

B-Cell Receptor Translocation to Lipid Rafts and Associated Signaling Differ between Prognostically Important Subgroups of Chronic Lymphocytic Leukemia

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Abstract

Chronic lymphocytic leukemia (CLL) is a highly heterogeneous disease in which interaction of the malignant cells with antigen is thought to play a key role. Individual CLL-cell clones markedly differ in their ability to respond to B-cell receptor ligation, but the mechanism underlying the frequent hyporesponsiveness is incompletely understood. Our aim was to further clarify the extent and cause of the B-cell receptor signaling abnormality in CLL and to assign pathophysiologic relevance to the presence or absence of B-cell receptor responsiveness. We show that extracellular signal-regulated kinase-2 phosphorylation, intracellular Ca^{2+} increases, CD79a phosphorylation, and translocation of the B-cell receptor to lipid rafts in response to ligation with anti-immunoglobulin M (as a surrogate for antigen) are features of CLL cells with relatively unmutated *VH* genes (<5% deviation from germ line) and a poor prognosis. B-cell receptor stimulation in these cases also promoted cell survival. In clones with mutated *VH* genes (>5% deviation from germ line), surface immunoglobulin M ligation failed to induce receptor translocation to rafts or to prolong cell survival. This failure of receptor translocation observed in mutated CLL cells was associated with the constitutive exclusion of the B-cell receptor from rafts by a mechanism involving src-dependent interactions between the B-cell receptor and the actin cytoskeleton. We conclude that exposure to antigen promotes the survival of unmutated CLL clones, contributing to the poor prognosis of this group. In contrast, hyporesponsive mutated CLL clones may have developed into a stage where continuous exposure to antigen results in relative tolerance to antigenic stimulation mediated by the exclusion of the B-cell receptor from lipid rafts. (Cancer Res 2005; 65(16): 7328-37)

Introduction

Chronic lymphocytic leukemia (CLL) is by far the commonest leukemia of adults and, despite advances in treatment, remains incurable. The disease is characterized by the accumulation of $CD5^+ CD23^+$ B cells with low expression of surface immunoglobulin. The current consensus is that CLL cells derive from antigen-activated B cells (1) and are more closely related to memory cells than to any other normal B-cell type (2, 3).

Because signals generated by interaction between antigen and the B-cell receptor (B-cell receptor) are crucial during B-cell

development (4), an understanding of B-cell receptor function in CLL is important in gaining insights into the pathogenesis of the disease. It has been known for a number of years that there is a marked case-to-case heterogeneity in B-cell receptor responsiveness in CLL, with the malignant cells being markedly hyporesponsive in a proportion of cases. Responsiveness has been related to progressive disease (5), to high CD38 expression (6), and, recently, to lack of extensive *VH* mutation (7). In contrast, it has been suggested that hyporesponsiveness may reflect an anergic state induced by chronic antigen exposure (8, 9).

Although B-cell receptor hyporesponsiveness is a well-recognized feature of some cases of CLL, its biological basis is unknown. Entry of the B-cell receptor into lipid rafts is an early event in B-cell receptor signaling and failure of this receptor to enter rafts is a feature of anergized normal B-lymphocytes (10). We therefore hypothesized that failure of the B-cell receptor to enter lipid rafts after cross-linking might be a feature of CLL clones which do not respond to B-cell receptor stimulation. Here we show that this is indeed so, and thereby show the presence of a very proximal defect in the B-cell receptor response in such cases. Also, we give our findings a pathophysiologic relevance by showing that, in B-cell receptor-responsive cases, antigen receptor ligation results in enhanced cell survival.

Patients, Materials, and Methods

Chronic lymphocytic leukemia patients. All the patients in the study had typical CLL (mature lymphocytes expressing CD5, CD19, CD23, and clonally restricted surface immunoglobulin) and were selected on the basis of having a WBC count mostly $>20 \times 10^9/L$ (Table 1). Peripheral blood samples were taken with informed consent and with the approval of the local Research Ethics Committee. Mononuclear cells were isolated by centrifugation over Lymphoprep (Life Technologies, Inc., Paisley, United Kingdom) before cryopreservation in liquid nitrogen.

Patient characteristics are given in Table 1. *VH* mutation analysis was done as previously described (11). The methods used to determine CD38 expression have also been detailed elsewhere (12).

B-cell receptor-induced extracellular signal-regulated kinase phosphorylation. Cryopreserved cells were thawed and resuspended at 1×10^7 cells/mL in RPMI supplemented with 1% bovine serum albumin (BSA), and then cultured for 2 to 3 hours at 37°C. Cells (1×10^7) were stimulated for 30 minutes with 10 μg F(ab)₂ fragments of goat anti-human immunoglobulin M (IgM; Fc_{5u} fragment specific) or with isotypic control (both from Jackson ImmunoResearch, West Grove, PA). The cells were washed twice and then lysed and sonicated in lysis buffer (1% SDS, 10 mmol/L Tris-HCl, pH 7.4), containing 1 mmol/L sodium orthovanadate, 0.1 mg/mL phenylmethylsulfonyl fluoride (PMSF), and 1 $\mu g/mL$ each of chymostatin, leupeptin, aprotinin, pepstatin A, and antipain. Fifty microliters of lysate (equivalent to 5×10^5 cells) were then submitted to SDS-PAGE and Western blotted with a mouse anti-phospho-extracellular signal-regulated kinase (ERK) monoclonal antibody (mAb; Santa Cruz Biotechnology, Santa

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Table 1. Characteristics of CLL cases studied

Case	Stage at diagnosis*	Stage at study*	Treatment [†]	Age/sex	Hemoglobin [‡] (g/dl)	WBC [‡] ($\times 10^9/L$)	VH [§]	CD38 (%)	Phospho-ERK [¶]	Calcium**	sgM ^{††}	Cell survival ^{‡‡}	Raft associated IgM ^{§§}
1	C/IV	C/IV	CLB	78/F	11.3	27	12.37	17	16.5	1.62	3.3	0	
2	C/III	C/III	None	65/M	9.7	99	11.1	0.7	0.94	1.12	1.3	-1	
3	A/I	A/I	None	81/F	12.3	97	10.64	0.1	1.93	1.16	1.7	-8.5	0.9
4	A/I	C/III	CLB	73/M	12.9	23	10.2	1.6	0.61	1.04	1.2	-1	1
5	A/I	A/I	None	83/M	14.2	53	8.4	4	0.88	1.37	1.2	1.5	42.8
6	A/0	A/0	CLB	70/M	14.6	88	7.48	1	1.73	1.01	1.11	-1	0.57
7	A/I	A/I	CLB + P	56/M	14.1	91	6.56	2	2.7	1.04	1.7	-3.5	0.7
8	B/III	C/IV	None	71/F	13.2	65	6.45	1.2	1.4	N/A	1.7	N/A	
9	A/0	A/0	None	81/F	13.8	41	5.68	6.2	6.4	1.1	1.4	-4.5	1.2
10	A/II	A/II	None	64/M	13	24	4.8	66	320.1	1.89	8.3	3.5	11.6
11	C/III	C/III	None	73/M	10.9	17	4.3	40	8.89	1.82	4.5	-0.5	
12	C/IV	A/0	CLB, CLB + P	74/F	11.5	16	4.15	40	107	1.72	2.4	32.5	
13	C/IV	C/IV	F + C	81/M	6	300	2.48	0.4	31.77	2.7	3	18.5	
14	B/II	B/II	CLB, F, CHOP	61/M	11.9	61	2	40	94.3	1.69	9.8	23.5	9.8
15	B/III	B/III	CLB	75/F	10.8	110	1.41	52	9.44	1.25	1.12	14	
16	A/I	C/IV	CLB	79/F	8.1	447	0.71	61	2.33	N/A	1.34	1.5	
17	C/III	A/I	CLB	68/M	11.9	136	0.66	4	198.9	2	2.2	11.5	36.2
18	A/I	C/III	CLB	38/M	10.3	136	0.42	66	0.9	1.13	1.8	-0.5	0.9
19	A/I	A/I	CLB, F	71/F	11.6	110	0.34	3.4	6.39	1.31	2.5	3	
20	B/I	B/I	CLB, F	61/M	11.6	231	0	6	24.9	1.1	1.2	1.5	4.2
21	B/II	B/II	CLB, F	63/M	14.4	248	0	51	13.7	1.07	2.7	4	
22	A/I	A/I	None	69/M	13.5	99	0	0.6	4.39	1.42	2.6	13.5	73.1
23	B/III	B/III	CLB	72/M	9.1	258	0	14	13.8	1.03	1.3	5	13.8

*Binet/Rai clinical stages.

