A ROLE FOR SHP-1 AND VAV IN THE
ABROGATION OF B CELL RECEPTOR SIGNAL TRANSDUCTION
BY LATENT MEMBRANE PROTEIN 2 (LMP2)

by

Mary-Ann M. Glasier

A thesis submitted in conformity with the requirements
for the degree of Masters of Science
Graduate Department of Immunology
University of Toronto

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A Role for SHP-1 and Vav in the Abrogation of B Cell Receptor Signal Transduction by Latent Membrane Protein 2 (LMP2)

M.Sc. 1997
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Abstract

Latent membrane protein 2 (LMP2) has been reported to block signal transduction through the B cell receptor (BCR) in Epstein-Barr virus-transformed lymphoblastoid cell lines (LCLs) through recruitment of Syk and Src-family protein tyrosine kinases. The purpose of this study was to investigate the role of the tyrosine phosphatase SHP-1 in this inhibition. SHP-1 activity was approximately ten times higher in resting LMP2+ LCLs as compared to LCLs lacking LMP2 (LMP2-), and this difference in activity did not significantly change after BCR stimulation. No association was detected between SHP-1 and LMP2 by co-immunoprecipitation and western blotting. In addition, CD22-associated phosphatase activity was observed only in resting LMP2+ LCLs and BCR stimulated LMP2- LCLs. Several potential substrates for SHP-1 in LMP2+ LCLs are discussed. Evidence is also presented supporting the inducible dissociation of Vav from LMP2, and the inducible tyrosine dephosphorylation of Vav in LMP2+ LCLs. These data suggest that LMP2 uses multiple mechanisms to inhibit the B cell's response to antigen.
ACKNOWLEDGMENTS

I would like to express my gratitude to:

My supervisor, Dr. Kathy Siminovitch, for her support in this study. This support included the acquisition of a studentship from the Medical Research Council of Canada (MRC).

My supervisory committee, Drs. Gillian Wu and Robert Rottapel, for their guidance especially in the construction of this thesis.

Post-doctoral fellows Giovanni Pani, for his guidance during the initial stages of this study, and Luis da Cruz for his many helpful discussions.

And, student Ally-Khan Somani for his expertise with graphics software.

This study was supported financially through an Ontario Graduate Scholarship and an MRC studentship.
<table>
<thead>
<tr>
<th></th>
<th>Section</th>
<th>Page(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Introduction</td>
<td>1-20</td>
</tr>
<tr>
<td></td>
<td>A. B Cell Receptor Activation</td>
<td>1-13</td>
</tr>
<tr>
<td></td>
<td>B. SHP-1</td>
<td>13-16</td>
</tr>
<tr>
<td></td>
<td>C. Epstein-Barr Virus Transformation</td>
<td>16-17</td>
</tr>
<tr>
<td></td>
<td>D. Latent Membrane Protein 2 (LMP2)</td>
<td>17-20</td>
</tr>
<tr>
<td>II</td>
<td>Thesis</td>
<td>21</td>
</tr>
<tr>
<td>III</td>
<td>Materials and Methods</td>
<td>22-26</td>
</tr>
<tr>
<td>IV</td>
<td>Results</td>
<td>27-45</td>
</tr>
<tr>
<td>V</td>
<td>Discussion</td>
<td>46-50</td>
</tr>
<tr>
<td>VI</td>
<td>References</td>
<td>51-63</td>
</tr>
<tr>
<td>Table/Figure</td>
<td>Page(s)</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>Table 1</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Figure 1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Figure 2</td>
<td>8</td>
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<td>Figure 3</td>
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<td>28</td>
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<td>Figure 6</td>
<td>29</td>
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<tr>
<td>Figure 7</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Figure 8</td>
<td>32 &amp; 33</td>
<td></td>
</tr>
<tr>
<td>Figure 9</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Figure 10</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Figure 11</td>
<td>38</td>
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<td>Figure 12</td>
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<tr>
<td>aa</td>
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<tr>
<td>BCR</td>
<td>B cell antigen receptor</td>
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<tr>
<td>C-</td>
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<tr>
<td>[Ca^{2+}]_i</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<td>FcγRIIb1</td>
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<td>GAP</td>
<td>GTP-ase activating protein</td>
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<td>gp</td>
<td>glycoprotein</td>
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<tr>
<td>Ig</td>
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<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
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<td>LMP2</td>
<td>latent membrane protein 2</td>
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<tr>
<td>LCL</td>
<td>lymphoblastoid cell line</td>
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<td>mAb</td>
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<td>MAPK</td>
<td>mitogen activating protein kinase</td>
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<td>PI-3K</td>
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<td>PKC</td>
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<td>pNPP</td>
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<td>SOS</td>
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<td>SD</td>
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<td>X</td>
<td>any amino acid</td>
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I. INTRODUCTION

The purpose of this study was to address the involvement of the hematopoietic non-receptor tyrosine phosphatase SHP-1 in the abrogation of B cell receptor signal transduction by the Epstein-Barr virus (EBV) latent membrane protein 2 (LMP2). Thus, background to several topics will be presented. These topics include B cell activation, SHP-1, EBV-transformation, and LMP2.

I.A. B Cell Activation Through Antigen Receptors

The activation of mature B lymphocytes is initiated by the binding of ligand to surface receptors specific for antigen. Antigen binding is transduced into the cell and initiates a program of activation, clonal expansion, and differentiation into either antibody-forming or memory cells (for review Gold & DeFranco, 1994; Cambier et al., 1994). Three well-documented early biochemical events in B cell antigen receptor (BCR) signaling are protein tyrosine kinase activation (Gold et al., 1990), intracellular calcium mobilization (Braun et al., 1979; Pozzan et al., 1982), and phosphoinositide hydrolysis (Coggeshall & Cambier, 1984; Bijsterbosch et al., 1985a).

I.A.1. Structure of the B Cell Antigen Receptor (BCR):

B lymphocytes express on their surface the same antigen-specific antibody secreted following activation and differentiation. As shown in Figure 1, each surface immunoglobulin molecule contains two antigen-binding sites (for review see Abbas et al., 1991). The amino-acid sequence comprising the antigen-binding site is highly variable and
Figure 1: The B cell receptor complex of the IgM class. Membrane immunoglobulin consists of two identical light (L) and two identical heavy (H) chains linked by disulfide bonds (–) as shown. Each H and L chain is composed of repeating immunoglobulin (Ig) domains of constant (□) and variable (△) amino-acid residues. The hypervariable regions within the amino-terminal variable Ig domain from one H and one L chain forms the antigen-binding site as indicated. Membrane Ig is expressed on the surface of the B cell in non-covalent association with disulfide-linked Ig-α/Ig-β heterodimers. Each chain of the heterodimer (Ig-α and Ig-β) contains one Immunoreceptor tyrosine-based activation motif (ITAM) which is responsible for transducing signals from mIg (Reth, 1989; Cambier, 1995) (D = aspartic acid; E = glutamic acid; Y = tyrosine; L = leucine; I = isoleucine; X = any amino acid).
results from somatic DNA recombination of discrete gene segments during B cell ontogeny (for review see Rolink & Melchers, 1991).

Membrane immunoglobulin (mIg) is not the complete antigen receptor. This was suggested early in BCR signaling studies by the small cytoplasmic tail of mIg. Further evidence for mIg-associated components came from the isolation of Ig–α and Ig–β by two independent research groups (Campbell & Cambier, 1990; Hombach et al., 1990a). These two proteins were later identified as products of the mb-1 and B29 genes respectively (Hombach et al., 1990b; Campbell et al., 1991) which had been previously cloned (Sakaguchi et al., 1988; Hermanson et al., 1988). Human mIg was also found to co-immunoprecipitate with Ig–α and Ig–β in weak detergents such as digitonin (Van Noesel et al., 1991; 1992; Clark et al., 1992a). The current model for the structure of BCR complex is one mIg molecule associating non-covalently with two Ig–α/Ig–β disulfide-linked heterodimers (Figure 1; for review see Reth, 1992).

The Ig–α/Ig–β heterodimer is responsible for signal transduction following the binding of antigen to mIg. This was shown by cross-linking experiments with chimeric proteins containing the cytoplasmic tails of Ig–α and Ig–β (Sanchez et al., 1993, Law et al., 1993; Kim et al., 1993; Burkhardt et al., 1994). The structural unit responsible for signal transmission is a conserved amino-acid sequence known as the ITAM (see Figure 1 for more detail). The ITAM is contained within other signal transducing components of immune recognition receptors such as the CD3 complex on T cells (for review see, Keegan & Paul, 1992). The two tyrosines within the ITAM are phosphorylated following BCR stimulation (Gold et al., 1991), and both are critical for signal transmission since conserved
mutation of either tyrosine ablates signaling (Romeo et al., 1992; Sanchez et al., 1993; Burkhardt et al., 1994; Flaswinkel & Reth, 1994).

