Phosphorylation of the B1 (CD20) Molecule by Normal and Malignant Human B Lymphocytes*

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The B1 molecule (CD20) is a phosphoprotein found only on B lymphocytes. Multiple isoforms of the B1 molecule are expressed with $M_r$ of 33,000, B1(33) and $M_r$ of 34,500-36,000, B1(35). In this study it was found that nonproliferating B cells did not incorporate $^{32}P$O$_4$ into B1 although phosphorylated class I histocompatibility molecules were easily detected. In contrast B1 isolated from proliferating or malignant B cells or B cell lines was heavily phosphorylated. Cross-linking B1 on the cell surface by antibody resulted in enhanced phosphorylation of B1 as did exposure to phorbol esters, and the membrane permeable diacetyl-glycerol analog 1,2-diacetoyglycerol. B1(33) and B1(35) produced identical peptide maps following limited proteinase digestion. However, B1(35) contained both phosphorysine and phosphothreonine, while B1(33) only contained phosphoserine. In addition alkaline phosphatase was able to remove the phosphate residue(s) that resulted in generation of the B1(35) form of B1 but was unable to remove the phosphorylation of B1(33). These results suggest that phosphorylation of B1 molecules is associated with proliferation and that the different $M_r$ forms of B1 result from the phosphorylation of B1 at different sites. Also, the finding that antibody binding to B1 generated a transmembrane signal may explain why antibody binding to B1 alters B cell function.

B1 (CD20) is a human cell-surface phosphoprotein expressed only by B lymphocytes (1-3). B1 is expressed from early pre-B cell development until final differentiation into plasma cells (4, 5). The binding of monoclonal antibodies reactive with B1 can either augment B lymphocyte function (6-8), inhibit B lymphocyte proliferation following mitogen stimulation (9-11), or inhibit B lymphocyte differentiation (8-11). Although the exact function of B1 remains unknown, these results suggest that B1 plays a central role in the regulation of the activation process required for B lymphocyte cell cycle progression and differentiation (11).

Phosphorylation and dephosphorylation of proteins is recognized as a major process for regulating cellular functions and serves a prominent role in signal transduction (12). The cross-linking of membrane immunoglobulin on B lymphocytes generates a signal across the plasma membrane resulting in protein phosphorylation events and physiologic responses (reviewed in Ref. 13). Although the direct physiologic significance of many phosphorylation reactions remains to be explored, numerous phosphate-acceptor proteins on B lymphocytes have been identified as physiological relevant membrane proteins such as transferrin receptors (14), and interleukin-2 receptors (15). Therefore, phosphorylation of specific cell-surface molecules may serve a pivotal role in the regulation of B lymphocyte proliferation. A cDNA clone for B1 has been isolated and the B1 protein shares no amino acid sequence homologies with other known proteins (16). However, the presence of several extensive hydrophobic regions suggests that B1 traverses the membrane numerous times similar to what occurs with several other membrane-associated phosphoproteins, such as rhodopsin and the $\beta$-adrenergic receptor (17, 18).

Recent studies have demonstrated that the B1 molecule is heterogeneous in that three forms of B1 with $M_r$ 33,000, 34,500, and 36,000 have been identified by SDS-polyacrylamide gel electrophoresis (3). The $M_r$ 33,000 species of B1 is the dominant form of the protein, while the two other forms of B1 represent less than 20% of the total surface B1 proteins. The $M_r$ 33,000, B1(33)$^+$ and 34,500-36,000, B1(35)$^+$ forms can be identified as electrophoretically distinct phosphoproteins (2, 3). In this study the association between phosphorylation of the different $M_r$ forms of B1 was examined in relation to B lymphocyte stimulation and proliferation. The heterogeneity in $M_r$ of B1 was found to result from the differential phosphorylation of B1 as has been found to occur with type II cAMP-dependent protein kinase and several other phosphoproteins (19-22). In addition the structural and regulatory basis for the differential phosphorylation of the different $M_r$ forms of B1 was examined.