[†] CLB, chlorambucil; P, prednisolone; F, fludarabine; C, cyclophosphamide; H, Adriamycin; O, vincristine.

[‡] Hemoglobin and WBC at time of study.

[§] Percent deviation from germ line.

^{||} Percentage of CD38⁺19⁺ cells.

[¶] Density of phospho-ERK band after BCR cross-linking as a ratio of that present in unstimulated cells.

** [Ca²⁺] in cells cross-linked with anti-IgM for 5 minutes as ratio of that present without cross-linking.

^{††} Mean fluorescence intensity of sIgM as a ratio of that of isotypic control.

^{‡‡} Change in percent viable cells following BCR cross-linking with F(ab')₂ anti- μ .

^{§§} Mean density of raft-associated IgM following BCR engagement (10 minutes) as a ratio of that present in unstimulated cells.

Cruz, CA). Membranes were then stripped and reprobed with a rabbit polyclonal antibody to ERK (Santa Cruz Biotechnology). Following densitometric analysis of the bands, phospho-ERK-2 was expressed as a fraction of total ERK-2. The ERK activation response to B-cell receptor stimulation was then expressed as the relative increase in phospho-ERK-2 following cell stimulation.

Dose response experiments in a representative responsive case using F(ab')₂ fragments of goat anti-human IgM to cross-link the B-cell receptor showed a maximal response with 10 μ g/10⁷ cells. Time-course experiments showed a maximal increase in ERK phosphorylation following incubation for 30 minutes with the antibody. In contrast, in nonresponsive clones, there was a failure of ERK phosphorylation at both early (5 minutes) and late (30 minutes) time points following B-cell receptor cross-linking.

Measurement of intracellular calcium. Cells were cultured at 1 \times 10⁷ cells/mL for 2 to 3 hours at 37°C in RPMI supplemented with 1% BSA before being washed thrice and resuspended in calcium assay buffer (120 mmol/L NaCl, 4.7 mmol/L KH₂PO₄, 1.2 mmol/L MgSO₄, 1.25 mmol/L CaCl₂, 10 mmol/L glucose, 20 mmol/L HEPES, pH 7.4; ref. 13) at a density of 1 \times 10⁷ cells/mL. The cells were then incubated with 3 μ g/mL of fura-2AM (Molecular Probes, Leiden, the Netherlands) for 30 minutes at 37°C with constant agitation, before being washed and resuspended at 1 \times 10⁷ cells/mL in the calcium assay buffer.

The intracellular calcium concentration was derived by measuring the fluorescence intensities at 505 nm in 200 μ L of fura-2AM-loaded cells in 96-well plates excited at 340 and 380 nm using a Gemini Spectramax plate reader (Molecular Devices, Sunnyvale, CA). Calibration was achieved by obtaining measurements of fluorescence from lysed cells in completely calcium-free medium (F_{\min}) and in medium with saturating concentrations of calcium (F_{\max}). To obtain F_{\min} , 200 μ L of fura-2AM-loaded cells at 1 \times 10⁷ cells/mL were resuspended in calcium assay buffer supplemented with 0.05% Triton X-100, 5 mmol/L EGTA, and 40 mmol/L Tris-HCl. F_{\max} was obtained in the same way except that cells were resuspended in calcium assay buffer supplemented with 0.05% Triton X-100 and 5 mmol/L CaCl₂. Simultaneous measurements were made on F_{\min} , F_{\max} , and the fluorescence obtained from cells stimulated with cross-linking antibody or isotypic control or from unstimulated cells. Fluorescence was measured for 10 minutes with readings taken every 11 seconds and with the cells maintained at 37°C. The calcium concentration was derived from the formula:

$$[\text{Ca}^{2+}] = K_d \times Q \times (R - R_{\min}) / (R_{\max} - R)$$

where R is the ratio of the fluorescence intensities measured at 340 and 380 nm in the test sample, R_{\min} is the ratio of F_{\min} measured at 340 and 380 nm, R_{\max} is the ratio of F_{\max} measured after excitation at 340 and

380 nm, K_d is the dissociation constant of fura-2 (224 nmol/L), and Q is the ratio of F_{\min} to F_{\max} following excitation at 380 nm.

CD79a phosphorylation. Cryopreserved cells were thawed and resuspended at 1×10^7 cells/mL in RPMI supplemented with 1% BSA and then cultured for 2 to 3 hours at 37°C. Cells were stimulated for either 2 or 10 minutes with 10 μ g F(ab)₂ fragments of goat anti-human IgM before lysis and immunoprecipitation of CD79a with a mouse anti-CD79a mAb (Serotec, Oxford, United Kingdom). Cells were lysed in ice-cold radio-immunoprecipitation assay buffer (RIPA; PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with sodium orthovanadate, PMSF, and other inhibitors as described above, and left on ice for 30 minutes. The samples were centrifuged at $13,000 \times g$ for 15 minutes at 4°C and the supernatants removed. Lysates were precleared by the addition of 20 μ L of washed rec-protein G beads (Zymed Laboratories, San Francisco, CA) for 60 minutes at 4°C. Samples were centrifuged at $13,000 \times g$ for 30 seconds and the supernatants incubated overnight with the immunoprecipitating antibody at 4°C. Twenty microliters of rec-protein G beads were added to the lysate and incubated for 60 minutes at 4°C. The lysates were centrifuged, the supernatant removed, and the sedimented beads washed thrice in RIPA buffer and boiled for 5 minutes in 100 μ L of double-strength sample buffer. Immunoprecipitates were subjected to SDS-PAGE and Western blotted with a mouse anti-phosphotyrosine mAb (ICN Biomedicals, Costa Mesa, CA). Subsequently, membranes were stripped of antibody by incubation with erasure buffer for 30 minutes at 65°C, following which membranes were reprobed with the anti-CD79a antibody.

Fluorescence-activated cell sorting analysis of surface immunoglobulin M. CLL cells were stained at 1 μ g/ 10^6 cells with anti-IgM [clone 145-8; immunoglobulin G1 (IgG1)] conjugated to phycoerythrin (Becton Dickinson, San Jose, CA). The intensity of expression was calculated as a ratio of the observed mean fluorescence intensity to that of phycoerythrin-conjugated nonspecific mouse IgG1 control (Becton Dickinson) using the Cell Quest software program (Becton Dickinson).

Cell survival. Cells were suspended in RPMI supplemented with 0.1% BSA at a density of 5×10^6 cells/mL and cultured for 48 hours in flat-bottomed 96-well plates coated with poly(2-hydroxyethyl methacrylate) (Sigma, Poole, Dorset, United Kingdom). The poly(2-hydroxyethyl methacrylate) coating provides a nonadhesive surface and, as a result, any rescue stimuli provided by adhesion are avoided (14). These culture conditions were chosen because previous work from this Department (15) has shown that, under these conditions, there is significant, but not major, loss of viability at 48 hours, allowing measurements of any proapoptotic and antiapoptotic effects of surface immunoglobulin cross-linking. The percentage of contaminating cells was similar in both unmutated and mutated subgroups (mean, 16% and 12% respectively; $P = 0.76$, Student's t test). After 48 hours of culture, mean baseline viability in the two subgroups was also similar (63% and 68% for unmutated and mutated CLL, respectively).

For testing the effect of B-cell receptor cross-linking on CLL-cell survival, surface IgM (sIgM) was cross-linked with F(ab)₂ fragments of goat anti-human IgM (at 10 μ g/ 10^7 cells). This concentration of antibody was found by titration in our studies and previous studies (16) to be sufficient for maximal rescue effects.

Cell viability was assessed using fluorescence-activated cell sorting (FACS) analysis of cell size and membrane integrity as measured by forward-light scatter and propidium iodide uptake (preincubation with 10 μ g/mL for 30 minutes), respectively (17). Dead cells display a decrease in forward-light scatter and an increase in propidium iodide uptake, and are visible as a separate cloud on propidium iodide versus forward-light scatter plots.

