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The Dual Role of OCA-B in B Cell Development and Signaling

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OCA-B (Oct coactivator from B cells) is a B-cell specific coactivator of transcription that acts in conjunction with the ubiquitously expressed activator, OCT-1 or the B cell-restricted OCT-2. OCT-1 or OCT-2 can bind an upstream octamer element with the consensus sequence 5'-ATGCAAAT-3' in target gene promoters and enhancers. At the octamer element, the OCT-1/2-OCA-B complex is able to regulate gene expression via interaction with the basal transcription machinery. OCA-B was originally identified as a nuclear transcriptional coactivator and then shown to be essential for antigen-driven immune responses, including secondary isotype expression, germinal center formation and BCR-dependent proliferation. These defects contribute to the severely impaired antigen-dependent immune responses of *Oca-b*$^{-/-}$ mice. The later identification of a membrane-bound, myristoylated form of OCA-B suggested additional, potentially unique functions in B cell signaling pathways and that OCA-B might function through both transcriptional and non-transcriptional mechanisms within the cell. Therefore, a strong focus was placed on identifying additional OCA-B target genes that could explain its role in the antigen-dependent immune response.

Importantly, this study has identified, by cDNA microarray, several genes that fail to be up regulated in the *Oca-b*$^{-/-}$ activated B cells. OCA-B deficient cells have a compromised response to BCR signaling and many of the identified OCA-B target genes (e.g. *Kcnn4*, *Lck*, *CyclinD3*, and *Cdc37*) play important roles in cell signaling; however, their specific functions in B cell development and activation are largely unknown. From multiple primary B cell stages, we have found that key OCA-B target genes are expressed and regulated throughout B cell development. This study has identified a novel developmental block in *Oca-b*$^{-/-}$ mice, which indicates that OCA-B also functions in the pre-B1 to pre-B2 cell transition by mediating pre-BCR signaling. Most surprisingly, OCA-B directly interacts with SYK, a tyrosine kinase critical for pre-BCR and BCR signaling. This unprecedented type of interaction -- of a transcriptional coactivator with a signaling kinase -- takes place in the cytoplasm and directly regulates SYK stability. Combined with the deregulation of OCA-B target genes, this may help explain both previously observed defects in the antigen-dependent immune response of *Oca-b*$^{-/-}$ mice and newly identified defects in early B cell development. This study indicates that OCA-B is required for a complete response to pre-BCR and BCR signaling at multiple stages of B cell development, through its non-transcriptional regulation of SYK.

To My Husband

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Δ	Deletion
Δ	When in an equation, change in
-/-	Knockout
Anti-	Antibody
BCR	B cell receptor
BP	Base pairs
Cα	Immunoglobulin constant region α
C_T	Threshold cycle, raw data for QPCR
E	Efficiency of a QPCR reaction
Eμ	IgH intronic enhancer
FACS	Fluorescence activated cell sorting
FSC	Forward scatter
GC	Germinal center
GFP	Green Fluorescent Protein
HS	Hypersensitive site
Ig	Immunoglobulin
IL-	Interleukin
KB	Kilobases
LPS	Lipopolysaccharide
MACS	Magnet activated cell sorting
NE	Nuclear extract
PCR	Polymerase chain reaction
PI	Propidium Iodide
pTYR	Phosphotyrosine
QPCR	Quantitative PCR
RT	Reverse transcriptase
S100	Cytoplasmic extract
SSC	Side scatter
TD	T cell-dependent
T_h	Helper T cell
TI	T cell-independent
V_κ	Immunoglobulin light chain promoter
V_H	Immunoglobulin heavy chain promoter
WCE	Whole cell extract
WT	Wildtype

CHAPTER 1

INTRODUCTION

B lymphocyte development is a complex process marked by characteristic changes in gene expression and cellular phenotype. The generation of mature/antigen-responsive B cells from undifferentiated bone marrow precursors is dependent on the transmission of signals from both the extracellular and intracellular environments. One of the major mechanisms that enables this process to occur is transcriptional regulation. The alteration of gene expression resulting from cell signaling is critical for the development and function of B cells by driving changes in cellular function. Furthermore, antigen-dependent B cell differentiation is a useful model to study the mechanisms by which cells mediate alterations in gene expression in response to extracellular signals. Related, transcription of immunoglobulin genes has served as a model for understanding complex gene regulation because immunoglobulin expression is highly controlled throughout B cell development. This control is two-fold, involving gene alterations (V(D)J recombination, somatic hypermutation, and isotype switching) and events at DNA regulatory regions (enhancers and promoters). Immunoglobulin transcriptional regulation is mediated by transcriptional activators acting in conjunction with components of the basal transcriptional machinery upon binding of proximal/promoter or distal/enhancer regulatory elements. Full activity of immunoglobulin promoters and enhancers require the function of multiple transcription factors, including OCT-1/2, PAX-5, PU.1, and NFκ-B. The nature of the

regulation of immunoglobulin and other immunologically relevant target genes by OCT transcription factors and the coactivator OCA-B in response to B cell signaling is the subject of this study.

Octamer-dependent Regulation of immunoglobulin Gene Expression

The promoters of immunoglobulin genes contain a TATA box at which the basal transcription machinery (RNA Polymerase II and general transcription factors) acts. In addition to the core promoter, the primary cis element that confers cell-type specificity of immunoglobulin gene expression is an essential upstream octamer motif with the consensus sequence, 5'-ATGCAAAT-3', or the reverse, 5'-ATTTGCAT-3' (Falkner and Zachau, 1984; Parslow et al., 1984, reviewed in Staudt and Lenardo, 1991). Subsequent studies have shown that there is an octamer element in all immunoglobulin promoters and enhancers that is typically 90-100bp upstream of the core promoter (reviewed in Staudt and Lenardo, 1991, Falkner and Zachau, 1984). The octamer element of immunoglobulin promoters has been extensively dissected by several mutagenesis studies to show that while some deviation can be tolerated, residues 4-6 are most critical for function in the context of immunoglobulin promoters (Cepek et al., 1996; Gstaiger et al., 1996). However, a consensus octamer element alone can not confer specificity of immunoglobulin expression because it is found in a diverse array of gene promoters such as the histone 2B (H2B), HSV immediate early, and

snRNA promoters (Sive et al., 1986; apRhys et al., 1989). B cell specificity could be mediated, in part, by regulation at additional functional elements (reviewed in Calame and Sen, 2004). For example, DICE, an IgH promoter element, was recently shown to be required for optimal V_H promoter activity (Tantin et al., 2004). Another recent study indicates that an 18bp element downstream of the Igκ transcription start site also effects octamer dependence (Casellas et al., 2002). However, the factors that bind to this site have yet to be identified.

Members of the POU (Pit, Oct, Unc) family of transcriptional activators are characterized by their ability to bind octamer elements via their POU domains. The family members, PIT-1, OCT-1/OCT-2, and UNC86 bind octamer elements in growth hormone and prolactin promoters, immunoglobulin promoters, and various *Caenorhabditis elegans* gene promoters, respectively (reviewed in Phillips and Luisi, 2000). Structural and biochemical studies indicate that these regulators are comprised of only two conserved domains, the POU specific and the POU homeodomains, which are joined by a flexible linker. The POU specific domain interacts with the ATGC octamer half site while the homeodomain interacts with other.

Of the POU proteins that activate immunoglobulin transcription, OCT-1 is ubiquitous, whereas OCT-2 is mainly lymphocyte specific (Landolfini et

al., 1986; Staudt et al., 1986; Scheidereit et al., 1987). Historically, OCT-2 was originally thought to be responsible for mediating B cell specificity of immunoglobulin expression; however, it was later shown to be dispensable for immunoglobulin transcription in vitro (Pierani et al., 1990; Luo et al., 1992). Subsequent analysis of *Oct-2*$^{-/-}$ mice confirmed that OCT-2 is not strictly required for immunoglobulin expression in vivo (Corcoran et al., 1993; Feldhaus et al., 1993). The first evidence for an additional factor being involved in immunoglobulin transcription came from the demonstration by Pierani *et al.* that addition of recombinant OCT-1 and/or OCT-2 was not sufficient to achieve high levels of IgH transcription in OCT depleted nuclear extracts. Furthermore, efficient IgH transcription was only produced in Hela cell nuclear extracts if purified OCT factors from B cells were added as opposed to recombinant OCT proteins. Thus, indicating that there was a unique factor present in purified OCT fractions from B cell nuclear extracts that could allow for high immunoglobulin transcription. This activity, which proved to be OCT-1-associated was purified and shown to function in conjunction with OCT-1 or OCT-2 to activate IgH transcription in vitro (Luo et al., 1992). This factor was designated OCA-B, for Oct coactivator from B cells (Luo et al., 1992). In the presence of OCT-1, addition of OCA-B to a non-B cell nuclear extract allowed for recapitulation of IgH expression, similar to that of a B cell extract. Importantly, OCA-B was found to have no effect on the octamer-containing *H2B* promoter (Luo et al., 1992).

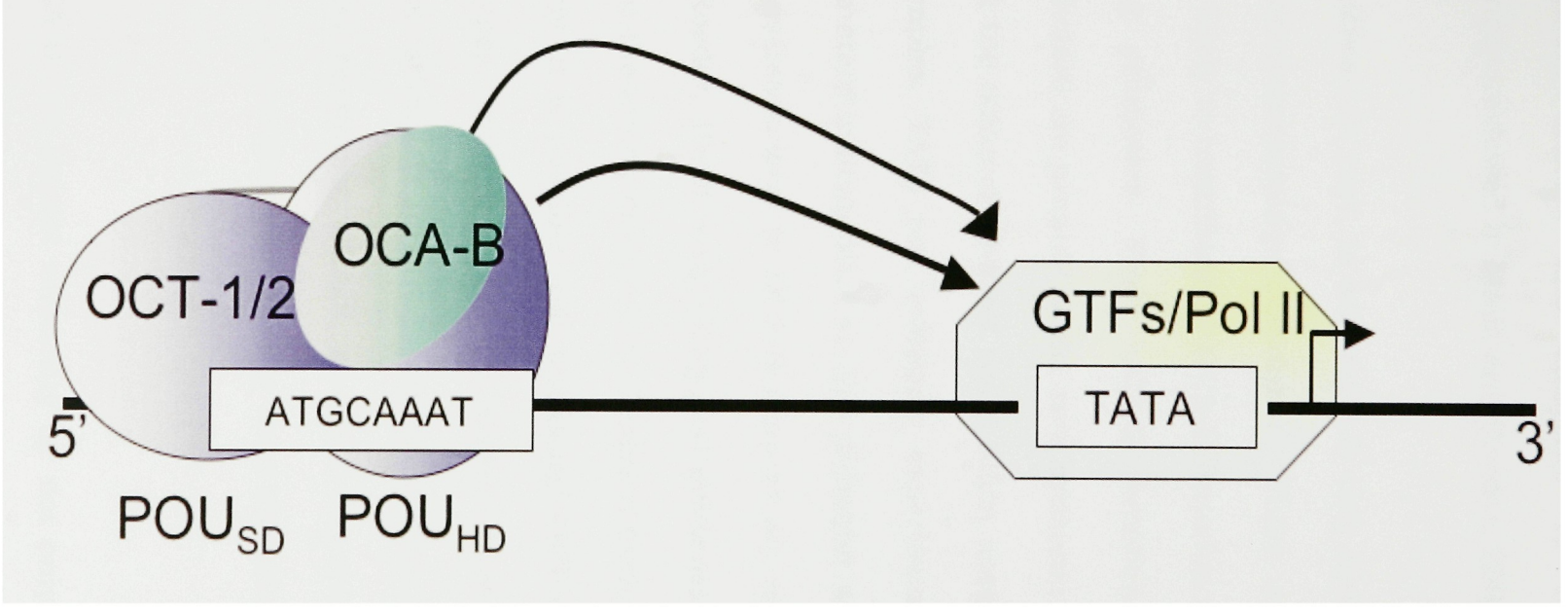
OCA-B:OCT:DNA Ternary Complex Formation and Transcriptional Activation (Figure 1.1)

Cloning and further analysis revealed that OCA-B is proline-rich, 256 amino acid protein with B cell-restricted expression (Luo and Roeder, 1995). Detailed biochemical analysis revealed that OCA-B functions efficiently with both OCT-1 and OCT-2 in vitro (Luo and Roeder, 1995; Luo et al., 1998). OCA-B was also cloned by yeast one-hybrid screens using OCT-1 as bait, and is also referred to as OBF-1 or BOB1 (Gstaiger et al., 1995; Strubin et al., 1995). While OCA-B interacts with both the POU-specific and POU-homeodomains of OCT-1/2, it does not interact with other members of the family (Luo and Roeder 1995; Gstaiger et al., 1996; Babb et al., 1997). The POU domains alone of OCT transcription factors are sufficient for recruitment and interaction with OCA-B but not for transactivation of IgH promoters, which indicated that OCA-B can not activate transcription alone (Luo and Roeder, 1995; Luo et al., 1998). However, provided that there is an A at position 5, OCA-B alone has a slight affinity for the octamer but is remains unable to activate transcription without OCT-1/2 (Luo and Roeder, 1995; Cepek et al., 1996; Chasman et al., 1999). Mutagenesis studies have identified the N-terminal 65 residues of OCA-B to be required for interaction with POU transcription factors (reviewed in Luo and Roeder, 1999). Conversely,

the transactivation domain of OCA-B is located within an acidic region of the carboxy terminus surrounding position 240 (Luo et al., 1998).

While the mechanism of OCA-B-mediated transcriptional activation has not yet been completely determined, full activation of IgH transcription in a reconstituted system requires both OCA-B and components of the general cofactor USA fraction (Meisterernst et al., 1991; Luo and Roeder, 1995). These studies were extended to show that PC4 depleted USA fractions could not support OCA-B function in a reconstituted system (Luo et al., 1998). However, PC4 depleted Hela cell nuclear extract could still support activated IgH transcription, which suggests that the extract has a redundant activity for PC4 that was not present in that USA fraction. The OCA-B/PC4 interaction was further demonstrated by GST-pulldown and localized to the activation domain of OCA-B, which is consistent with PC4 playing a role in OCA-B-mediated gene activation (Luo et al., 1998). GST-OCA-B pulldown has also revealed an interaction with TBP and TFIIB (Schubart et al., 1996a) but this finding has yet to be investigated further. Additionally, OCA-B-dependent activation was also shown to require PC2/Mediator (Luo et al., 1998). The mediator is a large multi-subunit complex that is the main link of

Figure 1.1 A Model of OCA-B Mediated Transcriptional Activation



See text for details.

transcriptional regulatory factors and cofactors to the general transcriptional machinery (reviewed in Malik and Roeder, 2000).

What still remains unclear, in part, is why OCA-B only functions from some consensus octamer-containing promoters. There are multiple mechanisms that are likely to contribute to OCA-B function on a subset of octamer-containing promoters. First, OCA-B function could be dependent on which factors are present at the core promoter and/or the sequence surrounding the octamer element could affect assembly of the OCA-B/OCT/DNA complex. TATA box swapping experiments between the H2B and IgH promoters indicate that an IgH promoter with an H2B TATA box becomes less responsive to OCA-B (Kim et al., unpublished; reviewed in Luo and Roeder, 1999), suggesting that elements at the core promoter play a role in the ability of OCA-B to function from an octamer element. Alternatively, it is possible that additional OCT coactivators exist in different cell types. Indeed, an activity, distinct from OCA-B has been identified that can stimulate IgH transcription in vitro (Fujii et al., unpublished data). Furthermore, OCA-S, an OCT coactivator that is necessary for H2B gene expression has recently been identified (Zheng et al., 2004).

It is possible that OCA-B can impart selectivity by not only facilitating interaction with the basic transcription machinery via transactivation,

but also by targeting specific octamer sequences. Mutagenesis and crystallization studies indicate that an A residue at the fifth position of the octamer element is critical for recognition by OCA-B. The crystal structure of the POU/OCA-B/DNA complex indicates that OCA-B makes contact with this residue (Cepek et al., 1996). Another, related mechanism that has been suggested to effect specificity is that OCA-B binding to promoter-bound OCT-1/2 could potentially be decided by the sequence immediately upstream of the octamer element (Tomilin et al., 2000; Remenyi et al., 2001). In this model, OCT proteins homo- or hetero-dimerize on the IgH promoter dependent on a heptamer and an octamer element separated by a few bases (Kemler et al., 1989; LeBowitz et al., 1989; Polleinger et al., 1989). According to this model, heptamer/octamer-containing promoters would not be activated by OCA-B. However, a heptamer/octamer-containing promoter is OCA-B-dependent as judged by in vitro transcription assays (Luo et al., 1992), indicating that this model may have limitations.

Yet another mechanism by which OCA-B promoter specificity can be obtained is indicated by the study of Casellas et al. which found that only some Vκ gene promoters require OCA-B for expression regardless of the type of octamer sequence they contain (Casellas et al., 2002). This study reopened investigation in to an 18bp element that lies downstream of the transcriptional start site because it is absent in the OCA-B

dependent Vκ promoter subset. It is speculated that this element, when present, could have a strong enough effect to make OCA-B dispensable for some Vκ promoters. In conclusion, it seems that OCA-B function at octamer-containing promoters is dictated by a combination of mechanisms and that this may be an important method by which the cell is able to tightly regulate immunoglobulin expression.

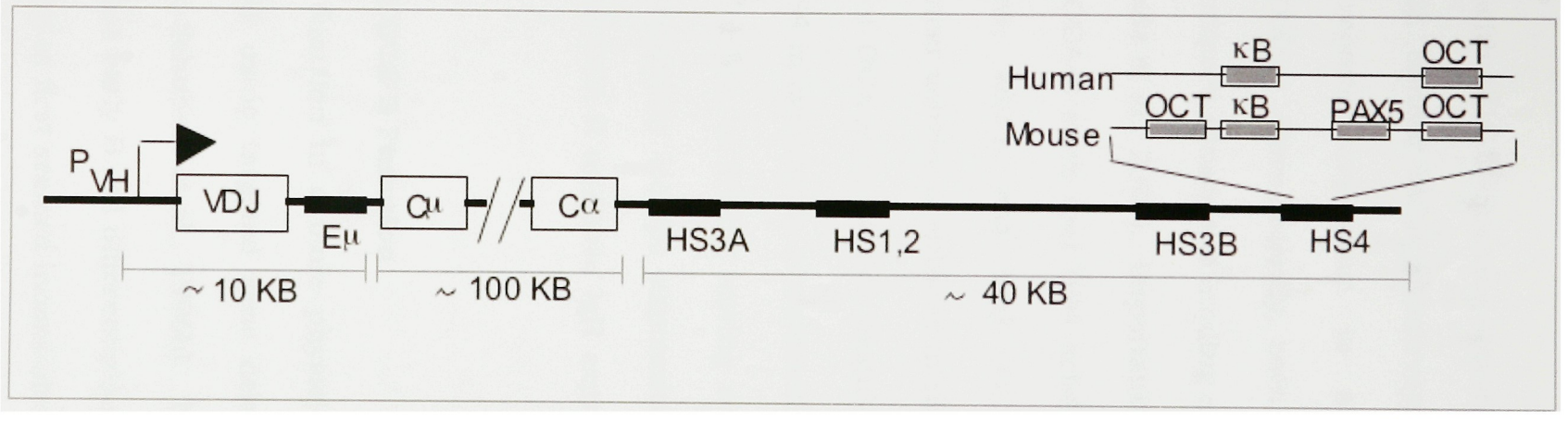
The Role of OCA-B in IgH Enhancer Function (Figure 1.2)

The IgH locus has two well-documented enhancers that, collectively, contain binding sites for NFκ-B, E2A, PAX-5, ETS proteins, and OCA-B/OCTs. The first enhancer (Eμ) is intronic and lies between the 3'-most V(D)J segment and the Cμ region. The contribution of this enhancer to immunoglobulin expression has been well characterized and it contains several consensus octamer elements (Neuberger, 1983; Tsao et al., 1988; Jenuwein and Grosschedl, 1991). The second IgH enhancer that lies 15kb downstream of the Cα region spans 40kb and is comprised of multiple hypersensitive sites (four in mice, three in humans) (reviewed in Khamlichi *et al.*, 2000). In mice the 3' enhancer elements are HS3a, HS1,2, HS3b, and HS4; which all contain consensus octamer elements (Matthias and Baltimore, 1993, Pettersson et al., 1990; Lieberson et al., 1991; Madisen and Groudine, 1994; reviewed in Khamlichi *et al.*, 2000). Originally, the HS elements of the 3' enhancer were thought to function separately; however, their synergism is required to achieve strong

promoter activation (Ong *et al.*, 1998). Although synergism between Eμ and the 3' enhancer has been demonstrated in vitro, the two enhancers are distinctly developmentally regulated in vivo (Ong et al., 1998; reviewed in Arulampalan et al., 1997). Eμ shows a constant level of activity throughout B cell development and is required for V_H gene arrangement and expression. Conversely, the activity of the 3' enhancer increases with B cell maturity and is required for the germline transcription that proceeds isotype switching (Stevens et al., 2000, reviewed in Arulampalam et al., 1997). Furthermore the 3' enhancer is also required for the expression of switched isotypes. For example, secondary isotype expression (IgA) is severely reduced in a plasma cell line that has lost the 3' enhancer (reviewed in Cogne et al., 2003). Studies on the individual contribution of the 3' enhancer elements show that HS3 and HS1,2 are more active in mature cells, whereas HS4 is active throughout B cell development. However, all of the elements contribute synergistically to the collective activity of the 3' enhancer (Michaelson et al., 1991; Chauveau et al., 1998; Chauveau et al., 1999; Anderson et al., 1999; Ong et al., 1998; Stevens et al., 1999).

OCA-B is likely to play a critical role in secondary isotype expression by functioning at immunoglobulin enhancers. While Eμ activity is less dependent on OCA-B, the 3' enhancer requires OCA-B for full activity,

Figure 1.2 Summary of the Immunoglobulin Heavy Chain Locus.



Black boxes indicate enhancer elements. Gray boxes indicate transcriptional regulatory elements that are suspected to be functional within HS4 (see text). Not to scale.

See text for details.

which is increased with OCA-B inducing signals such as CD40 and IL-4 (Stevens et al., 2000). Similar findings were reported in a study using an HS1,2-containing reporter transgene in an *Oca-b*$^{-/-}$ background (Anderson et al., 2000). More recently, both the murine and human HS4 were shown to require synergistic binding of NFκ-B and OCT-1/2 for full activity (Sepulveda et al., 2004). Importantly, HS4 is affected by the expression level of OCA-B such that HS4 activity is high in a Burkitt's lymphoma cell line, which has high OCA-B/OCT-2 expression. Conversely, 3' enhancer activity is reduced in plasma cell lines that have limited expression of OCA-B or OCT-2 (Sepulveda et al., 2004). The important role of HS4 in expression of switched isotypes is evidenced by targeted deletion of HS3a or HS1,2 having no effect on class switch recombination or secondary isotype expression (Manis et al., 1998). Thus, it is likely that OCA-B mediates IgH expression via HS4 or the 3' enhancer.

Genetic Analysis of OCA-B Function

To analyze OCA-B function in a more physiological setting, knockout mice were generated using targeted gene deletion (Kim et al., 1996; Nielsen et al., 1996; Schubart et al., 1996b). The phenotype of *Oca-b*$^{-/-}$ mice is striking in that early B cell differentiation is largely normal, as is IgM expression, which at first seemed inconsistent with established OCA-B function *in vitro*. However, normal IgM levels, is in accordance with

Oca-b$^{-/-}$ mice having largely unaffected antigen-independent phases of B cell development. As mentioned previously, an additional OCA-B-like activity has been identified that may prove to be responsible for V_H promoter activation in early B cells (Fujii et al., unpublished data). The most notable features of *Oca-b*$^{-/-}$ mice are the complete absence of germinal centers (reviewed in Maclennan, 1994, Ma and Staudt, 2001) and the severely reduced serum levels of secondary isotypes (IgA, IgG1, IgG2a, IgG2b, IgG3, and IgE), indicating that OCA-B is more important for later, antigen-dependent B cell development. Secondary isotype switching occurs normally in these mice but transcription of switched isotypes is impaired, which supports a role for OCA-B in heavy-chain transcription (Kim et al., 1996). As mentioned previously, the lack of switched isotypes in *Oca-b*$^{-/-}$ mice could be linked to OCA-B function at HS4 of the IgH 3' enhancer (Sepulveda et al., 2005). Additionally, *Oca-b*$^{-/-}$ B cells have reduced Calcium-flux and resulting proliferation in response to B cell receptor (BCR) cross-linking (Kim *et al.*, 1996, Schubart et al., 1996; Nielsen et al., 1996; Kim et al., 2000). Consistent with *Oca-b*$^{-/-}$ mice having normal T cell development and function, reconstitution experiments show that the defects of *Oca-b*$^{-/-}$ mice are B cell autonomous (Qin et al., 1998). Recently, a slight defect in early B cell differentiation, a reduced number of cells in transition from the bone marrow to spleen ($B220^{+}IgM^{hi}$) resulting from increased apoptosis, has been reported in *Oca-b*$^{-/-}$ mice (Hess et al., 2001). This defect may be

attributed in part to an impaired ability of *Oca-b*$^{-/-}$ mice to activate a subset of Vκ promoters (Vκ4 and Vκ3-833) (Casellas et al., 2002). Furthermore, there is also a reduction of transitional B cells in the spleen that may be linked to the skewed Igκ repertoire and/or defective signaling in *Oca-b*$^{-/-}$ mice (Jankovic et al., 2003). Additionally, *Oca-b*$^{-/-}$ mice have a pronounced reduction in recirculating B cells of the bone marrow (Schubart et al., 1996; Hess et al., 2001), which further suggests that these mice are defective in multiple signaling mechanisms.

In addition to these studies using *Oca-b*$^{-/-}$ mice, several double knockouts have been generated to better understand OCA-B function in B cell signaling pathways. These studies are of particular importance because full transcription of immunoglobulin genes requires the function of multiple transcription factors. However, *Oct-2*$^{-/-}$*/Oca-b*$^{-/-}$ mice show defects similar to those seen in *Oca-b*$^{-/-}$ mice (Schubart *et al.*, 2001) suggesting that neither factor is absolutely necessary for heavy chain gene transcription. *Btk* and *Oca-b* double null mice display an enhancement of the *Btk*$^{-/-}$ phenotype, in that these mice completely lack peripheral B cells (Schubert et al., 2004), which suggests that the two proteins have overlapping functions and may both be involved in BCR signaling. *Nfκ-b1*$^{-/-}$*/Oca-b*$^{-/-}$ mice maintain the *Oca-b*$^{-/-}$ phenotype in addition to an interesting reduction in the B-1a cell population and have further compromised conventional B-2 cell differentiation, which results

from a complete absence of T-independent and T-dependent immune responses (Kim *et al.*, 2000). Additionally, these mice have reduced IgM and secondary isotype levels. This suggests that OCA-B and NFκ-B have partially overlapping functions, playing cooperative roles in IgM production and B1 cell differentiation. As mentioned previously, a recent study has shown NFκ-B and OCA-B cooperativity at HS4 of the immunoglobulin 3' enhancer (Sepulveda et al., 2004).

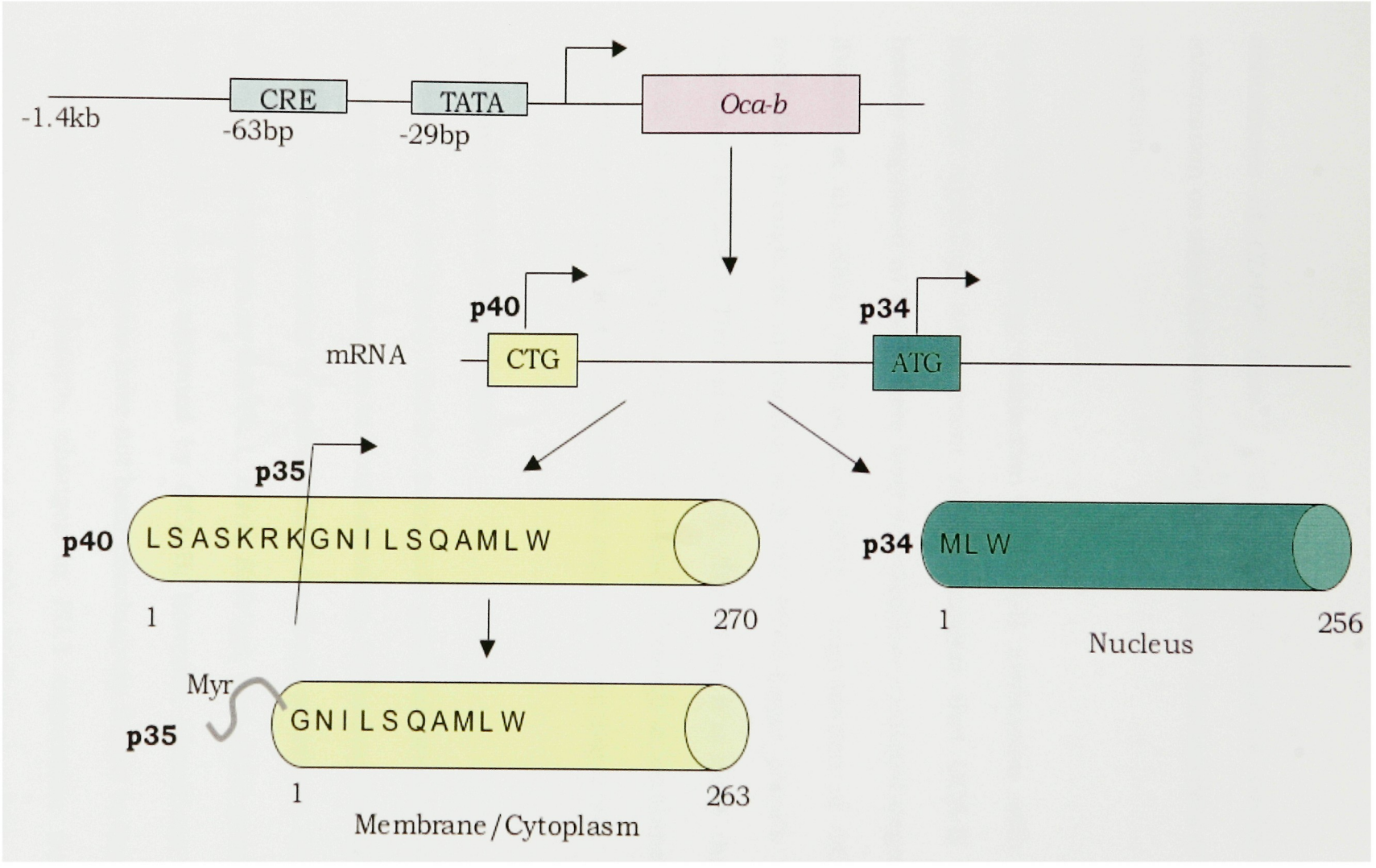
Regulation of OCA-B Expression (Figure 1.3)

Because most studies have focused on the function of OCA-B, the upstream signals mediating OCA-B expression are largely unknown despite that OCA-B expression is tightly controlled throughout B cell development and germinal center formation (Qin et al., 1998). Surprisingly, although OCA-B expression is normally restricted to B cells, its expression can be induced in T cells by stimulation with phorbol esters and ionomycin (Zwilling et al., 1997). However, due to the extreme method of stimulation used, the physiological relevance of OCA-B expression in T cells is unknown. However, these findings do suggest that OCA-B expression is differentially regulated by various signaling pathways such as those associated with BCR signaling and T cell help. Moreover, OCA-B can be induced in resting B cells by stimulation with LPS, anti-CD40 and IL-4 or BCR crosslinking (Qin et al., 1998). This finding was extended to show that in primary splenic B cells and

transformed B cell lines, OCA-B up-regulation by anti-CD40 and IL-4 is dependent on an intact CREB/ATF binding site (-64 to -57 BP) within its 1.4KB promoter region (Stevens et al., 2000). Interestingly, CREB sites are often targets of BCR-derived cell signaling (Xie and Rothstein, 1995).

Although induction of OCA-B is dependent on this site, the specific CD40-dependent pathway(s) mediating OCA-B induction is completely unresolved. This can be attributed to the magnitude and complexity of immune responses that are resultant of CD40-mediated signaling. The CD40-CD40L interaction on B cells results in the induction of many proteins other than OCA-B, such as those involved in the JNK/SAPK, NFκ-B, p38 protein kinase, and ERK pathways (reviewed in Grammer and Lipsky, 2000). *CD40-* and *CD40L*-deficient mice present defects in multiple aspects of the immune response such as T cell priming, dendritic cell migration, germinal center formation and secondary isotype switching (Kawabe et al., 1994; Xu et al., 1994). Both *CD40-* and *Oca-b*-deficient mice have a complete inability to form germinal centers and have severe defects in secondary isotype production. However, $CD40^{-/-}$ B cells do not undergo isotype switching, whereas $Oca\text{-}b^{-/-}$ B cells fail to express switched isotypes. Because there is some overlap between the $Oca\text{-}b^{-/-}$ and $CD40^{-/-}$ phenotypes, it would be useful to determine the extent that compromised OCA-B expression contributes to the

Figure 1.3 Transcription and Translation of OCA-B.



Transcription of *Oca-b* is dependent on a CRE site within its 1.4kb promoter region. OCA-B mRNA has two translation start sites from which the p40 and p34 isoforms are generated. P40 is then cleaved and myristoylated to become the p35 isoform. See text for details.

deficiencies of *CD40*$^{-/-}$ mice. This type of analysis could provide information on what CD40-mediated functions are downstream of OCA-B induction.

Despite the interesting connection of OCA-B expression with CD40-mediated signaling, more recent studies suggest that OCA-B is also heavily regulated at the protein level by ubiquitin-mediated degradation (Boehm et al., 2001; Tiedt et al., 2001). Degradation of OCA-B is mediated through its interaction with the RING-finger protein SIAH-1 (Boehm et al., 2001; Tiedt et al., 2001). Therefore, it is likely that tight control of OCA-B expression is maintained through a combination of transcriptional and post-transcriptional regulatory mechanisms.

Additional OCA-B Target Genes

As mentioned previously, *Oca-b*$^{-/-}$ mice lack germinal centers and have compromised responsiveness to B cell signaling, indicating that OCA-B is necessary for expression of genes critical for these processes. Octamer-containing promoters in the *Pu.1*, *Blr-1*, *B29/Igβ*, and *Bcl-2* genes have been reported to be activated by OCA-B; however, alterations in the expression of these genes have not been conclusively linked to the *Oca-b*$^{-/-}$ phenotype. For example, changes in PU.1 expression were not observed in *Oca-b*$^{-/-}$ B cells (Chen et al., 1996; Wolf et al., 1998; Malone and Wall 2002; Iotsova *et al.*, 1997; Kim et al., 2000). BLR-1 is a

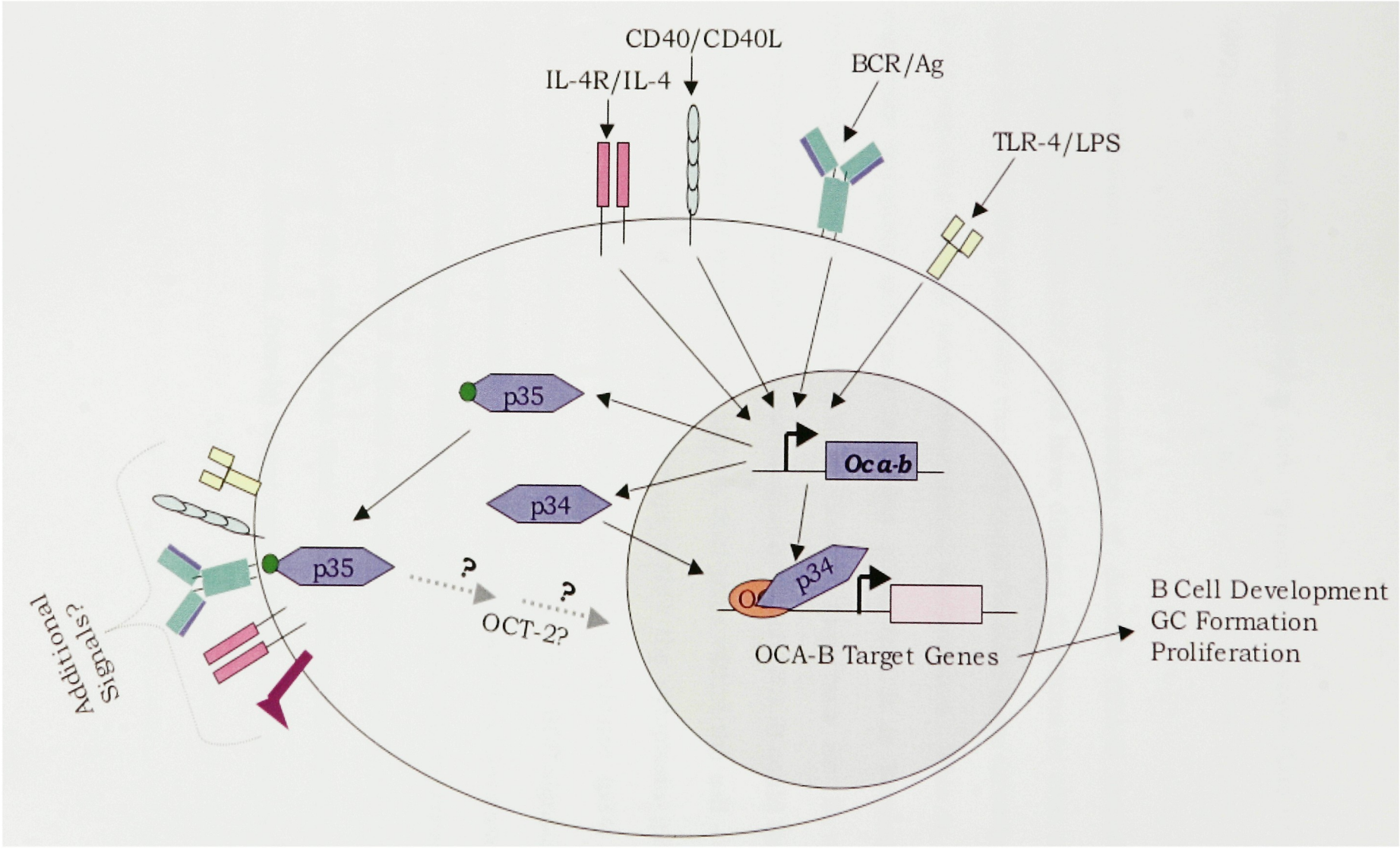
chemokine receptor that is involved in B cell homing and *Blr-1*$^{-/-}$ mice also lack germinal centers in some lymphoid organs (reviewed in Fu and Chaplin, 1999). However, the *Oca-b*$^{-/-}$ and *Blr-1*$^{-/-}$ phenotypes differ significantly in that *Blr-1*$^{-/-}$ mice can form germinal centers in the lymph node, have normal numbers of peripheral B cells, and have intact responsiveness to T-dependent antigens (Forster et al., 1996). Unlike *Oca-b*$^{-/-}$ mice, *Blr-1*$^{-/-}$ mice even develop ectopic germinal centers in the spleen upon immunization (Voigt et al., 2000). Therefore, reduction of BLR-1 expression does not fully account for the immune defects of *Oca-b*$^{-/-}$ mice. Transient transfection assays indicate that Igβ may also be an OCA-B target gene (Malone and Wall, 2002). Although it remains possible that reduced expression of Igβ contributes to the *Oca-b*$^{-/-}$ phenotype, further investigation is needed to determine if it is a direct OCA-B target gene in vivo. *Bcl-2* is an OCA-B target gene only in certain B cell subsets such as pre-B cells (Brunner et al., 2003). Furthermore, overexpression of BCL-2 in *Oca-b*$^{-/-}$ mice restores the transitional B cell defect in bone marrow but does not alter the mature B cell phenotype (Brunner et al., 2003). Similarly, removal of CD22, a negative regulator of BCR signaling, in *Oca-b*$^{-/-}$ mice only rescues the transitional B cell defect (Samardzic et al., 2002). Also, CD22 surface expression is increased in *Oca-b*$^{-/-}$ transitional B cells, which supports the view that OCA-B has an indirect role in CD22 expression. Thus, further study to identify

additional OCA-B target genes is necessary to understand OCA-B function in B cell differentiation and function.

