it must exhibit the following characteristics: 1) ligation of the co-receptor alone by monoclonal antibodies should inhibit cellular activation triggered through the receptor and 2) the co-receptor should be associated with signaling molecules (Janeway and Bottomly, 1994).

In T cells CD4 and CD8 act as co-receptors for the TCR/CD3 complex. Not only do they bind to the same MHC as the TCR, but they also physically associate with the TCR itself (Janeway, 1992). In addition, CD4 and CD8 bind to the protein tyrosine kinase lck (Veillette et al., 1988) and treatment of lectin induced T cells with antibodies to CD4 or CD8 inhibits mitogenesis in the absence of MHC recognition (Bank and Chess, 1985; Bekoff et al., 1985; Janeway, 1988; Wassmer et al., 1985; Rossoff et al., 1987; Tite et al., 1986), thus fulfilling the requirements of a co-receptor.

Like T cells, B cells also recognize antigen in the form of a complex which brings together the BCR/Igα/Igβ complex and its co-receptor to stimulate BCR signaling events (DeFranco, 1996). The co-receptor in B cells is a complex composed of CD19 and CR2 (CD23 + CD21) (DeFranco, 1996; Reth, 1995). Like CD4 and CD8, the CD19/CR2 complex fulfills the requirements for a co-receptor; it is associated with the PTK lyn (Cambier, 1992) and CD19 monoclonal antibodies will inhibit antigen-driven antibody responses (Barrett et al., 1990).
1.7.2 Adhesion molecules

In general, adhesion molecules serve to enhance pairing between less avid receptors and their ligands and may transmit signals that direct specific effector functions (Hogg and Landis, 1993). In terms of T lymphocytes, this enhanced pairing allows the TCR to come into contact with the MHC/Ag complex and signals passed through the adhesion receptors provide additional activation signals that synergize with the signals mediated through the TCR/CD3 (Springer, 1990; Hogg and Landis, 1993). Adhesion molecules are generally identified by the ability of monoclonal antibodies to inhibit or enhance homotypic or heterotypic adhesion (Dustin and Springer, 1991).

There are two main superfamilies involved in cellular adhesion during activation of lymphocytes; the integrins and the Ig superfamily (Hogg and Landis, 1993). The integrins are a large family that participates in adhesion through both cell-cell and cell-extracellular matrix interactions and have been found to be engaged in two-way signaling events across the cell membrane (Collins et al., 1994). In resting lymphocytes, integrins exist in a low avidity state; however, following activation, a high-avidity state is induced through a mechanism termed “inside-out” signaling. For example, the interaction between the integrin LFA-1 and its ligand ICAM-1 is mediated by inside-out signaling which serves to increase the avidity of LFA-1 upon TCR ligation thus leading to adhesion between the two receptors (Collins et al., 1994).

“Outside-in” signaling results in enhancement of TCR-mediated activation and has been demonstrated following ligand induced and antibody-mediated crosslinking of numerous integrins including LFA-1, VLA-4, VLA-5 and VLA-6 (Collins et al., 1994).
Because these integrins are capable of potentiating activation as well as mediating adhesion, they are often called costimulatory molecules as well as adhesion molecules.

The Ig superfamily is a functionally diverse family in which all members share a variable number of Ig domains. Members of this family that can be found on lymphocytes include the TCR, BCR, CD2, LFA-3, ICAM-1,2,3, CD4, CD8 and VCAM (Hogg and Landis, 1993).

One of the main T cell accessory molecules which contributes to adhesion as well as signaling between the T cell and an APC is CD2. In mice, CD2 ligation to its ligand CD48 (Kato et al., 1992) can mediate or enhance T cell activation and this activation can be blocked by antibodies against CD2 or CD48, suggesting that signals from this adhesive interaction are integrated with signals from the TCR during an immune response (Collins et al., 1994; Hahn et al., 1993).

1.7.3. Costimulatory molecules

Like co-receptors, costimulatory molecules have a number of common characteristics that can be used to identify them: 1) the biochemical characteristics of the costimulatory signal differ from those provided by the TCR/BCR, 2) costimulation in the absence of a TCR/BCR signal must not fully activate resting cells and 3) the costimulatory molecule may be linked to more than one signaling pathway (June et al., 1994).

The best studied costimulatory interaction between lymphocytes is that between CD28 and the B7 family (Lenschow et al., 1996). CD28 is expressed on all T cells and its expression increases 4-8 times upon activation (June et al., 1994). Its ligands, B7-1, B7-2 and B7-3 are all induced on activated B cells with varying peak induction times,
suggesting that the three ligands are used at different times during the immune response (Hathcock et al., 1994). B7-1 expression reaches a peak between 48 and 72 hrs, B7-2 is most highly expressed at 24 hrs and like B7-1, B7-3 expression is induced slowly (Hathcock et al., 1994).

CD28 activation in the presence of TCR stimulation augments T cell proliferation and lymphokine production and these reactions can be inhibited by antibodies to B7-1 (June et al., 1990; Linsley and Ledbetter, 1993). The importance of CD28 in lymphocyte function is apparent when CD28 knockout mice are examined. The peripheral T cells in these mice have impaired lymphokine secretion after mitogenic stimulation and are deficient in humoral responses suggesting that CD28 is involved in T-B cell interactions in vivo (Chen et al., 1993).

CD28 has been shown to activate a set of second messengers that complements those activated by the TCR and results in a functional synergy between the two receptors (Mondino and Jenkins, 1994). CD28 signaling is independent of increases in intracellular calcium or calcineurin activation and crosslinking of CD28 induces a pattern of tyrosine phosphorylation that is distinct from TCR-mediated tyrosine phosphorylation (Ledbetter et al., 1986; June et al., 1987; Vandenberge et al., 1992). Moreover, ligation of CD28 by B7 induces PI3K activity in T cells independent of TCR activation (Ward et al., 1993).

Another well studied interaction is that between CD40 and its ligand. CD40 has been implicated in numerous cellular events in B cells including homotypic adhesion, proliferation, germinal center (GC) cell survival and isotype switching (Kehry, 1996; Durie et al., 1994). Klaus et al. (1994) showed that α-CD40 monoclonal antibodies could induce B cells to form large spherical clusters and this clustering was important for
B cell activation via CD40. Furthermore, signals delivered via CD40 can influence cellular responses evoked by the BCR. For example, simultaneous ligation of sIg and CD40 in the presence of mouse L cells expressing FcγRII receptor results in the stimulation of resting B cells (Wheeler et al., 1993). The importance of CD40 has been confirmed by studies showing that mice lacking CD40 or CD40L exhibit defective T cell-dependent immune responses (Kawabe et al., 1994; Xu et al., 1994).

Like CD28, CD40 appears to be linked to more than one signaling pathway and the pathway used depends on the differentiation stage and type of B cell being triggered (Durie et al., 1994). Studies indicate that α-CD40 causes tyrosine phosphorylation of multiple substrates, PI turnover and activation of ser/thr specific protein kinases in activated, but not resting B cells (Uckun et al., 1991). In GC cells, α-CD40 triggers tyrosine phosphorylation similar to that induced by α-Ig but fails to increase IP₃ and intracellular calcium concentrations, suggesting that in the GC CD40 is coupled to PTK’s, but not the PI pathway (Knox and Gordon, 1993).

1.7.4 Two signal model

In order for proliferation and differentiation to effector cells to occur, activation of both naïve T and B lymphocytes requires at least two signals. The first confers specificity and is mediated via the antigen receptor and the second is costimulatory and is mediated by accessory molecules (see Table 1.2; sections 1.7.1 to 1.7.3)(Janeway and Bottomly, 1994; Weiss and Littman, 1994; Croft and Dubey, 1997). While it is known that engagement of the antigen receptor with foreign antigen is of critical importance for the initiation of a cellular response, signaling through the antigen receptor alone is insufficient to trigger full effector functions and will result in cell death or inactivation.
If antigen receptor engagement is combined with one or more costimulatory signals however; proliferation and differentiation into fully mature effector cells will occur. These costimulatory signals are neither antigen specific nor MHC restricted and can be provided by soluble factors such as the interleukins, adhesion molecules, co-receptors or costimulatory molecules (Collins et al., 1994; Collins et al., 1993; Jenkins, 1988; Green, 1994; Croft and Dubey, 1997).

In T cells, some of these molecules include LFA-1 (Siu et al., 1989), CD-2 (Collins et al., 1993), HSA (Liu et al., 1992), CD44 (Huet et al., 1989) and CD28 (June et al., 1994; Lane, 1997) and they may contribute to the T cell response by increasing adhesion between the T cell and the antigen presenting cell and/or by transmitting signals which then augment the TCR/MHC activation cascade (Table 1.2; sections 1.7.1 to 1.7.3)(Collins et al., 1994; Croft and Dubey, 1997).

Research indicates that each of these receptors probably acts in a distinct manner (Green, 1994; Croft and Dubey, 1997). Engagement of the adhesion molecule LFA-1 by its ligand ICAM-1, for example, serves to stabilize the interaction between the T cell and APC, which allows for effective engagement of low affinity Ag/MHC complexes on the APC with the TCR (Collins et al., 1994; Siu et al., 1989). CD28 signaling (see 1.7.3), on the other hand, appears to involve the induction of specific protein tyrosine kinases and this pathway is distinct from that induced through TCR stimulation (Vandenberghhe et al., 1992).

In addition to surface immunoglobulin receptors for antigen, a number of B cell accessory molecules have now been identified which may regulate activation and
adhesion of B cells; including CD40 (Gordon et al., 1994b), CD19 (Tedder et al., 1994), CD21 (Tedder et al., 1994) and CD22 (Law et al., 1994; Tedder et al., 1997) (Table 2.2). CD40 signaling (see 1.7.3) is involved in many aspects of B cell activation and effector cell function, such as homotypic aggregation (Klaus et al., 1994), isotype switching to IgE (Fuleihan et al., 1995), enhancement of B cell activation signals delivered through the antigen receptor (Wheeler et al., 1993) and transmission of signals to protect germinal center B cells from undergoing apoptosis (Clark et al., 1994). CD19 cross-linking experiments suggest that CD19 augments B cell proliferation in response to antigen binding to surface immunoglobulin (Carter and Fearon, 1992; Lankerster et al., 1996) and signaling via either CD21 or CD22 can also augment responses to antigen receptor cross-linking (Tsokos et al., 1990; Pezzutto et al., 1988).

1.8 IN VITRO METHODS TO STUDY MOLECULES INVOLVED IN ACTIVATION

Studying lymphocyte activation in vitro is complicated by the fact that each cell expresses a unique antigen receptor that is specific for only one antigen. Recently, activation studies have been performed using TCR or BCR transgenic mice, where every T or B lymphocyte is specific for one antigen and the antigen itself is used to activate the cells in vitro (McKnight et al., 1994; Croft et al., 1992; Cooke et al., 1994). Many conclusions about costimulation and activation, however, have been based on the analysis of polyclonal activation by lectins or antibodies and on studies with cloned cell lines (Dubey et al., 1995).

By definition, polyclonal activators stimulate B or T cells of many different antigen specificities and induce both proliferation and differentiation to effector cells. In many cases, polyclonal activation occurs independently of accessory cell signaling, but in
others, accessory cells, or specific signals from those cells, are absolutely required for full
activation (Severinson and Larsson, 1986).

Polyclonal activation is commonly used to study the mechanisms of lymphocyte
activation, proliferation and differentiation. The discovery that polyclonal activators
induce similar cellular responses as antigen specific activation (Gery et al., 1972; Habu
and Raff, 1977) suggests that these activators are appropriate model systems to study
lymphocyte function. Not only is it possible to activate large numbers of lymphocytes,
but cell culture techniques and in vitro assays have been improved so that they are simple
and reproducible (Severinson and Larsson, 1986).

Polyclonal activation now makes it feasible to study the role of cell surface
proteins for which there is no known function. Changes in protein expression during
activation and the role of the protein on proliferation, differentiation and effector cell
function can be studied using well established in vitro assays, such as flow cytometric
analysis, $[^3H]$-thymidine uptake, cytokine or immunoglobulin secretion, costimulation
assays and cytotoxic T cell assays (Severinson and Larsson, 1986). Some of these assays
were used to examine the role of SaBR in lymphocyte activation and are described in
Chapter 2.

*Polyclonal T cell activators*

1.8.1 concanavalin A

Antigen independent T cell responses can be studied by using plant lectins that
bypass the TCR/MHC requirement. T cell lectins are glycoproteins that bind specifically
to certain carbohydrate structures on the cell surface and induce proliferation of a
significant fraction of a T cell population in the presence of accessory cells (Peacock et
al., 1990). The best characterized lectin is concanavalin A (con A) which has high specificity for the sugar α-methyl-D-mannoside (Severinson and Larsson, 1986). This carbohydrate is abundant in many cell surface proteins and evidence suggests that the first step in activation by con A is the cross-linking of membrane proteins; in particular CD3 (Fleischer, 1984). Subsequent steps in the con A activation process include activation of the PKC pathway and induction of IL-2 production (Hunig et al., 1983).

Lectins are advantageous because they are simple to use, they will work on a variety of cell types and will mediate strong interactions. However, all lectins will cause a variety of other responses not necessarily due to activation, such as cytokine secretion and non-specific inhibition of membrane proteins (Peacock et al., 1990). Moreover, lectins lack specificity for membrane proteins and the responses elicited between lectins differ markedly. As a result, an accurate interpretation of the results, in terms of specific signaling events, can by difficult.

1.8.2 phorbol myristate acetate

Activation of lymphocytes induces the hydrolysis of phosphoinositides, which leads to the generation of the second messengers, inositol triphosphate (IP$_3$) and diacylglycerol (DAG). These act to increase the intracellular calcium concentration and activate PKC, respectively (Berridge, 1987). These early events induce cell cycle entry and proliferation and can be mimicked with agents that bypass the TCR: calcium ionophores, which mimic IP$_3$ (see next section) and phorbol esters, which mimic DAG (Truneh et al., 1985). Furthermore, phorbol esters can substitute for accessory cells in the response of purified T cells to lectins and induce IL-2 production and IL-2 receptor expression (Mueller et al., 1989).
Phorbol esters, such as phorbol myristate acetate (PMA) are incapable of stimulating full activation on their own however, and require another signal to elicit full activation (Isakov et al., 1987). This can be accomplished by using other activators in addition to PMA. Second signals can be supplied by ionophores, such as calcimycin (A23187), which will increase the intracellular calcium concentration or antibodies to specific accessory molecules, such as α-CD3, which will activate the specific signaling pathways associated with that molecule (Planelles et al., 1992).

Phorbol esters, alone or in combination with another activator, can be powerful tools for the dissection of signal transduction pathways during lymphocyte activation. PKC signal transduction and its integration with other pathways can be studied; however, the cellular responses may differ from those induced after antigen stimulation. It is important, therefore, that these facts be taken into account when interpreting results.

1.8.3 A23187

A23187 is a calcium ionophore that bypasses the TCR to increase membrane permeability to calcium, thus allowing extracellular calcium to enter the cell and increase intracellular calcium concentrations (Tsien et al., 1982). This increase in calcium concentration is required for lymphocyte proliferation and the induction of activation specific genes, but like PMA, A23187 is insufficient to fully activate T cells when used alone (Akerman and Andersson, 1984; Planelles et al., 1992). If combined with PMA, A23187 can induce a potent proliferative response (Akerman and Andersson, 1984; Planelles et al., 1992). Isakov et al. (1987) has shown that ionophores alone are capable of inducing the appearance of the IL-2 receptor, but in order to stimulate IL-2 production, a phorbol ester and an ionophore are required.
1.8.4 antibodies to CD3 and CD28

The polyclonal activators described so far have one major disadvantage: it is not possible to study or isolate the cell surface proteins involved in the activation of the various signaling pathways. In order to fully understand the how and whys of lymphocyte activation, the role of individual cell surface proteins must be ascertained. Antibodies that mimic the binding of specific cell surface protein counter-receptors or ligands (by cross-linking the receptor) have been shown to be indispensable in this regard.

It should be noted, however, that not all antibodies can mimic the physiologic ligand-induced cellular events of the native ligand. Some cell surface receptors require the ligand to bind to specific determinants or structural confirmations and often insufficient cross-linking can block the receptors ability to function and thus lead to cellular inactivation (Peacock et al., 1990).

The antibodies used in this thesis to study T cell activation included α-CD3 and α-CD28. α-CD3 mimics the binding of antigen to the TCR/CD3 complex and serves to activate specific signaling pathways, such as the PKC pathway. When used alone, it has very little effect on the proliferation of naïve T cells, but has a significant proliferative effect on memory T cells (Croft et al., 1994).

α-CD28 antibody cross-links the costimulatory molecule, CD28 and induces signal transduction pathways distinct from those induced by TCR ligation (June et al., 1994). Furthermore, in the absence of APC's, cross-linking of CD28 provides signals which can synergize with α-CD2, α-CD3, PHA and PMA to induce the expression of multiple lymphokines, including IL-2 and to stimulate proliferation in T cells (Linsley
and Ledbetter, 1993). Recently, Shanafelt et al. (1995) analyzed the cytokine profiles of helper T cell clones and showed that α-CD28 + α-CD3 activation was sufficient to stimulate IFN-γ production from Th1 clones, but was not enough to stimulate IL-4 production from Th2 clones, suggesting that different T cell subsets respond differentially to costimulatory signals.

Polyclonal B cell activators

1.8.5 Lipopolysaccharide

Mature B cells can be polyclonally activated by a variety of stimuli. These include lipopolysaccharide (LPS) (Morrison and Ryan, 1979), second messenger agonists such as PMA and ionomycin (Klaus et al., 1987), antibodies to surface immunoglobulin (Sieckmann et al., 1978), cytokines and antibodies to costimulatory molecules.

LPS is a phospholipid found in the outer cell wall of gram negative bacteria and is sufficient to induce all of the responses observed with living bacteria (Wright, 1995). Recent studies have described numerous proteins that may mediate cellular responses to LPS (Ziegler-Heitbrock and Ulevitch, 1993; Wurfel et al., 1994; Kielian and Blecha, 1995). Some of these proteins, such as CD14, have been shown to act as LPS receptors on macrophages and monocytes, while others (p73 and p38) are more likely to serve as receptors on lymphocytes (Kielian and Blecha, 1995). When added to purified B cells, LPS binds to its receptor and initiates signaling cascades resulting in cell cycle entry, cytokine secretion and antibody production (Kielian and Blecha, 1995).

1.8.6 IL-4

The T cell derived cytokine, IL-4, induces cell enlargement and increases the viability of mature B cells, but is unable to stimulate proliferation or cytokine production
if used alone (Parker, 1993). Furthermore, it upregulates the expression of ICAM-1 (Branden and Lundgren, 1993), MHC class II, and the costimulatory molecules, B7-1 and B7-2, which are involved in T cell activation (Stack et al., 1994). It is used in polyclonal activation in combination with other activators because it enhances the proliferative responses of B cells to costimulatory signals (Kehry et al., 1992) and drives isotype switching to IgE in the presence of CD40 signaling (Fuleihan et al., 1995).

1.8.7 antibodies to surface immunoglobulin

The B cell antigen receptor, in association with other membrane molecules, initiates signals for B cell activation. Binding of specific antigen can be mimicked by treatment with α-immunoglobulin (α-Ig) antibodies under defined culture conditions (Campbell et al., 1990). The majority of α-IgM and α-IgD antibodies available stimulate early activation events, such as increased expression of MHC class II molecules, membrane depolarization and cell cycle entry, but not all cells respond equally well to such treatment (Campbell et al., 1990). For example, after α-IgM treatment, early activation responses can be found in small resting mature B cells, while immature B cells will become anergic (Campbell et al., 1990). Furthermore, evidence suggests that sIg crosslinking alone does not promote proliferation and differentiation to antibody-forming cells, but requires additional signals in most cases (Bireland and Monroe, 1997). These signals are provided through direct T cell-B cell contact or via T cell derived cytokines (Bireland and Monroe, 1997).

1.8.8 antibodies to CD40

Contact-mediated signals delivered by T helper cells to mature B cells play a pivotal role in initiating B cell activation during antibody responses to T-dependent
antigens. These signals act in synergy with those delivered via cytokines, such as IL-4, and those transduced via the antigen receptor (Parker, 1993; Lane, 1995). CD40 is a costimulatory molecule that provides intracellular signals which synergize with those delivered through sIg and/or IL-4 (Gordon et al., 1994b; Kehry, 1996).

Stimulation of naïve resting B cells with α-CD40 results in a multitude of responses including: 1) homotypic aggregation, which is enhanced by costimulation with IL-4 or α-IgM (Klaus et al., 1994), 2) isotype switching to IgE when used in combination with IL-4 (Fuleihan et al., 1995), 3) B cell entry into the cell cycle and proliferation when used with a second signal (Gordon et al., 1994b; Wheeler et al., 1993) and 4) survival of germinal center B cells (Liu et al., 1989).

