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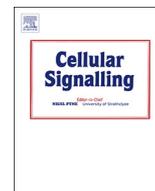
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The adaptor protein APS modulates BCR signalling in mature B cells

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ABSTRACT

Activation process of mature B cell is predominantly driven by specific BCR-mediated pathways, switched on and off all through late B cell differentiation stages. Mice deficient for APS, a member of the Lnk/SH2B family of adaptor proteins, showed that this adaptor plays a BCR-mediated regulatory role in mature B cells. However, the intermediates involved in this adaptor modulating functions in B cells are still unknown. In the present study, we investigated the role of APS in regulating BCR signalling notably through cytoskeleton remodeling in mature B cells. Herein, we showed that APS function is stage specific, as it exclusively intervenes in mature B cells. Upon activation, APS colocalizes with the BCR and associates with important regulators of BCR signalling, such as Syk and Cbl kinase. Importantly, APS interferes, as a scaffold protein, with the stability of Syk kinase by recruiting Cbl. This function is mainly mediated by APS SH2 domain, which regulates BCR-evoked cell dynamics. Our findings thus reveal that APS plays a regulatory role in BCR-induced responses by specifically modulating its interacting partners, which positions APS as a relevant modulator of BCR signalling in mature B cells.

1. Introduction

An appropriate activation of B cells is critical for their development and for immune responses, which, in mature cells, is dependent on the extensive coordination of intra- and intercellular communication in response to antigen (Ag) stimulation. B cell activation is initiated following the recognition of antigen by the B cell receptor (BCR) and results in B cell proliferation and differentiation towards antibodies (Ab) producing plasma cells. The BCR complex is composed of membrane immunoglobulin heavy (IgH) and light (IgL) chains, responsible for the extracellular recognition of antigen, and their associated signalling components, Ig α and Ig β , containing the immunoreceptor tyrosine-based activation motifs (ITAMs) [1]. Engagement of the BCR initiates phosphorylation of ITAM residues by spleen tyrosine kinase (Syk) and the subsequent assembly of a multiprotein complex, known as the signalosome. Positive and negative mediators are then recruited into the complex such as, guanine nucleotide exchange factor Vav1, the Bruton's

tyrosine kinase (Btk), the phospholipase C- γ 2 (PLC γ 2) or the E3 ligase Casitas B-lineage lymphoma (Cbl) [2,3]. B-cell activation does not result from unique ligand engagement of the BCR towards initiation, but also complex morphological changes. As such, an important role for the cytoskeleton as a point of integration through modulation of protein dynamics and clustering during B-cell activation has been described [4]. First, the role of actin cytoskeleton is indisputable for morphological changes as cellular spreading and contraction which helps B cells scan for antigens [5]. This is particularly critical for antigen presenting cell (APC)-dependent B cell activation [6]. Actin is also an important actor in BCR signalling starting from the molecular organization of the receptors in the cell membrane and the formation of the immunological synapse (IS) to the propagation of the signal from the signalosome [7–10]. Dynamic cytoskeleton reorganizations have also been implicated during later signalling events, in particular in driving Ag internalization into Major Histocompatibility Complex (MHC)-containing lysosomes [11] and in cooperating with clathrin to mediate efficient

Abbreviations: APC, antigen presenting cells; BCR, B cell receptor; BLNK, B cell linker; Btk, Bruton's tyrosine kinase; C-terminus, carboxyl-terminus; Cbl, E3 ligase Casitas B-lineage lymphoma; Glut4, glucose transporter type 4; IgH and IgL, immunoglobulin heavy and light chain; IS, immunological synapse; ITAM, immunoreceptor tyrosine-based activation motif; LLnL, N-acetyl-Leu-Leu-norLeucinal; pTyr, phosphotyrosine; N-terminus, amino-terminal; PH, pleckstrin homology; PLC γ 2, phospholipase C- γ 2; SH2, Src homology 2; SOCS, suppressor of cytokine signalling; Syk, spleen tyrosine kinase; TKB, tyrosine kinase-binding; WT, wild-type; Y, tyrosine; 3D, three-dimension

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attenuation of BCR signalling [12].

APS (also known as SH2B2, [13]) is a member of the adaptor family LNK/SH2B which includes Lnk (SH2B3) and SH2-B (SH2B1). These proteins share common protein-protein interaction domains and motifs. They possess a amino (N) terminal region containing a dimerization domain and proline-rich motifs, followed by a pleckstrin homology (PH) and a Src homology 2 (SH2) domains and a conserved tyrosine (Y) residue at their carboxyl (C) terminus [14]. Besides their significant structural similarities, their function appears to depend on their cell-specific expression, as exemplified during B-cell development with Lnk mainly involved in regulating early B progenitor's expansion, while APS modulates mature B cells, notably the B-1 subpopulation [15–17].

One common feature of the LNK family members is that they all play an important role as regulators of cytoskeleton organization in different cell types. In the case of APS, both APS-deficient and transgenic mice confirmed its role in the modulation of F-actin reorganization upon BCR capping and BCR signalling, leading to regulation of proliferation and survival of mature B cells [17,18]. Moreover, APS has been shown to regulate actin remodeling through interaction with specific partners. For instance, in the insulin pathway, APS associates with the actin binding protein Enigma to regulate insulin-induced Glucose transporter type 4 (Glut4) translocation in adipocytes [19]. However, the involvement of APS in the cytoskeleton reorganization in mature B cells is still unclear.

In the present study, we investigated the role of APS in regulating BCR-mediated signalling in mature B cells thus focusing on cytoskeleton remodeling. Our results showed that APS inhibitory function is stage specific as it is involved in BCR signalling in mature B cells exclusively. In these cells, APS colocalizes with the BCR, which facilitates its interaction with important signalling regulators such as Cbl and Syk kinase. To address the functional significance of APS interaction with these signalling molecules, we studied the contribution of APS to BCR-induced cytoskeletal rearrangement by expressing WT or APS SH2 mutant forms in the X16c mature B cell line and analyzed their functional effect on APS partners. We demonstrate that the SH2 domain of APS is important for BCR-induced dynamics. Furthermore, B cells over-expressing APS SH2 mutant form display enhanced expression and activation of the Syk kinase, suggesting that APS modulates the stability of this key BCR effector. Taken together, our findings indicate that, through modulation of its specific signalling partners, APS participates in the fine regulation of BCR-induced responses such as actin dynamics.

2. Materials and methods

2.1. Mice and cells

Wild-type (WT) and *Lnk*-deficient mice [15] were maintained at the Paris 13 University Animal facility and were used between 6 and 10 weeks (wks) of age. All animal procedures were conformed to institutional animal protocol guidelines and were approved by the Animal Care and Use Committee at the Paris 13 University. To isolate splenic B cells, single cell suspensions of splenocytes, after treatment with red blood cell lysis buffer (0.02 M Tris, 0.14 M NH₄Cl, pH 7.4) were incubated with anti-Thy1.2 mAb (Cedarlane Labs, Canada) and Low-tox-M-rabbit complement (Cedarlane Labs, Canada) to remove T cells and were then subjected to Ficoll density-gradient centrifugation. B cell purity (> 95%) was assessed by flow cytometry. X16c [20] (a kind gift from Dr. G. Sarmay, Lorand Eötvös University, Budapest, Hungary) and BAL17 mature B cell lines were cultivated in RPMI medium with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM β-mercaptoethanol.

