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Research paper

# Identification of new phosphorylation sites of CD23 in B-cells of patients with chronic lymphocytic leukemia

Martina Maďarová[a], Rastislav Mucha[a], Stanislav Hresko[a], Zuzana Makarová[a], Zuzana Gdovinová[b], Jarmila Szilasiová[b], Marianna Vitková[b], Tomáš Guman[c], Natalia Štecová[c], Tomas Dobransky[a,*]

[a] DB Biotech, Popradska 80, 04011 Kosice, Slovakia
[b] Department of Neurology, Faculty of Medicine Pavol Jozef Safarik University, L. Pasteur University Hospital Kosice, Trieda SNP 1, 04011 Kosice, Slovakia
[c] Department of Hematology and Oncohematology, Faculty of Medicine, Pavol Jozef Safarik University, L. Pasteur University Hospital, Trieda SNP 1, 04011 Kosice, Slovakia

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ABSTRACT

B-cell chronic lymphocytic leukemia (B-CLL) is the most common lymphoproliferative disorder in adults. Patients with B-CLL strongly express the CD23 – C type of lectin (low affinity IgE receptor, Fc epsilon RII), which is linked to B cell activation and proliferation. Phosphorylation in lymphocytes is tightly associated with regulation of protein activities, functional regulation and cell signaling, and may thus affect initiation and/or progression of the disease. Here we report changes in the phosphorylation of CD23 on threonine (pThr314) and two serine residues (pSer254, pSer265) in B lymphocytes of B-CLL patients, using a flow cytometry approach. The majority of tested patients with active forms of B-CLL presented a notable overexpression of CD23 along with pThr314, pSer254, and pSer265 CD23 phosphorylation positivity. Moreover, we have experimentally stimulated the CD23 phosphorylations in a subset of peripheral blood lymphocytes of healthy controls by phorbol-12-myristate-13-acetate treatment. This affects the activation of competent phosphorylation mediating kinases, resulting in the enhanced phosphorylation pattern. Together, these data confirm that CD23 protein is phosphorylated in B cells of B-CLL patients, report the identification of new CD23 phosphorylation sites, and suggest a possible role(s) of such phosphorylations in the activation of CD23 during the process of lymphocytic activation in B-CLL.

## 1. Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most common adult leukemia, characterized by the enhanced expression of CD23 in B cells within blood, bone marrow, and secondary lymphoid tissues [1,2]. Recent studies have shown that B-CLL is not only a static disease with the accumulation of resting long-lived lymphocytes, but also a dynamic process involving the proliferation and death of cells which require assistance from the surrounding microenvironment in order to be maintained and to progress [3,4]. B lymphocytes of patients with B-CLL typically overexpress the B cell activation marker CD23 [5]. Membrane associated CD23 and its soluble forms have been implicated in different functions, ranging from cellular adhesion, antigen presentation, growth and differentiation of B and T cells, rescue from apoptosis, release of cytotoxic mediators and regulation of IgE synthesis [6]. CD23 is a 45 kDa type II transmembrane glycoprotein, comprising of a large C-terminal globular extracellular domain that is very similar to C-type lectins, followed by a stalk region bearing several repeats that serve as a putative leucine zipper. The stalk region is followed by a short transmembrane sequence (a single hydrophobic membrane-spanning region) and a short N-terminal cytoplasmic domain [6–8]. Expression of CD23 in B lymphocytes promotes inositol lipid hydrolysis, calcium mobilization [9] and cAMP upregulation [10], all of which are important for the regulation of distinct cellular signaling pathways. Two isoforms of CD23 (CD23a and CD23b) have been described, which differ in their intracytoplasmic domains, and are linked to different signaling events [11]. CD23a is restrictively expressed on antigen activated B-cells, while CD23b is subjected to up-regulation following Epstein-Barr virus infection, stimulation by IL-4 in B cells or monocytes, or by engagement of CD40 on B cells, as well as also being inducible on a subset of T cells, polymorphonuclear leucocytes, follicular dendritic cells, intestinal epithelial cells and bone marrow stromal cells [7,12]. Both, CD23a and

* Corresponding author.
*E-mail address:* tdobransky@dbbiotech.com (T. Dobransky).

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CD23b isoforms are expressed by malignant B cells. In this context, the CD23a isoform predominates, and is involved in CLL cell survival and suppression of apoptosis. CD23b expression has been linked to active cell proliferation and growth stimulation [13,14]. Expression of the CD23 activated phenotype is not only limited to circulating B CLL lymphocytes, however. Pseudofollicles or proliferation centers found in lymph nodes and bone marrow are considered to be primary source of CLL proliferation, characterized by even greater densities of expression of CD23 and other proliferation-associated markers, such as CD38, CD71 and Ki-67 [15,16]. The leukemic cells are then released to the peripheral blood, where they become quiescent, unable to initiate their apoptotic program, and begin to accumulate in high levels [17,18].

CD23 has been linked to B cell activation and proliferation in various reports and its expression in B-CLL is regulated aberrantly compared to normal B cells [19]. Phosphorylation in lymphocytes regulates the activity of many proteins and may cause alternations in related signaling pathways, cellular activity or cell life cycle. Abnormal phosphorylation may thus induce pathological cellular events, and support the disease by affecting its progress in multiple ways. The switching of CD23 expression on and off occurs consistently in the early stage of normal B cell activation, suggesting a key role for CD23 in this process [5]. Here, we report the identification of new phosphorylation sites of CD23 – pThr314, pSer254 and pSer265 in circulating B cells of patients with B-CLL. These findings may play an important role in early, precise diagnostics of B-CLL, as well as present a potential target for a specific therapeutic approach.