1.9 CLONING OF SaBR-HISTORY

From 1987 to 1991, a group of investigators at the Terry Fox Laboratory, consisting of Dr. Keith Humphries, Dr. Rob Kay, Dr. Graeme Dougherty, Dr. Gerry Krystal and Dr. Peter Lansdorp, used expression cloning to identify novel cell surface proteins involved in the regulation of hematopoiesis. For this purpose, a panel of monoclonal antibodies was developed against B6SUTA, a murine IL-3 dependent multipotential progenitor cell line (Greenberger et al., 1983). B6SutA was used because it is one of only a very few cell lines capable of giving rise to both myeloid and erythroid lineages and is a good model system for the study of hematopoietic regulation. The antibody panel was analyzed by flow cytometry against murine hematopoietic cells in order to identify those antibodies with novel expression patterns. One of these antibodies, 11F6, was found to recognize an antigen expressed on multilineage progenitors and mature cells of all cell lineages and did not match the expression patterns.
of any known antibodies (Chapter 2, Table 2.1, Table 2.2). The expression pattern was deemed sufficiently interesting that 11F6 was used in a panning procedure to screen COS cells transfected with a B6SUtA cDNA library (Kay and Humphries, 1991). A single cDNA, which conferred 11F6 reactivity, was eventually isolated after multiple rounds of selection of transfected COS cells. Partial sequencing suggested that it encoded a novel cell surface protein, subsequently named SaBR.

1.10 THESIS RATIONALE AND OBJECTIVES

With the cloning of any new gene, there are many avenues to explore in order to gain a basic understanding of the gene and its protein product. When I began this study, the only information we had about SaBR was partial sequence data and some preliminary indications about its expression patterns within the hematopoietic system.

Of particular interest was the observation that SaBR expression appeared to be differentially expressed in lymphocytes within T cell subpopulations and between different tissues. These results suggested that SaBR may also be differentially expressed in other areas, such as during development or activation. Since the activation and development of lymphocytes has been well characterized and lymphocytes can be readily studied using \textit{in vitro} and \textit{in vivo} assays; I chose, as a first objective, to explore SaBR expression on developing and activated lymphocytes in greater detail. I hypothesized that changes in SaBR expression would reflect differences in the functional characteristics of lymphocytes. I hoped that by studying SaBR expression in depth, I would gain enough useful information to develop a more detailed hypothesis regarding SaBR function in lymphocytes. The results of these studies can be found in Chapter 2.
Since only partial sequence information was available on the SaBR cDNA clone, my second objective was to subclone, sequence and characterize this clone. Comparing a cDNA sequence with other proteins in the databases can often reveal homologies as well as matches to well characterized sequence motifs and protein families, which in turn can yield information about possible protein functions. Furthermore, I wished to use the cDNA as a probe to study the expression of SaBR mRNA and I wanted to express the cDNA in cell lines and transgenic mice in order to explore protein function. Chapter 3 describes the sequencing and initial characterization of the SaBR cDNA, as well as my attempts to examine the SaBR protein and clone the human homologue.

The sequencing of SaBR gave very few clues about the functional role of SaBR. It appeared to be highly expressed in various tissues, therefore, I embarked on a histochemical study of SaBR expression in hematopoietic and non-hematopoietic mouse tissues. I hoped that the pattern of SaBR expression within individual tissues and the identification of specific SaBR positive cells would give me more functional information and aid in developing a hypothesis regarding SaBR function. Therefore, my third objective was to use histochemistry on tissue sections to identify which cells and tissues were expressing SaBR. Chapter 4 outlines the results of these experiments and suggests possible functions for SaBR within non-hematopoietic tissues.

Transgenic mice are good model systems for manipulating gene expression and for studying the function of proteins in vivo. The results from Chapter 2 suggested that SaBR may be playing a functional role in B cell development and during lymphocyte activation; therefore, I chose to test this hypothesis by examining lymphocyte
development and activation in transgenic mice overexpressing SaBR within the lymphoid lineage. The results from these studies can be found in Chapter 5.

Finally, Chapter 6 summarizes my conclusions and outlines recommendations for future studies and the Appendix outlines methodologies developed to identify counter-receptors for SaBR.
CHAPTER 2
EXPRESSION ANALYSIS AND FUNCTIONAL CHARACTERIZATION OF SaBR IN LYMPHOCYTES

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2.1 INTRODUCTION

Activation of lymphocytes requires at least two signals. The first occurs after the binding of foreign antigen to the antigen receptor and subsequent signals are costimulatory and mediated by accessory molecules on the surface of the lymphocyte (Janeway and Bottomly, 1994; Weiss and Littman, 1994). These costimulatory signals are neither antigen specific nor MHC-restricted and can be provided by cytokines, adhesion molecules, co-receptors or costimulatory molecules (see Chapter 1)(Collins et al., 1994; Collins et al., 1993; Jenkins et al., 1988; Green and Thompson, 1994).

Our knowledge of lymphocyte development and activation has increased greatly over the last few years due to the cloning and characterization of an increasing number of accessory molecules; however, there are still many more accessory molecules that have yet to be identified. Preliminary analysis of SaBR within lymphocytes shows a differential pattern of expression within tissues and T cell subpopulations. This data suggests that SaBR may also be differentially expressed during activation or development; therefore, as a first objective, I decided to look at SaBR expression in developing and activated lymphocytes in greater detail.

I would like to hypothesize that differential expression of SaBR reflects functional changes in lymphocytes. The bulk of this chapter describes experiments designed to test this hypothesis. Flow cytometry was used to study SaBR expression during development, while polyclonal activation, flow cytometry, cell sorting and proliferation assays were used to study SaBR expression during activation. The beginning of this chapter describes the early flow cytometric experiments that led to the development of the hypothesis. The remainder of the chapter is devoted to studies
exploring SaBR expression in developing and activated lymphocytes. A preliminary
model of SaBR function in B lymphocyte activation is presented at the end of the chapter.

2.2 MATERIALS AND METHODS

**EXPRESSION ANALYSIS**

2.2.1 Animals

C57BL/6J and C57BL/6J GPI mice were bred and maintained in the animal
facility of the British Columbia Cancer Research Center (Vancouver, B.C., Canada). All
animals were used at 6-10 weeks of age and were kept under micro-isolators and
provided with sterilized food and water.

2.2.2 Antibodies for immunofluorescence analysis

The following monoclonal antibodies were used for flow cytometry of mouse
hematopoietic cells: 11F6 (α-SaBR) was developed at the Terry Fox Laboratory and was
labeled with biotin-succinimidyl-ester (Vector Laboratories, Inc., Burlingham, CA) by
standard procedures (van den Eertwegh and Claassen, 1991). Biotinylated rat IgG2b
antibody was obtained from PharMingen (San Diego, CA) and used as an isotype control.
α-CD32/CD16 (FcII,III receptor block) and fluorescein isothiocyanate (FITC)-labeled α-
CD43, L3T4 (α-CD4), Ly3.2 (α-CD8b), α-CD3, RB6-8C5 (α-Gr-1), Ter199 (erythroid
lineage marker) and 53-7.3 (α-Ly-1) were obtained from PharMingen (San Diego, CA).
FITC or phycoerythrin (PE)-labeled RA3-6B2 (α-B220) was also obtained from
PharMingen (San Diego, CA). FITC-labeled Thy1.2 was purchased from Boehringer
Mannheim Biochemicals (Indianapolis, IN). PE-labeled BP-1 and α-CD25 were
purchased from PharMingen (San Diego, CA). FITC-labeled α-IgM and PE-conjugated
steptavidin (Sa-PE) were obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL) and Jackson Immunoresearch (Bar Harbour, ME). FITC-streptavidin (Sa-FITC) was obtained from Sigma (St. Louis, MO) and allophycocyanin-conjugated streptavidin (Sa-APC) was purchased from PharMingen (San Diego, CA). Wheat germ agglutinin (WGA) was used either directly labeled with FITC (Molecular Probes) or biotinylated (Vector Laboratories, Inc, Burlingham, CA). FITC-labeled E13-161.7 (α-Sca-1) was provided by Dr. G. Spangrude (Rocky Mountain Lab, Hamilton, MT). FITC-labeled M1/70 (α-Mac-1) was obtained from American Type Culture Collection (ATCC) (Rockville, MD) and propidium iodide was obtained from Sigma (p-5264)(St. Louis, MO).

2.2.3 Cell preparation

Spleen, thymus, peripheral blood, bone marrow and lymph nodes from 6-8 week old mice were isolated, washed in Hanks Balanced Salt Solution (HBSS; StemCell Technologies, Vancouver, B.C) supplemented with 2% fetal bovine serum (HBSS/2%FBS) and solid organs passed through a fine wire mesh to isolate single cells.

2.2.4 Flow Cytometry

1x10^6 cells were preblocked on ice for 30 minutes with 1 µg/ml of α-CD32/CD16 in 100 µl HBSS/2%FBS. For staining involving only directly conjugated antibodies, optimal concentrations of the appropriate antibodies were incubated with the cells for 30 minutes on ice. For staining involving the biotinylated 11F6 antibody, 10 µg/ml biotinylated 11F6 Ab was added after the preblock and the cells incubated on ice for one hour. The cells were washed in HBSS/2%FBS and the secondary antibodies Sa-PE, Sa-FITC or Sa-APC were added for 30 min. on ice. If two or three color staining was
required, FITC, or PE conjugated antibodies to the second and third cell surface molecule were added at this time. Cells were then washed twice in HBSS/2% FBS and resuspended in the same buffer containing 1 µg/ml propidium iodide (PI). In this manner, dead cells were gated out by excluding those which stained positively with PI. In addition, isotype-matched rat antibodies were used as negative controls for staining. Flow cytometric analysis was performed using either a FACSort, FACScan or FACStarPLUS® flow cytometer (Becton Dickinson, Mountain View, CA) equipped with PC LYSIS II® software (Hewlett Packard Co., Palo Alto, CA).

LYMPHOCYTE ACTIVATION

2.2.5 Purification of primary splenic T and B cells

Spleens removed from 6-8 week old C57BL/6J GPI mice were washed in RPMI containing 2 mM L-glutamine and sodium bicarbonate (StemCell Technologies, Vancouver, B.C) and supplemented with 10% fetal bovine serum (RPMI/10%FBS), then passed through a fine wire mesh to make a single cell suspension. 1x10^8 cells were resuspended in a 2 ml volume and incubated with a nylon wool column for one hour at 37°C in accordance with the manufacturer’s instructions (PolySciences, Warrington, PA). The non-adherent T cell fraction was removed by washing the column with two volumes of RPMI/10% FBS. The column was then plunged 4 times using 1 volume of RPMI/10%FBS each time to remove the adherent B cell fraction. Both T and B cells were treated with ammonium chloride (StemCell Technologies, Vancouver, B.C) to lyse red cells, washed in RPMI/10%FBS and counted with a hemocytometer. T and B cells were plated into 6-well plates (Falcon 3046, Becton-Dickinson, Mountain View, CA) at a
density of 1x10⁶ cells/ml. T cells were plated in RPMI/10%FBS and B cells in RPMI/10%FBS plus 0.05 mM 2-mercaptoethanol (Sigma, St. Louis, MO).

T cell purity was determined using flow cytometry by analyzing the percentage of CD3+ cells and B cell purity was determined by analyzing the percentage of B220+ cells. In all experiments both T and B cell purity was greater than 80%.

2.2.6 Cell lines

The T28 T cell hybridoma and the R1.1 T cell lymphoma were also used in the following activation studies. T28 cells were generously supplied by Dr. Rock (Dana Faber Institute, Boston, MA) and R1.1 cells were purchased from American Type Culture Collection (Rockville, MD).

2.2.7 B cell activation

The following activators were used alone or in combination for the activation of nylon wool purified B cells or sorted B cells: IL-4 supernatant (5%) was kindly supplied by Dr. Sue Chappel (Terry Fox Laboratory, Vancouver, B.C), α-μ (10 μg/ml) was purchased from PharMingen (San Diego, CA), the IC10 hybridoma (α-CD40; 10 μg/ml) was generously provided by Dr. Maureen Howard (DNAX) and lipopolysaccharide (LPS; 100 μg/ml) was purchased from Sigma (St. Louis, MO). For each combination of activators described here, the optimal concentrations were determined using a proliferation assay dose response curve (see 2.2.9).

The activators were diluted in RPMI/10%FBS + 0.05 mM 2-mercaptoethanol containing 1x10⁶ cells/ml and added to 96 well round bottom dishes (Falcon 3077, Becton-Dickinson, Mountain View, CA) at a volume of 200 μl per well. The cultures were analyzed by flow cytometry 48 hours later for the expression of SaBR. To ensure
that the cells were activated, a [³H] thymidine incorporation assay to measure cell proliferation was performed simultaneously.

2.2.8 T cell activation

The following activators were used alone or in combination for the activation of nylon wool purified splenic T cells or sorted T cells: 10 μg/ml concanavalin A (ConA; Sigma, St. Louis, MO), 10 ng/ml phorbol myristate acetate (PMA; Sigma, St. Louis, MO), phytohemaglutinin (PHA; 1/100; Sigma, St. Louis, MO), 100 ng/ml A23187 (Sigma, St. Louis, MO) and 2 μg/ml soluble α-CD3 (PharMingen, San Diego, CA). Plate bound antibodies included α-CD3 at 0.25 μg/ml -0.5 μg/ml (PharMingen, San Diego, CA) and α-CD28 at 4 μg/ml (PharMingen, San Diego, CA). As for the activation of B cells, the optimal concentrations of each combination of activators was determined using a proliferation assay dose response curve (see 2.2.9).

The soluble activators were diluted in RPMI/10%FBS containing 1x10⁶ cells/ml and added to 96 well round bottom dishes at a volume of 200 μl per well. Plate bound antibodies were diluted in 50mM Tris (pH 9.5) (Gibco BRL, Canadian Life Technologies, Burlington, Ont.) and added to 96 well dishes at 50 μl per well overnight at 4°C. The wells were then washed twice with sterile phosphate buffered saline (PBS; StemCell Technologies, Vancouver, B.C) and 200 μl of cells added at a concentration of 1x10⁶ cells/ml.

Cells were activated on day 0 and analyzed by flow cytometry 48 hours later for the expression of SaBR. Double staining of SaBR and CD4 or CD8 was also carried out to further characterize SaBR expression. To ensure that the cells were activated, the expression of the IL-2 receptor was examined for each activator using the PE-labeled α-
CD25 Ab. Proliferation assays to measure cell proliferation of purified splenic T cells were also carried out simultaneously.

2.2.9 Proliferation assays

Two different proliferation assays were performed for these studies. To test for appropriate lymphocyte activation, $1 \times 10^5$ purified T or B cells were plated per well in 96 well round-bottom plates for 42 hours. The cells were pulsed with 1 μCi/well $[^3]$H thymidine (Amersham, Oakville, Ont) and harvested 6 hours later with an LKB 1295-001 cell harvester (Uppsala, Sweden). For the cell sorting experiments, equal concentrations of sorted cells were plated in 96 well dishes for 12 or 24 hours. The cells were pulsed with 1 μCi/well $[^3]$H thymidine at time 0 or 12 hours later and harvested 12 hours after pulsing. For both assays, proliferation was assessed by measuring isotope incorporation with a LKB 1205 Betaplate liquid scintillation counter (Uppsala, Sweden). All proliferation assays were performed in triplicate.

2.2.10 Fluorescent activated cell sorting

Spleens removed from 6-8 week old C57BL/6J GPI mice were washed in RPMI/10%FBS, then passed through a fine wire mesh to make a single cell suspension. $1 \times 10^7$ cells per sample were preblocked on ice for 30 minutes with 1 μg/ml of α-CD3/2/CD16 in 100 μl HBSS/2%FBS. The cells were then incubated on ice with biotinylated 11F6 for 1 hour and washed in HBSS/2%FBS. Sa-PE plus B220-FITC or CD4-FITC at optimal concentrations was then added for 30 minutes on ice. After washing twice in HBSS/2%FBS, the cells were resuspended at $1 \times 10^6$ cells/ml in the same buffer containing 1 μg/ml PI. The cell populations stained with 11F6 plus B220 or 11F6 plus CD4 were then gated on the live cell population and sorted into the following
subfractions using a FACSort equipped with PC LYSIS II® software: B cells: 1) B220+11F6+ 2) B220+11F6- 3) B220+ and T cells: 1) CD4+11F6+ 2) CD4+11F6- 3) CD4+

After sorting, the cells were washed with RPMI/10%FBS (T cells) or RPMI/10%FBS + 0.05 mM 2-mercaptoethanol (B cells) and counted with a hemocytometer. Equal numbers of cells for each of the three sort conditions were then plated in 96 well dishes at a final volume of 100 μl per well. After incubating at 37°C for 12 or 24 hours, proliferation assays were performed. In addition to each proliferation assay, the percentage of live cells and the percentage of cells expressing SaBR was determined using flow cytometry.

2.2.11 11F6 antibody assays

Two 11F6 antibody assays were performed. The first was a control for antibody effects on proliferation of sorted B cells and the second looked specifically at the effects of antibody on proliferation of nylon wool purified lymphocytes.

Assay #1: 1x10^5 B220+ sorted B cells/well were plated in 96 well dishes with 10 μg/ml purified 11F6 Ab or 10 μg/ml 11F6 Ab + 5 μg/ml RG7 crosslinker (mouse α-rat IgG2b Fc; American Type Culture Collection, Rockville, MD). The cells were pulsed with 1 μCi/well [³H] thymidine at time 0 or 12 hours later and harvested 12 hours after pulsing. Proliferation was assessed by measuring isotope incorporation with a LKB 1205 Betaplate liquid scintillation counter. All assays were performed in triplicate.

Assay #2: 5x10^5 nylon wool purified B or T cells per well were plated in 96 well dishes with 0-10 μg/ml soluble or plate bound 11F6 or rIgG2b control antibody. For the purified T cells, the IL-2R antibody was used as a positive control for inhibition of
proliferation. Furthermore, the purified lymphocytes were either added to the wells containing antibody just prior to activation or 12 hours after activation. The T cell activators used included conA, PHA, plate bound α-CD3, and α-CD3+α-CD28 (see 2.2.8 for details) and the B cell activators included IL-4+α-μ, IL-4+α-CD40 and IL-4+α-μ+α-CD40 (see 2.2.7 for details). The cells were pulsed with 1 μCi/well \[^{3}\text{H}]\) thymidine 12 hours after the addition of antibody and harvested 12 hours after pulsing. Proliferation was assessed by measuring isotope incorporation with a LKB 1205 Betaplate liquid scintillation counter. All assays were performed in triplicate.

2.3 RESULTS

2.3.1 SaBR is expressed throughout the hematopoietic system

11F6 is one of a large number of monoclonal antibodies (mAb) obtained from a screen for hybridomas specific to antigens expressed by the murine multilineage hematopoietic cell line, B6SUtA. Its unique expression pattern in the hematopoietic system indicated that 11F6 recognized a novel antigen (now called SaBR) which is differentially expressed throughout the hematopoietic system. In order to gain more information about SaBR, its expression within the hematopoietic system was examined in greater detail.

The majority of hematopoietic tissues examined stained positively with 11F6 antibody. Double staining of bone marrow with 11F6 and a combination of antibodies designed to delineate lineage positive (lin+) cells (α-B220, α-GR-1, α-Mac-1, Ter199, α-Ly-1), indicated that only 2% of early progenitors (Sca-1+lin-WGA+) express the protein, whereas 44% of mature lineage positive cells and 40% of multilineage
progenitors (Sca-1+) are positive for SaBR expression (Table 2.1; Vivienne Rebel, personal communication). Gating on cells that have the property of low light scatter and Sca-1 expression indicated a relatively high percentage of SaBR positive cells within the macrophage (Mac-1+), granulocyte (GR-1+) and B cell (B220+) lineages and a slightly lower percentage of SaBR positive cells in the T lymphoid (Ly-1+) and erythroid (TER199+) lineages (Table 2.1; Vivienne Rebel, personal communication).

The percentage of SaBR positive cells was variable when specific cell populations were examined within different hematopoietic tissues (Table 2.2). The bone marrow contained the highest percentage of SaBR positive lymphocytes followed by the spleen, peripheral blood and lymph nodes. SaBR positive granulocyte and macrophage subpopulations were most prevalent in the spleen and nucleated erythroid cells expressing SaBR were most abundant in the peripheral blood (Table 2.2).

The high percentage of SaBR B lymphocytes in the bone marrow, coupled by low expression in the peripheral blood, suggested that SaBR may be playing a role during B cell development. Moreover, the relative lack of SaBR expression in thymocytes suggested that while it may be playing a role in more mature T cells, it did not appear to be involved in thymocyte development. Since lymphocytes are fairly easy to study in vitro and their developmental pathways are well known, I decided to examine SaBR expression in mature lymphocytes and developing B cells more closely.

