Expression of ZAP-70 Is Associated with Increased B Cell Receptor Signaling in Chronic Lymphocytic Leukemia

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Abstract

We examined isolated leukemia B cells of patients with chronic lymphocytic leukemia (CLL) for expression of ZAP-70. CLL B cells that have non-mutated immunoglobulin (Ig) variable region genes (V genes) expressed levels of ZAP-70 protein that were comparable to that expressed by normal blood T cells. In contrast, CLL B cells that had mutated Ig V genes, or that had low-level expression of CD38, generally did not express detectable amounts of ZAP-70 protein. Leukemia cells from identical twins with CLL were found discordant for expression of ZAP-70, suggesting that B-cell expression of ZAP-70 is not genetically predetermined. Ligation of the B cell receptor (BCR) complex on CLL cells that expressed ZAP-70 induced significantly greater tyrosine phosphorylation of cytosolic proteins, including p72Syk, than did similar stimulation of CLL cells that did not express ZAP-70. Also, exceptional cases of CLL cells that expressed mutated Ig V genes and ZAP-70 also experienced higher levels tyrosine phosphorylation of such cytosolic proteins following BCR-ligation. Following BCR-ligation, ZAP-70 underwent tyrosine phosphorylation and became associated with surface Ig and CD79b, arguing that ZAP-70 is involved in BCR-receptor signaling. These data indicate that expression of ZAP-70 is associated with enhanced signal transduction via the BCR complex, which may contribute to the more aggressive clinical course associated with CLL cells that express non-mutated Ig receptors.
Introduction

The leukemia B cells of patients with chronic lymphocytic leukemia (CLL) can be classified into one of at least two major subgroups based upon the mutational status of their expressed immunoglobulin (Ig) variable (V) genes\(^1,2\). Patients with CLL cells that express non-mutated genes tend to have a relatively aggressive clinical course when compared with patients who have CLL cells that express Ig V genes with somatic mutations\(^2-4\). Moreover, patients with leukemia cells that express non-mutated Ig V genes generally are more likely to have CLL cells with atypical morphology, advanced clinical stage, and progressive disease\(^3\). However the mechanism(s) accounting for the differences in clinical behavior of these two types of CLL is unknown.

Recent studies on the gene expression profiles of isolated CLL B cells have revealed a set of genes that is differentially expressed between these two subgroups\(^5,6\). CLL cells from different patients share common expression levels of many genes and have a gene expression profile distinct from that of other B cell malignancies or normal B cells. However, CLL cells that express non-mutated Ig V genes can be distinguished from leukemia cells that have mutated Ig V genes through their differential expression of a small subset of genes. One of these genes encodes ZAP-70, a 70-kDa \(\zeta\)-chain CD3-receptor-associated protein tyrosine kinase (PTK) of T lymphocytes. In contrast to CLL cells that have mutated Ig receptors, CLL cells that use non-mutated Ig V genes express ZAP-70 RNA\(^5\).

The functional significance of ZAP-70 gene expression in this subgroup of CLL B cells is unknown. ZAP-70 is a PTK that is characterized by two tandem SH2 domains and a C-terminal catalytic domain\(^7,8\). Following ligation of the T cell receptor (TCR),
there is activation of src family PTK that in turn phosphorylates tyrosine-containing immunoreceptor tyrosine-based activation motifs (ITAMs) within the cytoplasmic tails of the accessory molecules of the TCR\(^9\). ZAP-70 is recruited to the phosphorylated ITAMs and subsequently becomes activated, in turn causing activation of Tec family PTKs and downstream signaling pathways, such as the phospholipase C\(\gamma\)/Ca\(^{2+}\) signaling pathway and the Ras/mitogen activated protein kinase (MAPK) pathway\(^{10}\). B cells generally lack ZAP-70, but instead use another related PTK, Syk, for signal transduction via the B cell receptor (BCR) complex\(^{11}\). Similar to ZAP-70, Syk is recruited to the phosphorylated ITAMs of the activated BCR complex where it subsequently becomes activated\(^{12}\). As such, ZAP-70 and Syk play similar roles in membrane antigen-receptor signaling pathways.