MATERIALS AND METHODS

Cell Culture Conditions and Labeling—The human B lymphoblasticoid cell lines Bjab, Daudi, SB, Namalwa, and Raji were maintained in RPMI 1640 medium (Gibco) containing 10% fetal calf serum, 2 mM L-glutamine, 50 units/ml of penicillin and 50 pg/ml of streptomycin. The cells were cultured at 37 °C in the presence of 6% CO$_2$ at densities between 1.0-1.5 X 10$^6$ cells/ml. Experiments were performed when cultures were at a density between 1.1-1.5 X 10$^6$ cells/ml and greater than 97% viable.

B lymphocytes were isolated from human blood and spleen as described (10) and greater than 90% expressed the B1 molecule on their cell-surface as determined by indirect immunofluorescence analysis. B lymphocyte stimulation with Staphylococcus aureus Cowan strain I was as described (9). Malignant blood lymphocytes were isolated from patient 1 who had chronic lymphocytic leukemia. Phenotype analysis of the patient's lymphocytes revealed that 80% of the cells were class II histocompatibility antigen positive, 61%
expressed the B1 antigen while less than 20% of the cells expressed T cell-surface antigens. Lymphocytes were also isolated from a lymph node removed from patient 2 with cell lymphoma. Thirty-four per cent of this patient's cells expressed class II histocompatibility antigens, 25% expressed the B1 antigen, 27% expressed B1 antigens, 25% expressed the B1 antigen, 27% expressed 10% (v/v) acetic acid, and 10% (w/v) trichloroacetic acid for 1 h at room temperature. Autoradiographs were made of dried gels using XAR-5 X-Omat film (Eastman) and light intensifying screens (Cronex Lightning Plus, Du Pont) with exposure at -70 °C. The amount of label contained in individual protein bands was determined by excising that portion of the dried gel, with quantification by scintillation counting.

**One-dimensional Peptide Analysis**—Limited peptide analysis of radiolabeled proteins was carried out as described by Cleveland (30).

**Analysis of Phosphoamino Acids**—Following autoradiography of fixed SDS-polyacrylamide gels, the B1(33) and B1(35) proteins were excised from the gel and electroeluted. The eluted materials were lyophilized and resuspended into 200 μl of 6 M HCl (Pierce Chemical Co.) and incubated at 110 °C for 2 h. Following dilution with water, the samples were lyophilized, resuspended in buffer containing 0.5% pyridine, 5% glacial acetic acid, pH 3.5, and mixed with phosphoserine, phosphothreonine, and phosphotyrosine as standards. The sample was analyzed by electrophoresis at pH 3.5 on cellular thin layer sheets (polyethylenimine-Cellulose F; EM Science, Cherry Hill, NJ) as described (31). The positions of the phosphoamino acid markers was determined by ninhydrin staining prior to autoradiography.

**Enzymatic Dephosphorylation of B1**—Washed immunoprecipitated material from 32P-labeled cells was incubated in 50 μl of 10 mm Tris-HCl, pH 8, containing 4 units of Escherichia coli alkaline phosphatase (Behring Diagnostics) for 60 min. Samples were resuspended in electrophoresis sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis.

**Statistical Analysis**—The paired Student's t test was used to determine the statistical significance of the results.

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**RESULTS**

**Phosphorylation of B1**—Immunoprecipitation of 32P-labeled B1 molecules from the Raji B lymphoblastoid cell line demonstrated that B1(33) and B1(35) were both phosphorylated (Fig. 1). Although the majority of B1 protein is found as the M, 33,000 form (3), the amount of 32P label found in the different M, proteins was variable. In 10 experiments B1(35) had 2.6 ± 1.8 times more label than B1(33) in all cases tested.