The Role of OCA-B in Signaling (Figure 1.4)

Given that impairment of germinal center formation in OCA-B deficient mice is most likely attributed to a signaling defect one must consider new roles for this protein *in vivo*. Originally, OCA-B was purified as two isoforms, p35 and p34, thought to reflect protein modifications; however, further investigation revealed an additional isoform, p40, that is present at very low levels and unstable in B cells (Yu et al., 2001). Interestingly, p40 and p34 arise from different translational start sites on a common mRNA (Yu et al., 2001). After translation, p40 is rapidly processed into p35 and myristoylated at the N-terminus. Transfection assays with the two isoforms (p34 and p35) indicate that the two proteins have distinct subcellular localization patterns (Yu et al., 2001). P35 is more prominent in the membrane/cytoplasm, whereas p34 is more heavily localized within the nucleus. Moreover, the localization of p35 is dependent on myristoylation because mutation of the myristoylation site results in complete nuclear localization. Of note, some p35 is also found in the nucleus and, reciprocally a small amount of p34 is found in the cytoplasm; however, it is unknown if this is functionally significant (Yu et al., unpublished data). Possibly related, p34 and p35 can both act as

Figure 1.4 A Model for OCA-B Function in B Cell Signaling Pathways



Transcription of OCA-B is induced by B cell activating stimuli. P34 regulates target gene expression within the nucleus and p35 receives additional receptor-derived signals. Thus, OCA-B may function in proximal and distal signaling events. See text for details.

transcriptional coactivators by in vitro transcription assays (Luo et al., 1992).

The identification and characterization of distinct OCA-B isoforms suggests that p34 and p35 could have different functions in vivo and raises the interesting possibility that p35 could function more directly in B cell signaling through non-transcriptional mechanisms. This is an especially intriguing and valid possibility given that it is becoming increasingly obvious that OCA-B is broadly important for B cell development and function. One mechanism by which OCA-B could have a more widespread impact is by playing dual roles within the cell – by functioning directly in signaling pathways within the cytoplasm (p35) and by acting as a conventional transcriptional coactivator (p34) to regulate target gene expression. A direct role for OCA-B in BCR signaling is supported by the multitude of B cell subsets that are developmentally or functionally affected by *Oca-b* deficiency. Validation of this hypothesis would require a significant amount of investigation because not only would one need to demonstrate that OCA-B functions directly in signaling but additional (non-Ig) target genes would also need to be identified.

OCA-B Expression in B Cell Lymphomas

The possibility that OCA-B contributes to oncogenic transformation was first indicated by the finding that OCA-B expression could be controlled in a temperature sensitive Abelson virus-transformed pre-B cell line, such that expression of v-ABL causes an increase in OCA-B expression (Qin et al., 1998). Similarly, the OCA-B level is elevated in many transformed B cell lines, irrespective of developmental stage, whereas its expression is tightly controlled throughout normal B cell development. Pathological studies of human tumor samples and cell lines have shown that OCA-B expression is compromised in the classical form of Hodgkin's lymphoma (Stein et al., 2001; Theil et al., 2001; Hertel et al., 2002). Hodgkin's Reed Sternberg (HRS) cells (classical Hodgkin's tumor cells) are thought to be crippled GC B cells that, through a complex mechanism, are able to evade apoptosis despite the lack of a functional BCR (reviewed in Kuppers, 2002). Recent studies have demonstrated that HRS cells lack expression of important B cell markers such as Igβ, TCL-1, IgH, IgL, OCA-B/OCTs due to epigenetic silencing (Ushmorov et al., 2004; Doerr et al., 2005). Importantly, ectopic expression of OCA-B and OCT-2 in HRS cell lines can partially restore immunoglobulin expression (Hertel et al., 2000), which not only indicates that OCA-B plays a role in regulating immunoglobulin gene expression, but also suggests that reduced OCA-B expression may be involved in the transformation of these cells.

Conversely, OCA-B expression is up-regulated in certain germinal center-derived B cell lymphomas such as Burkitt's lymphoma, follicular lymphoma, and diffuse large B cell lymphoma (Greiner et al., 2000). However, in these studies there is no comparison of the protein levels seen in the lymphoma samples versus their non-malignant counterparts, making it difficult to determine if there is actually up-regulation of OCA-B protein levels relative to normal germinal center B cells.

The stage has been set to consider a possible role for OCA-B in oncogenesis; however, there is little published data that distinguishes whether OCA-B expression levels contribute either to the initial transforming events or to the maintenance of tumorigenicity or whether its alteration is merely a by-product of transformation. However, a very recent study demonstrates increased OCT-2 and OCA-B expression in follicular lymphomas bearing a translocation of the *Bcl-2* gene to the immunoglobulin locus, t(14;18) (Heckman et al., 2005). Interestingly, siRNA of OCT-2 or OCA-B results in a significant reduction of BCL-2 expression and increased apoptosis of the infected cells (Heckman et al., 2005). This study indicates that OCA-B may be required for cell survival thus contributing directly to malignant transformation.

At the onset of this project, OCA-B appeared to be broadly important for B cell maturation and function through largely unknown mechanisms. The phenotype of *Oca-b*$^{-/-}$ mice, although well characterized, is somewhat surprising because IgM transcription is largely intact. However, mature B cell differentiation is severely defective, which highlighted the need to identify additional (non-Ig) OCA-B target genes. Equally important, was the need to examine the role that OCA-B might play in B cell signaling, especially given that the myristoylated-p35 isoform had recently been identified. It was the hope that information obtained from both of these approaches could be combined to explain the mechanism by which OCA-B functions in B cells.

CHAPTER 2

IDENTIFICATION OF OCA-B TARGET GENES INVOLVED IN ANTIGEN-DEPENDENT B CELL DIFFERENTIATION

2.1 PREFACE: ADDITIONAL OCA-B TARGET GENES?

Oca-b$^{-/-}$ mice have the striking inability to form germinal centers and B cells from these mice show diminished proliferation in response to B cell receptor (BCR) ligation (Kim et al., 1996; Schubart et al., 1996; Nielsen et al., 1996), indicating that OCA-B regulates additional target genes that function in B cell signaling pathways leading to proliferation and germinal center formation. Consistent with a role for OCA-B in mature B cell function, the level of OCA-B is tightly regulated during B cell activation and germinal center formation, both transcriptionally and post-transcriptionally (Qin et al., 1998; Stevens et al., 2000; Tiedt et al., 2001; Boehm et al., 2001). Apart from Ig promoters and the IgH 3' enhancer, the octamer-containing promoters in the BLR-1, PU.1, and Igβ/B29 genes were reported to be activated by OCA-B in transient transfection assays (Chen et al., 1996; Wolf et al., 1998; Malone and Wall, 2002). However, reduced expression of these genes does not fully account for the immune defects observed in *Oca-b*$^{-/-}$ mice. Therefore, identification of additional OCA-B target genes was expected to reveal genes that play critical roles in antigen-dependent B cell differentiation events including the germinal center formation. This portion of the study (done in collaboration) used cDNA microarrays to identify genes that are up-regulated upon stimulation of normal resting splenic B cells through the BCR alone or through BCR and helper T cell (Th) co-stimulation.

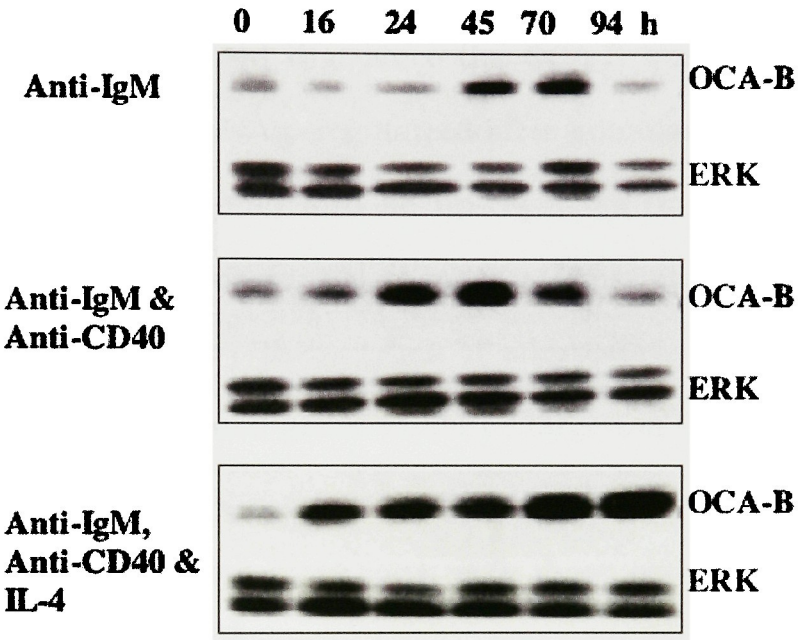
Additional studies then identified genes that are under-stimulated in *Oca-b*$^{-/-}$ B cells in response to these signals.

2.2 RESULTS

OCA-B Expression Profile During B Cell Activation

As mentioned previously, OCA-B is up-regulated in activated B cells (Qin et al., 1998). To characterize OCA-B induction further, we activated resting splenic B cells for up to 94 hours using BCR ligation or co-stimulation. OCA-B expression levels were analyzed in cells stimulated with anti-IgM alone or with a combination of anti-IgM, anti-CD40, and IL-4 for 16, 24, 45, 70, and 94 hours by immunoblot (Figure 2.2.1). OCA-B levels were highly up-regulated after 45 hours of stimulation with anti-IgM. During co-stimulation, the OCA-B level is up-regulated faster and to a greater degree than for anti-IgM stimulation. When stimulated with BCR alone, the OCA-B level decreased to that of the resting state by the 94 h point. However, the OCA-B level persisted and increased through the late time points during BCR/Th co-stimulation. These results indicate that OCA-B expression is differentially regulated by BCR alone and co-stimulation, and that OCA-B is likely to play a role in both early and late stages of antigen-dependent B cell development. The following cDNA array studies focused on time points at which OCA-B level was high.

Figure 2.2.1 Immunoblot Analysis of OCA-B Expression



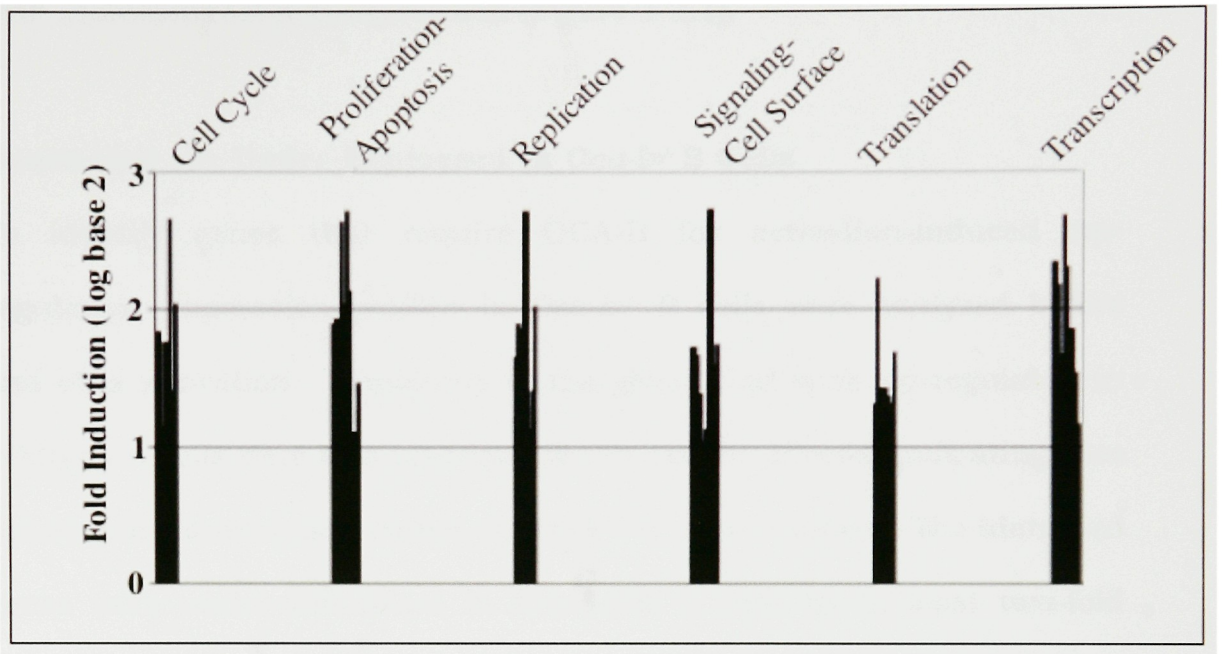
Resting B cells were stimulated with anti-IgM, anti-IgM and anti-CD40 or anti-IgM, anti-CD40, and IL-4. Whole cell extracts were made at the indicated time points and the expression of OCA-B was monitored by immunoblotting. ERK expression was monitored for normalization.

Gene Expression Profiles in Response to Signaling

After determining conditions for the optimal up-regulation of OCA-B, this information was used to analyze gene expression profiles through cDNA microarrays. We reasoned that since the OCA-B level was very low in resting B cells and highly up-regulateed after stimulation (Figure 2.2.1), OCA-B target genes should also be up-regulated in a similar pattern. However, there are a substantial number of other transcription factors that are up-regulated by these types of stimulation. Therefore, only a fraction of the up-regulated genes would be targets of OCA-B. In order to obtain an appropriate data set, we reasoned that when *Oca-b*$^{-/-}$ cells are stimulated with the same conditions, only those pathways that require OCA-B would be under-stimulated, whereas OCA-B independent pathways would be preserved. Thus a comparison of up-regulated genes in wildtype (activated/resting) and *Oca-b*$^{-/-}$ (activated/resting) was expected to result in the identification of genes that require OCA-B for expression. This approach has an advantage over a direct comparison of gene expression profiles (activated wildtype/activated *Oca-b*$^{-/-}$) because any populational and developmental differences in gene expression patterns between the two genotypes would be normalized. Thus, any differences that we identify in gene expression profiles would represent differences caused by the absence of OCA-B.

We analyzed the gene expression profile of wild-type B cells after 45h of BCR stimulation (high OCA-B expression). This analysis showed 180 up-regulated genes and 80 down-regulated genes by 2 fold or greater. We then wanted to identify genes that were up-regulated by at least 2 fold in the BCR stimulated wildtype cells (Figure 2.2.2). Of this class, many genes were involved in cell cycle, proliferation, signaling, and transcription (Figure 2.2.2). This indicates that the length of stimulus was appropriate and that the cells are still proliferating. Based on previous reports, the data is consistent with a B cell activation phenotype. The genes up-regulated with BCR stimulation included *cyclin D2* and *CD44* (Figure 2.2.2), which have been identified as B cell activation markers in published microarray analyses (Alizadeh et al., 2000; Glynne et al., 2000; Shaffer et al., 2001). Furthermore, many of the identified genes have previously been found to be induced in stimulated B cells using various techniques, including the small inducible cytokine subfamily A member 22 (*Abcd-1*) (Schaniel et al., 1998), neurogranin (Glynne et al., 2000), *Lck* (Glynne et al., 2000; Taieb et al., 1993), pre-B cell colony enhancing factor (*Pebf*) (Dadgostar et al., 2002), *Ki67* (Dadgostar et al., 2002), *Ezh2* (Raaphorst et al., 2000) and transferrin receptor (Kvaloy et al., 1984). These observations suggest that the B cell activation system used for these experiments is physiologically meaningful and that the cDNA microarray analyses have the potential to give valid OCA-B target genes. For the comparison of

Figure 2.2.2 Genes Induced by BCR Stimulation



Cyclin D3	Bcl212	Npm1	**Lck**	S6 kinase	Hp1a
Acdp1	CD27bp	Mcm4	**S100a10**	L12	**DbpA**
Gspt1	Lek1	Rpa2	Ppp1r14b	L7	Trim28
Cdc20	Stathmin	H2A.1	Ppp2ca	Fibrillarin	Ezh2
Cdc37	Ki 67	Primase 1	Cacybp	eIF-4A	Hmg-2
Cks1	Transf R	dUTPase	Neurogran	eIF-4E	Hmg-Y
	Odc	Pcna	Ywhae	eIF-2A	Paf67
	Srm		CD44		Rpa40
			Kcnn4		

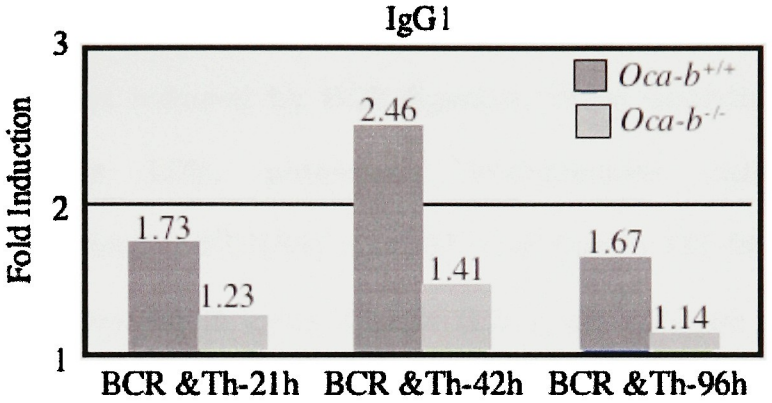
Genes whose expression level increased at least 2 fold after 45h of BCR stimulation were identified using cDNA array analyses and grouped according to function. OCA-B target genes are shown in boldface.

WT vs. *Oca-b*$^{-/-}$ cells, we analyzed gene expression profiles from 45h of BCR stimulation and 21h, 45h, and 92h of co-stimulation, coincident with increasing OCA-B expression (Figure 2.2.1).

Genes that are Under-Expressed in *Oca-b*$^{-/-}$ B Cells

To identify genes that require OCA-B for activation-induced up-regulation, expression profiles in *Oca-b*$^{-/-}$ B cells were analyzed before and after activation. A majority of the genes that were up-regulated in wildtype B cells were also up-regulated in *Oca-b*$^{-/-}$ B cells, indicating that the overall transcription status of *Oca-b*$^{-/-}$ B cells is intact. The identified genes were under-stimulated in knockout B cells by at least two-fold after activation in three replicate experiments and some of these genes are shown in bold of Figure 2.2.2. Thus, OCA-B appears to act on a restricted set of genes that play critical roles in B cell activation and differentiation (Table 2.2.1, first two columns). Also, the phenotype of *Oca-b*$^{-/-}$ mice was reflected in these experiments, in that the array data revealed a reduction of IgG1 expression in the knockout B cells upon activation (Figure 2.2.3). Furthermore, the array data was validated by both quantitative PCR (QPCR) and Northern blotting, indicating a false positive rate of 5%. OCA-B dependent genes were confirmed using both of these methods (Table 2.2.1). In general, the levels of induction observed with the Northern and QPCR were greater than those observed in the cDNA array analysis (Table 2.2.1). This tendency has been

Figure 2.2.3 IgG1 Expression after Co-stimulation is Reduced in Oca-b$^{-/-}$ B Cells

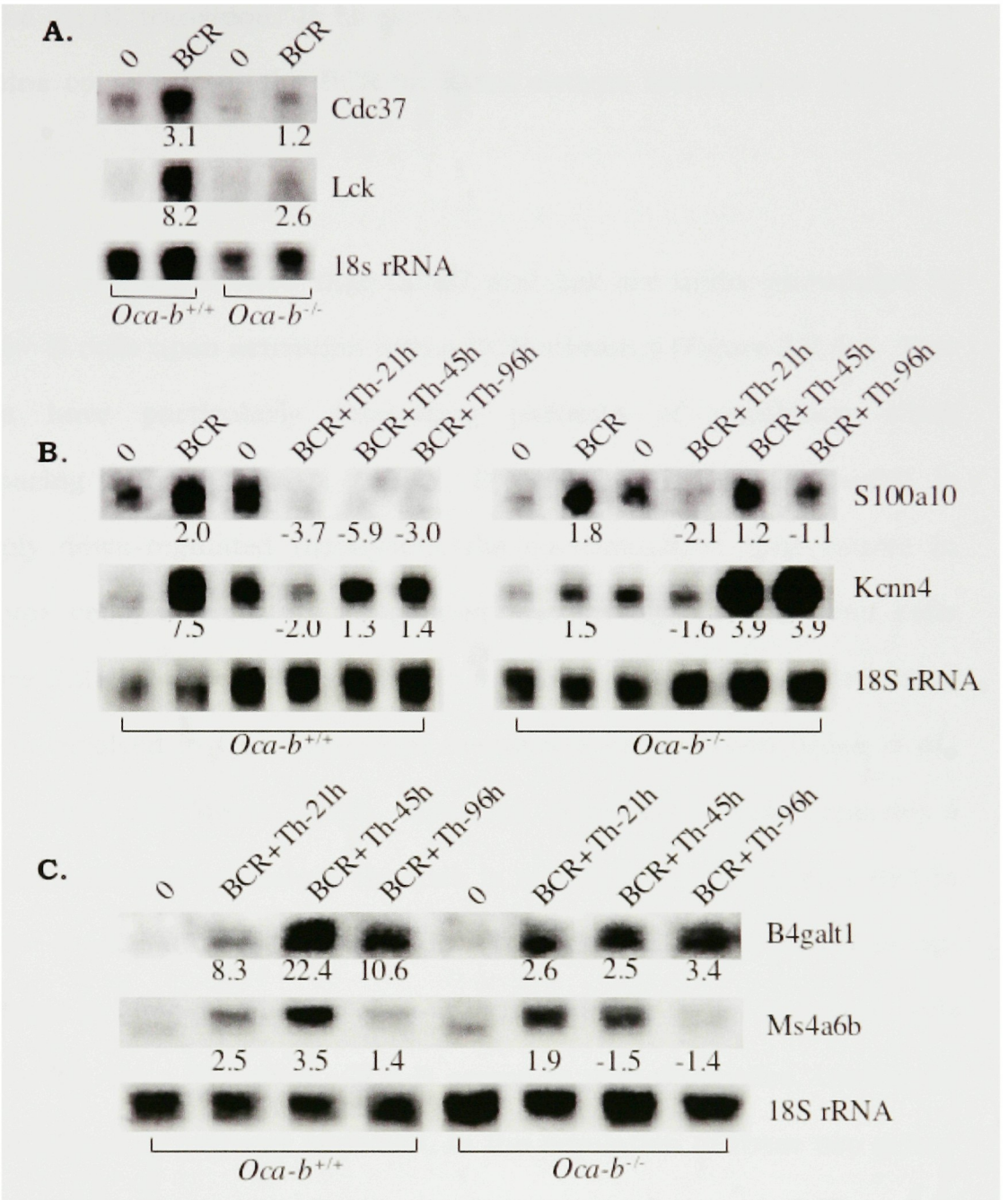


Induction of IgG1 (AI843944) expression upon co-stimulation in *Oca-b*$^{+/+}$ and *Oca-b*$^{-/-}$ cells was determined by cDNA array analyses. The average fold induction levels from five replicate experiments are shown.

reported before and may reflect the differences in kinetics of hybridization between solid-state- and solution-based reaction conditions (Taniguchi et al., 2001; Wurmbach et al., 2001).

Among the genes induced by BCR ligation, those encoding the protein tyrosine kinase LCK, potassium intermediate calcium-activated conductance channel (KCNN4), CDC37 and Cyclin D3 failed to be up-regulated in *Oca-b*$^{-/-}$ B cells (Table 2.2.1 and Figure 2.2.4). The mammalian homolog of yeast CDC37 is a molecular chaperone that targets HSP90 to cyclin-dependent kinase 4, making it a key regulator of cell cycle progression (Chen *et al.*, 2002). LCK is a tyrosine kinase originally identified in T cells but has also been implicated in B cell proliferation and lymphomagenesis (Jucker *et al.*, 1991; Allen *et al.*, 1992; Wildin *et al.*, 1995; Von Knethen *et al.*, 1997). Interestingly, the *Lck* gene contains two promoters that are used in a developmental- and cell-type-specific manner: a proximal promoter that is active in thymocytes and a distal promoter that is active in mature T and B cells (Wildin et al., 1995). Multiple studies have shown that the potassium channel KCNN4 is up-regulated in activated T cells and is required for sustained T cell activation by promoting Ca^{2+} influx (Khanna et al., 1999; Ghanshani et al., 2000). Cyclin D3 has been shown to be up-regulated in activated B cells (Solvason et al., 1996) and functions as a positive regulator of cyclin-dependent kinases 4 or 6, whose activity is required

Figure 2.2.4 Analysis of OCA-B-dependent Gene Expression by Northern Blot



A. Expression of the OCA-B target genes were analyzed in cells that had been stimulated with anti-IgM for 45 hours. B. Expression of the OCA-B target genes were analyzed in cells that had been stimulated with anti-IgM for 45h or anti-IgM, anti-CD40, and IL-4 for up to 96 hours. C. Expression of the OCA-B target genes were analyzed in cells that had been activated with co-stimulation for up to 96 hours. The values shown below the blots are normalized fold changes relative to the resting state (0). Target gene levels were normalized against 18S rRNA. A negative fold change denotes down-regulation.

for the G_1/S transition. It is expected that the genes encoding these proteins contribute to the BCR-mediated defects observed in *Oca-b*$^{-/-}$ B cells.

Northern blotting showed that *Cdc37* and *Lck* are under-stimulated in *Oca-b*$^{-/-}$ B cells upon activation with a BCR stimulus (Figure 2.2.4a). Two genes have particularly interesting patterns of regulation when comparing wildtype versus *Oca-b*$^{-/-}$ B cells. *S100a10* (calpactin) is sharply down-regulated throughout the co-stimulation time course in wildtype cells; whereas it is regulated less strongly in knockout cells (Figure 2.2.4b). The EF-hand family member, S100A10, is a structural protein involved in Ca^{2+} dependent membrane organization (Ruse *et al.*, 2001; Gerke and Mosse, 2002). Also, the *Kcnn4* gene, which encodes a calcium-activated potassium channel, is significantly under-regulated in *Oca-b*$^{-/-}$ B cells after BCR activation. In sharp contrast, *Kcnn4* is up-regulated at later time points of co-stimulation only in the knockout cells (Figure 2.2.4b). Therefore, OCA-B seems to play dual roles, contingent on the signaling pathway activated, in the regulation of these two genes. Namely, OCA-B is required both for their up-regulation by BCR and for their down-regulation by Th co-stimulation (see Discussion). In addition to Ig genes, this work has identified other OCA-B-dependent genes that are induced by co-stimulation signals (Table 2.2.1 and Figure 2.2.4). These include β1,4-galactosyltransferase (*B4galt1*), which is involved in

glycosylation of many immunologically important molecules such as IgG (Rudd et al. 2001), and a recently identified CD20-related transmembrane protein termed membrane-spanning 4-domains subfamily A member 11 (*Ms4a11* or *Ms4a6b*) (Ishibashi *et al.*, 2001; Liang and Tedder, 2001). These genes showed significantly reduced induction by co-stimulation in *Oca-b*$^{-/-}$ B cells and are likely to be involved in critical T-dependent B cell functions that are defective in *Oca-b*$^{-/-}$ mice.

Direct Regulation of OCA-B Target Genes

Some of the OCA-B-dependent genes identified by these experiments are likely to contain binding sites for an OCT/OCA-B complex in their promoters and/or enhancers and, thus, directly require OCA-B for full transcriptional activity. Others might be downstream, making them indirect targets of OCA-B. Indeed, database searches using TESS (transcription element search software www.cbil.upenn.edu/tess) indicated that some of these genes have upstream octamer elements, including both the distal promoter of *Lck* and the predicted promoter region of *Kcnn4* contain (Figure 2.2.5a). We analyzed the occupancy of these sites by OCT and OCA-B in the murine lymphoblastic B cell line A20 by chromatin immunoprecipitation assays. OCA-B and OCT-2 were found to bind specific octamer sites on the *Lck* (-396) and *Kcnn4* (-2932) promoter regions (Figure 2.2.5b). To verify this finding we also performed

Table 2.2.1 Verification Table to Summarize the Fold Induction of OCA-B Target Genes

		Oca-b $^{+/+}$			*Oca-b* $^{-/-}$		
Accession	Name	Array	Northern	QPCR	Array	Northern	QPCR
AI528742	*Cdc37*	2.49	3.14	15.3	1.09	1.22	0
AI413778	*Kcnn4*	3.3	7.57	34.2	1.27	1.51	1.2
AI573454	*Lck*	2.58	8.17	13.9	1.34	2.62	1.1
AI323871	*Cyclin D3*	2.23	nd	5.3	1.14	nd	0.8
AI506208	*s100a10*	2.68	2.02	5.36	1.29	1.43	0

		Oca-b $^{+/+}$		*Oca-b* $^{-/-}$	
Accession	Name	Array	Northern	Array	Northern
AI481551	*B4galt1*	3.38	22.4	1.07	3.49
AI835093	*Ms4a11*	2.55	3.45	1.32	1.53

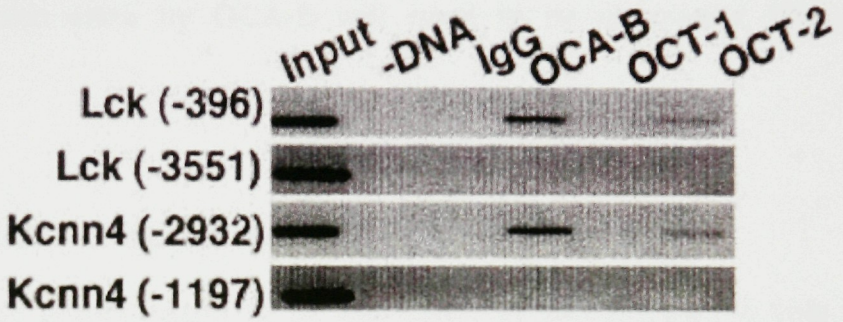
Northern blotting and QPCR were used to verify the data set obtained through cDNA array analyses. nd, not determined

Figure 2.2.5 Identification and Occupancy of Octamer Elements within OCA-B Target Gene Promoter Regions

A.

Lck
TAGCAAAT (-396) ATTTGCAT (-3551)
Kcnn4
ATTTGAAT (-2932*) ACTTCCAT (-1197*)

B.



C.

Name	Region Analyzed	Ocatamers	Position	5th Residue
S100a10	1 to -3437	ATGATAAT	-1855	not conserved
		ATGATAAT	-3178	not conserved
		TTAAATCA	-3229	not conserved
CyclinD3	1 to -2432	ATGATAAT	-1297	not conserved
		ACTTTGAT	-1425	conserved
		CTTTACAT	-1449	conserved
Cdc37	1 to -3636	ATGTAAAA	-2937	conserved
B4galt1	1 to -3971	ATGCTAAT	-2000	not conserved
Ms4a6b	1 to -3852	TAAAATTC	-755	not conserved

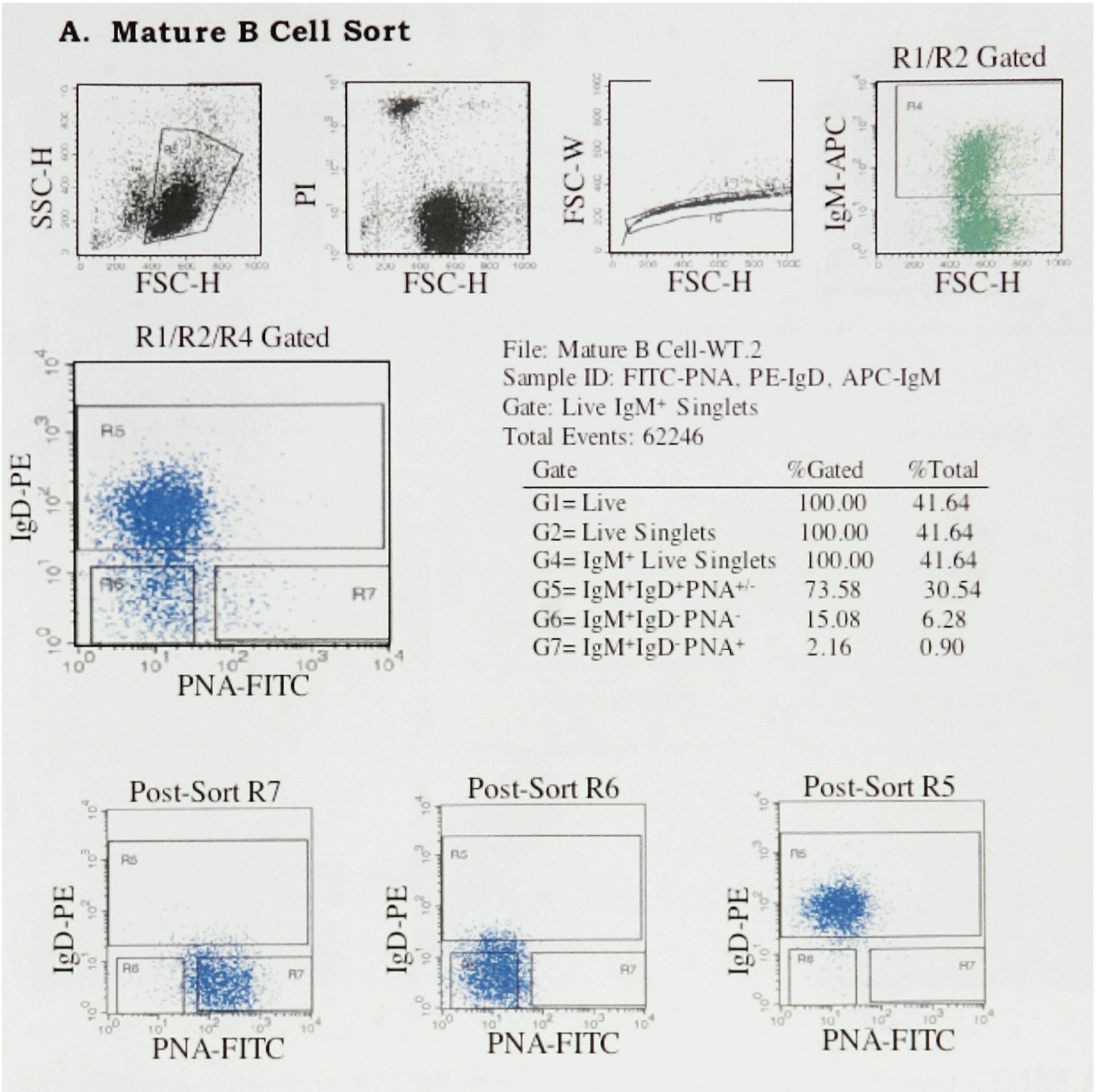
A. The octamer elements identified on the *Lck*- and *Kcnn4*-promoter regions and their positions relative to the transcriptional start site are shown. The A/T residue that is critical for OCA-B binding is underlined. The transcriptional start site for *Kcnn4* is estimated based on the EST clone and mouse genome alignment (www.ncbi.nlm.nih.gov). B. Chromatin immunoprecipitation assays were performed using the nuclei isolated from A20 cells. C. Identification of octamer elements within the putative regulatory regions of other OCA-B target genes.

the experiments in primary B cells freshly isolated by FACS (data not shown). The occupancy of these sites by OCT-2 and OCA-B was confirmed (data not shown). Octamer elements were located within the predicted promoter and enhancer regions of several of the other OCA-B target genes identified by the microarray analysis; however, the occupancy of these sites by OCA-B will need to be examined (Figure 2.2.5c).