Isolation of lipid rafts. CLL cells (1×10^8) were thawed and resuspended at 1×10^7 /mL in RPMI supplemented with 1% BSA before being cultured for 2 to 3 hours at 37°C. Cells were then stimulated for 10 minutes with F(ab)₂ fragments of goat anti-human IgM (10 μ g/ 10^7 cells). The cells were washed in ice-cold PBS before transfer to 200 μ L of ice-cold lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% Triton X-100, 1 mmol/L sodium orthovanadate, 0.1 mg/mL PMSF, and 1 μ g/mL each of chymostatin, leupeptin, aprotinin, pepstatin A,

and antipain). The cells were then disrupted by aspirating repeatedly with a syringe and 21 gauge needle and incubated on ice for 30 minutes. Four-hundred microliters of ice-cold 60% Optiprep (Nycomed, Oslo, Norway) were then added to the cell lysate, thereby adjusting the Optiprep concentration to 40%. The sample was overlaid with 1 mL of 30% Optiprep and 0.5 mL of 5% Optiprep before centrifugation at $250,000 \times g$ for 4 hours in a Beckman Optima TL Ultracentrifuge. Fractions of 150 μ L were removed from the top of the centrifuge tube and subjected to SDS-PAGE and Western blotting with a rabbit polyclonal antibody to Lyn kinase (Santa Cruz Biotechnology). Membranes were then stripped and reprobed with a mouse mAb to CD45 (Santa Cruz Biotechnology). Fractions 3, 4, and 5 were found to be enriched in Lyn and to exclude CD45, features consistent with the presence of lipid rafts (18). Fractions 10, 11, and 12 were relatively deficient in Lyn kinase and rich in CD45, features found in the Triton-soluble membrane and cytosolic compartments (18).

To confirm the presence of lipid rafts in fractions 3, 4, and 5, 1×10^8 CLL cells were prelabeled with 4.2 μ g/mL of horseradish peroxidase-conjugated cholera toxin B (Sigma) for 10 minutes before lysis and centrifugation as described above. A 10 μ L aliquot of each of the 13 fractions obtained following ultracentrifugation was incubated with 100 μ L of *o*-phenylenediamine buffer (Sigma) for 30 minutes at room temperature before stopping the reaction with 150 μ L of 0.67 mol/L H₂SO₄. The samples were read at 492 nm and increased absorbance was found in fractions 3, 4, and 5, denoting the presence of cholera toxin. As cholera toxin B binding is a feature of lipid rafts (18), this confirmed the presence of these structures in fractions 3, 4, and 5.

For all subsequent experiments, fractions 3, 4, and 5 were pooled and termed the raft fraction, whereas fractions 10, 11, and 12 were pooled and termed the soluble fraction. The pooled samples were subjected to SDS-PAGE and Western blotting with a mouse anti-phosphotyrosine mAb (ICN Biomedicals) or with rabbit antiphospho-Lyn polyclonal antibody (Santa Cruz Biotechnology). Membranes were then stripped and reprobed with either a goat anti-human IgM antibody (Sigma) or with rabbit polyclonal antibodies to Lyn, SH2-domain-containing protein tyrosine phosphatase 1 (SHP-1), or SH2 domain-containing inositol 5'-phosphatase (SHIP; Santa Cruz Biotechnology). The IgM content of lipid rafts was then assessed densitometrically.

For experiments using the src kinase inhibitor 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1; Alexis Corporation, San Diego, CA), cells were preincubated with 10 μ mol/L PP1 for 60 minutes at 37°C before being washed in culture medium and stimulated as indicated. For experiments using cytochalasin D (Sigma), cells were preincubated for 30 minutes at 37°C with 10 μ mol/L cytochalasin D before being washed in culture medium and stimulated as indicated.

Statistics. Data were tested for normality using Kolmogorov-Smirnov test. For normally distributed data, a t test or paired t test was used to evaluate the differences between sets. For non-normally distributed data, the Mann-Whitney U test was used. For the Fisher's exact test, B-cell receptor responsiveness was defined as a >2-fold increase in ERK phosphorylation following 30 minutes of incubation with anti- μ antibody. Regression plots were generated using the SPSS software package.

Results

Reduced Extracellular Signal-Regulated Kinase Phosphorylation and Ca²⁺ Fluxes in Response to B-Cell Receptor Cross-Linking Are Features of Mutated Chronic Lymphocytic Leukemia Clones

We used two measures of CLL-cell responsiveness to B-cell receptor stimulation: ERK-2 phosphorylation and [Ca²⁺] increases following cross-linking of sIgM. Both these processes are known to be regulated by the B-cell receptor (19).

Extracellular signal-regulated kinase-2 phosphorylation. The changes in ERK-2 phosphorylation, induced by B-cell receptor cross-linking with F(ab)₂ fragments of goat anti-IgM, in the

malignant cells of 23 patients are shown in Fig. 1 and Table 1. Following IgM cross-linking, there was a marked case-to-case variation in the induction of ERK-2 phosphorylation, ranging from no change to ~300-fold increase (Table 1; Fig. 1).

When B-cell receptor-induced ERK-2 phosphorylation was analyzed in relation to VH mutation (Fig. 1A and B), phosphorylation was more markedly increased in cases with <5% deviation from the germ line sequence (a median of 13.8-fold versus 1.7-fold for >5% mutation; $P = 0.005$, Mann Whitney U test; $P = 0.05$, Fisher's exact test).

Although we have previously found that >5% deviation from the germ line clearly defines a subgroup of CLL patients with a good prognosis (11) and others have used 4% for subgroup division (20), several studies have employed a level of 2% to identify mutated versus unmutated CLL (21–23). We therefore also analyzed our ERK-2 phosphorylation data using 2% VH mutation as the cutoff between mutated and unmutated CLL. When the data were examined in this way, there was no statistically significant difference in B-cell receptor-induced ERK-2 phosphorylation in the two subgroups (for VH < 2%, median change = 9.44-fold versus 4.6-fold for VH > 2%; $P = 0.57$). This indicates that cells with 2%

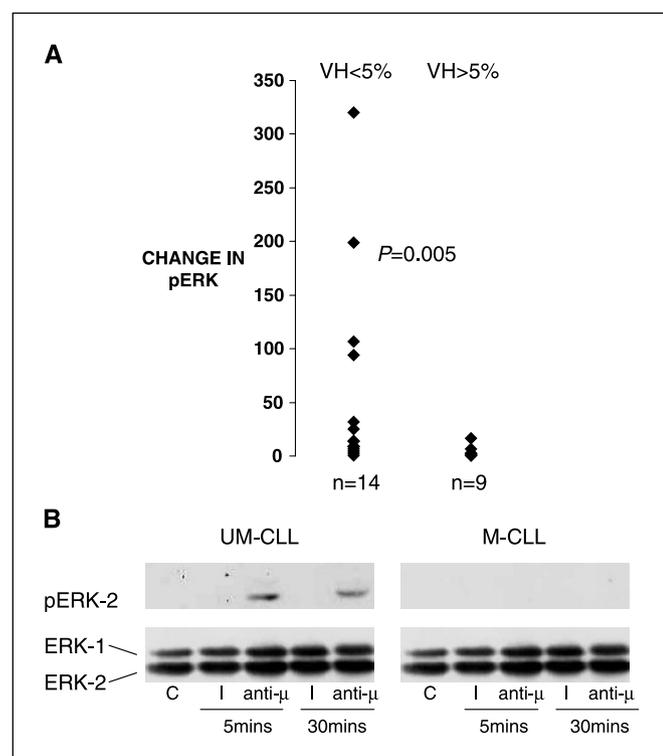


Figure 1. The relationship between changes in ERK phosphorylation in response to B-cell receptor cross-linking and VH gene mutational status. CLL cells (10^7) in 1 mL RPMI with 1% BSA were exposed to $10 \mu\text{g}$ of F(ab')_2 fragments of goat anti-human IgM antibody at 37°C . The cells were then washed twice, lysed, and sonicated in 1% SDS. Fifty microliters of lysate (equivalent to 5×10^5 cells) were then submitted to SDS-PAGE and Western blotted with a specific anti-phospho-ERK antibody. A, the change in ERK-2 phosphorylation following B-cell receptor cross-linking for 30 minutes was quantitated densitometrically (for details see Methods) and related to VH mutation. Points, mean of two separate estimations done on different occasions. Significance is calculated using the Mann-Whitney U test. B, Western blots for phospho-ERK-2 and total ERK in cell lysates of unmutated (UM-CLL) and mutated (M-CLL) CLL cells before and after 5 and 30 minutes of stimulation with F(ab')_2 fragments of goat anti-human IgM (anti- μ). F(ab')_2 fragments of goat IgG constituted the isotypic control (I). Lane C, cells were cultured for 30 minutes at 37°C in the absence of anti- μ antibody.