**Phosphorylation of the B1 Molecule**

![Flowchart](image_url)
protein contained 58 ± 11% (mean ± standard error of the mean) of the 32P label located within B1. In contrast to B1, the C3d/Epstein-Barr virus receptor (identified with the HB-5 antibody) was not constitutively phosphorylated while class I histocompatibility antigens are phosphorylated as described (25, 32). Exposure of B cell lines to PMA during the last 10 min of the 90-min labeling period resulted in significant changes in the phosphorylation of B1, such that B1(35) became the dominant form of B1 to contain phosphate (Fig. 1B). In eight experiments, the amount of label present in B1(35) significantly increased (p < 0.0025) by 225 ± 92% (mean ± standard error of the mean) while the amount of label present in B1(33) had a mean increase of only 34%. The amount of 32P labeled present in B1 significantly increased (p < 0.005) by 205 ± 49% after PMA exposure, similar to the 149 ± 34% increase that occurred with class I histocompatibility antigens. PMA exposure also resulted in the phosphorylation of the C3d/Epstein-Barr virus receptor as described (25), although the level of phosphorylation was considerably less than that of B1. Therefore, dramatic changes in the patterns of phosphorylation of B1 molecules occurred following exposure of B cells to phorbol ester.

The B1 Molecule Is Phosphorylated in Activated but Not in Resting B Lymphocytes—The phosphorylation status of B1 in resting and mitogen-stimulated B cells was examined by culturing the cells with 32PPO₄. The unstimulated cells were greater than 90% B1 antigen positive, and less than 3% of the cells expressed interleukin 2 receptors or the HB-7, B5, and T9 (transferrin receptor) activation antigens (10). Each culture was divided, and half of the cells were incubated with PMA. B1 immunoprecipitated from unstimulated B lymphocytes, either with or without PMA exposure, did not contain detectable 32PPO₄ label (even when the autoradiographs were extensively overexposed) (Fig. 2). In contrast phosphorylated class I molecules were easily detected. Proliferating B lymphocytes that had been stimulated with a polyclonal mitogen expressed phosphorylated B1 molecules, and the phosphorylation of B1(35) was increased following PMA exposure (Fig. 2). The same results were obtained with spleen cells from two donors and also with blood B lymphocytes. Therefore, the B1 molecule does not appear to be phosphorylated in resting B cells but becomes phosphorylated following mitogenic stimulation.

Malignant B Lymphocytes Express Phosphorylated B1—To determine the phosphorylation status of the B1 molecule in human B cell malignancies, tumor lymphocytes from two patients were cultured with 32PPO₄. One patient had chronic lymphocyte leukemia while a second patient had a B cell lymphoma, and the malignant cells expressed B1. In both cases 32P-labeled B1 was immunoprecipitated from the malignant cells, and the majority of 32P label was found as B1(35) (Fig. 3). The B cell lines, Bjab, Raji, Daudi, SB, and Namalwa which were derived from patients with B cell malignancies also constitutively expressed phosphorylated B1 (data not shown).

Structural Differences between B1(33) and B1(35)—Previous studies have suggested that the M₃, 34,500 and 36,000 forms of B1 result from differences in phosphorylation (3). To further examine this the different M₃ forms of B1 were immunoprecipitated from 32P-labeled Raji cells and separated by SDS-polyacrylamide gel electrophoresis. The individual proteins were isolated, acid hydrolyzed, and examined by thin layer cellulose electrophoresis to determine their phosphoamino acid composition. B1(35) isolated from cells with and without PMA exposure both contained 32P as phosphoserine and phosphothreonine in similar amounts (Fig. 4). In contrast B1(33) only contained phosphoserine residues (even when the autoradiographs were extensively overexposed). Therefore, differences in phosphorylation sites occur between B1(33) and B1(35).

The different M₃ forms of B1 were further examined by one-dimensional peptide mapping to determine their structural relationships. 32P-Labeled proteins isolated from SDS-polyacrylamide gels were subjected to limited digestion by chymotrypsin or V8 protease using the method of Cleveland (30). Analysis of the cleavage products by SDS-polyacrylamide gel electrophoresis showed that the labeled peptide fragments of the proteins were almost all indentical except for small differences in M₃. Therefore, differences in phosphorylation sites occur between B1(33) and B1(35). The different M₃ forms of B1 appear to be unique to the B1(35) protein, and these differences most likely reflected differences between labeling intensities in the two forms. Thereby, B1(33) and B1(35) appear to result from the differential phosphorylation of structurally similar, if not identical, proteins.