Most experiments investigating early activation events stimulate B lymphocytes with pepsin cleaved F(ab')\textsubscript{2} anti-Ig antibodies which cross-link surface antigen receptors independent of the receptor's specificity (for review see Klaus, 1990). This type of antigen is known as type 2 T helper (T\textsubscript{H}) cell-independent. Like other type 2 T-independent antigens, F(ab')\textsubscript{2} anti-Ig does not induce extensive proliferation and differentiation to antibody-secreting cells in the absence of T\textsubscript{H} cell cytokines (see also Paul et al., 1986). This contrasts with T-independent type 1 antigens, such as EBV, which can induce antibody secretion in the absence of T\textsubscript{H} cytokines.

\textbf{I.A.2. Protein Tyrosine Kinase (PTK) Activation:}

The most immediate event detectable following BCR cross-linking is the induction of protein tyrosine phosphorylation (Campbell & Sefton, 1990; Gold et al., 1990; Burkhardt et al., 1991). The ability of different tyrosine kinase inhibitors to block calcium mobilization and phosphoinositide hydrolysis emphasizes the importance of PTK activity to initial events in BCR signal transmission (Lane et al., 1991; Padeh et al., 1991). The PTKs involved in early events include Src family members p53/56\textsubscript{lyn}, p59\textsubscript{fyn}, p55\textsubscript{blk}, and p56\textsubscript{lck} (Burkhardt et al., 1991; Leprince et al., 1992; Lin & Justement, 1992), p72\textsubscript{syk} (Hutchcroft et al., 1992; Yamada et al., 1993), and Bruton's tyrosine kinase or Btk (Hinshelwood et al., 1995). Kinetic experiments indicate that Src-family PTKs are activated first and that this correlates with Ig-α/Ig-β tyrosine phosphorylation (Saouaf et al., 1994). The requirement of Src-family PTK activity for initial ITAM phosphorylation was also suggested by
experiments with BCR-transfected nonlymphoid AtT20 cells (Matsuuchi et al., 1992; see also DeFranco et al., 1995). These cells express Fyn but not Syk, and receptor stimulation induces phosphorylation of Ig-α and Ig-β but not calcium mobilization. Exactly which tyrosine of the ITAM is phosphorylated within seconds of BCR stimulation is not known. One study implicated initial Src-family activity in the phosphorylation of the first tyrosine in the ITAM of Ig-α (Flaswinkel & Reth, 1994). Src-family PTK activity is important to the activation of other PTKs. These other PTKs include Btk (Mahajan et al., 1995; Afar et al., 1996; Rawlings et al., 1996; Park et al., 1996) and Syk (Kurosaki et al., 1994; Takata & Kurosaki, 1995).

The physical link between PTK activation and antigen receptor ligation stems from their co-association in the resting state. A small fraction of both Src-family and Syk PTKs have been found to associate with the unligated BCR in both the mouse and human (Yamanashi et al., 1991; Burkhardt et al., 1991; Hutchcroft et al., 1992; Lin & Justement 1992; Clark et al., 1992b; Leprince et al., 1992; 1993; Law et al., 1993). The interaction between Src PTK and the resting BCR does not require phosphorylation of the ITAM (Pleiman et al., 1994a; Clark et al., 1994). BCR stimulation does increase the binding of Syk and Src-family PTKs to the phosphorylated ITAM via SH2-domain engagement (Law et al., 1993; Clark et al., 1994; Kurosaki et al., 1995).

The PTKs involved in BCR signaling possess modules for protein-protein interactions such as Src homology 2- (SH2) and 3- (SH3) domains (for review see Cohen, 1995). SH2-domains bind phosphorylated tyrosine-containing motifs with high affinity whereas SH3-domains bind proline-rich motifs with lower affinity (Pawson, 1995). Syk has
two SH2-domains and all Src family PTKs have one (for review see Bolen, 1995; Superti-Furga & Courtneidge, 1995).

Both Syk and Src-family PTKs are activated by binding to phosphorylated ITAMs (Flaswinkel & Roth, 1994; Pleiman et al., 1994a; Clark et al., 1994; Kurosaki et al., 1995; Rowley et al., 1995; Johnson et al., 1995). In resting cells, BCR-associated PTK activity is controlled by the activity of protein tyrosine phosphatases (PTPs). The role of PTP activity has been demonstrated with the PTP inhibitor sodium orthovanadate (Na₃VO₄) (Lin et al., 1992; Campbell et al., 1995; Wienands et al., 1996). Resting murine splenic B cells pre-incubated with Na₃VO₄ exhibit increased phosphorylation of Ig-α and Ig-β, as well as other proteins, on tyrosine (Lin et al., 1992). This effect can also be obtained by cross-linking CD45 (Lin et al., 1992). These and other data (Justement et al., 1991) have implicated CD45 in regulating BCR activation at the level of ITAM phosphorylation. SHP-1 is also thought to be involved in this regulation as both SHP-1 and CD45 have been shown to associate with the resting murine BCR (Brown et al., 1994; Pani et al., 1995) and dephosphorylate BCR subunits in vitro (Justement et al., 1991; Pani et al., 1995).

The resting cell also uses other mechanisms to regulate PTK activity. The most well-known example of this type is the phosphorylation of Src-family kinases by a PTK called Csk (Okada et al., 1991). Csk is the only known PTK capable of phosphorylating the carboxy-terminal tyrosine which negatively regulates Src-family kinase activity (Okada et al., 1991; Hata et al., 1994; for review see Cooper & Howell, 1993).

The current thinking is that the tyrosine-phosphorylated ITAM acts as a docking platform for the recruitment of SH2 domain-containing proteins like PTKs, the p85
component of phosphatidylinositol 3-kinase (PI-3K), SHC, RasGAP, and phospholipase C-γ (PLC-γ) (see Cambier et al., 1993; 1994). Syk is critical to the activation of many recruited effectors. Syk-deficient chicken B cells have impaired calcium flux and partial loss of protein tyrosine phosphorylation following antigen receptor stimulation (Takata et al., 1994), and Syk-, but not Src-, family PTKs trigger calcium mobilization when expressed as chimeras and aggregated (Kolanus et al., 1993). The existence of such multi-molecular complexes is supported by finding the accumulation of tyrosine phosphoproteins beneath the cross-linked BCR within 5 minutes of stimulation (Takagi et al., 1991).

I.A.3. Calcium Mobilization and the Phosphoinositide Pathway:

In B lymphocytes stimulated through antigen receptors, PLC-γ is activated by the coordinate activity of both Btk and Syk (Takata et al., 1994; Takata & Kurosaki, 1996). Activated PLC-γ initiates the phosphoinositide pathway by hydrolyzing membrane phosphatidylinositol 4,5-bisphosphate (PIP$_2$) generating second messengers inositol-1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG) (for review see Cambier et al., 1987a/b). The path followed by these second messengers is highlighted in Figure 2. IP$_3$, generated maximally about 1 minute after stimulation (Bijsterbosch et al., 1985b), binds to receptors on the endoplasmic reticulum and mediates rapid calcium efflux leading to increased intracellular free calcium ([Ca$^{++}$]$_i$) (Ransom et al., 1986). This is followed by a more prolonged influx of calcium from the extracellular space through plasma membrane calcium channels (for review, see Clapham, 1995). Both DAG and calcium are involved in the activation of protein kinase C (PKC). Optimal activation of PKC occurs following membrane translocation, within 2-4 minutes of receptor stimulation (Chen et al., 1986; Nel
Figure 2: A model of proximal events in B cell receptor (BCR) signaling. Following tyrosine phosphorylation of the ITAM, recruitment and activation of protein tyrosine kinases, the phosphoinositide and ras pathways are activated. See text for more detail. Key signaling intermediates include Shc, GAP, and Vav in the Ras pathway, and PLC-γ in the phosphoinositide pathway.
PKC activity has been implicated in diverse signaling roles including membrane-cytoskeletal interactions, the activation of mitogen-activated protein kinase (MAPK), and IL-2 gene expression (for reviews see Nishizuka, 1995; Jaken, 1996).

The phosphoinositide pathway in B lymphocytes can also be activated by G-proteins (Gold et al., 1987; Harnett & Klaus, 1988; Lane et al., 1991), and the level of PIP$_2$ hydrolysis is apparently equivalent to that induced by mIg cross-linking (Gold et al., 1990). The relative contribution of G-proteins and PTKs in early B cell activation is an unresolved issue (see Figure 2 and for review see Harnett & Rigley, 1992). In human B cells, one report provided evidence that PTK activity is also controlled by the activity of an uncharacterized G-protein (Melamed et al., 1992).