2.2. Cell sorting

Single-cell suspension from BM, obtained by flushing femur and tibia bones with PBS and from spleen from WT C57BL6 mice (10 wks)

were stained with a combination of fluorescence-conjugated antibodies and sorted by FACS Vantage cell sorter (BD Biosciences, San Diego, CA). The following monoclonal antibodies (all from BD Pharmingen, CA) were used: PerCP-Cy5.5-conjugated anti-B220 (RA3-6B2), PE-conjugated anti-CD43 (S7), FITC-conjugated anti-IgM (II/41), PE-conjugated anti-IgD (11-26c2a).

2.3. Retroviral transduction and transfection

WT APS and Lnk and mutated R364M form of APS cDNA were cloned into the MSCV-IRES-GFP (MIG) retroviral vector [21]. Retroviral supernatants derived from 293 Epstein Barr nuclear antigen (EBNA) expressing cells were used for the infection of X16c cells. The cells were incubated twice with retroviral supernatants in media containing 10 µg/ml polybrene for 48 h. After infection, GFP⁺ X16c cells were purified by cell sorting (Elite ESP, Beckman Coulter, Fullerton, CA) and maintained in appropriate media.

2.4. Cell growth assay

After infection, B62.1 were maintained in culture with IL-7 and from day 2, a portion of the cells was removed every day and subjected to flow cytometry to determine the proportion of infected cells as those that express GFP. The percentage of GFP⁺ cells relative to the infection rate at day 2 after infection was plotted.

2.5. Immunoprecipitation and western blot analysis

Cells were starved in medium with 0.5% FBS for 2 h and then stimulated with 5 µg/ml (X16c cells) or 10 µg/ml (splenic B cells and BAL17 cells) F(ab')₂ goat anti-mouse IgM, µ chain specific (Jackson ImmunoResearch, West Grove, PA) at 37 °C for the indicated times. When indicated, cells were treated or not with 50 µM *N*-acetyl-Leu-Leu-norLeucinal (LLnL) proteasome inhibitor (Sigma, Saint Louis, MO) for 15 min before activation. Cells were lysed in ice-cold 1% or 0.5% Nonidet-P40 (NP40) lysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 10% glycerol, 1 mM EDTA) containing phosphatases and proteases inhibitors for 20 min on ice. Insoluble material was removed by centrifugation and total cell lysates (TCL) were immunoprecipitated for 2 h at 4 °C. Immunocomplexes were solubilized in 2× SDS sample buffer, resolved on SDS-PAGE gels and subjected to immunoblotting. The following antibodies were used: anti-APS, raised against a GST-NPH (N-terminal plus PH domain) APS fusion protein (amino acid 1–307); anti-Lnk, raised against a NH₂-terminal synthetic peptide (amino acid 72–91) of mouse Lnk; anti-Cbl (Cell Signaling Technology, Beverly, MA), anti-phospho-tyrosine mouse monoclonal Ab (clone 4G10 Platinum) (Millipore, Billerica, MA), anti-Syk Ab (gift from Dr. U. Blank, Paris Diderot University, Paris, France), anti-phospho-Syk (Human Y323/ murine Y317) (Ab #2715; Cell Signaling Technology), anti-phospho-Syk Ab (Y348), anti-phospho Syk Ab (Y352) (Invitrogen-Thermo Fisher, Carlsbad, CA), anti-β-tubulin Ab (Sigma, Saint Louis, MO). Antibody complexes were detected with horseradish peroxidase (HRP)-conjugated sheep anti-mouse or anti-rabbit immunoglobulins (Cell Signaling Technology) and revealed by enhanced chemiluminescence (ECL; GE Healthcare, Little Chalfort, UK).

2.6. Fluorescence microscopy and colocalization analysis

B cells were starved and stimulated with 10 µg/ml (splenic B cells) or 5 µg/ml (X16c cells) Cy5-conjugated-F(ab')₂ goat anti-mouse IgM, µ chain specific antibody (Jackson ImmunoResearch) at 37 °C for different times. Cells were deposited on poly-Lysine coated slides (Sigma, Saint Louis, MO), fixed in PBS with 4% PAF for 10 min, permeabilized in PBS with 0.1% Triton for 5 min, and then blocked for 45 min in PBS with 5% milk. Cells were then stained for 1 h with the primary antibodies in PBS with 1% milk, washed and subsequently incubated for 1 h with the

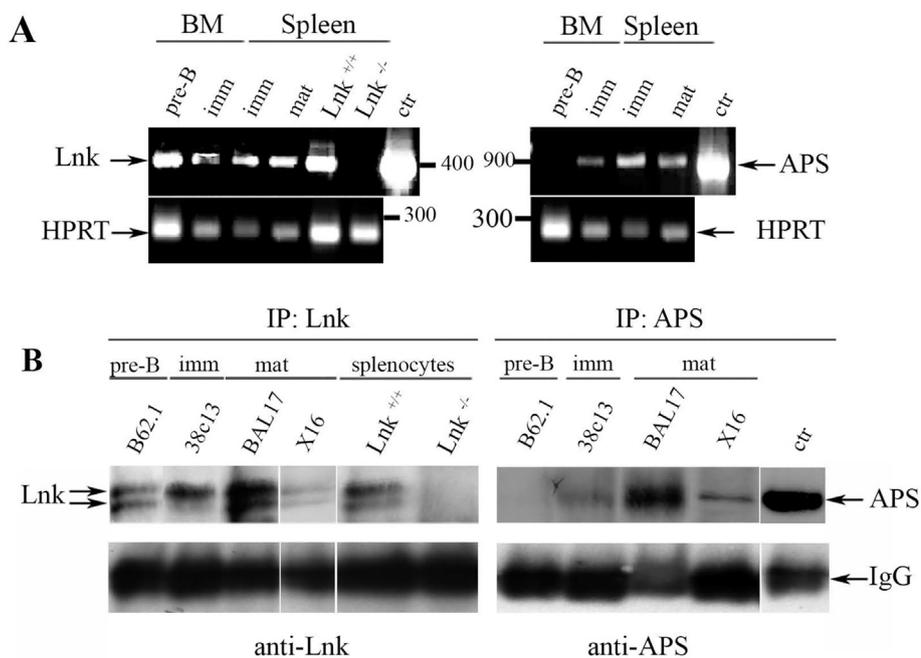


Fig. 1. APS and Lnk are differentially expressed during B cell differentiation. **A.** B cells from WT mice were sorted as follows: pre-B cells ($B220^+ CD43^- IgM^-$) and immature (imm) B cells ($B220^+ CD43^- IgM^+$) from bone marrow; immature B cell ($B220^+ IgD^- IgM^+$) and mature (mat) B cells ($B220^+ IgD^+ IgM^+$) from spleen. Total RNA was extracted and subjected to RT-PCR with specific primers for *Lnk* (left panel), *APS* (right panel), and *HPRT* (bottom panels) as control. Plasmids containing the full cDNA of *Lnk* or *APS* were used as respective positive controls (ctr). For *Lnk* and *HPRT*, RNA extracted from WT and *Lnk*^{-/-} splenocytes (*Lnk*^{+/+} and *Lnk*^{-/-}) was used as control. **B.** Cell lysates from the indicated pre-B, immature and mature cell lines were immunoprecipitated and immunoblotted with anti-Lnk (left panel) or anti-APS (right panel) antibodies. Cell lysates from WT and *Lnk*^{-/-} splenocytes (*Lnk*^{+/+} and *Lnk*^{-/-}) and from APS-overexpressing COS cells (ctr) were used as respective controls. White lines indicate repositioned gel lanes.