Treatment of B1 with alkaline phosphatase reduces the M₃ of the 34,500 and 36,000 forms of B1 to M₃, 33,000, consistent with the notion that phosphorylation results in the decreased electrophoretic mobility of B1 (3). This was examined further by extensively dephosphorylating 32P-labeled B1 with alkaline.
phosphatase from E. coli. Phosphatase treatment of B1 resulted in the elimination of label in B1(35) but did not remove the $^{32}$P label from B1(33) (Fig. 6). In addition 90% of the $^{32}$P label found in both B1(35) and B1(33) remained as label in B1(33). This suggests that a minor phosphorylation difference between B1(33) and B1(35) was responsible for the decrease in electrophoretic mobility that defined B1(35). Enzyme treatment also eliminated the phosphorylation increase induced by PMA exposure (Fig. 6). These results suggest that the decrease in electrophoretic mobility that defines B1(35) may result from the phosphorylation of B1 at a site(s) that is distinct from the site(s) where B1(33) is phosphorylated. These different phosphorylation sites also appear to have different susceptibilities to enzymatic dephosphorylation (Fig. 6).

Anti-B1a Antibody Binding Induces Increased Phosphorylation of B1—To determine whether binding of anti-B1 antibodies to B lymphocytes generates a transmembrane signal that affects B1 phosphorylation, the anti-B1a antibody was added to B cell lines during the last 10 min of culture with $^{32}$PO$_4$. Antibody binding resulted in a considerable increase in the amount of $^{32}$P label immunoprecipitated with B1 (Fig. 7A). In seven experiments the addition of anti-B1a antibody to the culture resulted in a significant increase ($p < 0.005$) of 115 ± 29% (mean ± standard error of the mean) in the amount of $^{32}$P label immunoprecipitated while PMA exposure resulted in a 217 ± 59% increase. The simultaneous addition of anti-B1a antibody together with PMA resulted in an increase in B1 phosphorylation greater than with either reagent added alone (Fig. 7A). The addition of anti-B1 antibody did not affect the amount of $^{32}$P label present in class 1 histocompatibility antigens (data not shown). It is unlikely that the greater than 100% increase in B1 phosphorylation occurred due to enhanced immunoprecipitation of B1 since in separate experiments greater than 90% of the B1 molecules were immunoprecipitated by the procedures used (data not shown). Also, treatment of the cells with anti-B1a antibody prior to cell lysis only resulted in the precipitation of 20% of the labeled B1 molecules (Fig. 7A). Therefore, the addition of anti-B1 antibody to cells appeared to result in a selective enhancement of the phosphorylation of B1.

To determine whether phosphorylation of B1 could be altered by other stimuli or was dependent on protein kinase inhibitors. The addition of an anti-IgM antibody to the cultures did not result in an alteration of B1 phosphorylation nor did an increase in Ca$^{2+}$ due to ionomycin exposure (Fig. 7B). In three experiments exposure of cells to the diacylglycerol analog, diC8, resulted in increased phosphorylation (78% increase) of B1 although the effect was consistently less than that following PMA exposure (156% increase). Both treatments preferentially increasing the amount of label in B1(35). The addition of a protein kinase inhibitor, H7, 5 min prior to diC8 exposure prevented an increase in phosphorylation (Fig. 7B) and also prevented an increase in phosphorylation following the binding of anti-B1a antibody (data not shown). The addition of H7 to cells during the entire labeling period caused a substantial reduction (91–88%) in the amount of $^{32}$P label in B1 (data not shown).