Expression Patterns of OCA-B Target Genes

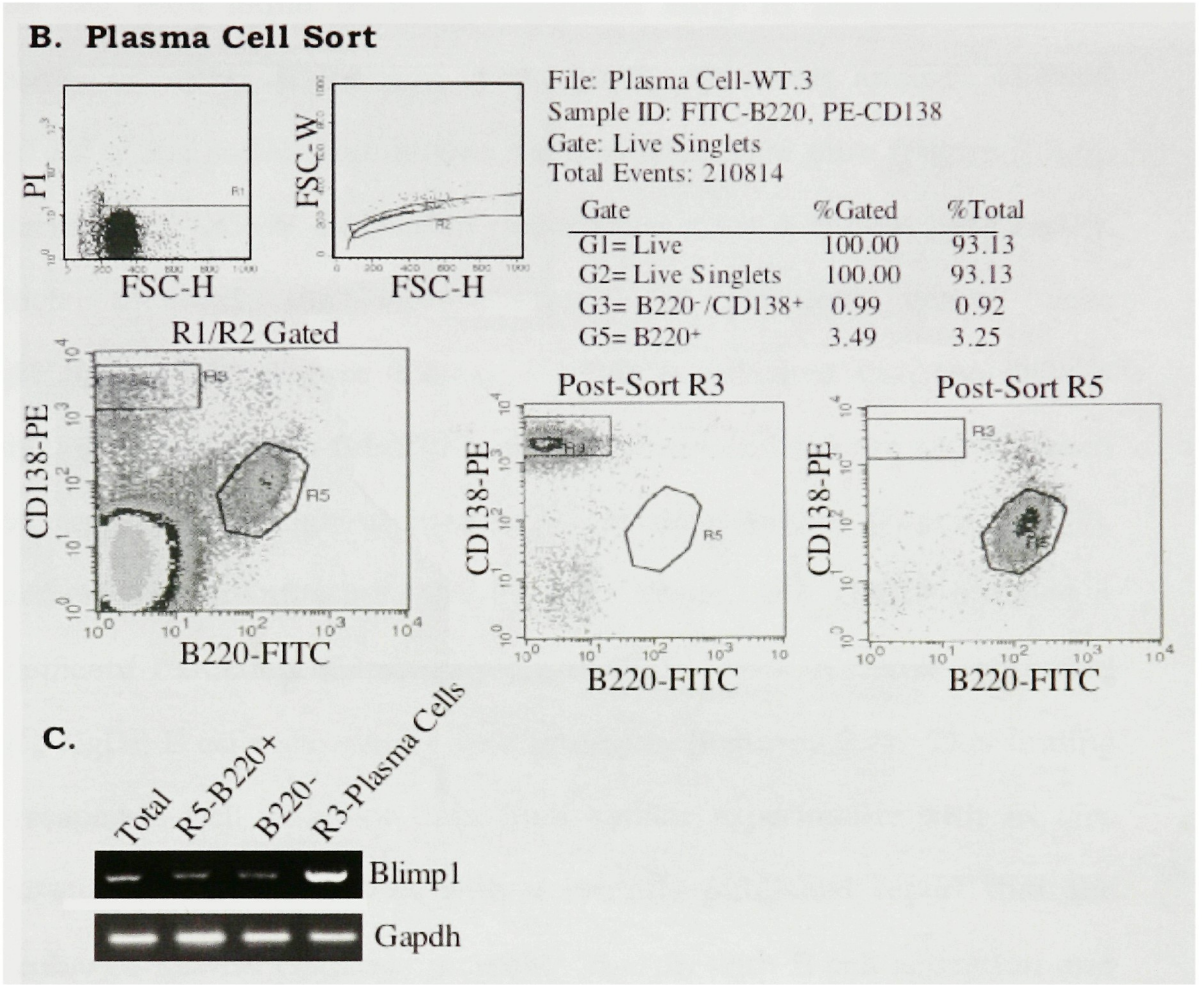
Since many of the genes found to be regulated by OCA-B play a role in cellular proliferation and signaling, they may be candidates to explain the severe defects observed in *Oca-b*$^{-/-}$ mice. However, the B cell-specific functions of these target genes such as *Kcnn4*, *Lck*, *Cdc37* and *Cyclin D3* are relatively uncharacterized. The first step toward understanding how the regulation of these genes may contribute to the *Oca-b*$^{-/-}$ phenotype was to profile their expression patterns in sorted primary B cells (this also will ensure that cells are live and free of contaminating populations) (Figure 2.2.6). Primary B cells at various stages of development were collected by FACS from spleens of wildtype mice. Germinal center B cells were collected from DNP-KLH-immunized mice from the IgM^{+}/IgD^{-}/PNA^{+} compartment (Figure 2.2.6a). Plasma cells were collected from the B220^{-}/CD138^{+} compartment and found by RT-PCR, as expected, to have

Figure 2.2.6 FACS to Collect Cells for Gene Expression Analysis



Various stages of splenic B cells from Wildtype and *Oca-b*$^{-/-}$ mice were collected by flow cytometry based on expression of key cell surface markers. A. Mature, activated and germinal center B cells were collected based on display of IgM, IgD, and PNA. In order to increase the percentage of germinal center cells, the mice were immunized with DNP-KLH. With the exception of the absence of a germinal center fraction, the overall profiles were similar for the *Oca-b*$^{-/-}$ mice (not shown).

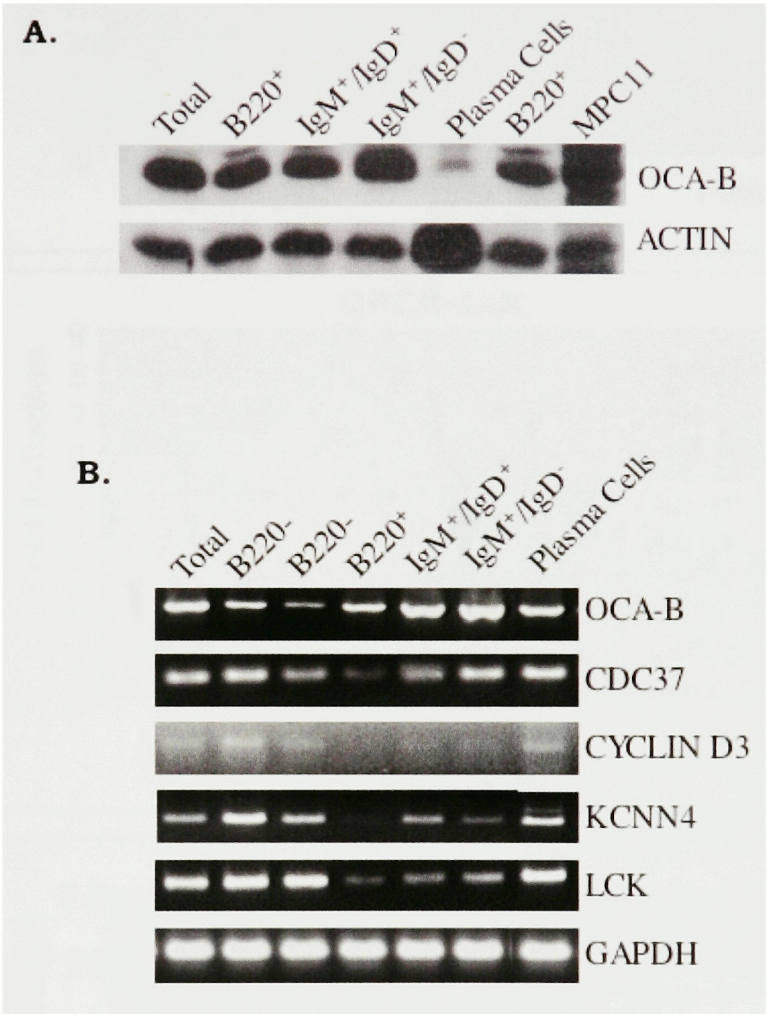
Figure 2.2.6- Continued



B. Plasma cells were sorted based on expression of B220 and CD138. Propidium iodide staining was performed for all flow cytometry experiments to exclude dead cells. C. Total RNA was prepared and RT-PCR for BLIMP-1 was performed using material from the cellular fractions shown in panel B.

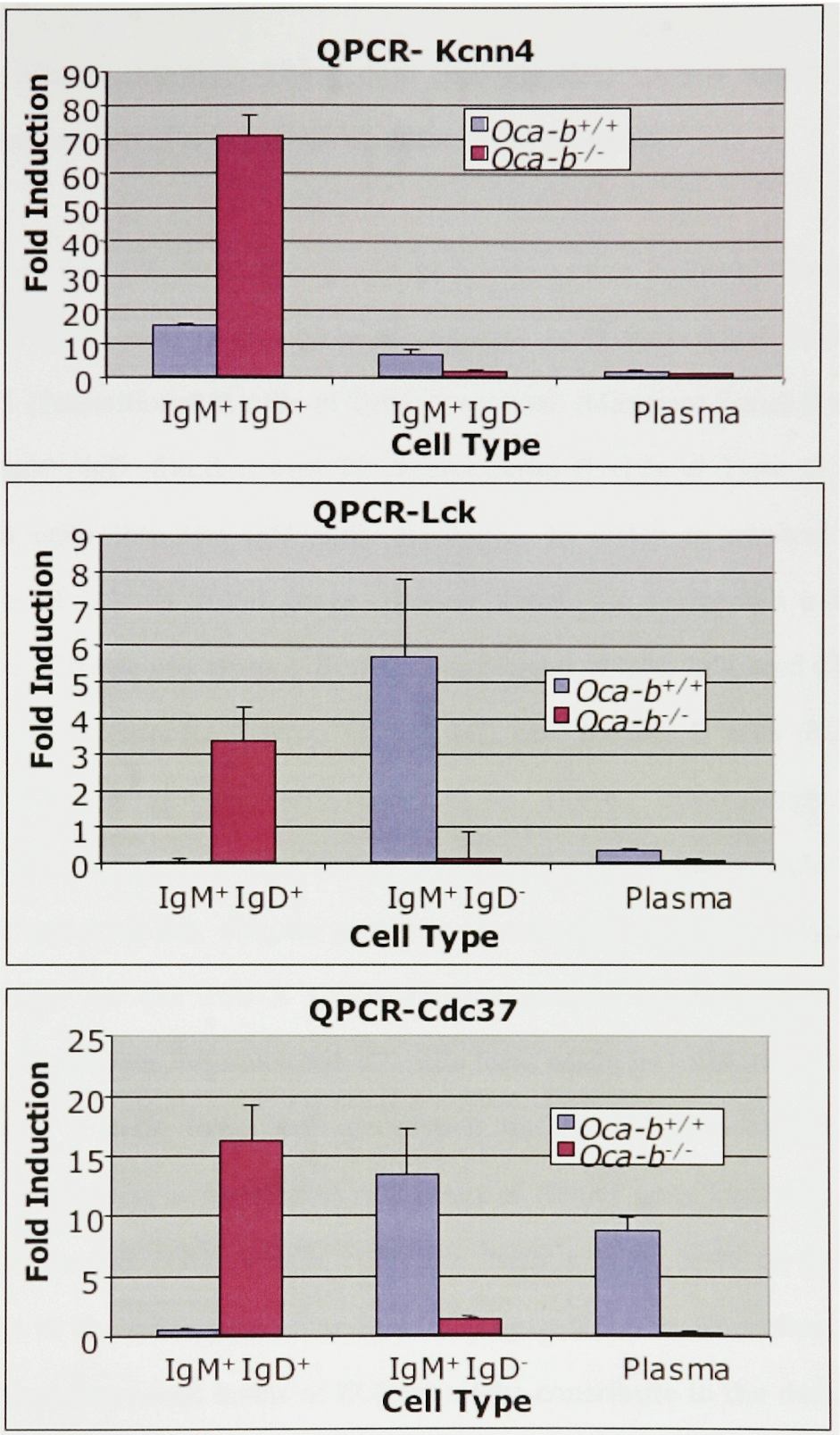
elevated expression of *Blimp-1* (Figure2.2.6b). BLIMP-1 is a transcriptional repressor that is critical for plasma cell differentiation and has been found to be up-regulated early in this differentiation process (reviewed in Johnson et al., 2005). Post-sort analysis showed that all of the sorted populations were at least 92% pure (Figure 2.2.6). Interestingly, OCA-B expression peaked in activated B cells (IgM^+/IgD^+), which included the earliest stage of germinal center cells ($IgM^+/IgD^+/PNA^+$) (Figure 2.2.7a). RT-PCR indicated that the OCA-B target genes analyzed, *Cdc37*, *Cyclin D3*, *Kcnn4* and *Lck* are all expressed and regulated throughout mature B cell development (Figure 2.2.7b). Furthermore, quantitative PCR for *Lck*, *Kcnn4*, and *Cdc37* revealed a significant reduction in transcription of these genes in *Oca-b*$^{-/-}$ activated (IgM^+/IgD^+) B cells isolated by flow cytometry (Figure 2.2.8). This finding corresponds well with our data from similar experiments with *ex vivo* activated B cells as well as with a recently published report that the number of KCNN4 channels increases sharply with B cell activation and that blockage of the this channel causes reduced B cell proliferation (Figure 2.2.4; Wulff et al., 2004). There were also significant reductions in the expression of the target genes in *Oca-b*$^{-/-}$ plasma cells. Interestingly, we also observed up-regulation of *Kcnn4*, *Lck*, and *Cdc37* in the *Oca-b*$^{-/-}$ mature B cell fraction (IgM^+/IgD^-) (Figure 2.2.8). Similarly, up-regulation of *S100a10* and *Kcnn4* was observed by Northern blotting in *Oca-b*$^{-/-}$ B cells that had been stimulated with anti-IgM, anti-CD40,

Figure 2.2.7 Expression Pattern of OCA-B and its Target Genes



A. Whole cell extract was prepared from the collected fractions (Figure 2.2.7) and used for Western blotting. B. The expression patterns of OCA-B target genes were examined by RT-PCR and normalized by Gapdh. The exponential phases of the reactions were determined by QPCR.

Figure 2.2.8 OCA-B Target Gene Expression

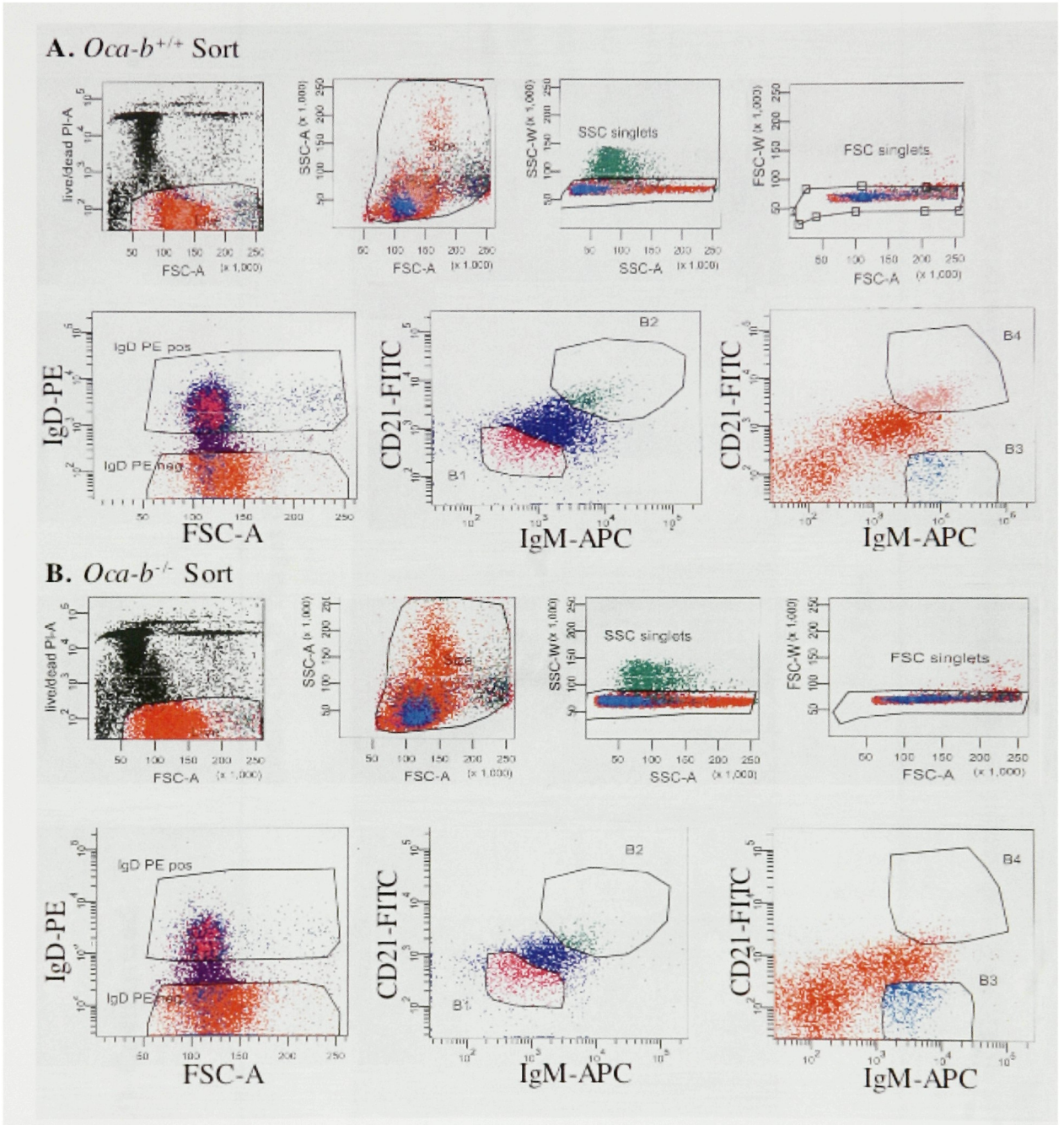


QPCR was performed using RNA from primary cells (Figure 2.2.6). The expression levels of the OCA-B target genes were monitored. The values shown were determined by relative quantification using Gapdh and are averages from at least three replicates.

and IL-4 (Figure 2.2.4b). These data indicate that OCA-B might also activate expression of a negative regulator of these genes.

Although we had observed changes in gene expression in freshly isolated *Oca-b*$^{-/-}$ B cells, the sorting scheme applied in Figure 2.2.6 broadly groups T1 (Transitional B cells of Type 1) and MZ (Marginal Zone) B cells into the IgM^{+}/IgD^{-} fraction and T2 (Transitional B cells of Type 2) and Mature B cells into the IgM^{+}/IgD^{+} fraction. In order to analyze the expression of OCA-B target genes in more detail, we performed a finer separation of these cell types. Surface expression of IgM, IgD, and CD21 can be used to separate splenic T1, T2, MZ, and Mature B cells (Figure 2.2.9a and Carsetti et al., 1995; Loder et al., 1999). Interestingly, the *Oca-b*$^{-/-}$ mice had a severe reduction in the MZ compartment (IgM^{+}IgD^{-} CD21^{+}) (Figure 2.2.9a), despite previous reports that this phenotype is not prevalent on the 129 X C57BL/6 background (Samardzic et al., 2002). Nevertheless, we collected MZ cells from wildtype mice and T1, T2 and Mature B cells from both genotypes for use in gene expression studies. There was a significant reduction of *Kcnn4* gene expression in *Oca-b*$^{-/-}$ mature B cells, which coincides with a high level of OCA-B expression in the wildtype mature B cells (Figure 2.2.9b). Therefore, it is possible that decreased levels of KCNN4 might contribute to the defective differentiation and function of *Oca-b*$^{-/-}$ mature B cells. Although we have a substantial amount of data to indicate that *Lck* is an OCA-B target

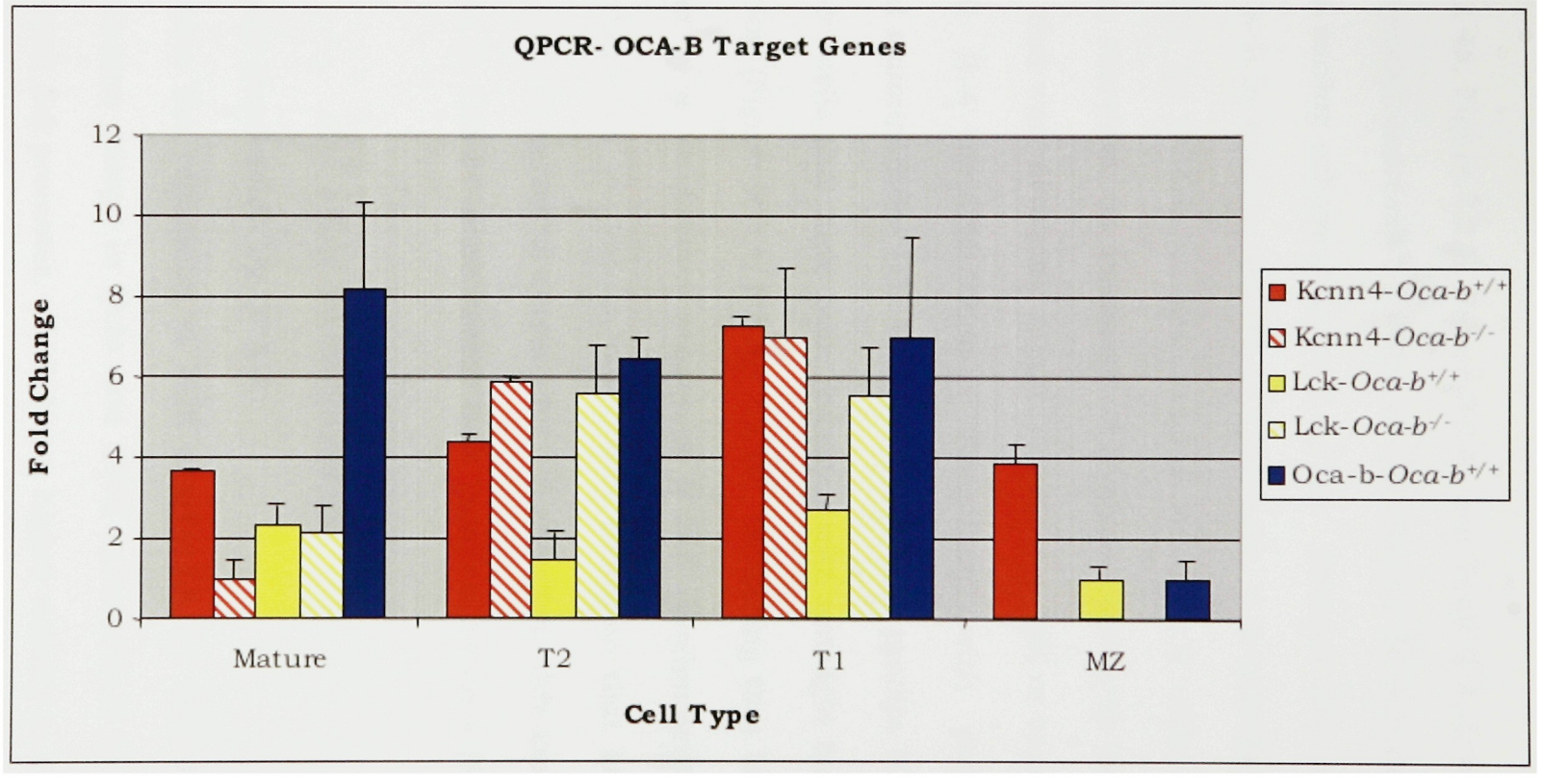
Figure 2.2.9 Target Gene Expression in *Oca-b*$^{+/+}$ and *Oca-b*$^{-/-}$ T1, T2, MZ, and Mature B Cells



A. The indicated fractions were collected by FACS based on surface expression of IgM, IgD, and CD21 from the spleens of *Oca-b*$^{+/+}$ and *Oca-b*$^{-/-}$ mice. B. Analysis of OCA-B and target gene expression by QPCR. Performed as described for Figure 2.2.8.

Figure 2.2.9- Continued

B.



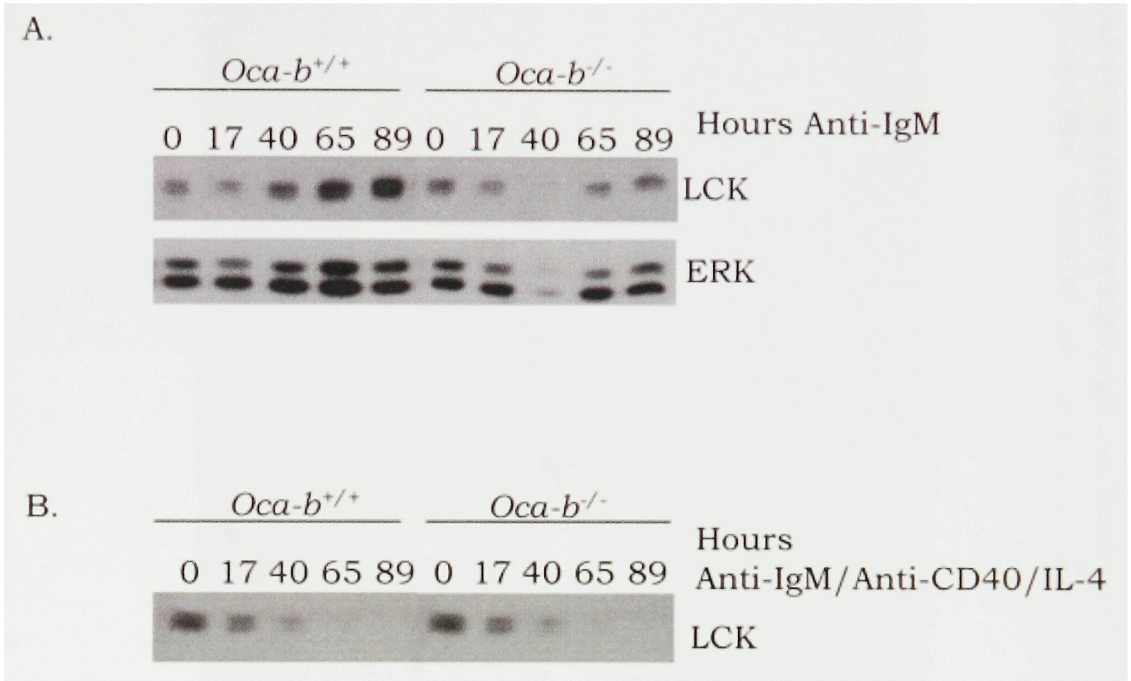
B. Analysis of OCA-B and target gene expression by QPCR. Performed as described for Figure 2.2.8.

gene (Figures 2.2.4a, Figure 2.2.5, Figure 2.2.8, and Table 2.2.1), we did not observe decreased expression in any of the *Oca-b*$^{-/-}$ B cell subsets collected by this sorting scheme (Figure 2.2.10b). Because LCK is not regulated by OCA-B in mature, MZ, T1 or T2 B cells, it is possible that regulation of LCK by OCA-B is more critical in early B cells or in activated B cells; therefore, we examined LCK protein levels in ex vivo cultured B cells that were activated in culture with anti-IgM or anti-IgM, anti-CD40, and IL-4 co-stimulation. Consistent with previous experiments that examined the LCK RNA level, the LCK protein level is reduced in *Oca-b*$^{-/-}$ cells that have been activated with anti-IgM but not with co-stimulation (Figure 2.2.4, Figure 2.2.10), indicating that LCK is an OCA-B target gene at distinct phases of B cell development and activation. Furthermore, immunohistochemistry indicates that LCK is highly expressed at the germinal center and, as expected, this staining pattern is absent in *Oca-b*$^{-/-}$ mice, which lack germinal centers (Figure 2.2.11).

Regulation of LCK Expression by OCA-B

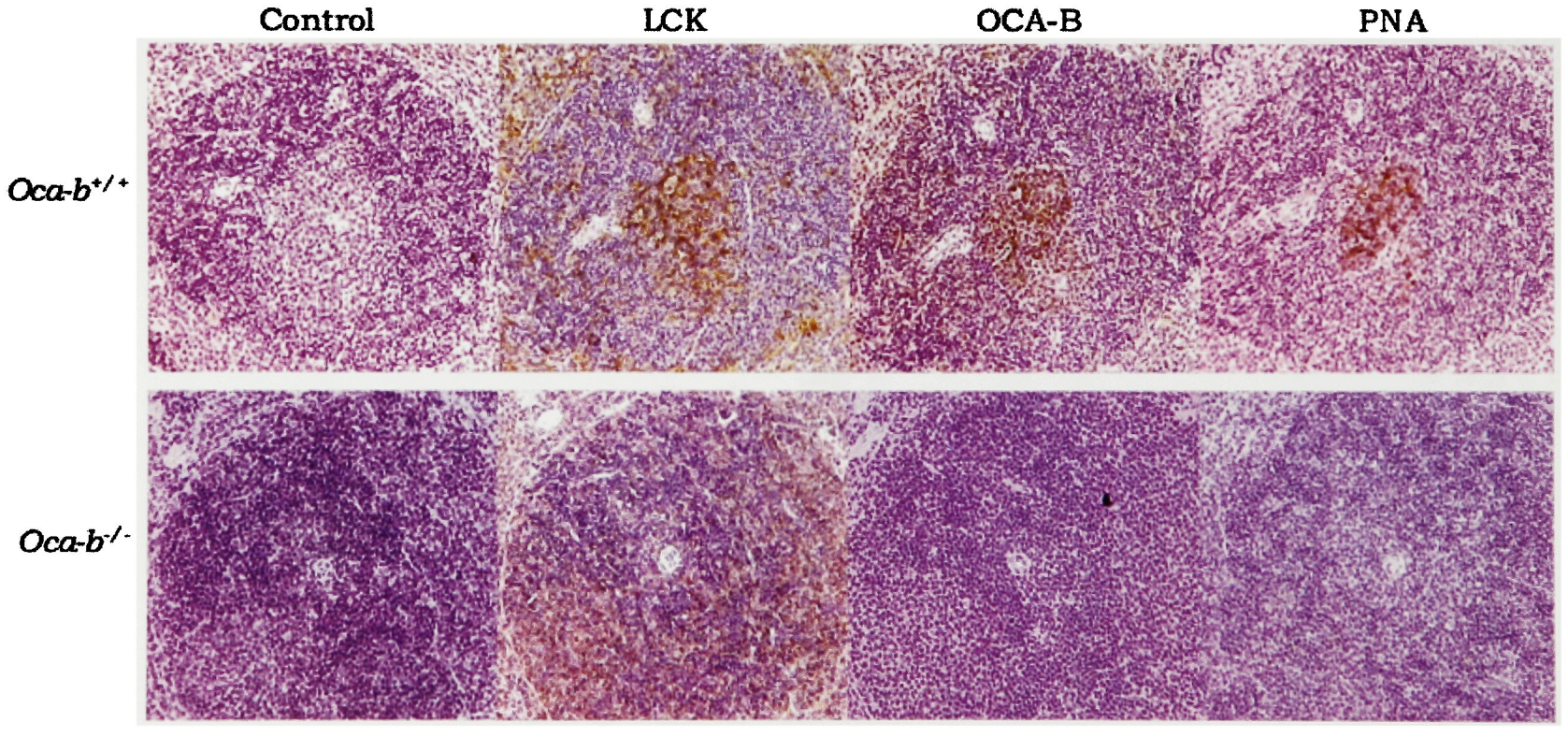
To further examine the regulation of LCK expression by OCA-B, we performed transfection assays in Hela and 293T cells with a reporter containing the distal *Lck* promoter, which has a higher activity in B cells than the T cell-specific proximal promoter (Wildin et al., 1995). Co-

Figure 2.2.10 Analysis of LCK Protein Levels in Activated B Cells



Total B cells were isolated from the spleens of 10 *Oca-b*$^{+/+}$ or *Oca-b*$^{-/-}$ mice. The cells were activated in culture with anti-IgM F(ab')$_2$ fragment (A), or with anti-IgM F(ab')$_2$ fragment, anti-CD40, and IL-4 (B) for up to 89 hours. Expression of LCK and ERK were analyzed by immunoblot. The activation experiments were performed three times for this study.

Figure 2.2.11 Immunohistochemistry to Examine the LCK Expression Pattern in the Spleen

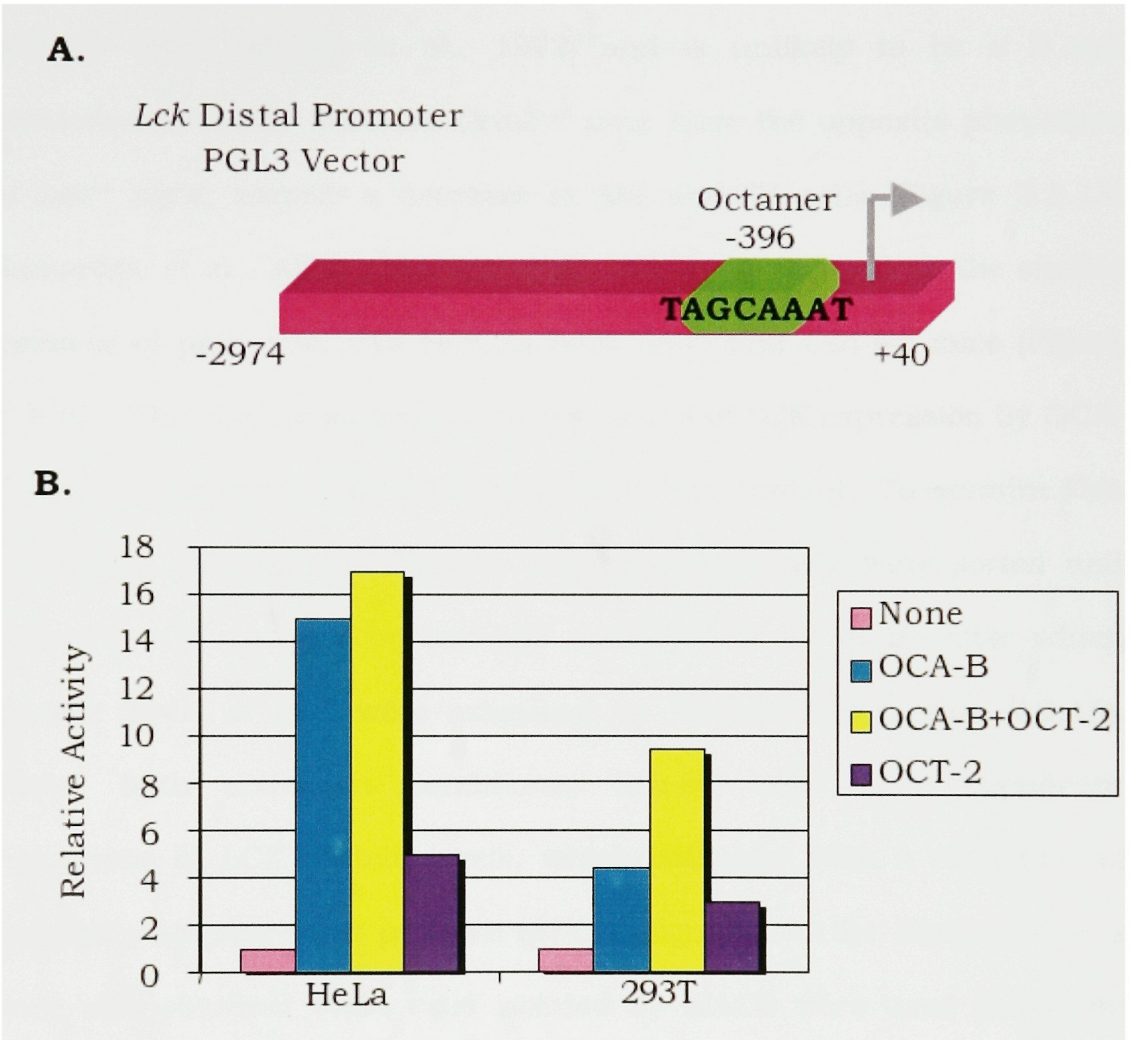


Immunohistochemistry was performed for LCK, OCA-B, or peanut agglutinin (PNA) on splenic sections from *Oca-b*$^{+/+}$ or *Oca-b*$^{-/-}$ mice. A representative germinal center is shown.

transfection of an OCA-B expression vector caused a sharp increase in *Lck* promoter activity, which could not be generated by OCT-2 co-transfection (Figure 2.2.12). Furthermore, OCA-B functions in conjunction with the endogenous OCT-1 or with transfected OCT-2 to regulate LCK expression (Figure 2.2.12).