Prior studies indicated that ZAP-70 could reconstitute BCR signaling in Syk-deficient B cells\(^{13}\). In addition, Syk apparently could effect TCR-signaling in patients deficient in ZAP-70\(^{14}\). However, it is not known whether ZAP-70 protein is expressed in CLL, let alone capable of undergoing phosphorylation or association with the CLL BCR complex upon surface Ig ligation.

In the present study we examined purified CLL B cells for expression of ZAP-70 protein and examined whether ZAP-70 potentially could play a role in BCR signal transduction.
Materials and Methods

Cell Isolation - Blood samples were collected from consenting patients who satisfied diagnostic and immunophenotypic criteria for common B cell CLL\textsuperscript{15}. The patients were not previously treated and had not received recombinant growth factors or exogenous cytokines. Blood mononuclear cells were isolated by density-gradient centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Cells were suspended in fetal bovine serum (FBS) containing 5\% dimethyl sulfoxide for storage in liquid nitrogen. The viability of the CLL cells was $\geq 85\%$ at the initiation of culture, as determined by flow cytometric analyses of cells stained with 5 $\mu$g/ml propidium iodide (PI; Molecular Probes, Eugene, OR) for 15 minutes at 37$^\circ$C. All samples contained more than 90\% CLL B cells, as assessed by flow cytometric analyses.

Flow cytometry – The cells were stained with mAbs conjugated with either fluorescein, phycoerythrin, allophycocyanin, or Cy-Chrome\textsuperscript{TM} (BD PharMingen, La Jolla, CA) for direct, four-color, multiparameter flow cytometry using a FACS-Calibur\textsuperscript{TM} (Becton-Dickinson, San Jose, CA), as described\textsuperscript{16}. The fluorescence intensity of each stained cell population was compared with that of the same populations treated instead with an isotype control, fluorochrome-conjugated mAb of irrelevant specificity. To measure for expression of CD38, we calculated the median fluorescence intensity ratio (MFIR). This is the ratio of the median fluorescence intensity of gated CD19$^+$/CD5$^+$ cells stained with CD38 versus that of CD19$^+$/CD5$^+$ cells stained with the isotype control mAb. In addition, we set a threshold fluorescence intensity with which to consider cells positive for CD38, such that $\approx 0.2\%$ of the CD19$^+$/CD5$^+$ cells treated with the isotype control mAb had
fluorescence greater than this threshold. CLL populations were considered positive for 
CD38 when > 20% of the CD38-stained CD19+/CD5+ cells had a fluorescence intensity 
greater than this threshold.

CLL B cells were isolated to >99% purity by flow cytometry using a FACS-Starplus
(Becton Dickinson) (data not shown). For this, the isolated blood mononuclear cells 
were stained with fluorochrome-conjugated mAbs for CD19. CD19-positive cells were 
isolated and then examined for purity by staining a test aliquot of cells with 
fluorochrome-conjugated mAbs specific for CD3, CD5, and CD56.