Therefore, it appears that B1 phosphorylation results directly from enzymatic protein kinase function. Although exposure of Daudi cells to interferons generates an increase in diacyl-
Phosphorylation of both Bl(35) and Bl(33) forms with a maximum of B1 on the cell surface at 1, 2, 4, 6, 12, 24 h as determined by indirect immunofluorescence staining with flow cytometry. The cells were treated with anti-Bla antibody or W6/32 antibodies. The washed immunoprecipitated materials were divided, and immunoprecipitated from proliferating lymphocytes incorporated a considerable amount of 32P label. Most significant was the finding that Bl from freshly isolated B cell tumors expressed Bl in the phosphorylated state as did malignant B cell lines (Fig. 3). Whether a direct causal relationship exists between Bl phosphorylation and B cell proliferation is also unknown but phosphorylation does serve a widespread role in regulating the function and structure of numerous plasma membrane receptors (34). The constitutive phosphorylation of Bl is unlikely to be a signal for internalization since the B1 molecule shows no tendency to be internalized in B cell lines (35). Treatment of normal B cells with PMA also failed to cause a measurable decrease in cell-surface Bl molecules over a 24-h period (data not shown). However, PMA exposure for 48-72 h results in decreased B1 expression by normal B lymphocytes and results in internalization of anti-B1 antibody-imunotoxins (36). Therefore, this delayed decrease in Bl from the cell-surface may be an indirect result of B cell

**DISCUSSION**

Although the physiological relevance of the phosphorylation of many cell-surface molecules remains to be established, the results presented here suggest that Bl phosphorylation may be associated with B cell activation and proliferation. In resting B cells, although large amounts of Bl antigen are expressed on the cell surface, none appeared to be phosphorylated at detectable levels (Fig. 2). In contrast Bl immunoprecipitated from proliferating lymphocytes incorporated a considerable amount of 32P label. Most significant was the finding that Bl from freshly isolated B cell tumors expressed Bl in the phosphorylated state as did malignant B cell lines (Fig. 3). Whether a direct causal relationship exists between Bl phosphorylation and B cell proliferation is also unknown but phosphorylation does serve a widespread role in regulating the function and structure of numerous plasma membrane receptors (34). The constitutive phosphorylation of Bl is unlikely to be a signal for internalization since the B1 molecule shows no tendency to be internalized in B cell lines (35). Treatment of normal B cells with PMA also failed to cause a measurable decrease in cell-surface Bl molecules over a 24-h period (data not shown). However, PMA exposure for 48-72 h results in decreased B1 expression by normal B lymphocytes and results in internalization of anti-B1 antibody-imunotoxins (36). Therefore, this delayed decrease in Bl from the cell-surface may be an indirect result of B cell
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Fig. 7. Anti-B1a antibody binding induces increased phosphorylation of the B1 molecule while protein kinase inhibitors down-regulate B1 phosphorylation. A, fifty-million Raji cells were labeled with 1 mCi of 32P04. The culture was then divided into four separate cultures and incubated for an additional 10 min with media alone, PMA (100 ng/ml), anti-B1a antibody (100 μg/ml), or anti-B1a antibody (100 μg/ml) + PMA (100 ng/ml). The cells were washed once, lysed, and the lysate precleared with an unreactive, isotype-matched monoclonal antibody. The lysates were then divided in half and immunoprecipitated with anti-B1a or W6/32 antibody. Immunoprecipitated materials were electrophoresed on a 12% SDS-polyacrylamide gel and autoradiographed.

activation or differentiation induced by PMA and may not result directly from the hyperphosphorylation of B1.

Functional studies have suggested that the binding of monoclonal antibodies to B1 inhibits mitogen-induced B cell proliferation by either blocking the generation of an essential transmembrane single or by generating a signal that disrupts B cell proliferation (11). Although antibody binding to B1 does not trigger an increase in intracellular Ca2+ (10), the finding that the binding of antibodies to B1 induced an increase in B1 phosphorylation indicates that antibody cross-linking of B1 does induce a transmembrane signal (Fig. 7A). The ability of anti-B1 antibodies to inhibit B cell entry into cell cycle following mitogen stimulation is similar to what occurs with monoclonal antibodies reactive with class II histocompatibility antigens and both may work through similar mechanisms (9, 10, 37). Cross-linking of class II histocompatibility antigens generates a transmembrane signal that causes protein kinase C to migrate from the cytosol to the nuclear membrane (38). This migration depletes the reservoir of protein kinase C in the cytoplasm that normally translocates to the plasma membrane following anti-IgM stimulation (38). Moreover, cAMP-generating signal transduction pathways can also induce the migration of protein kinase C from the cytosol to the nucleus (39). Signal generation through the B1 protein may also be similar to what occurs with the β-adrenergic receptor and rhodopsin which stimulate adenylate cyclase and cGMP phosphodiesterase, respectively, resulting in an increase in cAMP or a decrease in cGMP, respectively (34, 40). The general structure of these proteins is similar in that they all span the membrane multiple times, and they each appear to generate transmembrane signals that may involve phosphorylation. Thereby, cross-linking of B1 by antibody may generate a transmembrane signal that results in "heterologous desensitization" as occurs with β-adrenergic receptor-coupled adenylate cyclase (41). The findings that stimuli which activate adenylate cyclase inhibit lymphocyte function, but may under certain circumstances enhance function (reviewed in 40), may explain why both agonist and inhibitory effects have been found with anti-B1 antibodies (6–11).

