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Membrane IgM influences membrane IgD mediated antigen internalization in the B cell line Bcl1

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Abstract

Signalling through the B cell antigen receptor (BCR) is required for peripheral B lymphocyte maturation, maintenance, activation and silencing. In mature B cells, the antigen receptor normally consists of two isotypes: membrane IgM and IgD (mIgM, mIgD). Although the signals initiated from both isotypes differ in kinetics and intensity, in vivo, the BCR of either isotype seems to be able to compensate for the loss of the other, reflected by the mild phenotypes of mice deficient for mIgM or mIgD. Thus, it is still unclear why mature B cells need expression of mIgD in addition to mIgM. In the present paper, we used the B cell line Bcl1 and investigated the isotype-specific antigen internalization in dependence of co-stimulation of the reciprocal isotype and analysed whether the signal initiated from mIgM is modulated through signalling from mIgD and vice versa. We clearly showed that cross-linkage of mIgM decreases the rate of mIgD mediated antigen internalization and interpret this influence as a unilateral mIgM mediated control on signals initiated at mIgD.

Keywords: IgM; IgD; B lymphocytes; Signalling; Bcl1

1. Introduction

The B cell antigen receptor (BCR) is a multi-protein complex that steers the B cell through all stages of differentiation and development. During early B lymphopoiesis, the BCR mediates the expansion of only those B cells that have undergone productive VDJ-rearrangements and the deletion of self-reactive B cell clones [1]. In the periphery, the BCR on mature B cells provides either a maintenance signal, required for the long-term survival of these cells, or upon challenge with specific antigen, gives a signal that leads to activation or deactivation of the B cell. The deactivation signal normally leads to apoptotic death or at least functional unresponsiveness of the cell, a state termed anergy. The activation signal usually leads to the differentiation into plasma cells [2].

The BCR of mature B cells consists of two isotypes: IgM and IgD. Both classes of immunoglobulins are associated with two other transmembrane proteins CD79a (Igα) and CD79b (Igβ). Igα and Igβ form a sheath around the mIg molecule and are responsible for the coupling of the receptor to membrane proximal signalling elements [3]. The initiation of BCR signalling is still matter of speculation. According to the prevailing model, a signal is initiated upon oligomerization of two isolated BCR complexes through engagement by antigen [4]. This signal, however, has to interpret slightest changes in concentration and pathogenicity of the antigen in sense of activation or deactivation of the cell. How the B cell accomplishes this task is still largely unknown, but it is supposed that the balanced signalling through IgM and IgD receptors plays a huge role for initiation of the appropriate cellular response. Earlier studies have shown, that the signals initiated from either isotype differ concerning strength, duration and kinetics and that they are able to potentially induce differential cellular responses [5–8]. In addition, it could be shown that engagement of only one isotype receptor induces unresponsiveness of the reciprocal isotype, an effect designated receptor desensitization [9]. However, knock-out mice either lacking IgM or IgD exhibit no clear perturbation of B cell responses, so a definite explanation for the double expression of IgM and IgD on the surface of mature B cells is still matter of controversy.

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In the present paper, we used the B cell line Bcl1 to investigate, whether upon stimulation of both isotypes, the signal of one receptor isotype modulates the functions of the reciprocal one. We performed internalization experiments with isotype-specific antibodies and monitored the internalization efficiency of IgM (IgD) under simultaneous engagement of IgD (IgM). Using this experimental approach, we could show that anti-μ treatment of the cell line Bcl1 impairs IgD mediated signal transduction, reflected in a decrease of IgD mediated antigen internalization.

2. Materials and methods

2.1. Cells and reagents

Naive wild-type BALB/c mice were maintained at the central care facility at the University of Salzburg, according to the local guidelines for animal care. Spleens were minced in Dulbecco's PBS (Gibco, 0.02% KC1, 0.02% KH_2PO4, 0.8% NaCl, 0.144% $Na_2HPO_4{\cdot}2H_2O$, pH 7.4) and aggregated cells were sedimented for 5 min. The supernatants containing a monodisperse cell suspension were subjected to erythrocyte lysis. Therefore, cells were washed twice with RCLB buffer (0.834% NH_4C1, 0.084% $NaHCO_3$, 0.037% EDTA). Cells were cultured in RPMI-complete medium (RPMI 1460 (Gibco) containing 10% heat inactivated foetal calf serum (FCS, Gibco), 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM L-glutamine and 200 mM 2-mercaptoethanol). The mouse cell line Bcl1 clone 5B1b (BALB/c) was grown in the same medium.