2.3.2 SaBR is differentially expressed in lymphocytes

When SaBR expression within lymphocytes was examined in greater detail, differential expression was seen between tissues and within subpopulations of T
Table 2.1. Percentage of bone marrow cells expressing SaBR within different hematopoietic lineages at various developmental stages

A

<table>
<thead>
<tr>
<th>subpopulation</th>
<th>% SaBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>total BM</td>
<td>31</td>
</tr>
<tr>
<td>Lin+</td>
<td>44</td>
</tr>
<tr>
<td>Lin-</td>
<td>5</td>
</tr>
<tr>
<td>Sca-1+</td>
<td>40</td>
</tr>
<tr>
<td>Sca-1+Lin-</td>
<td>5</td>
</tr>
<tr>
<td>Sca-1+Lin-WGA++</td>
<td>2</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>subpopulation</th>
<th>% SaBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLS,Sca-1+, GR-1+</td>
<td>67</td>
</tr>
<tr>
<td>B220+</td>
<td>46</td>
</tr>
<tr>
<td>Mac-1+</td>
<td>58</td>
</tr>
<tr>
<td>Ly-1+</td>
<td>43</td>
</tr>
<tr>
<td>TER199</td>
<td>37</td>
</tr>
</tbody>
</table>

Single cell suspensions from C57Bl/6 bone marrow were stained with 11F6 and various other antibodies to specifically identify (A) the percentage of cells expressing SaBR at different developmental stages and (B) the percentage of progenitor cells from defined hematopoietic lineages that express SaBR (Dr. Vivienne Rebel, personal communication).
Table 2.2. **Mean percentage of SaBR positive cells (± SEM) within the hematopoietic system**

<table>
<thead>
<tr>
<th></th>
<th>% SaBR</th>
<th>Gr-1</th>
<th>Mac-1</th>
<th>B220</th>
<th>Ly-1</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>Ter199</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>0.57±0.27 (3)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>5.2</td>
<td>0.6</td>
<td>0.7</td>
<td>nd</td>
</tr>
<tr>
<td>bone marrow</td>
<td>31.0±9.6 (6)</td>
<td>23.9±10.2 (3)</td>
<td>23.0±14.2 (3)</td>
<td>30.2±6.2 (5)</td>
<td>61.0±1.4 (2)</td>
<td>nd</td>
<td>24.4</td>
<td>7.5</td>
<td>20.7±11.3 (3)</td>
</tr>
<tr>
<td>spleen</td>
<td>19.3±3.2 (4)</td>
<td>46</td>
<td>44</td>
<td>19.8±9.3 (4)</td>
<td>nd</td>
<td>nd</td>
<td>12</td>
<td>3.3±2.7 (4)</td>
<td>nd</td>
</tr>
<tr>
<td>peripheral blood</td>
<td>5.8±2.0 (3)</td>
<td>6.8±4.6 (3)</td>
<td>4.6±1.6 (3)</td>
<td>4.3±2.7 (3)</td>
<td>9.6±2.6 (2)</td>
<td>nd</td>
<td>12</td>
<td>2.6</td>
<td>44.5±3.1 (3)</td>
</tr>
<tr>
<td>lymph nodes</td>
<td>5</td>
<td>nd</td>
<td>nd</td>
<td>5</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

Single cell suspensions from different hematopoietic tissues were stained with 11F6 and a second antibody to specifically identify hematopoietic subpopulations, then analyzed by FACS. The number of experiments performed is indicated in brackets within the table. nd= not determined. Note: This table is based on experimentation by the author and Dr. Vivienne Rebel.
lymphocytes. As mentioned previously, the percentage of SaBR positive cells was highest in the bone marrow and spleen and considerably lower in the peripheral blood and lymph nodes (Table 2.2). In addition, the percentage of SaBR positive cells was three times higher amongst CD4+ T cells than CD8+ cells in all tissues examined (Table 2.2). Within B cells of the bone marrow, approximately 90% of pro-B cells (B220+Thy1.2+), 75% of pre-B (B220+IL-2R+) and 50% of immature and mature B cells (B220+IgM+) were found to express the antigen (Figure 2.1). These results, in combination with the lack of thymocyte expression, demonstrate that SaBR has a developmentally regulated pattern of expression in lymphocytes and may be involved in B cell development, particularly during the pro-B cell stage.

Since SaBR was found to be expressed on a higher percentage of lymphocytes in the spleen as compared to the peripheral blood and its expression was higher on CD4 T cells than CD8 cells, I wondered if it may be playing a role in either helper T cell function or activation of spleen cells. After lymphocytes receive activation stimuli, the expression of many functionally relevant cell surface proteins are either up or downregulated; therefore I chose to look at SaBR expression levels during activation. A change in expression levels would allow me to refine my original hypothesis to include a functional role for SaBR during lymphocyte activation.

2.3.3 SaBR is differentially regulated upon activation of purified splenic B cells

The expression levels of SaBR on activated splenic B cells was dependent upon the combination of activators used. Activation with stimuli that mimic complete antigen dependent activation (α-μ+IL-4 or α-μ+IL-4+α-CD40) resulted in a two fold decrease in both the mean fluorescent intensity (MFI) and the percentage of cells expressing SaBR.
Figure 2.1. Expression of SaBR during B cell development. Flow cytometric analysis of a single cell suspension of total bone marrow cells from C57Bl6 GPI mice. Cells were stained with biotinylated 11F6 antibody plus Sa-APC and antibodies to cell surface markers which distinguish different developmental stages A) B220-PE and Thy-1.2-FITC (early pro-B), B) B220-PE and CD43-FITC (pro-B), C) BP-1-PE and B220-FITC (late pro and pre-B), D) IL-2R-PE and B220-FITC (pre-B) and E) B220-PE and IgM-FITC (immature and mature B). Live cells were gated on their positive expression of both developmental markers (upper right hand quadrant) and the percentage of cells expressing SaBR was determined within the gated population and shown as a histogram. This figure consists of data from one experiment, but is representative of the results obtained from 4 independent experiments.
A 89%
early pro-B
(B220+Thy1.2+)

B 60%
pro-B
(B220+CD43+)

C 49%
late pro & pre-B
(B220+BP-1+)

D 74%
pree-B
(B220+IL-2R+)

E 53%
immature &
mature B
(B220+IgM+)
Activation with stimuli that are non-antigen dependent (IL-4+α-CD40), however, had the opposite effect and resulted in a 2 fold increase in the MFI and in the percentage of SaBR positive cells. No significant difference in SaBR expression was observed when LPS or the above activators by themselves were used (Figure 2.2).

It can be concluded from these results that SaBR expression is differentially regulated during B cell activation and that complete activation through the BCR results in downregulation of SaBR expression, while antigen independent bystander activation results in upregulation of SaBR expression. These results suggest that the presence of SaBR may be functionally relevant in terms of cellular responses following activation.

2.3.4 SaBR is upregulated upon activation of purified splenic T cells and the T cell lines T28 and R1.1

I reasoned that if SaBR expression was differentially regulated following B cell activation, then it may also be regulated following T cell activation. To test this hypothesis, I examined SaBR expression following treatment of splenic T cells with in vitro polyclonal activators.

Following a 48 hr treatment with polyclonal activators that bypass the TCR (PMA+A23187), the percentage of nylon wool purified splenic CD4 cells expressing SaBR increased 9 fold and the percentage of CD8 cells expressing the antigen increased by 30 times (Figure 2.3). Activation through the TCR complex (α-CD3), in addition to stimulation with the phorbol ester PMA, did not result in any significant change in SaBR expression on CD4 cells; however, there was approximately a 9 fold increase in the percentage of CD8 cells expressing SaBR (Figure 2.3). Treatment of purified splenic T cells with PMA, A23187 or α-CD3 alone, which were sufficient to upregulate IL-2R
Figure 2.2. **Expression of SaBR following activation of purified splenic B cells.** B cells were isolated from single cell suspensions of spleen cells by nylon wool purification, treated with various combinations of activators, incubated at 37°C for two days, then analyzed by flow cytometry for SaBR expression. Treatments included A) no activation, B) IL-4 + α-μ, C) IL-4 + α-CD40, D) IL-4 + α-μ + α-CD40, E) α-μ, F) α-CD40, G) IL-4 and H) LPS. The percentage of cells expressing SaBR is expressed as a histogram, with the unshaded and shaded peaks representing treatment with an IgG2b isotype control and the 11F6 antibody respectively. Each histogram is based on the results of one representative experiment and included in each figure is the percentage of SaBR positive cells for that experiment and the mean fluorescent intensity of the 11F6 staining for all experiments (n≥3).
no activation

A  
MFI=1438
33%

IL-4+α-μ

B  
MFI=827
17%

IL-4+α-CD40

C  
MFI=2350
63%

IL-4+α-μ+α-CD40

D  
MFI=965
12%

α-μ

E  
MFI=877
31%

α-CD40

F  
MFI=1979
35%

IL-4

G  
MFI=1688
45%

LPS

H  
MFI=1679
41%

SaBR
Figure 2.3. **Expression of SaBR following activation of purified splenic T cells.** T cells were isolated from single cell suspensions of spleen cells by nylon wool purification, treated with various combinations of activators, incubated at 37°C for two days, then analyzed by flow cytometry for A) SaBR expression within the CD4 subpopulation, B) SaBR expression within the CD8 subpopulation and C) the percentage of CD4 and CD8 cells expressing IL-2R. Treatments included: no activation, PMA + A23187, PMA+α-CD3, A23187, α-CD3, PMA and α-CD3+α-CD28. The percentages of CD4 and CD8 gated T cells expressing SaBR are shown as a histogram, with the unshaded and shaded peaks representing treatment with an IgG2b isotype control and the 11F6 antibody respectively. The percentages of CD4 and CD8 gated T cells expressing IL-2R are presented in a table. The table and histograms are based on the results of one representative experiment (n≥3) and included in each histogram is the percentage of SaBR positive cells within the CD4 or CD8 subpopulation for that experiment.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>% IL-2R</th>
<th>% IL-2R in subpop.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4</td>
<td>CD8</td>
</tr>
<tr>
<td>non-activated</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>PMA+A23187</td>
<td>55</td>
<td>50</td>
</tr>
<tr>
<td>PMA+α-CD3</td>
<td>71</td>
<td>61</td>
</tr>
<tr>
<td>A23187</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>α-CD3</td>
<td>54</td>
<td>41</td>
</tr>
<tr>
<td>PMA</td>
<td>37</td>
<td>35</td>
</tr>
<tr>
<td>α-CD3+α-CD28</td>
<td>56</td>
<td>47</td>
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</tbody>
</table>
expression, but not induce proliferation, did not affect SaBR expression (Figure 2.3). Activation of purified splenic T cells with physiologically relevant antibodies, by crosslinking of the TCR with α-CD3 in combination with α-CD28, also failed to show any significant changes in SaBR expression despite a strong proliferative response (Figure 2.3).

Further characterization of the role of SaBR in T cell activation was carried out using the cell lines, T28 and R1.1. T28 is a T cell hybridoma expressing low levels of SaBR and R1.1 is a lymphoma which normally does not express SaBR. Both cell lines were activated with a variety of polyclonal activators and assayed by flow cytometry for changes in SaBR expression. Like purified T cells, the percentage of T28 cells expressing SaBR increased more than two fold over non-activated controls after treatment with activators that bypass the TCR (PMA+A23187) (Table 2.3; Figure 2.4). Unlike purified T cells, however, treatment of T28 cells with conA resulted in a greater than two fold increase in SaBR positive cells over control populations (Table 2.3; Figure 2.4). In R1.1 cells, treatment with PMA, PMA+A23187 and A23187 alone resulted in a greater than two fold increase in the percentage of cells expressing SaBR, while treatment with conA, PHA, PMA+α-CD3 and α-CD3 had no significant effect on the expression of SaBR (Table 2.3; Figure 2.4).

Similar to the results obtained for B cell activation, stimulation of T cells or T cell lines with activators that bypass the TCR results in upregulation of SaBR expression. Unfortunately, due to the low basal levels of SaBR expression in both the T cells and cell lines it was not possible to detect potential decreases in SaBR expression upon activation through the TCR.
Table 2.3. Mean fold increase in SaBR positive cells (±SEM) after activation of purified splenic T cells and the cell lines, T28 and R1.1.

<table>
<thead>
<tr>
<th></th>
<th>ConA</th>
<th>PHA</th>
<th>PMA</th>
<th>PMA+A23187</th>
<th>PMA+α-CD3</th>
<th>A23187</th>
<th>α-CD3</th>
</tr>
</thead>
<tbody>
<tr>
<td>purified T cells</td>
<td>0±0</td>
<td>0.4±0.7</td>
<td>0±0</td>
<td>2.4±0.2</td>
<td>0±0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T28</td>
<td>10.6±7.5</td>
<td>3.6±3.8</td>
<td>2.3±1.3</td>
<td>22.2±13.0</td>
<td>6.8±5.7</td>
<td>2.3±1.1</td>
<td>1.3±1.5</td>
</tr>
<tr>
<td>R1.1</td>
<td>3.5±3.8</td>
<td>4.3±5.3</td>
<td>6.0±3.2</td>
<td>33.0±7.1</td>
<td>3.2±3.7</td>
<td>27.0±17.3</td>
<td>1.0±1.2</td>
</tr>
</tbody>
</table>

Nylon wool purified splenic T cells and the cell lines T28 and R1.1 were treated with various combinations of activators and incubated for two days at 37°C. Flow cytometric analysis was then performed to determine the percentage of cells expressing SaBR and the mean fold increase in SaBR positive cells (±SEM) over non-activated controls was determined. The number in brackets within the table indicates the number of experiments performed.
Figure 2.4. **Percentage of SaBR positive cells following activation of T28 and R1.1 cells.** The T cell lines (A) T28 and (B) R1.1 were treated with various combinations of activators for two days at 37°C, then analyzed by flow cytometry for SaBR expression. The percentage of SaBR positive cells for each individual experiment is indicated by closed circles within the figure. A horizontal line indicates the mean for each set of activators (n≥3).
2.3.5 Sorted B220+11F6+ cells replicate DNA at a higher rate than B220+11F6- cells

In sections 2.3.3 and 2.3.4, I demonstrated that SaBR expression is differentially regulated during lymphocyte activation and I proposed that SaBR may be playing a functional role during activation. If SaBR is functionally relevant, then the functional characteristics of SaBR expressing cells (e.g., ability to proliferate) may be different from SaBR non-expressing cells. To determine whether SaBR positive B cells are functionally different from their negative counterparts, spleen cells were sorted into B220+11F6+ and B220+11F6- fractions and activated with various combinations of stimuli. A proliferation assay was performed during the first 12 hours and during the 12-24 hour periods following addition of the activation stimuli. B220+11F6+ cells had a significantly higher $[^3]$H thymidine incorporation in the first 12 hours than the B220+ control or the B220+11F6- population when non-activated or activated with IL-4+$\alpha$-$\mu$, IL-4+$\alpha$-CD40 or IL-4+$\alpha$-$\mu$+$\alpha$-CD40 (Figure 2.5). There was no difference in $[^3]$H thymidine incorporation with $\alpha$-$\mu$, IL-4 or $\alpha$-CD40 alone (Figure 2.5). During the 12-24 hour activation period, no significant difference in proliferation was observed between any of the sorted populations (Figure 2.5). The $[^3]$H thymidine incorporation counts were very low, therefore, it appears that most of the cells were not dividing during the first 12 hours. Thus, the difference in proliferation was the result of only a very small population that was entering the cell cycle. These results suggest, that instead of directly resulting in proliferation, SaBR may be involved in priming the cells for proliferation competence.

It should also be noted that after 12 hours of activation, the B220+11F6- sorted fraction had already begun to express SaBR at levels equivalent to or higher than the
Figure 2.5. \[^{3}H\] thymidine incorporation of sorted splenic B cells. Splenic B cells were sorted into three subpopulations by fluorescent activated cell sorting; a) B220+11F6+, b) B220+11F6- and c) B220+. 5x10^4 cells/well of each sorted population were then incubated at 37°C with and without B cell activators and \[^{3}H\] thymidine was added at (A) time 0 or (B) 12 hours after sorting. Cells were harvested at time 12 hrs or 24 hrs and \[^{3}H\] thymidine incorporation determined for each population. The p values (based on a students standard T test) for sorted populations that are significantly different from the control (B220+) are indicated within the table.
starting B220+ cell fraction suggesting that a specific level of SaBR expression is somehow maintained at a population level on lymphocytes (Figure 2.6).

To account for the possibility that the increase in proliferation was induced by mAb crosslinking of SaBR on the B220+11F6+ sorted cells, rather than being intrinsic to the SaBR+ population, a proliferation assay was performed on B220+ sorted cells after treatment with 11F6 and control antibodies. Results indicated that the increased proliferation of B220+11F6+ sorted cells was not due to the presence of 11F6 antibody (Figure 2.6). Furthermore, when non-sorted activated and non-activated nylon wool purified lymphocytes were treated with the 11F6 antibody, no significant difference in \[^{3}\text{H}]\text{ thymidine incorporation was observed when compared to controls. This does not rule out the possibility, however, that other antibodies to SaBR may in fact mimic signaling and affect proliferation.}

Unlike B cells, when CD4 T cells were sorted into CD4+11F6+ and CD4+11F6- fractions and activated, no difference in \[^{3}\text{H}]\text{ thymidine incorporation was observed between the positive and negative fractions and the control during either time point (Figure 2.7). I must conclude, therefore, that while there appears to be differences in proliferation between SaBR+ and SaBR- B cells, there is no difference amongst SaBR+ and SaBR- T cells.

2.4. DISCUSSION

The 11F6 antibody was isolated from a screen of the murine IL-3 dependent hematopoietic cell line, B6SUtA and used in experiments to determine the expression of the 11F6 antigen (SaBR) in the hematopoietic system. Prior to my entry into the project
Figure 2.6. **SaBR expression in sorted B220+11F6- populations 12 hours after sorting.** The expression of SaBR on (A) non-activated and (B) activated sorted B220+11F6- cells was determined by flow cytometry 12 hours after sorting. SaBR expression is shown as a histogram and the percentage of SaBR positive cells is indicated within each figure. The horizontal line designates the portion of the histogram that is considered positive for SaBR expression based on isotype controls and SaBR expression at time 0. (C) The effect of 11F6 antibody on the proliferation of B220+ sorted B cells was also determined. B220+ sorted B cells were treated with 11F6 Ab or control rIgG2b Ab with and without the RG7 crosslinker for 12 hours, then a $[^3]$H thymidine incorporation assay was performed. The figure represents the mean CCPM±SEM for three independent experiments and a students standard T test revealed no significant difference between the control and the treated populations.
Figure 2.7. [³H] thymidine incorporation of sorted splenic CD4 T cells. Splenic CD4 T cells were sorted into three subpopulations by fluorescent activated cell sorting; a) CD4+11F6+, b) CD4+11F6- and c) CD4+. 1x10⁴ cells/well of each sorted population were then incubated at 37°C with and without T cell activators and [³H] thymidine was added at (A) time 0 or (B) 12 hours after sorting. Cells were harvested at time 12 hrs or 24 hrs and [³H] thymidine incorporation determined for each population. A Student’s standard T test revealed no significant difference between the control and the treated populations.
Dr. Vivienne Rebel (personal communication) examined the expression of SaBR during hematopoiesis by utilizing the expression of cell-surface markers which define specific developmental stages. In mice, the Sca-1 antibody has been shown to define stem cells and multipotential progenitor cells (Rebel et al., 1994; Spangrude et al., 1988). If bone marrow cells are double stained with 11F6 and Sca-1, 40% of the cells become double positive. Similar results are seen if the bone marrow cells are stained for 11F6 and markers which define lineage commitment (44% double positive).

However, staining for stem cells with the phenotype Sca-1+Lin-WGA+ yields only 2% positive cells, suggesting that 11F6 is only expressed on multipotential progenitors, committed progenitors that are beginning to express the lineage markers and mature cells and is unlikely to be involved in very early development of the hematopoietic system.

SaBR expression was also examined in different hematopoietic tissues and subpopulations of mature cells. Overall, the percentage of SaBR positive cells was highest in bone marrow and spleen, low in the peripheral blood and lymph nodes and almost absent in the thymus. As the main site of adult hematopoiesis, the bone marrow contains a higher proportion of progenitor cells than the blood (Metcalf and Moore, 1971). It also has a higher SaBR expression than other hematopoietic organs, suggesting that SaBR is found on a higher percentage of progenitors than mature cells. This observation is borne out when one notes the higher percentage of SaBR positive cells within bone marrow progenitors (LLS,Sca-1+)(Table 1B) of the macrophage/granulocyte, nucleated erythroid and B cell lineages as compared to the percentage of mature cells (Table 2.2) in those lineages (Dr. Vivienne Rebel, personal communication). The
exception appears to be the Ly-1+ cell lineage; where the percentage of LLS,Sca-1+Ly-1+ progenitors expressing SaBR in the bone marrow is less than the percentage of more mature cells expressing the antigen (Dr. Vivienne Rebel, personal communication). Ly-1, or CD5, is expressed on a small subset of B cells and all T cells (Ledbetter, 1980); therefore, since there are no immature T cells in the bone marrow, the LLS, Sca-1+Ly-1+ progenitors expressing the antigen in the bone marrow probably consists of only the small subset of B cells.