**Cell Treatment and Sample Preparation** - Frozen CLL cells were grown overnight in 
complete RPMI-1640 medium supplemented with 10% fetal bovine serum followed by 
2-3 hours of serum starvation before BCR-stimulation. The viability of the cells for each 
preparation always exceeded 90%, as assessed by trypan-blue exclusion. The cells 
were adjusted to 1 x 10^7 cells/ml in isotonic 0.1 M phosphate buffered saline (pH = 7.2). 
BCR-ligation was performed using biotinylated goat anti-human IgM F(ab)_2 (Southern 
Biotechnology Associates, Inc, Birmingham, LA) at 10 µg/ml. We incubated this reagent 
with the leukemia cells for 10 minutes on ice and then added Avidin (Sigma, St. Louis, 
MO) to a final concentration of 10 µg/ml. The cell mixture was incubated at 37° C for a 
defined number of minutes to effect BCR-signaling. Preliminary time course studies 
that examined for increases in tyrosine phosphorylation 1, 5, 10, 15, and 30 minutes at 
37° C revealed optimal stimulation at 10 minutes (data not shown). Therefore, in all 
subsequent experiments the anti-IgM/Avidin-treated leukemia cells were incubated for 
10 minutes at 37° C, placed on ice, and then immediately pelleted by centrifugation at 4°
C. Cell pellets were lysed in 200 μl of ice-cold 1% NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 10 mM NaF, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 0.8 μM aprotinin, 20 μM leupeptin, 36 μM bestatin, 15 μM pepstatin, 14μM leucylamido-(4-guanidino)butane (E-64)) for 20 minutes on ice. One-percent NP-40 lysis buffer (10mM Tris-HCl, pH 7.4, 5 mM ethylenediaminetetra-acetic acid (EDTA), 50 mM NaCl, 0.1% SDS, and 0.1% sodium deoxycholate) was used for co-immunoprecipitation experiments. Cell lysates were clarified by centrifugation at 20,000 x g for 15 minutes.

**V_H Gene Analyses** - Total cellular RNA was isolated from 5 x 10^6 CLL B cells using RNeasy (Qiagen. Valencia, CA), as per the manufacturer’s instructions. First strand cDNA was synthesized from one-third of the total purified RNA using an oligo-dT primer and Superscript II RT (Life Technologies, Grand Island, NY). The remaining RNA was removed with RNase H and the cDNA purified using QIAquik purification columns (Qiagen). The purified cDNA was poly-dG-tailed using dGTP and terminal deoxytransferase (Roche, Indianapolis, IN). The V_H gene expressed by the CLL B cells was determined by RT-PCR-ELISA technique\textsuperscript{17}. The cDNA from each sample was amplified using V_H family-specific primers for the sense strand of the gene of interest and an antisense C_μ consensus oligonucleotide primer. The PCR products were size selected by electrophoresis in 2% agarose containing 0.5 μg/ml of ethidium bromide (Life Technologies). The expected products were excised and purified using Geneclean III (BIO 101, Carlsbad, CA), as per the manufacturer’s instructions, and cloned into
pGEM-T (Promega, Madison, WI). Following transformation of XL-1 Blue competent cells (Stratagene, La Jolla, CA), plasmid DNA was isolated from overnight cultures of randomly selected bacterial colonies using QIAprep purification columns (Qiagen). Sequencing was conducted using a fluorescence-dideoxy-chain-termination method and an Applied Biosystems 377 automated nucleic acid sequence analyzer (ABI, Foster City, CA). Nucleotide sequences were analyzed using DNASTAR (Madison, WI) and by comparison to sequences deposited in the V BASE and Genbank sequence databases. At least 3 independent clones were analyzed for each sample. Somatic mutations were identified by comparison to the most homologous germline V<sub>H</sub> gene. The method of Corbett and colleagues was used to assign Diversity (D) genes of the longer gene families (D2 and D3) and 7 consecutive nucleotides were used for the shorter D gene families<sup>18</sup>.

**Immune Precipitation and Immunoblotting** - The protein concentration of each cell lysate was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Ten µg of total cell lysate were loaded onto 10% SDS polyacrylamide gels for electrophoresis. The size-separated proteins were transferred to Immobilon-P transfer membranes. Membranes were blotted with primary antibody at 4°C overnight or at room temperature for 2 hours. For analysis of phosphotyrosine proteins we used the phosphotyrosine-specific mAb 4G10 (Upstate Biotechnology, Lake Placid, NY). We incubated the blots with secondary antibodies that were conjugated with horseradish peroxidase for 1 hour at room temperature. Blots then were washed and prepared for enhanced chemiluminescence and subsequent autoradiography using Super RX film
(Fuji, Tokyo, Japan). Defined sections of the film were scanned for density measurements using a Scanmaker IISP (Microtek) that were analyzed using NIH Image software. To report the change in signal intensity following stimulation, we subtracted 1 from the ratio of signal intensity of the defined section for the stimulated sample over that of the same sample before stimulation and multiplied this number by 100 to derive the percent change in signal intensity.

