The Role of the *PTPN22* (Lyp/Pep) Phosphatase and its Disease-associated Variant in T-cell Signalling

by

Helen Miliotis

A thesis submitted in conformity for the requirements for the degree of Doctor of Philosophy

Institute of Medical Science

University of Toronto

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The role of the *PTPN22* (Lyp/Pep) phosphatase and its Disease-Associated Variant in T-cell signalling

Helen Miliotis, PhD Thesis

Institute of Medical Sciences, University of Toronto, 2012

The *PTPN22* gene encoding the Lyp/Pep protein tyrosine phosphatase has recently been described as a negative regulator of T-cell receptor signalling. Little biological information is available on this protein, but a variant allele in this gene conferring a R620W change has been associated with rheumatoid arthritis and other autoimmune disease states. To gain further understanding into the roles of Lyp, this work is aimed at identifying and characterizing Lyp interactions, and elucidating the effect of the variant Lyp in immunological disease. Specifically, the interaction of Lyp with the ubiquitin ligase Cbl was further examined and characterized to uncover its role in T-cells. Furthermore, the biochemical and functional differences of the variant Lyp were examined by utilizing a murine model of the variant, Pep R619W. This work led to novel findings on the stability of the protein and its resulting dysfunction, leading to cell hyperresponsiveness. Finally, a new role for Lyp in controlling cell migration was uncovered through its interaction with GRK2. The inhibitory properties of Lyp on cell migration are disrupted in the presence of the Lyp R620W variant, leading to dysregulation of GRK2 function and altered migratory properties of cells, particularly in the collagen-antibody induced arthritis model. Understanding the normal function of Lyp, as well as dysfunction of the variant, will provide new insights into normal T-cell signalling and aid in the understanding of the processes of autoimmunity.
ACKNOWLEDGEMENTS

The pursuit of a doctorate degree may be individual goal, but cannot be completed without the support, guidance and mentorship of a whole team of people. For me, this team included family, friends, and mentors that were instrumental in helping me to achieve one of my lifelong dreams.

First and foremost, I wish to thank my parents and family for always encouraging me to aim high and strive to be the best I can be, and for all those sacrifices they had to make to ensure that I had all the tools I needed to succeed. To my husband, for always being supportive and sitting through stories of failed experiments night after night at the dinner table. The love, encouragement and support of all of you have helped me to this point.

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As this work comes to a close and I look back at what has come to pass, I am truly grateful for not only the scientific skills but also the lifelong lessons that I learned from everyone along the way.

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>Activator protein - 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Tri-phosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cbl</td>
<td>Casitas B-cell lymphoma</td>
</tr>
<tr>
<td>COS</td>
<td>CV-1 (simian) in Origin, and carrying the SV40 genetic material</td>
</tr>
<tr>
<td>Csk</td>
<td>C-terminal Src kinase</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXC chemokine receptor 4</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal regulated kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>Fyn</td>
<td>Tyrosine kinase p59fyn</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GRK2</td>
<td>G-protein coupled receptor kinase 2</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HA</td>
<td>Hemoagglutinin</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin 2</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl b-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Lck</td>
<td>Lymphocyte-specific protein tyrosine kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Lyp</td>
<td>Lymphoid phosphatase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet-40</td>
</tr>
<tr>
<td>PAG</td>
<td>Phosphoprotein associated with glycosphingolipid enriched microdomains</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PEP</td>
<td>Pest-enriched phosphatase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>pNpp</td>
<td>para-Nitrophenylphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>PTP-HSCF</td>
<td>Protein tyrosine phosphatase hematopoietic stem cell fraction</td>
</tr>
<tr>
<td>PTP-PEST</td>
<td>Protein tyrosine phosphatase proline-glutamic acid-serine-threonine rich</td>
</tr>
<tr>
<td>pTyr</td>
<td>phosphotyrosine</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>Stromal derived factor 1-α</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SHP-1</td>
<td>Src homology region 2 domain-containing phosphatase-1</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>SLP-76</td>
<td>SH2 domain containing leukocyte protein of 76kDa</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>T1D</td>
<td>Type I Diabetes</td>
</tr>
<tr>
<td>TC-PTP</td>
<td>T-cell protein tyrosine phosphatase</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot</td>
</tr>
<tr>
<td>Zap-70</td>
<td>Zeta-associated protein 70</td>
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</table>
CHAPTER 1

INTRODUCTION
1.0 FOREWORD

Protein tyrosine phosphorylation is a fundamental mechanism in cell signalling. There is a fine balance between kinases and phosphatases in order to orchestrate and regulate these signals. Aberrations in phosphorylation can lead to profound consequences such as dysregulation in apoptosis or cell cycling, and the onset of malignancy. The human genome contains genes encoding for 90 tyrosine kinases, and at least 107 genes encoding tyrosine phosphatases (Alonso et al, 2004). Upon closer examination of these genes, only 85 of the kinase genes and 81 of the PTP genes are known to be functionally active and specific for phosphorytrosine. As the numbers between active kinases and phosphatases are similar, one can see how there may be interplay in their function towards their substrates.

1.1 PHOSPHATASES

Structure and classification

Human phosphatases can be divided into 4 distinct classes based on their structure and target substrate specificities (Alonso et al, 2004; Pao et al, 2007; Zhang, 2003) (see Table 1). Class I protein tyrosine phosphatases involve cysteine-mediated catalytic activity and represent the largest of the groups with 99 members. The so-called “classical”, phospho-tyrosine specific phosphatases are in this category, and they can be further divided into non-receptor, intracellular PTPs such as SHP1, SHP2, and the PEST family of phosphatases including PEP/Lyp, and the receptor-type, intracellular phosphatases such as CD45, CD148, and RPTPα. Non-receptor PTPs harbour only one catalytic domain, but
<table>
<thead>
<tr>
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<th>Type</th>
<th>Subtype</th>
<th>Phosphatase</th>
<th>Specificity</th>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Classified (26)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-Receptor (17)</td>
<td></td>
<td>PTP1B, TCPTP, PTPH1, PTP-HEF1, PTP-HEF2, PTPDO,</td>
<td>pv</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>PTPDO2, PTP-BAS, SHP1, SHP2, HsPTP, STEP, LYP/PEP,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PTP-PEST, PTP-HE6F, Typ-PTP, HPD-PTP</td>
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</tr>
<tr>
<td></td>
<td>Receptor (21)</td>
<td></td>
<td>CD48, LAR, DEP1, SAP1, CLEPP, PTPS31, PCTP, STEP,</td>
<td>pv, pt</td>
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<td></td>
<td></td>
<td></td>
<td>ICS, ICS2, RPTP α, RPTP γ, RPTP β, RPTP δ, RPTP α,</td>
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<td></td>
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<td>RPTP ε, RPTP α, RPTP α, RPTP γ, RPTP γ, RPTP β</td>
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<td></td>
<td>VH1-like (81)</td>
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<td>MAP kinase PTPs (11)</td>
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<td>PAC-1, MKP 1, MKP 2, MKP 3, MKP 4, MKP 6, MKP 7,</td>
<td>pv, pt</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>VHR, VHS, PYG1/2, MK - SYK</td>
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<td></td>
<td>Atypical dual specificity (19)</td>
<td></td>
<td>VHR, PR1, BDP1, TMDP, MKP 8, DSP 29, SKRIP, DSP 21,</td>
<td>pv, pt, mRNA</td>
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<td>MCR8, MGC 1136, HVH1, VHR, FMDP, VHS, VNY, VHS,</td>
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<td>CDC 14a, CDC 14b, KAP, PTPN2</td>
<td>p5, pt</td>
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<td>PTEN, TPIP, TPE, CI-TEK, kinesin</td>
<td>D3 phosphatases</td>
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<td>Mst family (10)</td>
<td></td>
<td>MTM1, MTM1-14T,</td>
<td>P208P3</td>
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<tr>
<td>CLASS II (1)</td>
<td></td>
<td></td>
<td></td>
<td>pv</td>
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<td></td>
<td>pv, pt</td>
</tr>
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<td>CLASS IV (4)</td>
<td></td>
<td></td>
<td></td>
<td>pv, p5?</td>
</tr>
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</table>
there are receptor PTPs that contain 2 catalytic domains in tandem, but only the one most proximal to the membrane is the active one (Andersen et al, 2001). Class I phosphatases also contain the VH1-like phosphatases, named as such due to their relationship to the vaccinia virus (Guan et al, 1991). These have very diverse specificities, such as for phospho-tyrosine and phospho-threonine for MAP kinase phosphatases (MKPs), for phospho-inositides in the case of PTEN (Wishart & Dixon, 2002), and for mRNA in the case of PIR (Deshpande et al, 1999).

Class II phosphatases only contain one member, the low-molecular weight PTP (LM-PTP) that essentially consists of a single-phosphatase domain. Class III phosphatases contain 3 members, cdc25A, cdc25B and cdc25C. These cysteine based PTPs dephosphorylate inhibitory tyrosines on CDKs to initiate the progression of cells through the cell cycle (Honda et al, 1993). The last group, known as Class IV PTPs function with a totally different catalytic mechanism. They use aspartic acid, and there are 4 members known as EyA but their biological function remains unknown (Tootle et al, 2003). The focus of this work will be on the Class I, classical tyrosine phosphatases of which PEP and Lyp are members.

**Class I PTPs: Mechanism of Catalysis**

Class I PTPs have a catalytic “signature” motif that contains the invariant cysteine residue and is defined by the sequence (I/V)HCXAGXXRX(S/T)G or more simply the CX₅R motif (Barford et al, 1995). The mechanism of catalysis for the protein tyrosine phosphatase PTP1B has been elucidated via crystallography and is considered as a general model for this class of phosphatases (Barford et al, 1994; Pannifer et al, 1998) but initial insights were also gained from the structure of the *Yersinia* tyrosine phosphatase (Stuckey et al, 1994). In the
tertiary structure of the phosphatase, the active site is lined by non-polar side chains, allowing for the formation of hydrophobic interactions with the phenyl ring of the phosphotyrosine residue on the substrate (Jia et al., 1995). The specificity for tyrosine-containing substrates to reach the active site is determined by the depth of the catalytic cleft, as the catalytic cleft in tyrosine phosphatases is relatively deeper than that of dual specificity phosphatases (9 Å which is the size of a phosphotyrosine residue, versus 3-6 Å, respectively) (Zhang, 2003). Once the substrate is in the proximity of the catalytic site, the engagement of the substrate induces a conformational change within the catalytic site among residues that form the WPD (Tryptophan, Proline, Aspartic Acid) loop (Zhang, 2003). These residues have been shown to be conserved among classical PTPs and are essential for the full enzymatic mechanism to be completed (Andersen et al., 2001). Once the substrate phosphate group enters the active site, the conserved arginine (in the CX₅R signature motif) forms two hydrogen bonds with two oxygens present in the substrate phosphate and thus secures it in place (Barford et al., 1994; Stuckey et al., 1994). During the process of securing the phosphate, the arginine rotates, allowing for the movement of the WPD loop (Stuckey et al., 1994). Specifically the rotation of the arginine allows the conserved tryptophan residue to move resulting in a twist in the backbone of the acid loop. Due to the rigidity of the conserved proline residue, the loop closes. This allows the aspartic acid to be in close proximity to the phosphate group (reviewed in (Zhang, 2003)). Once the substrate is in place, the mechanism of dephosphorylation involves two main steps (Figure 1) (reviewed in (Barford et al., 1998; Denu & Dixon, 1998; Zhang, 2003)). First, the cysteine in the catalytic domain carries out a nucleophilic attack on the phosphate group, while the aspartic acid acts as a general acid and donates its proton to the leaving group.
Figure 1

(Adapted from Barford et.al, 1998)
**Figure 1 Mechanism of Catalysis**

The cysteine in the catalytic domain of the phosphatase carries out a nucleophilic attack on the substrate phosphate group, while the aspartic acid on the phosphatase acts as a general acid and donates its proton to the leaving group oxygen, resulting in a phosphoryl-cysteine intermediate. At this point, the aspartic acid acts as a general base by removing a hydrogen atom from a water molecule. The remaining hydroxyl group finds the phosphoryl-cysteine intermediate and hydrolyzes it to release the free phosphate and restore the phosphatase.
oxygen, resulting in a phosphoryl-cysteine intermediate (Denu et al, 1996; Pannifer et al, 1998). In the second stage of the reaction, the aspartic acid now acts as a general base by removing a hydrogen atom from a water molecule. The remaining hydroxyl group finds the phosphoryl-cysteine intermediate and hydrolyzes it. At this stage the WPD loop opens up and the free phosphate is released (reviewed in (Denu & Dixon, 1998; Zhang, 2003).

**Substrate Trapping Mutants**

The properties of the catalytic domains can be manipulated by mutations in key residues of the enzyme in order to gain insights into phosphatase-substrate interactions (reviewed in (Blanchetot et al, 2005). The most common manipulation is a mutation of the catalytic cysteine to a serine residue. This C/S substitution results in a complete abrogation of phosphatase activity, while still retaining substrate binding (Jia et al, 1995). This can be used to “trap” the substrate into the catalytic pocket and may aid in the identification of substrates. Another strategy is to mutate the aspartic acid that is in the WPD loop, disrupting 2 events in the mechanism of dephosphorylation (Flint et al, 1997). Firstly, a D/A mutant cannot serve as an acid to the leaving group oxygen, and secondly, it cannot serve as a base in the final stages of the reaction when the WPD needs to open up and the substrate released. This results in incomplete dephosphorylation and an inability of the enzyme to release the substrate. These mutation strategies can be used in order to stabilize the often transient phosphatase-substrate interactions in order to facilitate further studies.

**PEST family of Phosphatases**

The PEST family of phosphatases are named as such due to their C-terminal regions that are proline-, glutamic acid-, serine- and threonine-rich (PEST) (reviewed in (Mustelin et al, 2005; Pao et al, 2007; Veillette et al, 2009). The three members of the family are PTP-
PEST, PTP-HSCF, and PEP/Lyp (Figure 2). They are intracellular phosphatases with similar structural characteristics. They each have a serine phosphorylation site at their N-terminus. Specifically these are S37 for PTP-PEST, S39 for PTP-HSCF and S35 for PEP/Lyp. These sites can be phosphorylated by PKA and PKC, and are thought to inhibit the phosphatase activity of these enzymes (Garton & Tonks, 1994; Habib et al, 1994; Yu et al, 2007). The catalytic domain resides at the N-terminus, followed by a central region that is quite unique to each member, and ending with a conserved carboxyl-terminal homology (CTH) domain. The CTH domain has been shown to bind the adapter PSTPIP1 and PSTPIP2 in both PTP-PEST and PTP-HSCF (Cong et al, 2000; Milkiewicz et al, 2006; Spencer et al, 1997; Wu et al, 1998), and may also do so in PEP/Lyp, though this has not been determined.

**PTP-PEST (PTPN12)**

PTP-PEST is expressed ubiquitously, but has very high levels in hematopoietic cells. Following its catalytic domain, PTP-PEST contains five proline-rich regions that bind to a wide number of interactors via their SH3 domains. These include binding to the adapter Grb2 (Charest et al, 1997), the inhibitory kinase Csk (Davidson et al, 1997), and scaffold protein p130Cas (Garton et al, 1996). The second proline rich region on PTP-PEST can also bind paxillin through its LIM domain (Shen et al, 1998). PEST knock-out mice were generated but ultimately died *in-utero*, around day 9.5. They displayed severe abnormalities in embryonic vascularization, mesenchyme formation, neurogenesis, and liver development (Sirois et al, 2006). This indicates that PTP-PEST plays an important role in critical cell processes, and experimental evidence suggests that this is due to PEST’s role in cytoskeletal organization. In order to study PTP-PEST, many groups have resorted to
Figure 2: PEST family of phosphatases

<table>
<thead>
<tr>
<th>GENE</th>
<th>MOUSE PROTEIN</th>
<th>HUMAN PROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTPN22</td>
<td>PEP</td>
<td>LYP</td>
</tr>
<tr>
<td>PTPN12</td>
<td>PTP-PEST</td>
<td>PTP-PEST</td>
</tr>
<tr>
<td>PTPN18</td>
<td>PTP-HSCF</td>
<td>BDP1</td>
</tr>
</tbody>
</table>
**Figure 2 PEST family of phosphatases**

The names and structures of the PEST family of phosphatases are shown. PTP: Protein tyrosine phosphatase, P: Proline rich region, S: Serine, CTH: C-terminal Homology
overexpression studies to elucidate its function. In fibroblasts, overexpression of PTP-PEST was found to promote cell migration by human memory T-cells, a reduction in IL-2 dephosphorylating key cytoskeleton – associated proteins such as paxillin, PSTPIP, FAK, Vav, and p190RhoGAP (reviewed in (Veillette et al, 2009)).

The role of PTP-PEST in the regulation of the cytoskeleton has been shown in cells of the immune system as well. In T-cells, the immunological synapse involves the rearrangement of the actin cytoskeleton by proteins such as WASp. This involves the phosphorylation of WASp by Fyn and the recruitment of CD2, CD2AP to the immunological synapse (Badour et al, 2003). Downregulation of this process involves the PSTPIP mediated recruitment of PTP-PEST, resulting in dephosphorylation of WASp and hence the termination of synapse formation and T-cell activation (Badour et al, 2004). Supporting its role as a negative regulator of T-cell activation, overexpression of PTP-PEST in Jurkat T-cells resulted in reduced production of IL-2, NFAT, and NF-kB levels, and in primary human memory T-cells, a reduction in IL-2 production was also seen (Arimura et al, 2008). Finally, Arimura et al have reported that, both in humans and in mice, TCR activation of naïve CD4+ and CD8+ T cells resulted in a reduction in the endogenous protein expression of PTP-PEST (Arimura et al, 2008). Furthermore, overexpression of PTP-PEST in the B-cell line A20 also resulted in a pronounced inhibition of BCR-induced IL-2 production, due to the dephosphorylation of a number of substrates, including Pyk2, Fak, Cas, and Shc (Davidson & Veillette, 2001).

Since the role of PTP-PEST in the cytoskeleton reorganization is established, it is likely that this protein regulates many other related processes in cells of the immune system,
such as antigen uptake/presentation, granule exocytosis, and phagocytosis (Veillette et al., 2009). Clearly this PEST-family member has important roles in the immune system.

**PTP-HSCF (PTPN18)**

PTP-HSCF (hematopoietic stem cell fraction) has also been referred to as brain-derived phosphatase 1 (BDP1), PTP20, PTP-K1, and fetal liver phosphatase 1 (FLP1). It is expressed in primitive hematopoietic cells (Cheng et al., 1996; Huang et al., 1996), and expression levels have also been detected in colon and brain tissues (Kim et al., 1996). Like the other PEST family members, PTP-HSCF also binds Csk, but via a different mechanism. There are 2 phosphorylated tyrosine residues on PTP-HSCF that bind the SH2 domain of Csk (Wang et al., 2001a). In transfection studies, this phosphatase is also found to be inhibitory by dephosphorylating the activating tyrosine on Src family kinases, but itself is also phosphorylated by Fyn (Wang et al., 2001a). It is not clear how these interactions function physiologically in the immune system. Overexpression in Ramos B cells also suppresses BCR signalling, possibly due to the interaction between PTP-HSCF and Tec kinase (Aoki et al., 2004). While there is evidence to suggest that PTP-HSCF is an inhibitory tyrosine phosphatase, it is the least studied of the PEST family members and its exact role remains to be elucidated.

**LYP/PEP (PTPN22)**

These members of the PEST family are the focus of this thesis. In humans, this phosphatase is known as Lymphoid phosphatase (Lyp) and in mice as PEST-enriched phosphatase (PEP). While these are widely thought of as homologues, there are significant differences between them in terms of their sequence, which may in turn reflect some
functional differences between them as well. For the remainder of this written work, the findings for Lyp and PEP will be described separately and in detail.

1.2 LYP/PEP

**Lyp Structure**

The Lyp N-terminus contains a serine phosphorylation site at position 35 (Yu et al, 2007) and a tyrosine phosphatase domain starting at position 27 to 291 (Cohen et al, 1999). The catalytic domain structure has been elucidated by three different groups (Barr et al, 2009; Tsai et al, 2009; Yu et al, 2007). They reveal the phosphatase signature motif responsible for catalysis (aa 226-233), a phosphotyrosine recognition loop (aa 54-60), and the WPD loop (aa 193-204) (Barr et al, 2009; Tsai et al, 2009; Yu et al, 2007). Furthermore, the catalytic domain of Lyp includes two additional cysteine residues (129 and 231) in very close proximity to the catalytic cysteine at 227, which may serve to regulate Lyp function (Tsai et al, 2009). These residues are conserved between Lyp and PTP-PEST and PTP-HSCF. The structure of Lyp’s catalytic domain as elucidated by Barr et al reveals that Lyp may contain a secondary substrate binding pocket, allowing for the dephosphorylation of substrates with 2 adjacent tyrosines, such as Zap-70 (Barr et al, 2009). This has not been determined experimentally.

Following the catalytic domain is a central region of about 300 amino acids that is largely uncharacterized. Bioinformatic analyses reveal a caspase-1 consensus cleavage site at amino acid 322, but its biological significance has not been shown. The interdomain of Lyp also contains a tyrosine at position 536 that is phosphorylated by Lck kinase (Fiorillo et al, 2010). The C-terminus contains four proline rich domains that are potential SH3 domain
binding sites, as well as a PEST rich region at aa 702-736 (Cohen et al, 1999). It has been suggested that Lyp may also contain a consensus sequence (XS/TPXK/R) between residues 741 to 745 that may be recognized by p34\(^{cdk2}\) (Cohen et al, 1999) but that has not been confirmed. Lyp contains two motifs, 468 to 471 (NF SY) and 496 to 499 (ENPY) that may bind phosphotyrosine binding (PTB) domains on other proteins (Cohen et al, 1999). Finally, Lyp has a consensus sequence for a C-terminal homology domain at aa 796 to 804 (Pao et al, 2007) but the functional relevance of this domain has not been confirmed (Figure 3).

The Lyp2 isoform described by Cohen \textit{et al} seems to be an alternate splice variant and as a result, has a different C-terminus (Cohen et al, 1999). An alternative stop codon results in a truncated protein having 7 different amino acids at the C-terminal end (Cohen et al, 1999). As a result, it is missing three proline-rich regions, the PEST region, and the CTH domain. No functional work has been carried out on this isoform.

A new variant of Lyp was recently identified and designated as Lyp 3 (Wang et al, 2010b). It results from alternative splicing and has a different C-terminus. Specifically, it lacks 28 amino acids between the first and second proline rich domains. The physiologic relevance of this isoform is unknown.

**PEP structure**

The PEP N-terminus contains a conserved serine phosphorylation site at position 35, and a phosphatase domain predicted at residues 23 to 288 (Matthews et al, 1992). The crystal structure for the PEP catalytic domain has not been eludicated as it has for Lyp but may be very similar as that region of the protein shares about 90% sequence identity with
Figure 3 Lyp and PEP Structures

Comparison of the main Lyp and PEP protein domains as indicated.
Lyp (Cohen et al, 1999) The C-terminus contains four proline rich sequences for protein-protein interactions (Cloutier & Veillette, 1996; Matthews et al, 1992), and also contains five separate PEST enriched sequences between residues 688-802 that may signal the protein for rapid degradation (Matthews et al, 1992). Putatively, PEP may have a nuclear localization signal (PVKRTK) at positions 446 to 451, but this has been deemed non-functional experimentally (Flores et al, 1994) and a casein kinase II site at positions 497 to 550 (Matthews et al, 1992). There are also three putative consensus sequences for phosphorylation by p34\(^{\text{cdc2}}\) kinase, at residues 667 to 671 (FTPSK), 735 to 741 (RTPGK), and 760 to 764 (SSPSK) (Matthews et al, 1992) Finally PEP also contains the consensus sequence for a CTH domain at its very end. Most of these putative motifs have not been confirmed biologically.

**PEP and Lyp as potential homologues**

Despite similarities in their sequences, Lyp and PEP exhibit marked differences. There is 89% identity between their catalytic domains, but only 61% identity in their non-catalytic domain (Cohen et al, 1999).

Both Lyp and PEP share a conserved serine at position 35, an N-terminal catalytic domain spanning the region approximately from residues 20-290. The inhibitory tyrosine at 536 is conserved among both, and they both share four proline-rich regions at their C-terminus. They share two of the three potential p34\(^{\text{cdc2}}\) sites, and one of the two potential PTB domain binding sites, followed by a CTH domain.

There are marked differences in the remainder of the protein. Lyp, but not PEP, has a caspase-1 cleavage site. PEP, on the other hand, contains five PEST regions compared to only one in Lyp. The putative nuclear localization signal and the casein kinase II sites are
also unique to PEP. These significant differences remain to be examined biologically. To date, this degree of homology is lower than any other described pair of mouse to human phosphatase homologues (Cohen et al, 1999).

**Tissue and Cellular expression**

**Lyp expression**

Expression of Lyp is believed to be restricted to cells of hematopoietic origin. Using mRNA, a number of groups have examined the distribution of the three Lyp isoforms in human tissues. In the first reported characterization of Lyp, Northern blot analysis was used to detect Lyp isoform 1 in human spleen, lymph node and thymus tissues, but not in prostate, small intestine, colon, testis or ovary (Cohen et al, 1999). Tissue distribution with a qPCR array confirmed that Lyp is expressed in major lymphoid tissues, but also secondary ones such as tonsil, intestine, testis, and others (Wang et al, 2010b) Lyp transcripts have been detected by semi-quantitative RT-PCR in normal human PBMC samples, with high expression in neutrophils, some expression in adherent PBMCs (such as monocytes) and a low level of expression in CD3^+ T cells (Chien et al, 2003). Analysis of further cell subtypes has shown the highest Lyp mRNA levels in natural killer cells and neutrophils, followed by expression in monocytes, dendritic cells, CD8^+ T cells, CD3^+ T cells, with the lowest levels in B cells and CD4^+ T cells (Begovich et al, 2004).

Expression in cell lines of Lyp1 has also been examined at the RNA level by either semi-quantitative RT-PCR (Chien et al, 2003) or Northern blot (Cohen et al, 1999; Hill et al, 2002). Lyp mRNA is detected at high levels in some myeloid cell lines such as KG-1 (very early myeloblasts) (Chien et al, 2003), HL-60 (myeloblast) (Chien et al, 2003; Hill et al, 2002) and ML-1 (myeloblasts) (Chien et al, 2003), U937 (myelomonoblasts) (Chien et al,
2003; Cohen et al, 1999), as well as SK-NO1 and KCL22 (CML blasts) (Chien et al, 2003). In contrast, very low to undetectable levels of Lyp transcripts have been reported in THP-1 (monoblasts) (Chien et al, 2003), K562 (erythroblasts) (Chien et al, 2003) (Cohen et al, 1999; Hill et al, 2002), Kasumi-1 (mature myeloblasts expressing the AML1-ETO fusion protein) (Chien et al, 2003), and Kasumi-3 (very early myeloblasts) (Chien et al, 2003). T-cell lines such as Jurkat (Chien et al, 2003) and Hut-78 express high levels of Lyp mRNA, while the B-cell derived Raji cell line expresses very low levels (Hill et al, 2002).

Levels of mRNA expression do not always correlate with protein expression. The study of endogenous Lyp protein levels has been challenging due to the lack of robust antibodies and the variation in protein migration observed on SDS-PAGE. Furthermore, it seems that Lyp protein is not highly expressed in resting cells. Cohen et al. cultured human PBMCs in the presence of αCD3 or PHA stimulus for 24 to 48 hours and reported an inducible expression of Lyp 1 protein at 110kDa after 24 hours, with a further increase at 48 hours with αCD3 stimulation (Cohen et al, 1999). Lyp1 protein expression was also shown in Jurkat (Cohen et al, 1999; Hill et al, 2002), Hut-78 (Hill et al, 2002) and the B cell lines Ramos and Daudi (Cohen et al, 1999), but not in the myeloid cell lines K562 or U937 (Cohen et al, 1999).

Lyp 2 transcript is detectable but at much lower levels relative to Lyp 1 (Cohen et al, 1999; Wang et al, 2010b). It is seen in thymus (Cohen et al, 1999; Wang et al, 2010b), human spleen, lymph node and was very high in fetal liver tissue (contains a large population of pre-B cells) (Cohen et al, 1999). It has also been detected in peripheral blood, and surprisingly in prostate (Wang et al, 2010b). At the protein level, Lyp 2 is detected in resting human PBMCs, but upon stimulation for 24 hours with either αCD3 or PHA, the 85kDa
protein interestingly disappears; the opposite to what is observed for Lyp1 (Cohen et al, 1999).

Lyp 3 mRNA expression was detected using qPCR arrays against human tissues (Wang et al, 2010b). While not to the same extent as Lyp 1, Lyp 3 is also expressed in major lymphoid tissues such as spleen, lymph node and thymus, as well as secondary lymphoid organs such as stomach and rectum, and surprisingly also in the retina (Wang et al, 2010b). Protein levels of Lyp 3 have not been examined to date.