5% VH mutation ($n = 5$) more resemble unmutated than mutated cells with regard to ERK phosphorylation in response to B-cell receptor cross-linking.

Ca²⁺ mobilization. As with ERK phosphorylation, there was marked case-to-case variation in Ca²⁺ mobilization following B-cell receptor cross-linking with F(ab')_2 fragments of anti-IgM (Fig. 2A-C). The [Ca²⁺] increase was significantly greater in unmutated versus mutated CLL clones as defined by >5% deviation from the germ line (Fig. 2C).

When >2%, rather than >5%, VH deviation from germ line was taken as a measure of hypermutation, the [Ca²⁺] increases in response to B-cell receptor stimulation were, as already observed with ERK phosphorylation, not significantly different between mutated and unmutated CLL-cell clones (not shown). These data therefore lend further support to the notion that cases with 2% to 5% VH mutation more resemble unmutated than mutated CLL. This explains why, when these cases are included in the mutated CLL group, the difference in the B-cell receptor responsiveness of cells in the unmutated and mutated groups becomes nonsignificant.

When the increase in intracellular [Ca²⁺] induced by anti- μ was related to the increased ERK-2 phosphorylation induced by B-cell receptor cross-linking, there was a strong correlation between Ca²⁺ increases and ERK responsiveness ($P = 0.019$; Fig. 2D).

Taken together, the above data indicate that both a failure to phosphorylate ERK-2 and the absence of an increase in intracellular [Ca²⁺] in response to IgM cross-linking are features of the B-cell receptor hyporesponsiveness selectively observed in mutated CLL.

However, in some cases, the activation of ERK was not associated with an increase in intracellular [Ca²⁺] (Fig. 2D). This indicates that, in a proportion of responding CLL clones, there is a dissociation between ERK phosphorylation and [Ca²⁺] increases and suggests that some additional heterogeneity in B-cell receptor responses exists among unmutated CLL clones.

Changes in the Extent of CD79a Phosphorylation following B-Cell Receptor Cross-Linking

B-cell receptor-induced calcium mobilization is an early signaling event and the lack of this response in mutated CLL cells points to a proximal defect in the B-cell receptor signaling pathway. We therefore next studied the ability of CLL cells to phosphorylate CD79a as another early event in B-cell receptor signaling. The phosphorylation of CD79a and CD79b precedes the activation of intracellular signaling molecules such as Lyn, syk, and phospholipase C γ (24). CD79a was chosen in preference to CD79b because mutations within the immunoreceptor tyrosine-based activation motifs of CD79b that could affect phosphorylation of this molecule have been described in CLL (25). As expected, in unmutated CLL cells, B-cell receptor cross-linking resulted in an increase in CD79a tyrosine phosphorylation. In contrast, in mutated CLL cells, CD79a was constitutively phosphorylated and subsequently became dephosphorylated following receptor ligation (Fig. 3). This shows that the outcome of B-cell receptor ligation differs between unmutated and mutated CLL at a very proximal stage of the B-cell receptor signal transduction pathway.

Relationship between B-cell Receptor Responsiveness and Surface Immunoglobulin M

CLL is characterized by weak surface expression of IgM, but the amount expressed varies from case to case (26). The expression of sIgM was weak but variable in all 23 cases examined. There was a

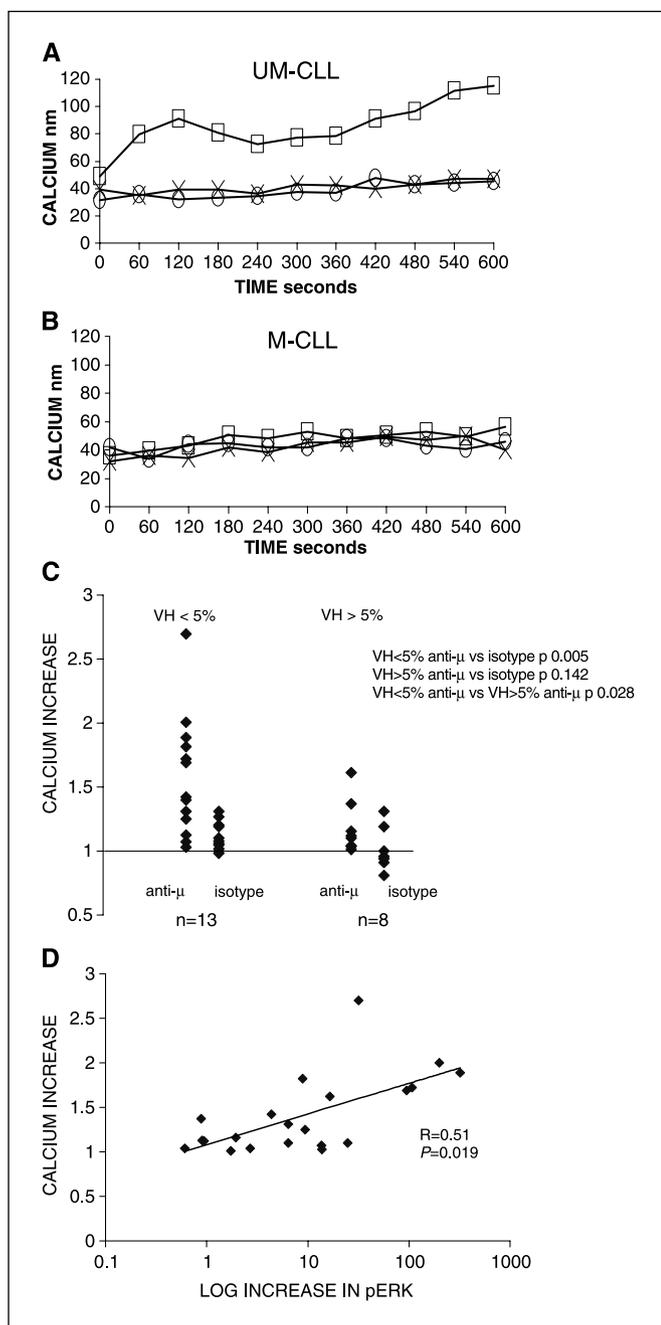


Figure 2. Changes in intracellular [Ca²⁺] following B-cell receptor cross-linking. Fura-2AM-loaded CLL cells were resuspended at 1×10^7 /mL in calcium assay buffer and incubated with either F(ab)₂ fragments of goat anti- μ antibody or F(ab)₂ fragments of nonspecific goat IgG as an isotypic control (both at a concentration of $10 \mu\text{g}/10^7$ cells). A Gemini Spectramax was used to measure fluorescence at 505 nm following excitation at 340 and 380 nm (see Methods). The intracellular [Ca²⁺] was measured continuously for 10 minutes following the addition of anti- μ or isotypic control antibodies. The control antibody had little or no effect on Ca²⁺ concentrations. **A** and **B**, [Ca²⁺] following B-cell receptor cross-linking (\square), compared with media (\circ) and isotypic controls (\times), in unmutated and mutated CLL clones respectively. Similar profiles were obtained in other responding and nonresponding cases (Table 1). **C**, [Ca²⁺] increases are related to VH mutation. The calcium increases were measured after 5-minute incubation with anti- μ antibody and expressed as a ratio of [Ca²⁺] in stimulated versus unstimulated cells. Significances were calculated using paired Student's *t* test. **D**, relationship between the change in phospho-ERK and intracellular [Ca²⁺] following B-cell receptor cross-linking. Linear regression analysis was done using the SPSS software package.

trend towards greater expression of sIgM on unmutated (<5% mutation) versus mutated clones (>5% mutation; $P = 0.054$). However, this trend was no longer observed when clones with 2% to 5% VH mutation were excluded from the unmutated group (Fig. 4). Moreover, the majority of responding unmutated clones closely resembled nonresponding mutated clones with regard to their sIgM expression (Fig. 4). We therefore conclude that, although the density of sIgM may have an effect on B-cell receptor responsiveness, the degree of expression of this molecule is not a critical determinant of the outcome of receptor ligation. B-cell receptor responsiveness was also not related to patient characteristics such as gender, age, and disease stage.