For immune precipitation studies, we incubated the lysates of 0.5-1 x 10⁷ cells with saturating amounts of mouse mAbs specific for ZAP-70 (29, Transduction laboratories, Lexington, KY), CD79b (C-19, Santa Cruz Biotechnology, Santa Cruz, CA), or human IgM (Southern Biotechnology Associates, Inc.), or affinity-purified rabbit antibodies to Syk (C-20, Santa Cruz Biotechnology). The lysates and antibodies were allowed to incubate either overnight at 4° C or for 2 hours at room temperature. Immune complexes were precipitated with 20 µl of Protein A agarose beads (Pierce, Rockford, IL) for an additional 2-hour incubation at 4° C. The beads were washed twice with PBS and once with lysis buffer. Bound proteins were eluted by boiling the samples in SDS sample buffer and then resolved by 10% SDS polyacrylamide gel electrophoresis.
Results

ZAP-70 expression in CLL B cells

We examined the leukemia B cells from 22 patients with CLL for expression of ZAP-70 protein. Twelve of the patients had CLL cells that expressed non-mutated Ig receptors, whereas the remainder had leukemia cells expressing Ig that had undergone somatic mutation (Figure 1).

![Figure 1](image-url)

**Figure 1** - Analysis of the somatic mutations in the Ig V\_H genes of 22 CLL patients. The distribution of replacement (▲) and silent (●) mutations in the complementarity determining regions (CDRs) and framework regions (FWs) of each heavy chain are indicated. Listed on the left margin are the designations for the heavy chain of each of the CLL samples. Indicated on the right margin are the V\_H genes, known D segments, and J\_H genes that have the highest homology with each heavy chain cDNA sequence.
A high proportion of cases expressed the V_{H}1-69 gene that prior studies had identified as being expressed at high frequency in B cell CLL\textsuperscript{1,19}. Two of the patients who had leukemia cells with mutated Ig V genes (CLL16 and CLL17) were monozygous twins who were concordant for CLL. Both twins were diagnosed at the age of 57 after they incidentally were found to have a leukocytosis of approximately 12,000/mm\textsuperscript{3} on a routine clinical exam. However, one twin (CLL16) subsequently experienced progressive disease, a lymphocyte doubling time of 2 years, and hyperlymphocytosis (>285,000/mm\textsuperscript{3}), whereas the other (CLL17) has had non-progressive disease and maintains a stable total white blood cell count of ≤ 17,500/mm\textsuperscript{3}. Cell lysates were prepared from CLL cells isolated to >99% purity using the fluorescence activated cell sorter.

ZAP-70 protein was detected by immunoblotting with anti-ZAP-70 mAb in lysates of all twelve CLL cell samples that expressed non-mutated Ig V genes (Figure 2 and data not shown). Moreover, the level of ZAP-70 protein detected in each of the ZAP-70

![Figure 2](image-url)

**Figure 2** - ZAP-70 protein expression in CLL cell samples that expressed non-mutated (samples 1 through 5) or mutated (samples 13 through 17) Ig genes. Ten µg of protein lysate from each sample were loaded onto separate lanes of a polyacrylamide gel for immunoblot analyses. Cell lysates were prepared from highly purified leukemia B cells (>99% purity), BJAB, or mixtures of BJAB and isolated normal blood T cells containing 100%, 33%, 10%, 3% or 1% T cells, as indicated at the top of the sample lanes. The immunoblots were probed with anti-ZAP-70 antibody to detect ZAP-70 protein, as indicated by the labeled arrow on the left-hand side of the figure. The blots were stripped and re-probed with antibodies specific for β-actin, as indicated by the labeled arrow of the bottom row.
70° leukemia B cell samples was comparable to that found in isolated blood T cells from normal adults (Figure 2). In contrast, CLL samples that expressed mutated Ig receptors generally did not have ZAP-70 protein at levels exceeding that found in control samples with 1% T cells (Figure 2). However, all samples contained equivalent levels of β-actin, indicating that all lanes had similar levels of protein loaded onto the gel (Figure 2).