**PEP Expression**

PEP was first detected using Northern blot in spleen, thymus, lymph node and bone marrow (Matthews et al, 1992). Expression in the thymus and spleen were also detected by in-situ hybridization (Flores et al, 1994). Using cDNA from different tissues as templates, PCR analysis determined PEP expression in bone marrow, fetal liver, spleen, lymph node, thymus, and tonsil (Gjorloff-Wingren et al, 2000).

While specific cell line names were not indicated, expression was reported in a T-cell hybridoma and a thymoma but not in macrophage cell lines or B-cell lymphoma (Matthews et al, 1992). However later on it was shown that stimulation of WEHI-231 immature B cells with IgM resulted in an increase of PEP mRNA level after 3-24 hours of stimulation (Hasegawa et al, 1999), reminiscent of the inducible Lyp expression reported in cells treated with anti-CD3 after 24 hours (Cohen et al, 1999).

An analysis of the murine PTP transcriptome was carried out by Arimura et al and they focused on phosphatases expressed in cells of the immune system (Arimura & Yagi, 2010). They reported that PEP is expressed at high levels in all cells of the immune system examined (T cells, B cells, immature dendritic cells, macrophages, mast cells, natural killer
cells, natural killer T cells, and neutrophils) with particularly high expression in NK and NKT cells (Arimura & Yagi, 2010). They observed no particular differences in expression of PEP within different T cell subsets (Arimura & Yagi, 2010). In B cells, in contrast to Hasegawa et al., they reported no expression changes upon 7 hours of IgM stimulation, but did report a decrease in PEP transcript upon LPS stimulation for 7 hours (Arimura & Yagi, 2010). Furthermore, treatment of dendritic cells with LPS for 48 hours resulted in a decrease of PEP expression (Arimura & Yagi, 2010). How these changes in expression across different cell types are functionally relevant are not entirely clear and further cell specific studies are needed.

PEP protein levels have been examined and due to the non-specificity of the available antibodies, tissues/cells were compared between WT mice and PEP \(-/-\) mice. PEP is stated to be present in T lymphocytes, particularly CD8\(^+\) T cells, and not in B cells (Hermiston et al, 2009).

**Cellular Localization**

Lyp and PEP cell localization studies have primarily been carried out using overexpression. The cellular localization of Lyp seems to be cytoplasmic. Transfection of Lyp into COS7 cells or KC22 myeloid cells displays perinuclear or cytoplasmic localization (Chien et al, 2003; Cohen et al, 1999)

For PEP, it was initially reported to reside primarily in the nucleus upon overexpression in HeLa cells (Flores et al, 1994), and mapping of the potential nuclear localization signal established that residues 788-802 at the extreme C-terminus of the protein were required (Flores et al, 1994). The nuclear localization of PEP was subsequently been challenged, however, in a study using overexpression of PEP into COS-1 cells that results in
The issue was re-examined by a third group that overexpressed PEP in Jurkat cells, and immunofluorescence staining revealed localization of PEP in the cytoplasm, near the plasma membrane, as well as the nucleus (Gjorloff-Wingren et al, 2000). Overexpression of the N-terminus of PEP, involving the catalytic domain alone, was observed to localize solely to the cytoplasm (Flores et al, 1994; Gjorloff-Wingren et al, 2000). However overexpression of the C-terminus yielded conflicting results: one study states that the C-terminus shows strictly cytoplasmic expression (Gjorloff-Wingren et al, 2000), in contrast to Flores’ et al findings that deemed this region as essential for nuclear localization (Flores et al, 1994). Gjorloff-Wingren et al concludes that the whole protein needs to be intact for the small amount of nuclear localization observed (Gjorloff-Wingren et al, 2000).

The reason for the discrepancy in these findings is not clear. The differences may partly be attributed to the different approaches that were used in the way the protein was overexpressed, the host cells that were used, and the different kinds of plasmids and tags that PEP was cloned in. Furthermore, cell fractionation studies carried out by several groups have established that PEP is largely localized to particulate fractions containing cellular membranes, and the cytoplasm, but not the nucleus (Cloutier & Veillette, 1996; Fournel et al, 1996; Gjorloff-Wingren et al, 2000; Hasegawa et al, 1999)

1.3 PEP KNOCK-OUT

Phenotype and Cell Mechanisms

Insights into the role of PEP can be gained through study of the murine knock-out, done by Hasegawa et al in 2004 (Hasegawa et al, 2004). Unlike the related phosphatase
PTP-PEST whose knock-out is embryonic lethal (Sirois et al, 2006), PEP deficient mice mainly show only an enhanced population of effector/memory T-cells (Hasegawa et al, 2004). Initial analysis of T-cell numbers and subsets did not show any discernible differences between wt and PEP $^{-/-}$ mice, except for a small increase in CD5 expression in DP thymocytes, and a small overall increase in the number of CD4$^+$ T-cells. Young PEP $^{-/-}$ mice (4-6 weeks) had no morphological differences compared to WT, but older mice (over 6 months) did present with larger lymph nodes and spleen (Hasegawa et al, 2004). In these older mice, the effector/memory T cell population was higher, particularly in CD8$^+$ T cells.

In order to determine the mechanism for this phenomenon, the authors checked naïve T-cell functions at first by assessing TCR mediated $[^3]$H]thymidine incorporation, cytokine production, mobilization of intracellular calcium concentration, expression of activation markers, and tyrosine phosphorylation of cellular proteins – all of which were normal between WT and PEP $^{-/-}$ cells initially (Hasegawa et al, 2004). To study effector cell functions, naïve T-cells were stimulated for 4 days, were rested for 2 days, and then restimulated through the TCR. Cells from PEP deficient mice showed increased proliferation, cytokine production, and Ca$^{2+}$ mobilization. Since PEP is thought to dephosphorylate Lck, the authors confirmed that PEP deficient cells had higher levels of phosphorylation on Lck’s activating tyrosine and attribute this as a mechanism for the hyperactivation of these effector T cells (Hasegawa et al, 2004). These findings in effector/memory T cells expanded in-vitro were consistent with functions of effector/memory T-cells in-vivo (Hasegawa et al, 2004). PEP deficient mice also had a higher number of germinal centres in the spleen and Peyer’s patches, but B cell signalling was deemed normal and the authors conclude that this phenotype is probably secondary to the
enhanced T cell functions in these animals (Hasegawa et al, 2004). Furthermore, PEP⁻/⁻ mice had no signs of autoimmunity as assessed by measurement of autoantibodies, or autoimmune mediated tissue damage. The resulting phenotype of PEP⁻/⁻ mice on the B6 background, the expansion of the effector/memory T cell population, is not sufficient to cause autoimmunity, and other mechanisms/defects in peripheral or central tolerance would be required to occur concurrently in order to progress to disease. This reflects the multifactorial nature of the genetics attributed to many autoimmune diseases.

**Importance of genetic background – phenotype and cell mechanisms**

To see if a different genetic background will provide a better environment to study PEP functions, the PEP deficient mice were crossed with CD45<sup>E613R</sup> mice (Hermiston et al, 2009). The substitution of Glutamate to Arginine at position 613 disrupts the dimerization and hence inhibition of the CD45 phosphatase (Majeti et al, 1998). The CD45<sup>E613R</sup> knock-in mouse displays a lupus-like syndrome, as characterized by enhanced lymphocyte proliferation and activation, the presence of autoantibodies, and immune complex glomerulonephritis, and has been attributed to a B-cell driven defect (Majeti et al, 2000). This phenotype, however, is background dependent. The autoimmunity is only seen on B6/129 F<sup>1</sup> background, not on a pure B6 background (Hermiston et al, 2009), indicating the importance of other genetic factors in leading to disease. In order to elucidate how subtle susceptibility loci might lead to autoimmunity, the PEP⁻/⁻ were crossed to the non-autoimmune B6 CD45<sup>E613R</sup> mice (Hermiston et al, 2009).

Indeed, the phenotype was a strong autoimmune one. The PEP⁺/CD45<sup>E613R</sup> mice had a larger spleen compared to WT littermates, and the number and size of the lymph nodes were particularly higher than that of each single mutant mouse alone (Hermiston et al, 2009).
The presence of autoantibodies to dsDNA was observed, as well as increased mortality by 12 months compared to WT (attributed to glomerulonephritis) (Hermiston et al, 2009). At the cellular level, effector/memory T-cells were increased, and B-cells were hyperresponsive (Hermiston et al, 2009). The authors attributed the T-cell phenotype to the role of PEP, and the B-cell phenotype to the role of CD45\textsuperscript{E613R}, based on the phenotypes of the single mutant mice (Hermiston et al, 2009). The combination of both of these defects is what ultimately results in a break in tolerance and the onset of autoimmunity. This model is more reflective of complex genetic backgrounds and microenvironments that exist in organisms.

1.4 LYP/PEP CELL FUNCTIONS

T-cell function

The role of Lyp on T-cell function has not been fully elucidated, but it is believed that Lyp acts to downmodulate T-cell activation. Silencing of Lyp by RNAi in Jurkat cells followed by antigen receptor stimulation resulted in a two-fold activation of signalling as measured by an \( NF\kappa B \) reporter assay (Begovich et al, 2004). In turn, overexpression of Lyp rather than silencing it, followed by antigen receptor stimulation resulted in a decrease of signalling as measured by IL-2 production (Abrahamsen et al, 2003; Hill et al, 2002). In the study by Hill et al., overexpression of the Lyp C227S catalytically inactive mutant or the R223M mutant could not inhibit activation (Hill et al, 2002), indicating that phosphatase activity is required for Lyp negative regulation of T cell function. NFAT production (Abrahamsen et al, 2003) and calcium mobilization (Abrahamsen et al, 2003; Hermiston et al, 2009) was also shown to be reduced upon antigen stimulation when Lyp was overexpressed.
PEP, in turn, has also been examined for its effect on T-cell activation. PEP overexpressed in the mouse cell line BI-141 was able to reduce the lymphokine secretion of the cells following TCR stimulation (Cloutier & Veillette, 1999). When PEP was overexpressed in Jurkat T-cells that were stimulated with αCD3/CD28 it reduced cell activation as determined by an NFAT/AP-1 luciferase reporter system (Gjorloff-Wingren et al, 1999). These inhibitory effects of PEP are also related to its phosphatase activity, as its catalytically inactive mutants could not exert this effect (Cloutier & Veillette, 1999; Gjorloff-Wingren et al, 1999). Interestingly, in a follow-up study, it was shown that PEP overexpression on a Western blot was hardly detectable as the expression was so low, but was still sufficient enough to suppress T-cell activation (Gjorloff-Wingren et al, 2000). In a cellular context, PEP is a very potent inhibitor relative to other phosphatases such as SHP1 and TCPTP (Gjorloff-Wingren et al, 2000). Other read-outs such as c-fos activation and JNK2 activation have also been shown to be attenuated upon PEP overexpression (Gjorloff-Wingren et al, 1999).

**B-cell function**

The expression of PEP in B cells has been shown by a number of groups, but has been disputed by others (Hermiston et al, 2009). The PEP knockout does not show any major B-cell specific defects other than enlarged germinal centres with an increase in GL-7⁺ B cells and CD23⁺ follicular B cells (Hasegawa et al, 2004), but there is some evidence that suggests PEP’s role in B cell signalling. It has been shown the PEP mRNA level increases two-fold upon BCR ligation by IgM after 24 hours (Hasegawa et al, 1999). This effect seems specific to this treatment and phosphatase, as other phosphatases such as PTP-PEST did not change their mRNA expression, and LPS stimulation did not have the same effect on
PEP mRNA levels (Hasegawa et al, 1999). In order to elucidate the role of PEP on B-cells, silencing was used to downmodulate PEP’s expression in WEHI-231 cells (Hasegawa et al, 1999). Normally IgM treatment of B-cells for 24 hours results in growth arrest of the cells and apoptosis, a phenomenon which was rescued by cells containing PEP antisense (Hasegawa et al, 1999). This indicates that PEP must contribute to signalling related to cell cycle arrest and apoptosis in these cells, but no further evidence at the molecular level has been provided to support this theory. Furthermore, treatment of human B cells with an inhibitor of Lyp against its catalytic domain, named I-C11(Yu et al, 2007), is stated to have no effect on BCR activation compared to untreated cells (Arechiga et al, 2009), thus implying that PEP does not have a very dramatic role in B cells.

Myeloid cell function

The role of Lyp has not been very thoroughly studied in myeloid cells, but there is one report to show that it may exert a biological effect using the KCL-22 cell line (Hill et al, 2002). These CML blast derived cells express the Bcr-Abl kinase. Overexpression of Lyp into these cells resulted in a decrease of overall tyrosine phosphorylation, as well as the total Bcr-Abl protein level (Hill et al, 2002). The biological significance of these findings is not clear.

1.5 LYP/PEP PROTEIN INTERACTIONS AND SUBSTRATES

Csk

The first and most characterized interaction involving PEP was with C-terminal kinase (Csk) (Cloutier & Veillette, 1996). It was discovered in a yeast two-hybrid screen using Csk as a bait protein, and was then confirmed using co-immunoprecipitations in COS-
1 cells following transfection of the two proteins, or endogenously in the hematopoetic cell lines BI-I41 (T cells), WEHI-231 (B cells) and RAW 264.7 (macrophages) (Cloutier & Veillette, 1996). Mapping of the interacting domains has established that the first proline rich region of Lyp (PPPLPERT) binds the SH3 domain of Csk (Cloutier & Veillette, 1996). Mutational analyses established that the second and fourth prolines, as well as the leucine, arginine and threonine residues were also important for binding (Gregorieff et al, 1998) and confirmed by structure determination of this region (Ghose et al, 1988). Furthermore, the P1 region on PEP is required but is not sufficient for Csk binding, as PEP residues I625 and V626, just after the P1 domain, are crucial in forming the right conformation needed for Csk binding (Ghose et al, 1988; Gregorieff et al, 1998), by allowing the positioning of the hydrophobic patch of PEP to be recognized and bound by Csk (Ghose et al, 1988). Furthermore, the interaction is a very strong one, with high specificity and affinity (Ghose et al, 1988). This has been confirmed biologically, with an estimated 50% of the total PEP found in the cell thought to be bound to Csk when co-immunoprecipitated from BI-141 cells (Cloutier & Veillette, 1996).

Functionally, the Csk-PEP interaction is thought to synergistically inhibit cell activation by acting on Src family kinases (Figure 4a) (reviewed in (Veillette et al, 2002)). These kinases are in an inactive state by an intramolecular interaction between their SH2 domains and a phosphorylated residue on their kinase domain. Csk negatively regulates Src-family kinases by phosphorylating the inhibitory tyrosine on their C-terminus. This family of kinases also contain an N-terminal activating tyrosine. It is well accepted that Csk and PEP work together to turn off these kinases, by Csk phosphorylating the inhibitory tyrosine while PEP dephosphorylates the activating tyrosine (Cloutier & Veillette, 1999).
Cloutier et al showed that PEP augments the inhibitory effect of Csk by overexpressing PEP in a T cell line and observing a reduction in lymphokine secretion and a reduction in tyrosine phosphorylation in molecules downstream of TCR activation (Cloutier & Veillette, 1999). Furthermore, the interaction with Csk was necessary for PEP to carry out its function in the cell, as deletions in the PI region, rendering PEP unable to bind Csk, were not as effective at downregulating lymphokine production of T cells (Cloutier & Veillette, 1999). Conversely, Csk may be necessary for Lyp/PEP to exert its full inhibitory effect. Overexpression of Lyp or PEP with Csk showed better inhibition of calcium mobilization and ERK activation than without Csk (Hermiston et al, 2009). Hence a model is generally accepted whereby Lyp/PEP together inhibit TCR activation (reviewed in (La Face et al, 1997; Veillette et al, 2002) and shown in Figure 4b.

In a resting T-cell, Csk is anchored to PAG/Cbp in the lipid rafts, and Lyp/PEP is in turn bound to Csk. Together they act to provide tonic inhibition of the Src kinases proximal to the membrane such as Lck. Upon antigen ligation, PAG/Cbp is dephosphorylated by CD45, resulting in Csk and PEP to be dissociated from the membrane. This allows for the Src kinases to be activated, by dephosphorylation by CD45 of their Csk targeted inhibitory tyrosine and the phosphorylation of their N-terminal, activating tyrosine (possibly by self-phosphorylation). Signalling is initiated downstream of the TCR. Post-activation, PAG/Cbp is re-phosphorylated by the active Src kinases, recruiting Csk and PEP back into the vicinity of the TCR complex where they can once again terminate signalling.

One may suspect that since PAG dephosphorylation is crucial for Csk and PEP to be released from it upon cell activation that PEP would be the phosphatase to carry out this
Figure 4

a)

Adapted from Veillette et al, Annual Review of Immunology 2002

b)

Adapted from Veillette et al, Annual Review of Immunology 2002 and Mustelin et al, Molecular Immunology 2004.
Figure 4 Regulation of Src family kinases by PEP/Lyp

a) Src family kinases such as Lck are regulated by tyrosine phosphorylation. They contain an inhibitory tyrosine at the C-terminus of the kinase domain, and an activating tyrosine in the N-terminus of the kinase domain. In the basal state, they are inhibited by an intramolecular interaction between their SH2 domain and the C-terminal phosphorylated tyrosine. This C-terminal phosphorylation is carried out by Csk, and any residual phosphorylation on the N-terminal tyrosine is dephosphorylated by Lyp. Upon activation, phosphatases such as CD45 dephosphorylate the C-terminal tyrosine, opening the molecule that self-phosphorylates its activating N-terminal tyrosine. b) In a resting T-cell, PAG is phosphorylated and bound to the SH2 domain of Csk, which in turn is bound to Lyp. Together, Csk and Lyp negatively inhibit Lck. Upon TCR activation, PAG is dephosphorylated, releasing Csk and Lyp from the complex. Lck is activated and initiates signalling. Post-activation, Lck phosphorylates PAG, thereby recruiting Csk and Lyp again into the proximity of the complex where they will further downregulate Lck activity.
effect. In fact, it has been shown that it is CD45, but not PEP or SHP1 that is responsible for PAG dephosphorylation (Fournel et al, 1996).

**Kinases**

The first direct substrates of PEP that were identified were Zap-70 and Fyn (Cloutier & Veillette, 1999). Overexpression of PEP into a T-cell line revealed downregulation of their phosphorylation, and further work including peptide mapping concluded that PEP directly dephosphorylates the activating Y417 on Fyn (but not Y528) (Cloutier & Veillette, 1999).

In order to find novel substrates of Lyp, Wu et al purified the catalytic domain of Lyp and its interactors and identified them by mass spectrometry (Wu et al, 2006). The interaction between a phosphatase and its substrate can be a very transient one, hence a substrate trapping mutant of the Lyp phosphatase domain was used consisting of D195A/C227S. The kinases identified as substrates of Lyp were Lck and Zap-70 (Wu et al, 2006). These were confirmed by overexpression of Lyp PTP WT or D195A/C227S into Jurkat cells, followed by immunoprecipitation and immunoblotting with the suspected interactors (Wu et al, 2006). Interestingly, none of these interactions could be detected with Lyp PTP (WT), but only with the substrate trap Lyp PTP (D195A/C227S) (Wu et al, 2006).

The specific tyrosines identified as direct substrates for Lyp were Lck Y394 and Zap-70 Y493 and not Y319 as proposed in the PEP knock-out (Hasegawa et al, 2004; Wu et al, 2006). The presence of hyper-phosphorylated Zap-70 Y319 on effector T cells from PEP-/- mice (Hasegawa et al, 2004) are likely the consequence of enhanced Lck activation in these cells and not direct dephosphorylation by PEP.
**TCR components**

Components of the TCR complex have also been shown to be associated with Lyp/PEP. In Wu’s study, using the phosphatase domain alone of Lyp mutated at D195A/C227S as bait, mass spectrometry revealed an interaction with CD3ε and the CD3ζ chain (Wu et al, 2006). No further work was carried out for CD3ε, but total phosphorylation of the CD3ζ chain was shown to be reduced in the presence of purified active Lyp, suggesting that the ζ chain is a direct Lyp substrate (Wu et al, 2006). PEP was previously shown to interact with the CD3ζ chain and downregulate its phosphorylation, but the authors suggested that it is not a direct association (Cloutier & Veillette, 1999). This discrepancy may be attributed to the different types of constructs used to overexpress and purify the cytoplasmic chain of ζ and its subsequent conformation, as well as the different types of trapping mutants employed (ie the Lyp PTP D195A/C227S used in Wu et al may detect direct binding more) (Wu et al, 2006).

**Cbl**

Cbl (also known as c-Cbl) is an E3 ubiquitin ligase that is expressed in all tissue types but has a particularly important role in immune function. Overexpression of PEP in a T-cell line showed downregulation of phosphorylation of a protein around 120kDa, thought to be Cbl (Cloutier & Veillette, 1999). The human form, Lyp, is also reported to interact with Cbl in Jurkat cells and COS-1 cells (Cohen et al, 1999). Overexpression of Lyp results in a downregulation of overall c-Cbl tyrosine phosphorylation (Chien et al, 2003; Cohen et al, 1999). It is not clear from the aforementioned studies if the Lyp and Cbl interaction is direct or mediated by other proteins such as Csk or the SFKs, or what the functional
consequences of this interaction is. A large portion of this thesis is focused on the understanding and characterization of this interaction.

**Grb2**

The adaptor Grb2 may also be present in a Lyp complex. When Grb2 was expressed as a GST fusion protein and used as bait to pull down potential new interactors in lymphocytes, Lyp was identified (Hill et al, 2002). Overexpression of both proteins in 293T cells confirmed the interaction, and even suggests that in the presence of Lyp and the Bcr-Abl kinase, Grb2 protein level is decreased, but it is not clear why (Hill et al, 2002). This interaction has not been confirmed for PEP, nor any work carried out on Grb2 with Lyp/PEP in immune cells.

**Others**

In the substrate trapping experiment followed by mass spectrometry carried out by Wu *et al*, other interactors that were identified were VCP (valosin containing protein) and Vav (Wu et al, 2006). Pull-downs with the substrate trapping PTP domain of Lyp and vanadate-treated Jurkat lysates confirmed the Vav interaction, but no other work was done to confirm the VCP interaction (Wu et al, 2006).

In another study by Hill *et al*, Lyp was overexpressed in COS cells along with the Bcr-Abl kinase (Hill et al, 2002). In the presence of Lyp, Bcr-Abl protein levels were reduced, and there was a reduction in phosphorylation of ERK1/2 and CrkL (Hill et al, 2002). The exact mechanisms of these observed effects have not been elucidated.

A major component of this thesis work is to find and characterize other novel Lyp interactions. This has led to the identification of G-protein Receptor Kinase 2 (GRK2) and
will be discussed in detail in later sections.

1.6 REGULATION OF PEP/LYP

Post-translational modifications

Post-translational modifications such as phosphorylation may be important in the regulation of Lyp/PEP activity. The PEST family of phosphatases contain a conserved serine residue at their N-terminus. It has been confirmed in Lyp that S35 is indeed phosphorylated, and that PKCδ is the responsible kinase (Yu et al, 2007). This is thought to destabilize the ability of the catalytic domain to recognize its substrate (Yu et al, 2007). Indeed, phosphorylated Lyp at S35 had lower activity than unphosphorylated Lyp in phosphatase assays using pNPP or c-Src as a substrate (Yu et al, 2007). Overexpression of a mutated S35E Lyp (to mimic constitutively phosphorylated serine) in Jurkat T-cells resulted in suppressed function of Lyp, as seen in an inability to suppress TCR activation as measured by an NFAT/AP1 luciferase reporter assay and hyperphosphorylation of pERK1/2 and Lck Y394 (Yu et al, 2007).

There is evidence to suggest that Lyp may also be negatively regulated by tyrosine phosphorylation. Lyp is tyrosine phosphorylated upon T-cell activation (Fiorillo et al, 2010). Overexpression of the catalytically inert C227S displayed enhanced tyrosine phosphorylation, indicating an innate capacity of Lyp to self-dephosphorylate (Fiorillo et al, 2010). Bottini’s group has identified a major site of Lyp phosphorylation at Y536 that is phosphorylated by Lck and may inhibit Lyp PTP activity. The model that has been proposed in this work is that the binding of Lyp to Csk promotes the recruitment of Lyp to the proximity of Lck where Lck phosphorylates Lyp on Y536 to turn off its activity in order to
potentiate T-cell signalling upon activation. This is supported by overexpression of Lyp Y536F resulting in reduced NFAT/AP1 luciferase reporter activation, enhanced CD69 expression in Jurkat cells and enhanced phosphatase activity of immunoprecipitated Lyp Y536F compared to wt (Fiorillo et al, 2010). The authors note that mutation of Y536 does not completely abrogate Lyp overall tyrosine phosphorylation, indicating that there are other sites that are also targets for tyrosine kinases, including Csk itself (Fiorillo et al, 2010). Evidence for this is provided by co-expression of Lyp with Csk resulting in enhanced Lyp phosphorylation (Fiorillo et al, 2010). Conversely, Lyp in thymocytes from Csk knock-out mice or cells with Csk siRNA contained reduced tyrosine phosphorylation, pointing to the possibility that Lyp may also serve as a Csk substrate (Fiorillo et al, 2010). The functional consequences of further phosphorylation sites on Lyp have not been determined.

**Intramolecular Inhibition**

A common form of regulation of tyrosine phosphatases is an intra-molecular inhibition of the catalytic domain, as described for SHP-1 and TC-PTP (Hao et al, 1997; Pei et al, 1994). There must also be some form of intra-molecular inhibition of the catalytic activity of Lyp/PEP, as the catalytic domain alone (aa 1-300) displayed more than 2-fold higher activity in phosphatase assays than the full-length protein (Gjorloff-Wingren et al, 1999; Hong et al, 2009). Liu et al examined this further by overexpressing and purifying sequentially shorter versions of Lyp from *Baculovirus* and assessing their activity (Hong et al, 2009). With full-length Lyp as a reference, a fragment corresponding to aa 1-423 showed a modest increase in activity, while aa 1-300 had the highest. Further truncation of this region concluded that a very short stretch (aa 301-320) just following the catalytic domain is largely responsible for the inhibition (Hong et al, 2009). While phosphatase
assays carried out comparing Lyp aa 1-300 compared to aa 1-320 do show that the latter has lower activity, these data do not show the full-length protein as a reference (Hong et al, 2009) Physiologically, even a modest increase in activity as originally seen with the fragment aa 1-423 compared to full-length Lyp could serve important biological functions. Also, as these Lyp fragments were purified from *Baculovirus*, the contribution of post-translational modifications in-vivo must also be taken into consideration.

The structure of the catalytic domain itself must be examined for possible roles into the regulation of its activity. As described in a previous section, the mechanism of dephosphorylation employed by the enzyme involves the movement of the WPD loop around the substrate. Recent structural analysis of Lyp has revealed that it has an atypical conformation of the loop (Barr et al, 2009). That is, the WPD loop contains an extra turn in one of the adjacent α helices, resulting in an atypically open but catalytically inactive conformation, as the aspartic acid in the WPD loop is moved out of the active site (Barr et al, 2009). This conformation was only observed in two other unrelated phosphatases, GLEPP1 and STEP (Barr et al, 2009), and may have an important regulatory role in the activity of these enzymes.

**Oxidation**

The catalytic domain of tyrosine phosphatases are sensitive to inactivation due to oxidation. This results in the loss of the nucleophilic properties of the catalytic cysteine, preventing the formation of the phospho-cysteine intermediate, and hence renders the enzyme inactive. Physiologically, oxidation of the phosphatase can occur due to the presence of reactive oxygen species (ROS) resulting from many different stimuli. For example, TCR signalling has been shown to produce ROS and negatively inhibit SHP2
activity (Kwon et al, 2005). There are mechanisms in place to protect the catalytic cysteine from such inactivation. A mechanism that exists for classical PTPs and that has been described primarily for PTP-1B utilizes the residue adjacent to the catalytic one (Salmeen et al, 2003; van Montfort et al, 2003). The catalytic cysteine forms a reversible sulfenamide ring with the main-chain amide of the next residue (usually serine) and thus it is protected from oxidation. Another mode of regulation is through a second cysteine residue in the PTP signature motif in the proximity of the catalytic cysteine. A disulfide bridge is formed between the two residues and the catalytic cysteine is reduced and reactivated. In classical PTPs, this second cysteine is thought to be within the P-loop, as has been described for the phosphatase LM-PTP (Chiarugi et al, 2001). Similar mechanisms are found in PTEN and cdc25 but the second cysteine is found outside the P-loop and is referred to as the “back-door” cysteine (Buhrman et al, 2005; Cho et al, 2004; Lee et al, 2002). In the case of Lyp, while it is thought to be a classical PTP, interestingly a sulfenylamide ring is not formed. Instead, a disulfide bridge is formed in oxidizing environments between the catalytic C227 and a “back-door” residue outside the P-loop, C129 (Tsai et al, 2009).