fluorescence-conjugated secondary antibodies. The following antibodies were used: anti-APS and anti-Cbl (Santa Cruz, Dallas, Texas), anti-Lnk, Alexa-488-conjugated anti-mouse IgG (H + L), Alexa-488 conjugated anti-rabbit IgG, Alexa-405 conjugated anti-rabbit IgG (Molecular Probes, Life Technologies, Carlsbad, CA), rhodamine-conjugated anti-goat IgG (Jackson Immunoresearch). The slides were mounted in Mowiol 4088 (Sigma, Saint Louis, MO) with Dabco anti-fading (Hoechst, Frankfurt, Germany). 3D image stacks were acquired by optical sectioning wide field microscopy using a Leica DMRXA2 microscope with a $100 \times 1.4NA$ objective (Leica Microsystems, Wetzlar, Germany) and a Photometrics Coolsnap HQ CCD, driven by Metamorph software (Molecular Devices, Sunnyvale, CA). 3D image stacks were restored using the PSF-based Modified Gold iterative constrained algorithm. Quantification of overlap regions of two fluorochromes (expressed as percentage of colocalization) was carried out on 3D images using the multidimensional image analysis (MIA) software using a wavelet-based spot detection algorithm. 3D surface rendering reconstruction was performed with the Amira (Mercury) software. Image acquisition and analysis were performed at the Nikon Imaging Centre at Institut Curie-CNRS (Paris).

2.7. BCR-induced B cells spreading

Glass coverslips were coated overnight at $4^\circ C$ with $4.2 \mu g/cm^2$ goat anti-mouse IgM antibody (Jackson Immunoresearch) and then blocked with PBS 2% BSA for 2 h. Cells were starved and then plated on the coated surfaces at $37^\circ C$ for the indicated times. When indicated, cells were incubated in the presence of $2 \mu M$ Syk inhibitor R406 (Selleck Chemicals, Houston, TX). They were finally fixed in 4% PAF/PBS and permeabilised in 0.1% Triton/PBS. Coverslips were incubated with rhodamine-phalloidin (Sigma) in 5% serum /PBS to visualize F-actin. Images were acquired with a fluorescent inverted microscope (Leica DMI6000) with a $100 \times 1.4NA$ objective and analyzed by Metamorph software. Each image was thresholded for light objects (total area in pixels) and shape factor was measured for each cell using Integrated Morphometry Analysis in Metamorph. The software calculated the shape factor using the formula $(4p \cdot area)/perimeter$, which returned a value between 0 and 1: a value of 1 being a perfect circle and near 0 a flattened object. Images acquisition was performed at the Imaging facility at Cochin Institute (Paris).

2.8. RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsband, CA) and reverse transcribed using the Superscript II first-strand synthesis system (Invitrogen). PCR was performed using specific primers for *APS*, *Lnk* and *HPRT* and the PCR products were visualized in a 1.5% agarose gel.

2.9. Statistics

P values were calculated using the unpaired *t*-test (two-tailed, 95% confidence interval) by the GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Statistical differences are shown as: ns, non-significant > 0.05 , * $p < .05$ and *** $p < .0001$.

3. Results

3.1. *Lnk* and *APS* are differentially expressed during B cell development

Analysis of *Lnk*^{-/-} and *APS*^{-/-} mice revealed the specific role of these SH2B family adaptors in the progenitor and mature B cell compartments, respectively [15–18]. One interesting observation in *Lnk*^{-/-} mice was that they exhibited a large increase in pro/pre-B cell numbers in comparison with mature B cell. This suggested that these proteins were either expressed or functional only at specific developmental stages, thus underlying a pivotal role in modulating B cell responses. To verify if *Lnk* and *APS* displayed a restricted expression profile during B cell differentiation, primary pre-B, immature and mature B cells were sorted from bone marrow and spleen of wild type (WT) mice. The sorted cells were then analyzed for *Lnk* and *APS* gene expression by RT-PCR. *Lnk* specific transcript was detected in all B cell subsets, while *APS* transcript was present in immature and mature B cells only (Fig. 1A). This was further confirmed at the protein level in B cell lines at different stages of differentiation by immunoprecipitation and immunoblotting with anti-Lnk or anti-APS specific antibodies (Fig. 1B). These results indicate that while *Lnk* is expressed all along B cell maturation, *APS* expression only appeared in immature and mature B cells.

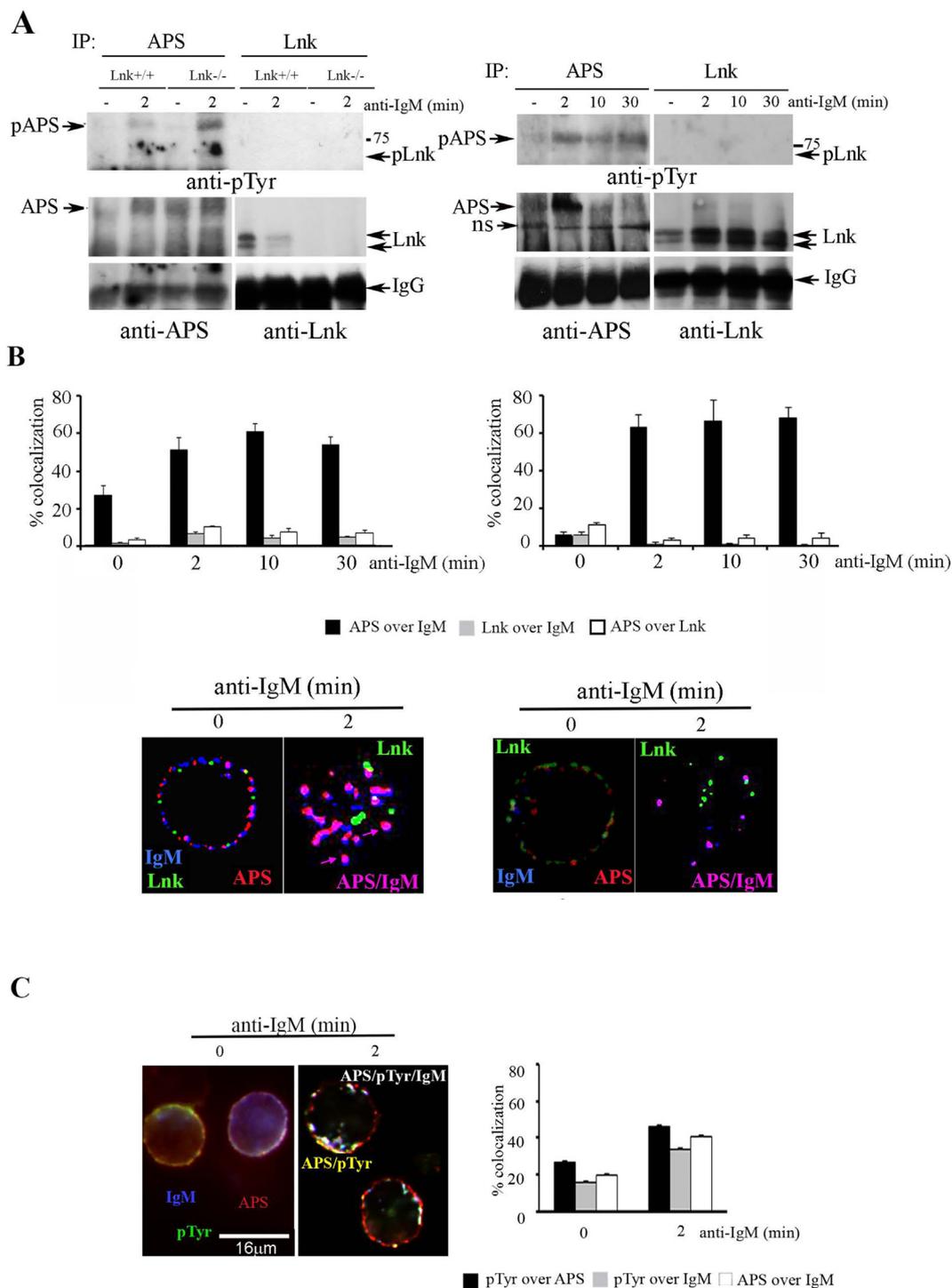


Fig. 2. Only APS adaptor is recruited to the BCR complex in mature B cells.