SaBR is also differentially expressed on mature cells. The most striking example occurs within the lymphocyte population. A large percentage of bone marrow lymphocytes are SaBR positive. This percentage drops approximately 50% in the spleen and even further in the peripheral blood and lymph nodes. If SaBR were involved in T lymphocyte homing to the bone marrow, it may explain the higher percentage of SaBR positive mature T cells in the bone marrow as compared to the spleen, peripheral blood and lymph nodes.

The higher percentage of SaBR positive B lymphocytes in the bone marrow can be mainly accounted for by the high percentage of B lymphoid progenitors that are present in the bone marrow. If these progenitors are indeed responsible for the majority of 11F6 staining in B cells, then it can be hypothesized that SaBR may be functionally involved in B cell development. In order to test this hypothesis, I examined SaBR expression at different stages of development using bone marrow derived B cells. I have shown that the greatest percentage of SaBR+ B cells in the bone marrow can be found within the progenitor pro-B cell subpopulation. In fact, 90% of pro-B cells express SaBR. For mature and immature B cells within the marrow this percentage decreases to
approximately 50%. In order to differentiate, pro-B cells must maintain contact with the stromal cell layer so that they can interact with extracellular matrix components, cell surface receptors and cytokines (Kincade et al., 1989; Cushley and Harnett, 1994). The high percentage of early B cells expressing SaBR suggests that it may be involved in early B cell development by mediating some of these interactions.

The percentage of CD4 and CD8 T cells expressing the antigen also appears to be different. There are approximately 2-3 times as many CD4+SaBR+ cells as CD8+SaBR+ cells in the bone marrow, spleen, peripheral blood and lymph nodes. The higher percentage of CD4+SaBR+ T cells suggests that SaBR may be playing a role in helper T cell function or that down-regulation of SaBR is required for the differentiation or cytotoxic function of CD8 cells.

Numerous cells within the spleen are involved in immunological responses to foreign antigen. SaBR is expressed on splenic lymphocytes and is differentially expressed within T cell subpopulations; therefore, it is reasonable to suggest that SaBR may be playing a role in the activation responses of splenic lymphocytes. In order to test and refine this hypothesis, SaBR expression was examined on activated lymphocytes following stimulation with in vitro polyclonal activators.

During stimulation, accessory molecules may become activated by either increasing or decreasing their expression on the cell surface (Mondino and Jenkins, 1994). I have shown that in B cells, complete activation by crosslinking of the BCR plus a costimulatory signal (IL-4 or α-CD40) results in the downregulation of SaBR expression, while activation with two costimulatory signals independant of signaling through the BCR results in upregulation. In CD4 and CD8 T cells, upregulation of SaBR
expression occurs after activation with stimuli that bypass the TCR (PMA+A23187). In CD8 T cells, upregulation of SaBR is also seen after signaling through the TCR complex (α-CD3) in conjunction with the second messenger agonist, PMA. These results suggest that the change in cell surface density of SaBR may be functionally relevant and affect how both B and T lymphocytes respond following activation. In fact, it is possible that SaBR is acting as an accessory molecule.

Accessory molecules expressed on lymphocytes may play many different functional roles; adhesion, costimulation, proliferation, or differentiation (Zuckerman et al., 1995; Collins et al., 1994). One means of identifying costimulatory or co-receptor function is by examining the effects of antibodies on the proliferation of lymphocytes expressing the antigen of interest. Antibodies to costimulatory molecules such as CD28 (Harding et al., 1992), CTLA-4 (Linsley et al., 1992; Walunas et al., 1994) and CD82 (Lebel-Binay et al., 1995) have all been shown to enhance lymphocyte proliferation. I have shown here and in other experiments that the 11F6 antibody alone or crosslinked has no effect upon proliferation of lymphocytes. This does not rule out the possibility that SaBR plays a costimulatory role, however. Different antibodies to the same cell surface protein may have different effects, depending on where the antibody binds; therefore, more antibodies need to be developed and tested in order to determine whether SaBR could indeed be costimulatory.

Antibodies have also been shown in many instances to change the adhesive properties of cellular interactions, thus implicating their corresponding antigens as possible adhesion molecules (Dustin and Springer, 1991). For example, antibodies to CD40 (Klaus et al., 1994) and CD43 (Sanchez-Mateos et al., 1995) increase adhesion,
while other antibodies, such as α-CTLA-4 and α-CD28 block adhesion (Linsley et al., 1992). In the case of SaBR, experimentation has shown that the 11F6 antibody does not alter the adhesion of lymphocytes with one another. Furthermore, transfection of 3T3 fibroblasts or COS cells with SaBR does not alter the adhesion of transfected cells to lymphocytic cell lines (Chapter 3).

If SaBR is playing a functional role in lymphocytes, then cells expressing SaBR may be functionally different from their negative counterparts. Differences in the ability of cell populations to proliferate in culture are easily measured; therefore, I decided to test this hypothesis by comparing $[^3]$H thymidine incorporation in B and T lymphocytes sorted on the basis of SaBR expression.

I observed that B cells sorted for SaBR expression have a higher $[^3]$H thymidine incorporation when placed in culture regardless of their activation status; therefore, in addition to lymphocyte activation, SaBR may also be involved in the process of cell cycle entry. Cell cycle status was determined for freshly sorted cell populations and in all cases almost 100% of the cells were in G$_0$/G$_1$, therefore SaBR positive cells were not actively in cycle upon sorting. Cell cycle status was also determined for SaBR positive cells 12 hours and 24 hours after being placed in culture; however, almost 100% were still in G$_0$/G$_1$, suggesting that only a very small percentage of SaBR positive cells were responsible for the increase in $[^3]$H thymidine incorporation seen after 12 hours. Moreover, this small actively cycling subpopulation may have been represented in the SaBR positive freshly sorted cells, but not detectable due to their small numbers. Furthermore, since an increase in $[^3]$H thymidine incorporation in the SaBR+ cells is seen in non-activated populations as well as in activated populations, the freshly sorted SaBR+
cells must be already “primed” for cell cycle entry, such that culture media alone is enough to cause a small population to begin proliferating.

No increase in $[^3]H$ thymidine incorporation was observed if an activator was used alone to stimulate any of the sorted populations. In vivo, lymphocytes will become anergic or die if only one activation signal is received, therefore these sorted cells could be undergoing anergy in response to the single activator. Because no proliferation is seen in any of the cultures stimulated with a single activator, the signals associated with anergy must be overriding or suppressing the positive proliferative effects of SaBR expression.

When CD4 T cells were sorted on the basis of SaBR expression, no difference in $[^3]H$ thymidine incorporation was observed between the two populations. This does not rule out the possibility that the two populations differ in other ways, such as cytokine production or the ability to provide T cell help. Assays examining these possibilities were not performed mainly due to the lack of reagents and technical expertise; however they will be important in the future to determine if there really is an intrinsic difference between SaBR+ and SaBR- T cells.

The cell sorting experiments described in this chapter indicate that SaBR expression appears to be maintained at a population level on a specific percentage of resting B and T splenocytes. B or T splenocytes sorted into SaBR positive and negative populations will begin to change their phenotypes (in regards to SaBR expression) back to unsorted levels within 12 hours. This is probably not due to the reversal of only a small sub-population because the reverted populations respond to the activators in the same manner as the pre-sorted populations. Since these results were obtained with liquid
cultures, the maintenance of SaBR expression must be controlled either by cell-cell interactions, soluble mediators or internal mechanisms.

A summary of the results in this chapter indicates that SaBR may be playing a role during B cell development and during lymphocyte activation. In addition, it appears to be involved in priming B cells for entry into the cell cycle and its expression is maintained at a population level on both B and T lymphocytes. At this point I'd like to propose a preliminary model regarding SaBR function in B cells. I will then suggest ways to test the model and briefly discuss SaBR function in T cells. Please keep in mind that this model is very preliminary and subject to change upon further experimentation. A schematic diagram of the model can be found in Figure 2.8.

Resting B cells constantly receive stimuli from their surroundings and other cells. I would like to propose a model whereby SaBR expression is a result of this stimulation and its presence serves to play a role in the subsequent cellular responses of these cells.

I propose that SaBR is expressed when a resting B cell receives a specific signal that primes the cell for entry into the cell cycle. These SaBR positive cells are now sensitive to cell cycle entry signals and will incorporate more \([3^H]\) thymidine than their negative counterparts if such signals are received. However, if no further signals are received, the cell will return to its earlier resting state and SaBR expression will be turned off. If, on the other hand, a SaBR positive cell receives the correct signals for cell cycle entry and progression, the cell will enter the cell cycle. As discussed earlier, it appears that only a small percentage of SaBR positive cells receive the correct stimuli to enter the cell cycle; the remainder will eventually return to a resting state and turn off SaBR.
Figure 2.8: Model depicting the role of SaBR in B cell activation. Filled in triangles represent SaBR expression.
expression. The constant turning on and off of SaBR in response to the presence or absence of external stimuli, may help explain why SaBR appears to be maintained on approximately 20% of splenic B cells.

Once the cells have entered the cell cycle, further signals are required for progression and activation. I propose that the presence of SaBR on the cell surface serves as a marker for future elimination or anergy if the cells do not receive proper activation stimuli through the BCR. When purified B cells are activated with a signal through the BCR in addition to one or more co-stimulatory signals (α-μ+IL-4 or α-μ+IL-4+α-CD40), SaBR expression is down-regulated. The cells are now no longer marked for elimination and will go on to proliferate and differentiate into antibody forming cells or memory cells.

However, if cells are activated with signals appropriate to give an activation response (eg. bystander activation), but the signals are not being passed through the BCR (IL-4+α-CD40), then SaBR is upregulated. The cells remain marked and unless rescued by other stimuli, will die or become anergic.

More experimentation is required to test the hypothesis that SaBR marks B lymphocytes for future elimination or anergy in the absence of appropriate signaling. The WEHI 231 B cell line could be used as a model system for future experimentation. This cell line has been used as a model of negative signaling through the BCR (Monroe and Seyfert, 1990). Stimulation of WEHI 231 cells with α-μ results in apoptosis; however the cells can be rescued by treatment with IL-4 or α-CD40 (Monroe and Seyfert, 1990). WEHI 231 cells are also SaBR negative; therefore transfection experiments can be performed to compare the apoptotic responses of SaBR positive and SaBR negative
WEHI cells following treatment with α-μ and rescue with IL-4 or α-CD40. If the above model is true then an increased percentage of apoptotic cells should be visible in the SaBR+ cells following activation and rescue. Similar experiments could be performed with the SaBR expressing T28 cell line to see what effects SaBR overexpression has on activation and apoptosis in T cells. It must be acknowledged that the components and processes involved in SaBR function may be missing or non-functional in vitro; therefore, it will be important to also perform in vivo experimentation, such as SaBR knockout mice (see Chapter 6 for more information).

In T cells, significant increases in SaBR expression were observed upon activation with PMA+A23187, both of which activate signaling cascades downstream of the TCR. The upregulation of SaBR expression during activation with PMA+A23187 was also seen with the T cell lines, T28 and R1.1, suggesting that the increase in SaBR expression seen with splenic T cells is real. These results can be correlated with the effect that two costimulatory signals have on SaBR expression during B cell activation. In both cases, activation stimuli that bypass the TCR/BCR result in upregulation of SaBR expression; suggesting that inappropriate activation in B or T cells leads to high SaBR expression, which in turn may lead to apoptosis or anergy, either directly or indirectly. However, splenic T cells and the T cell lines express very low basal levels of SaBR (T28) or none at all (R1.1), making it extremely difficult to detect potential decreases in SaBR expression upon stimulation through the TCR. More sensitive assays, such as quantitative RT-PCR, would need to be employed to determine if similar decreases in SaBR expression take place in T cells.
Interestingly, the combination of PMA+ionomycin does not result in any significant changes in SaBR expression. Both A23187 and ionomycin are ionophores that bypass the TCR and result in increases in intracellular calcium (Tsien et al., 1982); however, ionomycin has a higher specificity for calcium than A23187 and it is very pH dependent. If the pH in the media was not optimal for ionomycin, then it would not have worked as well as A23187. Alternatively, the increase in SaBR expression could have been the result of drug effects unrelated to the increase in intracellular calcium concentration. More experimentation on SaBR expression during T cell activation is required before a more relevant model describing SaBR function in both B and T lymphocytes can be proposed.
CHAPTER 3
SEQUENCING AND CHARACTERIZATION OF MURINE SaBR

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3.1 INTRODUCTION

In Chapter 1, I described the cloning of the 11F6 antigen, or SaBR, from a murine IL-3 responsive, multipotential progenitor cell line B6SUtA (Greenberger et al., 1983). Chapter 2 examined the expression pattern of SaBR in detail and the results indicated a potential role for SaBR in lymphocyte development and activation. Protein structure can also provide information regarding molecular function, which in turn can provide indications of biological function. In this chapter, I will describe the subcloning and sequencing of murine SaBR and perform a comparative analysis of its peptide sequence with other proteins in the databases. Having a cDNA clone allows for many experiments to be performed, including: 1) mRNA analysis 2) biochemical analysis of the SaBR protein 3) cell-cell adhesion studies 4) testing of the hypothesis that 11F6 reactivity in cells is due to SaBR expression and 5) cloning of the human SaBR cDNA, which would allow for the identification of preexisting antibodies from workshop panels. The results of these studies are discussed within this chapter.

3.2 MATERIALS AND METHODS

3.2.1 Southern analysis

Murine genomic DNA was cut with the restriction enzymes, PstI, PvuII, BamHI and EcoRI, run on a 1% agarose gel, capillary transferred to Hybond (Amersham, Oakville, Ont) overnight and cross-linked by a 2.5 minute exposure to ultraviolet irradiation in a Stratalinker (Stratagene, La Jolla, CA). The filter was prehybridized for 4 hours at 68°C in 250 mM NaPO₄ (pH 7.2), 1 mM Na-EDTA, 20% SDS, 5 mM sodium pyrophosphate, and 0.5% Boehringer-Mannheim blocking reagent, then hybridized for 16
hr at 68°C in the same solution containing denatured $^{32}$P labeled probe. Full-length SaBR cDNA was used as a probe. Filters were washed at 55°C twice in 25 mM NaPO$_4$, 150 mM NaCl, 5 mM sodium pyrophosphate and 1% SDS for 20 minutes each, then in 25 mM NaPO$_4$, 50 mM NaCl, 5 mM sodium pyrophosphate and 2% SDS for 20 min before placing on film.

3.2.2 DNA sequence determination of murine SaBR

Restriction fragments of SaBR cDNA were subcloned into Bluescript vectors and sequenced by the deoxy chain termination method, using denatured double-stranded templates and Circumvent DNA polymerase (New England Biolabs, Mississauga, Ont.) according to the Circumvent protocol for PCR thermocycle sequencing. The Sequenase protocol was also used for some templates (United States Biochemicals, Cleveland, Ohio). Both strands of the SaBR cDNA were fully sequenced, with overlapping sequence determined at all fragment termini.

Computer analysis was performed by using the GCG program (University of Wisconsin Genetics Computer Group, Madison, WI). Comparisons of the predicted SaBR sequence to other proteins in the GenBank and EMBL databases utilized the BLAST and FastA programs.

3.2.3 Sequencing of the EST clone 50776

The human cDNA clone 50776 was obtained from the Merck/Image Consortium (University of Washington, PA), subcloned into Bluescript and sequenced with an automated sequencer by the Nucleic Acid-Protein Service Unit (University of British Columbia, Vancouver, B.C). Both strands of the 50776 cDNA were fully sequenced, with overlapping sequence determined at all fragment termini.
3.2.4 PCR cloning

An HEL cDNA library, cloned into the pAX142 vector (Craig et al., 1993) and a Clontech human lung library in lambda gt10 (library # HL3004a), were used as templates in an attempt to amplify the 5' end of human SaBR using PCR. The following set of nested oligonucleotides, complementary to the 50776 clone and either side of the pAX142 and lambda gt10 vector cloning sites, were designed for PCR (Figure 3.1):

50776-3: 5' CGTGCTTGGGTTGGTACAGGAAGT 3'
50776-2: 5' GGAACCAGGAATCGTAGGTGAGCA 3'
pAX142-5': 5' TCATTCTCAAGCCTCAGACAGTGG 3'
pAX142-3': 5' AATCTGCCGTCATCGACTTCGAAC 3'
gt10-5': 5' AGCAAGTTTCAGCCTGGTTAAGT 3'
gt10-3': 5' TTATGAGTATTTTCTTCCAGGG 3'

PCR reactions, using the 50776-3 primer and the vector primers, were carried out using Elongase (Gibco BRL, Canadian Life Technologies, Burlington, Ont.) according to the manufacturer's instructions. After a predenaturation step at 94°C for 30 seconds, the next 30 cycles employed the following parameters: denaturation- 30 seconds at 94°C, annealing- 53°C for 30 seconds and elongation- 72°C for 2 minutes. The products of the PCR reaction were then used as targets in a second nested PCR which utilized the 50776-2 primer and the appropriate vector primer. Reaction conditions were the same, except that the annealing temperature was raised to 56°C. The products of the second PCR
Figure 3.1. **Location of primers used for HEL and lung library amplification of human SaBR.**

Amplification of clones containing human SaBR was carried out using primers 50776-3 and 50776-2, which are complementary to the 5' end of the human 50776 cDNA clone, and vector specific primers. A) Location of 50776 primers and pAX142 specific primers for amplification of clones within the HEL library. B) Location of cDNA primers and lambda gt10 vector primers for amplification of clones within the Clonetech human lung cDNA library.
reaction were cut with AccI and ApaLI for the pAX142 library and with EcoRI and ApaLI for the lambda gt10 library, the ends filled in with Klenow (Gibco, BRL, Canadian Life Technologies, Burlington, Ont.), and the DNA run on a 1.2% agarose gel. After staining with ethidium bromide, a Southern blot was performed (see 3.2.1) using a probe from the 5’ end of the 50776 clone in order to identify potential positive products. Positive bands were excised from the gel, eluted and the DNA precipitated. The DNA was then subcloned into the HincII site of Bluescript and sequenced (see 3.2.3).

3.2.5 Cell lines for flow cytometry

SaBR expression was determined in the following cell lines: B6SUTa cells were obtained from Dr. Gerry Krystal (Terry Fox Laboratory, Vancouver, B.C) and ABE-8, NFS-70, R1.1, and Yac-1 cells were purchased from American Type Culture Collection (Rockville, MD). T28 cells (Pyszniak et al., 1994) and MBL-2 cells (Takei et al., 1984) were generously supplied by Dr. Fumio Takei (Terry Fox Laboratory, Vancouver, B.C) and SVEC cells were supplied by Dr. Graeme Dougherty (Terry Fox Laboratory, Vancouver, B.C).

3.2.6 Flow cytometry

11F6 (α-SaBR) was developed at the Terry Fox Laboratory and was labeled with biotin-succinimidyl-ester (Vector Laboratories, Inc., Burlingham, CA) by standard procedures (van den Eertwegh and Claassen, 1991). Biotinylated rat IgG2b antibody was obtained from PharMingen (San Diego, CA) and used as an isotype control. α-CD32/CD16 (FcII,III receptor block) was also obtained from PharMingen (San Diego, CA). PE conjugated streptavidin (Sa-PE) was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL) and Jackson Immunoresearch (Bar Harbour, ME).
$1 \times 10^6$ cells were preblocked on ice for 30 minutes with 1 µg/ml of \(\alpha\)-CD32/CD16 in 100 µl Hanks balanced salt solution (HBSS; StemCell Technologies, Vancouver, B.C) containing 2% fetal bovine serum (HBSS/2%FBS). 10 µg/ml biotinylated 11F6 Ab was added after the preblock and the cells incubated on ice for one hour. The cells were washed in HBSS/2%FBS and the secondary antibody Sa-PE added for 30 min. on ice. Cells were then washed twice in HBSS/2% FBS and resuspended in the same buffer containing 1 µg/ml propidium iodide (PI). In this manner, dead cells were gated out by excluding those which stained positively with PI. In addition, isotype-matched rat antibodies were used as negative controls for staining. Flow cytometric analysis was performed using either a FACSort, or FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with PC LYSIS II® software (Hewlett Packard Co., Palo Alto, CA).