Another level of regulation seems to be added by another residue, C231, but surprisingly, in an opposite way to that of C129 (Tsai et al, 2009). This cysteine is within the P-loop, but may act to inhibit Lyp activity by preventing Lyp reactivation following oxidation (Tsai et al, 2009). Upon inactivation of Lyp by oxidation, a mutant form of Lyp C231S displayed much higher rates of reactivation than wild type, indicating that this residue may have a role in preventing the full reactivation of Lyp and keeping it inactive (Tsai et al, 2009). The authors suggested a novel “ying-yang” mechanism of redox regulation of Lyp by
these two non-catalytic cysteines (C129 and C231) (Tsai et al, 2009), adding to the complexity of this phosphatase and the importance of its regulation.

### 1.7 PHOSPHATASES AND AUTOIMMUNITY

To date, there have been a number of reports indicating that disruptions in genes encoding phosphatases can lead to perturbations in the immune system and subsequent autoimmune or autoinflammatory states. Autoimmunity represents very complex, multifactorial disease states. Often the genetic contributions of individual gene loci to disease susceptibility are quite low, but combinations of these genetic variants can increase disease risk.

**CD45 (PTPRC)**

One of the phosphatases implicated in autoimmunity is CD45 (PTPRC) (reviewed in Hermiston et al, 2009; Vang et al, 2008). This large, 180-220kDa transmembrane tyrosine phosphatase is expressed in very high abundance in all hematopoietic cell lineages and exists in different functional isoforms. It contains an extracellular domain, a transmembrane domain, and a cytoplasmic tail containing 2 consecutive PTP domains, only one of which is functionally active. It is autoinhibited by homodimerization. CD45 serves as a positive regulator of cell signalling by opposing Csk functions. It dephosphorylates PAG to release Csk, and also the inhibitory tyrosine residue on Src family kinases such as Lck and Fyn and renders them active. CD45 deficient mice display severe immunodeficiency, with greatly reduced numbers of T-cells (Byth et al, 1996; Kishihara et al, 1993). B-cell numbers were not greatly changed but their positive and negative selection was altered, leading to the generation of autoreactive B-cells in CD45−/− mice (Cyster et al, 1996; Huntington et al,
2006). Mice carrying CD45 mutation E613R in which dimerization of the molecule is disrupted present with a very strong autoimmune phenotype (Majeti et al, 2000).

In terms of human autoimmunity, people lacking CD45 suffer from severe combined immunodeficiency (Kung et al, 2000; Tchilian et al, 2001). Lower levels of CD45 have also been reported in T and B cells of patients with systemic lupus erythematosus (Flores-Borja et al, 2007; Jury et al, 2004). A polymorphism in CD45 conferring a C77G change that impairs dimerization of CD45 has been associated with increased risk for systemic sclerosis (Schwinzer et al, 2003), and autoimmune hepatitis (Vogel et al, 2003). This polymorphism has also been linked to patients with multiple sclerosis (Jacobsen et al, 2000), but subsequent studies have not been able to replicate this finding (Barcellos et al, 2001). There is another variant, C59A that alters alternative splicing in CD45 that has been shown to be present in a large family with many members with multiple sclerosis (Jacobsen et al, 2002). Both the mouse and human studies of impaired CD45 expression or function provide evidence that this phosphatase is important in the regulation of the immune system, and dysregulation of this phosphatase can result in autoimmune diseases.

**IA-2 (PTPRN) and IA-2β (PTPRN2)**

Other transmembrane phosphatases that have been implicated in human autoimmunity are IA-2 (PTPRN) and IA-2β (PTPRN2) (reviewed in (Vang et al, 2008). They are transmembrane with a cytosolic phosphatase domain, but they have not been extensively studied. Interestingly however, in Type I Diabetes, they were identified as being major autoantigens in pancreatic beta cells (Lu et al, 1996; Passini et al, 1995).
**TC-PTP (PTPN2)**

Another phosphatase that has been associated with autoimmunity is TC-PTP (PTPN2) (reviewed in (Gregersen & Olsson, 2009)). This phosphatase is actually expressed ubiquitously, and consists largely of a single phosphatase domain. It exists as two isoforms, and its substrates include STAT1 in the nucleus as well as Jak1 and Jak3. Mice deficient in TC-PTP have impaired B and T cell functions (You-Ten et al, 1997). Variants in PTPN2 have been reported to be associated with increased risk of Crohn’s disease and Type 1 Diabetes, while they may also be associated with Rheumatoid Arthritis and Graves’ disease (2007; Barrett et al, 2008; Todd et al, 2007).

**SHP1 (PTPN6)**

SHP1 (PTPN6) has been shown to be an important regulator in immune cell function and has implications to autoimmunity (reviewed in (Zhang et al, 2000); (Pao et al, 2007; Vang et al, 2008)). It contains two SH2 domains followed by the tyrosine phosphatase domain. SHP1 activity is auto-inhibited by a closed conformation of the protein with the N-terminal SH2 domain interacting with the catalytic domain and thus sterically blocking binding of a phosphorylated tyrosine. Upon cell activation, phosphorylated proteins interact with the SH2 domains on the protein and the intra-molecular interaction with the PTP domain is then disrupted, rendering the PTP domain open. Alternatively, the C-terminus of SHP1 itself may become tyrosine phosphorylated upon activation and form a new intra-molecular interaction with one of its SH2 domains, which also opens the PTP domain. Substrates of SHP1 include the Src family kinases and antigen receptor ITAMs.

The strong phenotypes of SHP1 mutant mice demonstrate the importance of this phosphatase in the immune system (Shultz et al, 1993; Tsui et al, 1993). The mice develop a
patchy hair loss, and are therefore known as *motheaten*. These mice have mutations in the SHP1 gene that disrupt alternative splicing of the mRNA. *Motheaten* mice have a total lack of SHP1 protein and die very young at 3 weeks from lung inflammation. The related strain, *viable motheaten* mice have mutations in which the SHP1 protein is expressed but is largely inactive. These mice survive to about 9 weeks (Green & Shultz, 1975).

Both *motheaten* strains display severe inflammatory phenotypes, consisting of increased circulating neutrophils and monocytes and subsequent tissue damage to liver, lungs and spleen. Signs of autoimmunity include the presence of autoantibodies and the onset of glomerulonephritis (Shultz & Green, 1976). It has been shown that *motheaten* mice have a significant increase in T regulatory cell populations (Carter et al, 2005), and B and T cell signalling is also defective (Pani et al, 1996; Pani et al, 1995). Defects in T cell development have been observed, and the thymus involutes at an early stage (Shultz et al, 1997). The phenotype of these mice point to important roles of SHP1 in immune function. Like CD45, lower levels of SHP1 protein have been detected in patients with SLE (Huck et al, 2001). To date, while polymorphisms in the SHP1 gene have been found in humans (Cao & Hegele, 2002; Matsushita et al, 1999), they have not been associated with autoimmune disease.

**PTP-PEST (PTPN12)**

When it comes to PEST family phosphatases, PTP-HSCF and PTP-PEST have not been associated with autoimmunity. Mutations in the adaptor PSTPIP that render it unable to bind PTP-PEST have been linked to PAPA syndrome and Familial recurrent arthritis, but PTP-PEST itself has not been directly implicated in these (Wise et al, 2002).
1.8 LYP’S ROLE IN AUTOIMMUNITY :C1858T (R620W)

Functional consequence of Lyp R620W

In recent years, one of the reasons that Lyp began to receive tremendous attention in the literature was because of its link to autoimmunity. This was due to a functional variant in the PTPN22 gene associated with Type I Diabetes, a single nucleotide polymorphism at C1858 to a T, conferring an arginine to a tryptophan change at position 620 (R620W) (Bottini et al, 2004). This corresponds to R619W in the mouse. Structurally, this positively charged residue is just after the first proline-rich domain of Lyp, and is important for positioning the hydrophobic residues in the P1 domain for interactions with its binding partners, such as the SH3 domain of Csk (Ghose et al, 1988). Substitution of the arginine for a tryptophan, a bulkier residue, may disrupt this positioning. Indeed, the R620W has been shown biologically to inhibit the formation of the Lyp-Csk complex, with the R620W essentially abolishing Csk binding (Begovich et al, 2004; Bottini et al, 2004). As Lyp and Csk are thought to cooperatively downregulate Src-family kinases (Cloutier & Veillette, 1999), the disruption of this interaction may lead to alterations in the regulation of cell signalling. To date, the R620W has only been shown to affect the binding to Csk and no other Lyp/PEP interactors.

As for the effect of the R620W on the phosphatase itself, very little is known. The Lyp protein has been shown to be tyrosine phosphorylated, and the R620W variant displays reduced tyrosine phosphorylation compared to WT (Fiorillo et al, 2010). One possible reason is reduced recruitment of Lyp R620W by Csk to the Lck kinase and hence reduced phosphorylation at Y536 (Fiorillo et al, 2010). Furthermore, the same group provided evidence that Lyp may be phosphorylated by Csk at another tyrosine, further supporting the
notion that reduced binding to Csk would hence reduce Lyp R620W phosphorylation (Fiorillo et al, 2010).

More work needs to be carried out in order to determine if the R620W substitution disrupts interactions with other binding partners, or alters the structure or post-translational modifications of the protein itself.

**Disease association**

Early reports of a link between the PTPN22 C1858T polymorphism and autoimmunity were provided by several groups in 2004 (reviewed in (Siminovitch, 2004)). The first was a case-control study involving Caucasian American and Sardinian populations and showed that patients with Type I Diabetes were more likely to carry this allele than healthy controls (Bottini et al, 2004). Specifically, in the Sardinian populations, 4% of controls carried the allele compared to 8% of patients, indicating that this allele predisposed people to a higher risk of developing the disease (Bottini et al, 2004). This was quickly confirmed for Type I Diabetes by other groups in the same year (Onengut-Gumuscu et al, 2004; Payne et al, 2004).

In another large case-control study in 2004 examining two populations, it was found that the same C1858T was associated with increased susceptibility to Rheumatoid Arthritis, as the allele was present in 17% of the control populations but in 28% of patients with Rheumatoid Arthritis (Begovich et al, 2004). The link to another autoimmune disease, Graves’ thyroiditis, was also established by Smyth et al showing the presence of the T allele in 10% of controls versus 14% of Graves’ patients (Payne et al, 2004) and Velaga et al showing the presence of the T allele in 15% of control versus 26% of Graves’ patients (Velaga et al, 2004).
Finally, in the same year, a link was found between this polymorphism and Systemic Lupus Erythematosus (Kyogoku et al, 2004). In this study, the T allele was found in 8% of controls compared to 12% of SLE patients (Kyogoku et al, 2004). In the above-mentioned studies, it was consistently observed that the homozygous genotype of the risk allele, TT, is extremely rare, found only in about 1% of cases. What the Kyogoku study found is that in the case of SLE, the homozygous CC genotype doubled the risk of developing the disease compared to carrying the heterozygous CT genotype (Kyogoku et al, 2004).

Since then, hundreds of reports supporting the link between the C1858T and a risk to autoimmunity have been published. The main ones are highlighted in Table 2.

Furthermore, in 2007, a groundbreaking genome-wide association study was carried out by the Wellcome Trust Case Control Consortium using 17,000 people and examining over 500,000 SNPs and their risk to disease (Peschard et al, 2007). Of the over half a million SNPs studied, this large study was able to robustly replicate the association of the PTPN22 C1858T risk allele to Rheumatoid Arthritis and Type I Diabetes (Peschard et al, 2007) further validating the importance of this locus. The association between the R620W and RA or T1D is particularly strong, conferring the most susceptibility risk after variants found in the MHC (reviewed in (Gregersen & Olsson, 2009) .

While the risk to many autoimmune diseases have been established, there are others that do not share this susceptibility locus, like Crohn’s disease (van Oene et al, 2005). In this large case controlled study of two Canadian populations, while the risk to Rheumatoid Arthritis was confirmed, the risk to Crohn’s could not be established (van Oene et al, 2005). Other autoimmune diseases where no association has been found include multiple sclerosis (Begovich et al, 2005), and primary biliary cirrhosis (Milkiewicz et al, 2006). These
Table 2: PTPN22 C1858T and Disease association

<table>
<thead>
<tr>
<th>Disease</th>
<th>Selected References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I Diabetes</td>
<td>(Bottini et al, 2004; Onengut-Gumuscu et al, 2004; Payne et al, 2004)</td>
</tr>
<tr>
<td>Rheumatoid Arthritis</td>
<td>(Begovich et al, 2004; Carlton et al, 2005; Orozco et al, 2005; van Oene et al, 2005)</td>
</tr>
<tr>
<td>Juvenile Idiopathic Arthritis</td>
<td>(Hinks et al, 2005; Viken et al, 2005)</td>
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<tr>
<td>Graves’ disease</td>
<td>(Skorka et al, 2005; Velaga et al, 2004)</td>
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<tr>
<td>Hashimoto’s thyroiditis</td>
<td>(Criswell et al, 2005)</td>
</tr>
<tr>
<td>Wegener’s Granulomatosis</td>
<td>(Jagiello et al, 2005)</td>
</tr>
<tr>
<td>Systemic Sclerosis</td>
<td>(Dieude et al, 2008)</td>
</tr>
<tr>
<td>Myasthenia Gravis</td>
<td>(Vandiedonck et al, 2006)</td>
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<tr>
<td>Generalized Vitiligo</td>
<td>(Canton et al, 2005)</td>
</tr>
<tr>
<td>Addison’s disease</td>
<td>(Skinningsrud et al, 2008)</td>
</tr>
<tr>
<td>Alopecia Areata</td>
<td>(Betz et al, 2008)</td>
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</table>
findings indicate that the PTPN22 C1858T is a common risk allele for some, but not all autoimmune diseases.

Interestingly, the R620W variant has been associated with reduced risk of developing tuberculosis, indicating one example of a protective effect of this variant (Gomez et al, 2005). This variant may also be protective in Crohn’s disease after all (Wang et al, 2010a), but further verification of this needs to be carried out. Presumably, while the R620W is a common risk allele for some autoimmune diseases, the modest protective effect that it provides against others may be a reason for its maintenance in human populations.

There are other SNPs for PTPN22 that have been identified, two of which are in the catalytic domain, conferring changes at R189X and R263Q (Carlton et al, 2005). Initially, neither of these SNPs were found to be associated with Rheumatoid Arthritis, but two other SNPs around the PTPN22 gene region showed weak associations to RA independent of R620W (Carlton et al, 2005). In a larger case-control study, the R263Q was later found to actually be associated with a lower risk of developing RA (Rodriguez-Rodriguez et al, 2010). The R263Q was studied at the functional level and Orru et al report that it has reduced phosphatase activity than the wt enzyme (Orru et al, 2009). This was determined by using phospho-flow cytometry of cells expressing R263 or Q263 and assessing SLP-76 phosphorylation status (Orru et al, 2009), (even though there are no reports of SLP-76 as a direct Lyp substrate).

These authors also assessed, however, reduced ability to downregulate ERK phosphorylation, NFAT activation, or in-vitro phosphatase activity of the Q263 compared to the wt (Orru et al, 2009). Structurally, the conformation adopted by the Q263 may interfere with substrate binding (Orru et al, 2009) and hence contribute to a loss-of-function.
Furthermore, the authors found an association between this variant and a reduced risk of SLE, indicating that the R263Q substitution may actually be protective against autoimmunity (Orru et al, 2009). A weak protective effect has been reported for R263Q in Addison’s disease (Skinningsrud et al, 2008), but not others such as Type I Diabetes (Zoledziewska et al, 2008) or systemic sclerosis (Diaz-Gallo et al, 2010).

**Gain or loss of function?**

The variant Lyp R620W has lost its capacity to bind Csk, and hence presumably, has lost its ability to act synergistically with Csk to downregulate Src-family kinases. At the level of this molecular interaction at least, this polymorphism may be considered a loss-of-function. At the cell or whole organism level however, there has been debate as to the true consequences of the presence of this polymorphism. Compelling evidence has been provided by different groups, but there are findings that need to be interpreted with caution.

**T-cell function**

The group that initially demonstrated that the C1858T was a risk allele for Type I Diabetes (Bottini et al, 2004) followed up with a report to draw attention to this matter, stating that this is a gain-of-function allele (Abrahamsen et al, 2003). To begin comparing the two variants, the authors purified T lymphocytes from T1D patients that were either C/C or heterozygous C/T, cultured them in the presence of αCD3/28 for 20 hours and measured IL-2 secretion. They found that patients heterozygous for the risk allele C/T had significantly lower levels of IL-2 secretion (Abrahamsen et al, 2003). According to the original characterization of the Lyp protein in 1999, culturing of PBMCs for 24 hours in the presence of CD3 can induce a significant increase in Lyp protein level (Cohen et al, 1999). This is not addressed in Vang’s report (Abrahamsen et al, 2003), and it is not clear if the
polymorphism may have an altered expression levels in response to stimulation, possibly affecting how the results were interpreted. The treatment of the same patient samples with PMA/ionomycin showed no difference in IL-2 production (Abrahamsen et al, 2003). This fits with the notion that under these conditions Lyp is inactive, as has been shown in Jurkat cells treated with PMA/ionomycin (Yu et al, 2007). Protein levels are shown to be equal, however, in Jurkat cells electroporated with plasmids encoding Lyp R or the variant W, the IL-2 secretion was shown to be reduced in the presence of the W variant (Abrahamsen et al, 2003). Jurkat T-cells were also electroporated with the different Lyps to be used in a luciferase assay using NFAT/AP-1, and initially the authors state that both the R or the W Lyp had an inhibitory effect, but after normalization of protein expression, the W Lyp had a more pronounced inhibitory effect (Abrahamsen et al, 2003). Similarly, an in-vitro phosphatase assay was carried out and found that the variant had higher activity, after normalization of protein levels. No data was shown as to what the original protein level was, why it was not an issue in previous electroporations, or how the normalization was calculated.

Phosphatase assays were once again carried out by the same group in a follow-up report but with different findings. When purified Lyp was overexpressed in Baculovirus and subjected to an in-vitro phosphatase assay, there was no significant difference between the WT or RW Lyp proteins, nor was there any difference with the addition of Csk (Fiorillo et al, 2010). This indicates that binding to Csk per se is not responsible for any direct effects on phosphatase activity in-vitro and that other post-translational modifications or interactions in the cell are needed to see some, if any, effect.
TCR signalling was also examined by Vang et al, results of which include a further reduction in pERK and phosphorylation of the CD3 ζ chain in Lyp W transfected cells. Lck is a known Lyp substrate, and supplemental information provided in this report showing phosphorylation status of pY394 shows an unconventional pattern of phosphorylation in control samples. Upon 0, 2, 5 minutes of αCD3/28 stimulation, this activating tyrosine of Lck should presumably be inducibly phosphorylated. Instead, in control samples, the opposite was shown. The vector control cells showed a huge phosphorylation signal in resting cells followed by a downregulation upon stimulation. The strongest phosphorylation signal in Lyp R or W cells was also at the resting time point (albeit lower than vector). Since the control untransfected samples did not show an expected inducible phosphorylation signal as a reference, it is difficult to assess subtle changes in dephosphorylation of a substrate by two variants of the same phosphatase. In summary, while there is evidence to support the possibility of an enhanced function of the W variant, in some experiments the expression was normalized and some it was not, thus possibly skewing some of the results. Needless to say, this study has prompted more related studies and in other cell types.

In support of the gain-of-function role of the Lyp W variant, Rieck et al examined T cells from human control subjects carrying either the C/C allele or were heterozygous for the C/T allele (Rieck et al, 2007). Calcium mobilization was measured in CD4^+ T cells with no difference observed in naïve cell compartments, but a lower calcium mobilization in heterozygous C/T samples in CD4^+ memory T cells (Rieck et al, 2007). When cytokine secretion was measured in CD4^+ cells, no significant difference in IL-2 or other cytokines was detected except for IL-10 which was reduced in C/T samples (Rieck et al, 2007). IL-10 is thought to be primarily an anti-inflammatory cytokine, and IL-10 deficient mice develop
autoimmunity ((Kassenbrock & Anderson, 2004). Hence a reduction in this cytokine would imply that there is in fact a defect in regulation of the immune system. No difference was observed in proliferation, but there was expansion of the CD4+ memory T-cell pool in people carrying the T allele (Rieck et al, 2007).

T-cell responses have also been measured in patients with Type I Diabetes. Aarnisalo et al reported that patients carrying the T allele showed reduced CD4+ T cell proliferation and calcium mobilization in response to CD3/28 stimulation compared to C/C controls (Aarnisalo et al, 2008)

There are groups, however, that challenge the gain-of-function arguments for the Lyp W variant in T cells. As up to 50% of PEP has been shown to be bound to Csk physiologically (Cloutier & Veillette, 1996), it is possible that when Lyp/PEP alone is overexpressed in cells, stoichometrically it may not be able to bind to its natural ligand, Csk, and hence may have off-target effects. To address this possibility, Arthur Weiss’ group co-expressed PEP/Lyp alongside Csk in Jurkat cells (Hermiston et al, 2009). They also co-transfected GFP to determine electroporation efficiency, and specifically determined the pERK signal in highly GFP expressing cells to ensure that different levels of expression did not complicate the result. Erk phosphorylation was determined following TCR or PMA stimulation. Following TCR stimulation, Lyp wt alone or in combination with Csk inhibited pERK to a greater extent than the Lyp W variant. The same experiment was done with PEP and Csk and the results were similar. No difference was seen with PMA stimulation, consistent with the notion that PMA may inhibit Lyp activity (Yu et al, 2007). Furthermore, PEP and Csk were overexpressed in Jurkat cells along with the cell surface marker CD16 and calcium flux was measured in CD16 expressing cells in order to ensure that successfully
transfected cells were examined. The results once again showed that PEP W is not as effective as wild-type PEP R at inhibiting calcium mobilization. Thus Zickerman et al demonstrate the Lyp R620W is in fact a loss-of-function allele, arguing that overexpression levels of the protein must be carefully controlled for and also studied in the context of Csk (Yu et al, 2007). While the overall electroporation efficiency can be determined in this way, it is not absolutely certain however, that the same cells have taken up CD16, PEP, and Csk at equal levels each. Also, while similar amounts of plasmid DNA may have been introduced into cells, it is not certain that this will correspond to equal amounts of protein ultimately in the cell.

A loss-of-function has also been reported in T-cells of patients with myasthenia gravis (Lefvert et al, 2008). PBMCs were isolated from patients, presented with auto-antigen, and IL-2 secretion was measured. Patients homozygous for the variant T allele showed a huge increase in IL-2, indicating that the Lyp R620W was not able to regulate T-cell activation (Lefvert et al, 2008).

**B cell function**

B cells were also briefly examined in the Rieck et al study. In support of the gain-of-function hypothesis, people carrying the T allele had a reduced pool of memory B cells and reduced BCR-mediated calcium mobilization (Rieck et al, 2007). B cell signalling was further examined by the same group using the same profile of human subjects (healthy donors with genotypes either C/C or C/T) (Arechiga et al, 2009). B cell proliferation was decreased, as well as total phosphorylation assessed by intracellular staining, in subjects carrying the C/T allele compared to the C/C allele (Arechiga et al, 2009).
Specifically, proximal B-cell signalling was examined by stimulation of B cells with IgM. Phosphorylation of Syk Y525, PLCγ Y759, AKT S473 and p38 MAPK T180/Y182 were all reduced in the sample heterozygous for the C/T compared to the homozygous wt C/C (Arechiga et al, 2009). The same analysis was carried out using a subject homozygous for the variant T/T allele, and while constant amounts of total Syk were demonstrated between wild type C/C subject and the variant T/T subject, the actual total amounts of Lyp were very different between these two samples (Arechiga et al, 2009). Though not addressed in the paper, the total levels of Lyp shown were much greater in the variant T/T (up to 5-fold higher perhaps). It is difficult to interpret the inherent phosphatase activity of one variant of Lyp to another when the protein amounts are so different in these samples.

There is an inhibitor of Lyp known as IC-11 that binds specifically to its catalytic domain (Yu et al, 2007). This was used by Arechiga et al to examine any changes in BCR signalling (Arechiga et al, 2009). This inhibitor has been used previously in Jurkat cells overexpressing Lyp and was shown to enhance phosphorylation of the Lyp substrate Y394 on Lck and subsequently pERK (Yu et al, 2007). The use of the inhibitor in the Arechiga study is justified to see if there is an enhanced phosphatase function encoded by the Lyp W variant (Arechiga et al, 2009). Treatment of B cells from human donors with IC-11 followed by IgM stimulation results in enhanced phosphorylation of PLCγ2 compared to vehicle treated cells, consistent with the notion that the phosphatase activity of Lyp is being inhibited, but no difference was observed between the C/C subjects compared to the C/T subjects (Arechiga et al, 2009). A difference is claimed to be observed in phospho Syk Y525/526 signalling, with enhanced rescue of this site of phosphorylation after inhibitor treatment of heterozygous C/T cells, but the authors present no data on the wt C/C subject.
treated with the inhibitor, rendering a direct comparison between the two impossible (Arechiga et al, 2009). Hence from the inhibitor data provided, it is possible to conclude that wt Lyp R has a role in inhibiting B cell signalling, but no evidence to support the notion that the phosphatase activity of the variant Lyp W is more than the wild type.

On the other hand, the variant W was shown to be unable to inhibit B cell function by another group (Lefvert et al, 2008). The production of autoantibodies as a read-out of B cell function was examined in myasthenia gravis patients carrying the T allele. Stimulation of PBMCs from patients with auto-antigen resulted in increased production of IgG against it, indicating that the variant is a loss-of-function (Lefvert et al, 2008).

A defect in B cell function in humans was also shown by another group (Magnifico et al, 2007). Antibodies from new transitional B cells of human subjects carrying the R620W variant were tested against antigens commonly reacted with in autoimmunity such as dsDNA and LPS. The R620W carriers were found to contain more reactive antibodies than wild type counterparts, indicating a defect in central B cell tolerance. Similarly, antibodies from mature naive B cells from the same subjects were also more reactive, indicating a defect in peripheral B cell tolerance. This led to overall higher frequency of autoreactive B cells. Furthermore, this study showed that R620W carriers also had different expression patterns of genes in different B cell activation pathways, such as the BCR, CD40 and TLR pathways. Naïve B cell stimulation of CD40 resulted in a significant increase in CD69 and CD25 in R620W carriers versus wild type (Magnifico et al, 2007). Overall, this report shows that the R620W variant interferes with the removal of autoreactive B cells, indicating a loss-of-function of this phosphatase.
Other cell types

Natural killer cells have been reported to express high levels of Lyp (Begovich et al, 2004). Natural killer cell expansion was examined in healthy subjects that were either homozygous wild type T/T or heterozygous C/T (Douroudis et al, 2010). Samples heterozygous at C/T had a significantly reduced ability to expand *in-vitro* (Douroudis et al, 2010) but the mechanism is not addressed.

To date, the functional consequences of the R620W variant in other cell types has not been published.

**Summary of Lyp R620W**

Clearly the functional consequences on the Lyp R620W mutant remain unresolved and the debate in the field ensues. The use of patient samples in addressing this issue may seem physiologically relevant, but human sample data can also come with inherent variability that may make results difficult to interpret.

At the biochemical level, it is well established that the W substitution alters binding to Csk, but not much else is known. It is possible that the actual protein expression between the wild type Lyp or its variant W may differ. While RNA transcripts seem to not be changed (Nielsen et al, 2007), there may be variations in protein amount in the cell due to differential RNA processing and protein synthesis or protein stability and degradation. Furthermore, the activity of Lyp may be regulated by further unknown mechanisms. When the wild type is in complex with Csk, it may be tightly regulated by another protein, but loses this regulation when it is unbound, hence possibly targeting other additional substrates, either activating or inhibitory. There may be another intra-molecular conformation observed in the
variant protein that may alter its phosphatase activity or result in disregulated substrate recognition. All these questions remain to be resolved.

One thing is clear – this phosphatase is a very important regulator in the immune system. The work presented in this thesis will attempt to delineate some of the mechanisms of Lyp and PEP and their respective R620W/R619W variants. Understanding the biological function of the normal protein, as well as the variant, will provide important insights into processes involved in immune cell function and autoimmunity.

1.9 HYPOTHESIS AND RESEARCH AIMS

Hypothesis

The PTPN22 Lyp/PEP protein represents a negative regulator of immune signaling, with the disease-associated R620W variant resulting in altered immune cell function.