A. Purified *Lnk*^{+/+} and *Lnk*^{-/-} splenic B cells (left panels) and BAL17 mature B cells (right panels) were stimulated or not with goat anti-IgM antibody (10 µg/ml) for the indicated times. Cell lysates were immunoprecipitated with anti-APS or anti-Lnk antibodies and blotted with anti-pTyr, anti-APS and anti-Lnk antibodies. White lines indicate repositioned gel lanes. B. Purified splenic B cells (left panels) and X16 mature B cells (right panels) were stimulated with Cy5-labelled donkey anti-IgM antibody for different times and plated on poly-lysine coated slides. Cells were stained with anti-APS and anti-Lnk antibodies and subsequently with secondary labelled antibodies. 3D image stacks were acquired by optical sectioning wide field microscopy (100× objective). Quantification of overlap regions of two fluorochrome signals was carried out on 3D images using the multidimensional image analysis (MIA) and expressed in the graphs as the percentage of colocalization between the labelled proteins. At least 25 cells were analyzed for each condition. In the panels, merged images after segmentation of cells stimulated or not for 2 min are shown. Lnk is shown in green, APS in red, IgM in blue and APS/IgM colocalization in magenta. All shown results are representative of at least three independent experiments. C. Purified splenic B cells were stimulated with 5 µg/ml Cy5-labelled donkey anti-IgM for different times. Cells were stained with anti-APS and anti-pTyr and subsequently with secondary labelled antibodies. 3D image stacks were acquired as described in B. Merged images after deconvolution of cells unstimulated or stimulated for 2 min are shown. pTyr is shown in green, APS in red, IgM in blue, APS/pTyr colocalization in yellow and APS/pTyr/IgM colocalization in white. Scale bar = 16 µm. All shown results are representative of at least three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

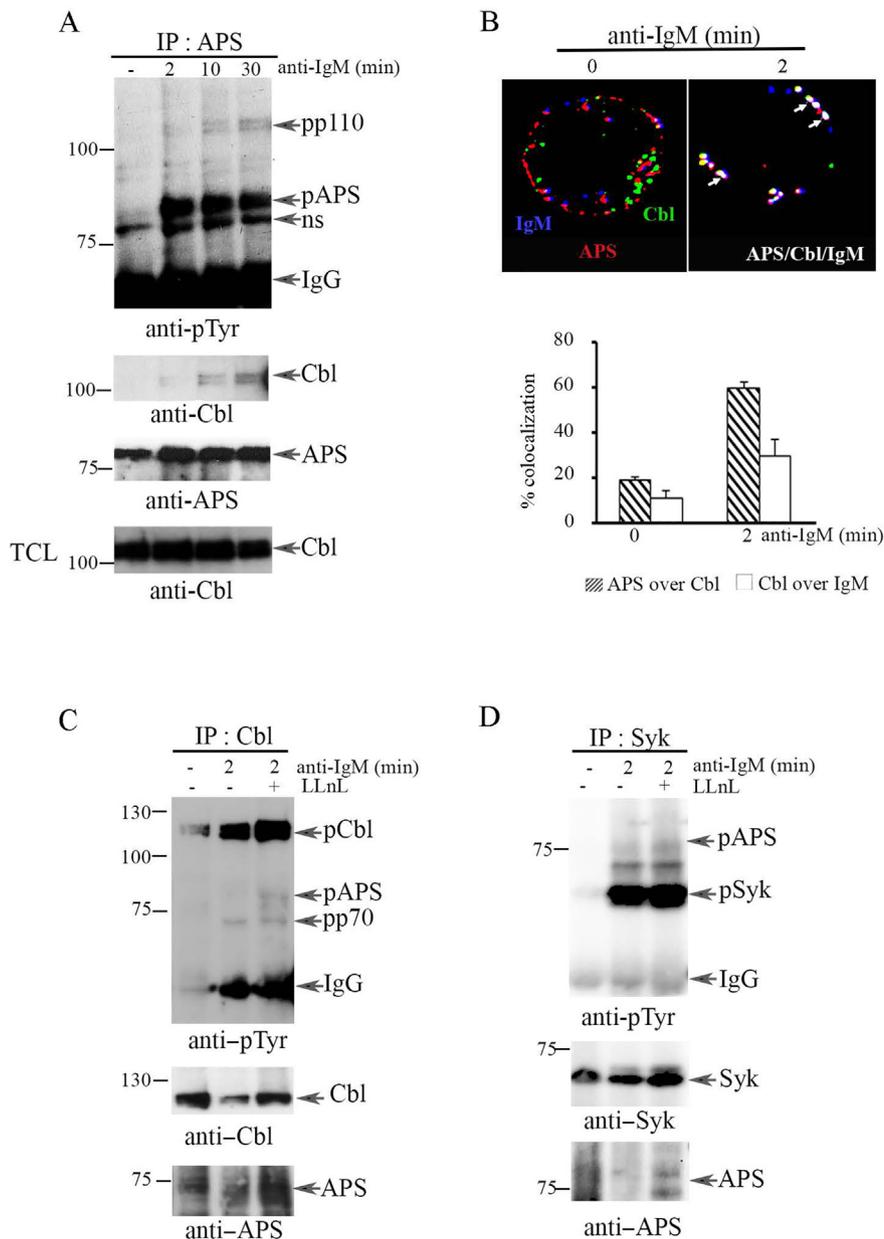


Fig. 3. APS colocalizes and interacts with partners of the BCR signalling cascade. **A.** BAL17 cells were stimulated with anti-IgM antibody (10 μ g/ml) for different times and cell lysates were immunoprecipitated with anti-APS and then immunoblotted with anti-pTyr, anti-Cbl and anti-APS antibodies. Total cell lysates (TCL) were used as control for Cbl size. ns = not specific. **B.** X16c cells were stimulated with 5 μ g/ml Cy5 labelled donkey anti-IgM antibody for 2 min and stained with anti-APS, anti-Cbl and secondary labelled antibodies. Images were acquired and analyzed as described in Fig. 2B. The percentages of colocalization between the labelled proteins are expressed in the graphs. APS is shown in red, Cbl in green, IgM in blue, APS/Cbl/IgM colocalization in white. **C-D.** BAL17 cells were treated or not with 50 μ M LLnL for 15 min before activation for 2 min with goat anti-IgM antibody (5 μ g/ml). Cell lysates were immunoprecipitated with anti-Cbl (C) and anti-Syk (D), followed by immunoblotting with anti-pTyr, anti-Cbl, anti-APS (C) and anti-pTyr, anti-Syk and anti-APS antibodies (D). All experiments have been performed at least 3 independent times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Only APS adaptor is recruited to the BCR complex in mature B cells

To address the functional significance of Lnk and APS expression in mature B cells, splenic B cells were first stimulated with anti-IgM antibody for 2 min, followed by Lnk or APS precipitation and their tyrosine phosphorylation status was then analyzed by western blot. While APS was clearly phosphorylated after 2 min of anti-IgM stimulation, no phosphorylation was detected on Lnk in the same stimulation conditions (Fig. 2A, left panels). Similar results were obtained in BAL17 cells, a murine mature B cell line displaying a strong BCR-activation which facilitated the study of BCR-dependent signalling pathways. In these cells, APS phosphorylation was maintained even at longer activation times, while Lnk remained unphosphorylated at any given time (Fig. 2A, right panels).