## 2. Materials and methods

### 2.1. Antibodies

Polyclonal anti-peptide antibody to C-terminal peptide of CD23 was raised against the peptide Glu294–Ser321 (EGSAESMGPDSRPDPDGRLPTPSAPLHS) in rabbit. Anti-rabbit phospho-specific antibodies recognizing specifically phosphorylated residues of CD23 – pThr314, pSer254, and pSer265, were raised against the phosphorylated peptides: Asp309–Ser321 (DGRLP**pT**PSAPLHS), Glu249–Val260 (EPTSR**pS**QGEDSV), and Cys259–Phe272 (CVMMRG**pS**GRWNDAF), respectively. Peptide immunogens were conjugated to maleimide activated keyhole limpet heamocyanin (KLH) at the N-terminal cysteine added to all the immunogenic peptides. The specific antibodies from crude antiserum were separated by immunoaffinity chromatography on NHS-Sepharose columns to which the peptides used as immunogen had been coupled. All antiserums produced to phosphopeptides were precleared first using the NHS-Sepharose conjugated related non-phosphorylated peptides. All immunoaffinity purified antibodies were conjugated with FITC and used for flow cytometry detection of CD23 and its phosphorylated forms in blood samples of B-CLL and control patients.

### 2.2. Patients

This study comprised 68 patients with B chronic lymphocytic leukemia (together 68 cases, men n = 42, median age 69; women n = 26, median age 74). The control population consisted of patients with diagnosed multiple sclerosis (30 cases) and healthy donors (60 cases). All samples were obtained from the Department of Hematology and Oncohematology, and from the Department of Neurology at the University Hospital in Kosice during a 3-year period. The CLL cases were diagnosed based on cytological and/or histological morphologic features in peripheral blood, bone marrow, or tissue biopsy, with the support of flow cytometric and/or immunohistochemical analysis provided by the Department of Hematology and Oncohematology of the University Hospital in Kosice, Slovakia. CLL diagnosis was classified according to the Rai staging system. Patients with all stages and different activity of the disease were selected for the present study.

### 2.3. Flow cytometry

Extracellular portion of CD23 on B lymphocytes was detected by specific antibody raised against CD23, phosphorylated forms of antigen by anti – pThr314-, pSer254-, and pSer265-CD23 (DB Biotech, Slovakia). Other lymphocyte selective markers applied in these studies were identified by mouse monoclonal antibodies to CD19, CD5 and CD3 (BD Bioscience San Jose, CA). Expression of CD23 and phosphorylated forms of CD23 was examined on cells co-expressing the CD19/CD5 markers. Immunophenotyping was performed by three or four-color cytometric analysis consisting of fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll (PerCP), and allophycocyanin (APC) combinations. Two ml of uncoagulated peripheral blood from each subject were obtained and processed within six hours by FACS Canto flow cytometer (BD Biosciences, USA). Uncoagulated blood (100 µl) was incubated with desired antibodies for 30 min, followed by lysis of red blood cells using FACS Lysing Solution (BD Biosciences, USA) and immediate analysis by flow cytometry. Isotype control (IgG-FITC) was used as negative control. Forward scatter (FSC) versus side scatter (SSC) was used to characterize the lymphocytes, monocytes and granulocytes. Expression of each marker was detected by one and two-dimensional method and data were displayed in selected population (gate). All results are expressed either as percentages of positive lymphocytes in the total lymphocyte population, or as the mean of fluorescence intensities (MFI, mean fluorescence intensity, shift in fluorescence intensity of a cell population) related to the entire lymphocyte population. A population was considered negative when the MFI showed no significant difference from the negative control. Sample analysis and acquisition were performed by FACS Diva flow cytometry analysis software (BD Biosciences, USA).

### 2.4. Blood sample preparation/stimulation

Whole peripheral blood of healthy patients was diluted in RPMI-1640 medium (Sigma-Aldrich, USA) and stimulated with 500 ng/ml of phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich, USA) for 30 min at 37 °C. Inhibitors (PhosSTOP, Roche, USA) were used to suppress phosphatase activity.

### 2.5. Preparation of recombinant CD23 and its mutants

Vector pCMV6-Entry (OriGene Technologies, USA) was used to generate constructs for the expression of recombinant truncated wild type form of human CD23 (P06734, residues Met194-Ser321). The insertion of the appropriate gene into the vector was performed by Blue Heron Biotech, USA. The QuickChange II site-directed mutagenesis kit (Agilent Technologies, USA) was used to generate plasmids containing mutated CD23 gene at the amino acids positions 254 (Ser → Ala), 265 (Ser → Ala) and 314 (Thr → Ala), respectively. All reactions were performed according to manufactureŕs conditions. Presence of correctly mutated DNA was verified by sequencing. All amplified and purified plasmids were used for transient transfection of HEK293 (ATCC® CRL-1573™) cells. The cells were cultured in Dulbeccós modified Eagle medium (DMEM, high glucose (4.5 g/L) with 1 mM sodium pyruvate), supplemented with 10% of heat inactivated fetal bovine serum (FBS), 1% of MEM non-essential amino acids, 100 units of penicillin, 0.1 mg of streptomycin and 0.25 µg of amphotericin B (all products from Sigma-Aldrich, USA). TurboFect transfection reagent (Thermo Scientific, USA) was used for transient transfection of HEK293. At the time of transfection, the growth medium was removed from culture flasks with HEK293 cells, the cells were washed with PBS, pH 7.4 (Gibco, USA), and serum-free and antibiotics-free transfection medium (DMEM supplemented with 1% of MEM non-essential amino acids) was added to the adhered cells. DNA was diluted in transfection medium and transfection reagent was added. The mixture was added drop-wise to the cells and cells were incubated at 37 °C and 5% $CO_2$ for four hours. After