1.4. Other Downstream Signaling Events:

The p21ras protooncogene is activated within 1 minute of receptor stimulation (Harwood & Cambier, 1993) and its activity has been linked to the activation of MAPK (Tordai et al., 1994). Ras can be activated through multiple mechanisms as represented in Figure 2. These mechanisms include membrane recruitment of two GDP/GTP exchange proteins, SOS and Vav, and one GTPase-activating protein called p120GAP (Trahey & McCormick, 1987; Feig et al., 1993; Gulbins et al., 1994; Lankester et al., 1994). Both Vav and GAP are tyrosine phosphorylated following BCR cross-linking (Bustelo & Barbacid, 1992; Gold et al., 1993). This apparently increases the GTP exchange activity of Vav (Gulbins et al., 1994; 1995); this has not, however, been corroborated by others (Bustelo et al., 1994). Ras activation through SOS proceeds via tyrosine phosphorylation of Ig-α.
recruited Shc which then recruits the Grb2/SOS complex to the membrane (Lankester et al., 1994; D’Ambrosio et al., 1996).

Lastly, PI-3K is also activated following BCR receptor stimulation (Gold et al., 1992; Pleiman et al., 1994b). PI-3K consists of a regulatory subunit (p85) and a catalytic subunit. The SH2 domains of p85 serves to translocate the catalytic subunit into the activated BCR complex. PI-3K phosphorylates the D-ring of inositol phospholipids and is involved in diverse processes such as mitogenesis, protein transport, and cytoskeletal rearrangement (for review see Kapeller & Cantley, 1994; Abraham, 1996).

**1.A.5. Regulation of BCR Activation:**

Other molecules can affect the outcome of BCR activation. Minimally, this list includes the Fc receptor for IgG (FcγRII or CD32), CD19/21 or the complement receptor 2 (CR2) complex, CD22, CD19, and CD40. Both CD22 and FcγRII are of particular importance to this study.

When FcγRII is co-ligated with mIg (for instance by intact anti-Ig antibody or antibody-coated antigen), the activation signal is aborted. This inhibition involves the premature termination of IP₃ generation and calcium influx (Bijsterbosch & Klaus, 1985b; Diegel et al., 1994; Choquet et al., 1993; Muta et al., 1994). In contrast to murine B lymphocytes which express only the b1 isoform of FcγRII, human B cells express all isoforms (a, b1, b2, and c) (for review see van de Winkel & Capel, 1993; Gergely & Sarmay, 1996). From studies with human B cells, apparently only the b isoform is involved in signal termination (Van Den Herik-Oudijketal et al., 1994). The ability of FcγRIIb to
inhibit calcium influx has been localized to a 13 amino-acid cytoplasmic sequence (Muta et al., 1994). Although both b isoforms contain this sequence, only the b1 isoform is tyrosine phosphorylated and co-caps with mIg in the human (Budde et al., 1994). The tyrosine in this motif is critical to inhibitory signaling in both human and mouse B cells (Muta et al., 1994; Budde et al., 1994; Van Den Herik-Oudijket al., 1994). Interestingly, this tyrosine is also critical to b2 inhibitory signaling even though it is not tyrosine phosphorylated (Budde et al., 1994; Van Den Herik-Oudijket al., 1994). In the mouse, tyrosine phosphorylation of this sequence has been shown to recruit SHP-1 (D’Ambrosio et al., 1995) and the inositol phosphatase SHIP (Ono et al., 1996). This sequence has since been called the ITIM for Immunoreceptor Tyrosine-based Inhibitory Motif (see Thomas, 1995; Isakov, 1997). The molecular mechanisms of FcγRIIb-inhibitory signaling have not yet been elucidated.

CD22 is a 130/140 kDa B-lineage specific transmembrane glycoprotein that associates with the resting BCR at low stoichiometry (Peaker & Neuberger, 1993; Leprince et al., 1993; for review see Law et al., 1994). CD22 is important to B lymphocyte responses to type II T-independent antigens that cross-link sIg (Otipoby et al., 1996; Sato et al., 1996; Nitschke et al., 1997). Although the exact role of CD22 has yet to be defined, the presence of CD22 does dampen the rise in [Ca^{2+}]_i following BCR cross-linking (Otipoby et al., 1996; Sato et al., 1996; Nitschke et al., 1997). Both CD22-deficient (CD22/-) murine splenic B cells and CD22 cross-linked human B cells display elevated levels of [Ca^{2+}]_i in response to anti-Ig stimulation (compare the result of Pezzutto et al., 1987 with those of Otipoby et al., 1996). Unlike CD22/- murine B cells however, human B cells treated with soluble or immobilized anti-CD22 exhibit enhanced, as opposed to depressed, proliferation
in response to anti-Ig (Pezzuto et al., 1987; 1988; Doody et al., 1995). CD22-/ B cells display a surface phenotype characteristic of anergic B cells. Mature CD22-/ B cells exhibit approximately 50 percent less sIgM as compared to wild-type and increased levels of MHC class II antigens (Sato et al., 1996; Nitschke et al., 1997). Other activation markers (B7-1/2), however, are not increased. The phenotype and response of CD22-/ B lymphocytes to anti-Ig also resembles that of SHP-1 defective splenic B cells. This aspect will be elaborated upon further in the next section on SHP-1.

CD22 performs multiple roles in BCR activation. In addition to synergizing with anti-Ig, CD22 was recently found to be required for heterologous desensitization. Sato and colleagues (1996) observed that CD22-/ B cells do not undergo desensitization following BCR cross-linking, unlike control B cells. Normally, and following BCR ligation, the B cell is desensitized to heterologous signals through unligated sIg receptors (Cambier et al., 1988: 1990; Lazarus et al., 1990; 1991; Rijkers et al., 1990). The function of CD22 in mediating receptor desensitization may be similar FcγRIIb inhibitory signaling as both recruit SHP-1 to an ITIM motif.

A model for CD22 signaling during early activation events is emerging. Following BCR cross-linking, CD22 is rapidly tyrosine phosphorylated (within 30 seconds) (Schulte et al., 1992; Campbell & Klinman, 1995; Law et al., 1996a) and subsequently recruits several effectors to the plasma membrane. These effectors include SHP-1, Syk, PLC-γ1, and PI-3K (Peaker & Neuberger, 1993; Leprince et al., 1993; Doody et al., 1995; Campbell & Klinman, 1995; Tuscano et al., 1996b; Law et al., 1996a/b). SHP-1 is recruited to three ITIM motifs within murine CD22 (Doody et al., 1995) and to possibly four ITIM motifs
within human CD22. The molecular mechanisms by which CD22 regulates intracellular calcium and receptor-induced desensitization have yet to be defined.

I.B. SHP-1

SHP-1 (also known as SH-PTP1, HCP, or PTP1C) is a 67 kDa cytosolic tyrosine phosphatase expressed predominantly in hematopoietic cells (for review see Bignon & Siminovitch, 1994). Recent data has linked the motheaten (me) and viable motheaten (meV) phenotypes to mutations in the gene encoding SHP-1 (Tsui et al., 1993; Schultz et al., 1993). Both me and meV mice display profound immunodeficiency and autoimmunity due to multiple hematopoietic abnormalities. The aberrant phenotype of these mice points to an important role for SHP-1 in hematopoietic cell development and function.

I.B.1. SHP-1 Signaling in B Lymphocytes:

SHP-1 has been shown to function as a negative regulator of lymphocyte activation (Pani et al., 1995; 1996; for review see Pani & Siminovitch, 1997). In B cells, SHP-1 associates with the resting antigen receptor and is thought to keep Ig-α dephosphorylated (Pani et al., 1995). Consistent with such a role, SHP-1 is capable of dephosphorylating Ig-α in vitro, and me splenic B cells proliferate maximally in response to 10-fold less anti-IgM antibody (Pani et al., 1995).

SHP-1 can be recruited and activated by three ITIMs in the cytoplasmic tail of CD22 (Doody et al., 1995). This ITIM is defined by the sequence I/VX(p)YXXL/V (for review see Pani & Siminovitch, 1997; Ulyanova et al., 1997). What follows from this event is currently being defined. CD22 may recruit SHP-1 only for the purpose of its
SHP-1 is also recruited by the ITIM sequence of FcγRIIb1 following its co-ligation with mIg (see Section I.A.5). Recently PLC-γ2 was shown to be tyrosine dephosphorylated after 5 minutes stimulation with intact anti-Ig antibody (Sarkar et al., 1996). Thus, SHP-1 may perform a similar role in both FcγRIIb inhibitory and CD22 signaling. Whatever the substrates for SHP-1 in FcγRIIb1-inhibitory signaling, SHP-1 is essential. This has been demonstrated by the inability of intact anti-Ig to inhibit the proliferation of me and me' splenic B cells (Pani et al., 1995; D'Ambrosio et al., 1995).