These results showed that despite the expression of both Lnk and APS in mature B cells, only APS was phosphorylated upon BCR stimulation.

In order to corroborate the exclusive stage-specific functionality of the two adaptors, we overexpressed WT Lnk or WT APS in the B62.1 pre-B cell line and we analyzed their IL-7-dependent proliferation. The

constructs were generated in the bicistronic MIG vector expressing the GFP protein that allows selection of Lnk or APS-expressing cells using GFP fluorescence. The proportion of GFP⁺ B62.1 cells was determined three days after infection and their fraction was assessed each following day and expressed relative to the initial GFP⁺ population (Fig. S1). IL-7-induced proliferation of cells over-expressing WT Lnk forms was dramatically inhibited (approximately 4-fold) at day 4 after infection as compared to vector. In contrast, expression of WT APS did not affect pre-B cells proliferation, demonstrating that this protein does not play a role at this developmental stage.

To examine if the absence of Lnk phosphorylation in response to BCR stimulation was due to a default of recruitment to the BCR complex, we analyzed Lnk and APS cellular distribution in response to BCR triggering. A quantitative method to analyze colocalization was used in immunofluorescence microscopy. For this, splenic B cells were stimulated with Cy5-conjugated- F(ab')₂ goat anti-Ig μ chain antibody for different times and then stained with specific anti-Lnk and anti-APS or anti-phosphotyrosine (pTyr) antibodies followed by fluorochrome-conjugated secondary antibodies. 3D image stacks were acquired by optical sectioning wide field microscopy, deconvolved and

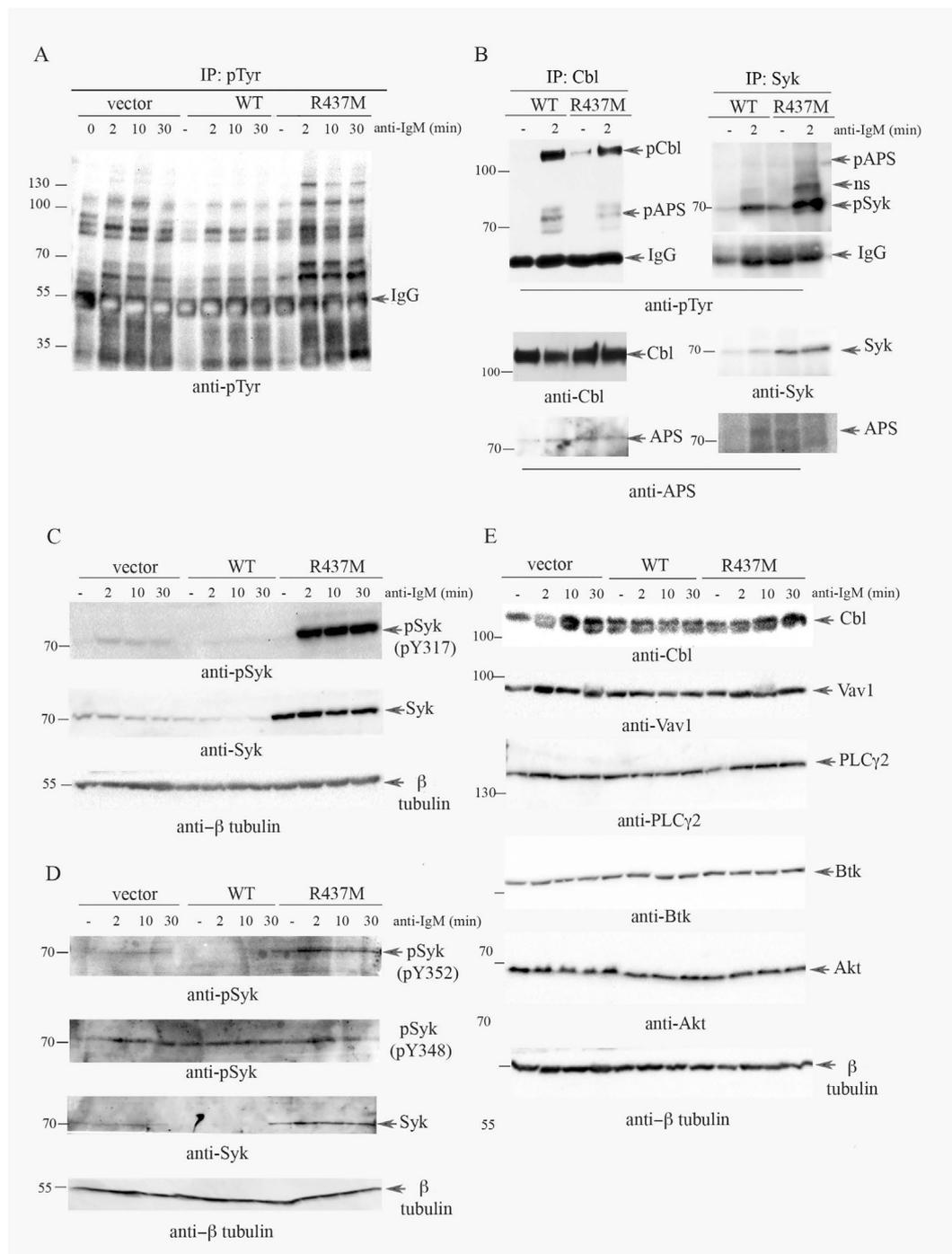


Fig. 4. APS SH2 domain is important for BCR-induced signalling.

A. X16c cells retrovirally infected with WT or mutant R437M APS constructs (indicated as WT and R437M) or vector alone were stimulated with goat anti-IgM antibody (5 μg/ml) for different times and cell lysates were immunoprecipitated and immunoblotted with anti-phosphotyrosin antibody. B. Infected cells were stimulated with goat anti-IgM antibody (5 μg/ml) for 2 min and cell lysates were immunoprecipitated with anti-Cbl (left panel) or anti-Syk (right panel) antibodies and immunoblotted with anti-pTyr, the anti-Cbl or anti-Syk respective antibodies and anti-APS antibody. C-E. Total cell lysates from infected cells, stimulated for different times, were immunoblotted with anti-phospho-Syk (pY317) (C), anti-phospho-Syk (pY352), anti-phospho-Syk (pY348) (D) and anti-Syk (C-D) or with the indicated antibodies (E). β-tubulin was used as loading control. All experiments have been performed at least 3 independent times.

the percentage of colocalization between two proteins was quantified from the overlap regions of two fluorescent signals. The mean percentage of APS-IgM and Lnk-IgM colocalization was calculated and compared for each activation time point. APS showed already colocalization with the BCR at the resting state (26%), and this value increased up to 60% after 10 min stimulation and remained constant up to 30 min. Instead, Lnk did not show colocalization neither with the BCR nor with

APS in unstimulated or stimulated samples. This was visualized by merged segmented images of the recruitment of APS to the BCR after 2 min stimulation where Lnk remained outside the complex (Fig. 2B, left graphs and panels). Similar results were obtained in murine X16c mature B cell line (Fig. 2B, right graph and panels). X16c cells were characterized by a slower BCR turnover than Bal17 cells, making them more suitable for analysis of cellular rearrangements using fluorescence

microscopy. Moreover, APS-BCR colocalized as discrete phosphorylated spots, as observed after staining with an anti pTyr antibody, suggesting the interaction of APS with phosphorylated signalling molecules present in the active BCR signalosome (Fig. 2C panels and graphs).