3.2.7 RT-PCR

Total RNA was isolated from the following cell lines (see also 3.2.5) using the guanidium isothiocyanate/phenol method (Chomcynski and Sacchi, 1987): B6SutA (multipotential progenitor), SVEC (endothelial), Yac-1 (T cell), NSF-70 (B cell), T28 (T cell), R1.1 (T cell), MBL-2 (T cell) and ABE-8 (B cell). First strand cDNA synthesis was performed using Superscript according to the manufacturer's instructions (Gibco, BRL, Canadian Life Technologies, Burlington, Ont.) and 5 µg of the first strand cDNA mix was used in a PCR reaction with 10 units of Elongase. The following SaBR specific PCR primers were used: forward primer 5' GATGCTGCTCTCAACAATACCAGGC 3' and reverse primer 5' CTCAAGATCTGTAGCTCCGCCCTGCTGA 3' giving a 2300 bp product. Denaturation was carried out at 95°C for 30 sec, annealing at 50°C for 30 sec
and elongation at 68°C for 4 minutes. 30 cycles were performed and Southern blot analysis was performed on the products of the PCR reaction as outlined in 3.2.1.

3.2.8 Vector construction

The CTV4 (Whitehead et al., 1995) and the CTV82 retroviral vectors (Rob Kay, personal communication) were used to create cell lines with long-term expression of SaBR. The full-length SaBR cDNA was inserted as a SalI fragment into the SalI site of CTV4 and CTV82.

3.2.9 Retroviral infection of 3T3 and T28 cells

BOSC23 cells were used as a packaging cell line for the infection of NIH 3T3 cells (American Type Culture Collection, Rockville, MD) and T28 cells by the CTV4-SaBR and the CTV82-SaBR retroviral constructs according to the protocol of Pear et al., (1993). Briefly, 25 μM chloroquine (Sigma, St. Louis, MO) was added to the BOSC23 media immediately prior to transfection with 6-10 μg CTV4-SaBR or CTV82-SaBR DNA that had been treated with calcium chloride and Hepes buffered saline solution (HBS; StemCell Technologies, Vancouver, B.C). The media was changed after 10 hours to remove excess chloroquine. 48 hours post-transfection, the supernatant was removed from the BOSC23 cells, filtered and added to NIH 3T3 cells or T28 cells along with 8 μg/ml polybrene (Sigma, St. Louis, MO) for 3-5 hours. Fresh media was added at this time and the cells harvested after 48 hours. Flow cytometric analysis was performed after 48 hours to confirm SaBR expression.

3.2.10 Cell lines for ligand binding assay

The following cell lines were used as test cells for the cell-cell binding assays described in 3.2.11 and 3.2.12: B6SUtA, ABE-8, NSF-70, R1.1, Yac-1, T28 and MBL-2
cells have already been described (see 3.2.5). A20, Bal17 and WEHI-231 cells were obtained from American Type Culture Collection (Rockville, MD). Til cells were generously supplied by Dr. Graeme Dougherty (Terry Fox Laboratory, Vancouver, B.C). 32D cells (Greenberger et al., 1983) and BaF3 (Palacios, 1985) cells were also used.

3.2.11 Visual cell-cell binding assay

CTV4-SaBR and CTV4 infected 3T3 cells were plated in 24 well dishes (Falcon, Becton-Dickinson, Mountain View, CA) until approximately 80% confluent. The cells were washed with fresh media to remove any dead cells or debris, then test cells (see 3.2.10) added at a concentration of 1x10^5 cells/well. Plates were incubated at 37°C or 4°C for 1/2 hour or 1 hour, then gently washed with either phosphate buffered saline (PBS; StemCell Technologies, Vancouver, B.C) or HBSS/2%FBS 4 times to remove non-adherent cells. Individual wells were then examined under the microscope and the number of bound cells was estimated for each well.

3.2.12 Fluorescence cell-cell binding assay

To confirm the results from the above assay and improve the sensitivity of the assay, another experiment was performed in which the test cells were labeled with a fluorescent dye and the resulting fluorescence determined after the treated test cells were added to the CTV4-SaBR or CTV4 infected 3T3 cells.

To fluorescently label the cells, a stock solution of 5 mg/ml fluorescein diacetate (Sigma, St. Louis, MO) was diluted 1:50 in PBS at room temperature, then one volume of the fluorescein diacetate suspension was added to 9 volumes of the test cell suspension. The cells were allowed to stand at room temperature for 20 minutes, after which they were washed in HBSS/2%FBS and counted with a hemocytometer. 1x10^5 cells were
added per well and allowed to incubate at 37°C or 4°C for ½ hour to 1 hour. The plates were washed gently 4 times in PBS or HBSS/2%FBS to remove non-adherent cells and the remaining fluorescence was determined with a CytoFluor™ 2300 fluorescent plate reader (Millipore, Fisher Scientific, Nepean, Ont).

3.2.13 Preparation of total cell lysates

CTV4 and CTV4-SaBR infected 3T3 cells were washed in PBS, then lifted from the plates with PBS plus 1mM EDTA (Gibco, BRL, Canadian Life Technologies, Burlington, Ont). After centrifugation, 10 volumes of PBS plus 1% NP40 (Sigma, St. Louis, MO) was added to the cells and incubated on ice for 10 minutes. The lysate was then centrifuged to remove any debris and the supernatant added to loading buffer for SDS polyacrylamide gel electrophoresis (SDS-PAGE). Two duplicate 7.5% SDS-PAGE gels were used in all cases; one gel was stained with Coomasie Blue to detect all proteins and the other was transferred to Immobilon (Millipore, Fisher Scientific, Nepean, Ont.) for Western blot analysis (see section 3.2.15).

3.2.14 Immunoprecipitation

1x10⁷ retrovirally infected 3T3 and T28 cells were washed three times with HBSS then resuspended in 0.5 ml biotin labeling buffer (138 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 0.4 mM MgSO₄, 0.5 mM MgCl₂, NaHCO₃ and 4.5 mM D-glucose (pH 7.4)). 40 μl of a 10 mg/ml NHS-LC-biotin solution (Sigma, St. Louis, MO) was then added and the cells incubated on ice for 10 min. After washing three times with DMEM, the cells were lysed with 1ml lysis buffer (1% Triton X-100, 1% BSA, 0.8% NaCl and 0.1% NaN₃ in 10 mM Tris (pH 7.5)) and centrifuged at maximum speed for 20 min. at 4°C. The supernatant was mixed with 5 μg of purified antibody (11F6, rIgG2b or α-LFA-1)
and incubated on ice for one hour. TIB-169 coupled beads (Dr. Fumio Takei, Terry Fox Laboratory, Vancouver, B.C.) were washed with lysis buffer three times, then a 50% suspension of beads was incubated with the cell lysate/Ab mixture overnight at 4°C with continual mixing. After washing the beads with lysis buffer without BSA and NaN₃ 4 times, SDS-PAGE sample buffer without reducing agent was added and the samples were boiled for 5 minutes. Half of each sample was then loaded onto a 7.5% SDS-PAGE gel and 50 mM DTT (Sigma, St. Louis, MO) added to the remainder. The remainder of each sample was boiled for 2 minutes and loaded onto a second 7.5% SDS-PAGE gel. After transferring to Immobilon, the blots were subjected to Western blot analysis (see section 3.2.15).

3.2.15 Western blot analysis

Blots were washed 4 times 10 min. each in PBS plus 0.1% Tween (BDH, Vancouver, B.C.), then blocked overnight at 4°C in 5% milk powder in PBS. After rinsing in PBS+Tween (PBS-T), 10 µg/ml b11F6 or control antibodies in PBS-T were incubated with the blots (not including the blots from the immunoprecipitation) with continual mixing for 2 hours at room temperature. The blots were washed 4 times 10 minutes each in PBS-T, then the streptavidin-peroxidase (Jackson Immunoresearch, Bar Harbour, ME) secondary antibody was added to all blots at a dilution of 1:1500 for one hour at room temperature. After another set of 4 washes of 10 minutes each in PBS-T, a 50:50 mix of Chemiluminescent substrate A and Chemiluminescent substrate B (LumiGLO; KPL laboratories, Gaithersburg, MD) were added to the blots for one minute, the excess substrate removed and the blots exposed to X-ray film and developed.
3.2.16 Northern Blot analysis

Total cellular RNA was isolated from thymus, stomach and bone marrow using the guanidium isothiocyanate/phenol method (Chomcynski and Sacchi, 1987), and poly(A)+ mRNA was selected by mixing with oligo dT cellulose beads before passing through a Millipore spin column (Millipore, Fisher Scientific, Nepean, Ont). mRNA was then electrophoresed through a 1% agarose gel containing 5% (v/v) formaldehyde, capillary transferred to Hybond overnight, and cross-linked by a 2.5 min exposure to ultraviolet irradiation. A murine Clontech filter (Bio/Can Scientific, Mississauga, Ont) containing mRNA from spleen, liver, kidney, brain, testis and heart, a human Clontech filter (Bio/Can Scientific, Mississauga, Ont) containing mRNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas as well as the above filter were prehybridized for 4 hours at 68°C in 250 mM NaPO4 (pH 7.2), 1 mM Na-EDTA, 20% SDS, 5 mM sodium pyrophosphate, and 0.5% Boehringer-Mannheim blocking reagent and then hybridized for 16 hr at 68°C in the same solution containing denatured 32P labeled probe. Full-length SaBR cDNA was used as a probe for the murine Northern and the full-length human 50776 cDNA was used as a probe for the human Northern. Filters were washed at 55°C twice in 25 mM NaPO4, 150 mM NaCl, 5 mM sodium pyrophosphate and 1% SDS for 20 minutes each, then in 25 mM NaPO4, 50 mM NaCl, 5 mM sodium pyrophosphate and 2% SDS for 20 min before placing on film.
3.3 RESULTS

3.3.1 SaBR is a unique transmembrane protein

Analysis of a genomic Southern blot of mouse DNA suggests that SaBR is a single gene (Figure 3.2). Sequencing analysis of the 2500 bp cDNA reveals a unique type 1 transmembrane protein of 820 a.a with a 22 a.a signal peptide (Figure 3.3). When the SaBR cDNA sequence was divided into sections and compared with other proteins in the database, a number of proteins exhibiting partial similarities to SaBR were revealed. The existence of regions of partial similarity divide the extracellular region of SaBR into 3 putative domains (Figure 3.3; Figure 3.4). The cysteine-rich amino-terminus is a somatomedin B-like domain. This domain has also been found in the extracellular matrix component vitronectin, the placental protein, PP1 and in the B cell protein, plasma cell glycoprotein 1 (PC-1) (Figure 3.4). It is also from this domain that SaBR gets its name (Somatomedin-B Related protein). The next domain has two blocks of sequence similarity to von Willibrand factor, as well as a putative sushi domain (Figure 3.4). The third domain is proximal to the transmembrane domain and has sequence similarity to the equivalent region of the selectins and the complement receptor family (Figure 3.4). The transmembrane domain spans the membrane once. The cytoplasmic domain is only 15 a.a and includes three serines and one threonine. Of these, only the first serine is in a context that would predict it to be the substrate for a known kinase, ie. protein kinase A. The sequence of SaBR confirms that it is a novel cell surface protein. The sequence does not give any useable indications of function, but does support the possibility that SaBR may function as a cell adhesion molecule.
Figure 3.2. Genomic Southern of SaBR. Murine DNA was cut with the restriction enzymes, PstI, PvuII, BamHI and EcoRI, run on a 1% agarose gel, transferred to Hybond and hybridized with a full length SaBR cDNA probe. A DNA marker ladder was used to determine relative sizes of hybridizing bands.
Figure 3.3. **Nucleotide and amino acid sequence of the SaBR cDNA clone.** Signal peptide is indicated by a box; the somatomedin B domain is single underlined and the conserved cysteines starred; von Willibrand factor similarity is indicated by a double overline; the selectin similarity has a single overline and the transmembrane domain is shown with a double underline.
Figure 3.4. SaBR amino acid alignment with similar proteins. A) somatomedin B domain alignment; PC-1=plasma cell membrane glycoprotein 1. Conserved cysteines are starred. B) von Willibrand factor alignment; vWF= von Willibrand factor. Also shown is the alignment with the portion of the C. elegans protein that has similarity to vWF. The cysteines involved in the putative sushi domain are starred. C) selectin and complement C3D receptor alignment. Blocked in sections indicate identical amino acids.
When the full length cDNA is compared with other proteins in the database, homology with a *C. elegans* protein of unknown function and a number of expressed sequence tags (ESTs) is found. The *C. elegans* cDNA clone exhibits 44% a.a similarity and 29% a.a identity to SaBR throughout its length, the Stratagene mouse skin cDNA EST clone 606458 is 100% identical to SaBR, the *S. scrofa* (pig) mRNA EST clone c14a08 shows 83% nucleotide identity to SaBR and the human endometrial tumor clone EST42818 shows 75% nucleotide identity (Figure 3.5). After subcloning and sequencing, a second human EST cDNA clone 50776 was shown to have 73% amino acid and nucleotide identity to SaBR over 1024 base pairs (Figure 3.5). The 50776 cDNA clone was obtained from a polyA primed human brain library. It was not a full length clone; therefore the RACE procedure (rapid amplification of cDNA ends) (Frohman, 1990) was used in an attempt to amplify the missing 5' end using an HEL and a human lung library as templates for PCR. These attempts were unsuccessful at obtaining a human cDNA clone encompassing more 5’ sequence than 50776; therefore, I was unable to proceed with experiments to identify anti-human SaBR antibodies in workshop panels.

### 3.3.2 SaBR is expressed in hematopoietic and non-hematopoietic cell lines

When hematopoietic and non-hematopoietic cell lines were examined by flow cytometry for expression of SaBR, low levels were observed in the multipotential progenitor cell line B6SUTA and the endothelial cell line, SVEC (Figure 3.6). Barely detectable levels were seen in the T cell lines Yac-1 and T28, and the B cell line NSF-70 (Figure 3.6). SaBR expression was not detectable in the cell lines R1.1, MBL-2 or ABE-8 (Figure 3.6). Because SaBR expression levels as detectable by flow cytometry were
Figure 3.5. **Comparison of the nucleotide sequence of pig, human and mouse SaBR.**
A) Nucleotide comparisons of the *S. scrofa* EST clone c14a08 and murine SaBR. B) Nucleotide comparisons of the human endometrial tumor cDNA clone EST42818 and mouse SaBR. C) Nucleotide comparisons of the sequenced human cDNA clone 50776 and murine SaBR. D) Amino acid comparisons of the sequenced human cDNA clone 50776 and murine SaBR. Blocked in sections indicate identical nucleotides or amino acids.
A

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B

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Figure 3.6. **Expression of SaBR in various cell lines.** A) Flow cytometric analysis was used to examine SaBR expression in the following cell lines: B6SUtA, SVEC, Yac-1, NSF-70, T28, R1.1, MBL-2 and ABE-8. Cells were stained with a biotinylated isotype control antibody plus Sa-PE (solid histograms) or the 11F6 antibody plus Sa-PE (open histograms). B) Southern blot analysis of an RT-PCR reaction involving the above cell lines. 5 µg of cDNA was used for each PCR reaction with the expected PCR product of 2300 bp.
low, RT-PCR was performed and the results verified the presence of SaBR message in the positive cell lines (Figure 3.6). These results imply that there is a correlation between the levels of 11F6 binding and the presence of SaBR in cell lines.

3.3.3 Transfection of SaBR cDNA confers 11F6 antibody binding

To determine if the isolated SaBR cDNA clone was capable of binding to the 11F6 antibody, SaBR was stably transfected into 3T3 and T28 cells using a retroviral construct. In both cell lines, flow cytometric analysis revealed 11F6 binding to the transfected cells; therefore, expression of SaBR confers 11F6 reactivity (Figure 3.7).

3.3.4 3T3 cells transfected with SaBR fail to bind hematopoietic cell lines

Since the protein structure of SaBR suggests that it may be involved in mediating cell adhesion, 3T3 cells were stably transfected with SaBR and used as target cells in a ligand binding assay with various hematopoietic cell lines. Upon visual inspection, CTV4-SaBR infected 3T3 cells failed to bind above background levels to the following hematopoietic cell lines: B6SutA, BaF3, A20, ABE8, Bal17, NFS70, WEHI-231, T28, Til, 32D, Yac-1, R1.1 and MBL-2 (Table 3.1). Similar results were obtained when test cells were treated with fluorescein diacetate, added to the CTV4-SaBR infected target cells and analyzed with a fluorescent counter. These results indicate that SaBR expression does not confer on 3T3 cells the ability to bind hematopoietic cell lines under the conditions tested.

3.3.5 SaBR cannot be immunoprecipitated or Western blotted using the 11F6 antibody

In an attempt to study the SaBR protein and determine its apparent molecular mass (expected molecular mass based on predicted amino acid composition is 120 kd),
Figure 3.7. **Expression of SaBR cDNA in T28 and 3T3 cells.** SaBR cDNA was inserted into the retroviral vector CTV82 and stably transfected into T28 and 3T3 cells. SaBR cDNA was then detected by flow cytometry using the 11F6 antibody. A) Histograms showing the expression of SaBR in T28 cells transfected with the CTV82 vector alone or CTV82-SaBR. B) Histograms showing the expression of SaBR in 3T3 cells transfected with the CTV82 vector alone (open histogram) or CTV82-SaBR (solid histogram). The percentage of SaBR positive cells is indicated within each figure.
A

T28CTV82

6%

T28CTV82-SaBR

72%

B

3T3CTV82-SaBR

21%
Table 3.1. Results of SaBR binding assays and FACS analysis of SaBR expression in cell lines.

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<td>--</td>
</tr>
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<td>Bal 17</td>
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<td>--</td>
</tr>
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<td>NFS-70</td>
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</tr>
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<td>WEHI 231</td>
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<td>Yac1</td>
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</tr>
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<td>R1.1</td>
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</tr>
<tr>
<td>MBL-2</td>
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</table>

Various non-adherent hematopoietic cell lines were added to a monolayer of 3T3 cells transfected with SaBR and incubated at 37°C for 1 hour. Non-binding cells were washed off and the SaBR expressing 3T3 cells were examined under the light microscope for visible binding above control levels. Positive binding is indicated in the table by a + sign and negative binding by a -- sign. The cell lines were also examined by flow cytometry for their expression of SaBR (see also Figure 3.6). Positively expressing cells are indicated by a + sign.
the 11F6 antibody was used for immunoprecipitation and Western blotting assays of whole cell lysates. Western blots using 11F6 as a primary antibody were unsuccessful in detecting SaBR within lysates of 3T3 cells expressing high levels of SaBR, therefore surface biotinylation followed by immunoprecipitation with the 11F6 antibody was employed. The immunoprecipitation of SaBR from 3T3 and T28 cells overexpressing the antigen was also unsuccessful; however, the control antibody against LFA-1 was capable of immunoprecipitating both chains of LFA-1 from T28 cells indicating that the immunoprecipitation procedure itself was working (Figure 3.8). Because these experiments were unsuccessful, it was not possible to determine the molecular weight of SaBR or its interactions with other proteins. SaBR does contain numerous potential N and O-linked glycosylation sites; therefore, it is possible that the protein is very large and was not entering the gel. Alternatively, the cell lysis conditions may have denatured the epitope. An alternative approach would be to bind the 11F6 antibody to the cells prior to lysis.

3.3.6 Expression analysis of mouse and human SaBR mRNA

In order to determine the pattern of SaBR mRNA expression in various mouse and human tissues, Northern blots were performed using Clontech mRNA filters and polyA purified mRNA. In the mouse, the blots revealed the presence of high levels of a 3.5 kb SaBR mRNA species in the kidney with lower levels in the heart, brain, lung, liver, skeletal muscle and stomach (Figure 3.9).
Figure 3.8. **Immunoprecipitation analysis of T28 cells transfected with SaBR.** T28 cells stably transfected with CTV82 (lanes 1-3) or CTV82-SaBR (lanes 4-6) were biotinylated, lysed and immunoprecipitated with either LFA-1 (lanes 1 and 4), the isotype control antibody rIgG2b (lanes 2 and 5) or 11F6 (lanes 3 and 6). Immunoprecipitates were run on a 7.5% PAGE gel, western blotted with the Sa-Per antibody, treated with chemiluminescent substrates and developed.
Figure 3.9. **Northern blot analysis of murine SaBR mRNA.** Northern analysis of a Clontech murine multiple tissue mRNA filter and three independently isolated mRNA tissues. 2 µg mRNA is present per lane. Full length SaBR cDNA was used as a probe and the filters were exposed to film for one month.
In contrast, human SaBR mRNA was highly expressed in all tissues examined, with the highest levels being in the lung and kidney (Figure 3.10). Furthermore, there were two mRNA species visible in each tissue, one at 3.5 kb and the other at approximately 4.0 kb. The larger mRNA product appears to be most prevalent in the lung, liver and kidney, while the smaller product is more prevalent in the heart, brain, placenta, skeletal muscle and pancreas (Figure 3.10).