I.B.2. SHP-1 Activation:

The structure of SHP-1 is shown in Figure 3. It contains two tandem SH2 domains and at least two sites for tyrosine phosphorylation. SHP-1 activity is regulated primarily by SH2 domain engagement (Pei et al., 1994). This releases the PTPase domain from a
Figure 3: The domain structure of human SHP-1. SHP-1 is a non-receptor protein tyrosine phosphatase with two amino-terminal SH2 domains, one carboxy-terminal catalytic domain, and at least two C-terminal sites of phosphorylation on tyrosines 538 and 566 as indicated (Shen et al., 1991). Tyrosine 538 is a consensus motif for the SH2 domain of Grb2 (Bouchard et al., 1994).
negative phosphotyrosine-independent interaction. Apparently the N-terminal SH2 domain functions specifically in the activation of SHP-1 whereas both domains participate in its recruitment (Pei et al., 1996). Other possible modes of SHP-1 activation include its association with anionic phospholipids (Zhao et al., 1993) and its tyrosine phosphorylation (Uchida et al., 1994). The role served by anionic phospholipids is particularly interesting because phosphatidic acid levels rise slowly following BCR stimulation, reaching maximum levels after 10 minutes (Coggeshall & Cambier, 1984). The possibility that phosphatidic acid activates SHP-1 in vivo is further enhanced by the recruitment of SHP-1 to the plasma membrane (via CD22) following BCR activation.

I.C. Epstein-Barr Virus (EBV) Transformation

EBV is a typical human herpesvirus and establishes two life cycles: a replicative or lytic life cycle in the oropharyngeal epithelium and a latent life cycle in B lymphocytes (for an extensive review of EBV, see Liebowitz & Kieff, 1993). During latent infection of human B cells in vitro only a small subset of EBV genes are expressed. This expression leads to the outgrowth of immortal lymphoblastoid cell lines (LCLs) in about 10% of the infected B lymphocytes.

EBV infection begins with viral attachment and B cell activation. The envelope glycoprotein (gp) 350/220 binds CD21 on the B cell (Fingeroth et al., 1984; Frade et al., 1985). Viral attachment to CD21 mediates internalization and the co-capping of CD21 with mlg (Tanner et al., 1987). CD21 has been shown to play an important role in the internalization of complement coated antigen and in setting the threshold for B cell activation (for review see Fearon & Carter, 1995). Following uptake, at least 11 latent gene
products are expressed: six nuclear antigens, two nonpolyadenylated RNAs, and three latent integral membrane proteins (LMP 1, 2A, 2B) (for review, see also Joske & Knecht, 1993). Of the nuclear antigens, EBNA2 is a key mediator of transformation. LMP1 is also important to EBV transformation (Kay et al., 1993). In fact, LMP1 has been shown to induce many of the phenotypic changes associated with transformation including the increased expression of adhesion molecules and activation markers such as LFA-1 and CD23 (see Liebowitz & Kieff, 1993). A significant fraction of LMP1 aggregates in the plasma membrane (Mann et al., 1985; Liebowitz et al., 1986). Recently LMP1 was shown to suppress transcription of the Igμ gene (Jochner et al., 1996).

I.D. Latent Membrane Protein 2 (LMP2)

Several different LMP2 deletion mutants of EBV were created by Longnecker and colleagues (1992; 1993a/b). In each case, the LMP2 gene product was found to be inessential for EBV transformation. A role for LMP2 in the maintenance of EBV latent infection was suggested by its ability to block calcium mobilization following treatment with either anti-MHC class II, anti-CD19, or anti-Ig antibody (Miller et al., 1993). Not long after, the absence of LMP2 was found to correlate with a higher frequency of lytic cycle gene induction (Miller et al., 1994). Thus, and based on these in vitro studies, LMP2 is thought to serve a role in maintaining EBV latency.

The LMP2 gene is simultaneously transcribed under the control of two promoters giving rise to two gene products: LMP2A and LMP2B (see Figure 4; for review see Longnecker, 1994). Both LMP2A and LMP2B are transmembrane-spanning proteins. LMP2B (40 kDa) is smaller than LMP2A (54 kDa) because it lacks an 119 amino-acid N-
Figure 4: The predicted structure of EBV latent membrane protein 2 (LMP2) gene products LMP2A and LMP2B. LMP2A and LMP2B both contain 12 hydrophobic transmembrane-spanning domains and a 27-amino-acid cytoplasmic carboxy-terminal domain. LMP2B initiates at the methionine as indicated and does not contain the 119-amino-acid cytoplasmic N-terminal domain unique to LMP2A. The numbers denote the locations of eight tyrosine residues in the amino terminus of LMP2A. Adapted from Fruehling et al., 1996.
terminal cytoplasmic domain. This domain of LMP2A contains eight tyrosines, two of which (74 and 85) conform to the ITAM consensus sequence. This ITAM-like sequence is thought to account for the ability of LMP2A to mobilize calcium when expressed as a chimeric protein and cross-linked (Beaufils et al., 1993; Alber et al., 1993). Table 1 presents data highlighting structural motifs present in LMP2A. LMP2A is constitutively tyrosine phosphorylated, both in vitro and in vivo (Miller et al., 1995). Exactly which tyrosines are constitutively and/or inducibly phosphorylated is not known. Tyrosine phosphorylation of LMP2A is thought to stem from the activity of both Syk and Src family PTKs based on co-immunoprecipitation studies (Burkhardt et al., 1992; Miller et al., 1995). LMP2A localizes primarily to the plasma membrane in association with LMP1 (Longnecker & Kieff, 1990). Many of the properties of LMP2A appear to be independent of other latent genes because transfected LMP2A also aggregates in the plasma membrane and is constitutively tyrosine phosphorylated in B lymphoma cells (Longnecker et al., 1991).

It is currently thought that LMP2 blocks signaling through the BCR by recruiting both Syk and Src family PTKs in a dominant-negative fashion. This hypothesis was presented in a recent publication by Miller and colleagues (1995). They showed that induction of protein tyrosine phosphorylation following receptor stimulation was blocked in B lymphocytes transformed by normal (LMP2+) EBV but not in lymphocytes transformed by a mutant virus lacking LMP2. Key signaling intermediates (p85, PLC-γ2, Vav, Syk) were constitutively tyrosine phosphorylated in LMP2+ LCLs but not LMP2- LCLs. The work of Miller et al. did not investigate other mechanisms for LMP2-mediated inhibition. Such mechanisms include the activation of negative regulators like SHP-1.
TABLE 1: Sequence Motifs of Latent Membrane Protein 2 (LMP 2)

A. Amino-Terminal Domain Motifs

<table>
<thead>
<tr>
<th>SH2 Domain Motifs*</th>
<th>Possible Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y₁₁₀PSA</td>
<td></td>
</tr>
<tr>
<td>Y₁₆₀EDP</td>
<td></td>
</tr>
<tr>
<td>Y₁₄QPL**</td>
<td>Syk, Src-family PTKs</td>
</tr>
<tr>
<td>Y₈₅LGL**</td>
<td>Syk, Shc, SHP-1, Src-family PTKs</td>
</tr>
<tr>
<td>Y₁₁₂EEA</td>
<td></td>
</tr>
<tr>
<td>Y₁₂DGG</td>
<td></td>
</tr>
<tr>
<td>Y₄₄WGN</td>
<td></td>
</tr>
<tr>
<td>Y₁₄₀SPR</td>
<td>Csk</td>
</tr>
</tbody>
</table>

* Fruehling et al., 1996; Songyang et al., 1994.

**Both Y74 and Y85 form a typical ITAM motif.

WW Domain Binding Motifs

| PPPPY₁₂₀           |
| PPPPY₆₁₀           |

1 See Staub & Rotin, 1996.

B. Carboxy-Terminal Domain Motif

RCCRYCCYYC Cysteine-rich region
Hypothesis

The purpose of this study was to test the hypothesis that the tyrosine phosphatase SHP-1 plays a role in LMP2’s abrogation of BCR signal transduction. It was initially thought that SHP-1 might bind to the phosphorylated motif Y85LGL which forms part of the ITAM-like motif in the amino-terminus of LMP2A. Following activation, SHP-1 was conceived to play a role in abrogating BCR signal transduction by functioning in manner similar to its role in FcγRII-mediated inhibitory signaling. Since substrates for SHP-1 in FcγRIIb1 signaling had not, at the time, been identified, this hypothesis also became a vehicle for the characterization of SHP-1 substrates.