Of the 10 CLL samples that expressed mutated Ig receptors, we found that only one (CLL16) expressed high levels of ZAP-70 protein. Through primary sequence analyses, we deduced that Ig heavy chain variable region of CLL16 had overall sequence homology of only 91.2% with the nearest fit germline sequences, namely Ig V\(_h\)3-33, D6-6, and J\(_{\mu}\)6 (Figure 1). Samples CLL16 and CLL17 were obtained from monozygotic twins who were concordant for CLL. Like CLL16, CLL17 also expressed mutated Ig genes with only 92% overall sequence homology with the nearest fit germline sequences, namely Ig V\(_h\)3-15, D1-26, and J\(_{\mu}\)4 (Figure 1). However, unlike CLL16, CLL17 did not have detectable levels of ZAP-70 protein (Figure 2).

We examined each leukemia cell sample for expression of CD38. Five of the 12 cases that used non-mutated Ig genes (CLL3, CLL5, CLL8, CLL9, and CLL11) had high-level expression of CD38 (average MFIR = 15 ± 8.6 S.D., with 76% (± 23% S.D.) of the leukemia cells on average found CD38°), whereas only 1 of the 10 cases with mutated Ig genes (CLL16) expressed this surface molecule (MFIR = 2.3 and 37% found CD38°). As such, a significantly higher proportion of the samples that used non-mutated Ig genes expressed CD38 (41%) than did samples with mutated Ig receptors (10%) (P < 0.05, Student’s t test). Furthermore, all samples found to express CD38 also had ZAP-70 protein, including CLL16. However, 7 of the 12 cases (59%) that used
non-mutated Ig genes and that also expressed ZAP-70 protein had insignificant expression of CD38 (average MFIR = 1.2 ± 0.4 S.D., with an average of 6.5% (± 6.5 S.D.) of the leukemia cells found CD38+). All nine cases other than CLL16 that expressed mutated Ig genes also had negligible expression of CD38 (average MFIR = 1.0 ± 0.2 S.D., with an average 0.9% (± 0.7 S.D.) found CD38+), including CLL17 obtained from the twin sibling of CLL16.

Functional Analyses of BCR Signaling

We examined whether ligation of the BCR could induce tyrosine phosphorylation of cytosolic proteins in the CLL cases examined for expression of ZAP-70. Each of the freshly thawed leukemia cell samples had comparable expression of surface IgM, allowing us to use anti-µ F(ab)₂ (abbreviated as “anti-µ”) as an agonist to effect BCR signaling (data not shown). We found that ligation of the BCR on CLL cell samples that expressed ZAP-70 (n =13) induced tyrosine phosphorylation of many cytosolic proteins (Figure 3 and data not shown). This was observed even for CLL16 that expressed mutated Ig genes. Moreover, the increase in detected tyrosine phosphorylation following BCR-ligation appeared greater in CLL cells that expressed ZAP-70 than in CLL cells that did not express ZAP-70 (Figure 3, and data not shown). In fact, ligation of the BCR on eight of the CLL cell samples that did not express ZAP-70, including CLL17, did not induce any apparent changes in tyrosine phosphorylation, as detected by the anti-phosphotyrosine antibody 4G10 (Figures 3 and 4, and data not shown).
To evaluate the relative increase in tyrosine phosphorylation, we measured the changes in density of bands identified on the immunoblot after BCR-ligation. For example, signal intensity measurements of a defined area that included proteins of 70-72 kDa on the immunoblot allowed us to compare the relative increase in the density of the bands following BCR ligation (Figure 4). Following BCR ligation, the 13 cases that
expressed ZAP-70 had an average 190% increase (± 120% S.D.) in band intensity. This was significantly greater than the 30% increase (± 30% S.D.) in band intensity observed for the 9 cases that did not express ZAP-70 protein (P < 0.01, Student’s t test) (Figure 4, left panel).