Overall, these results show that APS, but not Lnk, actively participates in the BCR complex formed upon antigen triggering. Furthermore, they underlined the sequential stage-specific functionality of these two adaptors in response to the respective main signalling pathways driving B cell differentiation.

3.3. APS interacts with signalling molecules involved in the regulation of BCR-mediated pathways

Since our results showed that APS localized at the BCR complex, we then investigated whether the adaptor could indeed interact with phosphorylated signalling molecules upon BCR-stimulation. For this, the BAL17 cell line was treated with anti-IgM antibody for different times and lysates were immunoprecipitated with anti-APS, followed by immunoblotting with anti-pTyr antibody. We observed a phosphorylated protein of approximately 110 kDa (pp110) coprecipitating with APS upon BCR triggering (Fig. 3A). By using antibodies recognizing signalling molecules known to participate in the BCR-mediated pathway and whose molecular weight was close to the one detected, we could identify p110 protein as the E3 ligase Cbl (Fig. 3A).

Association of APS with Cbl was further confirmed by immunofluorescence. X16c cells were stimulated for 2 min with Cy5-anti-IgM antibody, stained with anti-APS and anti-Cbl antibodies, imaged and then analyzed as previously described. The formation of a complex between APS/Cbl/IgM was detected in stimulated cells at sites where IgM was clustered (Fig. 3B, panels with arrows and graphs), while low levels of colocalization between APS/Cbl and Cbl/IgM were observed in unstimulated cells.

The functional implication of the APS/Cbl complex in either the endocytosis or degradation of the Insulin receptor and of positive signalling molecules has been previously shown [22,23]. Indeed, we also detected some APS at intracellular sites of IgM clustering (Fig. 2B, C and 3B). Therefore, we assessed the presence of other BCR-dependent signalling intermediates in the APS/Cbl complex that might be involved in degradation. For this, anti-IgM stimulated BAL17 cells were treated with *N*-acetyl-Leu-Leu-norLeucinal (LLnL), a proteasome inhibitor, and their lysates were immunoprecipitated with anti-Cbl antibody and then immunoblotted with anti-pTyr, anti-Cbl and anti-APS antibodies. These results showed an increase in both phospho-APS and APS protein coprecipitating with Cbl after LLnL treatment. Importantly, a second phosphorylated protein of 70 kDa (pp70) was detected associated with Cbl upon IgM-stimulation (Fig. 3C). This protein was identified as the Syk kinase after immunoprecipitation with a specific antibody which confirmed its association with APS upon BCR triggering (Fig. 3D). These results demonstrate that in mature B cells, APS interacts at the BCR complex with important regulators such as Cbl and Syk.

3.4. The SH2 domain of APS is involved in the regulation and the stability of key BCR-signalosome components

Previous studies reported the recruitment of APS to different signalling complexes notably via its SH2 domain [23–26]. To assess the functional implication of APS SH2 domain in BCR responses, we overexpressed APS WT (4.9 fold) or mutated at its SH2 domain (3.6 fold) in X16c cells using the MIG retroviral vector (Fig. S2A). In particular, the R437M mutation results in the disruption of the APS/ Insulin receptor interaction [27]. We first examined the impact of their expression on total protein phosphorylation. Vector alone, WT or SH2 mutant-overexpressing cells were stimulated with anti-IgM antibody for 2 to 30 min and total cell lysates were immunoprecipitated and immunoprobed with anti-pTyr antibody. The phosphorylation level of several proteins was significantly decreased in WT APS-overexpressing cells and

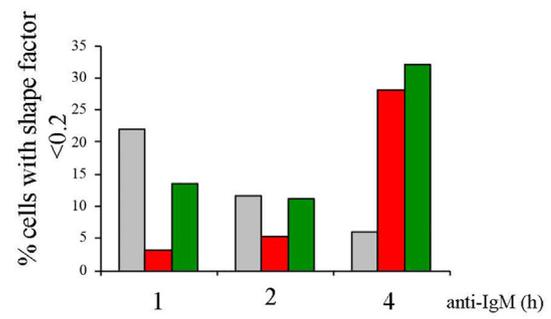
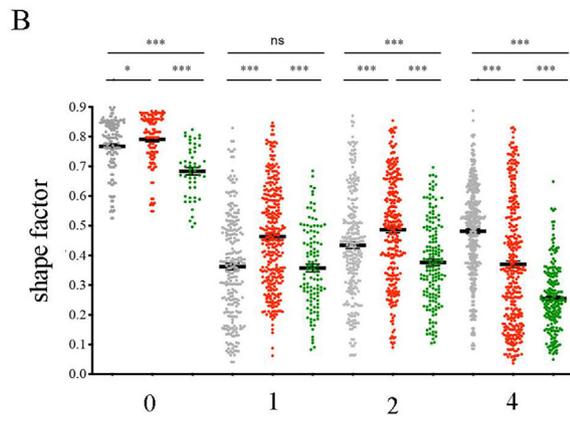
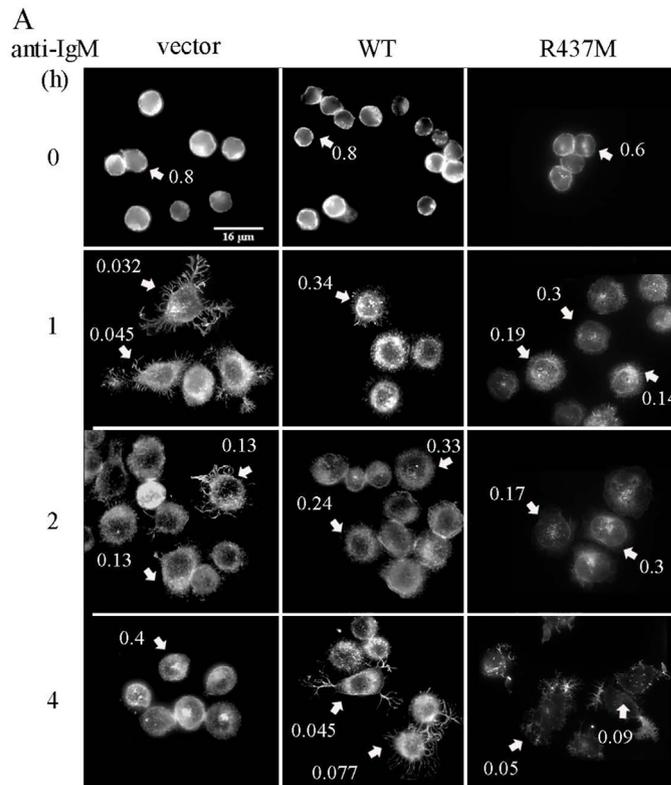
reversely, increased in R437M APS-expressing samples (Fig. 4A). Next, we examined whether inactivation of APS SH2 domain would affect its association with Cbl and Syk. Therefore, unstimulated or BCR-activated total cell lysates from the different APS-expressing cells were immunoprecipitated with anti-Cbl or anti-Syk specific antibodies and then immunoprobed with anti-pTyr or their respective antibodies. Cbl associated with WT phospho-APS and to a lesser extent with mutant phospho-APS upon BCR triggering (Fig. 4B left panel). In the case of Syk, coprecipitation with APS was weakly detected in every situation (Fig. 4B right panel), as shown previously in the absence of LLnL (Fig. 3D). However, we could not detect a direct interaction between pCbl and pSyk in both WT or mutant APS contexts, even in the presence of LLnL (Fig. S2B). Interestingly, we detected a significant increase in pSyk in R437M APS mutant-expressing cells as compared to cells expressing WT APS (Fig. 4B, right panel). This result was further confirmed when total cell lysates from R437M or WT APS-overexpressing cells were directly immunoprobed with anti-phospho-Syk antibodies directed to Tyr317 or Tyr352, specific target residues of Cbl activity or Lyn/Src kinase, respectively. Elevated levels of phosphorylation at residues Tyr 317 and Tyr 352 were observed in R437M APS-overexpressing cells stimulated for different times. Conversely, overexpression of WT APS strongly reduced these levels (Fig. 4C and D). In contrast, no difference at Tyr348 Syk auto-phosphorylation site was observed between the different extracts (Fig. 4D). Moreover, a reduced total Syk expression was detected in WT APS overexpressing cells; while levels of Cbl, as well as of other downstream effectors of the BCR signalling, such as Vav1, PLC γ 2, Btk and Akt were not modified in these cells. (Fig. 4E).