Rationale

The rationale for this study was based on the established function of SHP-1 as a recognized negative regulator and terminator of BCR signals (see Section 1.B.) and the presence of a potential ligand for SHP-1 in the amino terminal cytoplasmic domain of LMP2A.

Experimental System

The goal of this study was to compare SHP-1 binding and activity in two different EBV-transformed lymphoblastoid cell lines (LCLs). One LCL was transformed with a virus in which the LMP2 gene had been mutated; this LCL did not express LMP2A. The other LCL was transformed by a wild-type virus which contained and expressed LMP2.
III. Material & Methods

Cell Lines and Cell Culture

Both wild-type (LMP2+) and mutant (LMP2-) LCLs were obtained from Dr. R. Longnecker (Northwestern University, Chicago). Controls for some experiments included the Burkitt lymphoma Daudi (obtained from Dr. N. Bernstein, University of Toronto) and JS 181, an EBV-transformed LCL from a healthy donor created in this laboratory. The lines were maintained at 37 °C in RPMI 1640 supplemented with 10-20% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (100 μg/ml).

The two EBV-lymphoblastoid cell lines were clones that had been transformed by clonally derived EBV (Dr. R. Longnecker, personal communication). Briefly, the LMP2 null mutant virus was created by mutating a cloned segment of EBV genomic DNA containing LMP2 (Longnecker et al., 1993a). A 643 base-pair deletion (corresponding to the first 5 transmembrane domains of LMP2A and LMP2B) was replaced with a drug resistance gene. The recombinant virus was cloned, and cell-free virus from one clone was used to transform primary B lymphocytes from the peripheral blood of a healthy volunteer. These LCLs were also cloned, and specific clones were expanded. Expanded clones were checked for the absence of deleted DNA (by PCR), the presence of deleted DNA (by Southern blot hybridization), and the absence of LMP2A protein (by western blotting with a polyclonal anti-LMP2A) (Longnecker et al., 1993a). LMP2B should also not be expressed; this was not confirmed however.
Antibodies

Goat F(ab’), anti-human Ig and anti-IgM antibodies (both from Southern Biotechnology) were used in cross-linking experiments. Rabbit polyclonal anti-LMP2 sera was prepared to a purified *Escherichia coli* TrpE-LMP2 fusion protein (codons 19 to 311 of LMP2) (Longnecker & Kieff, 1990) and donated by Dr. R. Longnecker. Some of the antisera was affinity-purified (Longnecker & Kieff, 1990; Longnecker et al., 1991). The immune sera was primarily reactive to the amino-terminal portion of LMP2A (Longnecker & Kieff, 1990; Longnecker et al., 1991; 1993a). Rabbit polyclonal anti-murine SHP-1 was generated in our lab by injecting rabbits with a GST-SH2-domain fusion protein (codons 1-884 or both SH2 domains) (Kozlowski et al., 1993). It was tested for cross-reactivity to SHP-2 and found to be essentially non-cross-reactive. Rabbit polyclonal anti-Vav, rabbit polyclonal anti-SHP-1 (directed to the C-terminus), and mouse monoclonal anti-Syk (4D10) antibodies were purchased from Santa Cruz Biotechnology. The mouse monoclonal anti-human CD22 antibody (HIB22) was obtained from PharMingen, and the mouse monoclonal anti-phosphotyrosine antibody (4G10) from Upstate Biotechnology. Control antibodies, either normal rabbit serum or normal mouse IgG, were obtained from Sigma. The FITC-conjugated goat anti-mouse IgG, streptavidin-FITC, and biotinylated F(ab’)2 goat anti-human Ig and goat IgG were purchased from Jackson Laboratories.

Other Reagents

All chemicals were obtained from Sigma unless otherwise indicated.
Cultured cells were resuspended in phosphate-buffered saline (PBS) at 100 X 10^6 cells/ml just prior to stimulation. Typically cells (0.5 ml) were equilibrated for 2 min at 37 °C and then F(ab')_2 anti-human Ig (25 μg) was added. Stimulation was carried out for two min at 37 °C, unless otherwise indicated, and the reaction was stopped by brief microcentrifugation (5 sec at 1000g). The supernatant was removed and the cells were resuspended in ice-cold lysis buffer (1 ml buffer per 50 X 10^6 cells) containing 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10 mM NaF, 1 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethysulfonyl fluoride, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, and detergents of either 1% NP 40 or 1% digitonin. In some experiments, a combination of detergents was used in the stringent buffer RIPA (1% NP 40, 0.1% SDS, and 0.5% sodium deoxycholate). Stimulation was occasionally performed with pervanadate (at 1:25 per unit vol cells) which was prepared before use by mixing 100 mM sodium orthovanadate and 0.5% (v/v) hydrogen peroxide (final conc.) in a 1:3 (v/v) ratio. Cells were lysed on ice for 30 min with occasional vortexing. Detergent insoluble material was removed by centrifugation at 4 °C for 10 min at 10,000g.

**Immunoprecipitation and Immunoblotting**

Post-nuclear cell lysates (typically 0.3 ml) were incubated on ice for 1-2 h with the appropriate antibody (typically 1 μg affinity-pure antibody or 5 μl antiserum). Immune complexes were collected for 1 h at 4 °C with either Protein A or G sepharose (Pharmacia). Collected immune complexes were washed 4 times in lysis buffer and then eluted in either reducing or non-reducing SDS sample buffer by boiling (5 min). In the case of LMP2A
immunoprecipitations, proteins were eluted by heating for 15 min at 70 °C to prevent the aggregation found to ensue upon boiling (Longnecker et al., 1991). Proteins were separated by electrophoresis through polyacrylamide SDS gels (SDS-PAGE) and transferred onto nitrocellulose (Schleicher and Schuller Inc.). The resulting nitrocellulose blots were blocked for a minimum of 3 h in 200 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% Tween 20 (TBS-T) containing either 3% gelatin (Mallinckrodt Baker, Inc.) or 5% instant skim milk powder. Immunoblotting was carried out by exposing the nitrocellulose blot first to primary antibody for 2 h and then to a horseradish-peroxidase (HRP) linked secondary antibody (either protein A-HRP from BioRad or sheep anti-mouse Ig-HRP from Amersham) for 1 h, both at room temperature. Secondary HRP-linked antibodies were diluted (as recommended by the manufacturer) in TBS-T immediately before use. Extensive washing with TBS-T was performed between and following both primary and secondary antibodies. HRP-linked secondary antibodies were detected with enhanced chemiluminescence or ECL (Amersham). Immunoblots were frequently reprobed by first stripping bound antibody in a buffer containing 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7) for 30 min at 65 °C.

Assay of Phosphatase Activity

Immunoprecipitates were washed a further two times in phosphatase buffer (10 mM EDTA, 150 mM NaCl, 100 mM Tris, pH 7.4, and 10 mM dithiothreitol) and incubated with 8 mM p-nitrophenol phosphate (pNPP) overnight at 37 °C in 200 μl phosphatase buffer. Reactions were terminated by the addition of 0.8 ml of 0.2 M NaOH, and the absorbance was measured at 410 nm. Phosphatase buffer containing pNPP was used for background
measurements. In addition, absorbance measurements were extrapolated to μmol pNPP hydrolyzed using a standard curve prepared with p-nitrophenol. Equal quantities of precipitated protein were confirmed by western blotting if possible.

Calcium Measurements

Cells (20 X 10^6) were incubated in 2 ml media containing 5 μl of 1.5 M Indo-1 in dimethylsulfoxide for 30 min at 37 °C and then washed 3 times in PBS without calcium. Washed cells were resuspended in 10 ml PBS supplemented with 1 mM CaCl₂ (final cell conc. = 2 X 10^6/ml). Fluorometric analysis of intracellular calcium was undertaken with a LS50 luminescence spectrophotometer. Fluorescence was detected at an excitation wavelength of 336 nm and an emission wavelength of 430 nm. A stable baseline for intracellular calcium levels was established for 1 min prior to adding 10 μg F(ab')₂ goat anti-human Ig to 1 ml of cell solution. Maximum calcium levels (1 mM) were assessed by cell lysis with 1% Triton X-100.

