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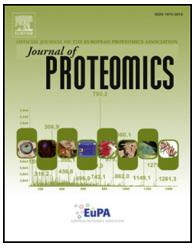

Phosphoproteomic analyses reveal that galectin-1 augments the dynamics of B-cell receptor signaling

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ABSTRACT

B-cell activation is important for mounting humoral immune responses and antibody production. Galectin-1 has multiple regulatory functions in immune cells. However, the effects of galectin-1 modulation and the mechanisms underlying the coordination of B-cell activation are unclear. To address this issue, we applied label-free quantitative phosphoproteomic analysis to investigate the dynamics of galectin-1-induced signaling in comparison with that following anti-IgM treatment. A total of 3247 phosphorylation sites on 1245 proteins were quantified, and 70–80% of the 856 responsive phosphoproteins were commonly activated during various biological functions. The similarity between galectin-1- and anti-IgM-elicited B-cell receptor (BCR) signaling pathways was also revealed. Additionally, the mapping of the 149 BCR-responsive phosphorylation sites provided complementary knowledge of BCR signaling. Compared to anti-IgM induction, the phosphoproteomic profiling of BCR signaling, along with validation by western blot analysis and pharmacological inhibitors, revealed that the activation of Syk, Btk, and PI3K may be dominant in galectin-1-mediated activation. We further demonstrated that the proliferation of antigen-primed B cells was diminished in the absence of galectin-1 in an animal model. Together, these findings provided evidence for a new role and insight into the mechanism of how galectin-1 augments the strength of the immunological synapse by modulating BCR signaling.

Biological significance
The current study revealed the first systematic phosphorylation-mediated signaling network and its dynamics in B cell activation. The comparative phosphoproteomic analysis on the dynamics of galectin-1 induced activation profiles not only showed that exogenously added galectin-1 augmented B-cell activation but also revealed its relatively enhanced activation in PI3K pathway. Together with proliferation assay, we further delineated that

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galectin-1 is important for B-cell proliferation in response to antigen challenge. Our phosphoproteomic study reveals a new role for galectin-1 in augmenting the strength of immunological synapse by modulating BCR signaling.

1. Introduction

B cells play crucial roles in mounting humoral antibody responses after encountering antigens. Whether glycan-binding proteins participate in the initiation of signaling pathways during the engagement of B cell antigen receptors is largely unknown. Galectin-1 belongs to a family of soluble lectins and contains conserved amino acid sequences in its carbohydrate-binding domain, and these residues are required for galectin-1's interactions with galactoside-containing glycans [1,2]. Galectin-1 plays important roles in regulating homeostasis and functions in multiple lineages of the immune system [3]. We previously showed that galectin-1 is induced during the differentiation of antibody-secreting plasma cells from mature B cells and promotes the generation of plasma cells. Extracellular galectin-1 more effectively binds to mature B cells than plasma cells [4]. Additionally, the level of galectin-1 can be upregulated in activated B cells in response to various stimuli, such as *Trypanosoma cruzi* infection [5]. Moreover, treatment with exogenously added recombinant galectin-1 (rGal-1), together with moderate cross-linking of B-cell receptors (BCRs), activates Syk and Erk1/2 phosphorylation and stimulates the proliferation of leukemic B cells [6]. However, the mechanism underlying galectin-1 modulation of normal B cell function has yet to be determined. Despite an increasing number of studies addressing the role of galectins in modulating immune system function, there is little understanding of how galectins regulate B cell function.

Extracellular galectin-1 promotes the formation of multivalent complexes with counter receptors, thereby facilitating the assembly of receptor complexes and the formation of galectin–glycan lattices [7,8]. The interaction of galectin-1 with cellular counter receptors helps to maintain the half-life of cell-surface receptors and modulate receptor-mediated signaling strength [7,9]. Several counter receptors of galectin-1 have been identified, including CD45, CD43, CD7, CD3, and CD4 on T cells, CD43 and CD45 on dendritic cells, and CD45, integrins, and pre-B-cell receptors on pre-B cells [10,11]. However, it remains to be determined if and how galectin-1 binds to mature B cells and triggers signaling cascades.

The global identification/quantification of thousands of *in vivo* phosphorylation sites has been achieved as a consequence of the rapid advances in mass spectrometry-based phosphoproteomic approaches [12], thereby facilitating the mapping of phosphorylation-mediated signaling pathways. For example, upon B-cell activation, the BCR signaling pathway is the most well-known signaling cascade that is initiated by the phosphorylation of v-yes-1 Yamaguchi sarcoma virus-related oncogene homolog (Lyn) and spleen tyrosine kinase (Syk) [13–15]. Lyn and Syk in turn activate the translocation of JNK to the nucleus, where it regulates the functions of transcription factors, such as Elk-1, ATF-2, and c-Jun, through phosphorylation [16]. Though the BCR-mediated signaling pathways induced by antigens are extensively studied, to date no studies have been published on the use of large-scale phosphoproteomic analyses to elucidate BCR signaling cascades. Using conventional two-dimensional electrophoretic analysis, an early proteomic study of differentiating B lymphoma cells demonstrated that the dynamic protein expression pattern started with the regulation of metabolic capacity and the expansion of the secretory machinery, which was followed by the mass production of immunoglobulin M (IgM) in response to stimulation in B cells [17]. However, the systematic phosphorylation-mediated changes associated with B-cell development or activation are largely unknown, especially at a global level.

As a preliminary step to gain a more thorough understanding of the signaling events that occur in B cell activation, we first applied label-free phosphoproteomics to *ex vivo* mouse splenic B cells for the proteome-wide identification of phosphorylation sites after treatment with an antibody against IgM (anti-IgM). To study the roles and mechanism of galectin-1 ligation on B cells, we further applied label-free phosphoproteomics to this *in vivo* model to systematically delineate the key phosphoproteins and galectin-1-dependent signaling pathways in B cells. To our knowledge, the current study presented the first comprehensive dynamic phosphoproteomic signatures of primary B-cell activation to date. We demonstrated that galectin-1 is involved in the BCR-mediated activation and proliferation of B cells. Together with validation from western blot analysis, pharmacological inhibitor studies, and proliferation assays, our findings also provided new insights into how galectin-1 participates in the activation and proliferation of antigen-primed B cells.

2. Materials and methods

2.1. Animal studies

B220 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used to isolate splenic B cells from 16- to 24-week-old C57BL/6 mice (purchased from National Laboratory Animal Center, Taiwan). *Lgals1*$^{-/-}$ mice (provided by the Consortium for Functional Glycomics) and littermate controls were obtained. *Lgals1*$^{-/-}$/MD4 mice were generated by crossing MD4 transgenic mice expressing BCRs with specificity for HEL in a C57BL/6 background (purchased from Jackson Laboratory) [18] with *Lgals1*$^{-/-}$ mice. For adoptive transfer experiments, 1×10^6 CFSE-labeled B cells were transferred into recipients together with 2×10^7 SRBCs conjugated with 2 μg of HEL (Sigma-Aldrich; HEL-SRBC) or SRBCs only (mock SRBC) in 200 μl of PBS, as previously described [19]. The protocol for conjugating HEL to SRBCs was described in a previous report [19]. For the *in vivo* labeling of cells with BrdU, mice were injected with 2 mg of BrdU (Sigma-Aldrich) in 200 μl of phosphate-buffered saline (PBS) daily by intraperitoneal (i.p.) injection. On day 2, splenocytes from BrdU/PBS-injected control mice were harvested and used for subsequent analyses, as previously described [20].