Failure of B-Cell Receptor Translocation into Lipid Rafts Is a Feature of Mutated Chronic Lymphocytic Leukemia

B-cell receptor translocation to lipid rafts is an important early event in the response of B cells to B-cell receptor engagement (18). Because the above results showed a failure in mutated CLL clones of two very early signaling events in response to ligation of the B-cell receptor (absence of intracellular calcium mobilization and CD79a phosphorylation), it seemed plausible that this failure might be associated with a lack of B-cell receptor translocation into lipid rafts. We therefore next examined whether unmutated and mutated clones differ in the composition and phosphorylation of raft-associated proteins before and after B-cell receptor ligation.

The IgM content of lipid rafts before and after cross-linking with anti- μ was used as a marker of B-cell receptor translocation into lipid rafts. In six of seven unmutated CLL clones examined, there was an increase in raft-associated IgM within 30 seconds of B-cell receptor cross-linking, and this was sustained for at least 10 minutes (Fig. 5). In contrast, in five of six mutated CLL cases studied, IgM was absent from rafts and did not translocate to this fraction at any time following B-cell receptor cross-linking (Fig. 5). When analyzed at 10 minutes following B-cell receptor cross-linking, the median increase in raft-associated IgM in unmutated CLL clones was 11.4-fold whereas in mutated CLL the median value was 1 (i.e., no change; $P = 0.001$, Mann-Whitney *U* test). The one case of unmutated CLL in which B-cell receptor cross-linking did not induce an increase in raft-associated IgM was also characterized by a failure of the cells to increase [Ca²⁺] and to phosphorylate ERK in response to B-cell receptor stimulation.

Phosphorylation of lipid-raft-associated Lyn kinase is one of the earliest events to occur following B-cell receptor translocation to lipid rafts (27). Translocation of the B-cell receptor to lipid rafts was significantly associated with increases in the phosphorylation

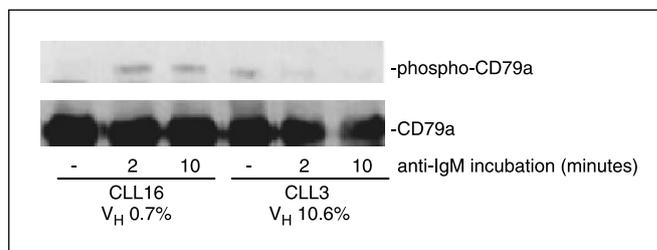


Figure 3. Changes in CD79a phosphorylation following B-cell receptor cross-linking. CLL cells (10^7) were incubated with either medium alone (-) or $10 \mu\text{g}$ F(ab)₂ anti-IgM for 2 or 10 minutes. CD79a was immunoprecipitated from RIPA lysates, subjected to SDS-PAGE, and Western blotted with an anti-phosphotyrosine antibody (phospho-CD79a). Membranes were then reprobbed with an anti-CD79a antibody (CD79a). Representative of six cases studied.

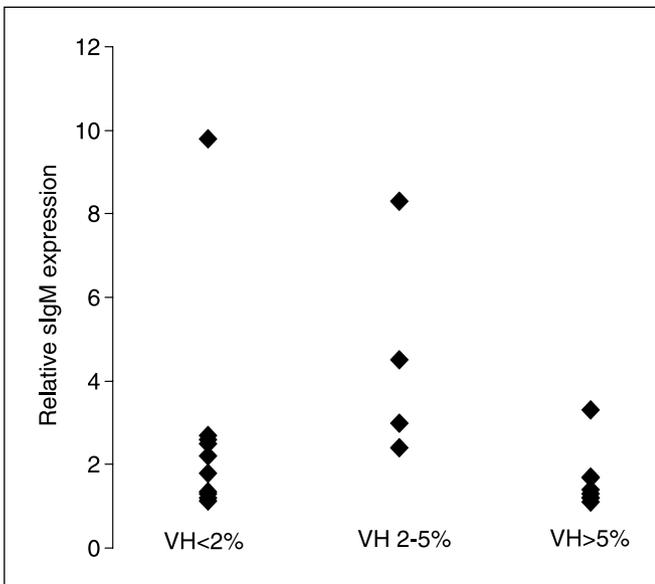
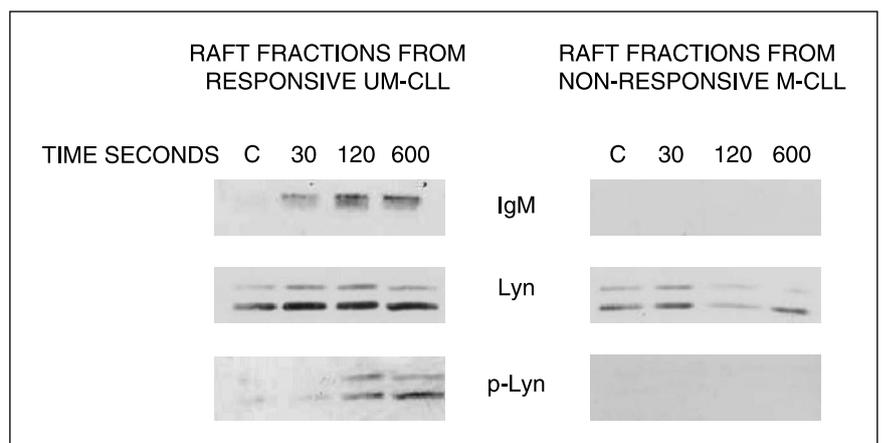


Figure 4. Relationship between sIgM expression and VH gene mutation. The intensity of sIgM was measured as the ratio of the mean fluorescence intensity of CLL cells stained with FITC-labeled mouse anti-IgM mAb (IgG1) as compared with their mean fluorescence intensity after incubation with labeled isotopic control. Results for 23 CLL cases with VH <2%, VH 2-5%, and VH >5% deviation from germ line.

of both the p53 and p56 Lyn kinase isoforms ($P = 0.001$ and 0.006 , respectively), whereas total Lyn remained unchanged (Fig. 5).

Because protein and lipid phosphorylations involved in B-cell receptor cross-linking are opposed by phosphatases (28, 29), we next examined the SHP-1 and SHIP content of the rafts of responding and nonresponding CLL cells, before and after B-cell receptor cross-linking. Before B-cell receptor stimulation, variable amounts of SHP-1 were detectable in the rafts from both unmutated and mutated CLL clones. After stimulation, there was a significant correlation between translocation of the B-cell receptor into lipid rafts and further recruitment of SHP-1 into these structures ($P < 0.001$; data not shown). Regarding SHIP phosphatase (which modulates phosphatidylinositol 3'-kinase-dependent signals generated by B-cell receptor stimulation; ref. 29), this enzyme was not detected in rafts in any CLL clones either before or after B-cell receptor stimulation, regardless of their responsiveness. The enzyme was, however, readily detectable in the

Figure 5. Effects of B-cell receptor cross-linking on the IgM and Lyn content of lipid rafts. CLL cells (10^8) were resuspended in 1 mL of RPMI containing 1% BSA, and then stimulated for 30, 120, or 600 seconds with 100 μ g of F(ab')₂ fragments of goat anti-human IgM. Cells were then lysed in 0.5% Triton-lysis buffer, and lipid rafts isolated as described in Methods. The contents of the rafts were then submitted to SDS-PAGE, transferred to nitrocellulose membranes, and Western blotted for IgM and total and phosphorylated Lyn (*p-Lyn*; visualized as a doublet representing the alternatively spliced p53 and p56 forms). Seven unmutated CLL clones and six mutated CLL clones were examined in this way. Representative examples of the findings for responsive unmutated versus nonresponsive mutated clones. C, control lane in which cells were simply cultured for 10 minutes without B-cell receptor stimulation.



residual Triton-soluble fraction of the cells (data not shown). Taken together, these results show that SHP-1, but not SHIP, is potentially involved in the attenuation of signals generated within rafts following B-cell receptor cross-linking of CLL cells.