Flow Cytometry

Cells (10^6) were incubated with 1 μg of appropriate primary antibody (as indicated in Figure legends) in 50 μl PBS supplemented with 1% FBS (staining medium) for 1 hr on ice, washed with staining medium, and further incubated for 1 hr with appropriate FITC-conjugated secondary antibody (1 μg in 50 μl; antibody as indicated in legends). Cells were washed and resuspended in staining medium (1 ml) supplemented with propidium iodide (1 μg/ml). Live cells (propidium iodide negative) were analyzed on a FACScan flow cytometer (Becton Dickinson).
IV. RESULTS

Confirmation of LCL Characteristics

The LCLs received from Dr. Longnecker were first examined for surface IgM (sIgM) levels and their response to BCR stimulation. As shown in Figure 5, both LCLs expressed a low level of sIgM as reported (Miller et al., 1994; 1995). LMP2 has been shown to prevent both calcium mobilization and the induction of protein tyrosine phosphorylation by comparing the responses of two different LCLs, one that expressed LMP2 (LMP2+) and one that did not (LMP2-) (Miller et al., 1993; 1995). Figure 6 is an anti-phosphotyrosine western blot of total cell lysates from LMP2+ and LMP2- LCLs following anti-Ig cross-linking for 1, 5, and 20 min. The absence of inducible PTK activity in LMP2+ LCLs is evident. SHP-1 was used as a control for equal loading (Figure 6B). Also, and in agreement with reported data, Figure 7 demonstrates that calcium is mobilized in LMP2- LCLs following BCR cross-linking but not in LMP2+ LCLs.

SHP-1 Activity in Wild-Type (LMP2+) and Mutant (LMP2-) LCLs

The simplest way to begin to define a role for SHP-1 in LMP2’s abrogation of BCR signaling was to compare SHP-1 activity in wild-type and mutant LCLs. SHP-1 activity was assessed first in resting LCLs lysed with RIPA buffer in order to disrupt non-covalent interactions between SHP-1 and other proteins. Equal volumes of lysate were immunoprecipitated with either anti-SHP-1 or normal rabbit serum (NRS), and the precipitates were incubated with p-nitrophenol phosphate (pNPP). Tyrosine phosphatases can dephosphorylate pNPP in vitro to p-nitrophenol which absorbs light at 410 nm.
Figure 5: LCLs derived from LMP2- (panel A) and LMP2+ (panel B) EBV were stained with either biotinylated F(ab')2 goat anti-human immunoglobulin (unshaded peaks) or normal F(ab')2 goat IgG (shaded peaks) followed by streptavidin-FITC as described in the Materials and Methods. FACScan analysis was performed on a Becton Dickinson flow cytometer.
Figure 6: LCLs (2 X 10⁶) derived from either LMP2+ or LMP2- EBV were stimulated with 2.5 μg goat anti-human IgM in 50 μl PBS for 1.5, or 20 min at 37 C. After a brief centrifugation (5 sec X 1000g), the cells were lysed in 1% NP 40 and separated on a 10% reducing SDS-PAGE gel. Following transfer, the immunoblot was probed first with anti-phosphotyrosine (α-P-tyr) (panel A) and then stripped and reprobed with polyclonal anti-SHP-1 (panel B).
Figure 7: Indo-1 loaded LCLs derived from either LMP2+ (panel A) or LMP2- (panel B) EBV were stimulated with F(ab')2 goat anti-human immunoglobulin (α-Ig) approximately 50 sec after beginning to monitor intracellular calcium levels. Triton X-100 was added to lyse cells and record maximum calcium.
Figure 8A shows that the activity of SHP-1 was 10-fold higher (SD = 4, n = 3) in LMP2+ LCLs as compared to LMP2-. Subsequent to the phosphatase assay, total SHP-1 protein was analyzed by western blotting. In all cases, the quantity of SHP-1 in immunoprecipitates of either LMP2+ or LMP2- LCLs was found to be the same. A representative western blot is shown in Figure 8C. These data suggested that the presence of LMP2 protein was sufficient to enhance SHP-1 activity. SHP-1 phosphatase activity was also measured following two minutes treatment with F(ab')2 anti-Ig antibody. The time point of two minutes was based on the kinetic profile of Figure 6. As shown in Figure 8B, net SHP-1 activity was relatively unchanged in both LCLs following BCR stimulation.

**LMP2 Immunoprecipitation**

On finding elevated SHP-1 activity in the LCLs which expressed LMP2 protein, it then became important to determine whether or not SHP-1 and LMP2 could be co-immunoprecipitated. Finding that LMP2A co-immunoprecipitated with SHP-1 would have suggested a physical basis for direct activation of SHP-1 by LMP2A. In this experiment resting LCLs were lysed in digitonin and precipitated with anti-LMP2A or normal rabbit serum (NRS) as a control. The resulting anti-phosphotyrosine western blot of anti-LMP2A precipitates is shown in Figure 9A. A tyrosine phosphoprotein (pp) of about 55 kDa co-precipitated with anti-LMP2. This blot was then stripped and reprobed with anti-LMP2A antibody, but no band above background could be discerned (data not shown). Not being able to detect LMP2A by Western Blotting contradicts results previously reported by Miller et al., 1995. The reason for my inability to detect LMP2A by Western Blotting is unknown. I did, however, notice that anti-LMP2A antibody reacted strongly only with LMP2A-
Figure 8: LCLs (20 X 10^6) derived from either LMP2+ or LMP2- EBV were lysed in RIPA buffer and immunoprecipitated with 20 µl of either normal rabbit serum (NRS) or polyclonal anti-SHP-1 serum (SHP). After washing sequentially in lysis buffer and phosphatase buffer, the precipitates were resuspended in 200 µl phosphatase buffer containing 8 mM pNPP (or 1.6 µmol) and incubated overnight at 37 °C. The reaction was stopped and formation of product was detected at 410 nm as described in the Materials and Methods. The result shown in panel A is the average (± SD) of three separate experiments. In two experiments the cells were stimulated prior to lysis (panel B), and background pNPP hydrolysis, as determined by immunoprecipitation with NRS, was subtracted from SHP-1 determined hydrolysis. All experiments were based on equal quantities of SHP-1 in the precipitates as assessed by Western blotting. A representative blot is shown in panel C.
Figure 9: LCLs (15 X 10⁶) from either LMP2+ or LMP2- EBV were lysed in 1% digitonin and immunoprecipitated with either 50 μl affinity-pure polyclonal anti-LMP2A (LMP) or 0.5 μl normal rabbit serum (NRS). Following fractionation of the precipitates on a 9% non-reducing SDS-PAGE gel and transfer, the resulting immunoblot (panel A) was probed with anti-phospho-tyrosine (α-P-tyr). This immunoblot was stripped and reprobed with polyclonal anti-SHP-1 (molecular weight of SHP-1 is 68 kDa; panel B). Figure 9 is representative of 3 experiments.
chimeric proteins (immunoprecipitated from transfected cells of Beaufils et al., 1993) from untreated but not from pervanadate-treated cells (data not shown). This suggested that anti-LMP2A antiserum preferentially reacted with non tyrosine phosphorylated LMP2A. This most likely reflects the fact that the antiserum was raised to a bacterially expressed fusion protein. Based on the strong phosphotyrosine content of pp55, it is likely a mixture of LMP2A (54 kDa) and p53/56lyn (Burkhardt et al., 1992; Longnecker et al., 1991).

Burkhardt et al. (1992) reported that LMP2A was the major tyrosine phosphoprotein in LMP2+ LCLs at 55 kDa by in vitro kinase assay and re-immunoprecipitation. To check for a physical association between LMP2A and SHP-1, the blot in Figure 9A was stripped and probed with anti-SHP-1. As can been seen in Figure 9B, SHP-1 did not co-precipitate with anti-LMP2A antibody. The high background in control (NRS) lanes arose from protein overloading and a long exposure time.