Research Aims

i. To further delineate and characterize the function of the Lyp-Cbl interaction in T-cells.

ii. To create a murine model of the disease-associated variant encoding Pep R619W in order to better understand its effects on immune cell function.

iii. To identify novel interactors and signaling pathways that Lyp is involved in to gain further insight into its function.
CHAPTER 2

PTPN22 ENCODING THE LYP TYROSINE PHOSPHATASE

DEPHOSPHORYLATES CBL ON Y371 TO DOWNREGULATE ITS E3 UBIQUITIN LIGASE ACTIVITY

Helen Miliotis, Jinyi Zhang, Katherine A. Siminovitch

Manuscript in Preparation

Author Contributions

H.M. designed and carried out all the experiments and wrote the manuscript, JZ and KAS assisted in project design, data analysis and manuscript preparation.
INTRODUCTION

Lyp is a cytosolic protein tyrosine phosphatase that is found in hematopoetic cell lineages and is encoded by the PTPN22 gene. It contains an N-terminal catalytic domain, and four putative proline-rich regions at the C-terminus for facilitating protein-protein interactions. The function of Lyp is unclear, but insights may be gained from its counterpart in mice. The murine form of Lyp (PEP) has been shown to inhibit T cell activation by interacting with a kinase, Csk, which also inhibits TCR signalling. This interaction is believed to provide dual regulation of Src family kinases through Csk phosphorylation of the C-terminal inhibitory tyrosine and Lyp dephosphorylation of the N-terminal activating tyrosine (Cloutier & Veillette, 1999). The knock-out of the mouse homologue, PEP, presents with normal naïve T-cell functions but some enhanced activation of effector/memory T cells, spontaneous development of germinal centres, elevated serum antibody levels, lymphadenopathy and splenomegaly (Hasegawa et al, 2004). Furthermore, a single nucleotide polymorphism in the PTPN22 gene encoding Lyp conferring an Arg620Trp (R620W) substitution has been associated with a number of autoimmune diseases. These include rheumatoid arthritis (Begovich et al, 2004; van Oene et al, 2005), Type I diabetes (Bottini et al, 2004), Graves’ disease (Smyth et al, 2004), and systemic lupus erythematosus (Kyogoku et al, 2004), but not others like Crohn’s disease (van Oene et al, 2005). The mouse PEP knock-out phenotype as well as the human genetic associations to autoimmunity suggests that this phosphatase is important in immunoregulation, but the mechanisms involved are largely unknown.

To further understand the role of Lyp in T-cell signalling, it is important to begin to further identify and characterize Lyp protein-protein interactions and their biologic sequelae.
One of the proteins that has been suggested to bind Lyp in myeloid cells and T-cells is c-Cbl (also known as Cbl) (Chien et al, 2003; Cohen et al, 1999). Members of the Cbl family are E3 ubiquitin ligases that have been shown to be critical negative regulators of the immune system by degrading components of signalling pathways as well as the immune receptors themselves (Dragone et al, 2006; Naramura et al, 2002; Panigada et al, 2002; Wang et al, 2001b). In addition, Cbl proteins contain multiple domains that facilitate their involvement in many signalling complexes and allow them to function as adaptor proteins. The N-terminus is composed of a tyrosine kinase binding (TKB) region that contains an SH2 domain capable of binding several phosphorylated proteins, including the Syk family kinases ZAP-70 and Syk (Lupher et al, 1998; Lupher et al, 1996; Meng et al, 1999). A RING finger domain responsible for its E3 ubiquitin ligase activity follows a short linker sequence (Joazeiro et al, 1999). Finally, the C-terminus contains polyproline rich regions and several phosphorylated tyrosines for further protein-protein interactions (reviewed in (Ryan et al, 2006; Thien & Langdon, 2001).

The regulation of Cbl ubiquitin ligase activity is poorly understood, but it is of great importance as there are Cbl mutants that are deemed oncogenic, namely v-Cbl, 70Z-Cbl lacking amino acids 366 to 382, and deletions in residues Y368 and Y371 (Andoniou et al, 1994). It is thought that tyrosine phosphorylation of residues in the linker region may be responsible for its activation, particularly Y371 (Levkowitz et al, 1998; Thien et al, 2001; Yokouchi et al, 2001). This has been confirmed by a structural analysis of that region (Dou et al, 2011). Studies have shown that in-vitro, deletions at Y368 and Y371 are oncogenic (Andoniou et al, 1994; Thien et al, 2001).
Of particular interest is the association of the Y371 mutation to human disease. A study using SNP analysis in a large cohort of patients with various myeloid malignancies found mutations in the Cbl linker region, including Y371 (Sanada et al, 2009). Another study of myeloproliferative neoplasms identified as many as 27 variants in Cbl, 12 of which were missense mutations that were clustered around the linker region and RING finger domain and included a Y371 mutation (Grand et al, 2009). A mutation conferring a Y371H substitution in Cbl was also deemed oncogenic in patients with acute myeloid leukemia (AML) (Fernandes et al, 2010). In patients with juvenile myelomonocytic leukemia (JMML), almost half of the mutations found in the Cbl gene corresponded to changes at Y371 (Loh et al, 2009), and the presence of a mutation at Y371 was also found in JMML patients in a separate patient cohort (Muramatsu et al, 2010). The link between mutations at Y371 and association with JMML was further confirmed and also shown to predispose children to possible developmental abnormalities and other disorders (Niemeyer et al, 2010). These aforementioned studies indicate that mutations in Cbl, especially at residue Y371, are tightly linked to myeloid disease states, and that the regulation of Cbl is critical in maintaining immune cell function.

In this report, we show for the first time that the N-terminus of Cbl is indeed tyrosine phosphorylated in human T-cells following TCR stimulation. Furthermore, we show that Cbl is a novel substrate of the Lyp phosphatase, as Lyp directly dephosphorylates Y371 on Cbl. Finally, the dephosphorylation of Cbl leads to its inactivation as an E3 ubiquitin ligase. Hence we show a novel interaction between a ubiquitin ligase and a tyrosine phosphatase, both of which are critical negative regulators of the immune system and aberrations in which have been associated with disease states in humans.
RESULTS

Lyp and Cbl endogenously bind in human PBMCs

The binding between Lyp and Cbl has been reported before (Chien et al, 2003; Cohen et al, 1999), but the functional relevance of this interaction has not been determined. In order to confirm the binding of Lyp to Cbl, we first sought to validate this interaction in human primary T-cells in order to ensure its physiological relevance in normal human cells. To this end, PBMCs were purified from human donors and cultured in the presence of the indicated stimuli. Expression levels of Lyp are clearly inducible after stimulation, consistent with previous findings (Cohen et al, 1999). As the level of Lyp was highest upon stimulation with PHA, this lysate was used to examine the endogenous interaction between Lyp and Cbl. Cbl was immunoprecipitated and immunoblotted with Lyp to assess their interaction. Figure 1b shows that this interaction exists in human primary PBMCs. Furthermore, we sought to examine the interaction under short term TCR stimulation. Human PBMCs were stimulated for 48 hours with PHA, followed by IL-2 for 24 hours. Cells were then serum starved overnight and then stimulated for the indicated time points with αCD3 and αCD28. Cbl was immunoprecipitated and immunoblotted for Lyp. The Lyp-Cbl complex seems to be inducible upon TCR stimulation.

As a T-cell model, Jurkat cells were used to assess the interaction between Lyp and Cbl. The endogenous level of Lyp in Jurkat cells was not easily detectable by immunoblotting. Hence, GFP-Lyp was overexpressed in Jurkat cells. The cells were serum starved and then stimulated with αCD3/αCD28 for the indicated time points. Results show that in Jurkat cells, the binding is inducible upon stimulation (Figure 1c). Confirmation of
Figure 1

a) LYSATES

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Figure 1. Lyp binds Cbl

a) Human PBMCs were harvested or cultured in media with the indicated stimuli. Cells were lysed and immunoblotting was carried out with the indicated antibodies (upper panel). Protein lysates of cells treated with PHA followed by IL-2 were subsequently used for immunoprecipitation of Cbl. Complexes were subjected to immunoblotting with the indicated antibodies (lower panel). b) PBMCs were stimulated for 48 hours with PHA, followed by IL-2 for 24 hours. Cells were then serum-starved overnight and then stimulated for the indicated time points with αCD3 and αCD28. Cbl was immunoprecipitated and immunoblotted with the indicated antibodies. c) Jurkat T-cells were electroporated with GFP-Lyp. Cells were lysed and GFP-Lyp was immunoprecipitated and immunoblotted with the indicated antibodies.
the Lyp-Cbl binding in hematopoietic cells prompted us to further characterize this interaction.

**The N-terminus of Cbl directly interacts with the interdomain of Lyp**

In order to begin the characterization of the Lyp and Cbl complex, we sought to map the interacting protein domains. To find the relevant binding domains of Cbl, domain deletions of the protein were used corresponding to amino acids 1-436, 437-646, 647-906 with an N-terminal GST tag, as previously described (Kirsch et al, 2001). When Lyp was co-transfected with domain deletions of Cbl, it bound to a fragment corresponding to amino acids 1-436 (Figure 2a). The N-terminus of Cbl is important for protein interactions as well as E3 ligase activity. It contains a modified SH2 domain that is flanked by a four-helix bundle and a calcium binding EF-hand subdomain (Meng et al, 1999). This is referred to as the tyrosine kinase binding (TKB) domain, and is the binding site for receptor tyrosine kinases as well as non-receptor Syk family tyrosine kinases. This domain is highly conserved, and all three subdomains are required for the correct folding to occur (Zheng et al, 2000). The TKB domain is followed by a short, 40 residue linker sequence that contains a conserved tyrosine among all five Cbl family members, Y371. This is followed by the RING finger domain which confers E3 ligase activity (Joazeiro et al, 1999).

Conversely, to pinpoint the region of Lyp that is relevant for Cbl binding, a series of truncation mutants were made. The Lyp protein was subcloned into pcDNA3.1 with a N-terminal Flag tag and serially truncated at its C-terminus. These included truncation of the proline-rich regions resulting in a fragment containing amino acids 1-613, and truncation of the interdomain resulting in the phosphatase domain alone in amino acids 1-299. This would determine the minimum length of Lyp that is required for binding Cbl.
**Figure 2. The N-terminus of Cbl directly binds the Lyp interdomain**

a) GST-Cbl constructs were overexpressed in COS-7 cells and then immobilized on glutathione sepharose beads. COS-7 cell lysate overexpressing GFP-Lyp equally divided and incubated with the indicated GST-Cbl fragments on beads, washed, and subjected to immunoblotting with the indicated antibodies.

b) Flag-Lyp truncation constructs were co-expressed with full-length HA-Cbl in COS7 cells. Lyp was immunoprecipitated and immunoblotted with the indicated antibodies (upper panel). Expression of the proteins in whole-cell lysates is indicated (lower panel).

c) His – Cbl 1-436 was produced in *E. Coli*, purified on nickel agarose and eluted by glutathione. GST-Lyp 1-674 was produced in *E. Coli*, immobilized on glutathione sepharose beads, and incubated with His-Cbl 1-436 purified eluate. The complexes were subjected to immunoblotting with anti-His, followed by GST.

d) GST-Lyp constructs were produced in *E. Coli*, immobilized on glutathione sepharose beads, and incubated with His-Cbl 1-436 purified eluate. The complexes were subjected to immunoblotting with anti-His, followed by GST.
The Lyp constructs were co-expressed in COS7 cells with full-length HA-tagged Cbl. Figure 2b indicates that while the fragment corresponding to 1-613 still binds Cbl, the phosphatase domain alone does not. This implies that the Lyp interdomain corresponding to amino acids 300-613 is important for binding Cbl. To date, this is the first report to indicate that this interdomain has protein binding capabilities.

While the association of Lyp and Cbl is now confirmed, it is not clear if the two bind directly or are in complex with other proteins. In order to determine if the two bind directly, recombinant fusion proteins were made and expressed in *E. Coli*. Since the N-terminus of Cbl was mapped to bind Lyp, this protein fragment corresponding to Cbl 1-436 was cloned into the pQE vector with a Histidine tag, grown in *E. Coli*, immobilized to nickel agarose and then purified by elution. The pGEX4T-2 vector was used to clone and produce GST-Lyp in *E. Coli* and bound to glutathione beads. The longest fragment of Lyp that could be purified in adequate amounts was Lyp 1–674, which contains the phosphatase domain, interdomain region, and first proline-rich region. Pulldowns of immobilized Lyp in the presence of purified Cbl indicates that this is indeed a direct interaction (Figure 2c). The Lyp interdomain was subsequently used in GST pulldowns to confirm that it is the relevant binding domain. Figure 2d shows that the interdomain, and specifically the region corresponding to amino acids 300-450, binds Cbl. This finding represents the first report of a direct interaction of Lyp that involves the region following the phosphatase domain.

**Lyp dephosphorylates Cbl Y371**

The establishing of the direct interaction between Lyp and Cbl prompted us to analyze further the possible role of this complex based on their well-characterized enzymatic
function. Cbl is a highly phosphorylated protein and as Lyp is a tyrosine phosphatase, one of the potential roles of this interaction is that Cbl serves as a Lyp substrate. Many of the studies on Cbl phosphorylation have focused on C-terminal tyrosines 700, 731, and 774 (Feshchenko et al, 1998), particularly in T-lymphocytes where they serve as binding sites for substrates targeted for ubiquitination. There are, however, multiple tyrosine residues on the N-terminus of Cbl, many of which are conserved. We turned our attention to the N-terminus of Cbl as this is the region that Lyp binds. To see if we can detect tyrosine phosphorylation at the N-terminus of Cbl, COS7 cells were transfected with the Cbl fragment corresponding to aa 1-436 and cells were subsequently stimulated through the EGF receptor. Lyp was also co-transfected to examine any differences in Cbl phosphorylation. Indeed, we see that there are N-terminal sites on Cbl that are inducibly phosphorylated upon EGF stimulation (Figure 3a). Furthermore, the addition of Lyp downregulates this phosphorylation, while the catalytically inactive Lyp C227S has no effect. This indicates that the phosphatase activity of Lyp is required for Cbl dephosphorylation.

Next we sought to elucidate which specific tyrosines may serve as a Lyp substrate. Residues Y268, 274, 291, 307, 337, 371 are conserved among all five Cbl family members (Kassenbrock & Anderson, 2004). In a previous report, the authors used mass spectrometry to test which residues actually become phosphorylated, and confirmed that Y371 becomes phosphorylated the most in that region (Kassenbrock & Anderson, 2004). In order to begin to elucidate which tyrosines may play a role in the actual function of Cbl, another group generated a series of point mutants and tested their activity in-vitro using a reconstituted ubiquitin system (Levkowitz et al, 1999). Tyrosine to phenylalanine substitutions at amino acids 92, 291, 274, 307, 337 and 368 did not impair Cbl’s ability to increase EGFR
ubiquitination and degradation, but Y371F did. The lack of activity of Y371F Cbl towards EGFR was also confirmed by Thien et al. (Thien et al, 2001), and has also been tested for activity towards c-Src (Yokouchi et al, 2001). This prompted us to focus our attention on whether Lyp dephosphorylates Y371.

The reduction in Cbl phosphorylation in the presence of Lyp can be attributed to two possibilities. First, Cbl may serve as a direct substrate of Lyp. Secondly, it is important to note that in the context of a COS7 cell where Cbl is phosphorylated by the EGFR and c-Src, a reduction in phosphorylation may simply reflect the downregulation of c-Src by Lyp. In order to address this possibility, we sought to dephosphorylate the N-terminus of Cbl or Y371F by Lyp \textit{in-vitro}. Cbl was overexpressed in COS7 cells and was phosphorylated \textit{in-vivo} by stimulation of the EGF receptor. Cells were lysed, and c-Src was first immunodepleted from lysates that overexpressed Cbl (Figure 3b). Immunoblotting with c-Src confirms the efficiency of this approach in removing c-Src from the lysates. Cbl was then immunoprecipitated, and served as a substrate for an \textit{in-vitro} phosphatase assay for Lyp. The Lyp in this case was produced in mammalian cells also, as the purified protein Lyp 1-674 from \textit{E.Coli} does not exhibit phosphatase activity. Lyp was overexpressed in COS7 cells that were also first immunodepleted of c-Src, then immunoprecipitated for Lyp. The two immunoprecipitated proteins, that is, Lyp and phosphorylated Cbl, were incubated together in phosphatase buffer at 30\textdegree C and the complexes were subsequently resolved on SDS-PAGE. In Figure 3c, immunoblotting with phosphotyrosine reveals that Cbl 1-436 is indeed tyrosine phosphorylated, while the Y371F mutant is not, indicating that this is the relevant substrate tyrosine of Lyp. The addition of Lyp completely abrogates the phosphorylation of Cbl, while the catalytically inert C227S has no effect. These results
**Figure 3**

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**Figure 3 Lyp dephosphorylates Cbl Y371**

a) COS7 cells were transfected with the N-terminal fragment of Cbl and the indicated Lyp constructs. Cells were stimulated with EGF ligand. The GST-Cbl fragment was immobilized on glutathione sepharose beads and subsequently immunoblotted for phosphotyrosine and GST.

b) COS7 cells overexpressing the indicated constructs were lysed and c-Src was immunoprecipitated. The efficiency of immunodepletion is indicated by immunoblotting cell lysates with c-Src before and after immunoprecipitation.

c) COS7 cells were transfected with a Myc-tagged N-terminal fragment of Cbl and cells were stimulated with EGF ligand. Conversely, Flag-Lyp was also overexpressed in COS7 cells. After cell lysis, c-Src was immunodepleted from lysates. Cbl 1-436 and Lyp were subsequently immunoprecipitated from the samples in which they were overexpressed. The two were then incubated together in phosphatase assay conditions. The reactions were stopped by the addition of 2x Laemmli buffer and immunoblotting was carried out as indicated (upper panel). Whole cell lysate expression of each protein is indicated (lower panel).
indicate that Lyp specifically dephosphorylates Y371 on Cbl. The identification of this novel Lyp substrate prompted us to further investigate the role of this Cbl tyrosine in T-cells.

**The role of Cbl Y371 in T-cells**

In T-cells, the majority of its phosphorylation has been shown to take place at its C-terminus, specifically at Y700, Y731, and Y774 (Feshchenko et al, 1998). While these three tyrosines have been shown to account for over 90% of tyrosine phosphorylation of Cbl in T-cells (Feshchenko et al, 1998), there may be a critical regulatory role for low stoichometry phosphorylation in T-cells of other tyrosines such as Y371. In order to see if there are N-terminal phosphorylation sites on Cbl that function in T cells, the N-terminus of Cbl was electroporated into Jurkat cells. Treatment with pervanadate for 5 minutes indicates that there are in fact N-terminal phosphorylation sites on Cbl (Figure 4a). Furthermore, mutation of tyrosine 371 to phenylalanine eliminates any phosphorylation, confirming that Y371 is the most functionally relevant tyrosine in that region of Cbl. This truncated portion of Cbl, however, lacks many other protein binding interacting domains; thus in order to see if phosphorylation occurs downstream of a more physiologic stimulus, we needed to use full-length Cbl. To this end, we utilized a construct of Cbl with point mutations at Y700F/Y731F/Y774F as described (Feshchenko et al, 1998). This was electroporated into Jurkat cells, followed by stimulation with αCD3/CD28 for the indicated time-points. Figure 4b confirms the presence of tyrosine phosphorylation at sites other than Y700, Y731 and Y774 downstream of TCR activation. Furthermore, addition of Lyp reduces the phosphorylation of Cbl (Figure 4c), likely at Y371 as we established that it was a direct substrate of Lyp (Figure 3b).
Figure 4 Cbl Y371 is necessary for Cbl activity in T-cells

a) Jurkat cells were electroporated with Myc-Cbl 1-436. Twenty-four hours post-electroporation, cells were stimulated with pervanadate for 5 minutes, and lysed. Myc-Cbl 1-436 was immunoprecipitated and immunoblotted for phosphotyrosine. b) Jurkat cells were electroporated with HA-Cbl Y700/731/774F. Twenty-four hours post-electroporation, cells were stimulated with αCD3/CD28 for the indicated time-points and lysed. HA-Cbl Y700/731/774F was immunoprecipitated and immunoblotted for phosphotyrosine. c) Jurkat cells were electroporated with HA-Cbl Y700/731/774F and Flag-Lyp as indicated. Twenty-four hours post-electroporation, cells were stimulated with αCD3/CD28 for the indicated time-points and lysed. HA-Cbl Y700/731/774F was immunoprecipitated and immunoblotted for phosphotyrosine. d) Jurkat cells were electroporated with HA-Cbl constructs. Twenty-four hours post-electroporation, cells were treated with MG132, stimulated with αCD3/CD28 for 2 minutes, and lysed. The endogenous CD3 ζ chain was immunoprecipitated and immunoblotted for ubiquitin (upper left panel), ζ (upper right panels). Whole cell lysate expression of HA-Cbl is indicated (lower panel).
Functionally, it has been shown that deletion at Y371 results in a loss of Cbl’s ubiquitin ligase activity (Levkowitz et al, 1999). The activity of Cbl as an E3 ubiquitin ligase is facilitated by the RING finger domain. It has been shown, however, that the linker region has important regulatory roles on Cbl function and that Y371 in this region may regulate the E3 ubiquitin activity of the molecule. We sought to determine if that was the case in T-cells, by testing Cbl activity towards a well-characterized substrate, the CD3 ζ chain (Myers et al, 2006; Wang et al, 2001b). As expected, overexpression of Cbl into Jurkat cells, followed by stimulation against CD3/CD28, resulted in increased ubiquitination of the ζ chain. Overexpression of Cbl harbouring a Y371F mutation, however, did not (Figure 4d). This confirms that the loss of Cbl phosphorylation at Y371 results in reduced Cbl ubiquitin activity in Jurkat T-cells. Hence, N-terminal Cbl phosphorylation does play an important functional role in T-cells, and Lyp downregulates this phosphorylation in T-cells.

**Lyp downregulates Cbl E3 ubiquitin ligase activity**

It is now established that Lyp dephosphorylates Y371 on Cbl, a residue that is functional in T-cells and is important for Cbl’s ubiquitin ligase activity. We next sought to determine if Lyp dephosphorylation of Cbl therefore downregulates Cbl E3 ligase activity. To this end, an *in-vitro* ubiquitin assay was carried out, using purified human E1, E2 UbcH5α, and immunoprecipitated Cbl fragment 1-436 as an E3 ligase. The substrate used in these reactions is purified S5α, a component of the 19S regulatory portion of the proteosome that recognizes ubiquitinated proteins and itself can become conjugated with ubiquitin (Uchiki et al, 2009). The reaction is then resolved on a gel and immunoblotted for ubiquitin. Results in Figure 5a show that Cbl is active as an E3 ligase in this system, but that a point mutation on Cbl Y371 abrogates its activity. When Cbl was immunoprecipitated from cells
**Figure 5 Lyp downregulates Cbl ubiquitin ligase activity** a) Myc-Cbl 1-436 and Flag-Lyp were co-overexpressed in COS7 cells. Cells were treated with MG132, stimulated with EGF for 5 minutes, lysed, and Myc-Cbl 1-436 was immunoprecipitated and served as an E3 ligase in an *in-vitro* ubiquitin reaction. The substrate in the reaction was S5a. Complexes were resolved on SDS-PAGE and immunoblotted with ubiquitin and Myc (upper panel). Immunoblotting using whole cell lysate samples indicates the expression of each protein (lower panel).

b) HA-Cbl and GFP-Lyp were co-expressed in COS7 cells. Cells were treated with MG132, stimulated with EGF for 5 minutes, lysed, and HA-Cbl was immunoprecipitated and served as an E3 ligase in an *in-vitro* ubiquitin reaction. The substrate in the reaction was immunoprecipitated Vav from TCR-stimulated Jurkat cells. Complexes were resolved on SDS-PAGE and immunoblotted with ubiquitin, HA, and Vav (upper panel). Immunoblotting using whole cell lysate samples indicates the expression of each protein (lower panel).

c) Myc-Cbl 1-436 and GFP-Lyp were co-overexpressed in COS7 cells. Cells were stimulated with EGF for 5 minutes, lysed, and the endogenous EGFR was immunoprecipitated and immunoblotted for ubiquitin (upper panel). Immunoblotting using whole cell lysate samples indicates the expression of each protein (lower panel).

d) Jurkat cells were electroporated with GFP-Lyp. Cells were treated with MG132, stimulated through CD3/CD28 for 10 minutes, and lysed. Endogenous Cbl was immunoprecipitated and immunoblotted for ubiquitin (upper panel). Immunoblotting using whole cell lysate samples indicates the expression of each protein (lower panel).

e) Jurkat cells were electroporated with GFP-Lyp. Cells were treated with MG132, stimulated through CD3/CD28 for 5 minutes, and lysed. Endogenous Vav was immunoprecipitated and...
immunoblotted with the indicated antibodies (upper panel). Immunoblotting using whole cell lysate samples indicates the expression of each protein (lower panel).
that co-expressed Lyp, its activity was decreased. This can be attributed to Lyp phosphatase activity as the catalytically inert C227S mutant had no effect. This *in-vitro* assay indicates that in the presence of Lyp, Cbl ubiquitin ligase activity is downregulated. Next the *in-vitro* ubiquitin assay was carried out by using a more physiologic substrate of Cbl, Vav (Miura-Shimura et al, 2003). In this case, the source of Vav was immunoprecipitated from Jurkat T-cells and co-incubated with Cbl in the reaction mixture. Indeed, in the presence of Lyp, Cbl has reduced ubiquitin activity towards Vav, but in the presence of Lyp C227S, it functions normally (Figure 5b). This confirms that the phosphatase activity of Lyp results in altered ubiquitination capacity of Cbl towards its substrates.

The *in-vitro* reactions indicate that Lyp turns off Cbl E3 ligase activity, and next it was determined if that occurs *in-vivo* in the physiologic context of a cell. To this end, we first tested activity of Cbl towards the EGFR, a well known and characterized Cbl substrate (Waterman et al, 1999; Yokouchi et al, 1999). Full-length Lyp was co-expressed with the N-terminus of Cbl in COS cells. Cells were stimulated with EGF ligand for 5 minutes and the EGFR was immunoprecipitated. Figure 5c shows that in the presence of Lyp, ubiquitin association to the EGFR receptor is greatly reduced. We next sought to replicate this effect in Jurkat T-cells. Cbl is known to auto-ubiquitinate itself along with other substrates. Upon overexpression of Lyp in Jurkat cells, there is a dramatic reduction in endogenous Cbl associated ubiquitin (Figure 5d).

Furthermore, endogenous Vav ubiquitination is also reduced *in-vivo*. Overexpression of full-length Cbl or full-length Y371F Cbl into Jurkat cells results in enhanced ubiquitination of Vav with Cbl wt, but not Y371F. Addition of Lyp reduces Vav associated ubiquitination, but the catalytically inert Lyp C227S restores it (Figure 5e). It should be
noted that Vav maintains the capacity to bind Cbl in the presence of Lyp, indicating that the reduction of Vav ubiquitination is not attributed to a disruption of the binding of the two, but a reduction in Cbl ligase activity. This clearly indicates that in a T-cell setting, Lyp is able to downregulate Cbl ubiquitin activity. This is directly attributed to dephosphorylation of Cbl Y371 by Lyp, as the two bind directly and Cbl is a direct substrate of Lyp.

**DISCUSSION**

In this work, we characterize a novel interaction between a tyrosine phosphatase, Lyp, and an E3 ubiquitin ligase, Cbl, both of which have been shown to be important players in immune cell function.

Cbl represents a novel substrate of Lyp whose functional consequences are now delineated. To date, only a small number of Lyp substrates have been identified (Wu et al, 2006). These include Src family kinases (Wu et al, 2006) which also interact with Cbl (Rao et al, 2002), so it was important to determine whether Lyp exerts its effect on Cbl directly, or indirectly via the kinases. In this work, it is established that the interaction between Lyp and Cbl is direct, and that Lyp can function on Cbl Y371 to dephosphorylate it. Presumably, *in-vivo*, however, there may be an interplay between all these different molecules. Lyp and its mouse homologue, PEP, are known negative regulators of Src kinases, by dephosphorylating their activating N-terminal tyrosines (Cloutier & Veillette, 1999; Wu et al, 2006). Hence *in-vivo*, Lyp may not only directly dephosphorylate Y371, but may also prevent its phosphorylation by inactivating upstream kinases. In HEK293 cells, the kinase shown to phosphorylate Y371 is c-Src (Yokouchi et al, 2001), but there are no reports of which specific kinase acts on this site in T-cells. Likely candidates are Lck or Fyn in T-cells, as
kinase activity of these enzymes has been shown to enhance their subsequent ubiquitination
by Cbl in T-cells (Andoniou et al, 2000; Rao et al, 2002). Furthermore, we did not test if the
regulation by Lyp is exclusive to Cbl or also takes place on the related molecule, Cbl-b. The
tyrosine at position 371 is conserved, so it seems likely that they may be regulated by Lyp in
a similar manner.

The phosphorylation of Cbl by c-Src has been shown to result in subsequently lower
levels of Cbl in the cell due to enhanced degradation (Bao et al, 2003). One of the major
roles of Lyp as a phosphatase is to oppose Src family kinase functions. In the scenario
described here, while kinases have been shown to phosphorylate and activate Cbl function,
Lyp opposes this by dephosphorylating and hence inactivating Cbl function. This may allow
for Cbl levels in the cell to not be degraded and be available for more subsequent inhibitory
functions in the cell.