Expression of ZAP-70 also appeared more relevant than expression of CD38 in defining the cases that responded to BCR ligation. The CLL cell samples that expressed CD38

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**Figure 4** – Increase in signal intensity of tyrosine phosphoproteins of 70-72 kD following BCR ligation. Each symbol represents one CLL sample. The height of each symbol corresponds to the percent increase in phosphoprotein signal intensity caused by BCR ligation, as indicated on left-hand axis. The graph on the left segregates the symbols of leukemia cell samples that did (ZAP-70⁺) or did not (ZAP-70⁻) express ZAP-70, whereas the graph on the right segregates samples based upon whether they expressed ZAP-70 and CD38 (ZAP-70⁺/CD38⁺, left group), or ZAP-70 without CD38 (ZAP-70⁺/CD38⁻, middle group), or neither ZAP-70 nor CD38 (ZAP-70⁻/CD38⁻, right group). The horizontal line drawn among the symbols of each group represents the average fold-increase in signal intensity for that group.
(n = 6) had on average a 220% increase (± 140% S.D.) in the intensity of bands in this area following BCR ligation. Linear regression analysis of the increase in band intensities relative to the CD38 MFIR for each of these six CD38+ cases did not reveal a significant relationship, providing a squared Pearson product-moment correlation coefficient (R²) of only 0.4661 (data not shown). Although the average increase in band intensity for these six CD38+ cases was significantly greater than the 30% average increase (± 30% S.D.) noted for the nine CLL cell samples that expressed neither CD38 nor ZAP-70 (P < 0.05, Bonferroni t test), it was not significantly greater than the 170% average increase (± 110% S.D.) noted for the seven CD38-negative CLL cell samples that expressed ZAP-70 (Figure 4, right panel). Moreover, following BCR-ligation the CLL cases that expressed ZAP-70 but not CD38 (n = 7) had a significantly greater increase in signal intensity (m = 170% ± 110% S.D.) than did the nine CD38-negative samples that did not express ZAP-70 (m = 30% ± 30% S.D.) (P < 0.01, Student’s t test).

We examined for BCR-induced increases in tyrosine phosphorylation of proteins of this size, namely p72Syk. For this, we performed Syk immune precipitation of leukemia cell lysates with anti-Syk antibodies before and after BCR-ligation and then probed the immunoblot with the anti-phosphotyrosine antibody 4G10 (Figure 5A). Following BCR-ligation, we noted an average 180% increase (± 40% S.D.) in the band intensity of P-Tyr Syk in the 5 examined cases that expressed ZAP-70. This fold increase was significantly greater than that induced in the 5 examined leukemia cells that did not express ZAP-70 (m = 40% ± 20% S.D.) (P < 0.05 Student’s t test). Stripping the blots and then re-probing them for Syk revealed that each of the 10 studied cases had similar amounts of Syk protein (Figure 5A, lower panel).
Similarly, we prepared immune precipitates from lysates of ZAP-70+ leukemia cases before and after BCR ligation using antibodies to ZAP-70. Immunoblot analyses of these samples with anti-phosphotyrosine antibodies revealed that the signal intensity of ZAP-70 increased following anti-µ stimulation (Figure 5B). ZAP-70 protein was found in each lane when the blots were stripped and then re-probed with anti-ZAP-70 antibody (Figure 5B, lower panel).

Figure 5 - Phosphorylation of Syk or ZAP-70 in CLL B cells after BCR ligation. Lysates were prepared before and 10 minutes after BCR-ligation with anti-µ F(ab)₂ and then used for immune precipitation with antibodies specific for Syk (panel A) or ZAP-70 (panel B). Each pair of lanes represents the lysates prepared from cells before (-) or after (+) BCR ligation, as indicated at the top of each sample lane. The numbers of the CLL samples are provided above each pair of lanes. CLL13 (“13”) and CLL14 (“14”) did not express ZAP-70, whereas CLL cases 1 through 4 were ZAP-70+. The top row in panels A and B provides the proteins of ~70-72 kD that were detected by anti-phosphotyrosine antibody 4G10, as marked by the left-hand arrow labeled “anti-P-Tyr”. The bottom rows in panels A and B provide the same bands identified on the stripped blots that reacted with anti-Syk (“Syk”) or anti-ZAP-70 (“ZAP-70”) antibodies, respectively, as indicated by the arrows on the left-hand side of the figure.