3.4 DISCUSSION

Structural analysis

Sequencing of murine SaBR revealed a unique 820 a.a type 1 transmembrane protein with no obvious sequence homologies or function. Based on partial sequence similarities, SaBR can be potentially divided up into 5 domains.

The amino terminus corresponds to a somatomedin B domain. These domains contain 8 conserved cysteines within a short segment of 45 residues and have been described in a variety of proteins from pea seed albumin 2 to human placental protein 11 (Jenne, 1991). The best studied of these proteins is the extracellular matrix component vitronectin and plasma cell glycoprotein-1 (PC-1). PC-1 is a type II transmembrane protein expressed in liver, kidney and on plasma cells during late B cell differentiation (van Driel and Goding, 1987; Rebbe et al., 1991). It contains two tandem repeats of the somatomedin B domain located in the extracellular region adjacent to the transmembrane domain (Jenne, 1991). The function of the somatomedin B domains in PC-1 has not been described, however.
Figure 3.10. **Northern blot analysis of human SaBR mRNA.**
Northern analysis of a Clonetech human multiple tissue mRNA filter.
2 μg mRNA is present per lane. The full length 50776 EST clone was used as a probe and the filter was exposed to film for 3 days.
Vitronectin is a plasma protein belonging to a group of adhesive proteins which includes fibronectin, fibrinogen and von Willibrand factor (Preissner, 1989). It is involved in the modulation of the complement system and contains a somatomedin B domain at the amino terminus. The function of the somatomedin B domain in vitronectin has been studied by Sieffert et al. (1994), who have shown it to be required for the binding and function of soluble plasminogen activator inhibitor 1 (PAI-1). More recently, Deng et al. (1996) defined the PAI-1 binding motif within the somatomedin B domain and suggested that this domain in other proteins may mediate binding to cell surface molecules or the extracellular matrix. It is therefore reasonable to speculate that the somatomedin B domain of SaBR may mediate the binding of a counter-receptor or soluble ligand.

The second domain has some sequence similarity to von Willibrand factor. Von Willibrand factor is synthesized by endothelial cells and has two main roles: it is required for the adhesion of platelets, via the integrin GPIIb-IIIa, to sites of vascular damage and it binds to and stabilizes factor VIII in the circulation (Sadler, 1991). SaBR has sequence similarity to a region of von Willibrand factor with no known function. This region, however, contains a putative sushi domain. Sushi domains were first identified in the plasma membrane β2 glycoprotein I and are composed of four cysteines comprising two staggered disulfide bridges (Ichinose et al., 1990). The domains are usually found as tandem repeats and have been described in more than 25 proteins; mainly proteins of the mammalian complement system (Ichinose et al., 1990). Unfortunately, no function has yet been described for sushi domains. One could speculate, however, that the three dimensional structure formed by the tandem repeats of
the domain plays a functional role in the binding of complement proteins to their receptors. SaBR only has one sushi domain, therefore, it may or may not be significant to SaBR function.

The third domain lies next to the transmembrane portion of the molecule. It has sequence similarity to the complement short consensus repeats of the selectin family and to the complement receptor family. It is known that the selectins mediate adhesive interactions between leukocytes and the endothelium and between leukocytes and platelets (Laskey, 1992; Rosen and Bertozzi, 1994). The complement receptors are also involved in binding; although instead of cell-cell or cell-endothelium interactions, they are involved in binding soluble complement during an immune response (Liszewski, 1996). SaBR’s similarity to proteins involved in adhesion, coupled with the presence of a somatomedin B domain and a putative sushi domain, suggests that SaBR may be involved in some sort of binding interaction, either with another cell, extracellular matrix components or soluble factors.

SaBR also contains a 23 a.a. transmembrane domain that appears to span the membrane once and a short (15 a.a.) cytoplasmic tail containing three serines and one threonine. Of these, only the first serine may serve as a substrate for a known kinase. Based on the size of the tail and the lack of residues suitable for phosphorylation events, it is unlikely that the cytoplasmic tail is directly involved in signaling. More experimentation, however, is required to determine if SaBR is capable of signaling, whether it be directly through phosphorylation of the serine or indirectly through a protein kinase intermediary.
Structural analysis of the SaBR cDNA and its similarities to other proteins does not provide any obvious indications of its molecular function. Given the similarities to vitronectin and the selectins, the most plausible inference about SaBR function is that it plays a role in cellular adhesion, possibly through interactions with the somatamedin B domain. To test the possibility that SaBR binds to a cellular ligand, ligand binding studies were performed using SaBR transfected fibroblasts as target cells and various hematopoietic cell lines as potential ligand-bearing cells. Retroviral infection was used to create a population of 3T3 cells where over 90% were stably transfected with the SaBR construct. Unfortunately, no significant binding of any of the test cell lines was observed. There are a number of possible explanations for this finding.

First, the test cell lines may not express the counter-receptor for SaBR or SaBR may not have a membrane bound counter-receptor. The cell-cell binding assay is only set up to detect membrane-bound counter-receptors; therefore another methodology would have to be developed to search for soluble ligands.

Second, SaBR may only function as a dimer and when expressed in 3T3 cells, it is missing its partner. Immunoprecipitation experiments would be useful in addressing this question. It is also possible that the binding of another pair of adhesion molecules is required to bring SaBR into contact with its counter-receptor and the 3T3 cells may not be capable of supplying the necessary interaction.

Third, SaBR may require specific post-translational modifications, such as glycosylation, that the 3T3 cells are unable to perform. Moreover, since SaBR expression is regulated during lymphocyte activation, it is possible that SaBR must undergo a conformational change upon activation in order to function. 3T3 cells would
be unable to supply the necessary activation signals; therefore other SaBR negative lymphocyte cell lines would have to be transfected in order to analyze this possibility.

Fourth, the assay itself may not be sensitive enough or the conditions may be suboptimal to detect weak binding, especially if other non-specific binding interactions are also occurring. If cations, such as calcium or magnesium are required for binding to occur, the presence or absence of these cations in the wash buffer can affect the results quite dramatically; therefore, experiments must be performed under both conditions. Some binding interactions require a specific temperature or incubation protocol. For example, Dianzani et al. (1994) showed that CD38+ B and T cell lines required a temperature of 4°C and a rocking motion to mimic leucocyte rolling on endothelial cells. If the interaction is weak, binding may take hours, rather than minutes, therefore it is important to check both long and short incubation times. In the studies described in this chapter, variations in the temperature, incubation times and wash buffer composition were all unsuccessful in achieving significant binding of SaBR expressing 3T3 cells to hematopoietic test cell lines. Although the presence of a membrane-bound ligand cannot be ruled out, it is possible that SaBR binds to a soluble ligand or an ECM component instead.

_Human vs mouse SaBR_

Sequence comparisons of the human EST clone 50776 and murine SaBR suggest that 50776 probably represents a partial cDNA of a human homologue of murine SaBR. Sequencing of 50776 reveals a 1560 bp cDNA clone representing all but approximately 1600 bp of 5' sequence. This partial human SaBR (hSaBR) clone exhibits 73% nucleotide identity with its mouse homologue.
Attempts to amplify the 5' end of human SaBR were unsuccessful. A 1000 bp PCR product was obtained from the HEL library, but the sequence similarity with murine SaBR ended within the overlapping region. This suggests that the primers specific for the 50776 clone may have started amplifying the human homologue, then fallen off and reannealed to a related gene. Alternatively, the cDNA clone could have undergone a translocation event with another cDNA in the library to create a fusion product that was subsequently amplified in the PCR reaction.

PCR amplification of the human lung library revealed numerous PCR products under 1000 bp, but failed to yield a PCR product of the expected size. In the future, subcloning and sequencing of all of these small products will be necessary in order to determine if the entire human cDNA is present. Alternatively, different human libraries could be used in the hopes that they contain larger fragments of human SaBR.

A comparison of mouse and human SaBR mRNA expression within tissues reveals a number of differences between the two species. Human SaBR has two mRNA species that appear to be differentially expressed within and between tissues. Sequencing of the 3' end of human SaBR (50776 clone) reveals a long 3' untranslated region; therefore, the two mRNA species found for human SaBR could represent alternative polyA splice products. Given that the two mRNA products are differentially expressed within a tissue, it is also possible that they represent an alternative splicing event within the coding region of the gene, thus producing two products with slightly different functions. Murine SaBR, on the other hand, has only one mRNA species per tissue and the expression level of the mRNA within a tissue varies from that found for human
SaBR. These differences suggest that the importance or even the role of SaBR may vary between species as well as between tissues.

Once the full length human SaBR cDNA has been cloned, it will be possible to do more complete comparative studies. For example, human SaBR could be expressed in COS cells and screened against antibodies from the leukocyte differentiation antigen workshop. If an antibody is found that recognizes human SaBR, there may be functional information about the antibody that will aid in designing future experiments. Antibodies to human SaBR could also be used in flow cytometry studies and histochemistry experiments to more fully compare expression patterns with murine SaBR. Finally, mapping of human SaBR may link the gene with a disease locus, thus supplying even more information about the possible function of the gene in humans.

Additional database searches have revealed that SaBR shows sequence homology throughout its length with a C. elegans protein of unknown function. The C. elegans protein is 1409 amino acids and can be found on chromosome 3. It contains a sushi domain and displays sequence similarity to von Willibrand factor in the same region as SaBR. Furthermore, the 317 bp portion of the S. scrofa EST that has been sequenced also shows sequence similarity to the von Willibrand factor like domain of SaBR, suggesting that this region in SaBR has been conserved over evolution and may have functional significance.

**Protein analysis**

In chapter 2, I demonstrated that the 11F6 antibody recognizes an antigen on hematopoietic cells and lymphoid cell lines; however subsequent mRNA analysis of hematopoietic tissues, primary cells and cell lines was negative, suggesting two
possibilities: 1) that the antigen recognized by 11F6 may be different from the antigen coded for by the cDNA or 2) expression levels of SaBR in hematopoietic tissues and cell lines are too low to be detected by polyA mRNA Northern analysis. There are a number of lines of evidence favoring the second possibility. Firstly, transfection of the full-length SaBR cDNA into 3T3 and T28 cells confers 11F6 binding. Secondly, expression levels of the SaBR antigen (as seen by flow cytometry) on various hematopoietic and non-hematopoietic cell lines corresponds roughly to the levels of RNA as seen by RT-PCR. Finally, both SaBR mRNA and protein were detectable in the same tissues. These tissues include kidney, heart, brain, lung, liver, skeletal muscle and stomach (Protein results can be found in Chapter 4).

To examine the SaBR protein in greater detail, immunoprecipitation and Western blot analysis were performed; however, both of these techniques proved to be unsuccessful. If SaBR is heavily glycosylated then it may not enter the gel and thus will not be seen during immunoprecipitation studies or Western blots. Alternatively, the 11F6 antibody may not be capable of binding SaBR in a denatured or soluble form; therefore, the immunoprecipitation protocol could be altered such that the 11F6 antibody is added to the cells prior to lysis. A larger panel of antibodies, capable of immunoprecipitation and Western blot analysis is required if more experimentation in this area is to be performed in the future.
CHAPTER 4
HISTOCHEMICAL ANALYSIS OF SaBR EXPRESSION IN MURINE TISSUES
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4.1 INTRODUCTION

Analysis of SaBR mRNA expression in Chapter 3 indicated that SaBR is widely expressed in many different tissues. Strong mRNA signals could be detected in tissues, such as kidney and stomach, suggesting that SaBR may be playing a role in these organs. The results from the preceding chapters do not allow the formulation of a hypothesis concerning the role of SaBR in non-hematopoietic cells. However, knowing which cells within a solid organ express SaBR may provide sufficient information to start making such hypotheses. In order to determine which cells within the individual organs were expressing SaBR, histochemical analysis was employed in the hopes that the cellular location of the SaBR protein would hint at its functional role. This chapter describes the results of these studies and discusses possible functional roles for SaBR in non-hematopoietic tissues.

4.2 MATERIALS AND METHODS

4.2.1 Histochemistry

Spleen, kidney, stomach, skeletal muscle, heart, liver, thymus and lung tissue were isolated from 6-8 week old C57/BL6 mice, washed in phosphate buffered saline (PBS; StemCell Technologies, Vancouver, B.C.), cut into small sections and fixed in 3% paraformaldehyde (BDH, Vancouver, B.C) for 1/2 hr at room temperature. The tissues were embedded in paraffin, sectioned on a microtome and mounted on poly-L lysine coated microscope slides (performed by the histochemistry lab, BCCA, Vancouver, B.C). After deparaffinizing in toluene (BDH, Vancouver, B.C) for 2 minutes, slides were
rehydrated by dipping in graded ethanol: 95,75, and 50%. Slides were then washed twice for 5 min. each in distilled H2O, and once in PBS for 5 minutes.

Preliminary blocking was accomplished by incubating the slides with 10% normal mouse serum (Jackson Immunoresearch, Bar Harbour, ME) in PBS plus 0.1% BSA (PBS/BSA) for 30 minutes in a sweat box. This was followed by an avidin-biotin blocking step for endogenous biotin or biotin-binding proteins. Slides were incubated with 1 mg/ml unconjugated egg-white avidin (Sigma, St. Louis, MO) for 20 min, rinsed in PBS and incubated with 0.1 mg/ml unconjugated biotin (Sigma, St. Louis, MO) for 20 min. Excess blocking reagent was drained and slides were incubated overnight at 4°C with 10 µg/ml biotinylated 11F6 mAb or 10 µg/ml biotinylated rat IgG2b isotype control mAb (PharMingen, San Diego, CA) diluted in PBS/BSA. Slides were dipped 3 times each in PBS/BSA, washed 3 times for 10 minutes in PBS/BSA and treated with 0.3% hydrogen peroxide (BDH, Vancouver, B.C) in methanol for 30 minutes to eliminate endogenous peroxidase. Slides were then incubated with 1 µg/ml streptavidin conjugated peroxidase (Jackson Immunoresearch, Bar Harbour, ME) for 30 minutes, dipped in PBS 3 times, washed in PBS 3 times for 5 minutes and added to a fresh solution of 0.6% diaminobenzadine (DAB; Sigma, St. Louis, MO) in PBS containing 0.03% hydrogen peroxide for a maximum of 4 minutes. After dipping in dH2O, slides were counterstained for 30 seconds in hematoxylin (Sigma, St. Louis, MO), airdried and mounted using polymount.
4.3 RESULTS

4.3.1 Histochemical analysis of SaBR

Histochemical analysis of mouse tissues embedded in paraffin and sectioned revealed SaBR protein expression in the majority of tissues examined. In terms of hematopoietic tissues, SaBR expression in the spleen was indistinguishable from the isotype control (Figure 4.1). Staining of red blood cells was visible throughout the spleen in both the 11F6 and isotype treated samples suggesting that this staining was non-specific (Figure 4.1). The thymus was also negative for the presence of SaBR (Figure 4.1), which was expected based on previous mRNA and flow cytometry results (see chapter 3).

SaBR expression was apparent in all non-hematopoietic tissues tested. In the kidney, expression appeared to be limited to the epithelial cells lining the convoluted tubules and collecting ducts of the cortex (Figure 4.2). Very little staining was visible in the medulla (Figure 4.2). Similar results were obtained for the stomach, where SaBR expression was largely restricted to the mucus cells lining the stomach (Figure 4.3). Protein expression was fairly uniform in the skeletal muscle, liver, heart and lung (Figures 4.4-4.7).

4.4 DISCUSSION

Northern blot analysis indicated that SaBR mRNA was being expressed in non-hematopoietic tissues with the strongest signals being present in the kidney and weaker signals found in the heart, brain, lung, liver, stomach, and skeletal muscle. To further
Figure 4.1. **SaBR expression in murine thymus and spleen.** Histochemical analysis of murine thymus (A and B) and spleen (C and D) stained with control antibody (A and C) and 11F6 antibody (B and D). Magnification=16x for spleen and 40x for thymus.
Figure 4.2. **SaBR expression in murine kidney.** Histochemical analysis of murine kidney cortex (A and B) and medulla (C and D) stained with control antibody (A and C) and 11F6 antibody (B and D). Magnification=40x. G=glomerulus, CT=convoluted tubule
Figure 4.3. **SaBR expression in murine stomach.** Histochemical analysis of murine stomach at 16x magnification (A and B) and 40x magnification (C and D) stained with control antibody (A and C) and 11F6 antibody (B and D). M=mucus cell.
Figure 4.4. **SaBR expression in murine skeletal muscle.** Histochemical analysis of murine skeletal muscle longitudinal sections (A and B) and transverse sections (C and D) stained with control antibody (A and C) and 11F6 antibody (B and D). Magnification=16x
Figure 4.5. **SaBR expression in murine liver.** Histochemical analysis of murine liver stained with A) control antibody and B) 11F6 antibody. Magnification=40x
Figure 4.6. **SaBR expression in murine heart.** Histochemical analysis of murine heart stained with A) control antibody and B) 11F6 antibody. Magnification=40x
Figure 4.7. **SaBR expression in murine lung.** Histochemical analysis of murine lung at 16x (A and B) and 40x magnification (C and D) stained with control antibody (A and C) and 11F6 antibody (B and D). B=bronchioles, A=alveoli
define which cells in these tissues were expressing the SaBR antigen, histochemical analysis was performed using paraffin embedded tissue sections. All tissues tested, with the exception of thymus and spleen, were positive for expression of SaBR above background levels.

The thymus was negative for SaBR expression, as expected, thus correlating with the flow cytometric results that show that SaBR is only expressed in mature single positive T cells (see Chapter 2). Since very few single positive T cells can be found in the thymus, it is reasonable that the histochemical analysis would be negative as well.

In the spleen, there were positive cells on both the control and 11F6 stained slides. These cells correspond to red blood cells, which non-specifically take up the peroxidase stain. Even though there appears to be slightly more staining in the 11F6 stained slide, the majority of these cells are red blood cells and very few splenocytes appear to be staining with the 11F6 antibody. Flow cytometric analysis (Chapter 2) has clearly shown that SaBR is expressed within the spleen; therefore, these results suggest that the lack of staining is due to a sensitivity problem, rather than non-expression of SaBR.

By Northern analysis, the kidney shows the highest expression of SaBR message. When histochemistry is performed, it can be seen that the majority of the staining is evident in the cortex, with little staining in the medulla. The cortex contains the renal corpuscles and convoluted tubules, where blood filtration takes place, while the medulla is composed primarily of collecting ducts that collect the urine and pass it onto the bladder for excretion (Kelly et al., 1984). There is no SaBR protein expression in the glomerulus; the tuft of capillaries within the renal corpuscle that provides the ultrafiltrate for the tubules (Kelly et al., 1984); rather the majority of the staining is evident within the
epithelial cells that comprise the lining of the convoluted tubules. When the ultrafiltrate enters the convoluted tubules from the glomerulus, small molecules such as glucose and amino acids are reclaimed by selective reabsorption and waste compounds are secreted (Kelly et al., 1984; Wheater et al., 1989). In addition, ions are transported across the tubule walls in order to maintain ionic homeostasis (Kelly et al., 1984). In order to accomplish these tasks, however; the epithelial cells of the tubules must contain many membrane bound receptors for selective transport and numerous enzymes to aid in the breakdown of large molecules for reabsorption (Kelly et al., 1984). It is possible that SaBR may be playing a role in this selective transport by acting as a receptor for specific compounds in the ultrafiltrate.

Furthermore, recent studies strongly suggest that tubular epithelial cells may play a role in the immune response. C4, a component of the complement system (Witte et al., 1991), and VCAM-1, an adhesion molecule (Lin et al., 1993) have been found to be expressed by these epithelial cells. In a review, Kelley and Singer (1993) discuss the capacity of these cells to function as antigen-presenting cells in that they express a variety of adhesion molecules (VCAM-1 and ICAM-1) and cytokines (TNF-α) which may enhance their interaction with blood-borne T cells. They suggest that the interaction of these cells with T cells may cause T cell activation or T cell unresponsiveness (Kelley and Singer, 1993). Based on SaBR’s expression and sequence identities, it is possible that it may be playing a role in this type of interaction by binding to a specific counter-receptor on T lymphocytes.

SaBR may also play a specific role in the stomach, where it is found to be expressed by gastric mucosal cells. According to Wheater, P.R. (1989), the main
function of these cells is to secrete an alkaline mucus which will protect the mucosal lining against the acidic environment of the lumen; although recent reports have indicated that these cells are also capable of responding to numerous growth factors (Kinoshita et al., 1995) and may play a role in protecting gastric epithelial cells against oxygen radicals (Hiraishi et al., 1993). SaBR is unlikely to be a growth factor receptor; however, it may play a functional role in protecting the stomach lining.