While the activation of Cbl by kinases has been established, its subsequent regulation
by tyrosine phosphatases has not been thoroughly studied. There are reports of an
interaction between Cbl and the phosphatase SHP-1 (Kant et al, 2002; Uddin et al, 1996).
Cbl may serve as a SHP-1 substrate, but the exact tyrosine that SHP-1 targets has not been
identified, nor if this affects Cbl activity directly or its association with other interactors.
Another recently identified Cbl interactor is STS-1 (Kowanetz et al, 2004) which later
proved to exhibit tyrosine phosphatase activity (Raguz et al, 2007). STS-1 dephosphorylates
Cbl as well as the EGFR, thereby preventing their interaction and subsequent
downregulation of the receptor (Raguz et al, 2007). Hence while there are phosphatases that
may modulate Cbl function in a cell, the exact mechanisms remain largely unknown. This is
the first report to describe in detail how a phosphatase, Lyp, regulates Cbl function.
The fact that we demonstrate Lyp specifically regulates the disease-associated Y371 residue on Cbl points to the importance of this interaction. Aberrations in Cbl function and mutations at Y371 have been tightly linked to multiple human myeloid disease states. By regulating this tyrosine, Lyp may serve to preserve Cbl function and inhibit the transforming potential of this residue.

In summary, we have provided further understanding of the mechanisms involved in Cbl regulation in lymphocytes by characterizing the interaction between Lyp and Cbl. This direct interaction results in a dephosphorylation of the disease-implicated Y371 on Cbl and a downregulation of its ubiquitin ligase activity. A better understanding of Lyp and its effector functions provides insight into the role of this phosphatase in the immune system.

**METHODS**

**Cell Lines and PBMC Isolation**

Jurkat E6 cells (ATCC) were maintained in RPMI 1640 medium with 10% FCS and 2mM L-glutamine, penicillin/streptomycin, 10mM sodium pyruvate, 100 mM HEPES pH 7.5, and 5mM D-glucose. COS-7 cells were maintained in DMEM medium with 10% FCS and 2mM L-glutamine, penicillin/streptomycin.

For PBMC isolation, heparinized blood was laid over Ficoll-Paque (GE Healthsciences) and centrifuged for 30 minutes to separate the PBMCs. Cells were adhered to plastic tissue culture plates for 1 hour at 37 C. The suspended cells were recovered and cultured in RPMI 1640 with 10% FCS and 2mM L-glutamine in the presence of OKT3(αCD3) at 5µg/ml and 9.3 at 2.5µg/ml (αCD28) the indicated time points up to 48 hours. Where indicated, dells were then washed with PBS and subsequently cultured in
growth medium containing IL-2 supernatant at 10µg/ml for 24 hours (provided by Dr. D. Branch, University of Toronto).

**Antibodies and Reagents**

Antibodies used were as follows. From Santa Cruz Biotechnology: α-GFP, α-Csk, α-HA, α-Ub, α-His, α-GST, α-c-Src, α-Vav, α-CD3ζ, α-c-Myc. From BD Biosciences, α-c-Cbl and α-EGFR. Others were α-Flag (Sigma Aldrich), α-pTyr (Millipore), and α-Lyp (Abnova). The ubiquitination components used were from Boston Biochem: Ubiquitin Activating Enzyme UBE1, UbH5α, Ubiquitin, His6-S5α.

**Plasmid Constructs**

The GST-tagged c-Cbl cDNA (Full-length, 1-436, 437-647, and 648-906) in the vector pEBG were generously provided by Dr. K. Kirsch (Boston University Medical Centre). The HA-tagged c-Cbl cDNA (Full length wt, Full length Y371F) in the pGEM 4Z vector backbone were generously provided by Dr. W. Langdon (University of Western Australia) (Thien et al, 2001) and were used to subcloned into vector pcDNA3. These were subsequently used to amplify Cbl fragment 1-436 wt and 1-436 Y371F into pcDNA3.1(-)C Myc/His. Tyrosine point mutations on Cbl were generously provided by A. Tsygankov. The Lyp cDNA was cloned into vector eGFP C1 or pcDNA3 with an N-terminal Flag tag. Site-directed mutagenesis using overlapping PCR was carried out to obtain the mutant C227S. Domain deletion constructs were generated by PCR corresponding to fragments 1-299, 1-613 and contained an N-terminal Flag tag.

**Immunoprecipitation/Immunoblotting**

Cells were lysed using NP-40 lysis buffer (1% NP-40, 50mM Tris pH 8, 150mM NaCl, 1mM EDTA) supplemented with 1mM PMSF, 1mM Na3VO4 and 1µg/ml each of
pepstatin/aprotinin/leupeptin. Where indicated, N-ethylmaleimide (Sigma Aldrich) was used to block de-ubiquitinating enzymes. After 30 minutes on ice, lysates were pre-cleared by centrifugation at 14,000 rpm for 10 minutes. The soluble portion was subjected to immunoprecipitation using 1-2 µg of the indicated antibodies for 2 hours at 4°C, followed by incubation by Protein G beads for 1 hour. The complexes were washed three times in lysis buffer and boiled in Laemmli buffer containing β-mercaptoethanol. The samples were then loaded onto SDS-PAGE and immunoblotted with the indicated antibodies.

**Transient Transfections and Electroporations**

COS-7 cells were grown to 90% confluency and DNA was transfected into them using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s directions. After 24 hours, cells were supplemented with 10µM MG132 (Sigma Aldrich) for 1-2 hours, and then stimulated with EGF ligand (100ng/ml) (Invitrogen) for the indicated time points. The media was aspirated and the cells were washed with ice-cold PBS before lysis. Electroporations of plasmids into Jurkat cells was carried out using the Amaxa Nucleofector as per the manufacturer’s directions. Briefly, 6 million cells were electroporated with 10µg of plasmid DNA. After 24 hours, cells were supplemented with 10µM MG132 (Sigma Aldrich) for 1-2 hours, stimulated as indicated, and lysed.

**Generation of Fusion Proteins and In-Vitro Binding**

Recombinant fusion proteins were generated by subcloning Lyp deletion constructs into pGEX4T-2 vector and transforming *E. Coli* Rosetta2 cells. Protein induction was carried out by growing cells to OD 1.0 and adding IPTG (Roche) at 0.1mM overnight at room temperature. The GST-Lyp proteins were immobilized on glutathione sepharose beads. Conversely, the His-Cbl fragment corresponding to amino acids 1-436 was cloned.
into pQE-30 and induced by growing the culture to OD 0.7 and adding IPTG at 0.5mM for 4 hours at 37°C. Protein lysates were incubated with nickel affinity beads and the protein was further eluted with 250mM Imidazole.

For the binding assays, 50µl bead slurry containing GST-Lyp fragments was incubated with 10µg of eluted His-Cbl 1-436 for 2 hours and 4°C. Complexes were washed and subjected to SDS-PAGE.

**In-vitro Phosphatase Assay**

Anti-Cbl immunoprecipitates were used as a source of substrates. COS-7 cells were transfected with the indicated Cbl constructs. After 24 hours, cells were stimulated with EGF ligand for 5 minutes, washed in ice-cold PBS, and lysed. In order to obtain Lyp, COS-7 cells were transfected with the indicated Lyp constructs for 24 hours and were lysed (in the absence of Na₃VO₄). Endogenous c-Src was immunodepleted for each sample by incubating with anti-Src antibody and protein G. The remaining lysate was removed and subjected to immunoprecipitation for Lyp or Cbl. The complexes were washed three times in lysis buffer, followed by twice in PTP buffer (100mM Bis-Tris pH 6, 150mM NaCl, 5mM DTT), and an equal amount of beads of Cbl or Lyp were combined together. Reactions in PTP buffer were carried out at 30°C for 60 minutes and were terminated with the addition of 2X Laemmli buffer containing β-mercaptoethanol and boiled.

**In-vitro Ubiquitination Assays**

Anti-Cbl immunoprecipitates were used as a source of E3 ligases. COS-7 cells were transfected with the indicated Cbl and Lyp constructs. After 24 hours, cells were stimulated with EGF ligand for 5 minutes, washed in ice-cold PBS, and lysed. After immunoprecipitation of Cbl, the complexes were washed three times in lysis buffer, followed
by twice in Ubiquitination buffer (50mM Tris pH7.5, 5mM MgCl$_2$, 20mM KCl) and then incubated with 30µl of the same buffer supplemented with E1 enzyme (50nm), E2 UbcH5α (500nm), ATP (4mM), DTT (1mM), purified ubiquitin (5µM), and the substrate S5α (1µM). The reaction was carried out for 90 minutes at 30°C and terminated with the addition of 2X Laemmli buffer containing β-mercaptoethanol and boiled
CHAPTER 3

THE AUTOIMMUNE DISEASE-ASSOCIATED PTPN22 VARIANT PROMOTES CALPAIN-MEDIATED LYP/PEP DEGRADATION ASSOCIATED WITH LYMPHOCYTE AND DENDRITIC CELL HYPERRESPONSIVENESS

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Author Contributions

J.Z. and K.A.S. designed the study. N.Z. and B.D. performed most of the flow cytometry analyses, Q.J. and A.C.P. derived the knock-in mice, H.M. and S.H. carried out the calpain and ubiquitination analyses. X.M., G.X., F.Q, and Z.H. carried out and/or derived reagents for other protein and RNA analyses. E.K., J.Z. and K.A.S. obtained and/or analyzed the human samples. J.Z., C.A.M., E.K., A.C.P. and K.A.S. played key roles in data analysis and manuscript preparation

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INTRODUCTION

An important role for the Lyp protein tyrosine phosphatase (PTP) and the Pep murine orthologue in immune cellular homeostasis is implied by the association of a variant in the Lyp-encoding \textit{PTPN22} gene with risk for multiple autoimmune diseases.\cite{Begovich2004, Bottini2004, vanOene2005, Velaga2004}. A member of the PEST domain-containing intracellular PTP family, Lyp/Pep is expressed only in haemopoietic cells and, in addition to catalytic and PEST motifs, contains four proline-rich regions, the most C-terminal of which mediates binding to the Src homology 3 domain of the Csk tyrosine kinase \cite{Cloutier1996, Cohen1999, Ghose1988, Gregorieff1998}. Lyp/Pep has been shown to negatively regulate T cell antigen receptor (TCR) signaling, an effect that involves a functionally synergistic Lyp/Pep-Csk interaction wherein Lyp/Pep dephosphorylates the activating tyrosine and Csk phosphorylates the inhibitory tyrosine on the Src family Lck and Fyn tyrosine kinases \cite{Cloutier1999, Gjorloff-Wingren1999, Hill2002}. Consistent with Lyp/Pep inhibitory effects on T cell responses, Pep-deficient mice manifest increases in TCR-driven T cell proliferation and expansion of the effector/memory peripheral T cell compartment, but these abnormalities are not associated with autoimmune disease \cite{Hasegawa2004}.

Although a regulatory role for Lyp/Pep in T cell activation is established, the impact of the autoimmune disease-associated Lyp620W variant on T cell responses remains unclear. Consistent with the dependence of the Lyp-Csk interaction on the position 620 arginine \cite{Cloutier1996, Ghose1988}, Lyp binding to Csk is severely impaired by tryptophan substitution at this site \cite{Begovich2004, Bottini2004}. However, studies of the variant’s effects on such TCR-directed cell responses as proliferation and IL-2
production have yielded conflicting results - some data showing enhanced responses in mutant compared to wild-type allele homozygote individuals, but other data revealing T and also B cell responses to be relatively reduced in risk allele homozygotes (Abrahamsen et al, 2003; Arechiga et al, 2009; Lefvert et al, 2008; Rieck et al, 2007). Similarly, data showing that Lyp620W has higher intrinsic phosphatase activity than Lyp620R and a greater inhibitory effect on TCR signaling diverge from a recent report showing TCR signaling to be relatively enhanced in Jurkat cells coexpressing variant versus wild-type Lyp/Pep and Csk, and also revealing a permissive effect of Pep deficiency on development of a lupus-like syndrome in mice carrying a CD45 activation mutant on a nonautoimmune (C57BL/6) background (Abrahamsen et al, 2003; Hermiston et al, 2009).

**RESULTS**

**Pep R619W mice present with T and dendritic cell hyperresponsiveness**

To better understand these discrepancies and delineate the impact of the Lyp/Pep variant on T cells, we used a targeting construct to generate C57BL/6 mice harboring a *Pep* allele encoding Pep619W (Figure 1a). Mice homozygous for the mutant allele were obtained at the expected Mendelian ratio and sequence and quantitative PCR (qPCR) analyses revealed their expression of Pep619W transcript levels comparable to those observed in wild-type mice (Figures 1b & c). In keeping with these findings, no differences in expression of the wild-type and mutant alleles were detected in cells from wild-type/mutant heterozygotes (Figure 1d).
**Figure 1**

(a) Schematic representation of the wild-type allele and the targeting vector. The vector is designed to replace the wild-type allele with a modified version.

(b) Electropherograms showing the DNA sequence of the wild-type allele (WT) and the 819W allele. The electropherograms indicate the presence of the mutation.

(c) Bar graphs illustrating the expression levels of the gene in lymph nodes, spleens, and thymi of WT and 819W mice under different conditions (Medium, α-CD3, TGF-β).

(d) Genomic DNA analysis showing the frequency of the mutation in Thymus and Lymph node samples.
Figure 1. Generation of Pep619W knock-in mice

a) Schematic showing the Pep619W targeting vector. The targeting vector was constructed by introducing a neomycin selection cassette flanked by two lox P sites in the intron between exons 13 and 14 (i.e. between nucleotides 103690266 and 103692435) upstream of a modified (C1967T) Pep allele. The lox P sites are 208 bp downstream of exon 13 and 1961 bp upstream of exon 14, with a total of 146 bp new DNA introduced into the targeted intron. This vector was electroporated into C57BL/6 embryonic stem cells and clones containing the mutant allele then identified by PCR and Southern blotting. Following transfection with a Cre recombinase expression vector to remove the neo cassette, ES clones containing the C1967T allele were injected into C57BL/6 blastocysts using the eight-cell laser injection method to generate chimeric mice carrying the targeted allele. Successful germline transmission were confirmed by Southern blotting analysis of PepR619W+/- mice. DTA: Diphtheria toxin A chain gene. = FRT (Flippase Recognition Target) sites; * = C1967T/R619W mutation; = Exon; = 3’ probe; = 5’ probe.  

b) Sequence analysis of Pep transcripts from PCR-amplified T cells of Pep619W knockin mice and wild-type littermates. c) qRT-PCR analysis showing relative levels of Pep and Pep619W in unstimulated lymph nodes, spleen and thymus (left panel) and in unstimulated and anti-CD3 or TGFβ-stimulated thymocytes (right panel) from wild-type and Pep619W mice. Values are expressed relative to GAPDH control and represent means (±SEM) of three independent experiments. d) Relative expression levels of the two Pep alleles as determined in genomic DNA and cDNAs from thymic and lymph node cells from a Pep619R/619W heterozygote mouse. Normalized allelic (C/T) expression ratios, calculated as described in Methods, are shown below each panel. These data are representative of three independent experiments.
Pep619W mutant mice appeared healthy, but manifested a progressive enlargement of the thymus and spleen beginning at approximately 2 months of age (Figure 2a). No other organ pathology was observed even in the oldest mice (10 months) examined. Evaluation of the bone marrow, thymus, splenic and lymph node populations in young (2-3 months) and older (8-10 months) animals revealed no abnormalities in early haemopoietic differentiation (data not shown) and no differences between mutant and wild-type littermates in terms of early T cell development (Figures 2b & c). However, despite normal proportions of CD4/CD8-defined thymocyte subsets and levels of TCR expression, T cell number in the thymus, spleen and lymph nodes were increased in Pep619W mutant compared to wild-type 619R homozygous mice (Figure 2d). There was also an obvious expansion of the peripheral effector/memory T cell compartment - the proportion and absolute numbers of CD44\(^{hi}\)CD62\(^{low}\) CD4\(^{+}\) (Figure 2e) and CD8\(^{+}\) (not shown) cells being higher in both spleen and lymph nodes of Pep619W compared to wild-type mice. Expression of the CD69 and CD25 activation markers was also increased in this population (Figure 2f) and in thymocytes (data not shown), suggesting enhanced activation of the memory/effector cell population in the context of Pep619W expression.

Given the importance of Lyp/Pep to TCR signaling and the upregulated expression of activation markers on Pep619W T cells, the mutant cells were assayed for their response to TCR stimulation. Compared to wild-type cells, Pep619W thymocytes showed increases in proliferative response to TCR stimulation and enhanced and prolonged Lck and ZAP70 tyrosine phosphorylation (Figure 2g & h). Studies of intrathymic selection in Pep619W mice carrying either the ovalbumin-specific OT-II TCR or the male H-Y antigen TCR
Figure 2. Enhanced T cell activation in Pep619W mice

a) Representative spleens and thymi from 3 month-old wild-type Pep619R (WT) and mutant Pep619W knock-in (619W) mice. b,c) Representative flow cytometric plots showing CD4 and CD8 staining patterns for thymocytes, lymph node cells and splenocytes from 3 month old wild-type (WT) and Pep619W mice. Numbers within each quadrant indicate percentages of total cells. The thymocyte samples from these mice were also co-stained for CD3ε or TCRβ and their staining profiles shown by representative histograms. d) Total thymocyte, spleen and mesenteric, axillary and inguinal lymph node cell numbers at 2-3 months age in WT and 619W mutant mice. Values represent means of 8 independent experiments. e) Flow cytometric analysis of splenocytes and lymph node cells from wild-type and Pep619W mice stained for CD4-FITC, CD44-PE, and CD62L-PerCp. Gated fractions represent naïve (CD44loCD62Lhi) and memory/effector (CD44hiCD62Llo) cell populations. Quantification of naïve and memory splenic T cells is shown on the right. Values are the means ±SEM of 7 independent experiments. f) Splenocytes were stained for CD4-FITC, CD44-PE and CD69-APC or CD25-APC and percentages of CD44hi cells expressing the CD69 or CD25 activation markers assessed. Quantification of CD69hi and CD25hi CD44hi CD4+ T cells is shown on the right. Values represent means ± SEM of 7 independent experiments. g) Thymocytes from 2 month old mice were stimulated for 2 days with the indicated concentrations (µg/ml) of anti-CD3, anti-CD3/CD28 antibodies, ConA or PMA/ionomycin (10ng/ml; 100ng/ml) and proliferative responses then determined after a 16-h [³H]thymidine pulse. Values represent means (±SEM) of triplicate cultures and are representative of 8 independent experiments. h) Thymocytes from 2 month old mice were stimulated with anti-CD3 and anti-CD28 antibodies for the indicated times, the cells then lysed and subjected to
immunoblotting analysis with anti-phospho-Lck (Y394) and anti-phospho-ZAP-70 (Y319) antibodies, followed by reprobing with anti-Lck or anti-Zap70 antibodies. Data are representative of seven independent experiments.
transgenes revealed increased positive selection of CD4\(^+\) thymic and peripheral T cells in OT-II mutant mice, but no significant change in negative selection, as assessed by elimination of H-Y TCR\(^+\) double and CD8\(^+\) single positive thymocytes in male H-Y mice (Figure 3a). These findings of increased thymocyte positive selection, TCR signaling and proliferation and expansion of the effector/memory T cell pool reveal the T cell phenotype of Pep619W mice to be similar to that of Pep\(^{-/-}\) animals (Hasegawa et al, 2004; Hermiston et al, 2009). TCR-induced proliferative response and effector (ZAP70 and Erk) activation were also enhanced in naïve splenic (Figure 3b & 3c) and lymph node (data not shown) CD4\(^+\) T cells from the mutant mice. However, as previously observed in Pep\(^{-/-}\) memory cells (Hasegawa et al, 2004; Hermiston et al, 2009), proliferative response was only marginally increased and effector activation essentially no different in the peripheral CD44\(^{hi}\)CD62\(^{lo}\) population of mutant compared to wild-type mice (Figures 3b-c).

In addition to the T cell abnormalities, Pep619W mice manifested modest increases in number and size of germinal centres formed in the spleen, in antigen-receptor, but not LPS-induced B cell proliferation, levels of some immunoglobulin isotypes, and both T cell-dependent and independent antibody responses as assessed in mice immunized with nitrophenyl-chicken globulin (NP-CG) or trinitrophenyl (TNP)-Ficoll, respectively (Figures 4a-d). By contrast, B cell development appeared normal (Figure 4e) and anti-DNA autoantibodies were not detected in the mutant mice (data not shown).

As dendritic cells play key roles in T cell activation and autoimmunity (Chung et al, 2008; Decker et al, 2006; Lowes et al, 2005; Villadangos & Schnorrer, 2007), effects of the Pep619W variant on DC maturation and function were also explored. Differentiation of dendritic cells appeared normal in the spleen (Figure 4f), bone marrow and lymph nodes
Figure 3

(a) OT-II TCR

- WT
- G139W

(b) Naive

- WT
- G139W

- CD3 (μg/ml)
- CD3/CD28

- P-value

(c) Naive CD4+ T cell

- CD4+ effector/memory T cells

- WT
- G139W

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**Figure 3. Increased positive selection and peripheral T cell activation in Pep619W mice**

a) Representative flow cytometric analyses of thymocytes and peripheral lymph node T cells from 2 month old OT-II TCR wild-type and Pep619W mice is shown in the upper two panels. Numbers indicate percentages of stained cells in each quadrant. Histograms in the third panel show KJI-26 (OT-II TCR) staining of gated CD4^+ cells. Numbers indicate percentages of CD4^+ cells showing high level expression of the OT-II TCR. A representative flow cytometric analysis of thymocytes from 2 month old male H-Y TCR wild-type and Pep619W mice is shown in the bottom panel. Numbers indicate percentages of cells in each quadrant. Results representative of three independent experiments are shown.

b) CD4^+ naïve (CD44^{hi}CD62^{lo}) and memory (CD44^{lo}CD62^{hi}) T cells purified from 2-3 month old C57BL/6 or C57BL/6 OT-II TCR wild-type and Pep619W spleen were stimulated for 48 hours with the indicated concentrations (µg/ml) of anti-CD3 antibody, anti-CD3/CD28 antibodies or Ova peptide and proliferative responses determined after a 16 h [³H] thymidine pulse. Values represent means (± SEM) of triplicate cultures and are representative of 5 independent experiments.

c) CD4^+ naïve and memory T cells purified from 2-3 month old wild-type an Pep619W spleen were stimulated for 2 min with anti-CD3 antibody and the cells then fixed, permeabilized, stained with anti-phospho Zap70 or anti-phospho-Erk antibodies and assessed by flow cytometry. Plots are representative of 5 independent experiments.
Figure 4. Enhanced activation and function of Pep619W B and dendritic cells

a) Histologic analysis showing splenic germinal centres (GC) of 3 month old wild-type and 619W mice (upper). Numbers of germinal centres/mm$^2$ and area of each GC (as averaged across 10 low power fields) are shown. ■ = 250µm. b) Splenic CD19$^+$ B cells from 2-3 month old wild-type and Pep619W littermates were cultured for 48 hr with anti-IgM antibody or LPS and proliferation evaluated after 16 hr $^3$[H]-thymidine pulse. Values represent means (±SEM) of quadruplicate cultures. c) Immunoglobulin levels were assayed by Luminex in sera from 8 month old mice. Values represent individual wild-type mice or Pep619W littermates. d) Anti-NP (T-dependent) and anti-TNP (T-independent) antibody titres from age-matched 2-3 month-old WT and Pep619W mice (four/group) immunized with NP-CG or TNP-Ficoll (with alum).

e) Representative flow cytometric analyses of lymph node cells from 2 month old wild-type or Pep619W mice showing IgM, IgD or CD5 relative to B220 staining. Numbers indicate percentages of IgM$^{hi}$, IgD$^{hi}$ or CD5$^{hi}$ B220$^+$ cells. f) Representative flow cytometry plot of splenic cells from 2 month old wild-type mice or Pep619W littermates stained for CD11c, CD11b and CD8α. Gated fractions represent CD11b$^+$ and CD8α$^{hi}$ CD11c$^+$ subsets with the percentage of total cells shown within each gate. Data shown are representative of 8 independent experiments. g) Graph showing mean fluorescence intensity (MFI) for CD40 and CD86 staining of unstimulated or LPS-stimulated bone marrow-derived dendritic cells from 3 month old mice. h) Histograms showing percentages and MFI of CD40-stained cells within the splenic and lymph node CD11c$^{hi}$ population. i) Quantitation of CD11c$^{hi}$ dendritic cell numbers in individual 3-8 month wild-type and 619W littermates. Values represent means ± SEM. j) Flow cytometric analysis of Pep619W and WT BMDCs pulsed
with ovalbumin or OVA$_{323-339}$ peptide and co-cultured for 4 days with CFSE-labelled CD4$^+$ OT-II T cells. Percentages of dividing cells are shown. k) WT and Pep619W CD11c$^+$ DCs were pulsed for 2 hr with OVA peptide, co-cultured for 4 days with CD4$^+$ OT-II T cells and assayed for $[^3]$H]thymidine incorporation. Values represent mean (±SEM) of three replicate cultures. l) WT and 619W Pep BMDCs were stimulated with LPS for 24 hours and the supernatants assayed for IL-12 levels. All data are representative of at least six independent experiments.
(data not shown) of the mutant mice. However, expression of the CD40 and CD86 costimulatory molecules in response to LPS was enhanced in bone marrow dendritic cells from the mutant mice, increased CD40 levels were detected on their splenic and lymph node dendritic cells and an increase in the absolute numbers of splenic dendritic cells was also apparent (Figures 4g & h). Consistent with these findings, both bone marrow-derived and splenic dendritic cells from the mutant mice were more effective than wild-type cells in inducing T cell proliferative responses to ovalbumin and/or Ova peptide and secretion of IL-12 by bone marrow dendritic cells in response to LPS was also higher in mutant compared to wild-type mice (Figures 4i-l). Taken together with prior data implicating Lyp in myeloid cell signaling (Chien et al, 2003), these data suggest that Lyp/Pep effects on immune and autoimmune responses may be subserved through the regulation of dendritic as well as lymphocyte activation and also imply a “loss-of-function” effect of the 619W variant.