Not only is *Lck* likely to be an important OCA-B target gene, but *Oca-b*$^{-/-}$ and *Lck*$^{-/-}$ mice may have partially overlapping phenotypes related to BCR signaling and B1a cell development. As mentioned previously, *Oca-b*$^{-/-}$ mice not only have defective BCR signaling and impaired TD immune responses also an enhancement of peritoneal B1a cells, a unique B cell subset that is involved in natural immunity (Kim et al., 2000). However, there have been conflicting reports as to the nature and extent of B cell-specific defects in *Lck*$^{-/-}$ mice (Ulivieri et al., 2003; Dal Porto et al., 2004; Frances et al., 2005). For these reasons it was important to further analyze B cell development in *Lck*$^{-/-}$ mice (obtained from Tak Mak) in order to determine the significance of LCK regulation by OCA-B. Unfortunately we were unable to identify any defect by flow cytometry in early B cell or plasma cell development using well-defined markers such CD25, CD43, CD117, CD138, B220 and IgM (Data Not Shown). Of note, we observed the recently reported defect in plasma cell (CD138^{high}/B220^{-}) differentiation of *Oca-b*$^{-/-}$ mice (Data Not Shown; Corcoran et al., 2005).

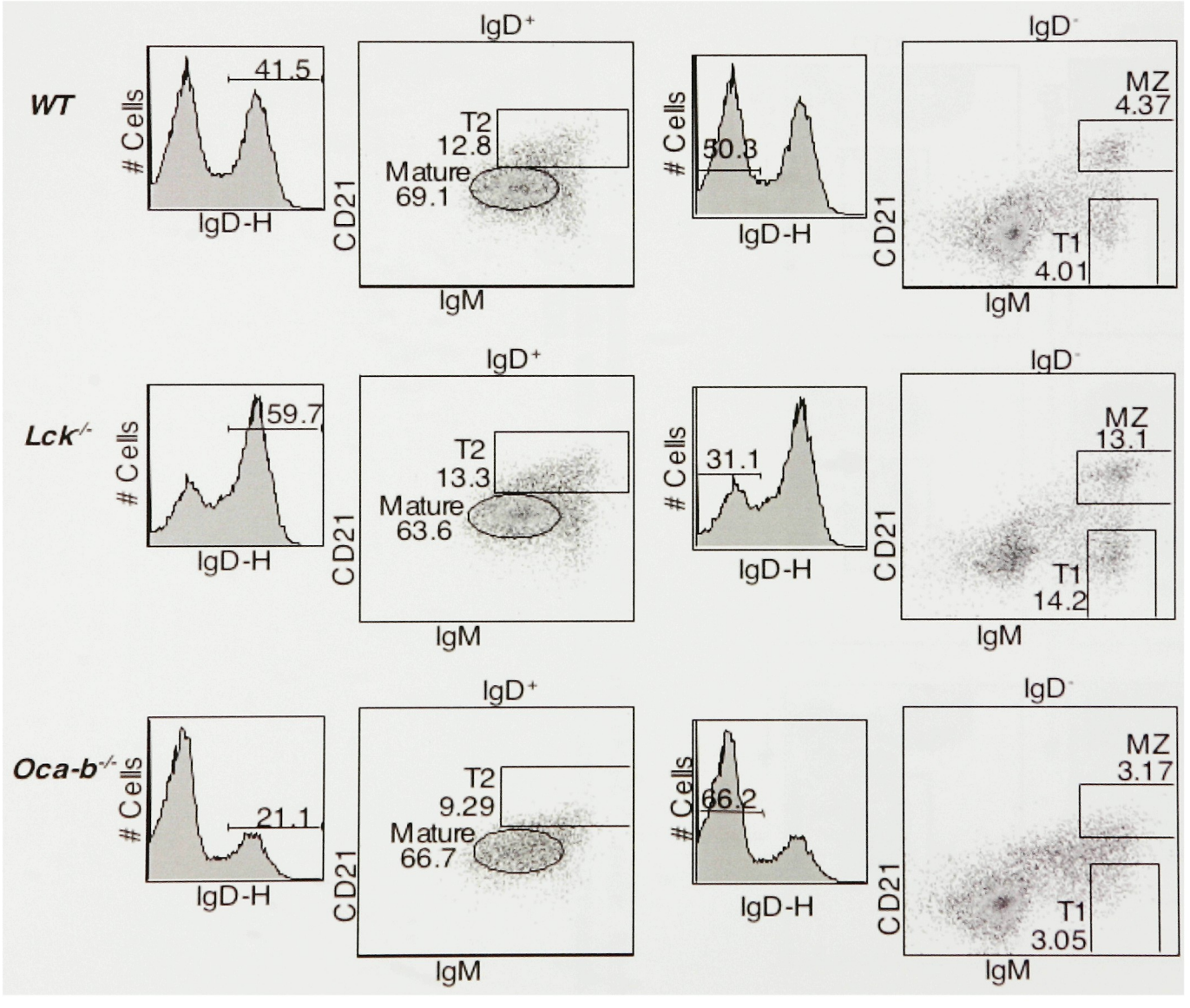
Figure 2.2.12 Analysis of *Lck* Distal Promoter Dependence on OCA-B



A. The responsiveness of the *Lck* distal promoter to OCA-B and OCT-2 was analyzed in HelaS or 293T cells that had been transiently transfected using Lipofectamine or Fugene reagents with an *Lck* distal promoter-luciferase vector, an OCA-B-CMV expression vector, and/or an OCT-2-CMV expression vector. PRL-CMV was used to normalize the luciferase activity.

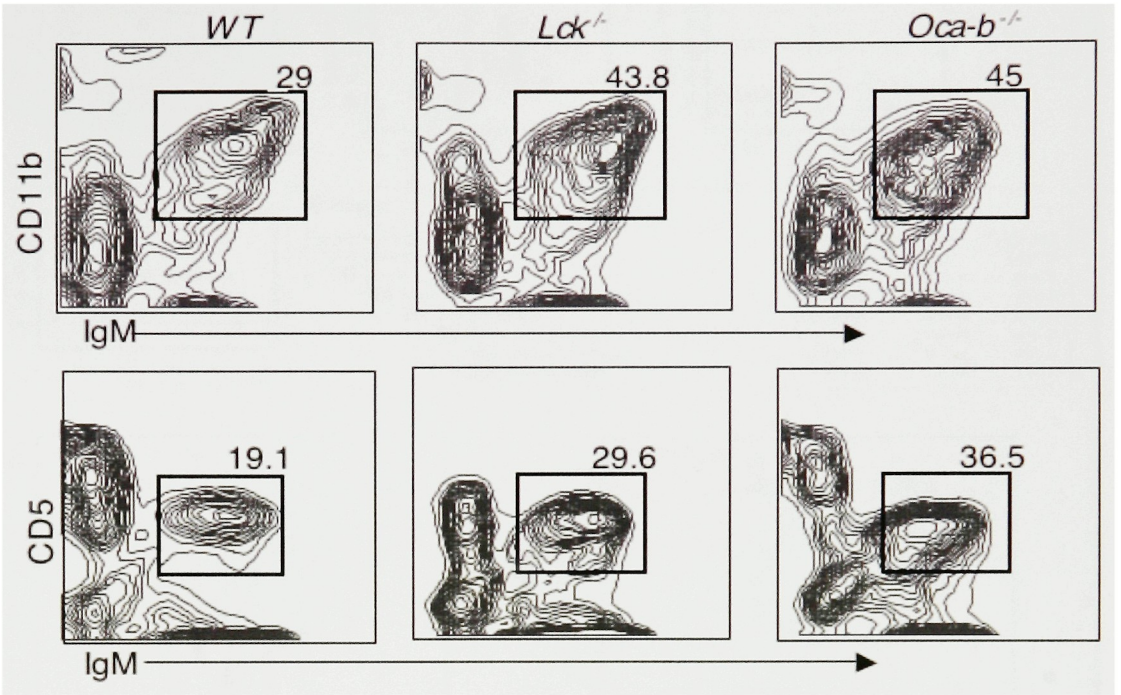
Lck$^{-/-}$ mice displayed an enhancement in the MZ and T1 compartments (Figure 2.2.13); however, this finding is most likely linked to defective T cell-dependent B cell activation caused by the absence of mature T cells in *Lck*$^{-/-}$ mice (Molina et al., 1992) and is unlikely to be a B cell autonomous defect. Because *Oca-b*$^{-/-}$ mice have the opposite phenotype of *Lck*$^{-/-}$ mice, namely a decrease in MZ and T1 cells (Figure 2.2.13; Samardzic et al., 2002; Jankovic et al., 2003), we focused on the similar increase of peritoneal B1a cells in both *Lck*$^{-/-}$ and *Oca-b*$^{-/-}$ mice (Figure 2.2.14). This finding suggests that regulation of LCK expression by OCA-B might be an important factor in B1a cell development. To examine this possibility, wildtype and *Oca-b*$^{-/-}$ peritoneal B1a cells were sorted and cultured with anti-IgM or anti-IgM and IL-4 for 48 hours after which protein levels of LCK were examined by immunoblot (Figure 2.2.15). Under both activation conditions, *Oca-b*$^{-/-}$ cells have significant reductions in LCK protein levels, which coincides with a reduction in tyrosine phosphorylated proteins (pTYR) (Figure 2.2.16). Similar results were also obtained when cells isolated by MACS were used (Data Not Shown). These data indicate that like conventional B2 cells, *Oca-b*$^{-/-}$ B1a cells also have compromised BCR signaling.

Figure 2.2.13 Distribution of Peripheral B Cell Subtypes in *Oca-b*$^{-/-}$ and *Lck*$^{-/-}$ mice



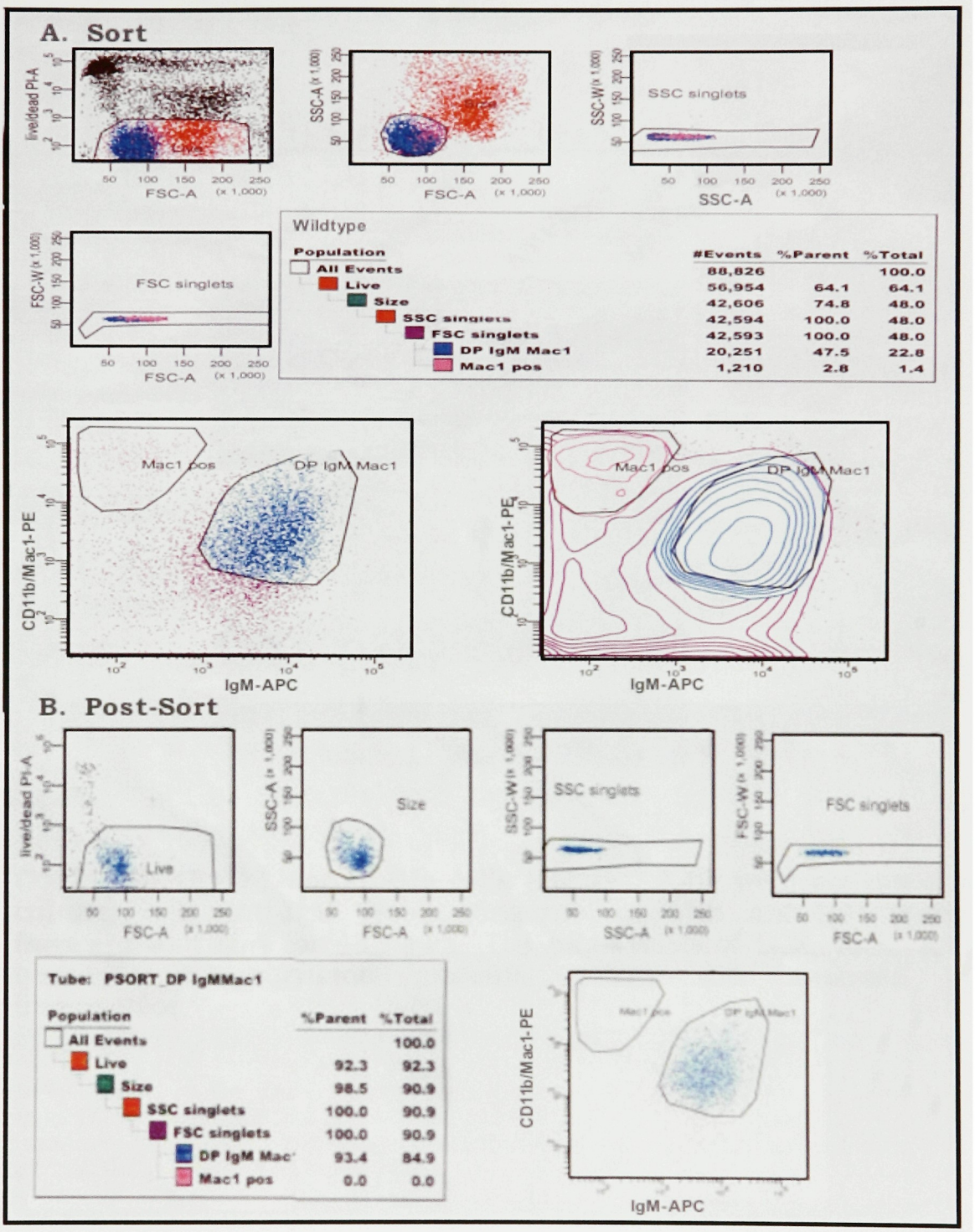
Splenocytes from *WT*, *Lck*$^{-/-}$, and *Oca-b*$^{-/-}$ mice were harvested and the indicated populations were analyzed by flow cytometry based on surface expression of IgM, IgD, and CD21. Transitional B cells of type 1 (T1), transitional B cells of type 2 (T2), mature B cells and marginal zone B cells (MZ) are gated.

Figure 2.2.14 Analysis of B1a cells in *Oca-b*$^{-/-}$ and *Lck*$^{-/-}$ mice



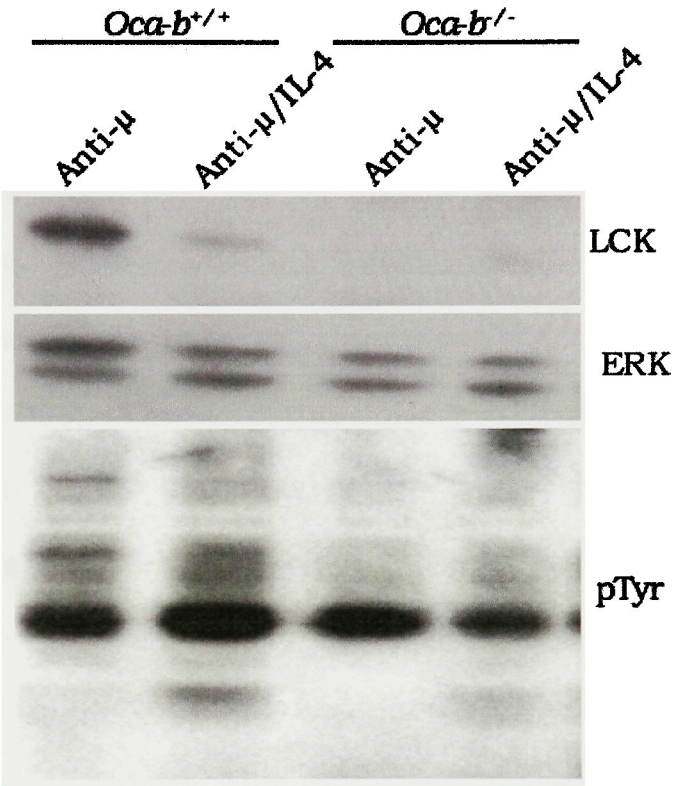
Cells from the peritoneum of *WT*, *Lck*$^{-/-}$, and *Oca-b*$^{-/-}$ mice were harvested and B1a cells were analyzed based on surface expression of IgM, CD11b, and CD5.

Figure 2.2.15 FACS to Collect B1a Cells for Gene Expression Analysis



The indicated fractions were collected by FACS based on surface expression of IgM and CD11b/Mac1 from the spleens of *Oca-b*$^{+/+}$ (shown) and *Oca-b*$^{-/-}$ mice. B. Post-sort analysis of the sorted IgM^{+}Mac1^{+} B1a population.

Figure 2.2.16 Analysis of LCK Protein Levels in Activated B Cells



Oca-b$^{+/+}$ or *Oca-b*$^{-/-}$ sorted B1a cells (Figure 2.2.16) were activated in culture with anti-IgM F(ab')$_2$ fragment or with anti-IgM F(ab')$_2$ fragment and IL-4 for 48 hours. The expression of LCK, ERK, and tyrosine phosphorylated proteins (pTYR) was analyzed by immunoblot.

2.3 DISCUSSION

Gene Expression Profiles in Normal B Cells

One aim of this study was to identify OCA-B target genes that are associated with B cell activation and maturation processes. To this end, we used a B cell culture system that allows stimulation by the BCR. Previous studies showed that resting splenic B cells cultured in the presence of an anti-IgM-derived $F(ab')_2$ fragment at 25–30 μg/ml induces a burst of cell proliferation that peaks around 48–72 h (Defranco et al., 1982; Kim et al., 1996). Therefore, this culture system likely mimics the activation process that accompanies a BCR–antigen interaction. Accordingly, the gene expression profiles showed that B cells stimulated with anti-IgM for 45 h up-regulated transcripts of many genes involved in cell cycle progression, DNA replication and repair, cell proliferation, translation, and transcription. This finding clearly demonstrates that B cells are actively proliferating and making new transcripts and proteins. Interestingly, this is the time period when the BCR-induced OCA-B level is maximal, indicating that OCA-B is involved in the B cell expansion phase. Most of the genes involved in cell cycle regulation, DNA replication and repair, cell proliferation, transcription, and translation induced by BCR ligation were also induced by co-stimulation but at an earlier time point (21 h vs. 45 h). The fact that OCA-B is expressed at early (21 h) as well as late (45–96 h) time points during co-stimulation

again indicates OCA-B involvement in the expansion, as well as the functional phase of antigen-dependent B cell differentiation.

Identification of OCA-B-Dependent Genes

This study has allowed identification of additional OCA-B target genes, such as *CyclinD3*, *Cdc37*, *Kcnn4*, and *Lck*, that are up-regulated in response to BCR ligation and likely to be involved in B cell proliferation and signaling. Additionally, *B4galt4* and *Ms4a6b* (*Ms4a11*) are downregulated in *Oca-b*$^{-/-}$ B cells that have been given Th co-stimulation. Because glycosylation is involved in a wide range of important processes (Rudd et al., 2001), defective glycosylation would have a significant impact on B cell function. Notably, the *B4galt4* activity is regulated primarily at the level of transcription (Lemaire et al., 1998). Although the function of *Ms4a6b* (*Ms4a11*) is currently unknown, the fact that it contains transmembrane domains that are conserved in CD20, HTM4, and the high-affinity IgE receptor β chain, suggests an involvement in signal transduction (Liang and Tedder, 2001; Ishibashi et al., 2001). Interestingly, OCA-B appears to be involved both in downregulation and in up-regulation of *Kcnn4* and *S100a10*. S100A10 (or calpectin) is a subunit of anexin 2, which is involved in Ca^{2+}-mediated lipid raft organization and vesicle movement (Gerke and Moss, 2002). The fact that *S100a10* and *Kcnn4* are up-regulated by BCR ligation and down-regulated by Th co-stimulation suggests that while they function in BCR-

induced expansion, they are not required for the functional phase of B cell differentiation. It also is possible that the failure to down-modulate these genes in *Oca-b*$^{-/-}$ B cells during the functional phase (i.e., BCR+Th, 45–96 h) may have inhibitory effects on B cell differentiation. Because OCA-B seems to be involved in both positive and negative regulation of the same genes, depending on the signaling pathway, it is an intriguing possibility that OCA-B may activate negative regulators of these genes. Furthermore, the direct OCA-B target gene, *Lck* has also been implicated, but not conclusively shown, to be involved in B1a cell development and cell signaling (Ulivieri et al., 2003; Dal Porto et al., 2004; Frances et al., 2005). Given that *Oca-b*$^{-/-}$ mice also show an enhancement of B1a cells (see Results; Kim et al., 2000) it will be important to determine the functional significance of LCK regulation by OCA-B. Nevertheless, this study has led to the identification and confirmation of direct OCA-B target genes, which may prove to be important for the differentiation and function of multiple B cell subsets. In conclusion, this study has identified genes involved in B cell proliferation and signaling as targets of OCA-B. Considering that the ability to proliferate and accurately process BCR- and Th-derived signals is critical for antigen-driven B cell differentiation, including the germinal center reaction (MacLennan et al., 1992), failure to appropriately regulate these genes provides a possible explanation for the severe defects observed in *Oca-b*$^{-/-}$ mice.

CHAPTER 3

THE ROLE OF OCA-B IN EARLY B CELL DEVELOPMENT AND PRE-BCR SIGNALING

3.1 PREFACE: OCA-B FUNCTION IN PRE-B CELLS?

As mentioned previously, studies with *Oca-b* knockout mice (*Oca-b*$^{-/-}$) showed that OCA-B plays a broad role in B cell development and function (Kim et al. 1996, Schubart et al., 1996, Nielsen et al. 1996). The most drastic defect in *Oca-b*$^{-/-}$ mice is observed in the peripheral lymphoid organs during antigen-dependent B cell maturation and effector function. These defects include dramatically diminished levels of IgH secondary isotypes, a complete lack of germinal centers in the lymphoid organs, and impaired antibody responses to both T-independent and T-dependent antigens (Kim et al. 1996, Schubart et al., 1996, Nielsen et al. 1996, Kim et al., 2000). The reduction in secondary isotype levels is caused by reduced expression of the switched genes and may be attributed in part to OCA-B function at the 3' enhancer (Kim et al., 1996; Tang and Sharp, 1999; Stevens et al., 2000). Surprisingly, IgM expression is largely normal in *Oca-b*$^{-/-}$ mice, although OCA-B is required for full activity of a subset of κ light chain promoters (Vκ4 and Vκ3-83) (Casellas et al., 2003). There is also a reduction in the transitional B cell compartment in the spleens of *Oca-b*$^{-/-}$ mice, perhaps as a consequence of the skewed Igκ repertoire and/or a B cell signaling defect (Casellas et al., 2003, Janovic and Nussenzweig, 2003).

At the cellular level *Oca-b*$^{-/-}$ B cells are deficient in processing B cell antigen receptor (BCR)-derived signals, as evidenced by their reduced proliferation following BCR ligation and an increased number of immature, apoptotic B cells in the bone marrow of *Oca-b*$^{-/-}$ mice (Kim et al., 1996; Hess et al., 2001). A recent study (see Chapter 2) has identified additional OCA-B target genes (e.g. *Kcnn4*, *Lck*, *Cyclin D3*, and *Cdc37*) that may be involved in these processes (Kim et al. 2003), although the exact mechanisms by which these genes contribute to B cell signaling and differentiation are not fully understood. Additionally, OCA-B is likely to play a unique role in B cell signaling as evidenced by the presence of two distinct OCA-B isoforms, one that is predominantly nuclear and another that is myristoylated and present in the cytoplasm (Yu et al., 2001).

Given that *Oca-b*$^{-/-}$ B cells are deficient in BCR signaling, it seemed important to determine whether these cells have a similar phenotype relative to pre-BCR signaling. This is a particularly interesting possibility since pre-BCR and BCR signaling pathways involve the action of many of the same proteins such as SYK, PLCγ2, FYN, BLK, LCK and LYN (reviewed in Wang and Clark, 2003). Furthermore, Igβ, which is the signaling component of both the pre-BCR and BCR, has been identified as an OCA-B target gene in mature B cells (Malone and Wall, 2002). Additionally, and significantly, the signaling proteins BTK and SLP-65,

which are also critical for BCR signaling and mature B cell differentiation, have recently been shown to play a role in early B cell development as well (Middendorp et al., 2002; Flemming et al., 2003; Kersseboom et al., 2003; Hendriks and Middendorp, 2004). Further supporting its role in pre-BCR signaling, OCA-B is highly expressed in large $B220^{+}IgM^{-}$ pre-B cells (Qin et al., 1998), which includes pre-BCR expressing cells (reviewed in Hendricks and Middendorp, 2004). Additionally, the OCA-B promoter was shown to be active in transiently transfected pre-B cell lines that also had endogenous OCA-B expression (Stevens et al., 2000).

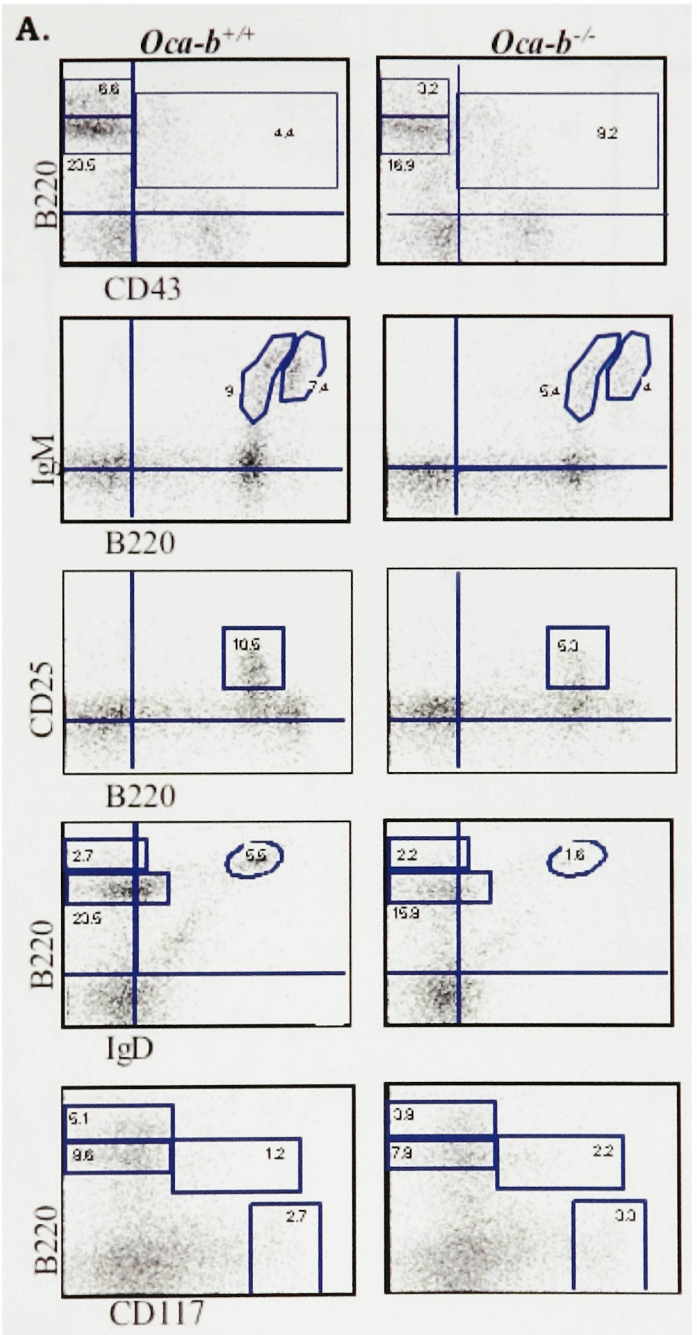
3.2 RESULTS

The Effect of OCA-B Deficiency on Early B Cell Development

In order to identify possible defects in early B cell development in *Oca-b*$^{-/-}$ mice, our first goal was to characterize the distribution of bone marrow B cell populations using flow cytometry (Figure 3.2.1). Because *Oca-b*$^{-/-}$ B cells have a compromised response to BCR signaling, we were particularly interested in analyzing cell stage transitions correlating with changes in pre-BCR expression. For this purpose, and to dissect the pre-B cell compartment of wildtype and *Oca-b*$^{-/-}$ mice, we examined the expression patterns of cell surface markers such as B220, IgM, CD43, CD117 and CD25. The knockout mice appear to have a partial

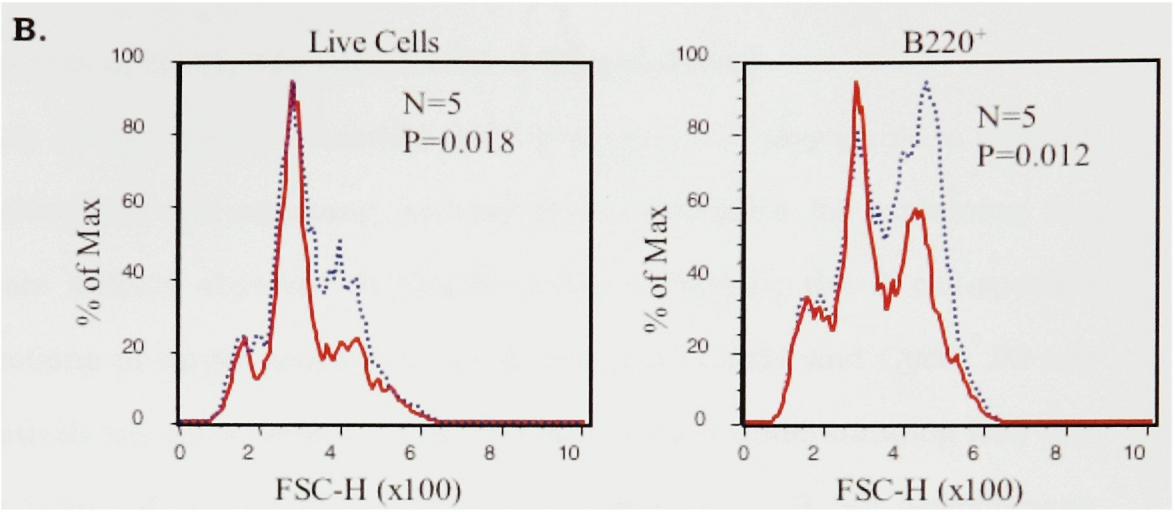
developmental block between the pre-B1 and pre-B2 cell stages. This is mainly indicated by a reproducible decrease (40-50%) in the $B220^+/CD25^+$ pre-B2 cell compartment and an increase of at least two-fold in cells at the preceding developmental stage ($B220^+/CD43^+$). A partial block is consistent with previous findings, in that a substantial number of B cells are able to reach maturity despite an overall reduction of peripheral B cells (Schubart et al., 1996). Consistent with an increased proportion of $B220^+/CD43^+$ pre-B cells in *Oca-b*$^{-/-}$ mice, there was also a significant increase (on average 25%) ($p=0.018$) in large cells (high FSC) within the bone marrow (Figure 3.2.1b, left panel). This increase in large cells was also apparent when examining the $B220^+$ compartment ($p=0.012$), where there was a similar increase of large cells relative to wildtype (Figure 3.2.1b, right panel). In addition, there was also a 40-50% decrease in $B220^{med}/\mu^+$ cells in the *Oca-b*$^{-/-}$ mice (9% vs. 5.4%) (Figure 3.2.1a). As previously reported (Schubart et al., 1996; Hess et al., 2001), *Oca-b*$^{-/-}$ mice also had a significant reduction in $B220^{high}IgD^+$ re-circulating B cells (5.6% vs. 1.6%) (Figure 3.2.1a). Overall, these findings suggest that OCA-B deficiency results in reduced developmental progression of B cells into the $B220^{med}/CD43^-/CD25^+$ pre-B2 compartment, which in turn leads to a build up of cells in the $B220^+/CD43^+/CD25^-$ pre-B1 compartment. This defect could be attributed not only to a potential defect in pre-BCR signaling but also to

Figure 3.2.1 ***Oca-b*$^{-/-}$ Mice Have an Altered Distribution of Bone Marrow B Cells**



A. Cellular populations from *Oca-b*$^{+/+}$ and *Oca-b*$^{-/-}$ bone marrow were analyzed by flow cytometry using antibodies against cell surface markers, as labeled. The numbers within the gates are percentages of live cells (low SSC). Data are representative of three experiments.

Figure 3.2.1- Continued



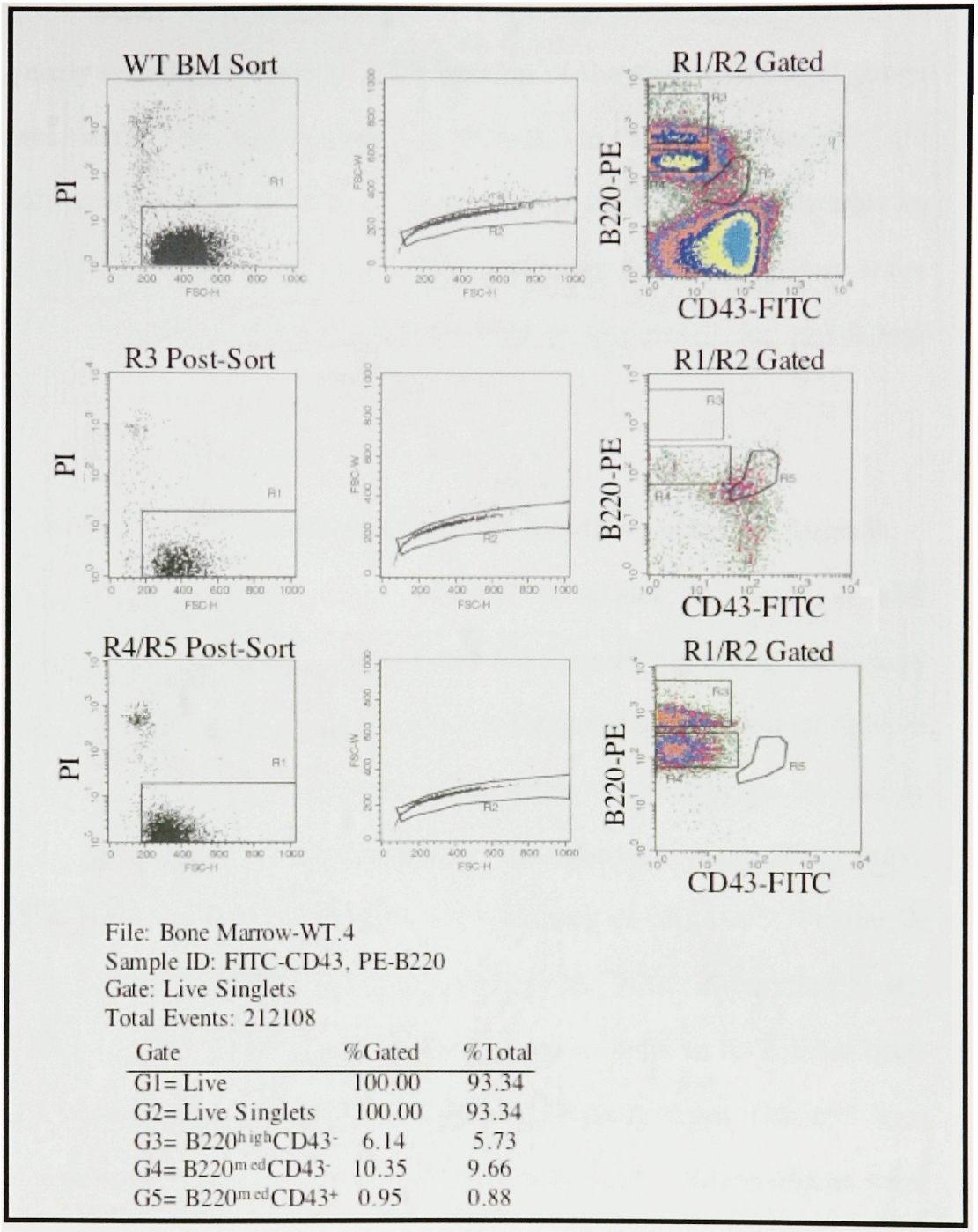
B. Size analysis of live cells (left) or B220^{+} cells (right) from bone marrow of *Oca-b*$^{+/+}$ (red) or *Oca-b*$^{-/-}$ (blue) mice.

the function of the newly identified OCA-B target genes that include *Cdc37*, *Cyclin D3*, *Kcnn4*, and *Lck* (Chapter 2; Kim et al., 2003).