The expression of SaBR in heart, skeletal muscle, liver and lung is fairly uniform, making it difficult to determine a potential role for the protein in these organs; therefore I will not speculate on SaBR function in these tissues at this time.

The wide spread distribution of SaBR in non-hematopoietic tissues is intriguing and one may speculate that it is playing a role in the immune response of these tissues. Most mature lymphocytes recirculate continuously throughout the body. Recirculation is regulated by cellular recognition events coupled with extravasation across the vascular wall thus targeting subpopulations of cells to specific organs (Butcher, 1996). Homing of lymphocytes also occurs within a tissue and like recruitment from the blood, must be regulated by a combination of adhesive, signaling and migratory events (Butcher, 1996). Each microenvironment within a tissue can therefore be characterized by a unique display of adhesive ligands and regulatory factors (Butcher, 1996). Based on the fact that the structural analysis and expression studies of SaBR suggest that it may be involved in adhesive interactions on lymphocytes, it is reasonable to speculate that SaBR may play a role in regulating lymphocyte homing into or within individual tissues.
CHAPTER 5
TRANSGENIC MICE

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5.1 INTRODUCTION

*In vitro* studies exploring the expression of SaBR during lymphocyte development revealed a differential pattern of expression in terms of developmental stage, location and activation status (Chapter 2). This information has allowed the formulation of a hypothesis concerning the role of SaBR in marking lymphocytes that receive antigen independent versus complete antigen dependent activation stimuli. One prediction of this model is that lymphocytes expressing SaBR should have a higher probability of being anergized or eliminated by apoptosis. Conversely, they may also have a higher propensity for proliferation in the absence of activating stimuli. Other results not covered by the model indicate that SaBR expression may affect lymphocyte development.

To determine the effects of SaBR expression on the development, proliferation and immunological competence of lymphocytes, I attempted to create transgenic mice which expressed high levels of SaBR throughout lymphocyte development, including mature cell populations. By comparing these mice to normal mice, I hoped to identify changes in lymphocyte populations or functions which could be attributed to SaBR overexpression. The nature of these changes could indicate which aspects of my hypothesis regarding SaBR function were valid or invalid.

Transgenic mouse technology is a powerful tool for the study of specific gene function in the context of the whole animal and has been used successfully in the past for the study of cell surface molecules involved in lymphocyte activation (Sharpe, 1995). The field has advanced rapidly in the past few years and today there are a number of different genetic manipulations that can be performed in order to answer questions about a gene product (Fassler *et al.*, 1995; Rink and Wenzel, 1996). Which experimental
system is chosen depends on a number of factors; the most important being what question is being asked. However, the target gene itself as well as the availability of appropriate technology, facilities, time and funding must also be considered.

In the classic transgenic mouse model, the cDNA of the gene of interest is incorporated into an expression vector and inserted randomly into the mouse genome. The vector generally contains tissue specific promoters and integration results in the constitutive expression of the cDNA product within the targeted tissues (Lo, 1996). These mice have a number of advantages; a genomic clone is not required, the techniques involved are not as technically challenging as those for other genetic manipulations and the gene product can be expressed in cells, tissues or during developmental stages when it is normally turned off. Mice constitutively expressing the gene of interest may give exaggerated responses in cells where it is normally expressed and may cause responses in cells where it is normally turned off, thus providing insight into normal gene function (Sharpe, 1995). One major disadvantage is that the endogenous gene is still functioning and its expression may mask the effects of the transgene.

Knockout mice, on the other hand, provide a definitive test for the contribution of a specific gene product to a biological process, provided the gene product is not redundant (Sharpe, 1995; Fassler et al., 1995). In these mice, gene function in murine embryonic stem (ES) cells is disrupted by insertion of an irrelevant sequence into an essential region of the targeted gene in the mouse genome, thus inactivating its expression (Lo, 1996; Capecchi, 1989). This is accomplished by using homologous sequences to help direct the specific integration of the disruptive sequence (Lo, 1996; Capecchi, 1989). Legitimate recombination events are then selected for and the positive
ES cells are transplanted into recipient females (Lo, 1996; Capecchi, 1989). This technique has been used to generate a great number of null mutant mouse strains, many of which exhibit distinct but expected phenotypes (Fassler et al., 1995; Pfeffer and Mak, 1994). Other null mutants exhibit unexpected phenotypes which reveal new functions for the disrupted gene (Fassler et al., 1995).

Newer techniques, such as “knock-in” technology and cell-type specific gene inactivation, allow for the introduction of precise modifications into an endogenous gene or the abolition of gene expression only in certain organs or cell types (Fassler et al., 1995). In addition to knockout mice, these newer techniques can allow one to address very specific questions about gene function. They also generate more useful information than standard transgenic mice; however, the creation of these specialized mice requires a genomic construct and is much more labour intensive and time consuming than the creation of a mouse that constitutively expresses the gene of interest.

Due to the lack of a genomic construct and lack of appropriate facilities, I chose to generate mice constitutively expressing SaBR within lymphocytes. These mice were generated by placing the SaBR cDNA under the transcriptional control of the TCR Vβ promoter and Ig μ enhancer. This transgenic vector was used because it has been employed successfully in the past to generate transgenic mice overexpressing HSA in both the B and T lymphoid compartments (Hough et al., 1994; Hough et al., 1996). This chapter describes the generation of SaBR transgenic mice that successfully integrated the transgene, but expressed an aberrant SaBR mRNA.
5.2 MATERIALS AND METHODS

5.2.1 Construction of the SaBR transgene

Construction of the LIT2 vector has been described previously (Hough et al., 1994). To construct the SaBR transgene, the full-length 2500 bp SaBR cDNA encompassing the complete open reading frame was subcloned into the BamHI site of the LIT2 vector under the transcriptional control of the TCR Vβ promoter and Ig μ enhancer. The human growth hormone (hGH) gene with a frame shift mutation in the coding region was inserted 3’ of the SaBR cDNA to provide introns that appear to enhance transgene expression (Figure 5.1).

5.2.2 Generation of Transgenic mice

A 7300 bp NotI fragment containing the transgene portion of LIT2-SaBR was purified by agarose gel electrophoresis, and injected into the pronuclei of (C57Bl/6J x C3H)F2 hybrid zygotes. Pups resulting from transplantation of injected zygotes into pseudopregnant females were analyzed for the presence of the transgene by Southern blot analysis of tail DNA (see 5.2.3).

(C57Bl/6J x C3H) hybrid mice were bred and maintained in the animal facility of the British Columbia Cancer Research Center (Vancouver, B.C., Canada). All animals were kept under micro-isolators and provided with sterilized food and water. Matings between transgenic littermates or controls were performed at 5 weeks of age and flow cytometry (see 5.2.5-5.2.7) and Northern blot analysis (see 5.2.4) were performed on mice at 6-7 weeks of age.
LIT2-SaBR

Figure 5.1. Schematic representation of the LIT2-SaBR construct. A 7300 bp NotI fragment of the LIT2-SaBR vector construct was used to inject pronuclei of (C57Bl/6 X C3H) F₂ hybrid mice. The hybridization locations of SaBR specific, hGH specific and SaBR-hGH probes used for Southern and Northern blot analysis are indicated by solid lines below the construct.
5.2.3 Southern blot analysis

In order to purify genomic DNA from transgenic mice, tail ends were isolated from 3 week old C57Bl/6 x C3H hybrid mice and dissolved at 50°C for 3 hours in 500 μl of tail buffer containing 10 mM Tris (pH 8), 10 mM EDTA (pH 8), 50 mM NaCl, 0.5% SDS and 20 μl proteinase K (Sigma, St. Louis, MO). The genomic DNA was then ethanol precipitated after 2 phenol and 2 chloroform extractions. To determine if transgene integration was successful, the DNA was cut with the restriction enzyme, PvuII, run on a 1% agarose gel, capillary transferred to Hybond (Amersham, Oakville, Ont) overnight and cross-linked by a 2.5 minute exposure to ultraviolet irradiation. The filter was prehybridized for 4 hours at 68°C in 250 mM NaPO₄ (pH 7.2), 1 mM Na-EDTA, 20% SDS, 5 mM sodium pyrophosphate, and 0.5% Boehringer-Mannheim blocking reagent, then hybridized for 16 hr at 68°C in the same solution containing denatured ³²P labeled probe. A 1700 bp PvuII fragment of SaBR cDNA was used as a SaBR specific probe, a 1000 bp PvuII fragment from the growth hormone region was used as a vector specific probe and a 1000 bp BamHI-BstXI fragment incorporating both GH and SaBR was used to identify both vector and insert (Figure 5.1). Filters were washed at 55°C twice in 25 mM NaPO₄, 150 mM NaCl, 5 mM sodium pyrophosphate and 1% SDS for 20 minutes each, then in 25 mM NaPO₄, 50 mM NaCl, 5 mM sodium pyrophosphate and 2% SDS for 20 min before placing on film.

5.2.4 Northern blot analysis

Total cellular RNA was isolated from the thymus and spleen of 6 week old transgenic mice and littermate controls using the guanidium isothiocyanate/phenol method (Chomcynski and Sacchi, 1987), and electrophoresed through a 1% agarose gel
containing 5% (v/v) formaldehyde, capillary transferred to Hybond overnight, and cross-linked by a 2.5 min exposure to ultraviolet irradiation. The filter was then prehybridized for 4 hours at 68°C in 250 mM NaPO₄ (pH 7.2), 1 mM Na-EDTA, 20% SDS, 5 mM sodium pyrophosphate, and 0.5% Boehringer-Mannheim blocking reagent and then hybridized for 16 hr at 68°C in the same solution containing denatured ³²P labeled probe. Filters were washed at 55°C twice in 25 mM NaPO₄, 150 mM NaCl, 5 mM sodium pyrophosphate and 1% SDS for 20 minutes each, then in 25 mM NaPO₄, 50 mM NaCl, 5 mM sodium pyrophosphate and 2% SDS for 20 min before placing on film.

5.2.5 Antibodies for immunofluorescence analysis

The following monoclonal antibodies were used for flow cytometry of transgenic and control spleen cells and thymocytes: 11F6 (α-SaBR) was developed at the Terry Fox Laboratory and was labeled with biotin-succinimidyl-ester (Vector Laboratories, Inc., Burlingham, CA) by standard procedures (van den Eertwegh and Claassen, 1991). Biotinylated rat IgG2b antibody was obtained from PharMingen (San Diego, CA) and used as an isotype control. α-CD32/CD16 (FcII,III receptor block) and fluorescein isothiocyanate (FITC)-labeled or PE-labeled L3T4 (α-CD4), Ly3.2 (α-CD8b) and RA3-6B2 (α-B220) were also obtained from PharMingen (San Diego, CA). PE-conjugated steptavidin (Sa-PE) was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL) and Jackson Immunoresearch (Bar Harbour, ME). FITC-streptavidin (Sa-FITC) was obtained from Sigma (St. Louis, MO) and propidium iodide was obtained from Sigma (p-5264)(St. Louis, MO).
5.2.6 Cell preparation

Spleen and thymus from 6-7 week old mice were isolated, washed in Hanks Balanced Salt Solution (HBSS; StemCell Technologies, Vancouver, B.C) supplemented with 2% fetal bovine serum (HBSS/2%FBS) and solid organs passed through a fine wire mesh to isolate single cells.

A.2.7 Flow cytometry

1x10^6 cells were preblocked on ice for 30 minutes with 1 μg/ml of α-CD32/CD16 in 100 μl HBSS/2%FBS. For staining involving only directly conjugated antibodies, optimal concentrations of the appropriate antibodies were incubated with the cells for 30 minutes on ice. For staining involving the biotinylated 11F6 antibody, 10 μg/ml biotinylated 11F6 Ab was added after the preblock and the cells incubated on ice for one hour. The cells were washed in HBSS/2%FBS and the secondary antibody Sa-PE or Sa-FITC added for 30 min. on ice. If two color staining was required, FITC, or PE conjugated antibodies to the second cell surface molecule were added at this time. Cells were then washed twice in HBSS/2% FBS and resuspended in the same buffer containing 1 μg/ml propidium iodide (PI). In this manner, dead cells were gated out by excluding those which stained positively with PI. In addition, isotype-matched rat antibodies were used as negative controls for staining. Flow cytometric analysis was performed using either a FACSort or FACScan flow cytometer (Becton Dickinson, Mountain View, CA). equipped with PC LYSIS II ® software (Hewlett Packard Co., Palo Alto, CA).
5.3 RESULTS

5.3.1 Construction of SaBR transgenic mice

In order to examine the effects of SaBR overexpression on lymphocyte differentiation and activation, transgenic mice were constructed using a SaBR transgene containing lymphocyte specific transcriptional elements. When tail DNA from the offspring of mice transplanted with oocytes injected with the SaBR transgene construct were examined, 4 samples were found to contain integrated transgene DNA (Figure 5.2). The DNA from two pups (376 and 379) contained multiple copies of the transgene, while the remaining pups' DNA (375 and 381) contained only one or two copies each (Figure 5.2). When these SaBR positive mice were backcrossed, mouse 381 proved to be sterile and mouse 375 failed to pass on the SaBR transgene to her offspring (Figure 5.3). Mouse 376 had 4 offspring containing the transgene, 3 males and 1 female and mouse 379 had 2 female pups and one male pup with the transgene (Figure 5.3).

5.3.2 SaBR transgenic mice do not express SaBR RNA or protein

Offspring from mice 376 and 379, that were positive for the SaBR transgene, were used in backcrosses to maintain the transgenic lines and in brother-sister matings to create SaBR homozygotes. The offspring from the various crosses were then used for flow cytometric analysis of SaBR protein expression and Northern blot analysis of SaBR RNA expression.

The transgene was targeted to lymphocytes, therefore SaBR RNA expression was examined in spleen and thymus of transgenic and control littermates. No SaBR RNA was detected in the normal or transgenic spleen of either transgenic line, suggesting that
Figure 5.2. Southern analysis of tail genomic DNA from offspring of mice transplanted with oocytes injected with a SaBR transgene. Genomic DNA was isolated from tail blood of 3 week old pups, cut with PvuII, run on a 1% agarose gel, transferred to Hybond and hybridized with a fragment of vector DNA specific for SaBR and GH. 10 μg of DNA is present per lane.
Figure 5.3. Southern analysis of tail genomic DNA from offspring of transgenic founders. Genomic DNA was isolated from tail blood of 3 week old pups, cut with PvuII, run on a 1% agarose gel, transferred to Hybond and hybridized with a fragment of vector DNA specific for SaBR and GH. 10 µg of DNA is present per lane.
endogenous SaBR expression is too low to be detected by Northern analysis and that transgenic animals, if expressing SaBR RNA, were not expressing at levels higher than normal (Figure 5.4). Normal thymus did not contain the 3.5 kb SaBR RNA as expected (based on FACS analysis in chapter 2), but the transgenic thymus of founder 379 contained a very large RNA species (over 10 kb) that was recognized by a vector probe as well as a probe for SaBR (Figure 5.4). The transgene should give a mRNA species of approximately 4 kb, which was not seen in normal or transgenic spleen or thymus (Figure 5.4).

When SaBR protein expression was analyzed by flow cytometry in the thymus and spleen of transgenic and control littermates, no significant differences in SaBR expression were observed (Table 5.1). Moreover, this was the case when examining SaBR heterozygotes or homozygotes (Table 5.1).

5.4. DISCUSSION

SaBR transgenic mice were generated using a vector containing lymphocyte specific transcriptional elements in order to determine if SaBR plays a functional role during lymphocyte development and activation. The SaBR transgene was successfully integrated into four mice, but only two of these mice were capable of passing the transgene on to their offspring. When these transgenic offspring and their control littermates were examined for RNA expression, no bands were visible in the spleen and a large ~10 kb RNA band was seen in the transgenic thymus. SaBR mRNA is expressed
Figure 5.4. **Northern analysis of SaBR RNA from transgenic mice.** Total RNA was isolated from spleen and thymus of transgenic mice and normal littermate controls from founder line 379 and 10 μg of each was run on a gel, transferred to Hybond and hybridized with a probe that recognizes the vector (GH) (top panel). The blot was then stripped and reprobed with a SaBR specific probe (bottom panel).
Table 5.1. Analysis of SaBR expression and lymphocyte composition of SaBR homozygote transgenic mice and littermate controls

<table>
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The spleens and thymuses of SaBR homozygote transgenic mice and their littermate controls for two different transgenic lines were examined by flow cytometry for a) the percentage of CD4+ T cells, CD8+ T cells, CD4-CD8- T cells, CD4+CD8+ T cells and B220+ B cells, b) the percentage of cells expressing SaBR and c) the percentage of cells within each subpopulation expressing SaBR. The table shows the results of a representative experiment in which one transgenic and normal mouse were examined for each of the two transgenic lines.
at levels too low to be detected by Northern blot analysis; therefore, only transgenic SaBR RNA would be expected to be visible on a total RNA Northern blot. The fact that I see no transgenic SaBR RNA in the spleen and no differences in SaBR protein levels could be explained by low expression levels of the transgene.

The ~10 kb RNA species found in the thymus (founder 379) may represent an aberrant integration, deletion or random mutation event. If a piece of DNA integrated into the middle of the transgene, an aberrant mRNA would be produced. Alternatively, if the promoter for the transgene was deleted and an upstream promoter was used, the mRNA would be bigger and would be unlikely to produce a functional protein. Furthermore, deletion of polyA sites or enhancers could also result in an aberrant mRNA product if up or downstream elements are being used instead.

Flow cytometric analysis of SaBR expression in the various subpopulations within the thymus indicated no difference between controls and transgenic animals, but overall SaBR expression was slightly higher in the transgenic thymuses of both lines. However, because the percentage of thymocytes expressing endogenous SaBR is so low to begin with (less than 1%) and only two sets of mice were examined, it is not possible to determine statistically if the difference between the control and transgenic animals is real. Furthermore, the fact that no transgenic SaBR RNA was visible in the thymuses of offspring from founder 376 and the RNA seen in the thymuses of offspring from founder 379 was aberrant, suggests that the difference seen in overall SaBR expression within the thymus is the result of experimental error or natural variation between mice.

The LIT2 vector has been used successfully in the past to create transgenic mice overexpressing HSA in both the B and T cell compartments (Hough et al., 1994; Hough
et al., 1996). This overexpression extended to those cells that expressed high endogenous levels of HSA (Hough et al., 1994; Hough et al., 1996), suggesting that the vector and the methods discussed in this chapter were unlikely to be the cause of my failure to produce transgenic mice overexpressing SaBR. The SaBR cDNA inserted into the LIT2 vector was identical to the SaBR cDNA used for expression in Cos, 3T3 and T28 cells; therefore the cDNA was capable of expressing a functional protein. However, the combination of LIT2 vector and SaBR cDNA was not directly tested for expressibility. This can be accomplished by transfecting the vector into T28 cells and should be performed before more transgenic mice are made.

It is also possible that there may have been selection against SaBR over-expression in the transgenic mice. This is unlikely because there was a good yield of transgenic pups from the first set of injections. A second round of injections was performed resulting in no transgenic pups; however, this appears to have been due to a general technical failure in the transgenic facility at that time. Furthermore, defects specific to lymphocytes typically have no effect on the viability of mice maintained in a sterile facility. Nonetheless, it is conceivable that a defect in lymphocytes (eg. overexpression of one or more cytokines) could have systemic effects that would prevent the birth of transgene-expressing pups. Another possibility is that expression from the LIT2 vector was occurring in a non-lymphoid organ, with SaBR overexpression in that organ being lethal.

The most likely explanation for my failure to get transgenic mice over-expressing SaBR was that not enough transgenic mice were generated in the first place. The use of
transgenic mice to test my hypothesis of SaBR function in lymphocytes is therefore still valid and should be pursued.
SUMMARY AND FUTURE DIRECTIONS

SaBR is a novel cell surface antigen that was cloned from the hematopoietic multipotential progenitor cell line, B6SUtA. SaBR is differentially expressed within the hematopoietic system in terms of cell lineage, location, developmental stage and activation status and as such is a candidate as a regulator of hematopoiesis. The objectives of the research presented in this thesis were designed to address this question.

My first objective was to analyze SaBR expression on developing and activated lymphocytes in greater detail. Flow cytometric analysis revealed that in the T lymphocyte lineage, SaBR was only expressed on mature single positive cells and was found on a greater percentage of CD4 cells than CD8 cells. In B cells, SaBR was expressed on a high percentage of progenitor cells within the bone marrow. Approximately 90% of early pro-B cells expressed the antigen as compared to 50% of immature and mature B cells.