That ZAP-70 undergoes tyrosine phosphorylation within minutes of BCR ligation suggests that ZAP-70 may associate with the surface BCR complex. To examine for this we prepared immune precipitates with anti-human IgM (Figure
6A) or anti-CD79b (Figure 6B) from ZAP-70⁺ cases before and after anti-µ stimulation. Immunoblot analyses with ZAP-70 antibodies revealed ZAP-70 in lanes with lysates prepared from anti-µ stimulated cases. Little or no detectable ZAP-70 signal was detected in the samples prior to BCR ligation. In contrast, both pre- and post-stimulated samples contained similar levels of µ (Figure 6A) or CD79b protein (Figure 6B), indicating that each immune precipitate had captured similar levels of target protein.

Figure 6 - Association of ZAP-70 with IgM and CD79b in CLL B cells after BCR ligation. Lysates were made before and 10 minutes after BCR-ligation with anti-µ F(ab)₂ and prepared for immune precipitation with antibodies specific for human IgM (panel A) or CD79b (panel B). Samples were loaded onto separate lanes of a polyacrylamide gel for immunoblot analysis with anti-ZAP-70, as indicated by the labeled arrow marked “ZAP-70”. Stripped blots were re-probed for anti-µ (panel A) or anti-CD79b (panel B) as indicated by the arrows marked “µ” or “CD79b”, respectively. Each pair of lanes represents the lysates prepared from cells before (-) or after (+) BCR ligation, as indicated at the top of each sample lane. The numbers of the CLL samples are provided above each pair of lanes.
Discussion

In this study, CLL B cells that expressed non-mutated Ig V genes were found to express levels of ZAP-70 protein that were comparable to that detected in blood T cells of normal adults. In contrast, leukemia cells that expressed mutated Ig receptors generally did not express detectable levels of ZAP-70 protein. These results agree with those obtained using microarray to assess the gene-expression profiles of CLL B cells.5 However, in this study we found that the association between the use of non-mutated Ig genes and expression of ZAP-70 was not absolute, in that one of the CLL samples that expressed mutated Ig receptors also expressed high-levels of the T-cell receptor PTK. Nevertheless, we note that a significantly higher proportion of the 12 cases with non-mutated Ig genes expressed ZAP-70 (100%) than did the ten cases with mutated Ig receptors (10%) (P < 0.01).

High-level expression of CD38 correlated with expression of ZAP-70. CD38 is a 45 kD type II transmembrane glycoprotein with ecto-enzymatic activity, which has been argued to play a complex role in B cell activation and proliferation.20-22 CLL-cell expression of CD38 has been associated with the use of non-mutated Ig V genes and more aggressive clinical disease.2,24 However, similar to other studies,25 we found that the expression of CD38 was not invariably associated with Ig mutational status. Indeed, one of the samples that expressed mutated Ig V genes (CLL16) also expressed CD38. Because this case also was ZAP-70+, it is conceivable that CD38 may play a role in expression of ZAP-70. However, because 7 of the 13 CLL cases that were ZAP-70+ had only negligible levels of CD38, expression of ZAP-70 appears neither dependent on CD38 nor sufficient to induce its expression in CLL.
The use of samples obtained from monozygotic twins concordant for CLL allowed us to examine for differences in leukemia cell populations that likely are due to somatic events. The CLL cells from both twins expressed mutated Ig \( \text{V}_{\text{H}} \) genes of the \( \text{V}_{\text{H}3} \) subgroup, but were discordant for expression of ZAP-70 and CD38. Because these twins were monozygous, this analysis suggests that the differences between patients in leukemia-cell expression of ZAP-70 and/or CD38 in CLL cannot be explained by genetic polymorphism.