It is already known that in normal B cells, B-cell receptor translocation into rafts is not dependent on the activities of src kinases or the actin cytoskeleton, although, once translocation has occurred, activation of the src kinase Lyn and cytoskeletal interactions are essential events in B-cell receptor signaling (19). Unmutated CLL clones, in which B-cell receptor cross-linking resulted in clear IgM translocation to rafts, were incubated with a src inhibitor (PP1) before B-cell receptor cross-linking and raft analysis. Although this incubation decreased tyrosine phosphorylation of most raft proteins (Fig. 6A), reduced Lyn phosphorylation (Fig. 7), and inhibited calcium mobilization and ERK-2 phosphorylation in response to B-cell receptor cross-linking (data not shown), it did not prevent B-cell receptor translocation to rafts (Fig. 6). Similarly, the inhibitor of actin polymerization, cytochalasin D, failed to prevent B-cell receptor translocation into the lipid rafts of unmutated CLL cells following IgM cross-linking (Fig. 6B). These results show that in unmutated CLL cells, as is the case in normal B-lymphocytes, B-cell receptor translocation into rafts following receptor cross-linking is a very early event that is not dependent on src activity or actin polymerization.

In contrast, incubation of mutated CLL clones with PP1 in the absence of anti-IgM resulted in spontaneous translocation of IgM to lipid rafts (Fig. 6A). Moreover, in contrast to what was observed in unmutated CLL cells, no such translocation occurred in mutated CLL clones after B-cell receptor cross-linking, either before or after treatment with PP1. This indicates that, rather than playing an active role in B-cell receptor translocation to rafts, in nonresponsive mutated CLL clones, constitutive src kinase activity actually excludes the B-cell receptor from the rafts.

The most likely process influencing the ability of B-cell receptor to translocate to rafts in mutated CLL is a src-dependent association of this receptor with the cortical cytoskeleton (30). To test whether such association is the cause of the failure of B-cell receptor translocation to rafts in mutated CLL clones, these cells were incubated for 60 minutes with cytochalasin D to depolymerize filamentous actin. Indeed, like the treatment of mutated CLL cells with PP1 (Fig. 6A), the disruption of the membrane cytoskeleton by cytochalasin D also caused translocation of IgM to lipid rafts in the absence of B-cell receptor ligation (Fig. 6B). As observed with PP1-treated cells, cytochalasin treatment did

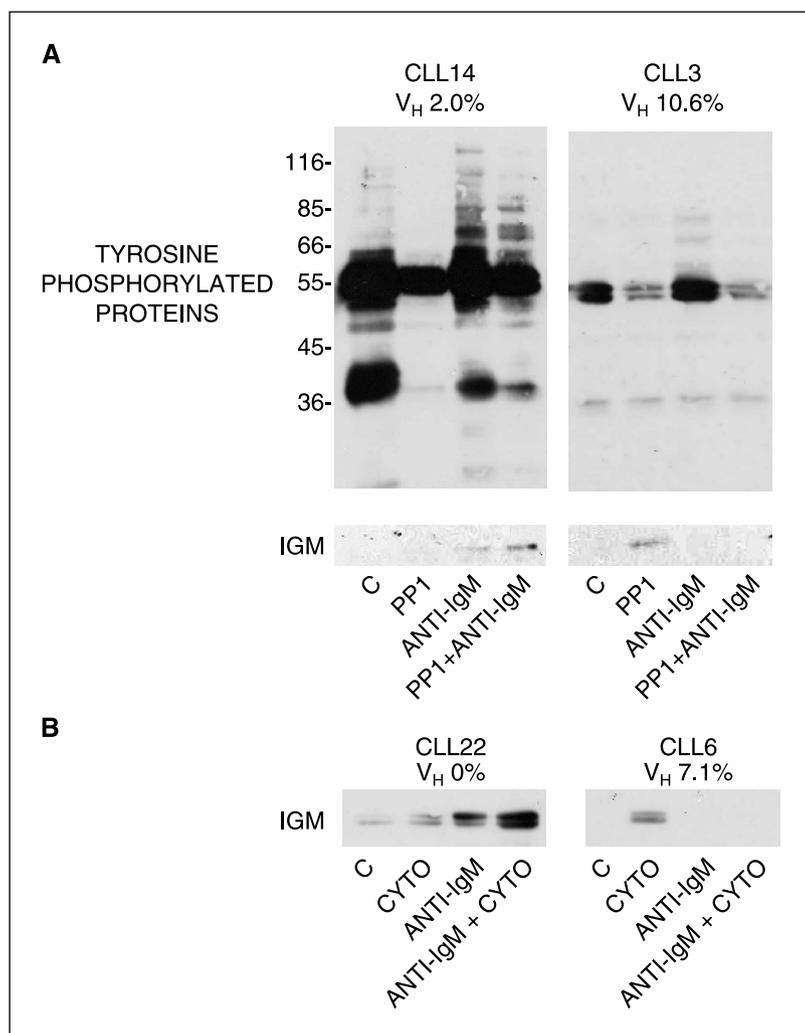


Figure 6. Effect of src kinase inhibition and actin depolymerization on IgM translocation to rafts. **A**, representative unmutated and mutated CLL clones were cultured for 60 minutes either alone (*C*, control; *lane 1*) or in the presence of the src-kinase inhibitor PP1 (10 μ mol/L; *lanes 2 and 4*). The cells were stimulated with F(ab')₂ anti-IgM for 10 minutes (*lanes 3 and 4*). Rafts were then isolated as before and analyzed for tyrosine-phosphorylated proteins with an anti-phosphotyrosine antibody (PY20) and for IgM as described in Fig. 5. **B**, IgM in the rafts of representative unmutated and mutated CLL clones cultured for 60 minutes either alone (*C*, control; *lane 1*) or in the presence of cytochalasin D (*CYTO*, 10 μ mol/L; *lanes 2 and 4*). The cells were stimulated with F(ab')₂ anti-IgM for 10 minutes (*lanes 3 and 4*). Rafts were then isolated and analyzed for IgM as described in Fig. 5. Representative of three separate experiments.

not restore the responsiveness to anti-IgM. This was as expected because signaling from rafts requires src kinase activity and because simple translocation of IgM to rafts through actin depolymerization is unlikely to mimic B-cell receptor stimulation secondary to receptor cross-linking. Moreover, cross-linking of the B-cell receptor by anti-IgM in cytochalasin-treated cells prevented cytochalasin-induced translocation. This indicates that, in mutated CLL cells, stimulation by anti-IgM results in an actin-cytoskeleton-independent exclusion of B-cell receptor from rafts not observed in unmutated clones.

These results suggest that interactions between the B-cell receptor and the actin cytoskeleton and exclusion from rafts play an important role in the hyporesponsiveness of mutated CLL cells to B-cell receptor ligation. The importance of src-dependent signals in the exclusion of the B-cell receptor from rafts is supported by the demonstration earlier in this article of the constitutive phosphorylation of CD79a in mutated, but not in unmutated, CLL cells.