Upon lymphocyte activation, SaBR expression was altered depending on the activator being used. In T cells, the combination of PMA+A23187, which bypasses the TCR, resulted in an increase in the number of cells expressing SaBR; both in the CD4 and the CD8 subpopulations. The combination of PMA+α-CD3 resulted in an increase in the percentage of CD8 cells expressing SaBR. When B cells were activated with stimuli that mimic antigen independent activation (α-CD40+IL-4), there was an upregulation in SaBR expression; however, activation through the BCR, with α-μ+IL-4 or α-μ+IL-4+α-CD40, resulted in downregulation of the antigen. Furthermore, I observed that B lymphocytes sorted for positive SaBR expression incorporated more [3H] thymidine when placed in culture than their negative counterparts. Based upon these
results, I proposed the hypothesis that SaBR plays a functional role during B cell
development and during activation. I hypothesize that SaBR primes lymphocytes for
entry into the cell cycle and serves to mark lymphocytes that receive antigen independent
activation stimuli, for anergy or apoptosis.

In order to test the hypothesis, I attempted to create transgenic mice
overexpressing SaBR within the lymphoid system; however, I was unsuccessful in
producing a mouse that expressed the transgene mRNA or protein.

The second objective of my research was to subclone, sequence and characterize
the SaBR cDNA clone. Protein sequence can provide information regarding molecular
function, which in turn can provide indications of biological function. Sequence
comparisons of different regions of murine SaBR with the databases revealed sequence
similarities to a number of proteins and protein domains; including the somatomedin B
domain, von Willibrand factor, sushi domains, the selectins and the complement
receptors. I also found a strong match to two human expressed sequence tag (EST)
clones. The 50776 clone was subsequently obtained from the Merck/Image Consortium
(University of Washington, PA), subcloned and sequenced. Because the clone was
missing the 5' end, it was used as a template for PCR cloning in an attempt to recover a
full-length cDNA. A number of small cDNA products were found; however, a clone
encompassing the 5' end was not recovered. At present, the human cDNA clone shares
73% nucleotide identity with its mouse homologue. In addition, it has two differentially
expressed mRNA products. This is in contrast to murine SaBR which has only one
mRNA product. I also attempted to perform some preliminary protein analysis using the
11F6 antibody for immunoprecipitation and Western blots; however, these studies proved to be unsuccessful.

Northern blot analysis revealed that SaBR was expressed in a number of non-hematopoietic tissues; therefore, my final thesis objective was to use histochemistry to analyze SaBR protein expression within individual cells in these tissues. I hoped that the cellular distribution of SaBR expression would provide indications of protein function within non-hematopoietic cells. The most interesting expression patterns were found within the kidney and stomach, where SaBR was found to be strongly expressed by the convoluted tubule endothelial cells and mucosal cells respectively.

Based on the expression studies described in this thesis, it appears that SaBR is playing a role in B lymphocyte development and activation. The evidence supporting a role for SaBR during development is based solely on its high expression in early pro-B cells; therefore, more studies need to be performed in this area. However, different techniques from those employed in this thesis will be required for the study of B cell progenitors. For example, Whitlock-Witte long term bone marrow cultures specifically support the proliferation of B cell progenitors and are invaluable for the study of B cell development in vitro (Whitlock and Witte, 1982). In fact, populations of pro-B cells and pre-B cells can be maintained in culture by growing bone marrow cells on an S17 stromal cell line (Faust et al., 1993; Kincade et al., 1989). Cells arising from these cultures could be sorted with respect to SaBR expression and the ability of the two populations to proliferate and differentiate could be studied. Alternatively, the cells could be treated with SaBR antibodies to determine the effect of the antibodies on proliferation, adhesion and colony formation.
There is also evidence to support a role for SaBR during B lymphocyte activation. The regulation of SaBR expression during activation and the differences in $[^3]$H thymidine incorporation between SaBR+ and SaBR- sorted B cells suggest that SaBR may serve as a marker and affect how SaBR expressing cells respond to future stimuli. Is SaBR acting as a costimulatory molecule? An adhesion molecule? A marker for cell death? The development of a larger panel of anti-SaBR monoclonal antibodies would be very useful in addressing these questions. Only the 11F6 antibody is available at the present time and studies employing the antibody have failed to implicate SaBR in adhesion or costimulation. This may be because 11F6 recognizes an epitope that is not involved in adhesion or costimulation; however, Parsons et al., (1996) have shown that it is now possible to select antibodies from a phage display antibody repertoire that recognize specific epitopes. This technique would be very useful in producing a panel of antibodies specific for different regions of SaBR. Furthermore, examining the effects of these multiple antibodies on cell proliferation, cytokine production, antibody production and effector functions would not only provide a more relevant answer to the question of SaBR function during activation, but would also supply us with much needed reagents for other studies, such as Western blot analysis and immunoprecipitation.

In T cells, SaBR is expressed only on mature single positive cells and can be found on a greater percentage of CD4 than CD8 cells, suggesting a potential role for SaBR in helper T cell function. A monoclonal antibody panel, such as that described above, would be very useful in addressing this observation. In vitro assays could be used to determine the effect of various SaBR antibodies on purified CD4 cells. After treatment with antibodies, CD4 cells could be examined for their ability to produce...
cytokines upon activation or to activate naive B cells (Bachmann et al., 1994). As with the B cells, antibody effects on adhesion and costimulation could also be performed.

In both T and B cells, it appears that specific signaling pathways are involved in the regulation of SaBR expression. To determine which pathways are playing a role, SaBR expression could be examined after treatment of activated and non-activated lymphocytes with specific inhibitors. These inhibitors could include the PKC inhibitor, staurosporin; the T cell activation inhibitor, cyclosporin A; Ca influx inhibitors, sphingomyelinase (Breittmayer et al., 1994), sphingosine (Breittmayer et al., 1994) and verapamil; or rapamycin, which prolongs the G1 phase of the cell cycle (Terada et al., 1995). If SaBR expression is altered from expected results after treatment, the inhibitor used would indicate which pathways are candidates for SaBR regulation.

SaBR is also expressed in non-hematopoietic tissues, in particular the mucosal cells of the stomach and the convoluted tubule endothelial cells in the kidney. As discussed in Chapter 4, both types of cells have been implicated in immune responses, gastric mucosal cells have growth factor receptors (Kinoshita et al., 1995) and the kidney endothelial cells express adhesion molecules and cytokines (Kelley and Singer, 1993).

Determining a role for SaBR in tissues other than the kidney and stomach, is complicated by the fact that SaBR is expressed equally throughout each of these tissues. However, since SaBR has been implicated in the immune function of the hematopoietic system, it is possible that it may be playing a similar role in the immune responses of non-hematopoietic tissues. Butcher and Picker (1996) review the evidence that a system of highly specialized immune microenvironments are present in most organs of the body.
and that interactions between these microenvironments and circulating immune cells regulates their function. Perhaps SaBR is playing a role in this regulation.

This thesis also explored the structure of SaBR and the results suggest that SaBR may be involved in an adhesive interaction. Whether this interaction involves a soluble or membrane bound ligand is predictable, however. Since the cell-cell binding assays described in Chapter 3 were unsuccessful, I recommend using the fusion proteins described in Appendix A for further experimentation. I also feel that immunoprecipitation experiments should be performed using a panel of new monoclonal antibodies, which would reveal the presence of associated proteins, whether they be cell-surface or intracellular. If SaBR is associated with a cell-surface protein with a known ligand, it is possible that the putative ligand for SaBR may be found on the same cell. Moreover, if SaBR is in a complex with a protein tyrosine kinase, the identity of the kinase may provide clues as to which signaling pathways could be involved in SaBR function.

The results presented in this thesis are encouraging and provide some useful information regarding the expression of SaBR in hematopoietic and non-hematopoietic tissues, but they tend to pose more questions than they answer. I propose that some of these questions could be addressed by creating more transgenic mice. Once the ability of the LIT2-SaBR vector to express SaBR in T28 cells is confirmed, more transgenic mice overexpressing SaBR in the lymphoid system could be made. These mice would be useful to test our present hypotheses and to study the function of SaBR in cells where it is normally not expressed, such as thymocytes. Complementation of Rag2-/- blastocysts (Spanopoulou, 1996; Chen, 1996) could also be used as an alternative route to study the
effects of SaBR overexpression in the lymphoid system, particularly if the transgene is being expressed in cells other than lymphocytes. SaBR could be overexpressed in ES cells and the ES cells could be injected into Rag2−/− blastocysts. The immune system of the resultant chimeras would be derived solely from the transgenic ES cells; thus allowing for the study of SaBR in lymphoid development and activation. Alternatively, a SaBR knockout mouse could be made (Capecchi, 1989). These animal models have provided researchers with a valuable means to study the functions of single genes in vivo. In fact, knockout technology has been used extensively to study lymphocyte differentiation and activation (Pfeffer and Mak, 1994; Fazekas de St. Groth et al., 1997; Shores and Love, 1996; Grewal and Flavell, 1997; Bluestone, 1996). If SaBR is indeed marking cells and affecting how they react to future stimuli, its effects may be too subtle to detect using transgenic mice where endogenous SaBR expression may hide the effects of SaBR overexpression. A SaBR knockout mouse, on the other hand, may show noticeable deficiencies in the development and/or function of the immune system. Furthermore, a knockout mouse would allow one to examine the effects of the null mutation on the physiology of various organs and perhaps provide clues as to SaBR function in non-hematopoietic tissues.
APPENDIX A
LIGAND DETERMINATION
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A.1 INTRODUCTION

The studies described in Chapter 2 imply that SaBR may be functioning as an accessory molecule during lymphocyte activation. Accessory molecules may function as adhesion molecules, co-receptors or costimulatory molecules (Zuckerman et al., 1995) and in all cases must bind to a soluble ligand or counter-receptor in order to function.

Moreover, sequence analysis of SaBR (Chapter 3) indicates that it shares sequence similarity to somatomedin B domain containing proteins. The somatomedin B domain of vitronectin is believed to function as the binding site for the plasminogen activator inhibitor-1 protein (Sieffert et al., 1994; Deng et al., 1996), therefore, it is possible that the somatomedin B domain of SaBR is also involved in binding. Fusion proteins are one way in which membrane bound counter-receptors can be found. This appendix describes the methods by which I created IgG Fc-SaBR fusion proteins.

A.2 MATERIALS AND METHODS

A.2.1 Vector construction

The CD5-IgG1 fusion vector was generously supplied by Dr. Brian Seed (Massachutes, MI) and used to create SaBR or ICAM-1 constructs minus the transmembrane and intracellular domains (Figure A.1). In all cases the CD5 signal peptide portion of the vector was replaced with the appropriate SaBR or ICAM-1 fragment containing its own signal peptide. The SaBR or ICAM-1 fragments were inserted in frame at the beginning of the IgG1 portion of the vector so that a proper soluble fusion protein would be transcribed with the extracellular domains of SaBR.
Figure A.1. **SaBR and ICAM-1 fusion proteins.** Schematic diagram of the ICAM-1 (A) and three SaBR (B, C and D) fusion proteins constructed using the CD5-IgG1 fusion vector. Putative domains are represented by boxes with different shading.
(A) ICAM-1 extracellular domain
ICAM-1-IgG

(B) somatomedin B domain
SaBR-IgG-1

(C) somatomedin B domain
von Willibrand factor-like domain
SaBR-IgG-2

(D) somatomedin B domain
von Willibrand factor-like domain
selectin-like domain
SaBR-IgG-3
or ICAM-1 at the 5' end and IgG1 sequences at the 3' end.

A.2.2 Preparation of the ICAM-1 fragment

PCR was used to amplify the extracellular portion of ICAM-1. A pUC1813-ICAM-1 construct was used as a source of DNA and the following primers were used in the PCR reaction.

Forward pUC: 5'GTAAAACGACGCTTCGACG 3'

ICAM-1 3': 5'CTCAAGATCTTTGAGAGTGGGACG 3'

The PCR reaction was carried out using Pfu in a thermocycle PCR machine (Idaho Technologies). After a predenaturation step at 95°C for 60 sec, the next 30 cycles employed the following parameters: denaturation - 95°C for 40 sec, annealing - 42°C for 40 sec, and elongation - 72°C for 2 minutes. The products of the PCR reaction were run on a 1.2% agarose gel, stained with ethidium bromide and the 1600 bp band corresponding to the extracellular portion of ICAM-1 excised from the gel and the DNA purified. After cutting with the restriction enzymes SalI and BglII (BglIII site was incorporated into the ICAM-1 3' primer), the resultant fragment was ligated into the XhoI - BamHI cut and filled in CD5-IgG1 fusion vector.

A.2.3 Preparation of the SaBR fragments

Three SaBR-IgG1 fusion proteins were constructed: SaBR-IgG-1 (1-810 bp) utilized the somatomedin B domain of SaBR, SaBR-IgG-2 (1-2025 bp) utilized the somatomedin B domain and Von Willibrand factor-like domain of SaBR and SaBR-IgG-3 (1-2250 bp) used the entire extracellular region of SaBR (Figure B.1). The following PCR primers were used to amplify up the relevant portions of SaBR from CTV4-SaBR:

CTV 5': 5' CCTCACTCCTTCTCTAGCTC 3'
SaBR-1: 5' CTCAAGATCTATCCTTCTCTCCT 3'
SaBR-2: 5' CTCAAGATCTCACATCTTCTGT 3'
SaBR-3: 5' CTCAAGATCTGTAGCTCCGCCCTGGCTGA 3'

The PCR reaction was carried out using Pfu in a thermocycle PCR machine. After a predenaturation step at 95°C for 60 sec, the next 30 cycles employed the following parameters: denaturation - 95°C for 40 sec., annealing - 55°C for 40 sec. and elongation - 72°C for 2 minutes. The products of the PCR reaction were run on a 1.2% agarose gel, stained with ethidium bromide and the bands corresponding to the correct extracellular portions of SaBR excised from the gel and the DNA purified.

*IgG-SaBR-1*: The SaBR-1 PCR fragment was cut with the restriction enzymes XhoI and BglII and ligated in frame to the CD5-IgG1 vector cut with the same enzymes.

*IgG-SaBR-2 and IgG-SaBR-3*: Because the restriction enzymes required to isolate the correct PCR fragments for ligation to the CD5-IgG1 fusion vector cut more than once for the IgG-SaBR-2 and IgG-SaBR-3 constructs, three piece in-frame ligations were required as follows: XhoI - PstI fragments of SaBR (from CTV4-SaBR) were ligated to PstI - BglII PCR fragments of SaBR (525 bp piece for SaBR-2 and an 850 bp piece for SaBR-3) and XhoI - BamHI cut CD5-IgG1 fusion vector.

A.2.4 Transfections

1-2x10^6 COS cells were transfected with 20 μg of each fusion construct using DEAE-Dextran with a 20% glycerol shock for 2 min and a 5 hour treatment with 0.1 mM chloroquine (Sigma, St. Louis, MO). After 48 hours, the media containing DMEM (StemCell Technologies, Vancouver, B.C.) plus 10% fetal bovine serum
(DMEM/10%FBS) was replaced with DMEM/1%FBS and after 72 and 96 hours, the supernatant was collected for analysis by Elisa.

A.2.5 Elisa

96 well flat bottom plates (Dynatech Immulon 1, Cantily, VI) were coated with 1 μg/well of the capture antibody, α-hIgG (Sigma, St. Louis, MO) diluted in 15 mM Na₂CO₃ plus 35 mM NaHCO₃ at pH 9.6. The plates were incubated overnight at 4°C, then washed three times in phosphate buffered saline (PBS; StemCell Technologies, Vancouver, B.C) containing 0.05% Tween (PBS/0.05%Tween; Sigma, St. Louis, MO). Blocking buffer composed of 5% skim milk powder in PBS was then added to the wells for 2 hours at 37°C. After washing 4 times in PBS/0.05%Tween, 200 μl of each sample (including media blank, negative controls, IgG standards and COS cell supernatants) was added to the appropriate well and incubated for 1.5 hours at 37°C. Triplicate wells were used for each sample. The wells were then washed three times in PBS/0.05%Tween and the detection antibody, α-hIgGFc-alkaline phosphatase (AP; Sigma, St. Louis, MO) added at a dilution of 1:2000 for 2.5 hours at 37°C. A final 3 washes in PBS/0.05% Tween were followed by the addition of the substrate, p Nitrophenyl phosphate disodium (pNPP; Sigma, St. Louis, MO) at a concentration of 500 μg/ml in substrate buffer (1 M diethanolamine (Sigma, St. Louis, MO) and 0.5 mM MgCl₂, pH 9.8) at 37°C until sufficient color development. The reaction was stopped with 5 M NaOH and the resulting absorbance read with an Elisa plate reader (Pharmacia, Baie d’Urfe, QB) at 405 nm.
A.3 RESULTS

A.3.1 Fusion protein assay

Three different SaBR containing Ig fusion proteins were constructed; full length SaBR, SaBR with a deleted selectin-like domain and SaBR with a deleted selectin-like domain and deleted von Willibrands factor domain (Figure A.1). To determine if functional proteins were constructed, all three fusion proteins, along with a control ICAM-Ig fusion protein, were tested in an Elisa assay for their ability to bind to human IgG Fc antibody. When the absorbance 405 nm for the supernatant containing each fusion protein was compared to an IgG standard curve, the SaBR-Ig fusion proteins and the ICAM-Ig fusion protein was found to be present in quantities less than 100 ng/ml (Figure A.2; Table A.1).

A.4. DISCUSSION

IgG1 Fc fusion proteins are useful for the identification of cells expressing ligands, provided the protein being used can function alone and in soluble form. Binding of the fusion proteins to test cells can be detected using fluorescently labeled antibodies specific for the Fc portion of the fusion protein. Cells that bind to the fusion proteins can then be sorted using flow cytometry. However, in order for positively binding cells to be detected above background levels, the fusion protein must be present in a high enough concentration to effectively saturate all the available ligands. Past experience has shown that successful cytometric analysis requires most antibodies to be present at concentrations
Figure A.2. **ELISA IgG standard curve.** Specific concentrations of IgG containing supernatant were added to ELISA plates containing human IgGFcR capture Ab and incubated at 37°C for 1.5 hrs. After washing, the human IgGFcR-AP detection Ab was added for 2.5 hrs at 37°C, then the alkaline phosphatase substrate added until distinct color was visible in the wells. The absorbance 405 nm was then determined for each well. The standard curve was used to determine relative concentrations of SaBR-Ig fusion proteins present in the COS cell supernatant after transfection.
Table A.1. Absorbance and concentration of ICAM-1-Ig and SaBR-Ig fusion proteins

<table>
<thead>
<tr>
<th>transfectant</th>
<th>mean absorbance 405 nm</th>
<th>concentration of fusion protein (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no DNA control</td>
<td>41</td>
<td>&lt;5</td>
</tr>
<tr>
<td>SaBR-1</td>
<td>253</td>
<td>≈25</td>
</tr>
<tr>
<td>SaBR-2</td>
<td>360</td>
<td>≈32</td>
</tr>
<tr>
<td>SaBR-3</td>
<td>777</td>
<td>≈95</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>785</td>
<td>≈98</td>
</tr>
</tbody>
</table>

Human IgGFc antibody was used as a capture antibody in an ELISA with human IgGFc-AP as the detection antibody in order to determine the concentration of SaBR-Ig fusion proteins present in the supernatants of COS cells transfected with pAX142 vectors containing SaBR or an ICAM-1 control. All supernatants were plated in triplicate and the absorbance 405 represents the mean. Concentration was determined by plotting the mean absorbance for each fusion protein on an IgG standard curve.
between 1 ng/μl and 10 ng/μl; therefore, fusion proteins should be used at concentrations of at least 1 ng/μl.

The concentration of ICAM-1-Ig and SaBR-Ig fusion proteins was less than 100 ng/ml (0.1 ng/μl), which is 10 times less than required. Although concentrating the proteins is an option, improvements to the COS cell transfection assay must also be made before these proteins can be used efficiently in a binding assay. There are a number of ways in which the transfection procedure could be altered in an attempt to improve transfection efficiency. The concentration of construct DNA could be altered, the supernatant could be collected and pooled over several days, or a different transfection procedure, such as lipofectin or electroporation could be used. Altering the DNA concentration and pooling the supernatant has since proven to be unsuccessful; however changing transfection procedures has yet to be tried.

Other variations to be attempted include altering the promoter or enhancer elements of the vector itself and using a different vector altogether, such as the pAX142 vector, both of which have been shown by Kay et al. (1991) to improve transfection efficiency.

Once fusion proteins can be produced in sufficiently high concentrations they can be used in different ways. As discussed here, they can be used to detect cells that bear putative ligands. Employing fusion proteins that encompass different regions of SaBR, may help to pinpoint which regions of SaBR are involved in ligand interactions. In addition, SaBR fusion proteins can be used to examine interactions between cells and between cells and the ECM. If SaBR is interacting with a ligand on another cell or ECM
component, the fusion protein may interrupt the interaction, thus implicating SaBR as a potential adhesion molecule.
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