**Levels and stability of Pep are reduced in Pep619W mice**

To delineate the molecular basis for the Pep619W cellular phenotype, the association of Pep with Csk was examined in mutant T cells and was observed to be profoundly reduced (Figure 5a). Unexpectedly, however, results of immunoblotting analysis revealed marked reduction of Pep levels in Pep619W heterozygous and homozygous (by about 50% and 80%, respectively) relative to Pep619R homozygous splenic cells (Figure 5b), lymph node and thymocytes (data not shown), a defect detected regardless of mouse age. Because Pep transcript levels are intact in mutant mice, the possibility that Pep619W is aberrantly degraded was explored by assessing Pep levels in pulse-labeled wild-type and mutant T cells.
Figure 5

a) IP: PEP

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d) Dendritic cells

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c) WT | 619W

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WT: О

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619W: О

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120

Chase (min)

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**Figure 5. Levels and stability of Pep are reduced in Pep619W mice**

a) Lysates prepared from thymocytes of wild-type (WT) and Pep619W (619W) mice were immunoprecipitated with anti-Pep or anti-Csk antibodies and then subjected to sequential immunoblotting analysis with anti-Pep and anti-Csk antibodies. 
b) Lysates prepared from thymus of WT, Pep619W homozygous and WT/Pep619W heterozygous mice and from bone marrow-derived dendritic cells of WT and 619W mice were subjected to sequential immunoblotting analysis with anti-Pep and anti-actin antibodies. 
c) Thymocytes from 6 week old wild-type and Pep619W mice were cultured for 24 hours with anti-CD3 and anti-CD28 antibodies and IL-2, pulse-labeled for one hour with $^{35}$S-L-methionine and chased for the indicated times in culture medium. Cells were then lysed and subjected to immunoprecipitation with anti-Pep antibody followed by SDS-PAGE and autoradiography. Actual band intensities as quantitated using NIH Image J Software are shown below each lane and relative band intensities expressed as a percent of band intensity at time 0 are shown in the graph. 
d) Thymocytes harvested from wild-type and Pep619W mice were cultured for 24 hours with anti-CD3/anti-CD28 antibodies and IL-2 and for an additional 16 hours with (+) or without (-) 0.5µM MG132 or 1µM ALLN. Cells were then lysed and the lysate proteins subjected to SDS-PAGE and sequential immunoblotting with anti-Pep and anti-actin antibodies. Numbers indicate relative band intensities expressed as the ratio of Pep band intensity to that of β-actin.
Interestingly, nascent Pep619W protein is degraded at a markedly accelerated rate, its level initially being comparable to that of 619R, but essentially disappearing at two hours after labeling compared to ~40% diminution of 619R levels over the same time (Figure 5c). Because PEST motif-containing proteins may be proteolytically cleaved by the calcium-dependent calpain protease and protein degradation is often proteosomally-mediated, the effects of MG132, a proteosomal inhibitor and ALLN, which primarily inhibits calpain, on Pep stability were explored (Bruzzaniti et al, 2009; Ji et al, 2003; Mykles, 1998). Treatment of splenic cells with either or both inhibitors increased Pep and Pep619W levels, suggesting that Pep degradation is influenced by both proteosomal and calpain activity (Figure 5d). Consistent with these findings, Pep coimmunoprecipitated with calpain 1 in T cells and in subsequent in vitro binding and cleavage assays, Pep was found to not only bind, but also to be cleaved by calpain 1, the interaction and extent of cleavage appearing greater for Pep619W than Pep619R (Figures 6a-c). In keeping with these observations, Pep was also inducibly ubiquitinated, a modification that targets proteins for proteosomal degradation, and Pep619W appeared more ubiquitinated than Pep619R (Figure 6d). Thus, the 619W variant appears to alter Pep binding to calpain as well as Csk, raising the possibility that the variant either directly or indirectly (e.g. by disrupting Csk binding) alters Pep structural properties so as to favor interaction with and subsequent cleavage by calpain. While the precise pathways underpinning Pep degradation require further investigation, our data implicate calpain- and proteasome-mediated proteolysis in the regulation of Pep levels and are consistent with a critical role for proteolytic degradation in reducing cellular levels of Pep619W.
Figure 6

a) IP: IgG PEP
   Blot: anti-calpain
   95kDa
   72kDa
   Blot: anti-PEP

b) IP: Myc
   M:EV
   N:WT
   M:619W
   72kDa
   133kDa
   95kDa

(c) Calpain 1
   Activated
   NA
   30 (min)
   PEP
   β-actin
   WT
   619W

(d) IP: PEP
   Stim: - +
   WT
   S10W
   WT
   S10W
   IgG
   Lithium
   100kDa
   150kDa
   200kDa
   PEP
**Figure 6. Pep binding to and cleavage by calpain is increased in Pep619W mice**

a) Lysates prepared from BI-141 T cells were immunoprecipitated with anti-Pep antibody followed by SDS-PAGE and sequential immunoblotting with anti-Pep and anti-calpain antibodies. b) Lysates prepared from Cos7 transfectants expressing myc (empty vector; M-EV), myc-tagged Pep619R (M-WT) or Pep619W (M-619W) were immunoprecipitated with anti-myc antibody and the immunoprecipitates then incubated for 1 hour at 4°C with 10µg calpain 1 in an *in vitro* binding assay. The complexes were then resolved by SDS-PAGE and subjected to immunoblotting analysis with anti-Pep and anti-calpain 1 antibodies. c) Lysates from Cos7 transfected cells expressing myc-tagged Pep619R or Pep619W were immunoprecipitated with anti-myc antibody and the immunoprecipitates incubated for 0-30 min at 37°C with 0.1U calpain 1 in CaCl$_2$-containing (activated) or non-activated (NA; no CaCl$_2$) buffer, resolved over SDS-PAGE and immunoblotted sequentially with anti-Pep and anti-actin antibodies. d) Lymph node T cells from WT and Pep619W mice were cultured with anti-CD3 (1µg/ml) and anti-CD28 (0.5µg/ml) antibodies for 48 hours and with 10µM MG132 for 2 hours prior to cell lysis. Lysates were adjusted to equalize amounts of Pep and Pep619W per sample and were then subjected to anti-Pep antibody immunoprecipitation, followed by sequential immunoblotting with anti-ubiquitin and anti-Pep antibodies. All data are representative of at least five independent experiments.
In view of the 619W variant association with reduced Pep stability, the possibility that the counterpart variant in Lyp also influences protein stability was explored in human T cells. Like Pep, wild-type Lyp (620R) was degraded at a slower rate than Lyp620W and Lyp620R and 620R levels in Jurkat cells were increased in the presence of MG132 and ALLN as is in keeping with calpain/ubiquitin-mediated proteolysis (Figure 7a). Lyp was also coimmunoprecipitated with calpain 1 *in vitro* and *in vivo* (Figures 7b & c) and again, the variant was more highly associated with calpain 1, more sensitive to calpain-mediated cleavage, and more ubiquitinated than wild-type Lyp (Figures 7c-e). In view of these findings, levels of Lyp expression were assessed by flow cytometry in unstimulated and ConA-stimulated peripheral blood mononuclear cells (PBMCs) from healthy or rheumatoid arthritis-affected subjects homozygous for either the risk or the wild-type Lyp-encoding *PTPN22* alleles. This analysis revealed Lyp levels to be markedly reduced in all risk allele homozygotes compared to wild-type homozygotes (Figure 8a). Diminished Lyp levels were observed in both naïve and memory Lyp620W-expressing T and B cells (Figure 8b), while proliferative responses to antigen receptor stimulation and signaling effector activation were increased in the Lyp620W compared to Lyp620R-expressing lymphocytes (Figures 8c & d). In keeping with calpain effects on Lyp, Lyp levels increased in both wild-type and mutant cells following calpain inhibitor treatment (Figure 6e).
Figure 7

(a) CHA+ (min): 0 30 60 120 120 0 30 60 120 120

(b) GFP GFP-LYP

GFP-LYP

anti-calpain

anti-GFP

(c) GFP-LYP GFP-620W

GFP-LYP

anti-calpain

anti-GFP

(d) Calpain 1

Activated NA

WT 620W

(e) IP: GFP

IP: IgG LYP 620W

ULys-lys LYP
Figure 7. Reduced stability and level of Lyp620W in human cells

a) GFP-Lyp (wild-type) and GFP-Lyp620W-expressing Jurkat cells were cultured for 30 min in methionine-free media, pulse-labeled with $^{35}$S-L-methionine and chased for the indicated times (0.5-2h). Chase was performed in the presence (+) or absence (-) of 10µM MG132 or 50µM ALLN. Cells were then lysed and the lysates immunoprecipitated with anti-GFP antibody followed by SDS-PAGE and autoradiography. Numbers below show band intensities. b) GFP (empty vector) and GFP-Lyp-expressing Jurkat cells were immunoprecipitated with anti-GFP-antibody and subjected to SDS-PAGE and immunoblotting with anti-Lyp, anti-calpain and anti-GFP antibodies. Bottom panel was cut to show GFP band in empty vector-transduced cells. c) Lysates from GFP-Lyp and GFP-Lyp620W-expressing Cos7 cells were immunoprecipitated with anti-GFP antibody and the immunoprecipitates incubated with calpain 1 in an *in vitro* binding assay, resolved over SDS-PAGE and subjected to immunoblotting analysis with anti-Lyp and anti-calpain antibodies. d) Lysates from GFP-Lyp and GFP-Lyp620W-expressing Jurkat cells were immunoprecipitated with anti-GFP antibody and the immunocomplexes incubated for 0-30 min at 37°C with 0.05U calpain 1 in CaCl$_2$ containing (activated) or non-activated (NA; no CaCl$_2$) buffer and immunoblotted sequentially with anti-Lyp and anti-actin antibodies. e) GFP-Lyp (WT) and GFP-Lyp620W-expressing Jurkat cells were cultured for 2 hours with 10µM MG132, stimulated for 5 min with anti-CD3/CD28 antibodies and then lysed. Lysates were then immunoprecipitated with anti-GFP antibody and subjected to immunoblotting with anti-Lyp and anti-ubiquitin antibodies. All data shown are representative of at least five
independent experiments. The circles denote individual CC genotype subjects and the triangles denote individual TT genotype subjects.
Figure 8. PBMC Lyp levels are reduced and activation increased in subjects homozygous for the risk allele

a) Peripheral blood mononuclear cells from healthy controls and rheumatoid arthritis (RA) patients homozygous for non-risk (CC) or risk (TT) *PTPN22*/Lyp genotypes were cultured for 48 hr with (STIM) or without (UNSTIM) ConA, and then fixed, permeabilized and stained with anti-Lyp antibody. Histograms representative of stimulated T cell Lyp staining patterns for each genotype and subject group are shown on the left with the percentage of positive (Lyp hi) cells indicated (shaded area represents isotype control). The graph shows MFI of Lyp staining for each subject. Subjects included 11 controls (7 CC and 4 TT genotypes) and 9 RA patients (5 CC and 4 TT genotypes). b) PBMCs from 11 healthy control subjects homozygous for the non-risk (CC) or risk (TT) *PTPN22*/Lyp genotypes were stained with antibodies to Lyp and to either CD4, CD45RA, CD45RO or CD20 and CD27. Graph shows MFIs of Lyp staining in naïve (CD45RA+) and memory/effector (CD45RO+) CD4+ T cells and in CD27− ( naïve) and CD27+ (effector/memory) CD20+ B cells. c) CD4+ T and CD19+ B cells from 10 subjects with CC and 7 subjects with TT *PTPN22*/Lyp genotypes were stimulated for 2 days with anti-CD3/CD28 or anti-IgM antibodies, respectively, and proliferative responses determined after a 16 h 3[H] thymidine pulse. Values represent means ±SEM of quadruplicate cultures. d) CD4+ T cells purified from 7 *PTPN22*/Lyp CC and 4 TT age-matched healthy controls were stimulated with anti-CD3 antibody, fixed, permeabilized and stained with anti-phospho-Erk antibody. Graph shows the post-stimulatory fold increase in phospho-Erk MFI relative to baseline level for each subject. e) PBMCs from CC or TT homozygous controls were cultured for 48 h with or without 0.5µM MG132 and 1µM ALLN and stained with anti-Lyp antibody. Staining
patterns for each genotype are shown for treated (light line) or untreated (dark line) cells.

Shaded area represents isotype control and numbers are percentages of Lyp$^{hi}$ cells.
DISCUSSION
Collectively, our results reveal a loss-of-function effect of the Lyp620W/Pep619W variant, the R→W substitution at this site rendering Lyp/Pep more susceptible to calpain- and proteosome-mediated cleavage and degradation so as to lower Lyp/Pep levels and diminish its inhibitory effects on T and to a lesser extent B cell activation. Our findings also identify a role for Lyp/Pep in dendritic cell activation and function and suggest that the Lyp620W/Pep619W variant influence on T cell responses and potentially autoimmune disease susceptibility may be subserved in part via effects on dendritic cell function. This possibility as well as the relative roles of calpain versus other proteolytic pathways in modulating Lyp/Pep stability require further investigation. However, our findings identify proteolytic degradation as a potentially important mechanism whereby the Lyp620W/Pep619W variant impairs Lyp/Pep capacity to modulate immune responses integral to the maintenance of immune tolerance. Our data also imply that Lyp/Pep variant acts as a hypomorph, inducing a lymphocyte/dendritic cell hyperresponsive phenotype which may not evoke overt autoimmune disease, but may instead create an immunologic milieu favoring the emergence of such disease.

METHODS
Mice
A Pep (PTPN22) gene targeting vector was derived by subcloning a 10.7kb Pep exon 12-15-containing genomic fragment isolated from the RPCI-23 C57BL/6 BAC library into the pG141 vector to create two Pep gene homology regions bracketing a loxP-flanked neomycin selection cassette. A C1967T mutation was introduced into exon 14 by site-
directed mutagenesis (QuikChangeII, Stratagene) and the targeting construct transfected into C57BL/6 embryonic stem (ES) cells derived as described (Casas et al, 2003). Following southern and PCR (Supplementary Table 1a)-based screening, selected ES cells were injected into CD-1 embryos (Poueymirou et al, 2007), the embryos transplanted into foster mothers and the chimeric progeny Pep genotypes confirmed by PCR (Supplementary Table 1b). Pep619W mice were bred to C57BL6 OT-II and H-Y TCR transgenic mice (Jackson Laboratory Bar Harbor) and the progeny backcrossed to generate Pep619W homozygotes carrying OT-II or H-Y TCR transgenes. Mice were maintained at the Ontario Cancer Institute Animal Facility according to approved ethical treatment of animal standards.

Reagents and Constructs

Reagents used included: antibodies specific for GFP, Csk, HA, Ub, His, Gst, c-Src, (Santa Cruz Biotechnology), for Lck, ZAP70 and phospho-Lck, -Zap70 and -Erk, c-Cbl and c-Myc and calpain (BD Biosciences and Cell Signaling); for Flag (Sigma Aldrich), pTyr (Millipore), Lyp (Abnova and R&D ) and Pep (from A. Veillette, McGill University); for CD3ε (2C11 hybridoma); and for CD3, CD4, CD8, CD11b, CD11c, CD19, CD25, CD40, CD43, CD45RA, CD45RO, CD49b, CD44, CD5, CD27, CD20, CD62L, CD69, CD86, CD8α, MHCII, TCRβ, IgM, IgD, KJ1-26 (OT-II TCR Vβ8.1/8.2) and T3.70 (H-Y TCR Vα3), conjugated to FITC, PE, APC, biotin, PerCP-Cy5.5 and PE-Cy7 (BD PharMingen and eBiosciences). [3H]-thymidine and [35S]-methionine were purchased from Dupont/NEN, LPS, Concanavalin A (ConA), PMA and ionomycin from Sigma Chemical Co., GM-CSF from PeproTech, interleukin 2 (IL-2) from ORF Genetics, and erythrocyte calpain 1 from Calbiochem. Expression constructs for functional studies were derived by subcloning full-length wild-type Pep (619R) or Lyp (620R) or mutant Pep (619W) or Lyp (620W) cDNAs
into the pEGFP vector and flag or myc-tagged wild-type or mutant Pep or Lyp cDNAs into pcDNA3.

**Histology**

Splenic tissue was fixed in 10% formalin for 2 days, embedded in paraffin, stained with H & E and the size and number of germinal centres measured using an Olympus BX61 microscope and Aperio Image Scope software.

**Cells and cell lines**

Lentiviral-transduced Jurkat T cells were produced by co-transfecting CGIP-Lyp620W and CGIP-Lyp620R constructs (vectors from J. Dick, University Health Network) with packaging plasmids into HEK293T cells and then infecting T cells with the virus-containing supernatants (Naldini, 1998). All cells and cell lines (Jurkat, BI-141 and Cos7) were maintained in 5-10% fetal bovine serum-supplemented RPMI-1640, with 50µM β-mercaptoethanol and 200U/ml GM-CSF added to BMDC cultures. Dendritic cells were purified from mouse splenic cell suspensions using EasySep CD11c^+^-PE selection kits (Stem Cell Technologies). Peripheral blood mononuclear cells (PBMCs) were obtained from peripheral blood by Ficoll-Hypaque centrifugation (General Electric Healthcare Biosciences), adhered to plastic for 1 hour at 37°C and the nonadherent cells cultured in RPMI 1640 with 10% FCS +/- 1µg/ml ConA. This study was approved by the Mount Sinai Hospital IRB.

**PTPN22 genotyping**

The human *PTPN22* C1858T variant (rs2476601) was genotyped from PCR amplicons (Supplementary Table 1c) using the Sequenom MassArray and SpectroCALLER software (Sequenom).
**Flow cytometry**

Cells were resuspended in staining buffer and incubated with fluorophor-conjugated antibodies for 30min at 4°C. To assess DC maturation or activation, BMDCs harvested after 8 day culture and stimulated for 24hrs at 5x10^5 cells/mL with LPS or CD11c^+ selected splenic cells, were stained with antibodies specific for mouse CD11c (N418), CD40 (FGK45.5) and/or CD86 (GL1) for 45min at 4°C. To assay Lyp expression, human PBMCs cultured for 48 hr in presence or absence of ConA (1µg/ml) were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences), incubated for 1 hour with 10% human serum and stained for 1 hour on ice with fluorophor-conjugated (Innova Biosciences) anti-Lyp antibody (R & D Systems) alone or with fluorophor-labeled anti-CD4, anti-CD45RA and anti-CD45RO (T cells) or anti-CD20 and anti-CD27 (B cells) antibodies. For antigen receptor signaling studies in mice, CD4^+ T cells purified from wild-type or 619W mouse thymus, spleen or lymph nodes (EasySep CD4^+ enrichment kit) were stimulated 2 min with anti-CD3 antibody (1µg/ml) followed by anti-hamster Ig cross-linking and the fixed cells then stained with antibodies to CD4, CD44, CD62L, phospho-Erk and phospho-ZAP70. For human studies, purified CD4^+ T cells and CD19^+ B cells (RosetteSep human CD4^+ T cell and EasySep human CD19^+ B cell kits) were stimulated for 5 min with anti-CD3 (5µg/ml) or anti-IgM (0.1µg/ml) antibodies, respectively, fixed and stained with anti-phospho-Erk antibody. Flow cytometry was performed using a FACS-Caliber TM (Becton Dickinson) and CellQuest Pro as well as FlowJo software.

**Proliferation assays**

Thymocytes, CD4^+ T cells or CD19^+ B cells from spleen or lymph nodes from age-matched Pep619R and Pep619W mice were cultured (2x10^5 cells/ml) for 48 hour with
varying concentrations of plate-bound or soluble anti-CD3ε antibody +/- anti-CD28 antibody (0.5-1.0µg/ml), ConA (1µg/ml), PMA plus ionomycin (10ng/ml and 100ng/ml, respectively), plate-bound anti-IgM F(ab)₂ antibody or LPS. To assess antigen-induced responses, CD4⁺ T cells (2.5 x 10⁴ cell/well) from OT-II wild-type or Pep619W mice were cultured for 48 hr with irradiated syngeneic wild-type splenocytes (2x10⁵ cells/well) and varying concentrations of Ova₃₂₃₋₃₃₉ peptide. Alternatively, splenic and lymph node cells from C57BL/6 or C57BL/6OT-II wild-type or Pep619W mice were stained with anti-CD4-FITC, anti-CD44-PE, and anti-CD62L-PerCP/Cy5.5 antibodies and naïve (CD4⁺CD44loCD62hi) and effector/memory (CD4⁺CD44hiCD62lo) T cells isolated by cell sorting (Becton Dickinson FACS ARIA), cultured for 48 hr with varying concentrations of anti-CD3 or anti-CD3/28 antibodies or Ova peptide-loaded irradiated splenocytes and then pulsed for 16 hrs with [³H]thymidine (1µCi/well). For assays of dendritic cell stimulatory activity, BMDCs from wild-type or mutant mice were incubated for 1hr with LPS (Sigma) and for 2hr at 37°C with 0.1mg/mL ovalbumin (Sigma). Cells were then washed and 1x10⁵ cells incubated for 4 days with 4x10⁵ CD4⁺ T cells from spleens of OT-II transgenic mice, labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE; Invitrogen), and numbers of dividing cells estimated by flow cytometry. Alternatively, purified CD11c⁺ splenic dendritic cells were cultured for 2 hr with LPS (100ng/ml) and Ova₃₂₃₋₃₃₉ peptide (1µg/ml) and for 3 days with 4x10⁴ purified OTII CD4⁺ T cells and pulsed during the last 16hrs with 1µCi/well [³H]thymidine. For human T and B cell proliferation assays, CD4⁺ T cells and CD19⁺ B cells isolated from PMBC using RosetteSep T cell and EasySep B cell purification kits were cultured in triplicate for 48 hrs in plates (2.5x10⁵ cells/mL) precoated with anti-CD3 (0.5µg/ml) or anti-IgM (0.1µg/ml) antibody and pulsed for the last 16 hours with [³H]
thymidine (1µCi/well). Cell incorporated radioactivity was measured by scintillation counting.

**Gene expression assays**

Total RNA (200ng) prepared (RNeasy kits, Qiagen) from unstimulated thymocytes, lymph node T cells, purified dendritic cells or anti-CD3 antibody (0.5µg/ml) or TGFβ (5ng/ml)-stimulated thymocytes cultured in IL-2-supplemented medium, was reverse transcribed with Superscript II (Invitrogen), and subjected to SYBR green-based quantitative RT-PCR (Supplementary Table 1) using the ABI7900 HT System (Applied Biosystems Inc). Relative quantities of mRNA were calculated by the comparative threshold method and expressed as relative fold increase compared to GADPH gene levels (Pfaffl, 2001). For allele-specific expression assays, genomic tail DNA and cDNAs prepared (as above) from thymus, lymph node and anti-CD3/CD28 antibody-treated lymph node T cells from Pep619W/619R mice were subjected to PCR amplification using primers flanking the C/T variant. Purified PCR products were resolved by capillary electrophoresis (ABI3100 DNA Sequencer) and the peak areas determined using ABI Gene Mapper Software. Ratio of the two transcript cDNA alleles was normalized based on the allelic ratio obtained from the genomic DNA analysis, such that allelic ratio=cDNA [peak area C allele/peak area T allele] / genomic DNA [peak area C allele/peak area T allele].

**Immunoblotting and immunoprecipitation**

Thymocytes, splenocytes or BMDCs (2x10⁶ cells) from Pep619R and Pep619W mice, Jurkat T or BI-141 cells were lysed for 30 min in cold lysis buffer and processed samples electrophoresed through 10% SDS-polyacrylamide and transferred to nitrocellulose (Schleicher and Schuell). After blocking, filters were incubated for two hours at room
temperature with relevant antibody and visualized by chemiluminescence (Amersham). For immunoprecipitation, cell lysates were precleared by preincubation with Protein A Sepharose 6B beads (Amersham Pharmacia Biotech) for 30min at 4°C followed by 2h incubation at 4°C with specific antibody or rabbit preimmune serum. The immune complexes were collected on Protein A sepharose 6B beads, washed and suspended in SDS sample buffer for immunoblotting analysis.

**Metabolic labeling**

Thymocytes cultured for 24 hours with anti-CD3 (2µg/ml) and anti-CD28 (0.2µg/ml) antibodies and IL2 (10µg/ml) or untreated GFP-Lyp620R/Lyp620W-transduced Jurkat cells (2x10^7 cells/ml) were incubated for 30 (Jurkat) or 60 (thymocytes) minutes at 37°C in cysteine and methionine-free medium, followed by 60 min pulse with 0.2mCi/ml [³⁵S]-L-methionine. Cells were then chased by incubation for varying times in medium containing 2mM nonradioactive cysteine and methionine, lysed and the lysates immunoprecipitated with anti-Pep or anti-GFP antibodies, followed by SDS-PAGE, autoradiography and quantification of band intensities using Image J software (NIH, Bethesda, MD).

**Assays for proteosome or calpain 1 activity**

Thymocytes from mutant or wild-type mice or GFP-Lyp620R/Lyp620W-transduced Jurkat cells (2x10⁶cells/mL) were cultured for 24 hours with anti-CD3 (2µg/ml)/anti-CD28 (0.2µg/ml) antibodies and IL2 (10µg/ml), incubated for 16 hrs with 0.5-10µM MG132 (Calbiochem) and/or 1-50µM N-acetyl-L-leucinal-L-leucinal-L-nonleucinal (ALLN; Calbiochem) and lysates then prepared and subjected to immunoblotting analysis with anti-Pep and/or anti-Lyp followed by anti-actin antibodies.
**In vitro calpain binding and calpain proteolysis assays**

Anti-Pep or anti-Lyp immunoprecipitates prepared from Pep619W or 619R or Lyp620W or 620R-expressing Cos7 cell lysates were incubated for 1 hour on ice with 10µg calpain 1 and subjected to SDS-PAGE, followed by immunoblotting analysis with anti-Pep/Lyp and anti-calpain antibodies. To assay calpain cleavage, Lyp or Pep immunoprecipitates from the same Cos7-transfectants were incubated for 0-30 min at 37°C with 0.1 (Pep) or 0.05 (Lyp) U human erythrocyte calpain 1 (Calbiochem) in activation buffer (40 mM HEPES (pH 7.2), 10mM dithiothrietol) with or without 2mM CaCl₂ and the samples then boiled and subjected to SDS-PAGE followed by anti-Lyp and anti-Pep immunoblotting analysis (Guttmann et al, 2001).

**Immunization protocols and ELISA and Luminex assays**

To assay *in vivo* immune responses, Pep wild-type and Pep619W mice were injected intraperitoneally with 100µg NP-CG (4-hydroxy-3-nitrophenyl)acetyl-coupled chicken γ-globulin or 100µg TNP (2,4,6-Trinitrophenyl)-Ficoll (Bioreresearch Technologies) with alum (Sigma) adjuvant. Sera was obtained at 0 and 21 days after immunization and anti-NP and anti-TNP antibody titres determined using NP and TNP-BSA ELISA (Southern Biotechnology). For IL-12 assays, BMDCs from WT and 619W Pep mice were stimulated for 24 hours with LPS (1ug/ml) and the supernatants collected and assayed for IL-12 expression using multiplex fluorescent bead immunoassay kits (LINCO Research). Sera from 6 month-old Pep619W and wild-type littermates were assayed for immunoglobulin levels using an immunoglobulin isotyping kit (*Millipore*) and for anti-double stranded DNA antibodies by ELISA (kits from USCN Life Science Inc). Luminex assay were performed according to the manufacturer’s protocol using a Luminex 100 Analyzer.
Statistical analysis

Data are expressed as the mean ± SEM unless otherwise specified. Unpaired two-tailed $t$ tests were used to compare mean values between two groups, with values of $p < 0.05$ considered to be statistically significant.
CHAPTER 4

THE PTPN22 PROTEIN LYP BINDS GRK2 TO MODULATE CHEMOKINE-MEDIATED CELL MIGRATION

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Manuscript in Preparation

Author Contributions

HM designed and carried out murine and human migration experiments, CAIA, a portion of
the biochemistry, analyzed data and wrote the manuscript, YS designed and carried out a
portion of the biochemistry experiments, SZ carried out the in-vivo trafficking experiments,
SH carried out a portion of the biochemistry experiments, XM carried out the pulse chase
experiments, QL assisted in the homing assays, MS carried out the mass spectrometry
analysis, JZ and KAS designed the project, analyzed data and helped in the preparation of
the manuscript.
INTRODUCTION

The Lyp protein tyrosine phosphatase and its murine homologue, Pep, have been shown to be important negative regulators of the immune system by downregulating signals downstream of the T-cell receptor (reviewed in (La Face et al, 1997; Veillette et al, 2002)). There are, however, multiple other signalling pathways in the immune system that Lyp may be downstream of. The variant of the protein conferring a Lyp R620W/Pep R619W change has been linked to autoimmunity (reviewed in (Siminovitch, 2004)). One of the important mechanisms in autoimmune disease states is altered cell chemotaxis/migration. In this work, we show that Lyp has a role in inhibiting cell migration, but that the R/W variant presents a loss-of-function in this role. This places Lyp downstream of chemokine signalling, and we sought to determine any novel interactions that facilitate this role. One of the new Lyp interactors that we present here is the G-protein coupled receptor kinase 2 (GRK2).

The GRK2 interactome reveals multiple effector functions of this kinase, but it has classically been described as a negative regulator of G-protein coupled receptor (GPCR) signalling. GRK2 is important in the regulation and desensitization of G protein coupled receptors (reviewed in (Penela et al, 2010; Penela et al, 2008; Premont & Gainetdinov, 2007; Vroon et al, 2006)). Upon agonist binding to the receptor, GRK2 is recruited and phosphorylates the cytoplasmic loops or C-terminal tail of the receptor. The phosphorylation recruits the arrestins to the receptor, which sterically block the interaction of the receptor to G proteins. This leads to desensitization of the receptor and hence a termination of further signalling. The receptor is then internalized via clathrin-coated pits for degradation or recycling.
GRK2 is ubiquitously expressed but is found at very high levels in leukocytes (De Blasi et al, 1995; Loudon et al, 1996). GRK2 has also been shown to very important in immune cell function, particularly in cell migration. Chemotaxis is facilitated by chemokine receptors on immune cells that are G-protein coupled, and many of them such as CXCR4, CCR5 and CCR1 have been shown to be GRK2 substrates (Balabanian et al, 2008; Busillo et al, 2010; Orsini et al, 1999; Tzeng et al, 2000; Vroon et al, 2004). T-cell and B-cell conditional knock-outs point to S1PR as a GRK2 substrate and show its role in migration and homing of lymphocytes into lymphoid organs (Aron et al, 2011). Lymphocytes from heterozygous GRK2+/- mice display enhanced migration towards the CCR5 and CCR1 ligands, consistent with reduced GRK2 levels leading to aberrant desensitization of the receptors and hence enhanced signalling (Vroon et al, 2004). Furthermore, these mice are more prone than their wild type littermates at developing experimental autoimmune encephalomyelitis, a multiple sclerosis model, due to enhanced lymphocyte migration into the brain (Vroon et al, 2005). Interestingly, altered expression levels of GRK2 have been found in physiologic contexts of disease. Patients with rheumatoid arthritis are reported to have decreased levels of GRK2 (Lombardi et al, 1999). This is also seen in normal human lymphocytes upon the addition of pro-inflammatory cytokines such as IFN-γ and IL-6 (Lombardi et al, 1999). Rats that are induced to develop acute adjuvant arthritis or allergic encephalomyelitis also have reduced GRK2 (Lombardi et al, 2001; Vroon et al, 2003). All of these studies point to the importance of GRK2 in maintaining and regulating immune cell function.

One of the receptors that GRK2 is thought to negatively regulate is the chemokine receptor CXCR4. It has been shown that overexpression of GRK2 promotes SDF-1α
mediated CXCR4 internalization (Orsini et al, 1999; Tzeng et al, 2000). Conversely, silencing of GRK2 followed by cell stimulation by SDF-1α results in enhanced CXCR4 signaling, as indicated by increases in CXCR4-mediated calcium mobilization and ERK1/2 signalling (Busillo et al, 2010).

GRK2 in turn, is subject to regulation. One such mechanism is by tyrosine phosphorylation by c-Src on residues 13, 86, and 92 (Fan et al, 2001; Penela et al, 2001; Sarnago et al, 1999). This leads to initial activation of GRK2, followed by its subsequent downmodulation by ubiquitination and degradation (Penela et al, 2001; Penela et al, 1998; Salcedo et al, 2006).