An accelerated electrophoretic migration following dephosphorylation is known to occur for neurofilaments, glycogen synthase, phospholamban, and type II cAMP-dependent protein kinase (19–22). It appears that phosphorylation of B1 at multiple different sites is responsible for the various M, forms of the B1 protein detected by SDS-polyacrylamide gel electrophoresis. Phosphorylation at one site does not detectably alter phosphorylation at a different location (Figs. 6 and 8). Moreover, B1 phosphorylation at multiple different locations (Figs. 6 and 8). Alkaline phosphatase removed the phosphate that produced the M, of B1 since in vitro synthesized B1 is identical in size with the M, 33,000 form of B1 (16). However, PMA treatment of cells resulted in enhanced phosphorylation and generation of the 34,500–36,000 forms of B1 suggesting that it induced phosphorylation at a different location or locations (Figs. 6 and 8). Alkaline phosphatase removed the phosphate that produced the M, 34,500–36,000 forms of B1 but did not alter the phosphorylation of the M, 33,000 form (Fig. 6). This pattern of dephosphorylation may reflect differential exposures of different phosphorylation sites on the surface of the B1 molecule. Different cytoplasmic environments or conformations that regulate access of the protein kinases to different
phosphorylation sites may explain these differences. Alternatively, subtle differences in the primary structure of the cytoplasmic regions of different B1 species might also account for the differences in phosphorylation and electrophoretic mobilities.

The protein structure of B1 deduced from a cDNA clone indicates that B1 shares no obvious structural similarities with known protein kinases (16). This suggests that antibody binding to B1 may result in the activation of a protein kinase that phosphorylates B1. In protein phosphorylation reactions, it is common for a single protein to serve as the substrate for more than one protein kinase (12). The B1 protein contains numerous potential phosphorylation sites for protein kinase C and cyclic GMP-dependent protein kinase (16), and the threonine residue at position 275 of the B1 protein appears to be a suitable site for phosphorylation by casein kinase II (42). Phosphorylation of B1 at different residues may occur since the cross-linking of cell-surface B1 or activation of B cells through its surface immunoglobulin resulted in preferential phosphorylation of B1(35) (Figs. 2 and 7) while PMA treatment resulted in preferential generation of the 34,500-36,000 forms of B1 (Figs. 1 and 7). Thereby, stimulation through surface immunoglobulin or B1 may preferentially activate protein kinases that phosphorylate B1 at sites that do not alter the $M_r$ of B1 while PMA treatment may activate different protein kinases or may result in hyperphosphorylation of B1. This is not unlikely since epidermal growth factor and PMA both induce epidermal growth factor receptor phosphorylation in different manners (43). Since B1(35) contained phosphothreonine while B1(33) did not, it is likely that enhanced activation of protein kinase C by PMA results in the increased phosphorylation of B1 at sites that are different from the sites where B1(33) is constitutively phosphorylated in cell lines (Fig. 4). In this respect it is significant to note that $^{32}$P-label B1 was preferentially found in malignant lymphocytes as the 34,500-36,000 form of B1. These differential properties of the various $M_r$ forms of B1 suggest that they may have distinct functional roles.

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