These results suggest that the APS SH2 domain is involved in Syk kinase turn over upon stimulation.

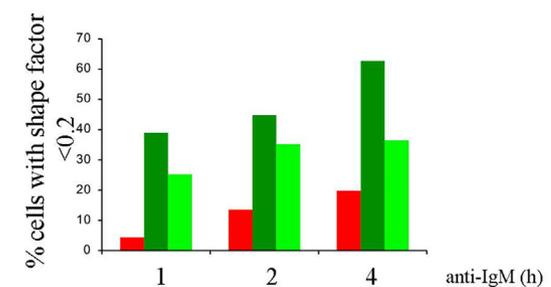
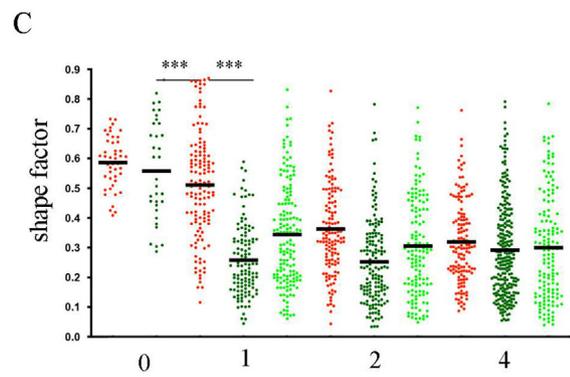
3.5. APS regulates cytoskeleton dynamics in mature B cells through its SH2 domain

The association of APS with proteins involved in BCR-evoked cytoskeleton remodeling suggested that APS may modulate these functions through these interactions.

Since the signalosome activation plays a key role in the regulation of the actin cytoskeleton in lymphocytes [28] we studied the impact of APS overexpression on BCR induced cytoskeleton remodeling. X16c cells overexpressing empty vector, WT or R437M APS were plated on coverslips coated with anti-IgM antibody and actin was visualized by phalloidin staining. In this way, we could assess dynamics as a specific response to the activation of the BCR, independently of the initial adhesion, as described in other studies [29]. We analyzed actin rearrangements in order to assess the implication of APS in the regulation of the signalling complex after its formation upon IgM stimulation. To quantify this mechanism, a shape factor was calculated from images using the Metamorph program. Round shaped cells were characterized by a shape factor near 1, while cells with more irregular shape are characterized by a value approaching 0. Indeed, after 1 h stimulation, an important cell spreading was observed in vector expressing cells, which showed an elongated or irregular shape with membrane extensions. Their mean shape factor decreased from 0.75 in unstimulated cells to 0.35. In contrast, WT APS overexpressing cells did not show a significant spreading while R437M APS-overexpressing cells displayed a spreading comparable to control cells. After 2 h, the number of vector expressing cells characterized by irregular shape decreased and returned upon 4 h to a more circular round shape (Fig. 5A, left panels; grey points in 5B left graph, black line in Fig. S2C). WT APS overexpressing cells showed a very delayed kinetics, with cells forming multiple F-actin-rich expansion after 4 h of activation only (Fig. 5A, middle panels). Accordingly, their mean shape value remained at higher levels than control cells during the first hours and decreased at 4 h of activation (red points in 5B left graph; red line in Fig. S2C). R437M APS-overexpressing cells showed more activated morphology at



● vector
● WT
● R437M
● R437M +R406



(caption on next page)

Fig. 5. APS affects BCR-induced cell spreading in mature B cells.

A. Vector-, WT- and R437M expressing X16c cells were plated on coverslips coated with anti-IgM antibody or BSA as control for the indicated times and F-actin was visualized with rhodamine-phalloidin staining. Images were acquired with a fluorescent inverted microscope (objective 100 \times , Leica) and analyzed by Metamorph software. Each image was thresholded for light objects and shape factor was measured for each cell using Integrated Morphometry Analysis in Metamorph. The shape factor indicates how closely a cell resembles a circle (with values close to 0 indicating an elongated cell and to 1 representing a perfect circle). Characteristic shape factor values are shown for several cells in the panels. B–C. The left graphs show the distribution of shape factor values for each time of stimulation and quantification of the mean values are shown. Each symbol represents a cell, thick horizontal lines indicate the means. The right graphs show the percentages of cells showing a shape factor value < 0.2. C. R437M expressing cells were treated or not with 2 mM Syk inhibitor R406. Statistical differences are indicated as: ns, non-significant > 0.05, * p < .05 and *** p < .0001. Similar results were obtained in six (B) and three (C) independent experiments. Scale bar = 16 μ m.

any time of stimulation compared to vector and WT APS expressing cells and significantly lower mean shape factor (Fig. 5A, right panels and dark green points in 5B left graph; dark green line in Fig. S2C). We then compared the percentage of cells characterized by a shape factor of 0.2 or less, thus displaying a high spreading phenotype. WT APS-overexpressing cells showed very low percentage of spreading after 1 h and 2 h of activation (3% and 5%) as compared to vector-expressing cells (22% and 12%) which reflected the observed delayed spreading. In contrast, R437M APS-overexpressing cells displayed high percentage of low shape factor values at all measured times, indicating a persistent spreading response in these cells particularly at 4 h (32%) (Fig. 5B right graph). Since R437M-expressing cells displayed higher Syk phosphorylation and expression (Fig. 4B–C–D), we investigated whether Syk activity impacted cytoskeleton remodeling. For this, we treated R437M-expressing cells with the Syk inhibitor R406. Indeed, we observed that the treated cells displayed a significantly higher mean shape value especially after 1 h stimulation and lower percentages of cell with low shape factor values indicative of a less activated morphology (Fig. 5C: light green points and histograms in left and right graphs respectively).

These findings show that APS interferes with BCR-induced cytoskeleton dynamics.

4. Discussion

Analysis of APS mutant mice showed the involvement of this adaptor in the development of the mature B cell compartment, with particular impact on cell proliferation and actin organization of B1 cells [17,18].