Primary antibodies for internalization experiments and for cell staining: Goat anti-mouse IgM (Southern Biotechnology), goat anti-mouse IgD (e-Bioscience), rabbit anti-mouse IgD (Bethyl Lab.), rat anti-mouse IgD (clone 11-26c), FITC labelled rat anti-mouse IgM (clone R6-60.2) and mouse IgG1 anti-mouse IgM (clone DS-1).
Secondary antibodies: Goat anti-mouse IgGl (Southern Biotechnology), unlabelled and FITC-labelled anti-rabbit IgG (Southern Biotechnology).
Further antibodies used: FITC-anti-CD19 (Serotec), PE anti-B220, PE-anti-CD24 and PE-Cy5 anti-CD5 (all Pharmingen).

2.2. Antigen internalization and flow cytometric measurements

Antigen internalization assays were performed according to Aluvihare et al. [10]. $5 \times l0^6$ cells logarithmically growing Bcl1 cells or primary lymphocytes were washed twice with PBS, and then incubated on ice with 3 μg/4×10^6 cells anti-μ and/or anti-δ antibodies for about 60 min in a total volume of 100 μl PBS/3% FCS (for internalization of anti-δ, we used 11-26c; for internalization of anti-μ, we used FITC labelled R6-60.2). Cells were washed twice with ice-cold PBS/3% FCS to remove unbound antibody. Each cell pellet was incubated with FITC-labelled secondary antibody (FITC anti-rat) on ice for additional 45 min in a total volume of 50 μl PBS/3% FCS. Cells were washed and re-suspended in 2 ml RPMI complete medium and equal aliquots of 0.5 ml were incubated at 37 °C in a 1.5 ml Eppendorf tube for the time indicated, followed by subsequent chilling on ice. The remaining fluorescence intensities were analyzed using flow cytometry (FACSCalibur, Becton-Dickinson). Cells were gated for living cells and geometrical mean fluorescence intensities were calculated using the CellQuest software (Becton-Dickinson). The relative fluorescence intensities were calculated by equating the fluorescence at time 0 with 100%. For calculating the amount of internalized antigen, the mean fluorescence at time 0 was set to 0%.

2.3. Measurements of apoptotic cells

Bcl1 cells were incubated with specific antibodies at a concentration of 0.3 μg/ml in a total volume of 1 ml in RPMI/10% FCS in flat bottom 48-well plates (Greiner). After 6 and 24 h, the percentage of apoptotic cells was analyzed using the CaspACE FITC-VAD-FMK (Promega) apoptosis assay, according to manufacturer's instructions.

2.4. Confocal microscopy

Cells were stained with FITC-anti-IgM (R6-60.2) and rabbit-anti-IgD serum (Bethyl Lab.) on ice for 60 min. Cells were washed and incubated with secondary antibodies (FITC anti-rat and PE-anti-rabbit) for further 60 min. Cells were washed twice and shifted onto 37 °C for various times. The cells were precipitated and fixed in 1 ml 3.7% paraformaldehyd/PBS for 30 min at 37 °C. The fixed cells were mounted onto a microscope slide and analyzed with confocal microscopy (Zeiss).

2.5. Cell extracts, Immunoprecipitation, surface biotinylation and Western blotting

For precipitation of the BCR, 5×10^7 Bcl1 cells or primary lymphocytes were washed twice with PBS, surface biotinylated with sulfo-NHS-LC-biotin (Pierce) at 2 mg/ml in PBS for 15 min at room temperature and washed twice with ice-cold PBS/15 mM Glycine and once with ice-cold PBS. Cells were stimulated on ice for 60 min with 3 μg anti-μ or anti-δ antibodies prior to cell lysis. Cells were lysed in 1 ml lysis buffer (50 mM Tris, 137 mM NaCl, pH 7.6) containing protease and phosphatase inhibitors (l mM Na_3VO_4 and complete mini tablets, Roche) and 0.1% thesit as detergent (Fluka) for 30 min on ice under constant agitation. The detergent soluble fraction was obtained by centrifugation for 10 min at 10,000 × *g*. Lysates from unstimulated cells were incubated with specific antibodies and protein-G sepharose (Protein G-4 fastflow, Pharmacia Biotech) over night at 4 °C under constant agitation. Lysates from stimulated cells were incubated with protein G sepharose to precipitate bound anti-μ or anti-δ antibodies. Precipitates were washed three times with ice-cold lysis buffer followed by washing with ice-cold PBS. Afterwards, the precipitates were boiled for 5 min in 50 μl of reducing SDS loading buffer and loaded onto a 12% SDS-PAGE (resolving gel: 2.8 ml H_2O, 2 ml 30% acrylamide 0.8% bisacrylamide mix, 3.2 ml lower buffer (500 ml: 90.85 g Tris, 20 ml 10% SDS, pH 8.8 with HCl), 4 μl TEMED, 24 μl 10% APS; stacking gel: 1.3 ml H_2O, 0.35 ml