Analysis of Newly Identified OCA-B Target Genes

Many of the recently identified OCA-B target genes play a role in cellular proliferation and signaling, making them candidates for explaining the severe defects observed in *Oca-b*$^{-/-}$ mice. However, the B cell-specific functions of target genes such as *Kcnn4*, *Lck*, *Cdc37* and *Cyclin D3* are relatively uncharacterized. The first step toward understanding how the regulation of these genes might contribute to early B cell development was to profile their expression patterns in primary B cells. Early B cells from the bone marrow of wild type mice were fractionated by FACS based on surface expression of B220 and CD43 (Figure 3.2.2). The populations analyzed included non-B cells (B220^{-}/CD43^{+}), pro-B cells (B220^{dull}/CD43^{+}), pre-B cells (B220^{med}/CD43^{-}), and immature B cells (B220^{high}/CD43^{+}) (Figure 3.2.2a). Post-sort analyses showed that all of the sorted populations were of sufficient purity (Figure 3.2.2). The OCA-B protein level was highest in bone marrow B cells at the pre-B cell stage, as evidenced by immunoblot (Figure 3.2.3c). Interestingly, the OCA-B protein level in pre-B cells was nearly equivalent to the amounts typically observed in activated (IgM^{+}/IgD^{+}) splenic B cells (data not shown). The expression patterns of OCA-B target genes *Cdc37*, *Cyclin D3*, *Kcnn4*, and *Lck* were analyzed by RT-PCR and Western blotting

Figure 3.2.2 FACS to Isolate Bone Marrow B Cells



File: Bone Marrow-WT.4
Sample ID: FITC-CD43, PE-B220
Gate: Live Singlets
Total Events: 212108

Gate	%Gated	%Total
G1= Live	100.00	93.34
G2= Live Singlets	100.00	93.34
G3= B220^{high}CD43^{-}	6.14	5.73
G4= B220^{med}CD43^{-}	10.35	9.66
G5= B220^{med}CD43^{+}	0.95	0.88

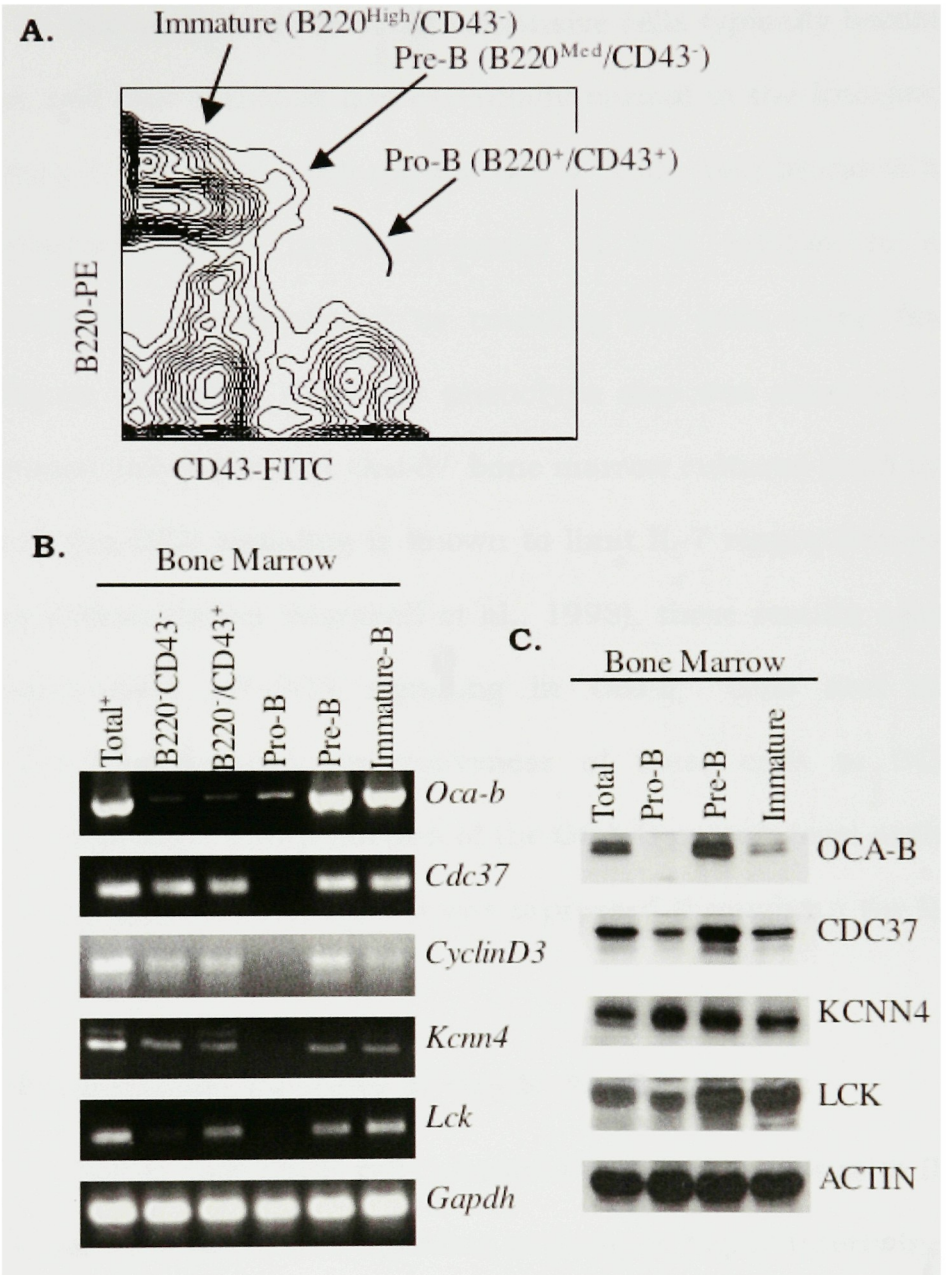
Bone marrow B cells were collected by FACS based on B220 and CD43 surface marker expression. PI-negative cells were gated as live cells (R1) and single cells (R2) were gated based on forward scatter. The R1 and R2 gates were used to collect the cellular fractions based on B220 and CD43 staining (as labeled- R3, R4, and R5). Post-sort analyses were performed to access the purity of the fractions.

(Figure 3.2.3a,b). All of these genes were expressed to varying levels during early B cell development. Expression of the analyzed target genes correlates with the expression of OCA-B, in that transcripts are minimally present in pro-B cells and show a substantial induction in pre-B cells (Figure 3.2.3 a and b). This indicates that OCA-B may serve to initiate a gene expression program that is important for pre-B cell development.

Oca-b$^{-/-}$ Early B Cells Aberrantly Respond to Differentiation Signals

Because OCA-B deficient mice showed a defect in early B cell development, we wished to look more closely at signaling pathways important for this transition and to determine if deregulation of OCA-B target genes could be contributing to the observed phenotype. A similar phenotype has also been described for the *Slp-65*$^{-/-}$ and *Btk*$^{-/-}$ mice, which also are hyper-responsive to IL-7 (Cheng et al., 1995; Turner et al., 1995; Jumaa et al., 1999; Middendorp et al., 2002; Flemming et al., 2003). To examine the responsiveness of *Oca-b*$^{-/-}$ cells to IL-7, total bone marrow B cells (CD19^{+}) were isolated magnetically from *Oca-b*$^{+/+}$ and *Oca-b*$^{-/-}$, mice and cultured for several days with IL-7. The cultures were sampled over a time course that extended to 168 hours of IL-7 exposure. In vivo, IL-7 is provided by stromal cells and serves to drive proliferation of interleukin receptor α (IL-7Rα) bearing B cells. Providing IL-7 to ex

Figure 3.2.3 Expression Pattern of OCA-B and its Target Genes in Bone Marrow B Cells



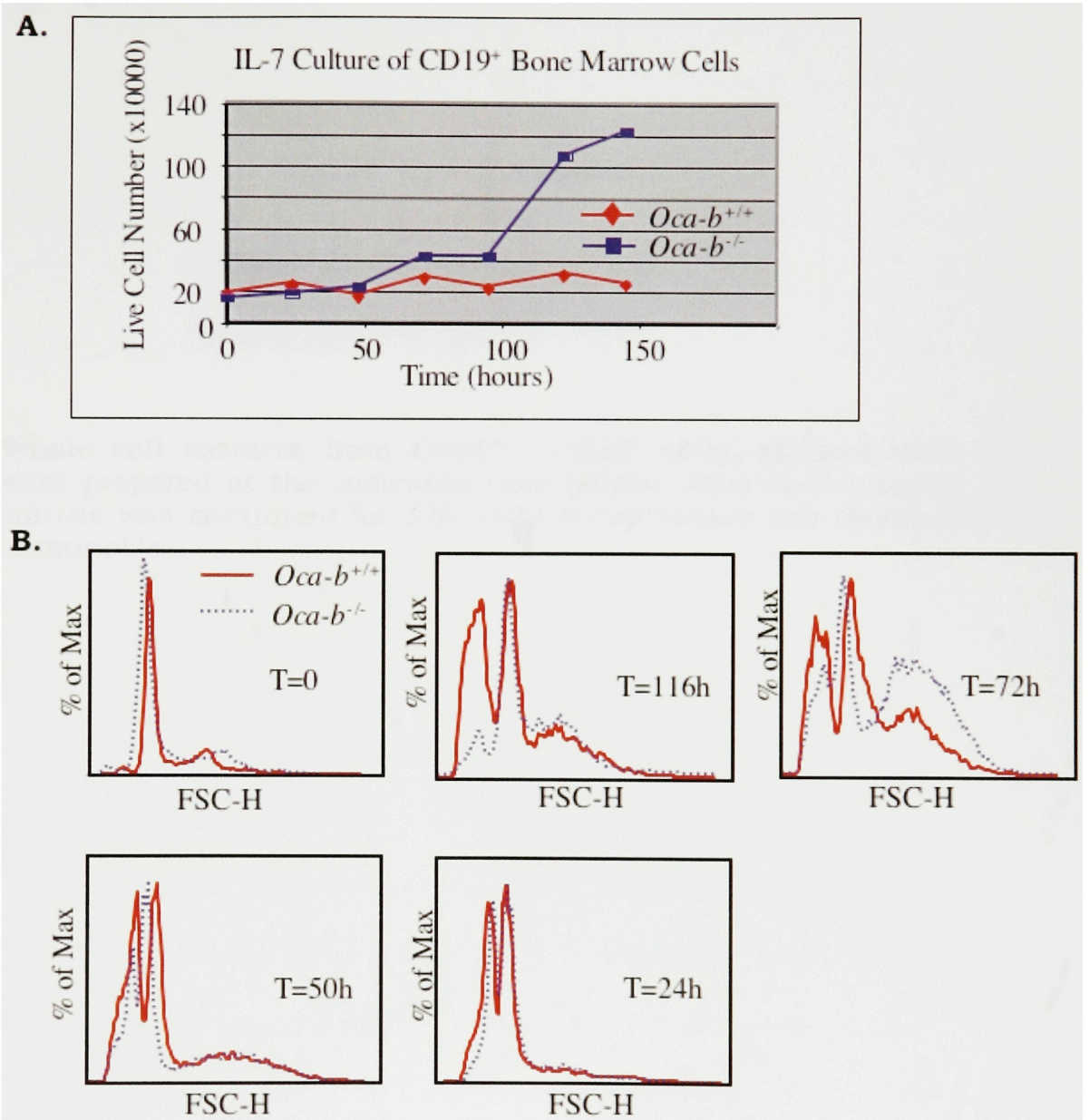
A. Bone marrow B cells from *Oca-b*$^{+/+}$ mice were collected by FACS based on surface expression of B220 and CD43, as shown in Figure 3.2.2. The populations collected were: B220^{-}/CD43^{-} (non-B) B220^{-}/CD43^{+} (non-B), B220^{+}/CD43^{+} (pro-B), B220^{med}/CD43^{-} (pre-B), and B220^{high}/CD43^{-} (immature B). B. Expression of OCA-B target genes were examined by RT-PCR. Total bone marrow cells were prepared prior to sorting. Gapdh expression was used to normalize the samples. All of the reactions shown are within the linear range of amplification for each target gene. C. Immunoblotting for OCA-B and its target gene products using whole cell extract from fractions shown in A.

vivo cultured CD19$^+$ cells stimulates differentiation of IL-7Rα bearing pro-B cells. In this culture system IL-7 responsive cells typically become larger in size, and this response was essentially normal in the knockout cultures (Figure 3.2.4b). In contrast, the *Oca-b*$^{-/-}$ cells were found to be markedly hyperproliferative in the presence of IL-7, relative to its wildtype counterpart, as measured by counting live cells using flow cytometry (Figure 3.2.4a). A similar phenotype also was observed in long-term stromal cell-dependent *Oca-b*$^{-/-}$ bone marrow cultures (data not shown). Since pre-BCR signaling is known to limit IL-7 responsiveness by promoting differentiation (Marshall et al., 1998), these results again indicate compromised pre-BCR signaling in *Oca-b*$^{-/-}$ cells and are consistent with the reduced responsiveness of these cells to BCR stimulation. Consistent with induction of the OCA-B protein level at the pre-B cell stage (Figure 3.2.3c), OCA-B was expressed throughout the IL-7 time course (Figure 3.2.5).

Defective Differentiation Capacity of *Oca-b*$^{-/-}$ Pre-B Cells

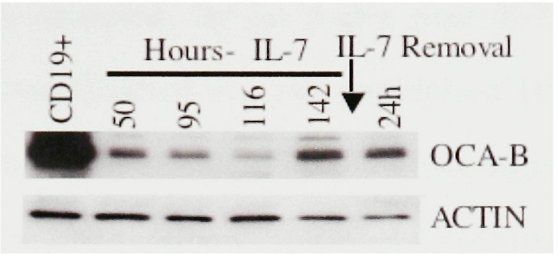
Given that the OCA-B deficient B cells showed an altered response to IL-7 and that responsiveness to this signaling pathway is highly intertwined with signals emanating from the pre-BCR (Hendricks and Middendorp, 2004), we further analyzed the developmental status of *Oca-b*$^{-/-}$ cells throughout a time course of IL-7 exposure and subsequent removal. IL-7 removal in this type of culture system serves to promote further

Figure 3.2.4 Altered Response of *Oca-b*$^{-/-}$ B Cells to IL-7



A. CD19^{+} cells were isolated from bone marrow of *Oca-b*$^{+/+}$ or *Oca-b*$^{-/-}$ mice and cultured with media containing IL-7 for up to 7 days. Live cells were counted using trypan blue exclusion to measure proliferation during the time course of IL-7 exposure (time points labeled). B. Analysis of cell size (FSC-H) during the IL-7 time course using FSC-H.

Figure 3.2.5 OCA-B Expression in IL-7 Cultured B Cells



Whole cell extracts from *Oca-b*$^{+/+}$ CD19^{+} cells cultured with IL-7 were prepared at the indicated time points. After IL-7 removal, the culture was continued for 52h. OCA-B expression was monitored by immunoblot.

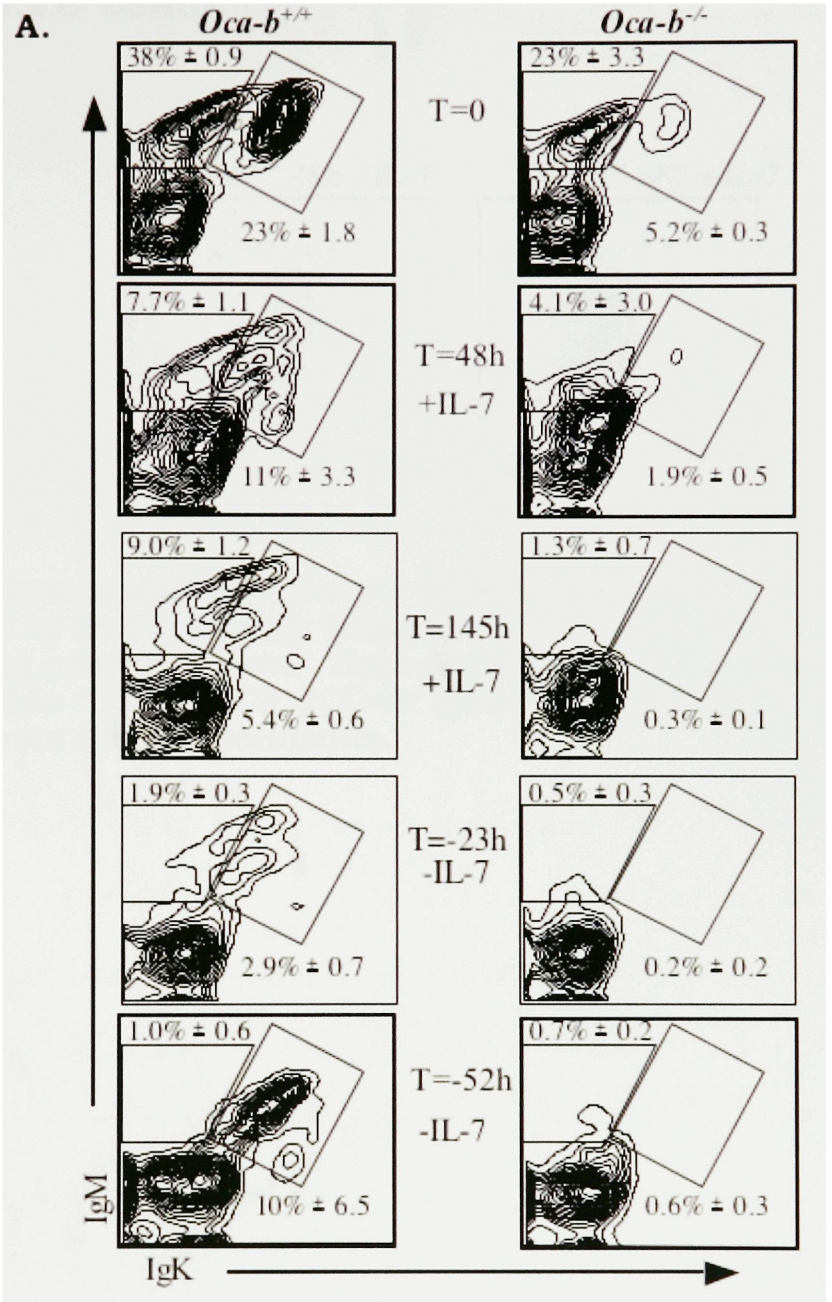
differentiation upon successful rearrangement of the immunoglobulin light chain (IgL), which is mediated by pre-BCR signaling (Rolink et al., 1991). The prevalence of surface kappa$^+$ cells following IL-7 removal can thus serve as an indicator of differentiation (Rolink et al., 1991; Ray et al., 1998). As expected, by 52 hours post-IL-7 removal, the wildtype culture had re-established a μ^+/κ^+ population (10%) (Figure 3.2.6a and Table 3.2.1). This differentiation step did not occur (within the given time period) in the rapidly proliferating *Oca-b*$^{-/-}$ culture such that, at 52 hours post-IL-7 removal, only 0.6% of live *Oca-b*$^{-/-}$ cells are $\mu+/\kappa+$ (Figure 3.2.6a and Table 3.2.1). A failure to produce surface kappa positive cells upon removal of IL-7 from the *Oca-b*$^{-/-}$ cells further suggests a defect in pre-BCR signaling, as opposed to an increased IL-7 sensitivity. It should be noted that both the wildtype and *Oca-b*$^{-/-}$ cultures display a similar decrease in viability upon IL-7 removal, as assayed by flow cytometry using propidium iodide (data not shown). As expected, the existing $\mu+/\kappa+$ cells that were present at the onset of the time course (T=0) died off at a similar rate due to lack of IL-7 responsiveness in both the wildtype and knockout cultures (T=0, T=48, T=145, Table 3.2.1). As previously reported (Hess et al., 2001), there were fewer IgM$^+$/Igκ^+ cells at T=0 in *Oca-b*$^{-/-}$ mice, which is due to a reduction of the re-circulating B cell pool. Additional analysis of cell surface marker expression over the time course showed that the *Oca-b*$^{-/-}$ culture had an increased percentage of cells expressing the pre-BCR (SL-156) after 48 hours of IL-

Table 3.2.1 Analysis of Cell Surface Marker Expression During the IL-7 Time Course

	IL-7 Time (hours)	CD43⁺/CD25⁻ Pro-B/Pre-B1	CD43⁺/CD25⁺ Pre-B1	CD43⁻/CD25⁺ Pre-B2	μ^{Det}/κ^{-}	μ^{Det}/κ^{+}
WT	0	3.9 ±0.7	5.2 ±0.9	42 ±2.1	38 ±0.9	23 ±1.8
Oca-b$^{-/-}$		6.9 ±0.3	6.3 ±0.3	31 ±2.7	23 ±3.3	5.2 ±0.3
WT	48	45 ±8.2	5.4 ±1.0	20 ±1.6	7.7 ±1.1	11 ±3.3
Oca-b$^{-/-}$		63 ±9.0	11 ±0.9	9.2 ±0.1	4.1 ±3.0	1.9 ±0.5
WT	145	58 ± 5.2	4.6 ± 1.7	10 ± 0 .6	9.0 ± 1.2	2.9 ± 0.7
Oca-b$^{-/-}$		36 ± 11	16 ± 4.0	8.9 ± 2.2	1.3 ± 0.7	0.3 ± 0.1
WT	-22	55 ±4.6	20 ± 6.2	21 ± 3.9	1.9 ± 0.3	5.4 ± 0.6
Oca-b$^{-/-}$		66 ±5.9	22 ± 3.2	9.3 ± 1.9	0.5 ± 0.3	0.2 ± 0.2
WT	-52	2.4 ± 1.8	3.0 ± 1.8	n.g	1.0 ± 0.6	10 ± 6.5
Oca-b$^{-/-}$		2.9 ± 0.2	2.2 ± 1.2	n.g.	0.7 ± 0.2	0.6 ± 0.3

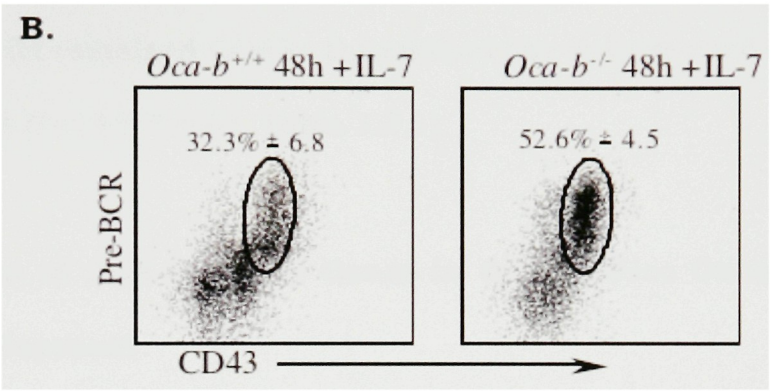
Samples were removed from the IL-7 cultures at the above time points and prepared for flow cytometry analysis. Antibodies against the above surface markers were used to analyze cellular populations within the WT and *Oca-b*$^{-/-}$ cultures. The values shown are averages from three experiments. Not gated (n.g.)

Figure 3.2.6 Defective Pre-BCR-mediated Differentiation of *Oca-b*$^{-/-}$ Cells



A. CD19^{+} cells were isolated from bone marrow by MACS from 10 *Oca-b*$^{+/+}$ or *Oca-b*$^{-/-}$ mice and cultured with media containing IL-7 for up to 142h. After IL-7 removal, the culture was continued for 52h. Ig κ positive cells were monitored by flow cytometry. The increase in IgM^{+}/Igκ^{+} cells upon IL-7 removal in the *Oca-b*$^{+/+}$ culture indicates further differentiation. The percentages are average values from three experiments.

Figure 3.2.6- Continued



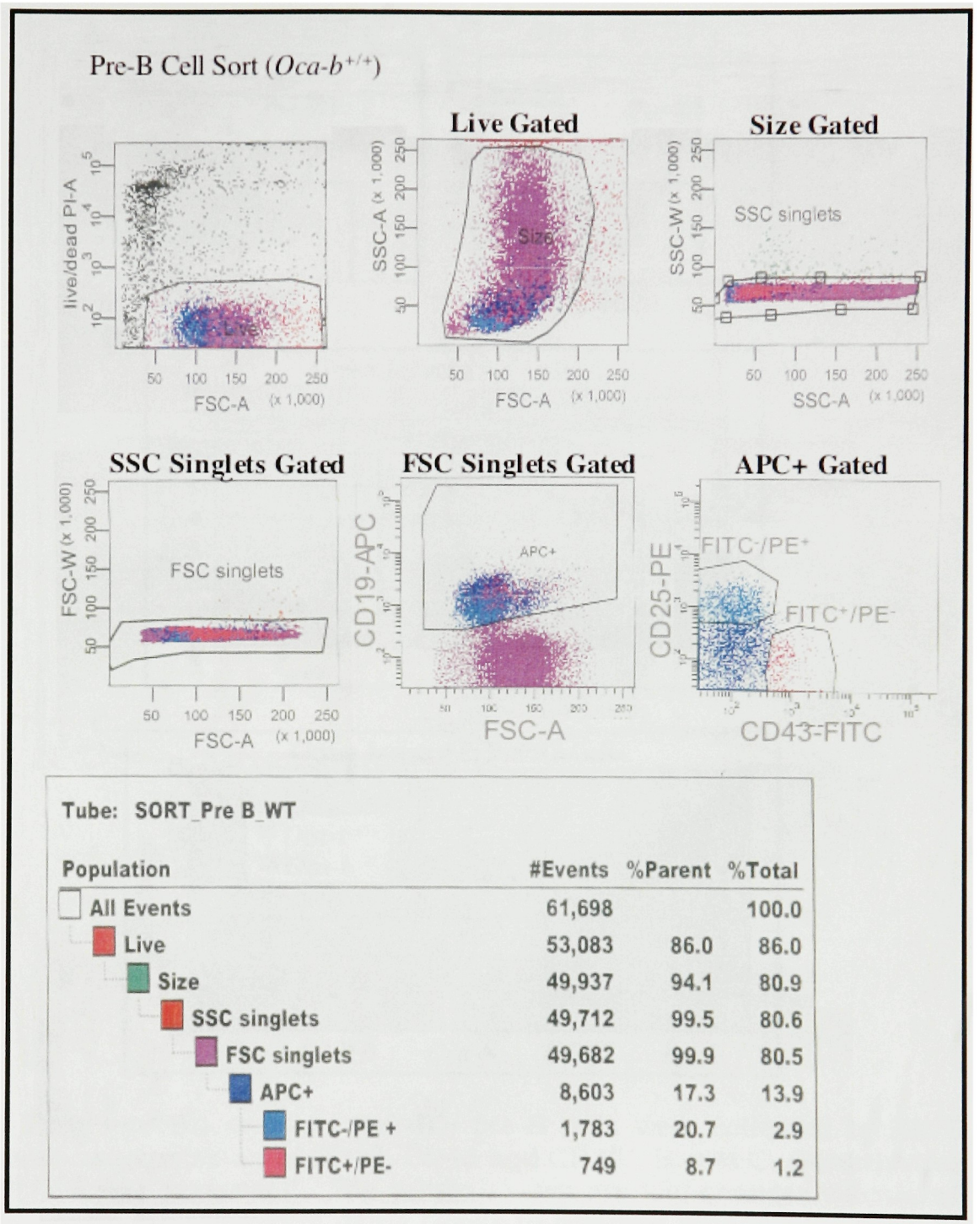
B. Surface expression of the pre-BCR during the time course was analyzed by flow cytometry using the SL-156 antibody; and the 48-hour time point is shown. The values shown are percentages of live cells within the pre-BCR$^+$/CD43$^+$ gate. The percentages are average values from three experiments.

7 exposure (31.2% vs. 52.4%) (Figure 3.2.6b). As expected, CD43 and the pre-BCR were co-expressed on the cell surface, indicating the specificity of the SL-156 antibody (Figure 3.2.6b). A greater proportion of *Oca-b*$^{-/-}$ cells remained CD43+ throughout the culture even after IL-7 was removed (T=145, T=-22, Table 3.2.1).

Deregulation of OCA-B Target Genes in *Oca-b*$^{-/-}$ Mice Occurs Early in B Cell Development

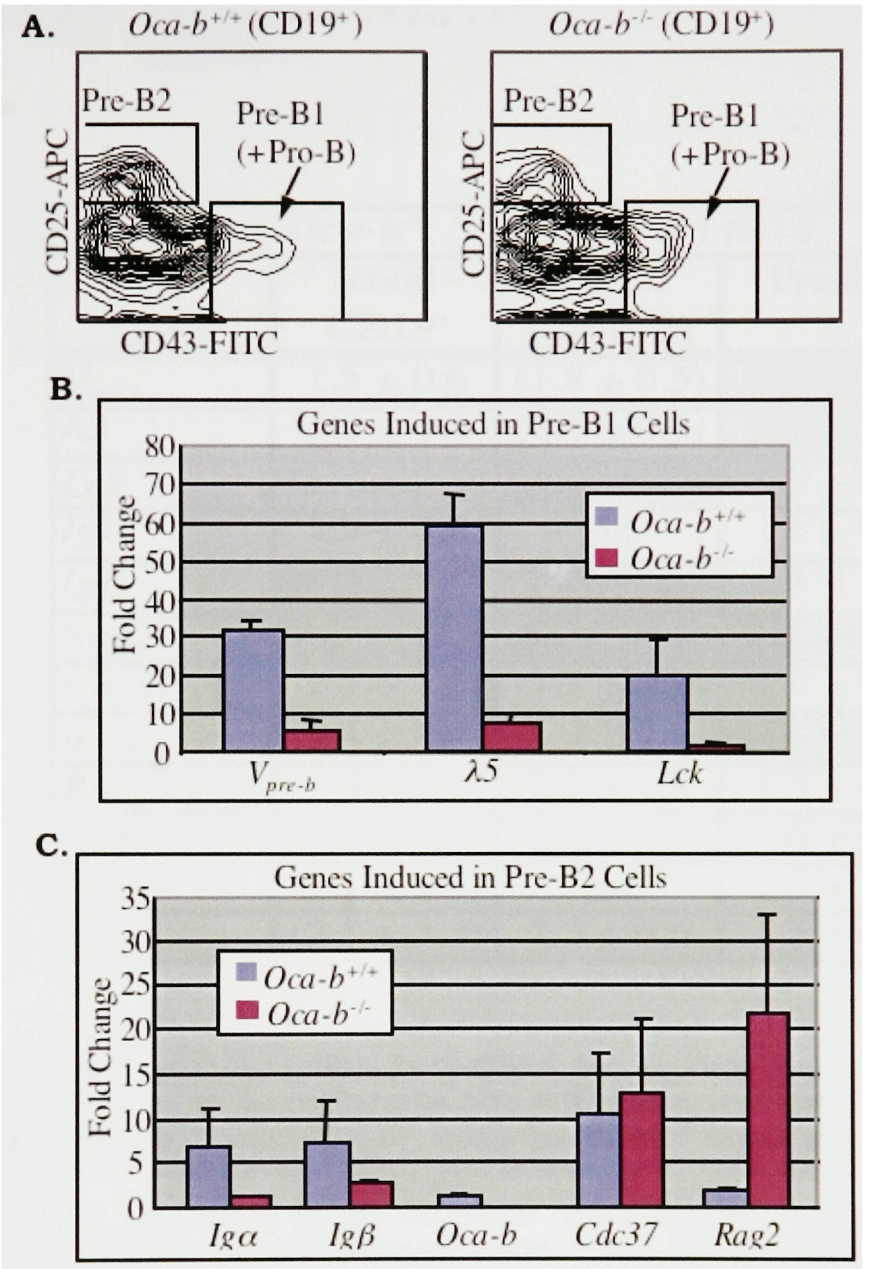
In order to further explain the impaired response of *Oca-b*$^{-/-}$ cells, we wanted to determine if the expression levels of key genes involved in pre-BCR signaling or other identified OCA-B target genes could be contributing to the effect. Pre-B1 cells (CD19+/CD43+/CD25-) and pre-B2 cells (CD19+/CD43-/CD25+) were fractionated from wildtype or *Oca-b*$^{-/-}$ mice by FACS (Figure 3.2.7). It should be noted that the markers used for fractionation of these two populations groups pro-B cells into the pre-B1 cell fraction; however, the developmental block remains apparent when this fractionation scheme is applied (Figure 3.2.8a). Quantitative RT-PCR was then used to determine the expression levels of $V_{pre\text{-}B}$, *λ5*, *Igβ*, *Igα*, $\mu_{constant}$, *Lck*, *Cdc37*, *Btk*, *Lyn*, *Fyn*, and *Syk* in the freshly isolated cells (Figure 3.2.8b,c and Table 3.2.2). As expected, up-regulation of and *λ5* was observed in the pre-B1 fraction relative to the pre-B2 fraction in both wildtype and *Oca-b*$^{-/-}$ mice (Figure 3.2.8b). There was also a substantial (20-fold) induction of *Lck* gene expression in the

Figure 3.2.7 FACS to Isolate Pre-B B Cells



Bone marrow B cells were collected by FACS based on CD19-APC, CD25-PE, and CD43-FITC surface marker expression. PI-negative cells were gated as live cells. The live cells were then gated for size, followed by gating for FSC and SSC singlets. These gates were used to gate APC+ cells from which the cellular fractions were collected based on CD25, and CD43 staining.

Figure 3.2.8 Expression Levels of Critical Genes for Pre-B Cell Signaling in *Oca-b*$^{-/-}$ Mice



A. Early (pre-B1) and late (pre-B2) pre-B cells were collected by FACS based on expression of CD19, CD43 and CD25. B. and C. Quantitative RT-PCR was performed. The values shown are fold changes calculated by relative quantification using *Gapdh* to compare RNA levels in pre-B1 versus pre-B2 cells for each genotype using the equations: ratio=$(E_{target})^{\Delta CT(Oca\text{-}b+/+preB1\text{-}Oca\text{-}b+/+preB2)}/(E_{GAPDH})^{\Delta CT(Oca\text{-}b+/+preB1\text{-}Oca\text{-}b+/+preB2)}$ or ratio=$(E_{target})^{\Delta CT(Oca\text{-}b-/-preB1\text{-}Oca\text{-}b-/-preB2)}/(E_{GAPDH})^{\Delta CT(Oca\text{-}b-/-preB1\text{-}Oca\text{-}b-/-preB2)}$. Genes that are induced in pre-B1 cells or pre-B2 cells are shown in B. and C., respectively. All of the values shown are averages (with standard deviations) generated from three to five independent experiments.

Table 3.2.2 Expression Levels of Genes Critical for Pre-B Cell Signaling

Oca-b$^{+/+}$ vs. *Oca-b*$^{-/-}$ (less in *Oca-b*$^{-/-}$) N=3-5			
Gene	Total CD19^{+}	Pre-B1 (+Pro-B)	Pre-B2
$V_{pre\text{-}B}$	1.5 ± 0.6	(1.8 ± 0.5)	3.6 ± 1.4
$\lambda 5$	1.5 ± 0.8	(2.8 ± 0.8)	4.1 ± 0.4
Lck	(1.7 ± 0.3)	(5.6 ± 1.7)	1.9 ± 0.7
Igα	1.0 ± 0.2	nd	1.6 ± 0.6
Igβ	(8.0 ± 3.8)	(5.0 ± 1.9)	(1.4 ± 1.0)
Rag2	21 ± 7.8	(5.2 ± 2.3)	3.2 ± 1.4
Cdc37	2.1 ± 1.2	(1.3 ± 0.1)	1.9 ± 0.7
$\mu_{constant}$	1.1 ± 0.4	1.2 ± 0.3	(3.8 ± 3.6)
Btk	(1.5 ± 0.7)	2.2 ± 1.8	1.3 ± 0.4
Lyn	1.9 ± 0.8	1.6 ± 0.9	1.0 ± 0.3
Fyn	1.1 ± 0.4	nd	2.3 ± 1.4
Syk	(2.1 ± 0.4)	2.3 ± 0.6	1.5 ± 0.5

Quantitative RT-PCR was performed as in Figure 3.2.8. A similar method was used to determine the difference in expression of genes between *Oca-b*$^{+/+}$ and *Oca-b*$^{-/-}$ cells using the equation: ratio=$(E_{target})^{\Delta CT(Oca\text{-}b+/+preB1\text{-}Oca\text{-}b\text{-}/\text{-}preB1)}/(E_{GAPDH})^{\Delta CT(Oca\text{-}b+/+preB1\text{-}Oca\text{-}b\text{-}/\text{-}preB1)}$ for each cell stage. A negative value denotes that *Oca-b*$^{-/-}$ cells have fewer transcripts relative to wildtype cells. All of the values shown are averages (with standard deviations) generated from three to five independent experiments.

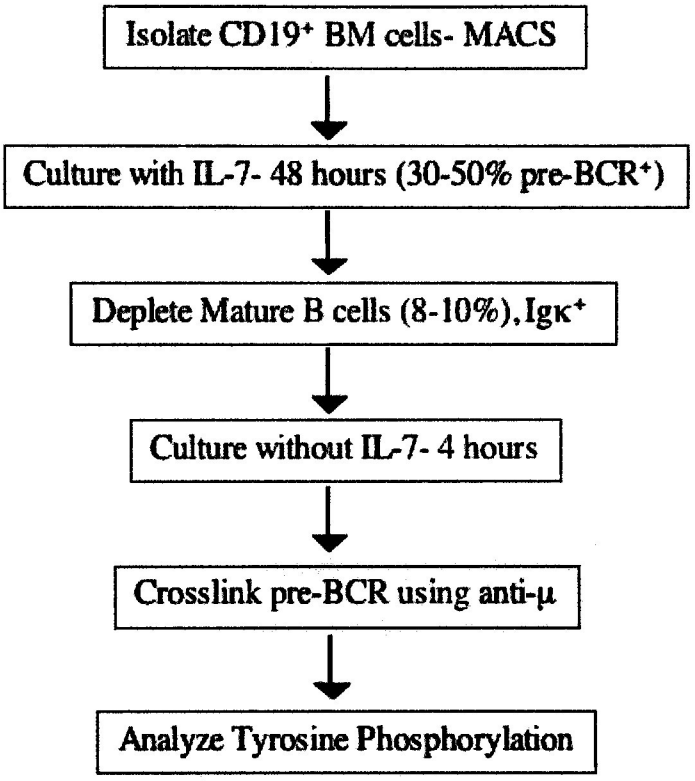
pre-B1 fraction, which was not apparent in *Oca-b*$^{-/-}$ cells (Figure 3.2.8b). Conversely, several of the analyzed genes were expressed more highly in the pre-B2 fraction. These included *Igα* (7-fold), *Igβ* (7-fold), and *Cdc37* (11-fold) (Figure 3.2.8c). OCA-B was expressed at a relatively similar level in the wildtype pre-B1 and pre-B2 fractions (Figure 3.2.8c). It is difficult to compare levels of induction between the pre-B1 and pre-B2 fractions of wildtype and *Oca-b*$^{-/-}$ mice because there may be differences in gene regulation at each stage of B cell development. In order to avoid this issue, we compared the relative expression (normalized to GAPDH) of these genes at each stage of development for wildtype versus *Oca-b*$^{-/-}$ mice (Table 3.2.2). Using this method of analysis, the *Oca-b*$^{-/-}$ pre-B1 fraction showed significantly reduced expression (shown by parentheses) of two genes, *Igβ* and *Lck* that are involved in B cell signaling mechanisms (Table 3.2.2). Two of the genes analyzed, $V_{pre\text{-}B}$ and *λ5*, were also over-expressed in *Oca-b*$^{-/-}$ pre-B2 cells (Table 3.2.2). The RNA levels of other kinases involved in B cell signaling, such as, BTK, LYN, FYN, and SYK, were relatively unaffected by the OCA-B deficiency (Table 3.2.2). Expression of *Cdc37*, a newly identified OCA-B target gene in peripheral B cells (Kim et al., 2003), was not affected at either stage of pre-B cell development (Table 3.2.2).