B-Cell Receptor Cross-Linking Has Different Effects on the *In vitro* Survival of Unmutated Versus Mutated Chronic Lymphocytic Leukemia Cells

Because B-cell receptor stimulation is central to B-cell development and survival (4) and because CLL cells are thought

to develop under the influence of antigenic stimulation (1) and are long-lived *in vivo* (31), we next examined the effect of B-cell receptor cross-linking on the survival of unmutated versus mutated CLL-cell clones. This seemed all the more important given the pronounced differences in the phosphorylation of ERK-2 observed

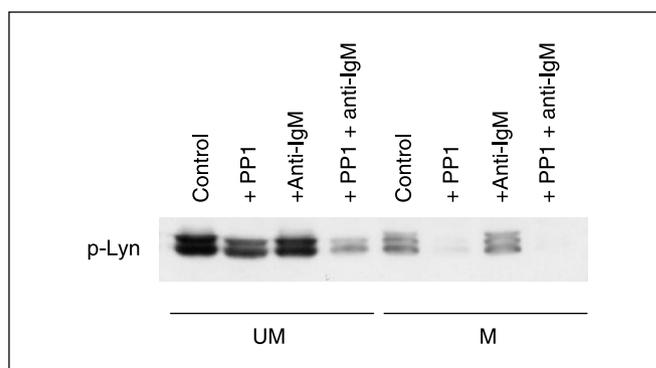


Figure 7. Effect of PP1 on Lyn phosphorylation. Representative unmutated and mutated CLL clones were cultured for 60 minutes either alone (*Control*) or in the presence of PP1 (*lanes 2 and 4*). The cells were stimulated with F(ab')₂ anti-IgM for 10 minutes (*lanes 3 and 4*). Cells were lysed in 1% SDS and aliquots of lysate subjected to SDS-PAGE and Western blotting with an anti-phospho-Lyn antibody.

above in unmutated versus mutated CLL cells because ERK activation can contribute to survival signals generated by B-cell receptor cross-linking (32). Figure 8 shows that cross-linking the B-cell receptor on unmutated CLL cells with F(ab')₂ fragments of anti- μ resulted in significant enhancement of the survival of these cells. In mutated CLL cells, in which B-cell receptor stimulation usually failed to induce ERK-2 phosphorylation and [Ca²⁺] increases, cross-linking the B-cell receptor had either no effect or caused a slight nonsignificant reduction in cell viability. This differential effect of B-cell receptor cross-linking on the survival of unmutated versus mutated CLL clones was highly significant ($P = 0.002$).

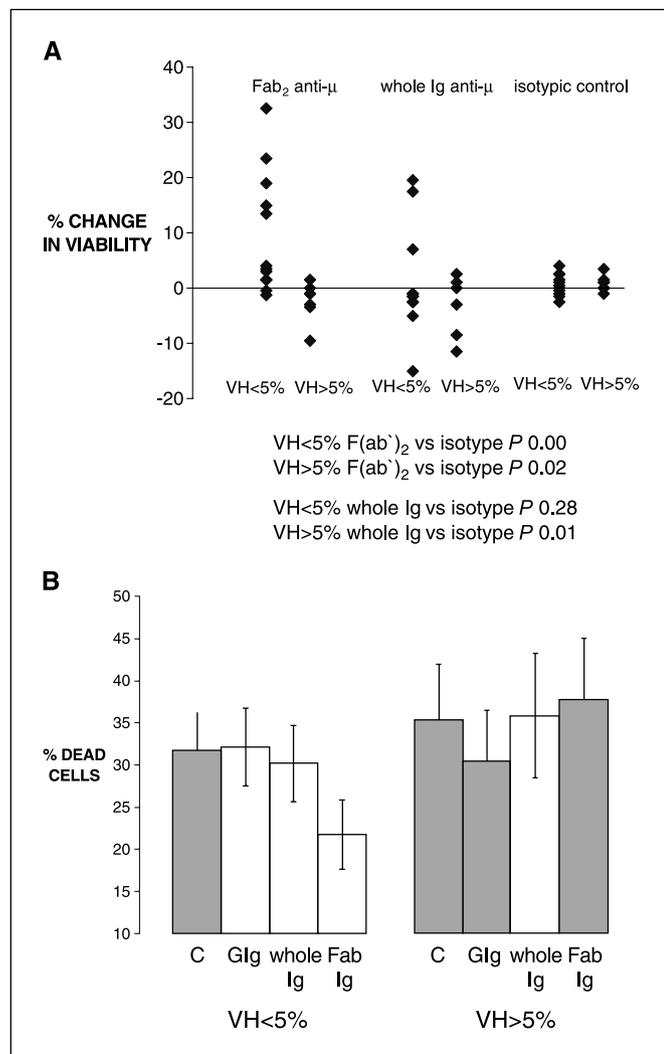


Figure 8. Regulation of CLL-cell survival by B-cell receptor cross-linking. CLL cells (1×10^5) were suspended in 200 μ L of RPMI with 0.1% BSA in 96-well plates coated with poly(2-hydroxyethyl methacrylate). The cells were then cultured for 48 hours at 37°C in the presence or absence of F(ab')₂ fragments or intact goat anti-human IgM, or with whole goat IgG as a control (all at 10 μ g/10⁷ cells). Cell viability was then measured by FACS analysis of cell size and propidium iodide exclusion (dead cells shrink and do not exclude propidium iodide). The effect of B-cell receptor cross-linking was determined by either calculating the percent change in the viability of stimulated versus nonstimulated cells (A) or by measuring the mean percent (± 1 SE) of dead cells (B) in unstimulated (C) versus the number of dead cells following incubation with nonspecific goat IgG (Glg) or with whole and F(ab')₂ fragments of goat anti- μ antibodies. The effects of B-cell receptor cross-linking were then compared in unmutated and mutated CLL cases. Significance was calculated using paired Student's t test.

We therefore conclude that when the B-cell receptor of unmutated CLL clones is cross-linked with anti-IgM, a significant enhancement of *in vitro* cell survival is observed.

Discussion

It has been known for some time that CLL is heterogeneous with regard to outcomes of B-cell receptor ligation (5, 8). However, the relationship of this heterogeneity to the degree of cell maturation/differentiation, and to the route(s) of development of different CLL clones, is unclear. Also, the signaling basis of responsiveness and unresponsiveness of CLL cells remains to be established.

We have addressed some of these questions by measuring ERK-2 phosphorylation and [Ca²⁺] increases following ligation of the B-cell receptor with anti- μ antibodies using clones derived from 23 subjects with B-cell CLL (Table 1). An increase in cytosolic [Ca²⁺] is an early consequence of such ligation and is already known to vary widely in cells from different patients (6). ERK-2 phosphorylation is a more distal signal known to play a role in the survival of B-lymphocytes (33). The results obtained were then analyzed with respect to VH gene mutation status of the cells, a key prognostic indicator in CLL.

There was a strong association between the presence of high levels of VH mutation and B-cell receptor hyporesponsiveness as indicated by the absence of both ERK phosphorylation and intracellular [Ca²⁺] increases in response to receptor cross-linking. Conversely, the VH-unmutated clones generated both these signals in response to B-cell receptor stimulation. A statistically significant correlation between VH mutation and B-cell receptor responsiveness was only observed when 5% deviation from germ line was used to define mutated cases of CLL. Cases with 2% to 5% VH mutation retained the ability to signal in response to B-cell receptor engagement. Thus, in terms of their B-cell receptor responsiveness, these cases more resembled unmutated than mutated CLL. Therefore, the present results lend support to the notion that 5% VH deviation from germ line may be more useful than 2% in discriminating between mutated and unmutated CLL in terms of their biological responses. Why this is so remains unclear, but it perhaps reflects the fact that different CLL clones may have developed by different routes. For example, peripheral B cells resembling CLL cells in being surface IgM⁺, IgD⁺, and CD27⁺ can acquire a certain, relatively low, frequency of mutation of their VH genes by a T-independent, extrafollicular route (34). It is therefore possible that CLL clones with up to 5% VH mutation may have developed by this route, whereas those with >5% mutation may have been subjected to T-cell-dependent, follicular influences.

Although the association between the presence of VH mutation and B-cell receptor responsiveness was strong, it was not absolute. Thus, one of the nine mutated CLL clones tested was B-cell receptor responsive, whereas 2 of 14 unmutated CLL clones were hyporesponsive. This indicates that, in a minority of cases, the extent of hypermutation cannot predict the outcome of B-cell receptor signaling. Furthermore, in two cases (both unmutated CLL), B-cell receptor stimulation was accompanied by ERK phosphorylation, but with no Ca²⁺ increases. These exceptions indicate that some additional heterogeneity in B-cell receptor signaling exists in both mutated and unmutated CLL clones.

Recently, two studies have shown a failure in mutated CLL to phosphorylate either Syk (7) or Zap-70 (35) following B-cell receptor ligation. The present studies, using different measures (ERK phosphorylation and Ca²⁺ mobilization) of B-cell receptor

responsiveness, confirm that such responsiveness is a feature of CLL with relatively low VH mutation.