Although no association between SHP-1 and LMP2A was found in resting LMP2+ LCLs, the possibility of an inducible association had yet to be tested. In the next experiment, LMP2+ LCLs were stimulated with either F(ab')2 anti-Ig or pervanadate. Pervanadate is a pharmacological agent used to inhibit tyrosine phosphatases and is reported to mimic many of the effects of receptor stimulation including IL-2 induction and mitogenesis (Evans et al., 1994; Campbell et al., 1995). Figure 10 shows the tyrosine phosphoproteins co-precipitating with anti-LMP2A. The band of 55 kDa was reproducibly found in both resting and stimulated wild-type LCLs. With pervanadate stimulation, a tyrosine phosphoprotein of 60 kDa (pp60) co-precipitated with anti-LMP2A. The identity of pp60 could not, however, be confirmed as SHP-1 by western blotting (data not shown).
**Figure 10:** Wild-type (LMP2+) LCLs (20 X 10⁶) were stimulated with either F(ab')₂ anti-immunoglobulin (α-Ig) or pervanadate (PerV), lysed in 1% digitonin, and immunoprecipitated with 15 μl of either anti-LMP2 serum (LMP) or normal rabbit serum (NRS). After separation (8% non-reducing gel) and transfer, the immunoblot was probed with anti-phosphotyrosine (α-P-tyr).
The activity of SHP-1 is known to be upregulated by SH2 domain engagement (see Section I.B.2). This prospect was investigated by immunoprecipitating SHP-1 from both resting and anti-Ig treated LCLs. As shown in the anti-phosphotyrosine western blot of Figure 11A, anti-SHP-1 co-precipitated pp140 in both resting and stimulated LCLs of wild-type or mutant origin. Although an anti-CD22 antibody for Western Blotting was not available within the time frame of this thesis, phosphoprotein pp140 likely represents CD22 because it has been reported to co-precipitate with SHP-1 by several groups using similar techniques (Lankester et al., 1995; Law et al., 1996a). Other than pp140, SHP-1 appeared to associate with fewer tyrosine phosphoproteins in LMP2+ LCLs as compared to LMP2- (see Figure 11B for verification of equal loading). For instance, pp130 inducibly associated with SHP-1 only in LMP2- LCLs. Finding fewer tyrosine phosphorylated proteins co-precipitating with SHP-1 in LMP2+ LCLs was consistent with its elevated activity. Aside from pp130, the only other discernible difference was the association of a 30 kDa tyrosine phosphoprotein with SHP-1 exclusively in LMP2+ LCLs. No specific tyrosine phosphoprotein could be correlated with elevated SHP-1 activity in wild-type (LMP2+) LCLs except perhaps pp30. The identity of pp30/32 is not known. Interestingly, since pp140 was inducibly tyrosine phosphorylated in wild-type LCLs, some tyrosine kinase must have been activated by BCR cross-linking.

Another possible regulatory feature of SHP-1 is its tyrosine phosphorylation state. To examine the phosphorylation level of SHP-1, the experiment described for Figure 11A was repeated except that the cells were lysed in RIPA buffer instead of digitonin. RIPA
**Figure 11:** LCLs (16 X 10⁶) derived from either LMP2+ or LMP2- EBV were either left unstimulated or stimulated with 20 μg F(ab')₂ anti-human immunoglobulin (α-Ig), lysed in 1% digitonin, and immunoprecipitated with 1 μg of either anti-SHP-1 (SHP) from Santa Cruz Biotechnology or normal rabbit Ig (NRS). Following fractionation on a 9% reducing gel and transfer, the immunoblot (panel A) was probed with anti-phosphotyrosine (α-P-tyr). After stripping, the blot was reprobed with polyclonal anti-SHP-1 (panel B). Figure 11 is representative of 3 experiments.
buffer contains 0.1% SDS and should disrupt non-covalent interactions between SHP-1 and other proteins. The absence of SHP-1 phosphorylation is evident in Figure 11A even though the LCLs were lysed in digitonin. Figure 12A repeats this observation and shows that SHP-1 is not tyrosine phosphorylated in wild-type LCLs with or without stimulation. Tyrosine phosphorylation could not, therefore, have accounted for the elevation of SHP-1 activity. Interestingly, the pp140 which appeared to be inducibly tyrosine phosphorylated in Figure 11A was not so induced in Figure 12A. This suggested that an inducible tyrosine phosphoprotein of 140 kDa interacted weakly with SHP-1 in LMP2+ LCLs.

**CD22 and SHP-1**

In an effort to address possible mechanisms mediating enhanced SHP-1 activity in LMP2+ LCLs, CD22 was examined. First, surface expression of CD22 was analyzed by flow cytometry (Figure 13). The two cell lines that lacked LMP2 (DAUDI and MUTANT) exhibited lower levels of CD22 surface expression than the two LCLs expressing LMP2 (JS181 and WILD-TYPE). These data suggested that the LMP2, or the coordinate interaction of LMP2 with other EBV latent genes, upregulated CD22 surface expression.

To explore the possibility that elevated SHP-1 activity stemmed from its association with CD22, CD22-associated phosphatase activity was assessed. CD22 was immunoprecipitated from resting or stimulated LCLs, and the precipitates were incubated with pNPP. Figure 14A illustrates the presence of protein tyrosine phosphatase (PTP) activity in resting LMP2+ LCLs but not in resting LMP2-LCLs. This observation reversed on BCR stimulation so that CD22-associated PTP activity was detectable only in mutant LCLs. CD22 immunoprecipitates were then blotted for SHP-1 (Figure 14B). SHP-1
Figure 12: This experiment repeated the experiment detailed for Figure 11 except the cells were lysed in RIPA buffer. The immunoblot (panel A) was probed with anti-phosphotyrosine (α-P-tyr), and then it was stripped and reprobed with anti-SHP-1 (panel B).
Figure 13: Cells from wild-type (LMP2+), mutant (LMP2-), or JS181 LCLs and Daudi were first stained with either normal mouse IgG or anti-human CD22 and then with FITC-conjugated goat anti-mouse IgG. Flow cytometry was performed as described in the Materials and Methods.
Figure 14: LCLs (50 X 10⁶) derived from LMP2+ and LMP2- EBV were either left unstimulated or stimulated with 50 μg F(ab')₂ anti-Ig (α-Ig), lysed in 1% NP40, and immunoprecipitated with 5 μg of either normal mouse IgG (IgG) or anti-human CD22. The precipitates were washed sequentially in lysis and phosphatase buffer, and then incubated with substrate pNPP as described in the Materials and Methods. The degree of pNPP hydrolysis as measured by absorbance (A410nm) for the two LCLs is presented in panel A. The precipitates were then subjected to electrophoresis and western blotted with polyclonal anti-SHP-1 (panel B).
protein could easily be detected in stimulated LMP2+ LCLs precipitates. Much less SHP-1 protein was detected in resting LMP2+ LCLs precipitates. This could indicate that a PTP different from SHP-1 associates with CD22 in LMP2+ LCLs. Alternatively, these data could be used to reinforce the idea that CD22-associated SHP-1 activity is very high in resting wild-type LCLs (activity is measured in product formed/unit enzyme/unit time) and that SHP-1 activity associates with another tyrosine phosphoprotein following receptor cross-linking.

**LMP2 and Vav**

This study was interrupted by the clonal outgrowth of LMP2+ LCLs. SHP-1 activity was almost as low as that observed in LMP2- LCLs. The experiments performed above were completed when SHP-1 activity was still elevated. In spite of low SHP-1 activity, these variant LMP2+ LCLs did not respond to BCR stimulation by induction of protein tyrosine phosphorylation (Figure 15B). As illustrated in Figure 15A, Vav co-precipitated with LMP2 from resting but not stimulated NP 40 lysates of LMP2+ LCLs. The Vav associating with LMP2 was weakly tyrosine phosphorylated and detectable only following plasma membrane enrichment (data not shown). In support of a role for Vav in LMP2-mediated abrogation of BCR signal transduction, Vav was tyrosine dephosphorylated in LMP2+ LCLs following cross-linking (Figure 16). This is opposite to the inducible phosphorylation normally found in B lymphocytes (Bustelo & Barbacid, 1992) and in the LMP2- mutant LCLs. Densitometric analysis of the both anti-phosphotyrosine and anti-Vav western blots revealed that Vav was inducibly dephosphorylated on tyrosine by 23% (as compared to resting levels) in LMP2+ LCLs and inducibly phosphorylated by 30% in LMP2- LCLs (also compared to resting levels).
Figure 15: Wild-type (LMP2+) LCLs (20 X 10⁶) were either left unstimulated or stimulated with 20 µg F(ab')₂ anti-Ig (α-Ig), lysed in 1% NP40, and immunoprecipitated with 10 µl of either normal rabbit serum (NRS) or polyclonal anti-LMP2 serum. After fractionation (9% reducing gel) and transfer, the blot (panel A) was probed with anti-Vav. In the same experiment, the degree of stimulation of wild-type LCLs, as compared to mutant, was checked by western blotting 2 µl of total cell lysate with anti-phosphotyrosine (α-P-tyr) (panel B). Figure 15 is representative of 2 experiments.
Figure 16: LCLs \((50 \times 10^6)\) derived from LMP2+ or LMP2- EBV were either left unstimulated or stimulated with 50 μg F(ab')2 anti-immunoglobulin (α-Ig), lysed in 1% digitonin, and immunoprecipitated with 5 μg of either normal rabbit Ig (NRS) or anti-human Vav. The immunoprecipitates were then fractionated (9% reducing gel). After transfer, the immunoblot (panel A) was probed with anti-phosphotyrosine (α-P-tyr). The blot was then stripped and reprobed first with anti-SHP-1 (data not shown) and then with anti-Vav (panel B). Figure 16 is representative of 2 experiments.
In this and previous studies, the function of LMP2 was investigated by comparing the responses of two different LCLs, one transformed with wild-type (LMP2+) EBV, and the other, with a LMP2-defective virus. Miller and colleagues (1995) established that LMP2 inhibited BCR signal transduction in a dominant-negative fashion by recruiting Syk and Src-family tyrosine kinases. In this study, data was presented showing that LMP2 protein expression in EBV-transformed LCLs enhanced the activity of SHP-1, a recognized negative regulator of BCR signal transduction.