It is not clear whether there are any tyrosine phosphatases that regulate GRK2 phosphorylation. In this work, we demonstrate that GRK2 represents a novel substrate of the Lyp tyrosine phosphatase. The Lyp R620W/Pep R619W variant has lost the ability to play this role on GRK2. This regulation of GRK2 by Lyp is needed to maintain steady-state levels of GRK2 in the cell in order for it to carry out its role in chemokine-mediated cell migration.

RESULTS

Pep R619W mice display higher levels of migration

The Lyp R620W variant has been linked to many autoimmune disease states. To date, this has partly been attributed to alterations in T-cell receptor signalling. To see if there are defects in other signalling pathways such as chemokine signalling, migratory and motility properties of lymphocytes from Pep R619W mice or their WT littermates were examined. Two-photon laser-scanning microscopic analysis of the motility of CSFE-labeled
PepR619W and CMTMR-labeled control T cells within the lymph nodes of recipient mice showed that Pep R619W T cells had longer track lengths and were migrating at dramatically faster median speeds than WT control T cells (Figures 1 a-c), resulting in a significant increase in area covered, shown as median square displacement (Figure 1d). However, PepR/W and WT T cells exhibited normal patterns with respect to turning angles between time points (Figure 1e) and the mean meandering index, a measurement of directionality which indicates the persistence of a cell to make a consistent forward movement in the same direction (Figure 1f). These data indicate that Pep R619W enhances the velocity of in-vivo T cell trafficking.

To see if this phenomenon translates to a possible homing defect in the mice, freshly purified naïve CD4+ T-cells from Pep R619W mice or their WT counterparts were labelled, mixed, and injected into a recipient mouse. One hour later, the recipient mice were sacrificed and their spleen, blood, and lymph nodes were examined for each donor population. Results in Figure 1g show more Pep R619W cells were recovered in spleen and lymph node than WT, indicating that these cells were able to migrate more to their target organs. Hence this in-vivo homing assay points to enhanced migratory properties of the Pep R619W cells.

To further explore any altered migratory properties of the Pep R619W cells, a transwell migration assay was carried out. The chemoattractant selected was SDF-1α, as it has been shown to have an important role in T-cell migration (Bleul et al, 1996b). Results are shown as the percentage of cells migrated compared to the number of input cells. Figure 1h shows that the Pep R619W lymphocytes show greater migration towards SDF-1α than
Figure 1

(a) WT vs. PepR619W

(b) Frequency (% of max)

(c) Speed (μm/sec)

(d) Mean Squared Displacement (μm²)

(e) Frequency (% of max)

(f) p = 0.059

(g) Recovered PepR619W SDF-WT cells

(h) % Cells Migrated

(i) % Cells Migrated

(j) WT vs. R619W

(k) Mean intensity fluorescence
**Figure 1 Pep R619W mice display higher levels of migration**

a) Lymph nodes from recipient mice were imaged 24 hrs later by time-lapse two-photon confocal microscopy. The migratory parameters of migrating T cells in time were calculated from the movies using Velocity software. Representative three-dimensional tracks of PepR619W and control T cells over a 15 min period are shown. Each coloured line represents a single T-cell track. b) Relative frequency of instantaneous velocities calculated from the net distance traveled during each time interval (displacement/time during a single time step) c) Average speeds calculated as velocity of a cell over 15 mins of PepR619W and control T-cells d) Frequency of mean square displacement (MSD), a measure of the average distance a given particle in a system travels. e) Frequency of mean turning angles from which a cell deviates between successive time points f) Mean meandering index, a measurement of directionality, calculated by dividing the distance of a cell traveled by the track length. Data are expressed as the mean values ± SEM and are representative of 4 independent experiments. g) A short term *in-vivo* homing assay was carried out by transferring naïve T-cells from Pep R619W and wild-type mice into a wild-type recipient. The homing efficiency into the indicated organs is shown relative to cotransferred WT T-cells. Results are representative of at least 3 experiments. h) Lymph nodes from Pep R619W mice or their WT littermates were harvested, and lymphocytes were subjected to a transwell migration assay with SDF-1α as a chemoattractant. Results are shown as the percent of cells migrated compared to the number of input cells, and are representative of at least 7 experiments. i) Freshly purified CD4+ cells from age and sex-matched human pairs that were homozygous for either the CC or the TT allele were subjected to a transwell migration assay using SDF-1α as a chemoattractant. Results are shown as the percent of cells migrated.
compared to the number of input cells. * p < 0.001 based on a paired two-tailed T-test, n=9 pairs (6 healthy control pairs and 3 patient pairs). j) Thymocytes from Pep R619W mice and their wild-type littermates were stimulated with SDF-1α for the indicated time-points. Total cell lysates were immunoblotted with phospho-ERK1/2 or total ERK2 as indicated.

i) Freshly isolated PBMCs from human donors were stimulated with SDF-1α for the indicated time-points and phospho-ERK1/2 was assessed by flow cytometry. Data is representative of the ERK activation observed in 5 human donor pairs as average Mean Fluorescence Intensity.
their WT counterparts. This is the first report to demonstrate that the variant Pep has a different role than the WT in cell migration.

In order to confirm the role of the Lyp autoimmune associated variant in T-cell migration, we recruited human donors that were homozygous for the C1858T PTPN22 substitution, conferring an R620W change. The donors were paired for age and sex, and their freshly purified CD4⁺ T-cells were subjected to a transwell migration assay using SDF-1α as a chemoattractant. Consistent with the mouse data, T-cells from human donors carrying the R620W displayed enhanced migratory properties compared to their wild-type counterparts (Figure 1i). These findings in the human samples confirm the role of Lyp in T-cell migration.

To see if the enhanced migration observed towards SDF-1α is reflected in signalling downstream of CXCR4, ERK activation was examined. Cells from mice or humans carrying the variant R/W allele were stimulated with SDF-1α for the indicated time-points and phospho-ERK1/2 was assessed by immunoblotting or flow-cytometry, respectively. Results show that pERK1/2 is enhanced in thymocytes from the Pep R619W mice, suggesting that CXCR4 signalling is hyperactivated (Figure 1j). Similarly, PBMCs from human subjects homozygous for CC or TT at nucleotide 1858 were stimulated with SDF-1α for the indicated time-points and pERK 1/2 was assessed by phospho-flow cytometry. Results indicate that there is higher activation of ERK in subjects carrying the AA allele (Figure 1k). This prompted us to further investigate the potential mechanisms that facilitate this phenomenon.
The mechanism of Lyp/Pep's role in migration is GRK2

To date, the Lyp interactome is limited to a small number of proteins, so we sought to determine new binding partners in order to gain more insights into Lyp function, especially cell migration. To this end, we utilized mass spectrometry to identify novel Lyp interactors. GFP- Lyp was overexpressed in Jurkat cells and immunoprecipitated. The resulting complexes were resolved on SDS-PAGE, the gel was stained, and the bands were excised and sent for mass-spectrometric analysis. This led to the identification of the G-protein receptor kinase 2 (GRK2). Confirmation of Lyp – GRK2 binding was first carried out in Jurkat cells overexpressing GFP-Lyp upon SDF-1α stimulation. Immunoprecipitation of endogenous GRK2 demonstrate that it binds GFP-Lyp, and that the binding is enhanced upon SDF-1α stimulation (Figure 2a). Hence the novel Lyp-GRK2 interaction identified by mass spectrometry is confirmed in cells.

GRK2 is an important regulator of immune cell migration, whose substrates include CXCR4, CCR5, and S1PR. We sought to determine if we observe migration alterations downstream of other GRK2 targets in the Pep R619W mice in addition to those seen downstream of CXCR4. Figure 2b shows that lymphocytes from Pep R619W mice show enhanced migration towards the GRK2 substrate CCR5, but not other receptors such as CCR7. The resting levels of chemokine receptors were not altered in mouse cells carrying the R/W variant (Figure 2c). Receptor levels in human PBMCs were also not altered in subjects carrying the TT alleles encoding Lyp R620W (Figure 2d). In mouse cells, upon SDF-1α stimulation, the GRK2 substrate CXCR4 does not get internalized as efficiently in the Pep R619W cells as in the WT, implying a defect in receptor internalization (Figure 2d). GRK2 signals to induce the eventual internalization of its target receptors.
Figure 2
**Figure 2 The mechanism of Lyp/Pep's role in migration is GRK2**

a) Jurkat cells expressing GFP-LYP were stimulated with SDF-1α for the indicated time points. GRK2 was immunoprecipitated and the complexes were resolved over SDS-PAGE, immunoblotted with anti-GFP (LYP) and anti-GRK2. b) Lymph nodes from PEP R619W mice or their WT littermates were harvested, and lymphocytes were subjected to a transwell migration assay with the indicated chemokines. For CCL4, cells were pre-activated for 48 hours with αCD3/αCD28. Results are shown as the percent of cells migrated compared to the number of input cells, and are representative of at least 5 experiments.

c) Cell-surface staining of the indicated receptors was carried out on lymphocytes from Pep R619W mice and their wild-type littermates. For S1PR, whole blood cells were also stained for CD4^+^ cells and the S1PR level was assessed. d) Cell-surface receptor staining of the indicated receptors was carried out on PBMCs from age and sex-matched human pairs that were homozygous for either the CC or the TT allele. n=8 pairs (5 healthy control pairs and 3 patient pairs) e) Lymphocytes from Pep R619W mice and their wild-type littermates were stimulated with SDF-1α for the indicated time-points. Cells were washed and cell surface receptor staining of CXCR4 was carried out and shown as the percentage of remaining receptors on the cell surface compared to the starting amount.
To begin to delineate the mechanisms involved in the Pep R619W cell migratory properties and ligand-induced receptor internalization, we turned our attention to further characterizing biochemically the Lyp/Pep and GRK2 interaction.

**The GRK2 kinase domain directly binds the Lyp interdomain**

In order to begin to elucidate the function of the Lyp-GRK2 complex, we sought to map the interacting domains. To this end, domain constructs of GRK2 were made consisting of amino acids 1-190 containing the RGS domain, 190-455 containing the serine/threonine kinase domain, and 456-689 containing the PH domain. These domain constructs were co-expressed and co-immunoprecipitated with GFP-Lyp in COS-7 cells. Results indicate that the relevant GRK2 binding region is between residues 190-455 containing the kinase domain. (Figure 3a) Conversely, the interacting Lyp domains were mapped for GRK2 binding. Serial truncation mutants of Flag-Lyp were made, consisting of 1-674 lacking the C-terminal proline rich regions, 1-613 lacking all proline rich regions, and 1-299 consisting of the phosphatase domain alone. These were co-expressed in COS-7 cells with full-length GRK2. Immunoprecipitation of GRK2 followed by immunoblotting reveals that the Lyp interdomain, the region between the catalytic domain and the first proline-rich region, is required for binding GRK2 (Figure 3b).

In order to elucidate if the binding of GRK2 and Lyp is direct or in complex with other proteins, recombinant fusion protein were made in *E. Coli*. Full-length GRK2 was expressed in the pQE-30 vector with a His-tag, immobilized on nickel agarose, and then eluted. The longest fragment of Lyp that was expressed as a GST-tagged fusion protein was to amino acid 674. GST- Lyp 1-674 was expressed and immobilized on glutathione sepharose and increasing amounts were incubated with purified GRK2. Figure 3c clearly
Figure 3

(a) GRK2-ΔC

(b) GRK2-ΔG2

(c) GRK2-ΔG2

(d) GRK2-ΔG2
Figure 3  The GRK2 kinase domain directly binds the Lyp interdomain

a) Schematic map of GRK2 domain structure and constructs (left panel). COS-7 cells were co-transfected with GFP-tagged Lyp and myc-tagged GRK2 constructs. GFP-Lyp was immunoprecipitated and the complexes were resolved by SDS-PAGE and sequentially blotted with anti-myc and anti-GFP antibodies.  
b) Schematic map of Lyp domain constructs (left panel). COS-7 cells were co-transfected with myc-tagged GRK2 and FLAG-tagged Lyp constructs. GRK2 was immunoprecipitated and the complexes were resolved by SDS-PAGE and sequentially blotted with anti-FLAG and anti-myc antibodies.  
c) Purified GST or GST-Lyp 674 pre-bound to Sepharose 4B-Glutathione beads was incubated with increasing amounts of purified His-GRK2 protein. After washing, the protein complexes were eluted from the beads with SDS sample buffer and subjected to SDS-PAGE. They were subsequently immunoblotted with anti-GRK2 and anti-GST antibodies (upper panel). GST, GST-Lyp 1-674, GST-Lyp 1-613 or GST-LYP1-299 pre-bound to Sepharose 4B-Glutathione beads were incubated with 2µg of purified His-GRK2 protein. After washing, the protein complexes were eluted from the beads with SDS sample buffer and subjected to SDS-PAGE. They were subsequently immunoblotted with anti-GRK2 and anti-GST antibodies (lower panel).  
d) COS-7 cells were co-transfected with myc-tagged CSK or myc-tagged GRK2 and GFP-tagged Lyp constructs. Overexpressed Csk or GRK2 were immunoprecipitated and the complexes resolved over SDS-PAGE. Immunoblotting was carried out with anti-GFP and anti-myc antibodies (left panels). The expression of the transfectants in whole cell lysates is shown (right panel).
demonstrates that this indeed a direct interaction (upper panel). Subsequent in vitro binding assays using purified GST-Lyp deletion constructs and His-GRK2 proteins confirm that the interdomain of Lyp is responsible for the binding (lower panel).

The variant of Lyp R620W alters the binding capacity of Lyp to Csk (Begovich et al, 2005; Bottini et al, 2004). We sought to confirm whether this affects indirectly the binding to GRK2. Figure 3d shows that while the Lyp R620W disrupts the binding to Csk (upper panel), it has no effect on the binding to GRK2 (lower panel).

The characterization of the mapping between Lyp and GRK2 unveils some new information about Lyp. We show a new, direct binding partner of Lyp in GRK2, mediated by the Lyp interdomain and the GRK2 central region that contains the kinase domain.

**Lyp dephosphorylates GRK2**

GRK2 has been shown to be tyrosine phosphorylated by Src kinase (Fan et al, 2001; Penela et al, 2001; Sarnago et al, 1999) but this has not been shown in the context of a T-cell. To see if this is the case, Jurkat T-cells were stimulated with SDF-1α for the indicated time points. Immunoprecipitation of GRK2 followed by immunoblotting shows that GRK2 is indeed tyrosine phosphorylated (Figure 4a).

There are no known tyrosine phosphatases at the moment that act to reverse this phosphorylation. As Lyp is a tyrosine phosphatase, we sought to determine if GRK2 is a Lyp substrate. Jurkat cells overexpressing GFP-Lyp wt or GFP-Lyp C227S were stimulated through the CXCR4 receptor and GRK2 tyrosine phosphorylation was assessed. The addition of Lyp downmodulates GRK2 tyrosine phosphorylation relative to the Lyp C227S mutant (Figure 4b). These findings indicate that GRK2 presents a novel substrate for the Lyp tyrosine phosphatase. To see if the Lyp R620W variant differentially regulates GRK2
Figure 4

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Figure 4 Lyp dephosphorylates GRK2

a) Jurkat cells were stimulated with SDF-1α for the indicated time-points to assess GRK2 tyrosine phosphorylation patterns. Cells were lysed, GRK2 was immunoprecipitated, and the complexes were resolved on SDS-PAGE. Immunoblotting was carried out for phosphotyrosine and GRK2. b) Jurkat cells stably expressing Lyp WT or C227S were stimulated with SDF-1α for the indicated time points. GRK2 was immunoprecipitated and the complexes were resolved on SDS-PAGE. Immunoblotting was carried out for phosphotyrosine and GRK2. c) Jurkat cells stably expressing Lyp WT or R620W were stimulated with SDF-1α for the indicated time points. GRK2 was immunoprecipitated and the complexes were resolved on SDS-PAGE. Immunoblotting was carried out for phosphotyrosine and GRK2. d) Thymocytes from PEP R619W or their WT littermates were stimulated with SDF-1α for the indicated time points. GRK2 was immunoprecipitated and the complexes were resolved on SDS-PAGE. Immunoblotting was carried out for phosphotyrosine and GRK2.
phosphorylation, Jurkat T-cells overexpressing Lyp WT or R620W were used. Figure 4c shows that GRK2 is hyper-phosphorylated in the presence of the Lyp R620W variant.

To further explore this, we turned to the mouse homologue of Lyp, Pep. Recent characterization of the disease-associated variant Pep R619W showed that the variant protein level is very low (shown in Chapter 3). If the Pep R619W protein level is very low, then we postulate that GRK2 tyrosine phosphorylation would be higher in these animals. To test this, thymocytes from Pep R619W mice or their WT littermates were stimulated with SDF-1α for the indicated time points. GRK2 was immunoprecipitated and the complexes were resolved on SDS-PAGE. Immunoblotting was carried out for phosphotyrosine and GRK2 (Figure 4d). Indeed, GRK2 is inducibly tyrosine phosphorylated upon stimulation, and phosphorylation levels are higher in the Pep R619W cells at the 3 hour time point. These findings demonstrate that the presence of Lyp/Pep is important in downregulating GRK2 tyrosine phosphorylation.

**Lyp regulates GRK2 protein levels**

We have now shown that Lyp binds GRK2 and downregulates its tyrosine phosphorylation. Tyrosine-phosphorylation of GRK2 has important functional consequences to the protein. It has been shown that tyrosine phosphorylation of GRK2 promotes its degradation by the proteasome (Penela et al, 2001; Penela et al, 1998; Salcedo et al, 2006). As Lyp dephosphorylates GRK2, this may result in the regulation of GRK2 protein levels. We wanted to see if the GRK2 protein degradation rate was changed by Lyp. In order to examine this, Jurkat cells overexpressing GFP-Lyp wt or GFP-Lyp R620W were metabolically labeled with ^35^S-methionine and then pulse-chased upon stimulation with SDF-1α. GRK2 protein gradually decreased in Lyp R620W-transfected Jurkat cells but was
retained at a higher level in Lyp WT-transfected Jurkat cells stimulated with SDF-1α, suggesting that Lyp inhibits SDF-1α induced degradation of GRK2 (Figure 5a).

To confirm this finding in the Pep R619W mice, freshly isolated thymocytes were labelled with $^{35}$S-methionine and then pulse-chased upon stimulation with SDF-1α. Similarly to the Jurkat cell data, GRK2 protein gradually decreased in Pep R619W cells but was retained at a higher level in their WT littermates (Figure 5b). The accelerated degradation of GRK2 should result in a lower observed total protein level. Indeed, in Jurkat cells overexpressing Lyp R620W or thymocytes from Pep R619W mice stimulated with SDF-1α, whole cell lysate immunoblotting revealed that the GRK2 protein level is reduced in the presence of the R/W variant upon stimulation (Figures 5c-d). It is clear from this data that the Pep R619W has lost the ability to regulate GRK2 protein level.

For the mechanism of the observed GRK2 protein instability, we turned our attention to ubiquitination. GRK2 has been shown to be ubiquitinated and degraded by the proteasome upon agonist stimulation (Penela et al, 2001; Penela et al, 1998; Salcedo et al, 2006). To see if the reduced GRK2 level seen in the presence of altered Lyp is due to ubiquitination, thymocytes were stimulated with SDF-1α in the presence of MG132, a proteasomal inhibitor. This will allow for the accumulation of ubiquitinated GRK2 but will prevent it from being degraded by the proteasome, thus allowing us to immunoprecipitate equal amounts of the protein. GRK2 was immunoprecipitated and then immunoblotted for ubiquitin. Figure 5e shows that upon stimulation for 3 hours, GRK2 is heavily ubiquitinated in the Pep R619W samples. These results conclude that the presence of Pep R619W results in higher ubiquitination and subsequent degradation of GRK2 upon SDF-1α stimulation.
Figure 5

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(e) IP: SDF-1α

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**Figure 5** Lyp regulates GRK2 protein levels

a) Jurkat cells stably expressing Lyp WT or R620W were metabolically labeled with $^{35}$S-methionine and then stimulated with SDF-1α for the indicated time points. GRK2 was immunoprecipitated, resolved on SDS-PAGE, and visualized by autoradiography (upper panel). Radioisotope-labeled GRK2 was quantitated by densitometry and plotted as a percentage relative to baseline level (lower panel). b) Freshly isolated thymocytes were metabolically labeled with $^{35}$S-methionine and then stimulated with SDF-1α for the indicated time points. GRK2 was immunoprecipitated, resolved on SDS-PAGE, and visualized by autoradiography (upper panel). Radioisotope-labeled GRK2 was quantitated by densitometry and plotted as a percentage relative to baseline level (lower panel). c) Jurkat cells stably overexpressing Lyp wt or Lyp R620W were stimulated with SDF-1α for the indicated time points. Whole cell lysate immunoblotting was carried out with GRK2 and β-actin. 

d) Thymocytes from Pep R619W mice or their WT littermates were stimulated with SDF-1α for the indicated time points. Whole cell lysate immunoblotting was carried out with GRK2 and β-actin. e) Thymocytes from PEP R619W mice or their WT littermates were stimulated with SDF-1α in the presence of the proteosomal inhibitor MG132. GRK2 was immunoprecipitated and the complexes were immunoblotted with ubiquitin and GRK2.
Altered Migration in the presence of Lyp/Pep R/W is attributed to levels of GRK2

GRK2 is important for regulating cell migration towards chemokine receptors. As we have shown lower levels of GRK2 in Pep R619W mice upon stimulation with SDF-1α, we propose that the altered migratory properties in the Lyp R620W/Pep R619W cells is due to lower GRK2 protein. This is consistent with findings that lymphocytes from heterozygous GRK2+/- mice display enhanced migration towards chemokine ligands (Vroon et al, 2004). In order to further confirm the role of GRK2 in this process, we attempted to “rescue” the GRK2 level to see how it influences migration. To this end, Jurkat T-cells stably overexpressing Lyp WT or Lyp R620W were then electroporated to overexpress GRK2 or its catalytically-inactive mutant, K220R. As shown in Figure 6, Jurkat cells overexpressing Lyp R620W show enhanced migration compared to cells overexpressing Lyp wt, consistent with the mouse data (lanes 1 and 4). The enhanced migration of the Lyp R620W cells is then lowered with the addition of GRK2 (lane 5). The addition of the inactive GRK2 K220R is not able to inhibit the migration as the GRK2 WT (lane 6). This confirms that the enhanced migration observed in the presence of the Lyp R620W variant involves GRK2.
Figure 6
Figure 6 Addition of GRK2 rescues migration inhibition.

Jurkat cells stably overexpressing Lyp wt or Lyp R620W and transiently overexpressing the indicated GRK2 constructs were subjected to a transwell migration assay with SDF-1α as a chemoattractant. Results are shown as the percent of cells migrated compared to the number of input cells.
Mice carrying Pep R619W are more susceptible to Collagen Antibody Induced Arthritis due to lower levels of GRK2

It has now been established that Lyp negatively regulates chemokine-mediated cell migration through its effect on GRK2. Cell migration is an important disease mechanism in autoimmune states such as rheumatoid arthritis. The Lyp R620W variant has been associated with predisposition to this and other diseases but the mechanisms are not completely understood. We turned our attention to GRK2, as patients with rheumatoid arthritis are reported to have decreased levels of GRK2 (Lombardi et al, 1999). As both of Lyp and GRK2 have been linked to rheumatoid arthritis, we wanted to determine if their role in cell migration affects arthritis onset or severity. To this end, we used a previously described model of Collagen Antibody Induced Arthritis (CAIA) (Nandakumar & Holmdahl, 2005) to induce the disease in the Pep R619W mice. Pep R619W mice or their wild-type littermates were induced and monitored for disease severity daily. The Pep R619W mice showed more severe gross limb and joint changes upon disease onset (Figure 7a). Compared to untreated littermates, the CAIA WT animals showed mild to modest paw swelling, but the CAIA Pep R619W mice had a higher incidence of severe joint and paw swelling and joint rigidity. The R619W mice overall had more pronounced hind paw thickness, and higher clinical scores (Figure 7b). We next sought to visualize any differences in lymphocyte migration in these animals. Representative knee sections were stained with hematoxylin & eosin or the indicated lymphocyte markers. There is more lymphocyte infiltration in the joints of the Pep R619W mice than their wild-type counterparts (Figure 7c). Quantitation of two histological sections per mouse show a significant increase in the CD3+ cells into the knee joint of Pep R619W mice compared to affected WT littermates. Cells stained with the
Figure 7

a) Normal

CAIA WT

CAIA R819W

b) Clinical Score vs Day of Treatment

Day of Treatment

0 2 4 6 8

WT

R819W

Ratio Change in Hind Paw Diameter

Normal CAIA WT CAIA R819W

**p = 0.005

c) H&E CD3 B220

Normal

CAIA WT

CAIA R819W

% CD3+ cells in image section

% B220+ cells in image section

p < 0.05

d) Spleen LN

WT R819W WT R819W

GRK2

actin
Figure 7. PepR619W mice present with more CAIA, enhanced cell migration, and lower GRK2 levels.

a) Age and sex-matched mouse littermates were subjected to collagen-antibody induced arthritis and monitored daily for disease onset. Representative images from gross mouse changes of hind paws on Day 6 are shown. b) Mice were monitored daily and clinical score was assigned as described in Methods ( * p < 0.05 based on a two-tailed T-test, n=10). At Day 5, clinical scores were WT Mean = 2.3, SEM ± 0.43, R619W Mean = 4.1, SEM ±0.57. Hind-paw thickness was measured by calipers daily and recorded in millimetres. The measurement for each paw at the peak of disease (Day 5) was expressed as a ratio compared to the same paw before CAIA induction (CAIA WT Mean = 1.06, SEM ±0.026, CAIA R619W Mean = 1.18, SEM ±0.034) (n=20) **p = 0.005 based on a two-tailed T-test (right panel). c) Histological staining of knee joints at Day 10 with hematoxylin-eosin, CD3, or B220 show enhanced amounts of cells into the joints. Quantitation was carried out by expressing the % of CD3⁺ or B220⁺ positive cells compared to the total number of cells in the indicated image sections. CD3⁺ cells: CTR Mean = 0.74, SEM ± 0.283 (n=12), WT Mean = 4.61, SEM ± 0.840 (n=20), R619W Mean = 9.41 SEM ± 1.644 (n=20). * p < 0.05 based on an unpaired, two-tailed T-test. (upper right panel) B220⁺ CTR Mean = 0.12 SEM ± 0.128 (n=12), WT Mean = 2.23 SEM ± 0.772 (n=20), R619W Mean = 3.59 SEM ± 0.662 (n=20) NS not significant. (lower right panel) d) Spleen or lymph nodes were harvested at Day 10 of disease onset. Whole cell immunoblotting with GRK2 and β-actin was carried out.
B-cell marker B220 are not very prominent in the joints. Finally, upon sacrifice of the study subjects, whole tissue lysates reveal that in the Pep R619W mice, the GRK2 protein level is down (Figure 7d). This is consistent with our results of lower GRK2 protein level in PepR619W upon stimulation with SDF-1α, but is more dramatic in this induced inflammatory state. Based on our previous results, we conclude that the inability of Pep R619W to regulate GRK2 leads to its reduced levels, and hence in enhanced migration of T-cells. This dysregulation can lead to more severe clinical outcomes in autoimmune disease states such as arthritis.

**DISCUSSION**

This report is the first to establish Lyp’s role in T-cell migration. This is largely facilitated by Lyp’s effect on a new interactor that we identified, GRK2, a well-characterized negative regulator of chemokine signalling. GRK2 represents a novel substrate of Lyp. Upon chemokine activation, the ability of Lyp to dephosphorylate GRK2 rescues it from subsequent proteasomal degradation, leading to its enhanced stability. The reduced capacity of the Lyp R620W variant to dephosphorylate GRK2 leads to its enhanced degradation and subsequent dysregulation in the attenuation of receptor desensitization and chemokine signalling.

A defect in cell migration has been shown to be an important mechanism in autoimmunity (reviewed in (Norman & Hickey, 2005)). Interestingly, Lyp has been shown to be associated with autoimmunity, and so is this new binding partner, GRK2. Patients with rheumatoid arthritis are reported to have decreased levels of GRK2 (Lombardi et al, 1999). Experimentally, rats that are induced to develop acute adjuvant arthritis or allergic
encephalomyelitis also have reduced levels of GRK2 (Lombardi et al, 2001; Vroon et al, 2003). Here we have uncovered a mechanism via Lyp/Pep for the dysregulation and protein instability of GRK2. The Pep R619W mice with CAIA have even lower levels of GRK2, leading to enhanced cell migration and a higher severity of disease. It would be interesting to be able to translate these findings to human subjects carrying the Lyp R620W versus non-carriers to see their GRK2 levels. Furthermore, we may be able to see if R620W patients with rheumatoid arthritis show even lower GRK2 levels than R620W carriers that are healthy.