Although CLL cells are characterized by low expression of sIgM, it is known that there is some case-to-case variation in the density of expression of this molecule (26). It therefore seemed important to relate the intensity of sIgM to VH mutational status and B-cell receptor responsiveness. In fact, mutated CLL clones were found to possess less sIgM than unmutated CLL cases. However, the level of sIgM was not critical for response because unmutated clones with low sIgM retained the ability to signal in response to IgM cross-linking. The observed combination of low sIgM and B-cell receptor hyporesponsiveness found here in mutated CLL is also a feature of anergized normal B cells (36, 37). It therefore seems plausible that in this group of CLL patients, the malignant cells may have been anergized through chronic antigen exposure.

We next looked at the mechanism of B-cell receptor hyporesponsiveness in mutated CLL. The absence of ERK phosphorylation and Ca^{2+} increases in response to B-cell receptor cross-linking pointed to a proximal signaling defect. This was supported by the selective induction of CD79a phosphorylation only in unmutated CLL cells following B-cell receptor cross-linking. This phosphorylation is mediated by Lyn kinase and requires B-cell receptor translocation to lipid rafts (30). The presence of already phosphorylated CD79a in lysates of mutated CLL cells indicates that these cells have already been stimulated, most likely by (auto)antigens *in vivo*. Interestingly, B-cell receptor cross-linking of these mutated CLL cells resulted in CD79a dephosphorylation, suggesting either activation of phosphatase(s) or induced association of the protein with an already activated phosphatase.

Because induction of B-cell receptor signaling normally occurs on translocation into lipid rafts (38), we next examined unmutated and mutated CLL cells for the presence of IgM in

lipid rafts before and after antibody-induced IgM cross-linking. We also analyzed the composition and phosphorylation of some relevant signaling components in the rafts of these cells. In unmutated CLL, cross-linking of the B-cell receptor resulted in the translocation of IgM to the raft fraction and in the phosphorylation Lyn kinase. Conversely, in mutated CLL (and the one case of unmutated CLL which failed to mobilize calcium and phosphorylate ERK in response to B-cell receptor cross-linking), stimulation of the B-cell receptor did not result in its translocation into lipid rafts.

Our finding of constitutive src-dependent interactions between the B-cell receptor and actin cytoskeleton in mutated CLL cells that exclude the B-cell receptor from lipid rafts was a novel and major finding in the present study, which points to a mechanism by which CLL cells can down-regulate their response to chronic antigenic stimulation. The active exclusion of the B-cell receptor from rafts may prevent the initiation of signaling in response to antigenic stimulation by sequestering the B-cell receptor away from rafts, thus preventing the initiation of effective signal transduction. This seems especially plausible given that B-cell receptor-hyporesponsive CLL clones have been compared with anergic B cells (9) and are widely believed to have developed under the influence of chronic antigenic stimulation (1). Anergic normal B-lymphocytes are characterized by a failure of translocation of the B-cell receptor to lipid rafts in response to B-cell receptor cross-linking (10). In addition, cytoskeletal interactions are believed to play an important role in the regulation of the B-cell receptor in these anergized cells (39, 40).

As regards our results with unmutated and mutated CLL cells, these are summarized in Fig. 9. This figure shows the composition of lipid raft and nonraft membrane fractions in the two cell types, both before and after B-cell receptor cross-linking, as well as the signaling response to such stimulation.

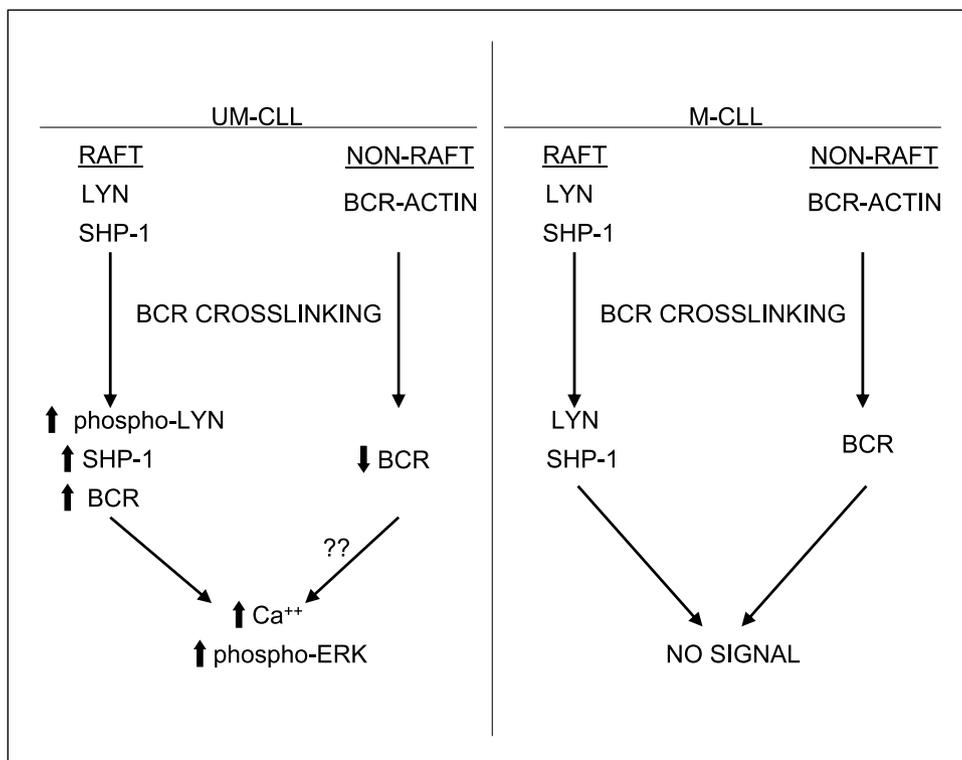


Figure 9. Flow chart summarizing the sequence of events following cross-linking of the B-cell receptor in unmutated and mutated CLL cells. In both unmutated and mutated CLL clones, before cross-linking, the B-cell receptor is present in the nonraft membrane fraction, whereas lipid rafts contain Lyn and SHP-1. Following cross-linking, the B-cell receptor and additional SHP-1 are recruited to rafts of unmutated, but not of mutated, CLL cells. In addition, following cross-linking, raft-associated Lyn kinase undergoes tyrosine phosphorylation in unmutated, but not in mutated, CLL cells. As a result, B-cell receptor cross-linking produces an increase in intracellular $[\text{Ca}^{2+}]$ and ERK phosphorylation in unmutated, but not in mutated, CLL.

To give our results a pathophysiologic relevance, we next looked at the effect of B-cell receptor engagement on CLL-cell survival in relation to VH mutational status, which is a strong prognostic indicator in the disease. We found that F(ab')₂ anti- μ consistently enhanced the survival of responsive unmutated CLL clones, but this treatment either had no effect or slightly reduced the survival of mutated CLL cells in which B-cell receptor cross-linking failed to induce ERK-2 phosphorylation. Therefore, in responsive unmutated CLL, chronic *in vivo* exposure to antigen might be expected to prolong the survival of the malignant cells and thereby contribute to their clonal expansion. We believe that the present findings provide at least a partial explanation of why patients with unmutated CLL have a poorer prognosis (21).

The present studies of B-cell receptor signaling support the notion that mutated CLL cells in some ways resemble anergic cells. Thus, like normal anergic B cells, mutated CLL cells express relatively weak sIgM and fail to recruit the B-cell receptor to rafts on stimulation. Therefore, we suggest that in both these cell types,

constitutive src-dependent interactions with the actin cytoskeleton prevent B-cell receptor translocation to rafts on ligation and therefore may serve to down-regulate the cellular response to receptor engagement. However, mutated CLL cells clearly differ from normal anergic B cells in their failure to generate ERK and Ca²⁺ signals on B-cell receptor stimulation outside rafts—responses still retained by normal anergic B cells.

In conclusion, the present work suggests that interactions between the B-cell receptor and lipid rafts are crucial in the modulation of CLL-cell responses to antigen and it is likely that a further understanding of these processes will result in a better understanding of the role of antigen in the pathogenesis of CLL.

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B-Cell Receptor Translocation to Lipid Rafts and Associated Signaling Differ between Prognostically Important Subgroups of Chronic Lymphocytic Leukemia

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