SHP-1 activity was elevated about ten-fold in resting LMP2+ LCLs as compared to LMP2- LCLs (Figure 8). Moreover, BCR cross-linking appeared to have little effect on total SHP-1 activity in either LCL. The upregulation of SHP-1 activity appeared to be an indirect consequence of LMP2 expression because no physical association between LMP2 and SHP-1 could be detected by conventional western analysis. This lack of association, however, cannot be regarded as definitive because of the low sensitivity of Western Blotting. In support of this, Syk was not detected in LMP2 precipitates (Figures 9 & 10) by Western Blotting whereas Miller et al. (1995) detected their co-association by in vitro kinase assay. In spite of the low sensitivity of western blotting, SHP-1 would not, theoretically speaking, likely bind tyrosine phosphorylated LMP2A. This argument is based on the fact that LMP2A does not possess the ITIM sequence recently defined by I/VX(p)YXXL/V (see Section 1.B.1). It is also based on the idea that SHP-1 associates with the resting and not activated BCR (Pani et al., 1995) and that LMP2A resembles an activated BCR. These structural similarities between LMP2A and an activated BCR include an ITAM-like
sequence, tyrosine phosphorylation, aggregation into patches in the plasma membrane, and co-clustering with tyrosine phosphoproteins (compare the results of Takagi et al., 1991 with those of Longnecker et al., 1991). Unlike the BCR however, LMP2A exhibits these features constitutively.

Elevated SHP-1 activity may have been a consequence of increased CD22 surface expression in LMP2+ LCLs, but increased CD22 expression may not have been a direct consequence of LMP2 expression. This hypothesis is based on data supporting the normal elevation of CD22 expression following BCR activation (Campana et al., 1985). The expression of LMP2 could, for instance, arrest the B lymphocyte at a point just following BCR cross-linking. Consistent with the idea that LMP2+ LCLs are a priori activated B cells, CD22 was constitutively associated with a more active PTP in LMP2+ LCLs as compared to LMP2- (Figure 14). Interpreting CD22-associated phosphatase activity is, unfortunately, complicated by the assay used in this study to assess phosphatase activity. CD22 is a substrate for SHP-1 (Law et al., 1996a). Consequently, the ability of associated phosphatases to dephosphorylate pNPP does not measure the release of inorganic phosphate from CD22. Measuring the release of inorganic phosphate would have been particularly important to the interpretation of CD22-associated phosphatase activity following BCR stimulation because SHP-1 inducibly associates with a number of tyrosine phosphoproteins. Thus, and by implication, CD22-associated PTP activity following receptor stimulation was likely underestimated (Figure 14A). This underestimation would also apply to total SHP-1 activity following receptor stimulation (Figure 8B). Although this study would have benefited from measuring the release of inorganic phosphate as opposed to the hydrolysis of pNPP, it is unlikely that the basic finding of constitutively elevated SHP-1 activity in
LMP2+ LCLs would have changed. In support of this, both LCLs exhibited a low level of constitutive tyrosine phosphorylation (see Figures 6, 11, & 12), and this low level would not likely have differentially affected the measurement of phosphatase activity.

One possible role served by the elevation of SHP-1 activity is the dephosphorylation of the induced pp140 following BCR engagement. If pp140 is CD22, these data would be consistent with the normal sequence of events following BCR cross-linking: CD22 is tyrosine phosphorylated and recruits SHP-1 (see Section I.A.5). But pp140 could also be, for instance, PLC-γ1. The association between SHP-1 and PLC-γ1 following stimulation would be consistent with SHP-1 counteracting any residual activity of Syk. The exact role served by activated SHP-1 in LMP2+ LCLs, however, requires further experimentation.

One method of determining SHP-1 substrates involves pre-incubating cells with a non-toxic dose of sodium orthovanadate (to inhibit endogenous phosphatase activity so that substrates are phosphorylated) and capturing SHP-1 binding proteins by immunoprecipitation. These data could then be analyzed in conjunction with those from untreated LCLs to determine which tyrosine phosphoproteins increase by pre-treatment with sodium orthovanadate.

Another more direct method involves transfecting catalytically inactive SHP-1 into LMP2+ LCLs. This would also address the effect of SHP-1 on LMP2-mediated abrogation of BCR signal transduction, in addition to defining the phosphoproteins held within the active site of the dominant-negative enzyme by the technique of substrate trapping.

Another possible role for SHP-1 activity in LMP2+ LCLs is the dephosphorylation of Shc. Miller et al. (1995) reported that Shc was not tyrosine phosphorylated in resting LMP2+ LCLs. This was interpreted as support for genetic data indicating that LMP2 was
not involved in MAPK activation and therefore EBV-transformation. The absence of Shc tyrosine phosphorylation seems highly unusual considering the increased phosphorylation of other key signaling intermediates such as PLC-γ, Vav, Syk, and p85. Consistent with the possibility that Shc is a substrate for SHP-1, macrophages from me mice show elevated levels of Shc tyrosine phosphorylation (Chen et al., 1996). Also consistent with this is the association between SHP-1 and other Shc binding proteins such as Grb2 (Pani et al., 1996; Kon-Kozlowski et al., 1996).

The association between LMP2 and Vav was introduced to support the contention that multiple mechanisms mediate LMP2’s inhibitory effects on BCR signaling. Data presented here suggested that LMP2 sequesters Vav (Figure 15). Although not shown, the Vav that LMP2 sequesters is only weakly tyrosine phosphorylated. This more than likely accounted for the absence of Vav in the anti-phosphotyrosine western blots of LMP2 precipitates (Figure 10). Data was presented indicating that Vav was tyrosine dephosphorylated in wild-type LCLs following receptor engagement (Figure 16). Although this could be mediated by SHP-1 in B lymphocytes, I was unable to detect an association between Vav and SHP-1 after two minutes receptor stimulation (data not shown). The purpose of LMP2-mediated recruitment of Vav may be to prevent its activation of p21ras and MAPK (for review of Vav-mediated Ras activation, see Bonnefoy-Berard et al., 1996). This would be in agreement with the modus operandi of LMP2 in EBV-transformation: to prevent lytic cycle gene induction and viral replication. The exact role played by Vav during proximal events in BCR signal transduction, however, awaits further investigation.
Beaufils et al. (1993) were unable to confirm LMP2A-mediated inhibition of BCR calcium mobilization using a CD8/LMP2A chimeric construct transfected into a murine B cell line. After first cross-linking CD8 and then stimulating through the BCR, Beaufils and colleagues observed that calcium mobilization was not inhibited in these transfectants. Miller et al. (1993), on the other hand, observed that LMP2A inhibited calcium mobilization in transfected Burkitt lymphoma cells following BCR cross-linking. One possible explanation for this discrepancy not previously considered is that the CD8/LMP2A chimera lacked the transmembrane and carboxy-terminal domains of LMP2. Another possibility is that the orientation of the N-terminus of LMP2A in the transfectants was upside down as compared to its orientation in LMP2+ LCLs. The chimeric proteins were created by splicing the end of the transmembrane domain of CD8α to the N-terminus of LMP2A. In so doing, the orientation of LMP2A was reversed. Experiments reported in this thesis support the idea that BCR signal transduction events are operating in reverse following receptor cross-linking. These events included the inducible dephosphorylation of Vav and the inducible dissociation of CD22-associated phosphatase activity.

In summary, LMP2 appears to utilize multiple mechanisms to interrupt signals through the BCR. In addition to the recruitment of Syk and Src-family PTKs as found by Miller et al. (1995), this study implicated SHP-1 and Vav in mediating some of LMP2's inhibitory effects. SHP-1 was more active in LCLs expressing LMP2 as compared to those not expressing LMP2. This activity was postulated to be for the purpose of pp140 and Shc dephosphorylation. The purpose of LMP2 recruitment of Vav merits further examination. LMP2 may sequester Vav to prevent the unligated BCR sufficient access to critical signaling intermediates as already suggested by Miller et al., 1995.
VI REFERENCES