However, the signalling partners and mechanism by which APS modulates BCR signalling and cytoskeleton dynamics are still scarcely described. Here, we showed that APS is phosphorylated and recruited to the receptor complex upon BCR triggering, where it interacts with BCR regulators, notably the Cbl E3 ligase and the Syk kinase. Our results demonstrate that APS, partly via its SH2 domain, plays a role as scaffold protein in the stability of the Syk kinase, a key signalosome effector. We further show that the SH2 domain of APS modulates the regulation of BCR-induced spreading, thus pinpointing a relevant role for this adaptor in the cytoskeletal rearrangement in mature B cells.

B cell lineage is the only one in which two Lnk family members, namely Lnk and APS, participate to B cell development, not only by their differentiation-stage specific expression, but also at the functional level. Our results showed that Lnk is expressed at every stage of the B cell development, however its deficiency affects primarily IL-7-mediated expansion of B cell progenitors, without altering the number and the functionality of mature B cells (this work and [15]). Indeed, Lnk is neither recruited to the BCR signalling complex nor phosphorylated by this signalling cascade despite its expression in the cells. Previous results with transgenic *Lnk* mice suggested a potential role of Lnk in BCR-mediated pathways, but this has only been shown in *Lnk* highly-expressing cell lines [30]. Accordingly, we also observed Lnk phosphorylation in response to BCR triggering only in cell line in which Lnk was overexpressed (data not shown). These observations suggest that the recruitment of Lnk and its potential functional implication might be dependent on its expression levels. This also argues for a potential competitive recruitment of the two adaptors according to their expression levels. Conversely, overexpression of APS in pre-B cells has not

effect on IL-7 dependent proliferation confirming that Lnk is unique in achieving this function at this differentiation stage. Altogether, our results show that in physiological conditions Lnk and APS rather play sequential inhibitory functions at different developmental stages of the lineage, through the modulation of IL-7- and BCR- driven signalling cascades, respectively.

Our biochemical analysis of mature B cells overexpressing APS revealed reduced levels of protein phosphorylation in response to BCR stimulation, which suggested that APS regulates negatively the signalosome, possibly by binding to signalling molecules acting either as positive or negative effectors. Indeed, our coimmunoprecipitation experiments indicated that APS is activated and recruited to the receptor complex upon BCR triggering in mature B cells. Additionally, APS colocalizes and interacts with Cbl and Syk, two important inhibitors and activators respectively of the BCR signalling cascade. This result was not observed in splenic B cells from APS transgenic mice that might be due to the concentration of stimulatory anti-IgM antibody used. Another possible explanation might be that Iseki et al. used a reverse strategy with Cbl immunoprecipitation in absence of proteasome inhibitor [18].

Our results argue for an adaptor negative function of APS in bridging Cbl-mediated ubiquitination of the Syk kinase, leading to the proteasomal degradation of the signalling activator. This model is supported by the observation of a higher coprecipitation between APS/Syk and APS/Cbl in the presence of a proteasome inhibitor. Conversely, we could not observe any interaction between Cbl and Syk, even in the presence of proteasome inhibitor. This suggests that APS may play a structural bridge role in this complex by approaching Cbl and Syk proteins in the absence of their direct interaction.

In adipose tissue APS was also able to interact with Asb6, a protein containing ankyrin repeats and a SOCS box that regulates ubiquitination [31]. Together with our results on the interaction between Cbl and APS, these data indicate that ubiquitin ligases are potential specific partners of APS.

The main mechanism by which APS adaptor may interact with its different partners is through its distinct functional domains and motifs. Crystallography and biochemical studies using site-directed mutagenesis have shown that the tyrosine kinase-binding (TKB) domain of Cbl interacts with APS Y618 once phosphorylated either by the insulin or the EPO receptor. The sequence surrounding Y618 at the C-terminus of APS is strikingly conserved in SH2B and Lnk, the other members of the LNK family [23,35]. On the other hand, several studies demonstrated that the SH2 domain of APS is the main mediator of its regulatory function. For this reason, we analyzed whether APS SH2 domain might also play a role in APS interactions and function at the BCR signalosome. Our results showed that inactivation of the domain results mainly in enhanced expression and activation of the Syk kinase, in contrast to Cbl or several other effectors of the signalling pathway. These results underlie the importance of this interacting domain in Syk stability and allow to propose a model in which, upon recruitment to the BCR signalosome, APS through its SH2 domain would bring Syk in a close vicinity to Cbl, bound via APS pY618. This would facilitate the ubiquitination and the proteasomal degradation of the kinase. Conversely, inactivation of APS SH2 domain hinders the Syk/Cbl contact, resulting in significant increase of Syk expression. This model does not exclude the possibility of an APS-independent mechanism mediating Syk

degradation by Cbl in specific B cell conditions [32].

Functional evidence of the role of APS in B cells was demonstrated with the generation of *APS*-disrupted animals [17]. *APS*^{-/-} mice displayed increased CD5⁺ B-1a cell number, whose development is enhanced upon sustained BCR signal [33]. Indeed, *Cbl*^{-/-}/*Cblb*^{-/-} mice showed also a substantial increase in B-1 cells [34]. This suggests that APS association with its interacting partners may control important developmental checkpoints by modulating the intensity or the duration of BCR signalling.

A second important feature shared by APS and its partners is their involvement in the regulation of cytoskeleton rearrangement either through their association with actin filaments or actin associated proteins. Indeed, previous studies suggested a role for APS in the regulation of F-actin content in B cells [17,18] and in other cell types such as mast cells [35], fibroblasts and adipocytes [19,36]. As for APS partners, Syk has been shown to play a critical role in the reorganization of the actin cortex during BCR-induced signalling [37]. Similarly, Cbl has been reported to regulate cytoskeletal dynamics in B cells [38] partly by modulating the ubiquitination of Syk [39,40]. Upon activation, B lymphocytes spread and protrude when scanning for antigens and finally form immune synapses upon contact with antigen presenting cells. In our experiments, we showed that over-expression of WT APS slow down BCR-driven dynamics; at the opposite APS SH2 mutant form sustained them. Indeed, after internalization of the receptor complex, in the presence of the mutated form, Syk is not degraded and this is a reasonable explanation for the observed sustained cell spreading. On the contrary, in the presence of WT APS, Syk is degraded and thus rearrangements are delayed. We could detect an elevated level of Syk phosphorylation in the presence of mutated APS form and, particularly, on specific residues, Tyr352 and Tyr 317. Interestingly, Tyr352 is phosphorylated by LYN kinase, an essential kinase of the initial BCR signalosome, which facilitates subsequent phosphorylation of the activation loop of Syk [41]. These results underscore the scaffolding role of APS in the regulation of BCR-mediated activation and actin dynamics. These processes are central also in late BCR-dependent B cell development, notably in the B1 compartment.

In conclusion, our studies identified for the first time Cbl and Syk as partners/ effectors of APS in the modulation of BCR signalling and cytoskeleton organization in mature B cells. APS/Cbl association allows the adaptor to play the role of scaffold protein for the stability of a key BCR signalling effector, the Syk kinase. Although the exact molecular mechanisms should still be identified, it is tempting to speculate that APS, after its recruitment to the BCR signalosome, facilitates Cbl function on signalling effectors, enhancing their degradation and thus playing a pivotal role in the attenuation of the signal. These functions are mainly mediated by APS SH2 domain, which also plays an active role in the regulation of BCR-driven dynamics.

Author contribution

ED performed all the experiments, analyzed data and wrote the paper. JBS designed and developed the quantitative method to analyze colocalization. NVB and LV supervised the project and wrote the paper. The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellsig.2020.109673>.

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