We found that leukemia cell samples that expressed ZAP-70 had a greater increase in tyrosine phosphorylation following BCR-ligation than did leukemia cell samples lacking expression of ZAP-70. Prior studies demonstrated that CLL cases were heterogeneous in their ability to respond to BCR cross-linking\(^{26,27}\). Furthermore, CLL cells that expressed CD38 appeared more responsive to BCR-ligation\(^{28}\). This association between CD38 and signaling intensity also was observed in the leukemia cell samples examined in this study. However, this just may be a reflection of the noted association between CD38 and expression of ZAP-70. All cases that expressed ZAP-70 were noted to have an increase in tyrosine phosphorylation following BCR-ligation. Moreover, the extent of the average BCR-induced increase in signal intensity for ZAP-70 cases was significantly higher than that noted for samples that did not express ZAP-70, even in ZAP-70\(^+\) cases that had negligible expression of CD38 (Figure 4).

One of the proteins that is phosphorylated after BCR-ligation is Syk. Syk is an important PTK in the BCR-signaling pathway\(^{12,29,30}\). Prior studies noted that the CLL cells of different patients varied in the extent to which Syk was phosphorylated following BCR-ligation\(^{31,32}\). Moreover, the extent to which Syk underwent tyrosine-
phosphorylation appeared to be associated with the ability of the leukemia cells to respond to BCR-ligation\textsuperscript{32}. However, as noted in this and a prior study\textsuperscript{32}, the differences between CLL cases in the levels of Syk phosphorylation following BCR-ligation were not apparently related to differences in the levels of Syk protein found in each of the CLL cell populations. Instead, we found that the leukemia cells that were better able to respond to BCR-ligation expressed ZAP-70 in addition to Syk. Moreover, the average level of BCR-induced Syk phosphorylation in such cases was significantly greater than that observed in cases that did not express ZAP-70.

It is not clear whether expression of ZAP-70 contributes to the enhanced BCR-signaling capacity noted for CLL samples that express this protein. Prior studies indicated that Syk has an approximately 100-fold greater intrinsic PTK activity than does ZAP-70\textsuperscript{33}. Also, in contrast to ZAP-70, Syk can undergo stimulation in the absence of src-related kinases, initiate immunoreceptor signaling, and promote tyrosine phosphorylation of ITAMs in the BCR complex\textsuperscript{34}. In addition, normal B cells do not express ZAP-70 and yet respond to BCR-ligation as well as that of the CLL cases that have the highest response to stimulation via the BCR complex\textsuperscript{32}. As such, ZAP-70 is not necessary for effective BCR-signaling, at least by normal B cells. Nevertheless, the association between expression of ZAP-70 and magnitude of tyrosine phosphorylation induced by surface Ig crosslinking in CLL suggests that this PTK may enhance the signaling capacity of the BCR complex in B cell CLL.

Consistent with this notion, we found evidence that ZAP-70 may function in BCR-signaling. Following ligation of the BCR we found that ZAP-70 undergoes tyrosine phosphorylation and complexes with the proteins of the BCR complex. Conceivably,
expression of ZAP-70 may lower the threshold for BCR signaling. ZAP-70 has been reported to promote tyrosine phosphorylation of the T cell receptor signaling motifs in immature CD4+8+ thymocytes in response to TCR crosslinking\textsuperscript{35}. As such, ZAP-70 may function to enhance phosphorylation of such motifs in CLL B cells and thereby lower the threshold for Syk phosphorylation following BCR ligation. Alternatively, expression of ZAP-70 in CLL cells may enhance the stability of phosphorylated Syk, allowing accumulation of the functional form of Syk in CLL following BCR ligation. Transfection of leukemia cells that lack ZAP-70 may be required to test these hypotheses. Nevertheless, expression of ZAP-70 appears most closely associated with the relative capacity of the BCR-complex to effect signaling in the CLL B cell. Such differences may contribute to the noted clinical heterogeneity among patients with this disease.
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Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia

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