Analysis of Pre-BCR Signaling in *Oca-b*$^{-/-}$ Cells

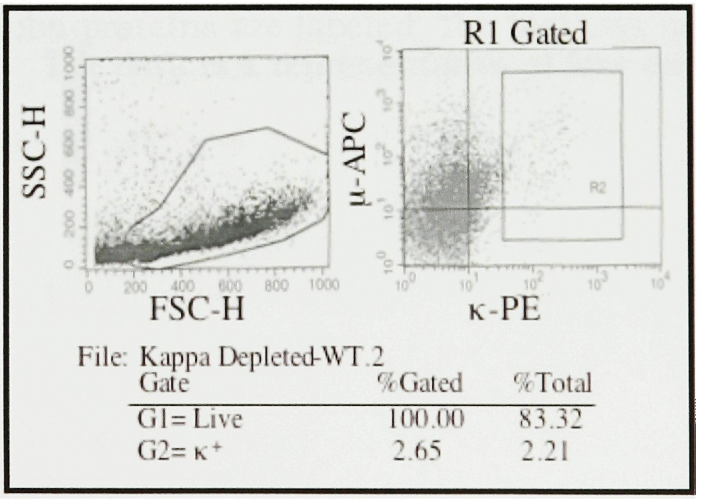
Although we did observe alterations of gene expression in *Oca-b*$^{-/-}$ mice, it remained possible that *Oca-b*$^{-/-}$ mice could be defective in cell signaling mechanisms; therefore, we directly analyzed pre-BCR function in *Oca-b*$^{-/-}$ cells. To study pre-BCR signaling capacity, the receptor was cross-linked using anti-μ after 48 hours of IL-7 stimulation, which allowed for the accumulation of pre-BCR^{+} cells (30-50% CD19^{+}/CD43^{+}/SL-156^{+}) (Figure 3.2.6b and Figure 3.2.9). To avoid cross-linking the mature BCR (up to 20% of wildtype cells) (Figure 3.2.9), kappa^{+} cells were depleted prior to stimulation with anti-μ. Flow cytometry analysis of the depleted population confirmed a near absence of mature cells after depletion (98% purity) (Figure 3.2.9b). As expected, pre-BCR signaling, as measured by global tyrosine phosphorylation, peaked at 5 minutes in the wildtype samples (Figure 3.2.9c). The *Oca-b*$^{-/-}$ samples showed reduced tyrosine phosphorylation in response to pre-BCR cross-linking. Additionally, a reduction in phosphorylation was observed in the knockout culture at time 0 (Figure 3.2.9c). These data indicate that OCA-B may function more directly in B cell signaling pathways; however, given that LCK is an OCA-B target gene (Chapter 2 and Kim et al., 2003), it is possible that reduced LCK expression could also contribute to the early B cell defect of *Oca-b*$^{-/-}$ cells. In order to examine this possibility we generated an LCK retroviral expression vector (Figure 3.2.10a) and infected CD19^{+} bone marrow B cells with LCK-expressing virus; however, expression of LCK

Figure 3.2.9 Analysis of Pre-BCR Function in *Oca-b*$^{-/-}$ Cells

A. Procedure to Analyze Pre-BCR Signaling



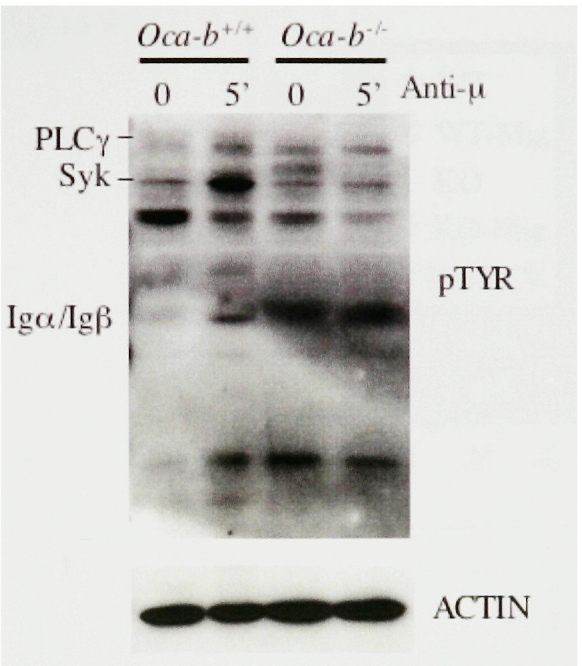
B. Igκ-depleted Fraction



A. Procedure to analyze pre-BCR signaling. B. After depletion of kappa^{+} cells, anti-IgM F(ab')$_2$ fragment was used to crosslink the pre-BCR for various lengths of time.

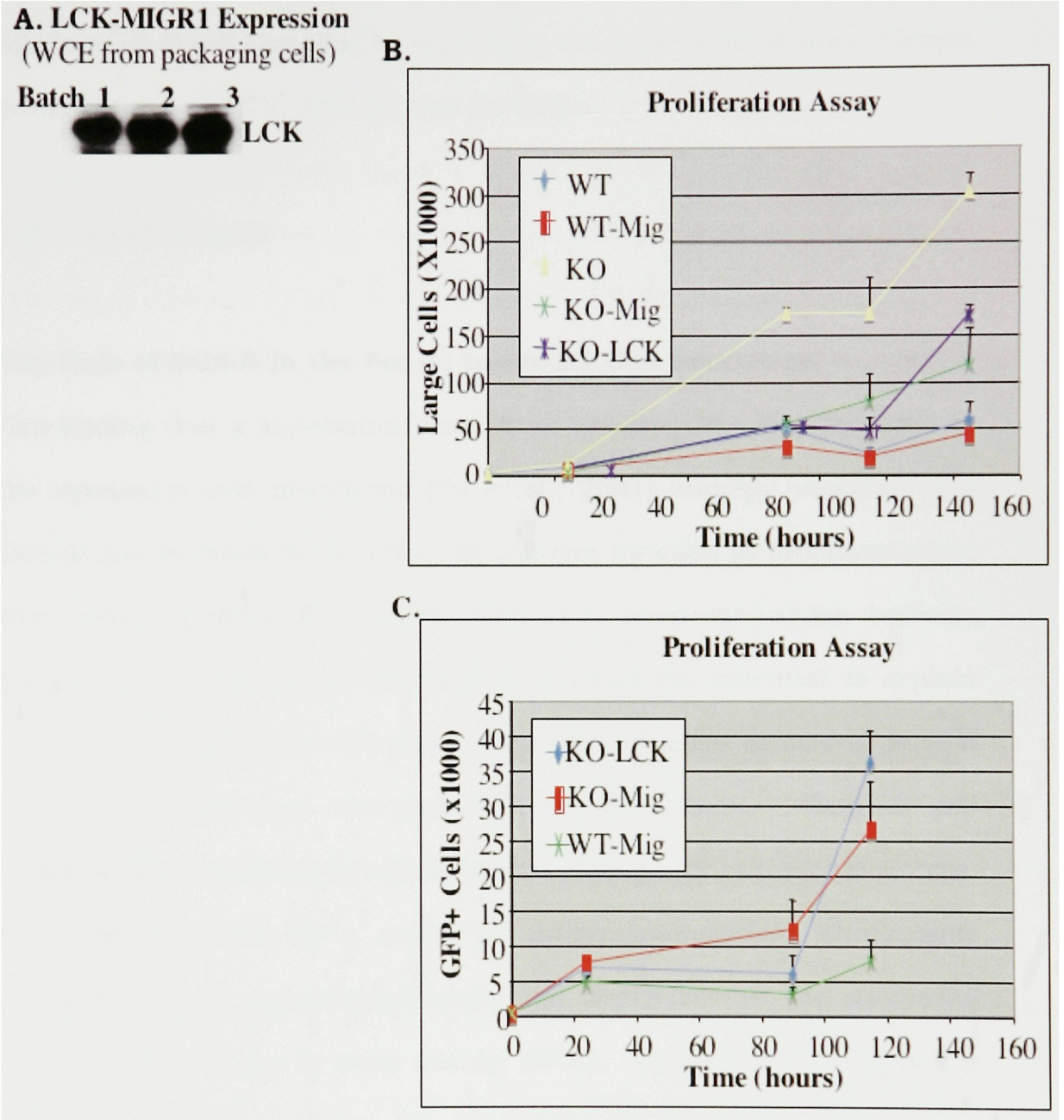
Figure 3.2.9- Continued

C. Global Tyrosine Phosphorylation



C. Tyrosine phosphorylation in the cellular extract was monitored using anti-phosphotyrosine (pTYR). The bands corresponding to relevant phospho-proteins are labeled. The blot was normalized using ACTIN. The data is a representative of four experiments.

Figure 3.2.10 Analysis of *Oca-b*$^{-/-}$ Cell Proliferation After Infection with LCK-MIGR1



A. Expression of LCK protein from the retroviral construct was confirmed by immunoblot. B and C. CD19+ cells from WT or *Oca-b*$^{-/-}$ (KO) mice were isolated as previously described. The cells were infected with LCK-GFP-MIGR1 (LCK) or empty-GFP (MIGR1) virus and cultured as previously described. Proliferation of large cells (panel B) or GFP+ cells (panel C) was monitored by flow cytometry.

was unable to prevent the hyperproliferation of *Oca-b*$^{-/-}$ cells in response to IL-7 (Figure 3.2.10b,c). These data indicates that OCA-B may play a role in early B cell signaling by regulating the expression of more relevant proteins such as BTK, SLP-65, and/or SYK.

3.3 DISCUSSION

The Role of OCA-B in the Pre-B1 to Pre-B2 Cell Transition

The finding that a substantial fraction of cellular OCA-B is localized to the cytoplasm and membrane (Yu et al., 2001) strongly suggests that OCA-B has multiple roles in the cell and can function in processes other than transcription. The identification of a novel interaction between OCA-B and SYK supports this notion and has the potential to explain many aspects of the *Oca-b*$^{-/-}$ phenotype-- including the defects in early B cell development that are presented in this study. Early B cell development is marked by characteristic changes in cellular phenotype, that are regulated by a series of antigen-independent checkpoints mediated by cell signaling pathways and dependent on the successful rearrangement of the Ig locus (Hardy, 2003). Expression and signaling from the pre-BCR mediates the progression of developing B cells, first into large (cycling) pre-B1 cells and then into small pre-B2 cells in which light chain rearrangement occurs.

The pre-B1 to pre-B2 transition is marked not only by a change in cell size but can be defined by changes in the display of cell surface markers such as CD43, CD117, CD2, pre-BCR, and CD25 (IL-2R) (Hardy and Hayakawa, 2001). By comparing the display of cell surface markers on wildtype and *Oca-b*$^{-/-}$ bone marrow B cells, we have found that knockout mice have a significantly increased proportion of large B220^{+} cells. Likewise, these mice have an increased B220^{+}CD43^{+}CD25^{-} compartment (mostly large cells) and, reciprocally, a decreased B220^{+}CD43^{-}CD25^{+} compartment. This change represents a partial block in the pre-B1 to pre-B2 stage of B cell development in *Oca-b*$^{-/-}$ mice. This defect is similar to that observed in mice deficient in *Btk*, *Slp-65*, or interferon regulatory transcription factors *Irf4* and *Irf8* (Khan et al., 1995; Jumaa et al., 1999; Pappu et al., 1999; Hayashi et al., 2000; Xu et al., 2000; Middendorp et al., 2002; Lu et al., 2003; Flemming et al., 2003). More importantly, this type of block, although more severe, has also been described in *Syk*$^{-/-}$ mice (Cheng et al., 1995; Turner et al., 1995; Saijo et al., 2003).

OCA-B and IL-7 Responsiveness

OCA-B is known to play a role in BCR signaling, as evidenced by studies showing that *Oca-b*$^{-/-}$ cells have reduced responsiveness to this signaling pathway (Kim et al., 1996). Additionally, many of the OCA-B target genes (such as *Lck*, *Cyclin D3*, *Cdc37*, and *Kcnn4*) that are induced by

BCR stimulation have now been shown to be expressed in pre-B cells. With this in mind, it seemed likely that the newly identified developmental block could be related to pre-BCR signaling. To examine this issue we utilized ex vivo cultured cells in the presence of IL-7. In mice, IL-7-mediated signaling promotes cellular proliferation and survival of IL-7Rα bearing B cells (Carsetti et al., 2000). It should be mentioned that mice with a targeted deletion of this gene have arrested B cell development at pro-B cell stage (impaired VDJ rearrangement), which occurs much earlier than the block described in this study, excluding the possibility that *Oca-b*$^{-/-}$ mice are defective in IL-7R signaling, per se. Highly relevant to this study, it is suspected that pre-BCR signaling eventually leads to the termination of IL-7-induced proliferation via an SLP-65-BTK-dependent pathway (Marshall et al., 1998; Flemming and Paige, 2002, Hendriks and Middendorp, 2004).

As reported for *Btk*$^{-/-}$ and *Slp-65*$^{-/-}$ cells in the IL-7 culture system (Middendorp et al., 2002; Flemming et al., 2003), we have found *Oca-b*$^{-/-}$ cells to be markedly hyper-proliferative in the presence of IL-7. Additionally, we have shown that there is a substantial increase and persistence of pre-BCR^{+} cells within the *Oca-b*$^{-/-}$ culture. This finding not only mimics the phenotype observed in vivo, but also further suggests that there is a build up of these cells due to inefficient mediation of IL-7Rα signaling by the pre-BCR in the deficient cells. As mentioned

previously, another major outcome of pre-BCR signaling is to promote Ig light chain rearrangement, which, if successful, is followed by display of the mature BCR on the cell surface; and SYK exerts a major function in this signaling process. In the IL-7-driven culture system, this process occurs in wildtype cells following IL-7 removal, at which point Igκ$^+$ cells can be observed. Not only does this process fail to occur in the *Oca-b*$^{-/-}$ cells, but these cells also maintain a surface phenotype characteristic of pre-B cells.

Contribution of Changes in Gene Expression to the *Oca-b*$^{-/-}$ Early B Cell Block

There are two possibilities that could explain the incomplete block of OCA-B deficient cells at the pre-B1 cell stage. The first, and less likely, situation is that these cells are simply delayed and can eventually correct themselves for transition into the pre-B2 compartment but at less than 100% efficiency. The second possibility, which is more common in the case of deficiencies affecting lymphopoeisis, is that some cells can progress developmentally despite their altered state. One way to address this issue was to analyze gene expression patterns in pre-B1 and pre-B2 cells of wildtype versus *Oca-b*$^{-/-}$ mice. In the first scenario, one would expect to observe gene expression changes only in the pre-B1 fraction. However, significant alterations in gene expression also were observed in *Oca-b*$^{-/-}$ pre-B2 cells, indicating that they cannot efficiently turn off

surrogate light chain (SLC) expression despite having cell surface markers characteristic of pre-B2 cells. Downregulation of SLC is an outcome of pre-BCR signaling and is coincident with termination of proliferation in response to IL-7 (Hendriks and Middendorp, 2004).

As mentioned previously, an increase in pre-BCR$^+$ cells was shown in the *Oca-b*$^{-/-}$ IL-7 culture, which could be due to their enhanced proliferative capacity or to an enhanced expression of pre-BCR components. We have shown that the latter possibility is unlikely because we did not find increased expression of the pre-BCR light chains ($V_{pre\text{-}B}$ and $\lambda 5$) in *Oca-b*$^{-/-}$ pre-B1 cells. Nor was there increased expression of $\mu_{constant}$ in the knockout cells, which is expected since OCA-B is a coactivator of immunoglobulin gene transcription. A pronounced decrease was observed in the reported (Mallone and Wall, 2002) OCA-B target gene Igβ. While the LCK RNA level was reduced in *Oca-b*$^{-/-}$ cells, importantly, other kinases involved in pre-BCR signaling such as, BTK, SYK, FYN, and LYN are largely unaffected at the RNA level by OCA-B deficiency in both pre-B1 and pre-B2 cells; however, it remains possible that OCA-B functions to regulate unidentified target genes involved in pre-BCR signaling. The mechanism underlying the defective pre-B cell transition and signaling in *Oca-b*$^{-/-}$ mice will be explored further in the next chapter.

CHAPTER 4

Non-transcriptional Regulation of SYK Expression by the Coactivator OCA-B is Required for Signaling at Multiple Stages of B Cell Development

4.1 PREFACE: THE MECHANISM BY WHICH OCA-B FUNCTIONS IN PRE-BCR AND BCR SIGNALING?

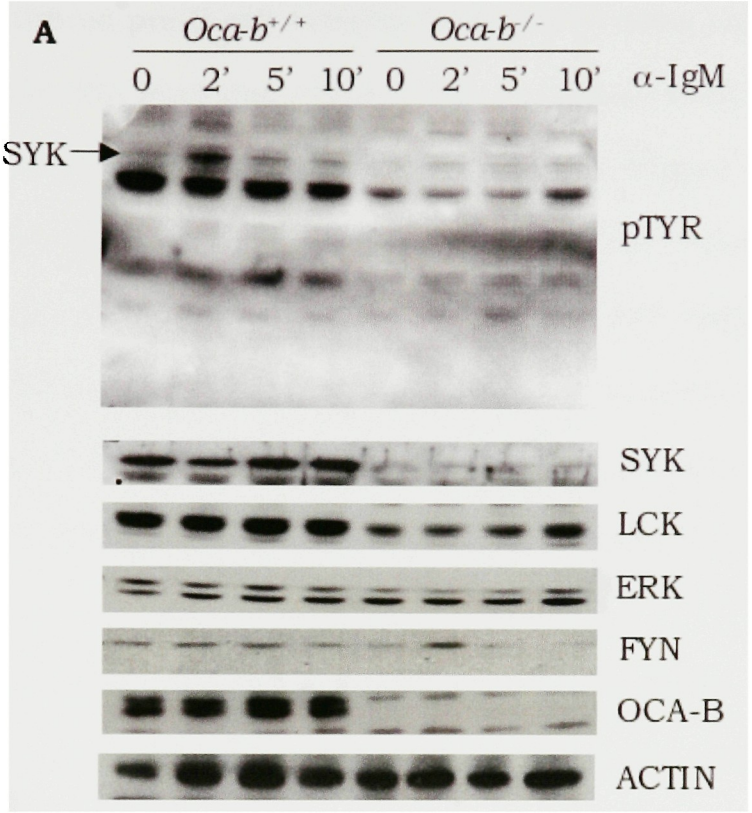
As shown in the previous chapter, analysis of early B cell surface marker expression and proliferative capacity of *Oca-b*$^{-/-}$ bone marrow B cells have revealed defects in the pre-B1 to pre-B2 cell transition, in pre-BCR signaling, and in IL-7 responsiveness. Expression studies of the recently identified OCA-B target genes, as well as, genes involved in pre-BCR signaling, have also been performed in order to access possible contributions to the observed phenotype. This analysis has revealed a reduction of the LCK and Igβ RNA levels in early B cells from *Oca-b*$^{-/-}$ mice. A reduction of the LCK RNA level was also observed in various mature B cell subsets. While the study presented in the previous chapter led to the characterization of LCK expression and transcriptional regulation by OCA-B, we suspected that OCA-B might function in pre-BCR/BCR signaling through additional mechanisms. Although LCK might be involved in B cell development, it is thought to be of marginal importance for BCR signaling in mature splenic B cells. Furthermore, the finding that a substantial fraction of cellular OCA-B is localized to the cytoplasm and membrane (Yu et al., 2001) strongly suggests that OCA-B has multiple roles in the cell and can function in processes other than transcription.

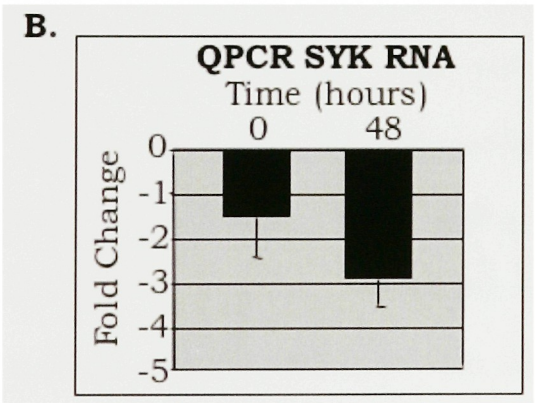
4.2 RESULTS

Further Analysis of Pre-BCR Signaling in *Oca-b*$^{-/-}$ Cells

As discussed in the previous chapter, because we had observed that *Oca-b*$^{-/-}$ pre-B cells are hyperproliferative in response to IL-7 and have reduced expression of a pre-BCR signaling component (Igβ), we then analyzed pre-BCR function in these cells. The *Oca-b*$^{-/-}$ samples showed reduced tyrosine phosphorylation in response to pre-BCR cross-linking (Figure 3.2.9). In order to further examine this defect, we performed additional pre-BCR crosslinking studies using a time course of stimulation (Figure 4.2.1a). As expected, pre-BCR signaling peaked at 2 minutes in the wildtype samples (Figure 4.2.1a, upper panel). As before, a lower initial phosphorylation was observed in the knockout culture (Figure 4.2.1a, upper panel). This interesting finding led us to examine the total protein levels of relevant kinases, and this analysis revealed that SYK expression is severely reduced in *Oca-b*$^{-/-}$ pre-B cells even in the absence of pre-BCR activation (Figure 4.2.1a, lower panel). The expression of LCK, the product of an OCA-B target gene, also was reduced, albeit less dramatically, while FYN and ERK expression levels were unaffected (Figure 4.2.1a, lower panel). Since the SYK protein level is heavily regulated by ubiquitin-mediated proteolysis (Duan et al., 2004), we wanted to determine if the SYK RNA level is also affected in the IL-7 cultured cells. Cultured CD19^{+} cells were sampled over a 96-hour

Figure 4.2.1 Analysis of Pre-BCR Function and SYK Expression in *Oca-b*$^{-/-}$ Cells





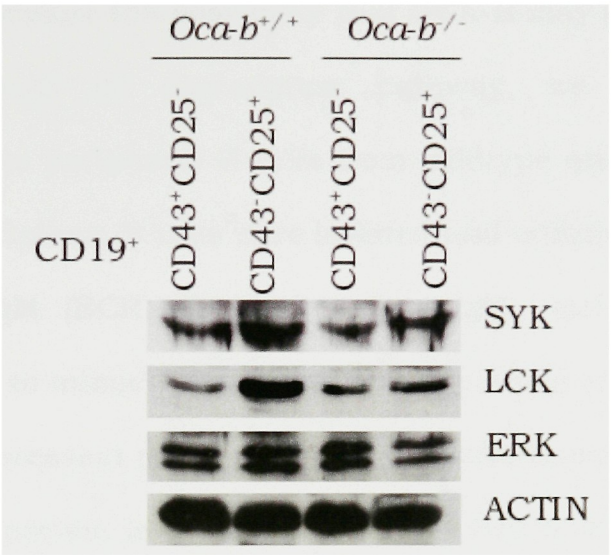
A. CD19^{+} cells were isolated from bone marrow of 12 mice and cultured in IL-7 for 48h. After depletion of kappa^{+} cells, anti-IgM F(ab')$_2$ fragment was used to crosslink the pre-BCR for various lengths of time. Tyrosine phosphorylation in the cellular extract was monitored using anti-phosphotyrosine (pTYR). The band corresponding to SYK is labeled. The total protein levels of SYK, LCK, ERK, FYN, and OCA-B were also examined. The blot was normalized using ACTIN. The data is a representative set from four experiments. B. QPCR was used to analyze SYK RNA levels in the cells that were used for the experiment in panel A. Relative quantification was used to compare the genotypes, a negative value denotes less in transcript in *Oca-b*$^{-/-}$ cells. The values shown are averages generated from three of the experiments performed for panel A.

time course of exposure to IL-7. Quantitative PCR was then used to compare the amount of SYK mRNA in *Oca-b*$^{-/-}$ versus wildtype IL-7-cultured pre-B cell samples (normalized using GAPDH). Interestingly, the SYK RNA level was reduced only marginally (2.9 fold), relative to the SYK protein level, after 48 hours of culture (Figure 4.2.1b). This coincides with the moderate reduction (2.6-fold) observed in freshly isolated pre-B cells (Table 3.2.2). Importantly, significant reductions in the SYK and LCK proteins levels were also observed in freshly isolated *Oca-b*$^{-/-}$ early B cells (Figure 4.2.2a,b), which indicates that the reduction of SYK in *Oca-b*$^{-/-}$ cells is not a by-product of the IL-7 culture system.

Regulation of SYK Expression in *Oca-b*$^{-/-}$ Cells

Since the SYK protein level was dramatically reduced in *Oca-b*$^{-/-}$ cultured pre-B cells and since it is difficult to directly compare quantitative PCR values with immunoblot data, we wished to determine if *Syk* is a direct OCA-B target gene. Two non-consensus octamer sites were identified in the predicted *Syk* promoter region. However, occupancy of these sites by OCT or OCA-B was not observed in IL-7 cultured primary cells or in the murine lymphoblastic A20 cell line by chromatin immunoprecipitation assays (not shown). Given that the chromatin immunoprecipitation assays were negative and that QPCR showed no difference in SYK RNA levels in vivo (Table 3.2.2) and only marginally during the IL-7 culture, we conclude that SYK is not a transcriptional target of OCA-B.

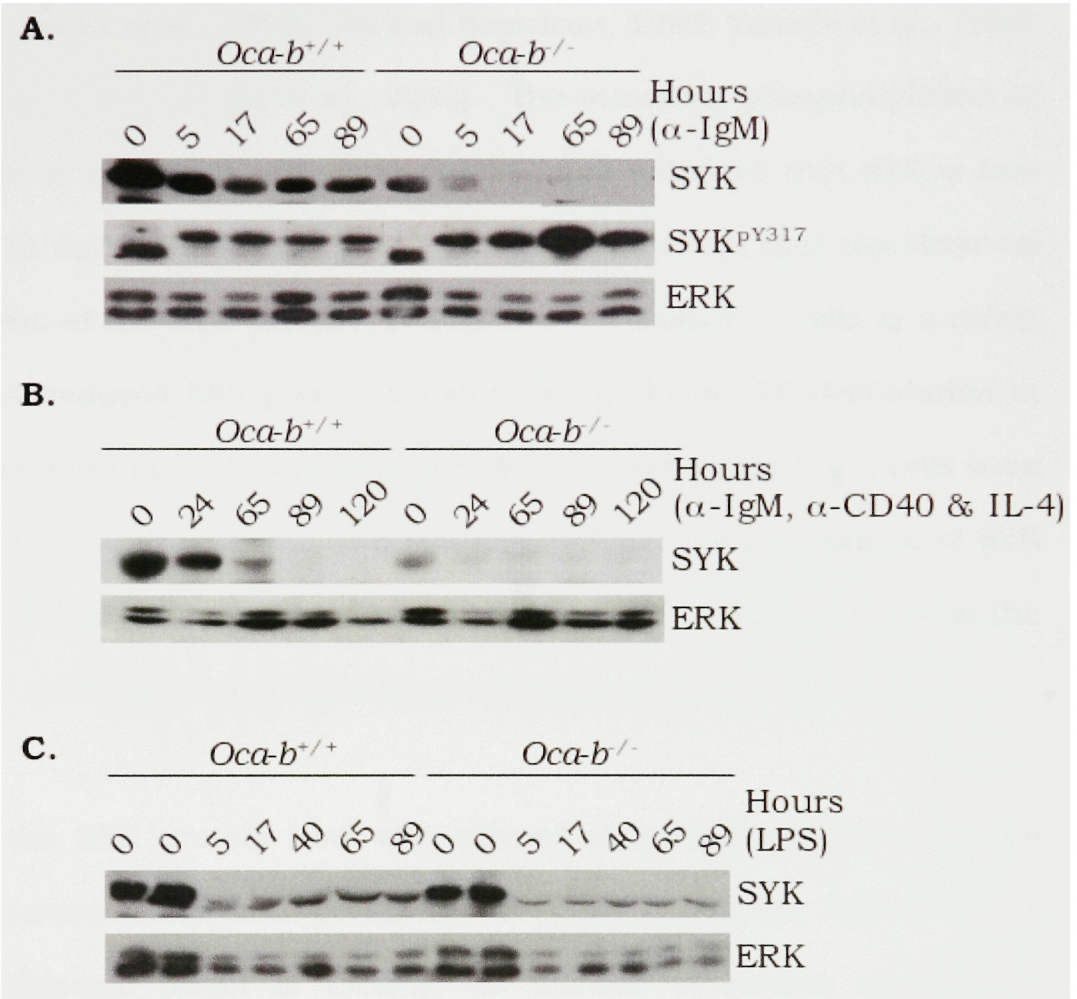
Figure 4.2.2 Analysis of SYK Protein Levels in Sorted Bone Marrow B Cells



A. Early (pre-B1) and late (pre-B2) pre-B cells were collected from 10 mice/genotype by FACS based on expression of CD19, CD43, and CD25. B. Immunoblotting was performed for SYK, LCK, ACTIN and ERK. The data is representative of two experiments.

Importantly, SYK is heavily regulated at the level of protein stability via proteosome-dependent pathways in many hematopoetic cell types (Yankee et al., 1999; Paolini et al., 2001; Paolini et al., 2002; Sohn et al., 2003). To explore the possibility that OCA-B may affect only SYK protein levels through its degradation pathway, we first examined SYK expression in peripheral B cells from wildtype and *Oca-b*$^{-/-}$ mice (Figure 4.2.3a-c). Splenic B cells were isolated and cultured for up to 120 hours with anti-IgM (BCR stimulation), anti-IgM, anti-CD40, and IL-4 (co-stimulation to mimic T cell-dependent signaling) or with LPS. Analysis of the SYK expression patterns by immunoblot revealed marked reductions in the SYK protein level for all of the activation conditions tested (Figure 4.2.3a-c). However, a less pronounced reduction of SYK expression in *Oca-b*$^{-/-}$ cells was observed when the cells were cultured in the presence of LPS as compared to what was observed for cells cultured in the presence of anti-IgM or anti-IgM, anti-CD40, and IL-4. Importantly, SYK is dynamically regulated in the wildtype cells activated with anti-IgM, anti-CD40, and IL-4, which is consistent with this type of stimulation promoting both proliferation and differentiation, as opposed to proliferation alone during activation with anti-IgM or LPS. In contrast, the protein levels of other kinases such as LYN, FYN and ERK were not affected by the OCA-B deficiency (Figure 4.2.3a-c and data not shown). There was a significant increase of SYK that is tyrosine phosphorylated in the linker region at residue 317 in the knockout cells (Figure 4.2.3a).

Figure 4.2.3 Analysis of SYK Protein Levels in Mature B Cells

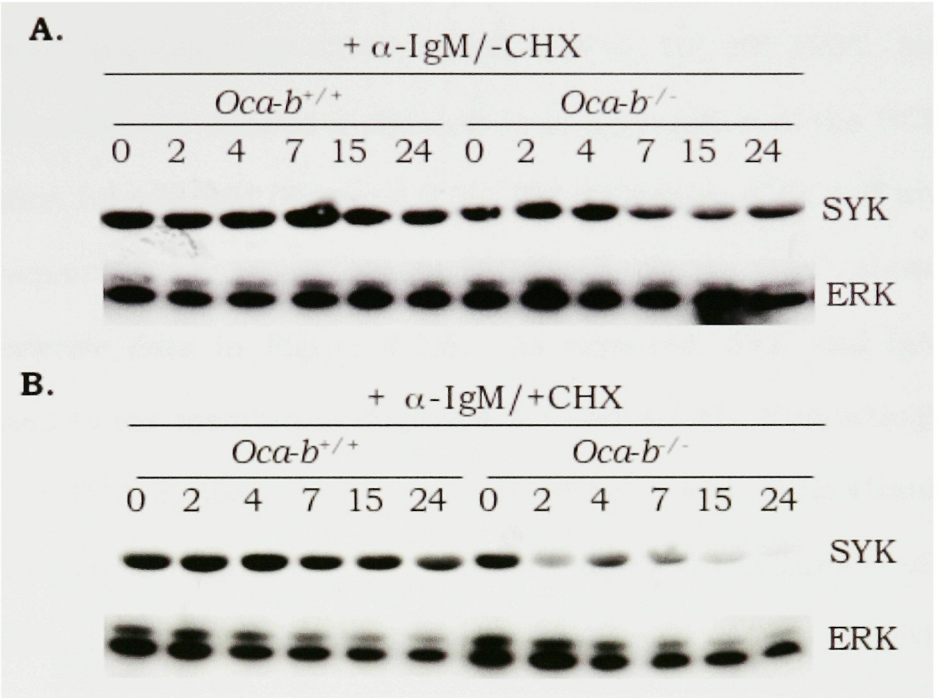


Total B cells were isolated from the spleens of 10 *Oca-b*$^{+/+}$ or *Oca-b*$^{-/-}$ mice. The cells were activated in culture with anti-IgM $F(ab')_2$ fragment (A), anti-IgM $F(ab')_2$ fragment, anti-CD40, and IL-4 (B), or LPS (C) for up to 120 hours. Expression of SYK, phosphorylated SYK at tyrosine 317 (SYK^{pY317}) and ERK were analyzed by immunoblot. The activation experiments were performed three times for this study.

SYK is phosphorylated at this residue by LYN, which then allows SYK to be targeted for ubiquitin-mediated degradation by CBL (Keshvara et al., 1998; Lupher et al., 1998; Ota and Samelson, 1999; Yankee et al., 1999; Rao et al., 2001; Hong et al., 2002). The increased phosphorylation at this site in mature B cells from *Oca-b*$^{-/-}$ mice indicates that SYK is less stable in the absence of OCA-B. In order to confirm that the observed reduction of the SYK protein level in *Oca-b*$^{-/-}$ mature B cells is a direct result of reduced SYK protein stability, we analyzed SYK degradation in the presence of cycloheximide (Figure 4.2.4). Mature resting B cells were isolated and cultured with anti-IgM and cycloheximide. Analysis of SYK protein levels by immunoblot indicated that SYK is highly unstable in the *Oca-b*$^{-/-}$ cells relative to the wildtype cells (Figure 4.2.4).

Since the SYK protein level was affected at multiple stages of B cell development (early and peripheral B cells) and since *Syk* is not a transcriptional target of OCA-B, we wanted to further explore the mechanism by which OCA-B regulates SYK stability. OCA-B is present in the cell as two isoforms, arising from different translation start sites (Yu et al., 2001). The larger form, p35, is myristoylated and is localized predominantly to the cytoplasm and plasma membrane (Yu et al., 2001); therefore, it is plausible that OCA-B and SYK could interact in the cytoplasm or at the membrane. To investigate this possibility, we performed immunofluorescence microscopy to determine if OCA-B and

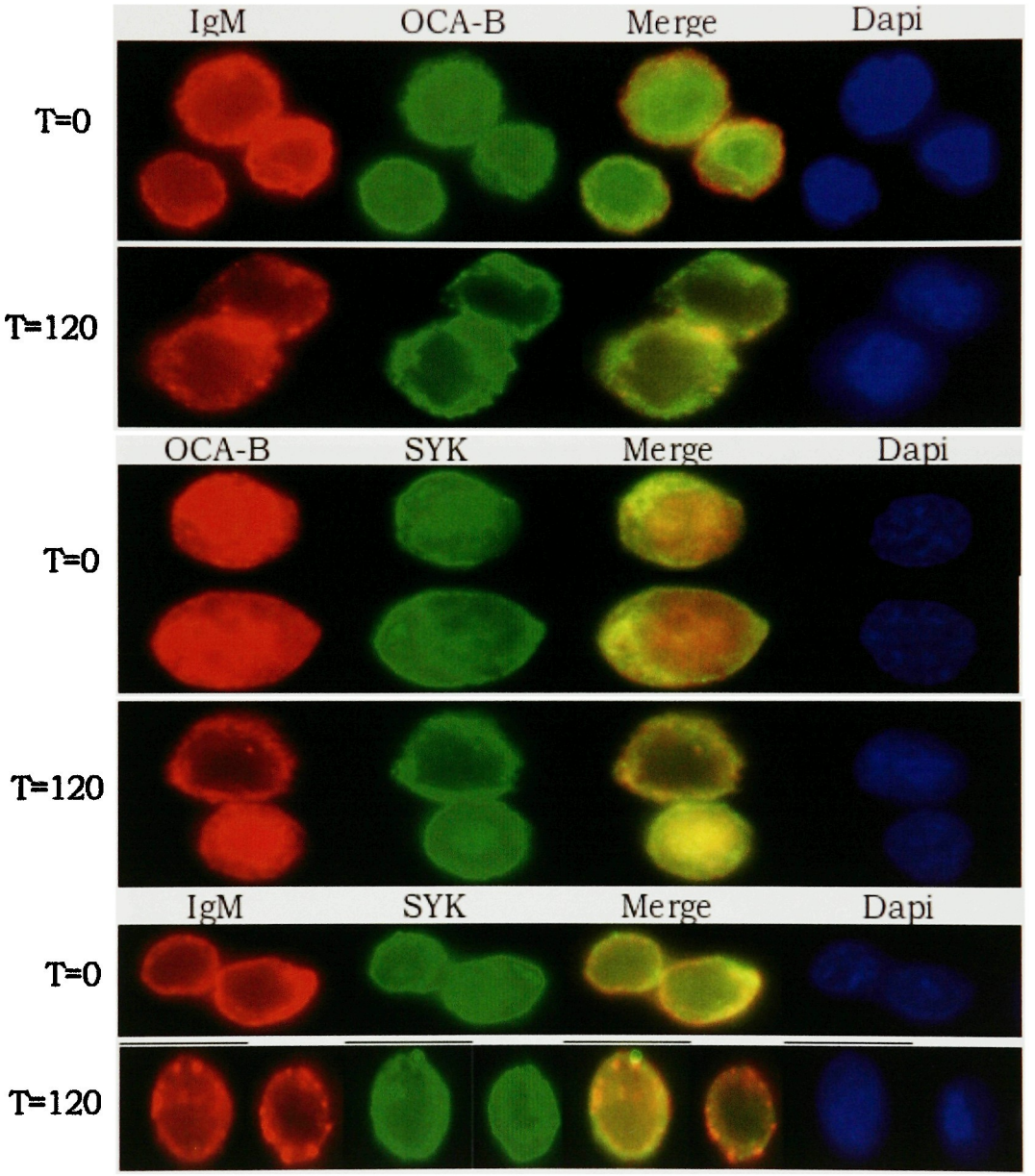
Figure 4.2.4 SYK Stability in *Oca-b*$^{-/-}$ Splenic B Cells



Resting B cells were isolated from spleens of *Oca-b*$^{+/+}$ or *Oca-b*$^{-/-}$ mice and activated with anti-IgM F(ab')$_2$ fragment for up to 24 hours either in the presence (+CHX, panel B) or absence (-CHX, panel A) of cycloheximide. The stability of SYK and ERK were monitored by immunoblot.