One of the ways that GRK2 protein levels are regulated is by phosphorylation, followed by ubiquitination. These mechanisms have been studied previously in other cell types, but not in-depth in the context of T-cell signalling. The kinase responsible for GRK2 tyrosine phosphorylation is known to be c-Src (Fan et al, 2001; Penela et al, 2001; Sarnago et al, 1999). In a T-cell however, the exact protein kinases have not been determined, but likely candidates are Lck or Fyn. These are shown to be Lyp targets (Cloutier & Veillette, 1999; Hasegawa et al, 2004). While we show that Lyp downregulates GRK2 tyrosine phosphorylation, it is not clear if this is a direct dephosphorylation or a consequence of Lyp inactivating the Src family kinases. Also, it is not known if all three tyrosines that Src phosphorylates, 13, 86, and 92, are relevant in chemokine signalling, or which specific one Lyp targets.

Tyrosine phosphorylation of GRK2 precedes its subsequent ubiquitination. The only reported ubiquitin ligase to date to carry out this function is Mdm2 (Salcedo et al, 2006), but this is shown upon β2-adrenergic receptor stimulation. It has not been confirmed if this is the actual ligase responsible for GRK2 ubiquitination in T-cells upon chemokine stimulation.
Other important RING finger ligases in the immune system are Cbl and GRAIL. The work presented here provides a framework to build upon the GRK2 interactome further in the context of T-cells.

As GRK2 function has been studied in many other hematopoietic cell types, we can begin the examine the role of Lyp in other cells and downstream of other receptors. These include other chemokine receptors implicated in GRK2 function, such as CCR5 and CXCR2 (Fan & Malik, 2003; Vroon et al, 2004) Other receptors that are upstream of GRK2 include Toll-like Receptors (TLRs). Stimulation of TLR2, 3, 4 and 7 upregulate GRK2 protein expression in macrophages (Loniewski et al, 2008). In neutrophils, upregulation of GRK2 by TLR2 has been shown to lead to increased internalization and reduced signalling of CXCR2 (Alves-Filho et al, 2009). Changes in GRK2 protein level have also been observed in inflammatory or septic disease states (Alves-Filho et al, 2009; Arraes et al, 2006; Fan & Malik, 2003). Thus the observed role that GRK2 plays in many different cell types opens up the possibility that Lyp in complex with GRK2 is involved in other pathways and cell types as well.

Cell migration is a complex process involving many different mechanisms. While we show compelling evidence that GRK2 is the effector responsible for Lyp’s role in cell migration, there could be other mechanisms that play a part as well. Migration is facilitated in part by Src-family kinases, which are known Lyp substrates. We show in Chapter 3 that the Lyp/Pep R/W variants cannot effectively regulate Src kinases in-vivo. It is entirely possible that this dysregulation also plays a role in the enhanced migration observed in the R/W variants. Hyper-activated Src kinases would also lead to enhanced downstream signalling, including that involving Rho GTPases and cytoskeletal reorganization. These are
all inter-connected mechanisms stemming from dysregulated Src kinases that could be involved and may be explored further.

In summary, we have shown that the Lyp phosphatase negatively regulates T-cell migration. This is facilitated in part by Lyp’s stabilization of a newly identified interactor, GRK2. The enhanced migratory properties in patients and mice carrying the variant Lyp R620W/Pep R619W allele through the Lyp-GRK2 interaction provide a new mechanism to the development of arthritis. This new protein complex also provides a framework in which to further study both GRK2 and Lyp in the context of other cell types and pathways. This will provide insights into the important roles of these molecules in the immune system and the onset of autoimmunity.

**METHODS**

**Antibodies and Reagents**

Antibodies used were as follows. From Santa Cruz Biotechnology: α-GFP, α-Csk, α-GRK2, α-Ub, α-GST, α-His, α-c-Myc. From eBioscience: α-CXCR4 (APC), α-CCR5 (PE), α-CCR7 (PE). Others were α-Flag (Sigma Aldrich), α-pTyr (Millipore), and α-Lyp (Abnova). Mouse and human recombinant SDF-1α were from R&D Systems.

**Plasmid Constructs**

GRK2 cDNA was cloned into vector pcDNA3 with a C-terminal Myc tag, or vector pQE-30. Domain deletion constructs were generated by PCR and contained a C-terminal Myc tag. The Lyp cDNA was cloned into vector eGFP C1 or pcDNA3 with a N-terminal Flag tag. Site-directed mutagenesis using overlapping PCR was carried out to obtain the mutant C227S or R620W. Domain deletion constructs were generated by PCR
corresponding to fragments 1-299, 1-613 and contained an N-terminal Flag tag. Similarly, domain deletion constructs were generated by PCR and subcloned into pGEX3T-2.

**Immunoprecipitation/Immunoblotting**

Cells were lysed using NP-40 lysis buffer (1% NP-40, 50mM Tris pH 8, 150mM NaCl, 1mM EDTA) supplemented with 1mM PMSF, 1mM Na$_3$VO$_4$ and 1µg/ml each of pepstatin/aprotinin/leupeptin. After 30 minutes on ice, lysates were pre-cleared by centrifugation at 14,000 rpm for 10 minutes. The soluble portion was subjected to immunoprecipitation using 1-2 µg of the indicated antibodies for 2 hours at 4°C, followed by incubation by Protein G beads for 1 hour. The complexes were washed three times in lysis buffer and boiled in Laemmli buffer containing β-Mercaptoethanol. The samples were then loaded onto SDS-PAGE and immunoblotted with the indicated antibodies.

**Cell culture, Transient Transfections and Electroporations**

COS-7 cells were grown to 90% confluency and DNA was transfected into them using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s directions. After 24 hours, cells were stimulated with EGF ligand (100ng/ml) (Invitrogen) for the indicated time points. The media was aspirated and the cells were washed with ice-cold PBS before lysis. Electroporations of plasmids into Jurkat cells was carried out using the Amaxa Nucleofector as per the manufacturer’s directions. Briefly, 6 x 10$^6$ cells were electroporated with 10µg of plasmid DNA. After 24 hours, cells were stimulated as indicated, and lysed.

Freshly isolated murine lymphocytes or thymocytes were lysed immediately or subjected to stimulations were indicated.

Human PBMC were purified for CD4$^+$ T-cells using the Rosette Sep Human T-cell Enrichment Kit (Stemcell Technologies) as per the manufacturer's directions.
**Generation of Fusion Proteins and In-Vitro Binding**

Recombinant fusion proteins were generated by subcloning Lyp aa 1-674 into pGEX4T-2 vector and transforming *E. Coli* Rosetta2 cells. Protein induction was carried out by growing cells to OD 1.0 and adding IPTG (Roche) at 0.1mM overnight at room temperature. The GST-Lyp proteins were immobilized on glutathione sepharose beads. Conversely, GRK2 was cloned into pQE-30 and induced by growing the culture to OD 0.7 and adding IPTG at 0.5mM for 4 hours at 37°C. Protein lysates were incubated with nickel affinity beads and the protein was further eluted with 250mM Imidazole. For the binding assays, 50µl bead slurry containing the GST-Lyp fragments was incubated with eluted His-GRK2 for 2 hours and 4°C. Complexes were washed and subjected to SDS-PAGE.

**Mass Spectometry**

GFP-Lyp immunoprecipitates were resolved on SDS-PAGE, stained with Coomassie Blue, and the resulting bands were excised. Gel slices were subjected to trypsin digestion and MALDI-TOF analysis, followed by MS-MS analysis.

**Pulse chase assay**

Thymocytes or Jurkat cells (2x10^7 cells/ml) were incubated for 1 hour at 37°C in cysteine and methionine-free culture medium followed by 30min pulse with 0.5mCi/ml [³⁵S]methionine (NEN). Cells were then either lysed in cold lysis buffer containing 1% Nonidet P-40 or chased by a hour incubation in culture medium containing nonradioactive cysteine and methionine (1mM final concentration) for the times indicated. GRK2 was then immunoprecipitated from cell lysates, separated by SDS-PAGE and detected by autoradiography.
Two-photon microscopy

Pep WT or Pep R619W T-cells (5 x 10^6) were labeled with CSFE (5,6-carboxyfluorescein diacetate succinimidyl ester, 2 µM) and CMTMR (chloromethylbenzoylaminotetramethylrhodamine, 10µM) at RT for 10 minutes and the CSFE labeled cells were effluxed by adding 10mL of ice cold FBS and left on ice for 10 min. CMTMR labelled cells were resuspended in RPMI 1640 with 10% FBS and incubated at 37°C for 20 minutes. Cells were washed 3 times with RPMI 1640 with 10% FBS after efflux and resuspended into 300 µL PBS. Recipient B6 mice were injected intravenously with 200-300µL of mixed CSFE and CMTMR labeled Pep WT or Pep R619W T-cells. The recipient mouse was sacrificed 24 hours after injection and superficial cervical and axillary lymph nodes were collected and placed in the perfused medium (RPMI 1640 without phenol red was prewarmed at 37°C and perfused in 95% O_2/5% CO_2) at 37°C. Lymph nodes were imaged in a 37°C heated stage with Zeiss LSM 510 META NLO with the FLUAR 20×/0.75 NA objective lens and image acquisition was done using the accompanying software (Zeiss). CSFE and CMTMR were excited using the Chameleon pulsed femtosecond laser set at 810nm. Emission wavelengths of 500-550nm (CSFE) and 565-615nm (CMTMR) were acquired. For 3D time lapse imaging, each xy plane spanned 256×256 µm at 3µm spacing and 60µm depth (20 xy planes in each z-stack). Each z stack was imaged 40 seconds apart for 15 minutes. Data sets produced by the microscope are a time series of three-dimensional (3D) image stacks containing one or more fluorescence intensity channels and analyzed using Velocity software (PerkinElmer) to yield quantitative data on cell movements. Cell tracking was performed by identifying the centroid of each cell within the 3D image at successive time points manually.
Transwell migration assay

Transwell migration chambers were purchased from Corning (5µm) and used according to manufacturer’s directions. Briefly, membranes were left uncoated, and were blocked for one hour at 37°C in PBS with 2% BSA. Freshly isolated murine lymphocytes or human CD4+ cells were seeded at a total of 5 x 10^5 cells in 100µl RPMI + 0.5% BSA on the upper chamber. The lower chamber was filled with 600 µl RPMI + 0.5% BSA containing the indicated chemo-attractant. After 2-4 hours, the migrated cells in the bottom chamber were collected and quantified with calibrite beads (Becton Dickinson) using flow cytometry.

In-vivo Homing Assay

Mouse naïve CD4+ T-cells were isolated from spleen as per manufacturer’s directions (R&D Systems). Cells were labelled with either CFSE (eBioscience) or CMTMR (Invitrogen) and injected into a C57BL/6 recipient mouse. One hour later, the mouse was sacrificed, the organs harvested, and the donor populations were analyzed by flow cytometry. Results are shown as the ratio of PepR619W recovered cells compared to wild type.

Collagen Antibody Induced Arthritis

An experimental model of arthritis was utilized as previously described (Nandakumar & Holmdahl, 2005). Animals 8-10 weeks of age were injected intravenously with 3mg ArthroMab antibody cocktail (MD Biosciences). At day 3, mice were boosted intraperitoneally with 50µg of LPS. The mice were monitored and scored blindly by adopting previously described simple scoring scales (Doppler et al, 2008; Holmdahl & U., 1998) for a total of 3 points per limb as follows: 0 = normal, 1 = mild swelling or erythema of paw and/or digits, 2 = moderate swelling and erythema of paw and/or ankle and/or more
than one digit, 3 = severe swelling and involvement of entire paw, ankle and more than one digit. There are a total of 12 possible points per mouse.

**Histological Analysis**

Upon sacrifice of mice, limbs were dissected and fixed in 4% paraformaldehyde for 24 hours, followed by decalcification in EDTA for 3 weeks. Samples were then paraffin embedded and stained for hematoxylin-eosin or the indicated antibodies. Quantitation is expressed as the number of positive cells for the indicated marker relative to the total number of cells in that section.

**Statistical Analysis**

Where indicated, a two-tailed Student’s T-test was used to determine statistical significance.
CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS
Overview of new insights into Lyp biologic sequelae

The work presented in this thesis is a significant contribution towards a better understanding of the PTPN22 phosphatase Lyp/Pep and its functional variant R620W/R619W. When this work began, there was very little knowledge of Lyp’s binding partners, cellular contexts, signalling pathways, or the effects of the variant form of this protein. A summary of what was known at the beginning of this work is shown in Figure 1a. Lyp had been shown to be downstream of the TCR, where along with Csk, it negatively regulated Src-family kinases (Cloutier & Veillette, 1996). The Grb2, CD3ζ, and Cbl interactions were reported, but no function was attributed to them (Cohen et al, 1999; Hill et al, 2002; Wu et al, 2006). The Lyp R620W variant had been shown to be associated with autoimmune disease and lose its binding to Csk (Begovich et al, 2004; Bottini et al, 2004) but it was not known what the effect of the variant was to the function of the phosphatase itself. In this thesis, we have greatly increased our understanding of the Lyp phosphatase signalling interactions, as shown in Figure 1b. The Cbl interaction is now thoroughly characterized to include the identification of Cbl as a Lyp substrate. We now have expanded the Lyp interactome to include calpain and GRK2, and show Lyp downstream of the chemokine receptor CXCR4. Furthermore, the thesis provides novel mechanisms in the function of the wild type and autoimmune-associated variants of Lyp in the context of T-cells, B-cells, and even dendritic cells.
Figure 1
Figure 1. Functions of Lyp a) The understanding of Lyp function in 2004. b) The contribution of this thesis work to the current understanding of Lyp function.
Lyp and Cbl

The first objective of this thesis was to further characterize the Cbl-Lyp interaction. We have now identified Cbl as an actual substrate of Lyp. It was shown that the two proteins bind directly, and that Lyp dephosphorylates Cbl specifically at Y371. This residue is important for Cbl function and disease association. We re-iterate the importance of Y371 in a T-cell context, and show that dephosphorylation of Lyp inhibits Cbl ubiquitin ligase activity. Hence we now better understand the role of these two important players in immune cell function.

The effect of the autoimmune associated Lyp R620W was not examined in the context of Lyp and Cbl. Presumably, as the binding is mediated through the Lyp interdomain, the binding between them may not be affected, as the R620W mutation is in the Lyp proline-rich region. In view of the findings presented later on in the thesis regarding the reduced stability of the Lyp R620W protein, it may not be as efficient as the wild-type in downregulating Cbl phosphorylation and activity. For example, as Lyp R620W was shown as a loss-of-function in terms of protein levels, that would leave unregulated, phosphorylated Cbl in the cell. This then may lead to tighter regulation by cell signalling by Cbl. Conversely, phosphorylated Cbl also leads to enhanced Cbl auto-ubiquitination and degradation. It is entirely possible that this may lead to overall less Cbl in the cell. In order to address these possibilities, we can begin by determining if there are any changes in the protein levels of Cbl in the presence of Lyp R620W, followed by ubiquitination assays to assess any changes in Cbl activity. If we do these experiments in settings where Lyp R620W is overexpressed, we may not detect a lot of difference in comparison to the effect of the Lyp wt, as the variant may not be regulated in the same way in-vitro in the same way as in-vivo.
For this, we can utilize the Pep R619W mouse models. We can examine for protein levels of Cbl, followed by in-vitro ubiquitination assays by immunoprecipitating Cbl from mouse cells. Also, the ubiquitin associated with physiologic substrates of Cbl such as Vav can be assessed in the mouse model. These experiments may provide clues as to the role of the Lyp variant in terms of its regulation of Cbl.

The site on Cbl that we show Lyp targets is Y371. Mutations at this residue rendering a loss-of-function in terms of Cbl ubiquitin activity are linked to a number of myeloid neoplasms. It would be interesting to see if the R620W is associated in any way to similar malignancies as Cbl. If Cbl is more active as a ubiquitin ligase in the presence of Lyp R620W this may lead to tighter regulation by Cbl and hence lead to a protective effect against malignancies. If however, active Cbl also leads to accelerated auto-ubiquitination and degradation, the profile of malignancies that may be associated with R620W may be the same as when Cbl is inactive. As large-scale population studies have been carried examining Lyp association with autoimmunity, it may be worth exploring any linkage possibilities to other pathologies such as the myeloid neoplasms that Cbl is associated with.

There are other members of the Cbl family of ubiquitin ligases that Lyp may interact with. The amino acid Y371 on Cbl is conserved as Y363 on Cbl-b, and phosphorylation of that site may increase its activity as it does with Cbl. Mutations conferring Y to E substitutions at both Cbl Y371 and Cbl-b Y363 to mimic phosphorylated states were found to increase ubiquitin ligase activity of both Cbl and Cbl-b (Kassenbrock & Anderson, 2004). Since that site is conserved between Cbl and Cbl-b, it is possible that Lyp may also dephosphorylate Y363 on Cbl-b. Assays such as in-vitro and in-vivo ubiquitination assays as described in Chapter 2 can be undertaken to begin to explore this possibility. While Cbl and
Cbl-b share many of the same substrates, they also have distinct roles in immune signalling. One of the major signalling pathways that Cbl-b regulates is downstream of CD28 (reviewed in (Loeser & Penninger, 2007). Hence, alterations of Cbl-b ubiquitin ligase activity may have different consequences that can be examined.

**Insights into the RW disease-associated variant using a mouse model**

To better understand how Lyp is associated with autoimmunity, we utilized a knock-in murine model of Pep R619W containing the variant of the human homologue Lyp. We show that these mice manifest with enhanced activation of T-cells, B-cells, and dendritic cells – a new cellular context for Lyp function. Surprisingly, both humans and mice harbouring the RW variant show significantly reduced protein levels. A few potential mechanisms for this phenomenon were revealed. While transcript levels of PTPN22 remain the same in the variant, the RW variant associates more with calpain, a proteolytic enzyme. This leads to cleavage and subsequent degradation of the protein. The phosphatase is simply not present in adequate amounts in order to efficiently regulate its cell targets, essentially resulting in a loss-of-function.

We also began to explore the effect of the RW variant to the processes of autoimmunity. While we used a CAIA model to study arthritis in the Pep R619W mice, other genetic mouse backgrounds may allow for more physiologic, spontaneous development of autoimmunity. A permissive genetic background has previously been used to uncover Pep function when it was crossed with the CD45\(^{E613R}\) mice (Hermiston et al, 2009). The CD45\(^{E613R}\) mice have hyperresponsive B cells, characteristic of human and murine SLE (Majeti et al, 2000). When crossed with Pep knock-out mice, the resulting strain displayed enlarged lymph nodes, spleen, the presence of autoantibodies, and glomerulonephritis (Hermiston et
al, 2009). Similarly, if we study the Pep R619W in a different background, we may uncover additional functions. Defects in T-cell migration are not only characteristic to arthritis, but to SLE as well (Takeuchi et al, 1993). One such possibility is the B6.SLE1 mice. These mice are a congenic strain that spontaneously develop a lupus-like syndrome and have commonly been used to study the disease (El Chami et al, 2005). Perhaps crossing our Pep R619W mice with the B6.SLE1 strain may allow for the presentation of the disease and the study of T-cell migration in a more physiologic context.

**A new role for Lyp: regulation of cell migration**

The novel interaction of Lyp and GRK2 also reveals new roles of Lyp in other signalling contexts. The identification of GRK2 as a new substrate puts Lyp downstream of chemokine signalling, specifically CXCR4. This receptor not only becomes serine phosphorylated, but also tyrosine phosphorylated (Vila-Coro et al, 1999). It is possible that Lyp may not only regulate CXCR4 through GRK2, but directly as well. There are other phosphatases such as Shp1 that have been shown to associate with CXCR4. It would be interesting to see if Lyp associates with CXCR4 and if the receptor also serves as a potential substrate.

Chemokines play an important role in the immune system and in immune cell migration. We show here that Lyp is a key regulator of cell migration through GRK2. Lyp dephosphorylates GRK2 and hence prevents it from being degraded by the proteasome. The stabilization of GRK2 leads to enhanced negative regulation by GRK2 of CXCR4 upon ligand binding. This could be due to modulation of receptor levels by GRK2. Receptor levels on the cell surface can have important consequences on cell migration, but on other processes as well. For example, the CXCR4 receptor serves as a co-receptor for entry of the
HIV virus into T-cells (Bleul et al, 1996a). If Lyp regulates CXCR4 signalling or the receptor level, it may have consequences on HIV infection. This can be tested first by infecting cells *in-vitro* that express Lyp or the Lyp R620W variant and observing any differences in the rates of infection. Based on the results, it may be worth examining the *PTPN22* C1858T polymorphism in populations that are HIV-positive to see if it is associated with any differences in the rates of infection. A similar study examining people that tested positive for *Mycobacterium Tuberculosis* infection revealed that the Lyp variant was in fact protective against the onset of active disease (Gomez et al, 2005). Through these studies we can expand our understanding of the variant allele in not only autoimmune diseases but infectious diseases as well.

Interestingly, just as Lyp has been linked to rheumatoid arthritis (Begovich et al, 2004), so has GRK2. Levels of GRK2 have been shown to be reduced in both human and mice with different forms of arthritis (Lombardi et al, 2001; Lombardi et al, 1999). The mechanism for the reduction of GRK2 has not been delineated. In this work, we provide at least one explanation for the reduction of GRK2, especially in the presence of the Lyp R620W variant. The resulting low levels of Lyp R620W does not allow for full regulation of GRK2. That is, more GRK2 in the cell remains tyrosine phosphorylated and hence ubiquitinated and degraded. In the Pep R619W mice, upon induction of CAIA, we show much less resulting GRK2. This leads to a dysregulation in cell migration. We also show enhanced migration in people with the Lyp R620W variant. To confirm the role of GRK2 in human samples, it would be interesting to see if indeed the GRK2 level is down in the presence of the Lyp variant in both resting and chemokine stimulated human cells.
A better understanding of Lyp protein binding and regulation

We present in this work the first reported instances of protein binding capabilities of the Lyp interdomain, spanning amino acids 300-613. We show that this region is responsible for binding both Cbl and GRK2. Specifically for Cbl binding, we narrowed down the region to amino acids 300-450. On Cbl, Lyp binds the N-terminus. This region contains what is known as a TKB domain that includes an SH2 domain, presumably for binding phosphorylated tyrosines. Examination of the Lyp sequence between 300-450 reveals a possible tyrosine residue that may be phosphorylated for Cbl binding. The residue at Y437 is conserved between Lyp and its mouse homologue Pep. It may conform to a potential SH2 binding site, as its motif is YxNx (Kimber et al, 2000). This remains to be tested experimentally.

In terms of how the Lyp interdomain binds GRK2, this remains unknown. The GRK2 serine/threonine kinase domain spans 265 amino acids in the middle of the protein with multiple potential protein interacting motifs. Of these, not many are obvious regions for binding Lyp based on our current understanding of the Lyp interdomain. Perhaps a peptide-scanning array or similar technique can be utilized in order to delineate the exact residues involved in this interaction. This may provide more insights into some of the functional motifs situated in this largely uncharacterized region.

While significant strides have been taken in the understanding of Lyp, much still remains unclear. It is often assumed, for example, that Lyp and its homologue Pep act the same. This however, may not be entirely correct, as the interdomain and C-terminus of the two differ substantially at only 61% homology. This is the lowest than any other described pair of mouse to human phosphatase homologues (Cohen et al, 1999). To date, there is no
biological function attributed to the interdomain, and no other known protein interactions other than the ones we report for Cbl and GRK2. It would be interesting to see what other proteins bind this region and if this interdomain had different binding capabilities between Lyp and Pep.

This interdomain may contain regions for the regulation of Lyp function. In this work, we largely described how Lyp regulates other proteins, such as Cbl and GRK2. We do not fully understand, however, how Lyp itself is regulated. It has been established that there is an inhibitory tyrosine phosphorylation site in the interdomain at Y536, targeted by Lck (Fiorillo et al, 2010). The authors of this work note that mutation of Y536 does not completely abrogate Lyp overall tyrosine phosphorylation, indicating that there are other sites that are also targets for tyrosine kinases, even Csk itself (Fiorillo et al, 2010). To further explore this possibility, mutations of other tyrosines of Lyp can be introduced to see if they are residues that are phosphorylated. Bioinformatic analysis of the Lyp sequence reveals potential tyrosine phosphorylation sites at Y339, Y437, Y471 and Y499. To test if any of these are in fact Csk substrates, we can introduce combinations of mutations at these tyrosine residues in Lyp or the Lyp R620W form. Since Csk does not bind Lyp R620W, less phosphorylation in the Lyp R620W variant may lead to the identification of Csk targets. These may also be confirmed by using Csk-deficient T-cells to see if Lyp is phosphorylated in the presence of Csk or not. It is possible that if Csk also targets Lyp, it may regulate its localization and catalytic activity. In turn, since the R620W variant cannot bind Csk, it will not be subject to the same regulation, leading to another mechanism for aberrant cell signalling.
Ubiquitination is another post-translational modification that we show is important in Lyp regulation, with the RW variants being more ubiquitinated than their wild-type counterparts. It is not clear which ubiquitin ligase carries out this function. One obvious possibility is Cbl. Though we show that Lyp turns off Cbl activity, it is entirely possible that at certain times of cell activation, Cbl in turn downregulates Lyp. Other possibilities include ligases such as Itch or NEDD4. In recent years it has become apparent that there is a lot of cross-talk between the E3 ligases themselves, as Cbl itself is ubiquitinated by Itch and NEDD4 (Magnifico et al, 2003). The other Lyp interactor, GRK2, is also ubiquitinated, in this case by Mdm2 (Salcedo et al, 2006). It would be interesting to see if any of the aforementioned ligases is responsible for Lyp ubiquitination, either directly or in complex with Cbl and/or GRK2.

The interdomain also contains possible cleavage sites that may be important for Lyp activity or localization. In this report, we show that calpain is responsible for cleaving Lyp. The consequence of this is not entirely understood. Cleavage may lead to protein instability and degradation, but it may release certain protein domains to take part in other interactions. The interdomain on Lyp may also contain a caspase-1 consensus cleavage site at position 322 (LQAD) (Earnshaw et al, 1999). This has not been confirmed biologically. Caspase-1 activation has been shown to take place downstream of the inflammasome, and has important roles in inflammation and infection (reviewed in (Li et al, 2008). As autoimmunity often involves inflammatory states, perhaps Lyp is somehow regulated by caspase-1 cleavage. In order to test this, Lyp can be incubated in-vitro with recombinant, active caspase-1 to see if it results in Lyp cleavage products. The putative cleavage site is just after the catalytic domain. This may release the catalytic domain in finding its targets and exerting its
phosphatase activity. A similar phenomenon has been observed with the related phosphatase PTP-PEST (Halle et al, 2007). In the case of PTP-PEST, cleavage by caspase-3 releases the catalytic domain and increases its phosphatase activity. Perhaps a similar effect on Lyp activity may be observed after cleavage with caspase-1. It should be noted that the catalytic domain has been shown to have many protein binding capabilities on its own without needing the C-terminus (Wu et al, 2006).

The notion that the C-terminus may somehow hinder the phosphatase domain is supported by evidence showing sequential C-terminal deletions of Lyp result in higher catalytic activity (Hong et al, 2009). It is entirely possible that there exists an intramolecular interaction in Lyp that regulates its interactions/activity. Intramolecular interactions are common regulatory mechanisms in protein tyrosine phosphatases, such as SHP1 and TC-PTP (Hao et al, 1997; Pei et al, 1994). SHP1 activity is auto-inhibited by the binding of its N-terminal SH2 domain to the catalytic domain. This is then released upon cell activation. It may be possible that similar mechanisms may be in place for Lyp, that is, the C-terminus is somehow interacting with and interfering with the catalytic domain. As the Lyp R620W variant may have a higher intrinsic catalytic activity (Bottini et al, 2004) then perhaps it adopts a more open conformation. While there is structural information available for the Lyp catalytic domain, no such information exists for the interdomain or C-terminus. Protein crystallography of this region would help provide some insights into more functional characteristics of the remainder of the Lyp protein.
GENERAL CONCLUSION

Overall, this body of work has contributed significantly to the understanding of the Lyp phosphatase and its autoimmune associated variant, R620W. The research aims outlined at the beginning of the work involving the Lyp-Cbl interaction, the generation of the murine model of the disease-associated variant, and the discovery of new interactors and signalling pathways were successfully carried out and improved our understanding of this phosphatase. First, we characterized Cbl as a new substrate of Lyp at Cbl Y371 and show that this leads to downregulation of Cbl ubiquitin ligase activity. We then generated a murine Pep R619W model in order to examine the effect of the variant to immune function. We showed compelling evidence in favour of the Lyp R620W/PepR619W variant as a loss-of-function systemically. The altered protein stability of the variant makes it unable to regulate its substrates, leading to enhanced cell activation. Finally, we established Lyp/Pep’s role in cell migration by identifying a new interactor GRK2. By regulating GRK2 stability, Lyp/Pep plays a role in the mechanisms involved in cell migration in humans as well as mouse models of arthritis. In support of the initial hypothesis, overall we show that indeed Lyp/Pep acts to attenuate cell signalling, and that the presence of the disease-associated variant results in dysregulation of its targets and hyperactivation of cells. The new findings of Lyp in the context of new interactors, cell types and signalling pathways provide a better understanding of this important regulator of immune function.
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