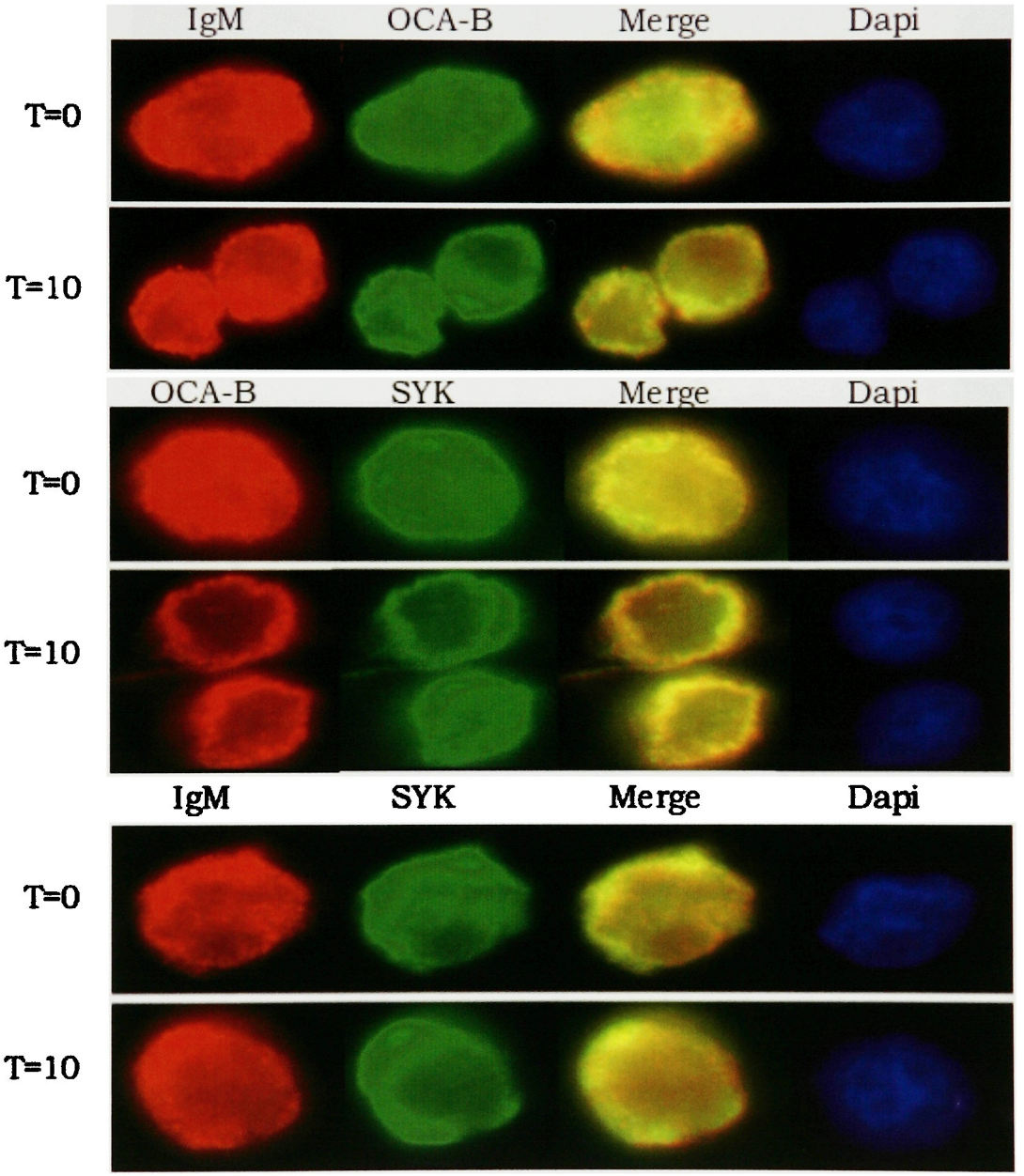
SYK colocalize. Namalwa cells, a human Burkitt's lymphoma B cell line, were used for these experiments. The localization of IgM, SYK, and OCA-B was analyzed over a time course of BCR (anti-IgM) stimulation or co-stimulation (anti-IgM, anti-CD40, and IL-4) (T=0, 10', 40', 120'). Analysis of IgM fluorescence showed a characteristic aggregation of the BCR with stimulation by anti-IgM (Figure 4.2.5). Colocalization of OCA-B and IgM was frequently observed upon BCR stimulation, as shown by representative data in Figure 4.2.5. As expected, SYK and IgM also colocalized to the membrane (Figure 4.2.5 and 4.2.6). Interestingly, the pattern of OCA-B distribution was altered both with BCR stimulation and with co-stimulation such that a significant number of cells ($p=0.0057$) showed a strong cytoplasmic localization of OCA-B by the end of the anti-IgM stimulation time course (Figure 4.2.5, 4.2.6, and 4.2.7blue). The significant increase of cells with OCA-B in this configuration during co-stimulation was more rapid, occurring within 10 minutes ($p=0.004$) (Figure 4.2.7b, red), which suggests that the redistribution of OCA-B is signal-dependant. Furthermore, colocalization of SYK and OCA-B could be observed in cells that had strong cytoplasmic staining of OCA-B (Figure 4.2.5 and 4.2.6). Similar results were also obtained by imaging Daudi cells, another Burkitt's lymphoma cell line (data not shown). Interestingly, some of the cells showed both nuclear and cytoplasmic staining for SYK (Figure 4.2.5). This correlates with Namalwa nuclear extracts having a significant amount SYK, as detected

Figure 4.2.5 Immunofluorescence Microscopy to Analyze Localization of IgM, SYK, and OCA-B upon BCR Stimulation



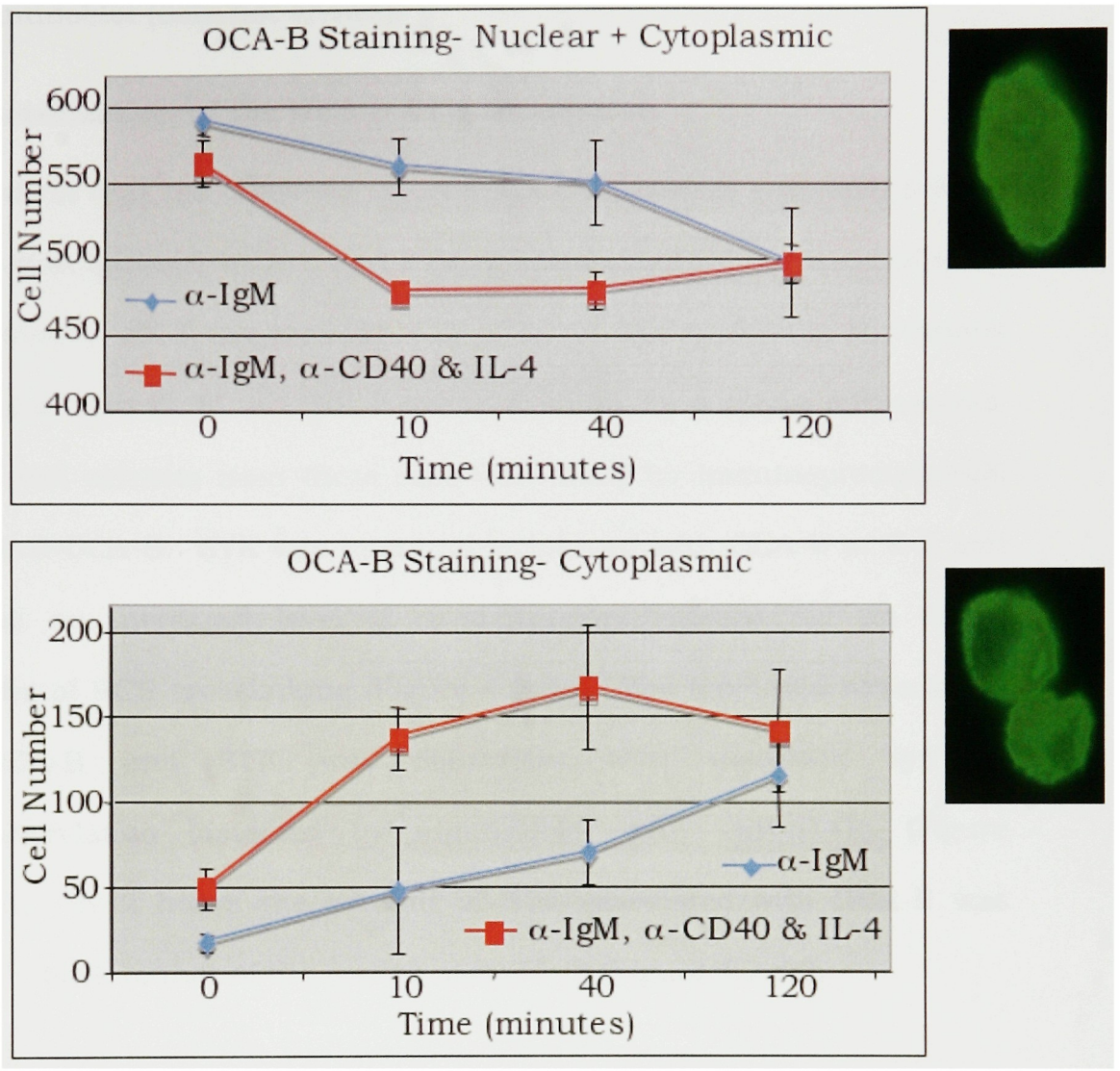
Namalwa cells were stimulated with anti-human IgM for 120 minutes (T=120) or left unstimulated (T=0). After fixation and permeabilization the localization of IgM (red-AlexaFluor546), OCA-B (green-AlexaFluor488), and SYK (green-AlexaFluor488) was analyzed. To show co-localization the red and green channels were merged (merge). DAPI was used for nuclear staining. The data is representative of three experiments.

Figure 4.2.6 Immunofluorescence Microscopy to Analyze Localization of IgM, SYK, and OCA-B upon Co-Stimulation



Namalwa cells were stimulated with anti-human IgM, anti-human CD40 and recombinant human IL-4 for up to 120 minutes or left unstimulated (T=0), the 10 minute time point is shown (T=10). The cells were stained and imaged as in panel A. The data is representative of three experiments.

Figure 4.2.7 Statistical Analysis of OCA-B Localization Patterns



The occurrence of Namalwa cells with cytoplasmic staining of OCA-B (as visualized by immunofluorescence microscopy) during a time course of stimulation is presented. For each time point, 600 cells were scored for having either nuclear and cytoplasmic (A) or mainly cytoplasmic (B) staining for OCA-B. Counting was performed for three independent experiments in order to generate the averages and standard deviations shown. With anti-IgM stimulation (shown in blue), the decrease in cells having nuclear plus cytoplasmic staining between 0 and 120 minutes has a significance of p=0.0131 (left panel) and the increase in cells having cytoplasmic staining has a significance of p=0.0057 (right panel). During co-stimulation (shown in red), the decrease in cells having nuclear plus cytoplasmic staining between 0 and 10 minutes has a significance of p=0.005 and the increase in cells having cytoplasmic staining has a significance of p=0.004.

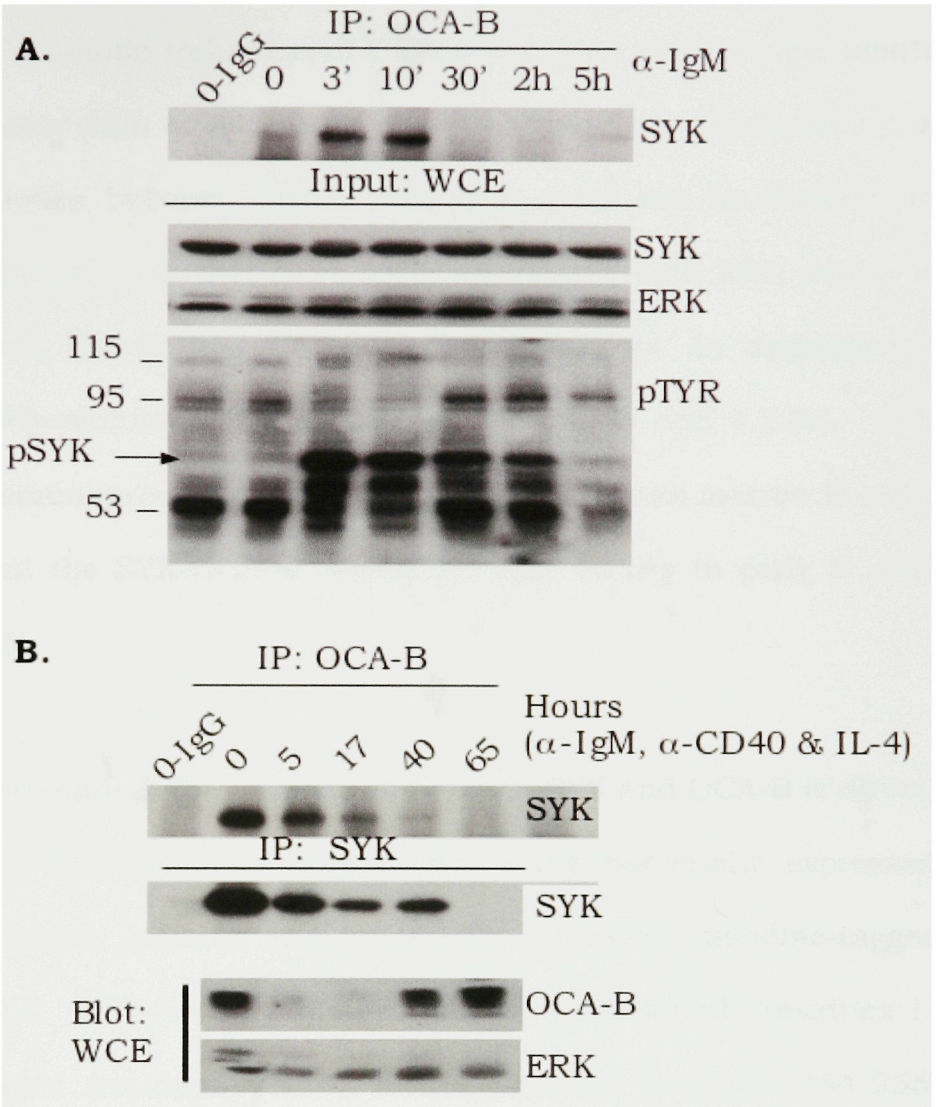
by immunoblot (data not shown).

Characterization of the OCA-B-SYK Interaction

To confirm that the observed colocalization represents a protein-protein interaction between OCA-B and SYK, we performed immunoprecipitation experiments. BCR crosslinking with anti-IgM was performed for various lengths of time (0, 3', 10', 30', 2 hours, 5 hours) on wildtype splenocytes. Whole cell extracts from these cells were used for immunoprecipitation with anti-OCA-B. SYK was immunoprecipitated with OCA-B at T=0 and showed an increased level of co-immunoprecipitation for up to 10 minutes of BCR crosslinking (Figure 4.2.8a). The increased association of OCA-B and SYK was coincident with increased tyrosine phosphorylation (detected by immunoblot with anti-pTYR) (Figure 4.2.8a). By 2 hours the amount of SYK associated with OCA-B was roughly back to that of the resting state (T=0) (Figure 4.2.8a).

Because SYK expression was reduced in *Oca-b*$^{-/-}$ mature B cells that have been activated in culture with anti-IgM, anti-CD40 and IL-4 for long periods of time, we wanted to analyze the interaction between OCA-B and SYK in this context. For this, splenocytes were co-stimulated with anti-IgM, anti-CD40, and IL-4; and an immunoblot analysis of the derived extracts revealed that the interaction decreased with time (Figure 4.2.8b). By 40 hours the association of SYK and OCA-B was marginal compared

Figure 4.2.8 Identification of the OCA-B-SYK Interaction



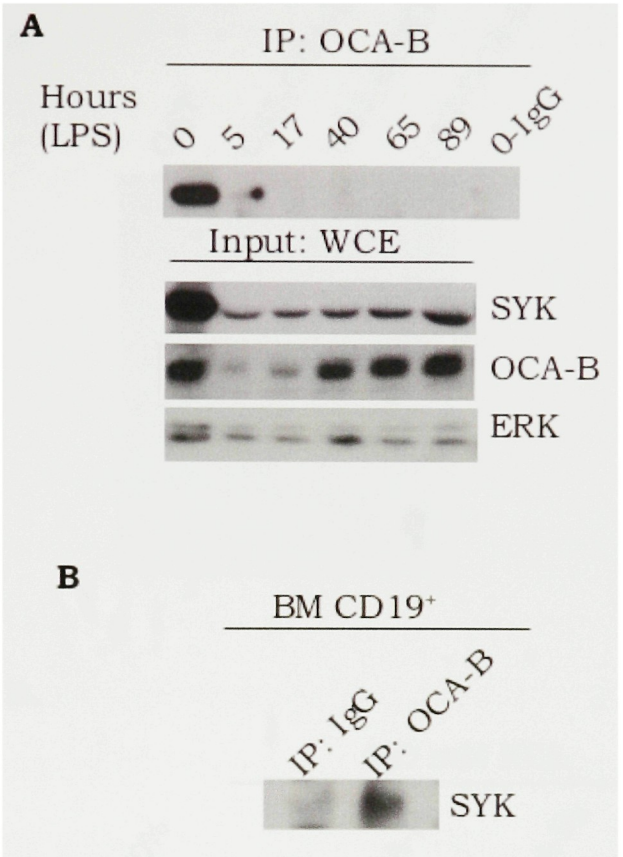
A. *Oca-b*$^{+/+}$ splenic B cells were activated in culture with anti-IgM F(ab')$_2$ fragment for a time course from T=0 to 5 hours. Immunoprecipitation (IP) was performed using anti-OCA-B or IgG (control) and whole cell extract. The presence of SYK in the IP was monitored by immunoblotting. The total level of SYK, ERK and tyrosine phosphorylated proteins (pTYR) in the whole cell extract (WCE) was also examined. B. *Oca-b*$^{+/+}$ splenic B cells were activated in culture with anti-IgM F(ab')$_2$ fragment, anti-CD40, and IL-4. Immunoprecipitation of OCA-B or SYK was detected by immunoblot. The total protein level from whole cell extract (WCE) is also shown for OCA-B and ERK (bottom panels). All of the immunoprecipitation experiments were reproduced at least four times each.

to T=0 and by 65 hours SYK protein was no longer present (Figure 4.2.8b). The dramatic induction of OCA-B expected at later time points of co-stimulation (Kim et al., 2003) also was observed. Interestingly, a lasting interaction between OCA-B and SYK could not be detected in wildtype splenic B cells that have been stimulated with LPS, which is consistent with the finding that SYK levels are not as dramatically affected in LPS-stimulated *Oca-b*$^{-/-}$ cells (Figure 4.2.9 and 4.2.3c). SYK was also coimmunoprecipitated with OCA-B in total bone marrow B cells, indicating that the SYK-OCA-B interaction also occurs in early B cells (Figure 4.2.9b).

In order to determine if the interaction between SYK and OCA-B is direct, we performed GST-pulldown experiments using bacterially expressed GST-OCA-B fusion proteins and bacterially expressed histidine-tagged full length SYK. GST-fusion proteins containing full-length (residues 1-256), N-terminal (residues 1-154), and C-terminal (residues 154-256) OCA-B were purified and normalized for the pulldown according to the amount of full-length product (Figure 4.2.10a). The GST-pulldown showed a direct interaction between SYK and OCA-B, with SYK having the highest affinity for the C-terminus of OCA-B (Figure 4.2.10b).

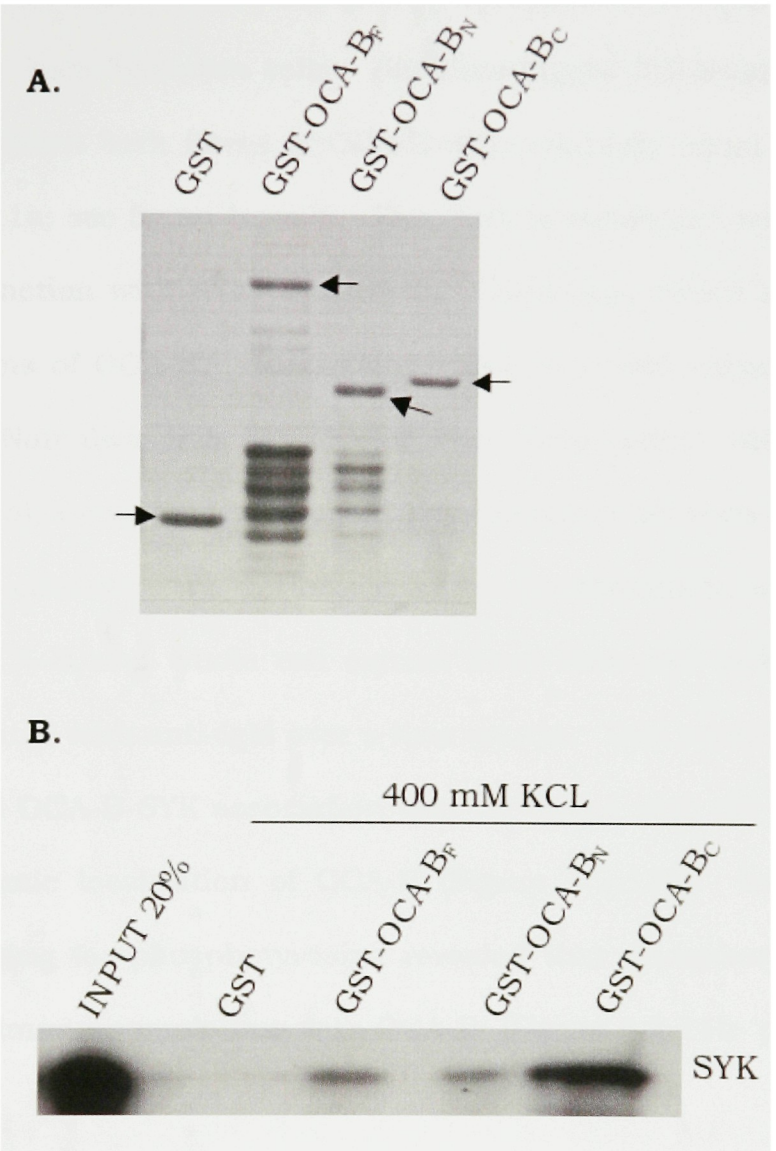
Since we observed colocalization of OCA-B and SYK in the cytoplasm (Figure 4.2.5), it was necessary to determine if SYK preferentially

Figure 4.2.9 Characterization of the OCA-B-SYK Interaction



A. *Oca-b*$^{+/+}$ splenic B cells were activated in culture with LPS for a time course from T=0 to 89 hours. Immunoprecipitation (IP) was performed using anti-OCA-B or IgG (control) and whole cell extract. The presence of SYK in the IP was monitored by immunoblotting. The total level of SYK, OCA-B, and ERK in the whole cell extract (WCE) was also examined (bottom panels). B. CD19^{+} bone marrow B cells from *Oca-b*$^{+/+}$ mice were purified by MACS and whole cell extract was prepared. Immunoprecipitation of SYK was detected by immunoblot.

Figure 4.2.10 Characterization of the OCA-B Domain Required for Interaction with SYK

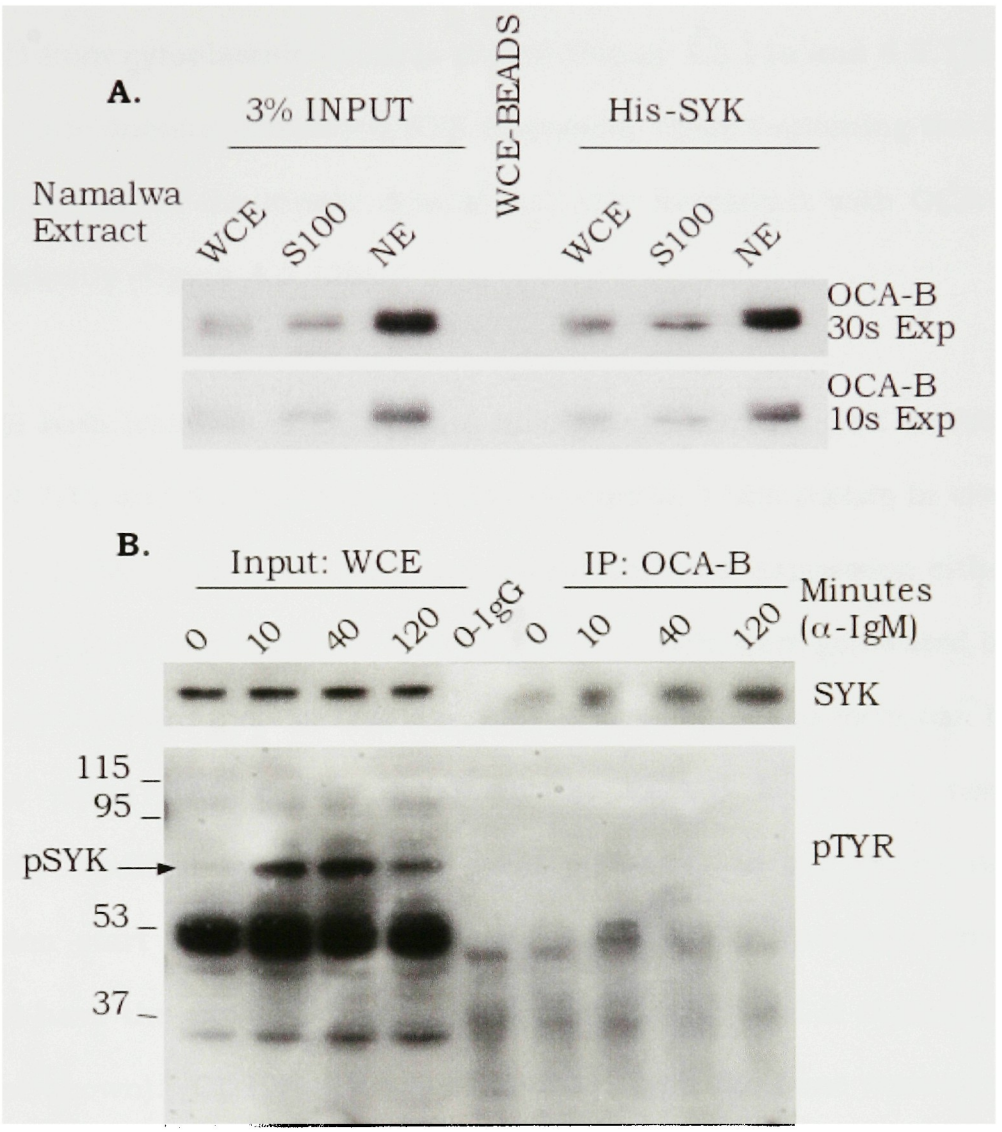


A. Coomassie blue-stained SDS-gel showing purified GST or GST-OCA-B proteins (products maked by arrows). B. Purified His-tagged SYK was incubated with immobilized GST or GST-OCA-B proteins and bound SYK was monitored by immunoblotting. This experiment was performed at least three times using up to 500 mM KCL.

associates with OCA-B p35, the myristoylated form. We performed Ni-NTA pull down assays using bacterially expressed histidine-tagged full length SYK and either whole cell (WCE), cytoplasmic (S100), or nuclear (NE) extracts from Namalwa cells. Histidine-tagged full-length SYK was able to pull down both forms of OCA-B with relatively equal efficiencies (Figure 4.2.11a; see figure legend). This data is consistent with a strong OCA-B interaction with SYK through its C-terminus, which is the same for both forms of OCA-B. Because we have observed reduced nuclear staining in Namalwa cells for OCA-B upon stimulation with anti-IgM (Figure 4.2.7a), we wanted to analyze the interaction between OCA-B and SYK in this context. We performed immunoprecipitation experiments with anti-OCA-B and whole cell extract from Namalwa cells that had been stimulated with anti-IgM over a time course. This analysis revealed an increased OCA-B-SYK association that correlated with the increase in the cytoplasmic localization of OCA-B (Figure 4.2.11b). Interestingly, immunoblotting for phosphotyrosine revealed that phosphorylated SYK does not co-immunoprecipitate with OCA-B (Figure 4.2.11b- pTYR, lower panel).

To examine the interaction between OCA-B and SYK in more detail, we mapped the SYK domain that interacts with OCA-B. Histidine-tagged-SYK proteins were generated in a manner consistent with the domains of

Figure 4.2.11 Analysis of SYK Interaction with p34- Versus p35-OCA-B and Examination of SYK Phosphorylation State

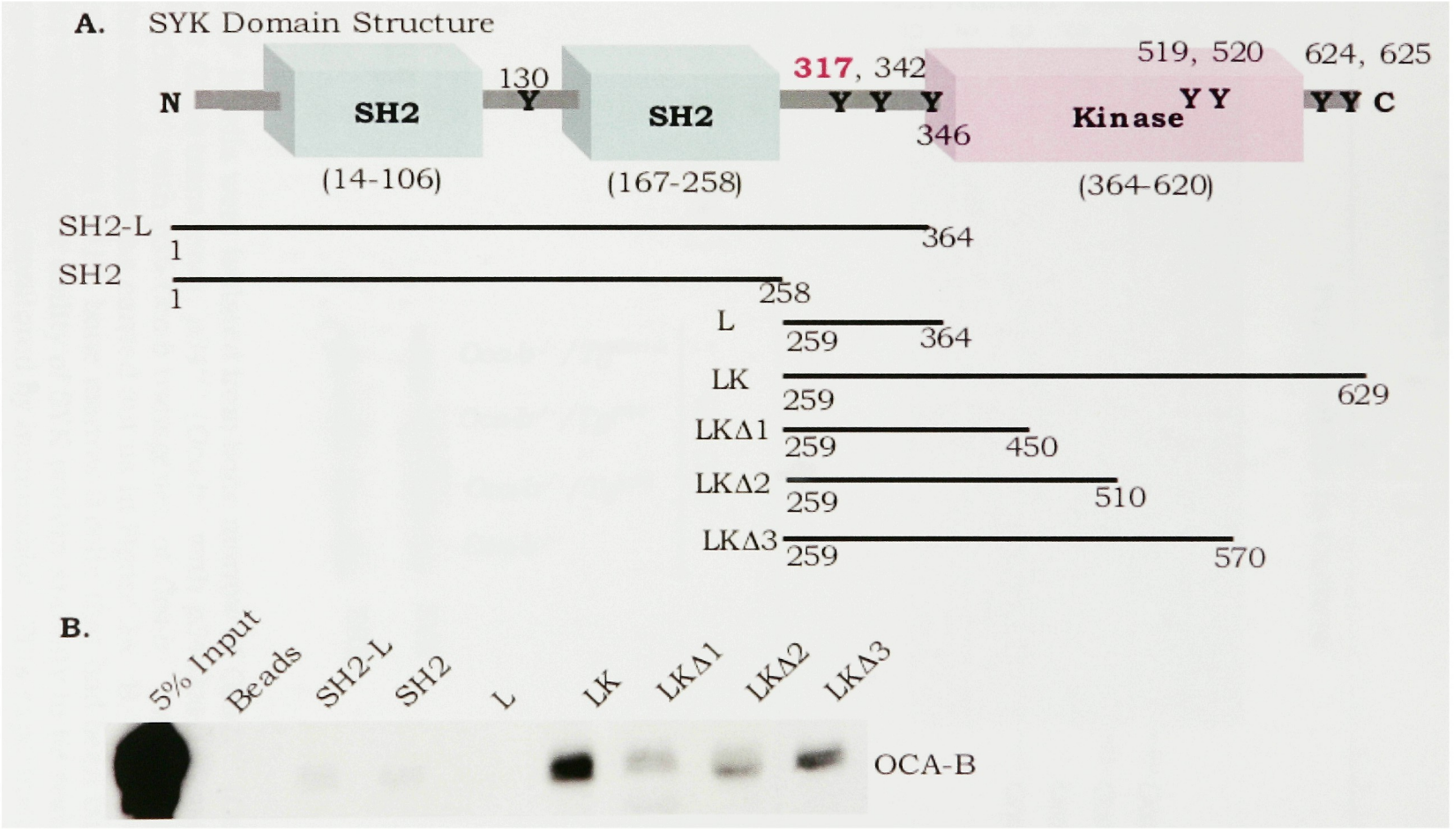


A. Purified His-tagged SYK or Ni-NTA beads alone (control) were incubated with Namalwa cellular extracts. The binding of OCA-B was monitored by immunoblotting. Long and short exposures are shown to distinguish OCA-B p34 and p35. This experiment was reproduced five times. B. Whole cell extracts from Namalwa cells that were stimulated with anti-IgM for a time course were used for immunoprecipitation with anti-OCA-B. The presence of SYK was analyzed by immunoblot. The presence of tyrosine phosphorylated proteins (pTYR) was monitored in the WCE and IP by immunoblot. The band that corresponds to SYK is labeled. This experiment was performed twice.

SYK (Figure 4.2.12a). Other than full-length SYK, only SYK fragments containing the kinase domain are able to efficiently pull-down both forms of OCA-B from cytoplasmic extracts (S100) (Figure 4.2.11a and 4.2.12b). Of the kinase domain-containing SYK fragments, those containing the C-terminal portion of the kinase domain are able to interact with OCA-B most efficiently (Figure 4.2.12b).

Although both isoforms of OCA-B are able to interact with SYK in vitro (Figure 4.2.11 and 4.2.12), we wanted to determine if this occurs in vivo. To this end, BAC-transgenic mice that specifically lack expression either of OCA-B/p35 or expression of OCA-B/p34 in B cells were generated on the *Oca-b*$^{-/-}$ background. In this way, loss of function of one form can be assessed using BAC clones in which the OCA-B locus has been genetically modified by introducing point mutations in one of the two translation start sites. We have confirmed that the transgenic mice obtained have the expected pattern and selectivity of OCA-B expression (data not shown). CD19^{+} cells were harvested from the bone marrow of these mice and cultured in the presence of IL-7 (Figure 4.2.13). All of the transgenes (*Oca-b*$^{+/+}$, *p34*$^{+/+}$, *p35*$^{+/+}$) were able to reverse the hyperproliferation defect of *Oca-b*$^{-/-}$ mice (Figure 4.2.13s). Consistent with the in vitro interaction data, the OCA-B p34 and p35 transgene products were both able to stabilize SYK in *Oca-b*$^{-/-}$ mice (Figure 4.2.13b).

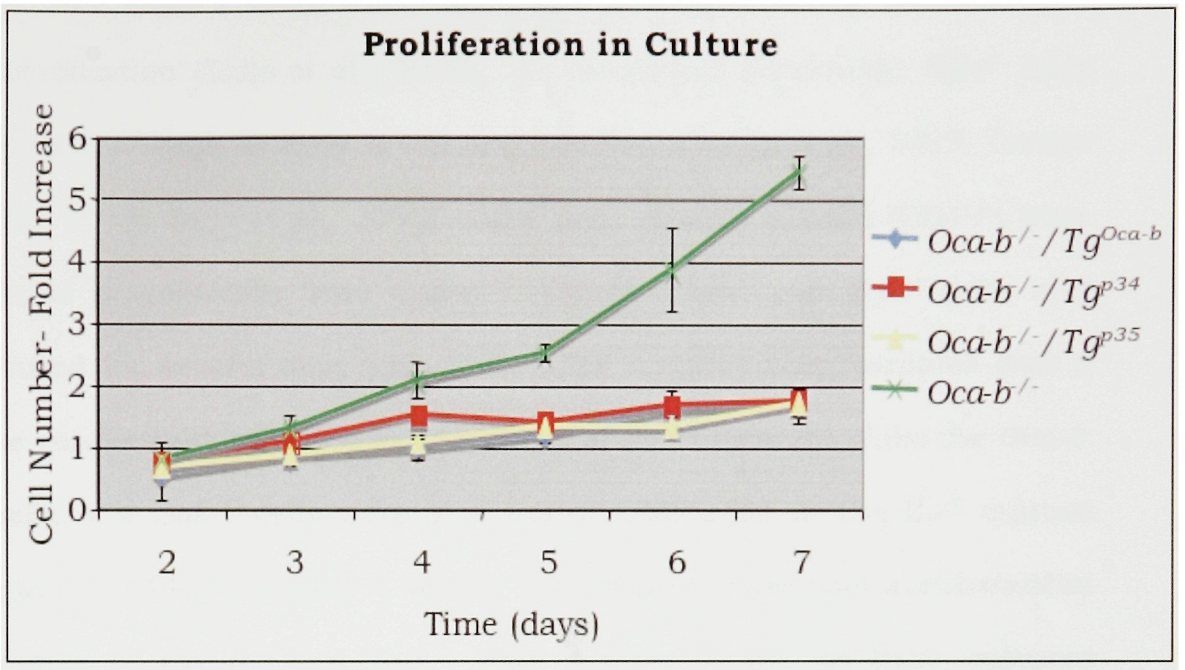
Figure 4.2.12 Characterization of the SYK Domain Required for Interaction with OCA-B



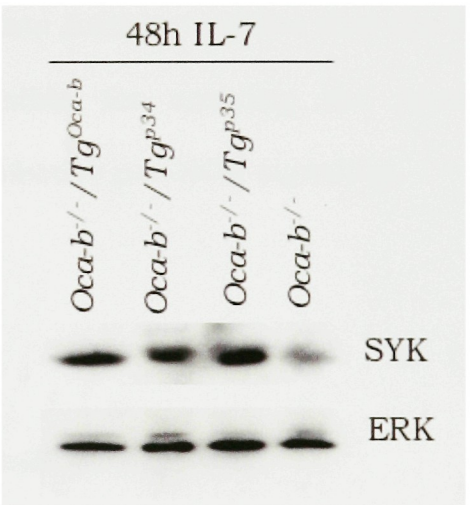
A. Histidine-tagged protein expression constructs were generated based on the SYK domain structure. The constructs used in the experiment are shown. B. Purified histidine-tagged SYK proteins were incubated with S100 (cytoplasmic extract) from Namalwa cells. The binding of OCA-B was monitored by immunoblotting. This experiment was performed twice.

Figure 4.2.13 Rescue of *Oca-b*$^{-/-}$ Mice with OCA-B-Expressing Transgenes

A.



B.



A. CD19^{+} B cells were isolated from bone marrow of *Oca-b*$^{+/+}$ (*Oca-b*$^{-/-}$ with wildtype *Oca-b* transgene), *p34*$^{+/+}$ (*Oca-b*$^{-/-}$ with *p34-Oca-b* transgene), *p35*$^{+/+}$ (*Oca-b*$^{-/-}$ with p35-*Oca-b* transgene), or *Oca-b*$^{-/-}$ (no transgene) mice and the experiment was carried out as in Figure 2A. B. Whole cell extract was prepared from CD19^{+} bone marrow B cells that had been cultured in IL-7 for 48 hours. The ability of SYK protein stability to be restored in the transgenic mice was monitored by immunoblot. This experiment was performed twice.

In order to further develop the analysis we used *Syk* conditional knockout (*Syk*$^{-/-}$) mice that had been generated using Cre-mediated recombination (Saijo et al., 2003). As mentioned previously, *Syk*$^{-/-}$ mice also have a block in early B cell development (Cheng et al., 1995; Turner et al., 1995; Saijo et al., 2003). Total bone marrow B cells (CD19^{+}) were isolated magnetically from *Oca-b*$^{+/+}$, *Oca-b*$^{-/-}$, *Syk*$^{+/+}$, or *Syk*$^{-/-}$ mice and cultured for several days with IL-7. The cultures were sampled over a time course that extended to 168 hours of IL-7 exposure. Like the *Oca-b*$^{-/-}$ cells, the *Syk*$^{-/-}$ cells were also hyperproliferative in the IL-7 culture (Figure 4.2.14a). By day 7 of the time course there was a substantial increase of *Oca-b*$^{-/-}$ and *Syk*$^{-/-}$ cells relative to the wildtype cultures (Figure 4.2.14b). These data further indicate that the absence of SYK in *Oca-b*$^{-/-}$ mice is responsible for creating the block in pre-B cell development caused by defective pre-BCR signaling.

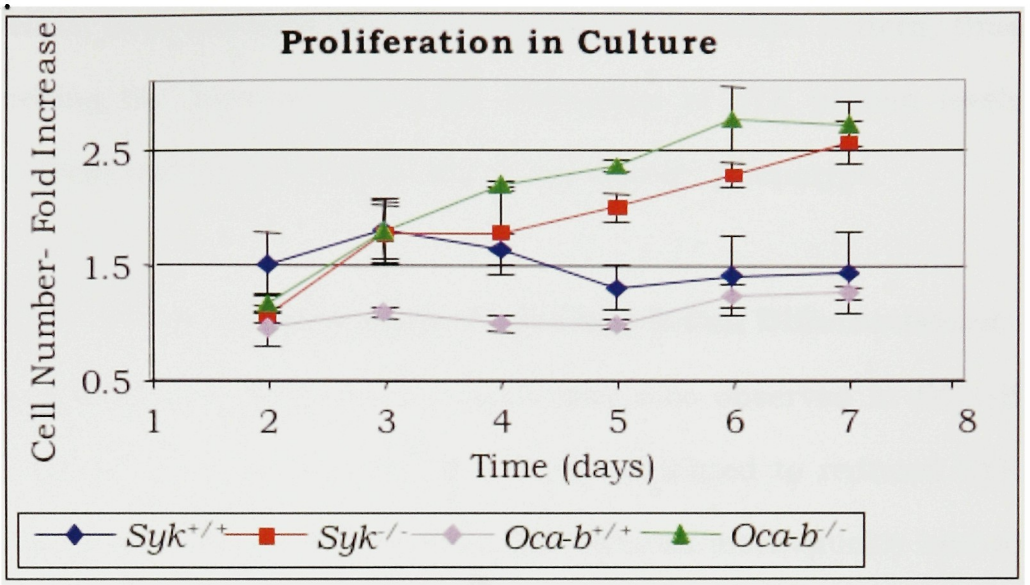
4.3 DISCUSSION

The Role of OCA-B in Pre-BCR Signaling

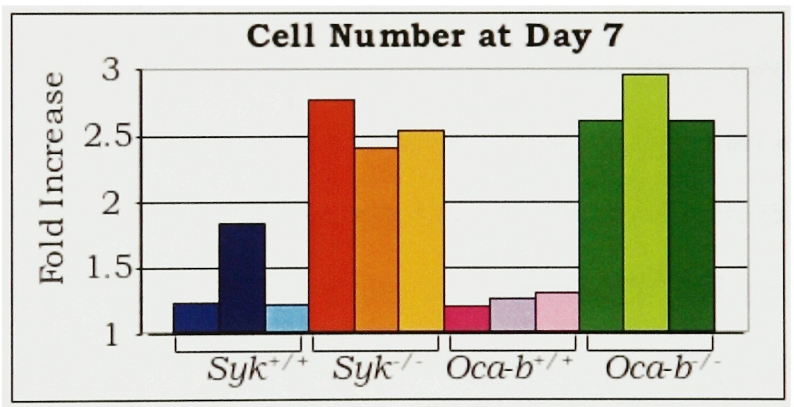
We have shown that pre-BCR signaling capacity is reduced in *Oca-b*$^{-/-}$ mice as a direct result of a severe defect in SYK expression. Mechanistically, this explains the developmental block observed in vivo as well as the hyperproliferation of *Oca-b*$^{-/-}$ cells in response to IL-7. As

Figure 4.2.14 Characterization of *Syk*$^{-/-}$ Cells in the IL-7 Culture

A.



B.



CD19^{+} cells were isolated from bone marrow of *Syk*$^{+/+}$, *Syk*$^{-/-}$, *Oca-b*$^{+/+}$ or *Oca-b*$^{-/-}$ mice and cultured with media containing IL-7 for up to 7 days. Live cells were counted using trypan blue exclusion to measure proliferation during the time course of IL-7 exposure (time points labeled). Each point represents the average fold increase for three mice.

mentioned, SYK expression appears to be less severely affected in freshly prepared (FACS) cells than in cells that have been cultured in IL-7. Nevertheless, both methods of analysis indicated the same pattern, thus strengthening the argument that the disruption of SYK protein levels plays a critical role in the development of the *Oca-b*$^{-/-}$ phenotype.

OCA-B-SYK Interaction in Antigen-Dependent B Cell Differentiation

A severe reduction in SYK protein levels was also observed in OCA-B deficient splenic B cells and can be directly attributed to reduced SYK stability in *Oca-b*$^{-/-}$ cells. SYK protein was reduced more quickly and to a greater extent in knockout cells stimulated with anti-IgM or a combination of anti-IgM, anti-CD40 and IL-4. *Oca-b*$^{-/-}$ mice display a significant reduction in follicular B cells and also have a general reduction of peripheral B cells (Schubart et al., 1996, Kim et al., 2000). Interestingly, *Syk*$^{-/-}$ mice can develop immature B cells in the periphery, but they fail to enter follicles or the recirculating B cell pool (Turner et al., 1997). Therefore, it is possible that the reduced SYK stability and the resulting decrease in protein level in *Oca-b*$^{-/-}$ B cells contributes to the decrease in follicular and recirculating B cells in *Oca-b*$^{-/-}$ mice. In the case of activation with anti-IgM, anti-CD40, and IL-4, which mimics T cell-dependent stimulation, wildtype cells showed a reduction in SYK by 65 hours, a change that correlates with differentiation of these cells as evidenced in part by increased IgG1 transcription (Kim et al., 2003). The

more dramatic reduction of SYK protein levels in the knockout cells may contribute to the compromised antigen-dependent immune response of *Oca-b*$^{-/-}$ mice.

Mechanism of OCA-B-SYK Interaction

The mechanism by which OCA-B effects SYK protein levels is unexpected, since SYK is not a transcriptional target of OCA-B as judged by QPCR and chromatin immunoprecipitation assays. Because of this, alternative functions of OCA-B, both direct and indirect, need to be considered. The most obvious evidence to support a direct effect by OCA-B on SYK protein stability is that the two proteins strongly interact, as shown both in vitro by assays with bacterially expressed (unmodified) forms at high salt concentration and in vivo by co-immunoprecipitation. Also, SYK is heavily regulated at the protein level by ubiquitin-mediated degradation (Keshvara et al., 1998; Lupher et al., 1998; Ota and Samelson, 1999; Yankee et al., 1999; Rao et al., 2001; Hong et al., 2002) and, importantly, we have demonstrated that SYK stability is reduced in the *Oca-b*$^{-/-}$ IgM-cultured mature B cells. This regulation is mediated by CBL, a ring finger ubiquitin ligase that binds to SYK that has been tyrosine phosphorylated in its linker region (residue 317); phosphorylation at this site is critical for SYK degradation (Keshvara et al., 1998; Lupher et al., 1998; Yankee et al., 1999; Sada et al., 2000; Hong et al., 2002; Sohn et al., 2003). Significantly, this phosphorylated

form of SYK was increased in *Oca-b*$^{-/-}$ cells that had been stimulated with anti-IgM; however, OCA-B interacts with unposhorylated SYK. This increase in phosphorylated SYK and the decrease in total SYK levels due to SYk instability in the knockout cells may be a result of OCA-B being unavailable to interact with and stabilize SYK. This stabilization most likely effects the inactive form of SYK because OCA-B interacts with unphophorylated SYK through its kinase domain. This would be a unique and novel mechanism by which SYK protein levels are maintained.

Another interesting aspect of the interaction between SYK and OCA-B is that the interaction increases with BCR signaling and can occur with either form of OCA-B (Figure 6A, E and F, Figure 7). Consistent with the C-terminus of OCA-B being most important for interaction with SYK, pull-down and immunoprecipitation assays showed that SYK can interact with both the myristoylated-p35 and p34 forms of OCA-B and rescue experiments in *Oca-b*$^{-/-}$ mice showed that both forms of OCA-B are able to stabilize SYK in vivo and to rescue the IL-7 hyperproliferation defect. Additionally, immunofluoresence analyses showed co-localization of OCA-B and SYK in Namalwa cells that had been stimulated with anti-IgM. The Namalwa cells showed responsiveness to BCR signaling, in that IgM became more aggregated with treatment. The significantly increased incidence of cells with "nuclear exclusion" of OCA-

B following BCR stimulation could be related to its interaction with SYK because increased association between OCA-B and SYK was detected by immunoprecipitation using extracts from these cells (Figure 5C and 6F). Possibly related to this idea, there was also a fraction of cells that had strong nuclear staining for SYK. Nuclear localization of SYK has been reported previously and is dependent on a nuclear localization signal in the linker region (Ma et al., 2001; Wang et al., 2003), although the functional significance of this finding has not been fully determined. Further investigation into the translocation of OCA-B within the cell could provide yet another example of the unique behavior of this transcriptional cofactor and could also reveal alternative functions for SYK.

Dual Function of OCA-B in B Cell Signaling

Although OCA-B is best known for its function as a transcription coactivator, this study has provided further evidence that OCA-B can function by multiple mechanisms within the cell (Figure 4.3.1). As a transcription factor, OCA-B activates a subset of genes that are important in cell signaling (Kim et al., 2003). Although there is also a severe reduction in LCK RNA and protein levels in *Oca-b*$^{-/-}$ pre-B cells, *Lck*$^{-/-}$ mice display a normal pre-B1 to pre-B2 transition and grow normally in an IL-7 culture (data not shown). Therefore the reduction in LCK is not likely to be responsible for the defects in pre-B cell

differentiation in *Oca-b*$^{-/-}$ mice. However, regulation of OCA-B target genes has been shown to be important for other aspects of B cell differentiation, in particular mature B cell function (Teitell, 2003). Interestingly, the OCA-B-SYK interaction occurs early during B cell activation when OCA-B expression is relatively low. When OCA-B expression is at the highest level, later in the activation program, not only is the OCA-B-SYK interaction no longer apparent but the transcriptional targets of OCA-B start to be induced (Figure 4.2.8b and 4.2.9a and Kim et al. 2003). These findings indicate that OCA-B plays dual roles in the cell, making it broadly important for B cell signaling. It not only can regulate early events in intracellular signaling by direct interaction with signaling components (SYK), but it can also function classically as a transcription coactivator on downstream target genes at later stages of B cell activation.

CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

Multi-Functional OCA-B

OCA-B is a protein of many surprises. Historically, OCA-B was the first cell-type specific coactivator identified (Pierani et al., 1990; Luo et al., 1992; Luo and Roeder et al., 1995). OCA-B was found to function specifically either with the ubiquitious transcription factor OCT-1 or with the related cell-type restricted transcription factor OCT-2 to mediate B cell restricted activation of target genes (reviewed in Luo and Roeder, 1999). Although initially characterized for its function at Ig promoters, OCA-B was later found to function at the IgH 3' enhancer (Tang and Sharp, 1999; Stevens et al., 2000; Sepulveda et al., 2005). Furthermore, the phenotype of *Oca-b*$^{-/-}$ mice highlights a broader role for OCA-B in B cell differentiation and function. Even more interesting was the finding that OCA-B is one of a relatively small group of proteins that makes use of two in-frame translation initiation start sites. Furthermore, one of the resulting OCA-B isoforms (p35) is myristoylated and localized to the cytoplasm/membrane (Yu et al., 2001). This finding raises yet another interesting possibility, that OCA-B could function directly in signaling pathways. Indeed, this study has uncovered the highly unique two-fold role that OCA-B plays in B cell development and proliferation. OCA-B functions in an unprecedented manner – not only by regulating expression of its transcriptional targets within the nucleus as a classic coactivator but also by functioning directly in signaling pathways by regulating kinase protein stability. Collectively, these findings explain

why OCA-B is critical for many aspects of B cell differentiation and function such as pre-BCR and BCR signaling, pre-B cell differentiation, germinal center formation, cellular proliferation, secondary isotype expression, plasma cell differentiation and maintenance of the re-circulating B cell pool.

OCA-B in Early B Cell Development and Signaling

Although *Oca-b*$^{-/-}$ mice are most obviously defective in antigen-dependent B cell development, more subtle yet, important, defects are apparent in early B cell development. This study has identified the first block in B cell development that occurs as a result of OCA-B deficiency. This defect is caused by the failure of pre-BCR signaling, which occurs by a two-fold mechanism. First, expression levels of OCA-B target genes such as Igβ and LCK are reduced in *Oca-b*$^{-/-}$ pre-B cells. Although the specific role of LCK in B cells remains to be determined, Igβ is the signaling component of the pre-BCR and its reduced expression is likely to contribute to the early B cell defects of *Oca-b*$^{-/-}$ mice. Furthermore, it is likely that unidentified OCA-B target genes play a role in mediating pre-BCR responsiveness. The second and more direct mechanism by which OCA-B mediates pre-BCR signaling is through stabilization of the protein tyrosine kinase SYK. As evidenced from this study and previous reports (Cheng et al., 1995; Turner et al., 1995; Saijo et al., 2003) SYK is critical for early B cell development and pre-BCR signaling.

Despite the role of OCA-B in pre-BCR signaling, pre-B2 cells are present in *Oca-b*$^{-/-}$ mice at half of the normal amount. However, *Oca-b*$^{-/-}$ B cells once again fail to develop normally at the immature stage such that reduced Vκ promoter usage causes a reduction in splenic transitional B cells (Casellas et al., 2002; Jankovic et al., 2003). This defect is due to the classic function of OCA-B as a transcriptional coactivator on a subset of octamer-containing Vκ promoters.

The Possible Function of OCA-B in B1 Cell Development

This study also demonstrates that *Oca-b*$^{-/-}$ mice have an increased population of B1a cells in the peritoneum, a phenotype that is also observed in *Lck*$^{-/-}$ mice. Furthermore, we demonstrate that LCK is a direct OCA-B target gene that is expressed in B1a cells. However, because there are conflicting reports as to the nature of the B1a cell defect in *Lck*$^{-/-}$ mice (Ulivieri et al., 2003; Dal Porto et al., 2004; Frances et al., 2005), these findings will need to be developed further in order to investigate the functional significance of LCK expression and regulation by OCA-B in B cells. Analysis of tyrosine phosphorylation showed that *Oca-b*$^{-/-}$ B1a cells have reduced responsiveness to B cell signaling pathways and it is likely that reduced SYK stability contributes to the signaling defect of *Oca-b*$^{-/-}$ B1a cells.

OCA-B Function in Mature B Cell Differentiation and Activation

Multiple peripheral B cell subtypes are affected by the absence of OCA-B, which can be largely attributed to defective BCR signaling resulting from SYK instability and reduced expression of target genes in *Oca-b*$^{-/-}$ mice. This study has identified and validated OCA-B target genes in mature B cells through a combination of cDNA array analysis, QPCR, blotting, immunohistochemistry, and ChIP assays. While the specific contribution of reduced expression of these genes to the phenotype of *Oca-b*$^{-/-}$ mice will need to be determined by mutagenesis studies, they are likely to play important roles in B cell function (Chapter 2 and Kim et al., 2003). This study, amongst others (Samardzic et al., 2002) has found *Oca-b*$^{-/-}$ mice to be deficient in marginal zone B cells. It is possible that this defect could be attributed to a reduced B cell migration caused by reduced expression of OCA-B target genes such as BAFFR and BCMA (Samardzic et al., 2002). However, it is also likely that defective BCR signaling caused by SYK instability contributes to the reduction of marginal zone B cells in *Oca-b*$^{-/-}$ mice.

As mentioned previously, a multitude of studies have established a function for OCA-B in a wide range of processes such as germinal center formation, B cell activation, BCR signaling, and plasma cell differentiation (Kim et al., 1996; Nielsen et al., 1996; Schubart et al., 1996b; reviewed in Teitell, 2003). The failure of *Oca-b*$^{-/-}$ mice to

efficiently undergo antigen-dependent B cell differentiation can be explained by the sequential two-fold function of OCA-B. First, OCA-B mediates proximal BCR signaling by directly regulating SYK protein stability. The reduction of SYK protein levels provides an explanation for the reduction of BCR-induced Calcium-flux in *Oca-b*$^{-/-}$ cells. This is particularly interesting given that this study has demonstrated that OCA-B target genes are activated at later stages of B cell activation, post-OCA-B induction. Conversely, the interaction between OCA-B and SYK occurs early in B cell activation when OCA-B protein levels are relatively low. Interestingly, we find that OCA-B and SYK protein levels are inversely correlated; however, we observe a redistribution of OCA-B immediately following BCR stimulation. The alteration of OCA-B localization throughout B cell activation may be one way the cell is able to control the mode of OCA-B function.

CHAPTER 6

MATERIALS AND METHODS

Animals

8-10 week old female *Oca-b*$^{-/-}$ mice (Kim et al., 1996) and C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were used for all experiments. *Syk* conditional knockout mice were also used (*Syk*$^{-/-}$) (Saijo et al., 2003). All mice were maintained under specific pathogen-free conditions at the laboratory animal research center within The Rockefeller University.

Cell Lines

MPC11, a mouse plasmacytoma cell line, was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine and 10% Horse Serum. Namalwa, a human burkitt's lymphoma cell line, was maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. Daudi, a human burkitt's lymphoma cell line, was maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. A20, a mouse lymphoblastic B cell line, was maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, and 10% Fetal Bovine Serum. HelaS, a human carcinoma cell line and 293T, a human firbroblast cell line were maintained in DMEM supplemented with 2 mM L-glutamine and 10% Fetal Bovine Serum.

Flow Cytometry Analysis and Cell Sorting

Bone marrow cells were flushed from the femurs and tibia of 5-8 mice using PBS supplemented with 10% Fetal Bovine Serum. Splenocytes were obtained by crushing spleens in RPMI 1640 supplemented with 10% Fetal Bovine Serum. B1a cells were obtained by peritoneal lavage with PBS. The cell suspensions were prepared for staining according to standard procedure (Kim et al, 2000). The samples (1 X 10^6 cells) were analyzed on a FACsort (BD Biosciences, Mountain View, CA) using CellQuest software (BD Biosciences, Mountain View, CA) until 2 x 10^5 events were acquired. Cell surface markers were detected using the following antibodies (all from BD PharMingen, San Diego, CA): B220-PE (clone RA3-6B2), B220-FITC, CD43-FITC (clone S7), CD117 (clone 2B8), IgM-APC (clone II/41), CD19-PE (clone 1D3), CD19-APC (clone 6D5), CD25-APC (clone PC61), CD25-PE (clone PC61), IgD-PE (clone 11-26c.2a), pre-BCR (clone SL-156)-streptavidin-PE, κ-PE (clone 187.1), CD138-PE (clone 281-2), CD21-FITC (clone 7G6), CD11b/MAC-1-PE, and CD5-PE. Cell size analysis was performed from five independent experiments. The large cells were gated (FSC-H > 375) and the percentages obtained were used in a Student's T Test to determine if the values obtained from the wildtype samples were statistically distinct from that of *Oca-b*$^{-/-}$. Total bone marrow cells from 10-12 female mice were isolated and stained (as described above) for cell sorting using the FACS Vantage SE (BD Biosciences, Mountain View, CA). Dead cells and

doublets were excluded based on propidium iodide (BD PharMingen, San Diego, CA) incorporation and laser scatter patterns, respectively. Post-sort analysis was performed to access the purity of all the fractions collected. For Figure 2.2.7, five to ten mice were immunized 5 days prior to sorting with 100 μg of DNP-keyhole limpet hemocyanin (KLH, Calbiochem, La Jolla, CA).

Quantitiative PCR and RT-PCR

For the quantitative PCR experiments presented in Chapter 2, DNase-treated total RNA preparations (2 mg) were reverse transcribed using Superscript II reverse transcription kit (Invitrogen, Carlsbad, CA). The 20 ml reverse transcription reaction was first diluted 10-fold followed by 3-fold serial dilutions over a 250-fold range to generate standard curves. The same RNA stock was used for both quantitative PCR and cDNA microarray. Primers (Sigma, USA) were designed for each gene using the Primer Express program (Applied Biosytems, Foster City, CA). The primer sequences are as follows: *Pol2E* sense, TCAAGGTATACTGCCAGCGCA, and antisense, GTCCACCAGGAACT GCTTGG; *Kcnn4* sense, ATCGGACTCATGGTTCTGCAC, and antisense, GCAGTGGACAGGGTGATCAA; *Cdc37* sense, CCACCTGGTGTGTGA GGAAA, and antisense, TCTGGTGAGCTACCTGCTCC; and *Lck* sense, CCATGATGGAGACTTGGGCT, and antisense, CTTCTTGGCCAGTCG TCAGG. Primer quality (lack of primer-dimer amplification) was

confirmed by melting curve analysis using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The final 20 ml reaction consisted of 0.425-100 ng template, 1X Quantitect SYBR Green master mix (Qiagen, Valencia, CA), and 30 ng gene-specific primers. All solutions were distributed to 384 well plates using the Hydra96 HTS automatic dispensing system (Robbins Scientific, USA). The PCR was as follows: 2 min 50° C, 10 min at 95° C, and then 40 cycles of 15 sec at 95° C followed by 1 min at 60° C. Relative quantification based on standard curves was used for data analysis. The relative abundance was calculated using the efficiency-calibrated equation described previously (Pfaffl, 2001, Soong et al., 2000). Briefly, the slope of each standard curve was used to calculate PCR efficiency ($E=10^{-1/slope}$). The final ratio was generated using the following equation: Ratio= $[(E_{ref})^{Ctsample}/(E_{target})^{Ctsample})/ ((E_{ref})^{Ctcontrol}/(E_{target})^{Ctcontrol}]$. RNA polymerase II (*RBP5*, AW413738) gene was found to be unregulated in all of the array experiments and was used as a reference gene.

For the quantitative PCR experiments presented in Chapters 3 and 4, DNase-treated total RNA preparations were reverse transcribed using the Superscript II reverse transcription kit (Invitrogen, Carlsbad, CA). Subsequent RT-PCR was carried out according to standard protocols using cycle numbers within the linear range of amplification for each gene, as determined by quantitative PCR (Kim et al., 2003). Quantitative

PCR was performed as described above and in Kim et al., 2003 with minor modifications. For these experiments the reference gene was *Gapdh*, although similar results were obtained using the RBP5 subunit of RNA polymerase II. Additional primer sequences are as follows: *Cyclin D3* sense, CACAGATGTCACAGCCATTC, and antisense, ATTGTTCTAG AGGCAGGGAGGG; *Oca-b* sense, CGGAGTATGTGTCCCATGAA, and antisense, TGATGAGTGGTGGAGAAGCA; *Gapdh* sense, TCAACT ACATGGTCTACATGTTCGAG, and antisense, ACCATTCTCGGCCTTG ACTGT; $V_{pre\text{-}b}$ sense, CCCAGGTTCCTGCTGAGATA, and antisense, TGCTCAGATACCCCAGGTTC; *λ5* sense, GTCCCTGTCACTCAGGG TGT, and antisense, GAGGCATCCACTGGTCAGAT; *Igα* sense, TCATA CGCCTGTTTGGGTC, and antisense, AGGGTTCCTGCCATTGTTT; *Igβ* sense, CCTCCTCATCATCCTCTTCATC, and antisense, GGTGGCTGT CTGGTCAATG; *Rag2* sense, ATGGATTTGGAAGAACGCAC, and antisense, GGAGGGATTTCATTGGAGGT; $\mu_{constant}$ sense, TAAATGTGT TCGTCCCACCA, and antisense, GAGCTTCCCATCCTTTAGCC; *Btk* sense, AAGTTTCCAGTTCGGTGGTCTC, and antisense, GCAATGTGTT CTGCTGTCTCACT; *Lyn* sense, GTATCAGCAGCAACCGTAGTGG, and antisense, CATCAGGAACCAGGAACACAGA; *Fyn* sense, GACCTCCA TCCCGAACTACAAC, and antisense, ATCTTCCGTCCGTGCTTCATAG; *Syk* sense, ATGAGAAGATGCCCTGGTTCC, and antisense, TGTCCCT GTCAATGCGGTAGT; *Blimp-1* sense, ACACAGTTCCCAAGAATGCCAAC AG, and antisense, CGCAAAGTCCCGACAATACCACAC.

Northern Blotting

CDNA clones of various OCA-B target genes were digested with restriction enzymes for the generation of radio-labeled probes that were hybridized to RNA blots according to standard procedures. The samples were normalized using the 18s rRNA signal. The data was quantified using a phosphorimager.

Immunoblotting

Immunoblotting was carried out with whole cell extract according to standard procedure. The antibodies employed were: anti-OCA-B (C-20), anti-SYK (N-19) anti-LCK (2102), anti-CDC37 (E-4), anti-ERK2, anti-FYN (fyn3) (all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphoSYK (Tyr323) (Cell Signaling Technology, Beverly, MA), anti-phosphotyrosine (4G10, Upstate Biotechnology, Charlottesville, VA) anti-Actin (Sigma, USA), and anti-KCNN4 (a gift of K. Nehrke).

Transient Transfection

HelaS or 293T cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA) or Fugene (Roche, USA). *Renilla* Luciferase activity was analyzed 48 hours-post transfection using a luciferase assay kit that included PRL-CMV for normalization (Promega, Madison, WI).

Primary B Cell Culture

For the experiments presented in Chapter 2, resting splenic B cells were purified as described before (Defranco et al., 1982, Kim et al., 1996) using complement-mediated lysis of Thy-1$^+$ cells followed by Percoll (Amersham, Piscataway, NJ) gradient centrifugation to isolate high density cells. Splenocytes from 20 female mice (5-8 weeks old, in *B6 x 129Sv* background) per genotype were pooled in order to obtain sufficient numbers of purified resting B cells and to normalize any variations in gene expression among the mice. For a given experiment, 60% of the cells were designated as resting B cells and used to make RNA or whole cell extract without stimulation. The remaining cells were cultured in RPMI supplemented with heat inactivated 10% fetal bovine serum in the presence of 30 mg/ml anti-mouse IgM $F(ab')_2$ fragment (Pierce Biotechnology, Rockford, IL) for BCR stimulation. For co-stimulation, 5 mg/ml anti-mouse IgM $F(ab')_2$, 10 mg/ml anti-CD40 antibody (BD Pharmingen, San Diego, CA) and 10 ng/ml recombinant mouse IL-4 (Sigma, USA) were used. Typical preparations contained at least 90% B220+ cells (not shown). Cell suspensions containing B1a cells were obtained by peritoneal lavage with PBS. For the experiments presented in Chapter 3, CD19$^+$ B cells from bone marrow were isolated by MACS (Miltenyi Biotec, Auburn, CA) and cultured without feeder cells using RPMI supplemented with 20% FBS, 0.05 mM β-ME, 100 U/ml P/S, and 5 ng/ml recombinant IL-7 (Sigma, USA). Fresh IL-7 was added every 72

hours. Flow cytometry analysis was performed, as above, to analyze cellular proliferation, size, and cell surface marker expression over a time-course of IL-7 exposure. For the experiments presented in Chapter 4, splenocytes from 10 female mice were harvested and splenic B cells were enriched by complement-mediated lysis of Thy-1$^+$ cells followed by a Percoll gradient (Defranco et al., 1982) to separate dead cells and debris, or to fractionate resting B cells (Figure 4G). Preparations were determined to contain at least 92% live B220$^+$ cells by flow cytometry (not shown). The cells were stimulated as described previously (Kim et al., 2003). To analyze protein stability, cycloheximide was added in some cases at a concentration of 10 μg/ml.

cDNA Microarray

Microarray assays used an indirect labeling method using 5-(3-aminoallyl)-dUTP and amino-reactive fluorescent dyes Alexa 555 and Alexa 647 (Molecular Probes, Carlsbad, CA). Glass cDNA array slides containing 9,800 PCR-amplified mouse cDNA clones (Research Genetics, Huntsville, AL) were from the Rockefeller University Gene Array Core Facility. Hybridization and washing were done in a Hybstation (GeneTAC, Ann Arbor, MI) and slides were scanned in a 428 Glass Array Scanner (Affymetrix, Santa Clara, CA). For data analyses and clustering, data acquired by GENEPIX PRO 3.0 (Amersham Pharmacia, Piscataway, NJ) software were directly uploaded into the TANGO (Transcriptome ANalysis

of GenOmes) program (Altmann et al., 2001). Quality control experiments were carried out to determine optimal RNA concentrations, and yellow test experiments in which duplicate RNA samples from a single source were labeled with Alexa 555 or Alexa 647 dye were performed to determine the inherent noise levels. For expression profiling of the WT B cells in response to BCR stimulation, four replicate probe labelings and hybridizations were carried out. Three replicate experiments were done with *Oca-b*$^{-/-}$ B cells. The TANGO query program was used to select genes whose intensity values in each channel were above two standard deviations of background and whose stimulated-resting values were at least 2. The output was further filtered by selecting for genes whose expression changed in at least three replicate experiments. For costimulation experiments, we carried out dye-flip replicates (in which reverse combinations of dyes were used to label the resting and stimulated samples) for each time point. Genes whose expression changed in both of the two replicates were selected. The filtered genes were clustered by using Profile- and K-means clustering (P = 0.05) (Tavazoie et al., 1999). Verification of representative genes by quantitative real-time PCR and Northern blot indicated that the frequency of false positives was 5%.

Pre-BCR Crosslinking

$CD19^+$ cells were cultured in IL-7-containing media (as above) for 48 hours. Cultures were then depleted of $Ig\kappa^+$ cells using biotinylated anti-κ (BD Pharmingen, San Diego, CA) conjugated to streptavidin dynabeads (Dynal Biotech, Brown Deer, WI). Depleted fractions were washed and cultured for 4 hours without serum or IL-7 and then stimulated at 37°C with 10 μg/ml anti-IgM $F(ab')_2$ (Pierce Biotechnology, Rockford, IL). The cells were washed with PBS/1 mM Na_3VO_4 and lysed with NP40/Vanadate buffer (1.5% NP40, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, and 20 μM Leupeptin). Following electrophoresis and transfer, tyrosine phosphorylation was accessed using anti-phosphotyrosine (4G10, Upstate Biotechnology, Charlottesville, VA). Blots were stripped and then probed with antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA) to SYK (LR), LCK, FYN (fyn3), ERK, or ACTIN (Sigma, USA).

Protein-Protein Interaction Studies

For immunoprecipitation, lysates were incubated with anti-OCA-B, anti-SYK, or rabbit IgG (control) for two hours followed by incubation with Protein A-Sepharose (Amersham, Piscataway, NJ). The immuno-precipitate was then washed four times with lysis buffer. The precipitated proteins were resolved by SDS-PAGE and analyzed by immunoblot. cDNAs encoding OCA-B residues 1-256, 1-154, and 154-

256, or SYK 1-629 were inserted into the GX41 or PET28a plasmids, respectively. The proteins were expressed and purified from E. coli according to standard procedures. For the GST-protein-protein interaction experiments, 10μg of GST-OCA-B protein was bound to glutathione-Sepharose (Amersham, Piscataway, NJ) and incubated with approximately 250 ng of purified 6x-Histidine-tagged SYK for three hours at 4°C. The beads were washed 6 times with binding buffer [20 mM Tris-HCl (pH 7.4), 20% Glycerol, 0.2 mM EDTA, 0.1% NP40, 0.2 mM PMSF, and 400 mM KCl]. The bound proteins were resolved by SDS-PAGE and analyzed by immunoblot. For the Ni-NTA pull-down experiments, 10 ug of purified his-tagged SYK was incubated with extracts from Namalwa cells for three hours in binding buffer [20 mM Tris-HCl (pH 7.4), 10% Glycerol, 30 mM Imidazol, 0.1% NP40, 1.0 mM PMSF, 0.2 mg/ml BSA and 150 mM NaCl]. The beads were washed 6 times with binding buffer and bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting. Cytoplasmic and nuclear extracts were prepared according to standard procedure (Dignam et al., 1983).

Immunofluorescence Microscopy

Namalwa cells were treated with goat anti-human IgM $F(ab')_2$ (Southern Biotechnology. Birmingham, AL) or with anti-human IgM $F(ab')_2$, anti-human CD40 and recombinant human IL-4 for various lengths of time before fixation with 2% paraformadehyde. Cells were permeabilized

using 0.5% saponin and incubated with anti-SYK (4D10) (Santa Cruz Biotechnology, Santa Cruz, CA) and/or anti-OCA-B. The secondary antibodies used were AlexaFluor488-conjugated chicken anti-rabbit IgG, AlexaFluor488-conjugated chicken anti-mouse IgG, AlexaFluor546-conjugated goat anti-rabbit IgG, AlexaFluor546-conjugated donkey anti-goat IgG (Molecular Probes, Carlsbad, CA).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays were performed as described (Shang et al., 2000) with minor modifications. Antibodies used included OCA-B (C-20), OCT-1 (C-21), and OCT-2 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA). The primers used were as follows: *Kcnn4* sense, CACAGGACACAGAGTGGGAAGGAGTTGTAA, and antisense, GAA CGTGACTCTGGGAATAGGATGTTGTGT; *Lck* sense, GGCTCTCTTTGG ATCTCTTACCCAGGAGTAT, and anti-sense, GTTCTGGAGGTGAAGAC AGGAATGGCTA.

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